Conserved regulators of the cell separation process in *Schizosaccharomyces*

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Running title. Regulators of cell separation in *Schizosaccharomyces*

ABSTRACT

The fission yeasts (*Schizosaccharomyces*) representing a highly divergent phylogenetic branch of Fungi evolved from filamentous ancestors by gradual transition from mycelial growth to yeast morphology. For the transition, a mechanism had been developed that separates the sister cells after the completion of cytokinesis. Numerous components of the separation mechanism have been characterised in *Sch. pombe*, including the zinc-finger transcription factor Ace2p and the fork-head transcription factor Sep1p. Here we show that both regulators have regions conserved within the genus. The most conserved parts contain the DNA-binding domains whose amino-acid sequences perfectly reflect the phylogenetic positions of the species. The less conserved parts of the proteins contain sequence blocks specific for the whole genus or only for the species propagating predominantly or exclusively as yeasts. Inactivation of either gene in the dimorphic species *Sch. japonicus* abolished cell separation in the yeast phase conferring hypha-like morphology but did not change the growth pattern to unipolar and did not cause extensive polar vacuolation characteristic of the true mycelium. Neither mutation affected the mycelial phase, but both mutations hampered the hyphal fragmentation at the mycelium-to-yeast transition. Ace2p^{Sj} acts downstream of Sep1p^{Sj} and regulates the orthologues of the Ace2p-dependent *Sch. pombe* genes *agn1*⁺ (1,3-alpha-glucanase) and *eng1*⁺ (1,3-beta-glucanase) but does not regulate the orthologue of *cfh4*⁺ (chitin synthase regulatory factor). These results and the complementation of the cell separation defects of the ace2⁻ and sep1⁻ mutations of *Sch. pombe* by heterologously expressed ace2^{Sj} and sep1^{Sj} indicate that the cell separation mechanism is conserved in the *Schizosaccharomyces* genus.

Key words: Septum, cell division, transcription factor, dimorphism, hypha, fission yeast, evolution

1. Introduction

Fungi display a variety of propagation modes in their vegetative growth phases. Most species propagate either as unicellular yeasts or as multicellular filamentous organisms, but many fungi can alternate between two (dimorphic fungi) or several modes (polymorphic fungi) to adapt to different substrates and changes of the environmental conditions. As the hyphae and pseudohyphae of the di- and polymorphic species are frequently invasive (Sipiczki et al., 1998b; Sudbery et al., 2004; Wong et al., 2010), the ability to switch to filamentous morphology is often considered to be necessary for virulence and/or for penetration into solid substrates like tissues. All types of growth strategies involve septation (a kind of cytokinesis characteristic of cell-walled organisms) that separates segregated sets of chromosomes and organelles after the completion of mitosis. The elaboration of the fungal septum is a highly complex process that involves precisely organised cellular processes governed by multiple intra- and extracellular signals (reviewed in Goyal et al., 2011; Walther and Wendland, 2003; Wolfe and Gould, 2005). In yeasts and in the yeast phase of di- and polymorphic species, the septum and a narrow band of the mother cell wall that surrounds its edge have to be degraded to allow the sister cells to separate physically (for recent reviews see Cabib, 2004; Sipiczki, 2007).

The yeast septum consists of layers of different structures. Due to the differences in their chemical composition, the enzymes of the separation machinery specifically degrade one of the layers (usually called primary septum) without affecting the flanking layers (also called secondary septa). In the budding yeasts (such as *Saccharomyces* and *Candida*) the degradable layer mainly consists of chitin (Kuranda and Robbins, 1991), whereas in the fission yeasts *Schizosaccharomyces* it is composed of linear 1,3-beta-glucan (Humbel et al, 2001). Thus the dissolution of the primary septum requires chitinase activity in the budding yeasts (Kuranda

and Robbins, 1991) and 1,3-beta-glucanase (Eng1p) activity in *Sch. pombe* (Martin-Cuadrado et al., 2003). As the composition of the cell wall differs from that of the septum, its site-specific degradation requires different enzyme activity(ies). In *Sch. pombe*, the Agn1p 1,3-alpha-glucanase participates in this process (Dekker et al., 2004), but other enzymes may also be involved because the inactivation of *agn1*⁺ does not abolish sister cell separation, only delays it.

The production, delivery and activation of these enzymes are under the control of complex networks of genes and proteins in both yeast systems. In *Sch. pombe* a regulatory cascade of specific and general transcription factors controls cell separation in which the transcription factors Ace2p and Sep1p play essential roles (reviewed in Sipiczki, 2007).

The *Sch. pombe* zinc-finger protein Ace2p regulates numerous genes that act in the M-phase of the cell cycle, among them genes that encode the cell separation enzymes Agn1p and Eng1p (Alonso-Nunez et al., 2005; Dekker et al., 2006). Its counterparts in the budding yeasts *S. cerevisiae* and *C. albicans* also regulate cell separation genes including the chitinase genes *CTS1* and *CHT3* (Doolin et al., 2001; Kelly et al., 2004). The activity of the *Sch. pombe* *ace2*⁺ gene is controlled by the fork-head transcription factor Sep1p (Alonzo-Nunez et al., 2005; Bähler, 2005; Rustici et al., 2004) and by numerous other regulators (Batta et al., 2009; Buck et al., 2004; Lee et al., 2005; Petit et al., 2005). In contrast, the *S. cerevisiae* protein Hcm1p showing the highest sequence similarity to Sep1p is not directly involved in the regulation of the *S. cerevisiae* *ACE2* gene. It contributes to cell separation indirectly, through the timely activation of *FKH1* and *FKH2* (Pramila et al. 2006). The fork-head-type transcription factors encoded by these genes are G2/M phase-specific activators for a set of M/G1 genes (Sic1 cluster) that includes *ACE2* (Spellman et al., 1998). The *S. cerevisiae* Fkh1p and Fkh2p proteins also bind to the promoters of the Ace2p-dependent genes to prevent the activation of these genes by the Ace2p-paralogue Swi5p (Voth 2007). The Fkh2p

orthologue in *Sch. pombe* binds to Sep1p and the PCB (Pombe cell Cycle Box) promoter motif to repress the activity of the genes that have this motif in their promoters (Papadopoulou et al., 2008). *ace2*⁺ is one of them (Buck et al., 2004).

In the budding yeasts, the Ace2p and Fkh2p orthologues are also involved in the regulation of pseudohyphal growth (Baladron et al., 2002; Kelly et al., 2004; King and Butler, 1998). The pseudohyphae are chains of bud-like cells connected by intact septa. Obviously, the transition from the yeast phase to the pseudohyphal stage is associated with the inactivation of the septum-cleaving machinery that separates the bud from its mother cell in the yeast phase. Since the fission yeasts do not propagate by budding, they can not form pseudohyphae. This, however, does not prevent them from producing multicellular structures. The dimorphic *Sch. japonicus* forms yeast cells in liquid substrates (yeast phase) and produce invasive mycelium (mycelial phase) consisting of true hyphae in solid substrates (Sipiczki et al., 1998b). Changes in the culturing conditions (e.g. availability of nitrogen) evoke transitions between the growth morphologies. *Sch. japonicus* is supposed to be the closest relative of the latest common ancestor of all extant fission yeasts that was most probably a filamentous fungus (Sipiczki, 2000). The other species of the genus are essentially yeasts but certain *Sch. pombe* strains can also form short cell chains reminiscent of true hyphae under very specific culturing conditions (Amoah-Bauhin et al., 2005; Prevorsevsky et al., 2009). In the true hyphae of *Sch. japonicus* neither the septum nor the adjacent cell wall is degraded (Sipiczki et al., 1998b), suggesting that the cell-separation machinery is switched off like in the pseudohyphae of the budding yeasts. Inactivation of *sep1*⁺ and *ace2*⁺, the positive regulators of the cell separation in *Sch. pombe*, causes a filamentous morphology (chains of cells with intact septa) (Alonso-Nunez et al., 2005; Sipiczki et al., 1993) suggesting that transitions between the growth-phases could also be evoked by modulating the activity of these genes. However, the mutant cell chains are not equivalents of the mycelial phase

(Sipiczki, 2007) because they do not show certain characteristic features of the invasive true hyphae, such as the strictly unipolar growth, the intense vacuolation at the non-growing cell ends and the high rate of apical extension at the growing cell ends.

In this study, we demonstrate that putative orthologues of the *Sch. pombe* Ace2p and Sep1p transcription factors can be found in other fungal species and they have evolved parallel with the evolution of the genomes of the organisms. The orthologues of both transcription factors have specific structural elements characteristic of all *Schizosaccharomyces* species but missing in other fungi. The phenotypes of the deletion mutants in the dimorphic *Sch. japonicus*, the fission yeast species most distantly related to *Sch. pombe*, and the complementation of the *Sch. pombe* mutations by heterologously expressed *Sch. japonicus* genes indicate that all fission yeasts share the Sep1p-Ace2p-regulated cell separation machinery.

2. Materials and methods

2.1. Yeast strains and growth conditions

Yeast strains used in this study are listed in Table 1. The composition of the rich culture media YEA (yeast extract agar), YEL (YEA without agar) the minimal media SMA (synthetic minimal agar), SML (SMA without agar) EMMA (Edinburgh minimal medium agar) and EMMML (EMMA without agar) were described previously (Sipiczki and Ferenczy, 1978; Mitchison, 1970). For culturing auxotrophic strains, the minimal media were supplemented with nutrients according to the auxotrophy of the strains. For suppression the *nmtI*⁺ promoter, the media were supplemented with 15 μ M thiamine.

2.2. Plasmids and DNA manipulations.

The cloning vectors used and the plasmids constructed in this study are listed in Table 1. The *Sch. pombe ura4⁺* cassette for gene disruption was obtained from the plasmid KS-ura4 (Bähler et al., 1998). Transformation of *Escherichia coli* cells, plasmid recovery from bacterial cells and basic recombinant DNA methods were performed by standard techniques described in Sambrook et al. (1989). Genomic DNA was isolated from exponential-phase yeast cultures with the glass bead method (Hoffman and Winston, 1987). Transformation of yeast cells of exponential-phase cultures grown in YEL was performed either with electroporation or with the lithium acetate method (Ito et al., 1983). Transformants were selected and maintained on SMA plates. Amplification of DNA sequences from genomic DNA and purified plasmid preparations was carried out with primers listed in Table 2. The PCR reaction parameters were optimised for each reaction individually. Digestion of DNA with restriction endonucleases and ligation of DNA molecules were performed according to the instructions of the manufacturers of the enzymes used.

2.3. Inactivation of the *Sch. japonicus* genes *ace2^{Sj}* and *sep1^{Sj}*

For in-vitro gene-disruption, the *ace2^{Sj}* and *sep1^{Sj}* genes were amplified from genomic DNA of the wild-type strain 7-1 with primer pairs complementary to chromosomal sequences that flanked the coding regions of the genes and contained recognition sites for the enzymes *NdeI* and *SalI* (Table 2). The PCR products were cloned into pJET1.2 (Table 1) with blunt-end ligation. The inserts were then excised from the resulting pJET1.2.*ace2^{Sj}* and pJET1.2.*sep1^{Sj}* plasmids with *NdeI* and *SalI* and cloned into pUC18 (Table 1). Using the same enzymes for opening pUC18 removed its *HindIII* site from the plasmid and allowed us to use

the *HindIII* sites of the cloned *Sch. japonicus* sequences for in-vitro gene disruption. As the *Sch. pombe ura4⁺* gene complements the uracil auxotrophy of *Sch. japonicus* 7-252 (Furuya and Niki, 2009), we used this gene to construct cassettes for gene disruption and as a marker gene for the identification of the transformants.

To construct the *ace2^{Sj}* disruption cassette, the *ace2^{Sj}* gene was amplified from the pUC18-*ace2^{Sj}* plasmid with a pair of primers that had *HindIII* recognition sites at their 5' ends and hybridized to different parts of the *ace2^{Sj}* gene with their 3' parts (Table 2). *ace2disfor* was complementary to a position located behind the start and *ace2disrev* was complementary to a position located before the end of the *ace2^{Sj}* coding region. The amplified DNA was cut with *HindIII* to generate sticky ends and ligated with the *Sch. pombe ura4⁺* cassette excised with the same enzyme from the plasmid KS-ura4 (Table 1). After ligation, the DNA was transformed into *E. coli* and bacterial colonies were selected that contained plasmids carrying the *Sch. pombe ura4⁺* gene flanked with the terminal parts of the *Sch. japonicus ace2^{Sj}* gene (partial deletion of *ace2^{Sj}*). The purified plasmid preparation was digested with *NdeI* and *SalI* and the linear disruption cassette obtained was used to transform the uracil auxotrophic mutant 7-252 of *Sch. japonicus*.

For the construction of the *sep1^{Sj}* disruption cassette, the pUC18-*sep1^{Sj}* plasmid was digested with *HindIII* to cut the only recognition site for this enzyme located in the *sep1^{Sj}* coding sequence. The linearised plasmid was ligated with the *Sch. pombe ura4⁺* cassette obtained with the same enzyme from KS-ura4. The ligated DNA was transformed into *E. coli* and bacterial colonies were selected containing plasmids with *sep1^{Sj}* disrupted with *ura4⁺*. The plasmid preparation was digested with *NdeI* and *SalI* and the linear disruption cassette obtained was used to transform 7-252 *Sch. japonicus* cells. Prototrophic yeast colonies growing on the minimal medium EMMA were isolated from both transformation experiments and checked for the integration site of the disruption cassettes

with primers complementary to the *ura4⁺* cassette (KS-ura4F or KS-ura4R) and with primers (ace2revT or sep1revT) hybridizing to chromosomal sequences located outside the regions amplified from the wild-type strain for the in-vitro construction of disruption cassettes (Table 2). When DNA prepared from the wild type and from the transformants was used as template, PCR products were obtained only from the reactions with the transformants, demonstrating that the wild-type genes were replaced with disruption cassettes in the transformants.

2.4. Measuring the transcription level by quantitative RT-PCR.

To determine the transcription level of genes, the yeast cultures were grown in YEL or EMMML (+ supplements) at 30°C until mid-log phase. Cells of the cultures were collected by centrifugation and used for total RNA isolation using the method described previously (Chomczynski and Sacchi, 1987). cDNA synthesis was carried out with the ImProm-II Reverse Transcriptase system (Promega, A3802) according to the manufacturer's instruction. 0.5 µg RNA was used as template. For the RT-PCR (Bio-Rad IQ5 real-time PCR system) reaction, the SsoFast EvaGreen supermix (Bio-Rad, 172-5200) reagent was used with final primer concentration of 0.2 µM. Serial dilutions of wild-type *Sch. pombe* (0-1) and *Sch. japonicus* (7-1) DNA (1/10, 1/100, 1/1000, 1/10000) were prepared to generate a standard curve for each reaction. The reaction conditions were as follows: 94°C for 2 minutes, 40 cycles at 94°C for 2s, 57°C for 20s. All PCR reactions were normalised to *sce3⁺* or *sce3^{Sj}* transcription data. The experiments were repeated at least twice using cDNA from different biological repeats.

2.5. Microscopic examination

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221 For morphological observation of yeast cells and hyphae, phase contrast microscopy and
222 fluorescent microscopy were used. To examine the morphology in the yeast phase, the cells
223 were grown in YEL or on MMA agar plates at 30°C. Cell number was determined by
224 counting in a haemocytometer. To visualise primary septa and nuclei, cells were stained with
225 calcofluor and DAPI, respectively (Sipiczki et al., 1998a). The transitions between the yeast
226 and mycelial phases and the growth of hyphae were observed in thin (~1 mm thick) agar films
227 (YEA) sandwiched between microscope glass slides and cover slips (Sipiczki et al., 1998b).
228 To prepare the film, a sterile microscope glass slide was placed into a sterile glass Petri-dish
229 and warmed up to around 40°C. Hot molten agar medium was then carefully poured in the
230 dish until it covered the microscope glass. After the medium had solidified, yeast cells were
231 inoculated on its surface with a sterile loop. The yeast cells were then carefully covered with a
232 sterile cover slip. The yeast cells could propagate under the cover slip and deprived the thin
233 layer of medium of nutrients very fast and entered the mycelial phase. The narrow space
234 forced the hyphae extend horizontally. They could be easily examined by placing the Petri-
235 dish under the objective of a microscope. To induce the hypha-to-yeast transition, 2-5 µl of
236 YEL was dropped onto the surface of the medium at the edge of the cover slip. The “old
237 ends” and the “new ends” of the yeast cells were distinguished on the basis of their
238 fluorescence intensity after calcofluor staining (Sipiczki et al., 1998). By definition, the old
239 end of a cell is the end which was one of the ends of the mother cell, whereas the new end is
240 formed from the septum layer called secondary septum during the division of the mother cell.
241 Due to the difference between the chemical composition of the cell wall and that of the
242 secondary secondary septum, the old end is bright and the new end is dark. Later, when the
243 new end starts growing, it develops a bright cap (formed by newly synthesised cell wall on the
244 tip of the old end) which is separated by a dark band (ring) (rest of the former secondary

septum) from the so-called division scar (the edge of the mother cell wall cleaved at the last cell division). In chains of *ace2^{Sj}* and *sep1^{Sj}* mutants, the old and new cell ends cannot be distinguished by calcofluor staining because the septa are not cleaved (the secondary septum remains within the intact septum). In the case of the first cell and the last cell of a chain, the tip is the old end, and the end at the uncleaved septum is the new end. In the second cell and the last-but-one cell of a chain, the cell end located closer to the chain end is the new end. The identification of the old and new ends of the internal cells of cell chains is described in Sipiczki et al, 1993. The yeast cells and the hyphae were examined with an Olympus DX-40 microscope and the images were captured with an Olympus DP-70 camera.

2.6. Bioinformatics

For identification of putative orthologs of *Sch. pombe* proteins, reciprocal Blastp search was performed in the databases *Schizosaccharomyces pombe* GeneDB (<http://old.genedb.org/genedb/pombe/blast.jsp>), Broad Institute (<http://www.broadinstitute.org/scientific-community/data>), *Saccharomyces* Genome Database (<http://www.yeastgenome.org/cgi-bin/blast-sgd.pl>), Génolevures (<http://www.genolevures.org/blast.html#databases>), *Candida* Genome Database (<http://www.candidagenome.org/cgi-bin/compute/blast-sgd.pl>), *Aspergillus* Genome Database (http://www.aspergillusgenome.org/cgi-bin/compute/blast_clade.pl), *Histoplasma* genome at Genome Institute, Washington University (http://genome.wustl.edu/genomes/view/histoplasma_capsulatum/) and the National Center for Biotechnology Information (NCBI <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The best hit for each protein was extracted and used for comparative sequence analysis. To determine overall similarity of proteins a modified Needleman-Wunsch global alignment algorithm (Needleman

and Wunsch, 1970) was used with the tool available at <http://mobyli.pasteur.fr/cgi-bin/portal.py?#forms::needle> (gap opening penalty: 5; gap extension penalty: 1). To localise conserved regions, pairwise blast bl2seq (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) alignments and multiple Clustal W 2.0 (Larkin et al., 2007) and MAFFT v. 6.850 (Katoh and Toh, 2008) alignments were generated. Conserved domains were localised in the proteins by scanning their sequences against the PROSITE protein profile database of protein domains (<http://ca.expasy.org/prosite/>) using the ScanProsite tool (<http://ca.expasy.org/tools/scanprosite/>). WebLogos (Crooks et al., 2004) for the conserved domains were generated from Clustal W multiple alignments with the tool provided at <http://weblogo.berkeley.edu/logo.cgi>. The multiple sequence alignments were also used for phylogenetic tree construction with the Neighbor-Joining algorithm available in the PHYLIP package Version 3.67 (Felsenstein, 2007). Branch support was estimated from bootstrap analysis (111 replications; SEQBOOT and CONSENSE of the PHYLIP package). *Rhizopus oryzae* sequences were used as outgroups.

3. Results

3.1. Identification and sequence analysis of fungal orthologues of Ace2p and Sep1p

A common procedure of searching for orthologous proteins is based on the identification of reciprocal best BLAST hits. We used this method to look for the putative fungal orthologues of the Ace2p and Sep1p transcription factors of *Sch. pombe* in the genomes of all *Schizosaccharomyces* species and certain phylogenetically more distant fungi. First we identified the most similar proteins in the searched databases then used these proteins for reciprocal BLAST searches in the *Sch. pombe* genome database. The proteins that gave the

highest scores with Ace2p and Sep1p in the reciprocal search were considered the orthologues of the *Sch. pombe* proteins. To identify putative orthologues from species for which complete genome sequences are not yet available, we performed general BLAST searches in the NCBI database. The identified proteins with the highest scores were then tested with reciprocal BLAST. In many species, the reciprocal search did not identify Ace2p or Sep1p as the most similar *Sch. pombe* proteins. These species and proteins were not considered further. The gene IDs for the identified orthologues are listed in Table 3. Both groups of proteins were diverse in size and sequence similarity.

3.2. Comparative sequence analysis of the Ace2p and Sep1p orthologues

All Ace2p orthologues contained a characteristic C2H2 region consisting of two complete copies and one incomplete copy of the C2H2 zinc finger domain (Fig. 1). In the *Schizosaccharomyces* proteins these domains were parts of a conserved C-terminal region (marked with I in Fig. 1) of 116 to 118 residues in length (sequence identity ranging from 72 to 96%). All fission-yeast proteins contained two shorter conserved regions (II and III in Fig. 1) that were not detected in the other fungal sequences. None of these regions and no other parts of the fission-yeast proteins showed sequence similarity to the 29 amino-acid long stretch of the *S. cerevisiae* Ace2p protein required for nuclear export (NES) (Jensen et al., 2000) and the region necessary for nuclear localisation (NLS) (O'Conallain et al., 1999). NLS is located on the C-terminal side of the zinc-finger domain in the *S. cerevisiae* protein. In the proteins of three *Schizosaccharomyces* species (Ace2p^{Sp}, Ace2p^{So} and Ace2p^{Scr}) the zinc-finger domains are located at the very end of the sequences, leaving no room for such a domain. The *Sch. japonicus* orthologue Ace2p^{Sj} has a short extension behind the zinc-finger domain but its sequence is very different from that of NLS. No part of the

Schizosaccharomyces proteins showed any similarity to the 169 amino-acid long region of the *S. cerevisiae* protein that specifically activates the *CTS1* promoter (McBride et al., 1999) either.

In global pair-wise sequence comparison of the fission-yeast Ace2p proteins, the *Sch. japonicus* orthologue had the most divergent sequence. It showed 32-33 % identity with the other proteins that exhibited 40-71% identity with each other.

The Sep1p orthologues also showed considerable size variability and great sequence diversity outside the DNA-binding domains. Within the *Schizosaccharomyces* group, the *S. japonicus* protein (Sep1p^{Sj}) had the most divergent sequence again: 31 % global identity with the other sequences that showed 44-73 % identity with each other. In the variable regions, all fission-yeast proteins contained two conserved blocks that had no counterparts in the other fungal proteins (marked with I and IV) and one region (marked with III) that was also detectable in the Sep1p orthologues of the Pezizomycotina species but not in the Saccharomycotina proteins (Fig. 2). The conserved block marked with II in Fig. 2 was present in three fission-yeast proteins and absent in all other proteins including Sep1p^{Sj}.

3.3. Phylogenetic analysis of the Ace2p and Sep1p orthologues

Although most proteins selected by the reciprocal best hit search were hypothetical and experimentally uncharacterized, we assumed that they could have homologous functions and could be involved in the regulation of genes encoding proteins for cytokinesis and cell separation. Further we also assumed that the phylogenetic analysis of their sequences might allow an insight into the history of the separation process. However, the high sequence variability outside of the DNA-binding domains did not allow meaningful multiple alignments of entire sequences required for the phylogenetic analysis. To overcome this

problem we used only the DNA-binding domains with short flanking sequences. For Ace2p analysis we used the region located between the residues 416 and 527 of the *Sch. pombe* protein and the corresponding sequences of its orthologues. For Sep1p analysis the sequence between the amino acids 122 and 217 and the corresponding regions in the orthologous proteins were used. The Weblogos generated from Clustal multiple alignments presented in Figs 1 and 2 show the conservatism of each position.

The phylogenetic analysis of these regions generated trees showing topologies consistent with the phylogenetic positions of the species. Pezizomycotina and Saccharomycotina were clearly separated from each-other and the fission yeasts formed monophyletic groups branching off from basal positions in both trees (Fig. 3). Their lineages then split, separating the dimorphic *Sch. japonicus* from the rest of the genus. The deep positions of the branching points indicate that these separation events took place early in the evolution of the fungal tree of life.

3.4. Defective cell separation and growth morphology in the yeast phase of the *Sch. japonicus* disruption mutants

The effect of the inactivation of *ace2^{Sj}* and *sep1^{Sj}* on cell separation was examined in exponential-phase yeast cultures grown on solid agar medium or in liquid medium. The cells of the wild-type cultures were physically separated and rarely contained more than one septum. In contrast, both the *ace2^{Sj}::ura4^{Sp}* (7-258) and the *sep1^{Sj}::ura4^{Sp}* (7-254) mutant cells formed long, branching chains (Fig. 4). DAPI-staining visualized single nuclei in their cells. The septa between the nuclei were bright when stained with calcofluor, demonstrating that the cell separation machinery, which degrades the calcofluor-positive primary septum in the wild-

type cells (Sipiczki et al., 1998a), was inactive in the mutants. Occasionally, the mutant cell chains broke, resulting in cell ends covered with bright material, indicating that the breakage was generated by physical forces rather than through enzymatic dissolution of the primary septum. This morphology corresponds to that of the hypha-like cell chains of the *ace2⁻* and *sep1⁻* mutants of *Sch. pombe* (Alonso-Núñez et al., 2005; Sipiczki et al., 1993) but differs from the morphology of the hyphae produced in the mycelial phase of *Sch. japonicus*. In contrast to the mycelial-phase hyphae, the cells of the *ace2⁻* and *sep1⁻* hyphae showed no extensive vacuolation, were not invasive (did not grow into the agar medium) and grew bipolarly like the yeast-phase cells. However, their bipolar growth differed from that of the yeast-phase cells because the unsplit septa covering their ends did not allow polar extension. As their ends were not free, the mutant cells shifted the sites of growth initiation to the edges of the septa and grew in lateral directions producing branches along the chains as described for *sep1⁻* cells in *Sch. pombe* (Sipiczki et al. 1993). The cell separation defect was more pronounced in *ace2^{Sj}::ura4^{Sp}* than in *sep1^{Sj}::ura4^{Sp}* as shown in Fig. 5. Monitoring the changes of the proportions of single cells, cell clumps (consisting of 2-to-unknown numbers of cells), cell pairs connected with intact septa (cells with single septa), cell chains consisting of countable numbers of cells (cell chains with 2-to-several septa), clumps of cell chains (consisting of several cell chains) in cultures grown in the liquid medium YEL revealed that the *sep1^{Sj}::ura4^{Sp}* mutant had an intermediary phenotype between the wild-type and the strongly chain-forming *ace2^{Sj}::ura4^{Sp}* mutant (selected sets of results are shown in Fig. 5). In the *sep1^{Sj}::ura4^{Sp}* cultures, but not in the *ace2^{Sj}::ura4^{Sp}* cultures, the proportion of cells with cleaved septa increased with time (age of culture).

3.5. Growth morphology in the mycelial phase of the *Sch. japonicus* disruption mutants

The ability to produce invasive mycelium was tested on solid media. The wild type and the mutants did not differ in the timing of transition from surface yeast propagation to invasive growth and their hyphae looked essentially alike. Invasive mycelium appeared around their yeast colonies after 5 to 6 days of incubation and consisted of unipolarly growing, extensively vacuolated and branched true hyphae (Fig. 6A). The branches shot out from behind the septa (on the non-apical sides) laid down in the dividing tip cells of the hyphae.

To examine of the effect of the mutations on the mycelium-to-yeast transition, the medium was supplemented with nitrogen in front of the growing front edge of the mycelium. It was shown earlier that the abrupt increase of the level of nitrogen sources in the medium halts the growth of the hyphae and converts them into yeast cells (Sipiczki et al., 1998b). The elevation of the nitrogen level in the medium stopped hyphal growth and triggered recurrent rounds of septum formation both in the wild-type (7-1) and in the *ace2^{Sj}::ura4^{Sp}* (7-258) and the *sep1^{Sj}::ura4^{Sp}* (7-254) mycelium (Fig. 6B-E). The septate wild-type hyphae then split their septa and broke up into separate fragments (individual cells), whereas the mutant hyphae did not cleave their septa. Shortly after septation, both the separating fragments of the wild type and the non-separating fragments of the mutants begun to grow at both of their poles, demonstrating that they were switching from the unipolar mycelim-type growth to the bipolar yeast-type growth. The bipolar growth of the mutant resulted in lateral extensions (branches) on sides of the non-split septa. Certain extensions converged, generating physical tension that ripped the septum flanked by them (e.g. Fig. 6D).

3.6. The effect of the inactivation of *sep1^{Sj}* and *ace2^{Sj}* on putative cell-separation genes

In the cell-separation cascade of *Sch. pombe*, the Sep1p transcription factor regulates the activity of the *ace2⁺* gene that codes for an activator of numerous downstream genes involved

in the cell separation machinery (for a review see Sipiczki, 2007). To investigate the functional relationship between *sep1^{Sj}* and *ace2^{Sj}*, we compared the *ace2^{Sj}* mRNA level by RT-PCR in exponential phase yeast cultures of the wild type (7-1) and the *sep1^{Sj}::ura4^{Sp}* (7-254) mutant. The mutant cells had drastically reduced *ace2^{Sj}* mRNA level (Fig. 7), demonstrating that Sep1p^{Sj} is a positive regulator of *ace2^{Sj}*.

Then we asked whether Sep1p^{Sj} and Ace2p^{Sj} do have a regulatory function similar to that of their *Sch. pombe* counterparts. To examine their role in cell separation, we first searched the *Sch. japonicus* genome for orthologues of the *Sch. pombe* genes coding for the cell separation enzymes endo-1,3-alpha-glucanase (*agn1⁺*), endo-1,3-beta-glucanase (*eng1⁺*) and the Cell Separation Ring component Cfh4p (*cfh4⁺*) by the reciprocal best hits method. The following putative proteins were identified: SJAG_06167.4 (Agn1p^{Sj}), SJAG_00667.4 (Eng1p^{Sj}) and SJAG_06027.4 (Cfh4p^{Sj}). Then gene-specific primers (Table 2) were designed for each gene and the activity of the genes was compared by RT-PCR in exponential-phase wild-type (7-1), *ace2^{Sj}::ura4^{Sp}* (7-258) and *sep1^{Sj}::ura4^{Sp}* (7-254) mutant cultures (Fig. 7). The mRNA levels of *agn1^{Sj}* and *eng1^{Sj}* were lower in the mutants than in the wild type. The reduced transcriptional activity proves that Sep1p^{Sj} and Ace2p^{Sj} are positive regulators of *agn1^{Sj}* and *eng1^{Sj}*. However, they appear to play no role in the regulation of *cfh4^{Sj}* because its mRNA level was not reduced in the mutants.

3.7. Activity of *sep1^{Sj}* and *ace2^{Sj}* in *Sch. pombe* cells

The similarity of protein sequences, domain structures and mutant phenotypes in the most distantly related *Schizosaccharomyces* species (Sipiczki, 2000) suggest functional conservatism for both transcription factors in the genus. The functional homology can be verified by testing the genes of one species for activity in the other species. The availability of

cloned genes from *Sch. japonicus* and deletion mutants in *Sch. pombe* provided a possibility for the examination of functional equivalence by testing ability of the *Sch. japonicus* genes for rescuing the cell separation defects of the *Sch. pombe* *sep1*⁻ and *ace2*⁻ mutants.

To construct plasmids for heterologous expression of the *Sch. japonicus* genes in *Sch. pombe* mutants, the coding regions of *ace2*^{Sj} and *sep1*^{Sj} were excised with *Nde*I and *Sal*I from the pJET 1.2.*ace2*^{Sj} and pJET 1.2.*sep1*^{Sj} plasmids, respectively, and the fragments obtained were ligated into pREP expression vectors opened with the same restriction endonucleases at the thiamine-repressible *nmt1*⁺ promoters. The pREP-*ace2*^{Sj} and pREP-*sep1*^{Sj} constructs were then transformed into uracil auxotrophic *Sch. pombe* mutants defective either in *ace2*⁺ (2-1043) or in *sep1*⁺ (2-1407). Prototrophic transformants were selected on minimal medium supplemented with thiamine that suppressed the activity of the *nmt1*⁺ promoters to which the coding regions of the *Sch. japonicus* genes were fused.

The pREP expression vectors contain full strength (pREP2) or attenuated versions (pREP42 and pREP82) of the thiamine-repressible *nmt1*⁺ promoters (Basi et al., 1993; Maundrell, 1993). The *ace2*⁻ *Sch. pombe* cells transformed with the low strength pREP82-*ace2*^{Sj} plasmids showed intermediary phenotypes. The culture contained both single, non-septate cells and short cell chains (Fig. 8D). The medium strength pREP42-*ace2*^{Sj} and the high-strength pREP2-*ace2*^{Sj} restored nearly wild-type cell separation. Cell chains were no longer visible in the culture but cell pairs showing *eng1*⁻-like delayed septum degradation were quite frequent (Fig. 8E). To examine the efficiency of gene activation by the plasmid-borne *ace2*^{Sj} gene, the mRNA levels of *ace2*⁺ and Ace2p-dependent genes *adgl*⁺, *cfh4*⁺, *eng1*⁺ and *mid2*⁺ were compared by RT-PCR in exponential-phase cultures of the wild type (0-1), the *ace2Δ* mutant (2-1043) and the *ace2Δ* + pREP42-*ace2*^{Sj} transformant cultivated in thiamine-free medium (Fig. 9). *ace2*⁺ mRNA was detected neither in the mutant nor in the transformant. The mRNA levels of all Ace2p-dependent genes were much higher in the

transformant than in the *ace2Δ* mutant but lower than in the wild-type cells. The intermediate mRNA levels in the transformants were consistent with the incomplete rescue phenotype. Expression of the *Sch. japonicus* gene from the high-strength pREP2-*ace2^{Sj}* plasmid caused cell lysis upon prolonged cultivation in thiamine-free medium (Fig. 8H). The *sep1⁻* *Sch. pombe* cells transformed with the pREP1-*sep1^{Sj}*, pREP41-*sep1^{Sj}* and pREP81-*sep1^{Sj}* plasmids had very similar phenotypes: the medium-strength expression of *sep1^{Sj}* almost completely restored the wild-type cell-separation morphology (Fig. 8F) whereas its strong overexpression caused cell lysis.

4. Discussion

Ace2p and Sep1p are transcription factors of the fission yeast *Sch. pombe* that regulate the process of sister cell separation after the completion of cytokinesis (reviewed in Sipiczki, 2007). Both proteins seem to have multiple functions because they also regulate the activity of genes that are not involved or are not involved directly in the cell separation process (Rustici et al., 2004). This functional complexity makes it rather difficult to identify their functional equivalents in other organisms. The zinc-finger type transcription factor Ace2p is the activator of 24 genes in *Sch. pombe*, from which only a few (e.g. *agn1⁺* and *eng1⁺*) have been shown to have direct roles in cell separation (Alonso-Nunez et al., 2005; Rustici et al., 2004). Its orthologue in the budding yeast *S. cerevisiae* regulates the expression of at least 20 genes (Doolin et al., 2001). The groups of Ace2p-dependent genes in these organisms share similar (orthologous) members (e.g. genes encoding enzymes involved in cell separation) and also includes genes that are controlled by Ace2p only in one or the other species (e.g. the meiosis-specific gene *RME1* in *S. cerevisiae* and *cfh4⁺/chr1⁺* encoding a chitin synthase regulatory factor in *Sch. pombe*) (Alonso-Nuñez et al., 2005; Doolin et al., 2001).

Nevertheless, both proteins are required for proper cell separation after the completion of cytokinesis. The genes that code for the Ace2p proteins are controlled by sophisticated regulatory mechanisms that are very different in *Sch. pombe* and *S. cerevisiae*. The key regulator of the *Sch. pombe ace2⁺* gene is the fork-head protein Sep1p (Rustici et al., 2004). It has no obvious functional equivalent in *S. cerevisiae* because the *S. cerevisiae* protein most similar to it in amino acid sequence (Hcm1p) plays only an indirect role in *ACE2* regulation. Hcm1p does not interact with the *ACE2* promoter but regulates the timing of the activity of its activator gene *FKH2*. Since Fkh2p is also a fork-head-type transcription factor, it could be, in principle, considered as an alternative counterpart of Sep1p. However, it contains an FHA (fork-head associated) domain of specific functions missing in Sep1p and Hcm1p. Besides, the *S. cerevisiae* Fkh2p is more similar in sequence to *Sch. pombe* Fkh2p than to Sep1p (Szilagyi et al., 2005).

Additional difficulties in searching for functional equivalents of *ace2⁺* and *sep1⁺* are caused by the fact that different organisms have different numbers of transcription factors containing zinc-finger or fork-head domains. For example the number of the C2H2 zinc-finger proteins is 13 in *Sch. pombe* and 30 in *S. cerevisiae* according to the Pfam list in the Sanger Institute GenDB database (<http://old.genedb.org/>). The peculiarity of the *S. cerevisiae ACE2* is that it has a paralogue, the *SWI5* gene that arose from a genome duplication event during the evolution of the lineage leading to *Saccharomyces* (Wolfe and Shields, 1997), and the two paralogous transcription factors recognise similar promoter signals (Doolin et al., 2001) but only Ace2p regulates cell separation.

Assuming that it is the amino-acid sequence and protein structure rather than the sets of regulated genes that reflects the phylogenetic history of the transcription factors, we searched databases by the reciprocal best hits method for putative orthologues of *Sch. pombe* Ace2p and Sep1p in all *Schizosaccharomyces* species and in representatives of other groups of

Fungi. All proteins identified share high degree of sequence identity in and around the DNA-binding domains and much lower or negligible similarity in other parts of the proteins.

The phylogenetic analyses of the domains and their flanking sequences grouped the fission yeast proteins together and confirmed the divergent position of *Schizosaccharomyces* on the fungal tree of life proposed by molecular phylogenetic studies (reviewed in Sipiczki, 2000). There are two main subphyla within the phylum Ascomycota: the Pezizomycotina (which includes hyphal fungi) and the Saccharomycotina (which includes yeasts and is sometimes called the hemiascomycetes). The third subphylum Taphrinomycotina (Archiascomycotina), containing the fission yeast genus *Schizosaccharomyces*, is a small outgroup to both of these taxa (Hedges et al., 2004; James et al. 2006; Sipiczki, 2000). These three subphyla diverged about 798-1166 million years ago, shortly after their separation from Basidiomycota (Hedges et al., 2004). The Ace2p and Sep1p trees are fully consistent with this view because the *Schizosaccharomyces* proteins comprise a deep-rooting branch in both trees. It is notable that, although both trees reflected the phylogenetic relationships of all species analysed, the zinc-finger domains gave a tree with much better statistical support. Besides, their tree positioned the early diverging *Yarrowia* branch correctly, whereas the fork-head domains clustered it incorrectly with Pezizomycotina. Phylogenetic analyses based on sequences of conserved genes and proteins and genome comparison have unanimously demonstrated that *Yarrowia lipolytica* represents a phylogenetic lineage that branched off from Saccharomycotina at an early stage of evolution (e.g. Cornell et al., 2007; Yun and Nishida, 2011) exactly as shown in the Ace2p phylogeny.

It is hypothesized that all present-day ascomycetous taxa, including *Schizosaccharomyces*, evolved from filamentous ancestors, and the unicellular yeast growth forms evolved independently in various fungal lineages as an evolutionary response to specific environmental conditions (e.g. Berbee and Taylor, 1993). Gradual transition from

mycelial growth to yeast growth must have happened also in the fission-yeast lineage. According to a model proposed for fission yeast phylogenesis (Sipiczki, 1995), *Sch. japonicus* had branched off from the lineage already before the yeast morphology became dominating and has retained both forms of growth to the present. In the other branch the mycelial form gradually had declined by the time of the next ramification that separated *Sch. pombe* and *Sch. octosporus*, two true yeast species. Recent results refined this view by showing that certain *Sch. pombe* strains can be forced to form hypha-like structures under very specific culturing conditions (Amoah-Buahin et al., 2005; Prevorovsky et al., 2009) and by describing a new species very closely related to *Sch. octosporus* (Helston et al., 2010). The phylogenetic trees deduced from the conserved zinc-finger and fork-head domains of the Ace2p and Sep1p orthologues are perfectly consistent with these data. In both trees the dimorphic *Sch. japonicus* branches off first from the lineage, then *Sch. pombe* separates from the *Sch. octosporus* - *Sch. cryophilus* pair, for which no mycelial forms have been described.

The exact correspondence of the Ace2p and Sep1p trees with the phylogenetic positions of the species involved in the analyses indicates that these transcription factors evolved parallel with the evolution of the genomes of the organisms and were not specifically invented for cell separation or acquired as xenologues by horizontal gene transfer. The presence of the Ace2p orthologues in strictly mycelial species implies that these transcription factors had evolved to control other processes and became involved in cell separation only in the lineages that adopted yeast, di- or polymorphic modes of propagation. As the need to separate cells after septation appeared independently in numerous groups of fungi, the acquisition of cell separation function(s) must have occurred multiple times during the evolution of Fungi. Since the composition and structure of the septa varied in these lineages, the novel target genes also varied. For example, the dissolution of the primary septum requires the activation of the endo- β -1,3-glucanase gene *eng1*⁺ in *Sch. pombe* (Martin-

570 Cuadrado et al., 2003) and the chitinase genes *CTS1* and *CHT3* in budding yeasts (Doolin et
571 al., 2001; Kelly et al., 2004). In filamentous fungi, where septation is not followed by cell
572 separation, the orthologues of the *Schizosaccharomyces* Sep1p and Ace2p do not have to
573 regulate cell separation processes, but may have other regulatory functions which the
574 *Schizosaccharomyces* proteins also have. For example, the Sep1p orthologue of *Aspergillus*
575 *nidulans* (MCNB) is a positive regulator of the gene that encodes the mitosis-specific NIMA
576 protein kinase (Ukil et al., 2008). The *Sch. pombe* orthologue of the *nimA* gene, *fin1*⁺,
577 belongs to a cluster of Sep1p-controlled genes that are also activated in the M-phase of the
578 cell cycle (Buck et al., 2004). The putative *ace2*⁺ orthologue of *Aspergillus nidulans* is an
579 experimentally uncharacterised ORF. In the related pathogenic fungus, *Aspergillus fumigatus*,
580 the inactivation of *ace2* modifies conidiation and reduces virulence (Ejzykowicz et al., 2009),
581 but its involvement in cell-cycle regulation has not been investigated yet. Further
582 investigation is needed in more fungal species to identify the ancestral regulatory module of
583 these transcription factors and trace the changes of the module in diverging phylogenetic
584 lineages.

585 The regions of the Ace2p proteins that are N-terminal to the zinc-finger domains may
586 ensure specific target gene recognition and interactions with other effectors. Evidence for
587 different functional specificity of different segments of the N-terminal part has already been
588 found for the *S. cerevisiae* Ace2p protein, where a short region is required for the activation
589 of the chitinase gene *CTS1* (McBride et al., 1999). It is tempting to surmise that the conserved
590 segments shared by all *Schizosaccharomyces* Ace2p proteins and missing in the proteins of
591 the other species involved in this analysis probably have similar functions. Consistent with
592 their closer phylogenetic relationships, the *Sch. pombe*, *Sch. octosporus* and *Sch. cryophilus*
593 proteins share two more conserved regions that are not present in Ace2p^{Sj}. These are most
594 probably not essential for the regulation of the cell separation genes because their absence in

595 Ace2p^{Sj} does not prevent septum cleavage in the yeast phase of *Sch. japonicus* and does not
596 prevent the rescue of the *ace2Δ* separation defect in *Sch. pombe* cells. Thus, Ace2p and
597 Ace2p^{Sj} are functionally homologous in spite of the considerable difference in their amino
598 acid sequence. Consistent with this, the heterologous overexpression of *ace2^{Sj}* caused
599 rounding and lysis of the *Sch. pombe* cells, a phenotype also observed in cells overexpressing
600 the native *ace2⁺* gene (Petit et al., 2005) and indicating excessive production of enzymes
601 degrading cell wall components. There is, however, a remarkable difference between the
602 regulatory roles of Ace2p and Ace2p^{Sj}. The deletion of *ace2^{Sj}* did not reduce the mRNA level
603 of *cfh4^{Sj+}*, the putative orthologue of the Ace2p-dependent *Sch. pombe* gene that codes for a
604 component of the Cell Separation Ring formed in the cell wall of the dividing *Sch. pombe* cell
605 (Alonso-Nunez et al., 2005). The inactivation of the *Sch. japonicus sep1^{Sj}* gene conferred
606 essentially the same phenotype and the heterologous expression of the wild-type *sep1^{Sj}* gene
607 complemented the cell-separation defect of the *sep1⁻* cells of *Sch. pombe*. These results
608 demonstrate that *sep1^{Sj}* and *sep1⁺* play homologous, virtually identical roles in the regulation
609 of the separation of yeast cells as well.

610 The observed congruence between the functions of the Sep1p-Ace2p pairs in two
611 phylogenetically distantly related fission yeast species suggests that they may act alike in the
612 entire genus. As the *Sch. octosporus* and *Sch. cryophilus* orthologues are more similar in
613 sequence and structure to the *Sch. pombe* proteins, it is likely that the entire cell-separation
614 cascade (*sep1*→*ace2*→cell separation genes) is conserved in all fission yeasts.

615 The inactivation of the genes of either of these transcription factors confers a
616 filamentous morphology in both species. These cells chains are similar to the true hyphae and
617 thus their formation in the mutant cultures could be interpreted as a proof for the involvement
618 of the Ace2p and Sep1p orthologues in the regulation of dimorphism. This interpretation
619 would be consistent with the proposed role of Ace2p in the regulation of pseudohyphal

growth of the budding yeasts *Saccharomyces* and *Candida* (Kelly et al., 2004; King and Butler, 1998). However, the comparison of the structure, morphology and growth patterns revealed that the true-hypha-like cell chains of the *Sch. pombe* cell separation mutants are not equivalents of the true hyphae of *Sch. japonicus* (Sipiczki, 2007). Here we find that the cell chains of the *Sch. japonicus* mutants defective in *ace2^{Sj}* or *sep1^{Sj}* are also different from the true hyphae of the mycelial growth phase. While the hyphae grow unipolarly and extend fast due to extensive vacuolation at the non-growing poles (Sipiczki et al., 1998b), the cells of the mutant cell chains grow bipolarly, with much slower extension rate and do not form large polar vacuoles when cultured under conditions supporting yeast growth in the wild type. Thus, the mutant cell chains are non-separated yeast cells rather than true hyphae.

In the wild-type *Sch. japonicus*, the cell separation mechanism is inactivated at the yeast-to-hypha transition and reactivated at the hypha-to-yeast transition (Sipiczki et al., 1998b). In principle, this can be achieved by programmed switching off and switching on the Sep1p^{Sj}- Ace2p^{Sj} cascade or by phase-specific binding of negative regulators on the promoters of Ace2p^{Sj}-target genes. The complete shut down of *ace2^{Sj}* at the yeast-to-hypha transition is conceivable because the inactivation of *ace2^{Sj}* by gene disruption allows normal transition and normal hyphal growth without noticeable side effects. At the transition in the opposite direction, Ace2p^{Sj} is essential only for splitting the septa of the fragmenting hyphae and seems to be dispensable for the rest of the transition events such as the termination of high-speed cell extension, the switching to bipolar growth and the reduction of the intensity of vacuolation. Several possibilities are conceivable for inactivation and reactivation of the *ace2^{Sj}* function at growth-phase transitions. In *S. cerevisiae*, the Ace2p activity is regulated by phosphorylation in regions implicated in nuclear export and localisation of the protein (Jensen et al., 2000; O'Conallain et al., 1999). A similar mechanism is also conceivable for Ace2p^{Sj}, although no apparent counterparts of NES and NLS were identified in its sequence.

Alternatively, the suppression of septum cleavage might be achieved by factors that negatively regulate the Ace2p-controlled cell-separation genes. In *C. albicans*, Efg1 is a negative regulator of Ace2p target genes involved in cell separation and the phosphorylation of Efg1 by Cdc28-Hgc1 downregulates these genes during hyphal growth (Wang et al., 2009). Further research could elucidate the mechanism of activation and inactivation of the cell-separation machinery at growth-phase transitions of the dimorphic *Sch. japonicus*. However, when planning molecular biological experiments with hyphae, one has to bear in mind that the *Sch. japonicus* mycelium grows only in solid substrates and apart from its growing front edge, it mainly consists of dead cells and resting arthrospores (Sipiczki et al., 1998b).

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(Figure legends)

Fig. 1. Ace2p sequence analysis. The upper part of the figure shows the location of conserved domains and blocks in the *Schizosaccharomyces*, *Saccharomyces cerevisiae* and *Aspergillus nidulans* proteins. Sp, *Sch. pombe*; So, *Sch. octosporus*; Scr, *Sch. cryophilus*; Sj, *Sch. japonicus*; Sc, *S. cerevisiae*; An, *A. nidulans*. The length of each protein is given in brackets behind the species symbol. The numerals above the conserved regions show their location in the amino-acid sequences of the proteins. The lower part of the figure shows the Weblogo generated from the Clustal alignment of the conserved regions of all proteins (listed in Table 3) containing the zinc-finger motifs. The residues cysteine, histidine and phenylalanine are shown in black. The bottom box shows the location of the characteristic cysteine and histidine residues of the complete and incomplete C₂H₂ units and the phenylalanine residues that contribute to the specification of a precise DNA-binding surface. I, II and III are conserved regions in *Schizosaccharomyces* orthologues (see text for explanation. NES: nuclear entry signal. CTS binding: region required for binding to *CTS1* promoter. NLS: nuclear localisation signal.

Fig. 2. Sep1p sequence analysis. The upper part of the figure shows the location of conserved domains and blocks in the *Schizosaccharomyces*, *Saccharomyces cerevisiae* and *Aspergillus nidulans* proteins. Sp, *Sch. pombe*; So, *Sch. octosporus*; Scr, *Sch. cryophilus*; Sj, *Sch. japonicus*; Sc, *S. cerevisiae*; An, *A. nidulans*. The length of each protein is given in brackets behind the species symbol. The numerals above the conserved regions show their location in the amino-acid sequences of the proteins. The lower part of the figure shows the Weblogo generated from the Clustal alignment of the conserved regions of all proteins (listed in Table 3) containing the fork-head domains. The bottom box shows the location of the fully

conserved residues. The fork-head domain signatures 1 {[K R] - P - [P T Q] -
[F Y L V Q H] - S - [F Y] - x (2) - [L I V M] - x (3 , 4) - [A C] - [L I M] } and 2 { W -
[Q K R] - [N S D] - [S A] - [L I V] - R - H } are highlighted with black background.

Fig. 3. Phylogenetic trees generated from protein sequences. (A) Tree of Ace2p orthologues.
(B) Tree of Sep1p orthologues. Numbers given at nodes are the percentage of frequencies
with which a given branch appeared in 100 bootstrap replicates. Values lower than 50 are not
shown.

Fig. 4. Cell separation in the yeast phase of *Sch. japonicus*. (A-C) Wild-type cells of 7-1. (D-
H) Cells of 7-258 *ace2^{Sj}::ura4⁺*. (J-L) Cells of 7-254 *sep1^{Sj}::ura4⁺*. Phase contrast (A, D, E
and J), calcofluor-stained (B, F, G and K) and-DAPI-stained (C, H and L) images are shown.
Arrowheads mark cell ends covered with undissolved primary septa. + and - mark old-end
and new-end cell growth, respectively, in the branching cell chains.

Fig. 5. Proportion of cell types in wild-type and mutant cultures growing in liquid medium.
(A) Exponential phase cultures. (B) Stationary-phase cultures. (C) Examples of microscopic
morphology of types. 1, single cells; 2, clumps of cells; 3, cell pairs (separated by single
septa); 4, chains of three cells (separated by two septa); 5, chains of multiple cells; 6, clumps
of cell chains. The data are average values from three experiments.

Fig. 6. Cell separation during mycelium-to-yeast transition in *Sch. japonicus*. (A)
Morphology of the invasive hyphae of the wild type 7-1. (B) Septation and fragmentation of
wild-type hyphae. (C and D) Septation and fragmentation of the 7-258 *ace2^{Sj}::ura4⁺* invasive

hyphae. (E) Septation and fragmentation of the 7-254 *sep1^{Sj}::ura4⁺* invasive hyphae. The separating hyphal fragments are clearly visible in the phase-contrast image (B), but the integrity of the uncleaved septa is better demonstrated when phase-contrasting is not used (C, D and E). The lower resolution of phase-contrast images is caused by the four glass and medium layers, through which the light has to pass (see Materials and methods). The quality of the phase-contrast images is further reduced by hyphae that grow above or below the hypha which is in focus (see the background in B). + marks cell growth in direction towards the apex of the hypha. – marks growth in the opposite direction. Only examples of both types of growth are marked. v, vacuole.

Fig. 7. Transcription of orthologues of Sep1p/Ace2p-dependent cell separation genes in *Sch japonicus* wild-type cells (7-1), *ace2^{Sj}::ura4⁺* (7-258) and *sep1^{Sj}::ura4⁺* (7-254) mutant cells.

Fig. 8. Effects of the heterologous expression of *Sch. japonicus* genes in *Sch. pombe* mutants. (A) Cells of the *Sch. pombe* wild-type strain 0-1. (B) Cell chains of the *Sch. pombe ace2Δ* strain 2-1043. (C) Cell chains of the *Sch. pombe sep1Δ* strain 2-843. (D) Partial restoration of cell separation in the *Sch. pombe ace2Δ* strain 2-1043 transformed with pREP82-*ace2^{Sj}*. (E) Cell separation in the *Sch. pombe ace2Δ* strain 2-1043 transformed with pREP82-*ace2^{Sj}* after depletion of thiamine. (F) Cell separation in the *Sch. pombe sep1Δ* strain 2-843 transformed with pREP82-*sep1^{Sj}* after depletion of thiamine. (G) Swelling of *Sch. pombe ace2Δ* cells transformed with pREP2-*ace2^{Sj}* after depletion of thiamine. (H) Lysis of *Sch. pombe ace2Δ* cells transformed with pREP2-*ace2^{Sj}* after a prolonged period of incubation in thiamine-free medium.

916 **Fig. 9.** Transcription of Ace2p-dependent cell-separation genes in *Sch. pombe* wild type (7-1),
917 *ace2Δ* mutant (2-1043) and *ace2Δ* mutant transformed with pREP82-*ace2^{Sj}*. The
918 transformant was cultivated in thiamine-free medium for 16 h.
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Table 1
Strains and plasmids used in this study

Strain/plasmid	Description	Source
<i>Sch. japonicus</i> strains		
7-1	Wild type: type strain CBS 354, CCY-44-5-1	CCY ^a
7-252	<i>mat</i> ^{Sj} - P2028 <i>ura</i> ^{Sj} -D3	Furuya and Niki, 2009
7-254	<i>sep1</i> ^{Sj} :: <i>ura4</i> ^{Sp} <i>ura4</i> ^{Sj} -D3	This study
7-258	<i>ace2</i> ^{Sj} :: <i>ura4</i> ^{Sp} <i>ura4</i> ^{Sj} -D3	This study
<i>Sch. pombe</i> strains		
0-1	Wild-type strain L972 h ⁻	U. Leupold
2-843	<i>sep1</i> :: <i>ura4</i> ⁺ <i>ura4</i> -D18 h ⁺	Zilahi et al., 2000
2-1043	<i>ace2</i> ::kanMX4 <i>ura4</i> -D18 h ⁻	Alonz-Nunez et al., 2005
2-1243	<i>leu1</i> -32 h ⁻	U. Leupold, Bern collection
2-1407	<i>sep1</i> :: <i>ura4</i> ⁺ <i>ura4</i> -D18 <i>leu1</i> -32 h ⁻	Recombinant from 2-843 x 2-1243
Plasmids		
pJET1.2	Cloning vector	Fermentas
pJET 1.2. <i>ace2</i> ^{Sj}	Contains the cloned <i>ace2</i> ^{Sj} gene	This study
pJET 1.2. <i>sep1</i> ^{Sj}	Contains the cloned <i>sep1</i> ^{Sj} gene	This study
pUC18	Cloning vector	GenScript
pUC18- <i>ace2</i> ^{Sj}	Contains the cloned <i>ace2</i> ^{Sj} gene	This study
pUC18- <i>sep1</i> ^{Sj}	Contains the cloned <i>sep1</i> ^{Sj} gene	This study
KS-ura4	Contains the <i>ura4</i> ⁺ cassette for gene disruption	Bähler et al., 1998
pREP1	Expression vector with high-strength <i>nmt1</i> promoter and <i>LEU2</i> marker gene	Maundrell, 1993
pREP2	Expression vector with high-strength <i>nmt1</i> promoter and <i>ura4</i> ⁺ marker gene	Maundrell, 1993
pREP41	Expression vector with medium-strength <i>nmt1</i> promoter and <i>LEU2</i> marker gene	Basi et al. 1993
pREP42	Expression vector with medium-strength <i>nmt1</i> promoter and <i>ura4</i> ⁺ marker gene	Basi et al. 1993
pREP81	Expression vector with low-strength <i>nmt1</i> promoter and <i>LEU2</i> marker gene	Basi et al. 1993
pREP82	Expression vector with low-strength <i>nmt1</i> promoter and <i>ura4</i> ⁺ marker gene	Basi et al. 1993
pREP2- <i>ace2</i> ^{Sj}	High-strength expression of <i>ace2</i> ^{Sj}	This study
pREP42- <i>ace2</i> ^{Sj}	Medium-strength expression of <i>ace2</i> ^{Sj}	This study
pREP82- <i>ace2</i> ^{Sj}	Low-strength expression of <i>ace2</i> ^{Sj}	This study
pREP1- <i>sep1</i> ^{Sj}	High-strength expression of <i>sep1</i> ^{Sj}	This study
pREP41- <i>sep1</i> ^{Sj}	Medium-strength expression of <i>sep1</i> ^{Sj}	This study
pREP81- <i>sep1</i> ^{Sj}	Low-strength expression of <i>sep1</i> ^{Sj}	This study

^aCCY: Culture Collection of Yeasts, Bratislava, Institute of Chemistry, Slovak Academy of Sciences

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Table 2
List of primers

Primer	Sequence (5'-3') ^a	Remarks
For gene cloning		
Sjpace2F	CCC <u>CATATG</u> ATGTCTTTTCG	For amplification of <i>ace2^{Sj}</i>
Sjpace2R	CCCGTCGACTTAAGCAGCAAG	For amplification of <i>ace2^{Sj}</i>
Sjpsep1F	CCC <u>CATATG</u> ATGAGCAAAAAA	For amplification of <i>sep1^{Sj}</i>
Sjpsep1R	GGGTCGACTTAGGGAAGGTGGA	For amplification of <i>sep1^{Sj}</i>
For gene disruption		
Sjace2disfor	CCC <u>AAGCTT</u> TGGATCCTCGAGCCC	For disruption of <i>ace2^{Sj}</i>
Sjace2disrev	GGG <u>AAGCTT</u> ATTATCCAATCTCAT	For disruption of <i>ace2^{Sj}</i>
Sjace2revT	TAA CCG AAA TTA CCG AAA CC	For checking the disruption of chromosomal <i>ace2^{Sj}</i>
Sjsep1revT	ACTGTTACACATCCAATCCT	For checking the disruption of chromosomal <i>sep1^{Sj}</i>
KS-ura4F	GCCAATGAAAGATGTATGTAG	For checking the disruption of chromosomal <i>ace2^{Sj}</i> and <i>sep1^{Sj}</i>
KS-ura4R	CACAAGATAGAATGGATGTT	For checking the disruption of chromosomal <i>ace2^{Sj}</i> and <i>sep1^{Sj}</i>
For RT-PCR		
Sjace2Frt	AACCGGCGTACTTCCTTCTT	For <i>ace2^{Sj}</i>
SjaceRrt	AAACTGGCCATTGTTTCAGG	For <i>ace2^{Sj}</i>
Sjsep1Frt	CTCCGAAATCCATGTGTCTT	For <i>sep1^{Sj}</i>
Sjsep1Rrt	TCATCGGGGAAAAGAATGAG	For <i>sep1^{Sj}</i>
Sjadg1RTF	ATC CCC TTC TGA CTC GAC CT	For <i>adg1^{Sj}</i>
Sjadg1RTR	GCG AGA AGG AGG TAG CAA TG	For <i>adg1^{Sj}</i>
Sjagn1Frt	TGCTCCTGTCTCTCCTTGTT	For <i>agn1^{Sj}</i>
Sjagn1Rrt	ATGTCAGGCTGCATGTCAAG	For <i>agn1^{Sj}</i>
Sjcfh4RTF	GCT AAA CAG CAC CGT CAT CA	For <i>cfh4^{Sj}</i>
Sjcfh4RTR	CCG CAT TGG GAA CAG ATA GT	For <i>cfh4^{Sj}</i>
Sjeng1RTF	CCT TCA CCG GCT TTA TTC AA	For <i>eng1^{Sj}</i>
Sjeng1RTR	TGA GTC TTC CGT GTG ACC AG	For <i>eng1^{Sj}</i>
Sjmid2RTF	AAG GCT TGG TGG ATG ATG AC	For <i>mid2^{Sj}</i>
Sjmid2RTR	CTT CTG GAC GAG CAA CAT CA	For <i>mid2^{Sj}</i>
Sjsce3RTF	GTCCGAGGGTGAGATTACCA	For <i>sce3^{Sj}</i>
SjsceRTR	GAAGTCAACGTAGGCGAAGC	For <i>sce3^{Sj}</i>
Space2RTleft	TTA CAA TGA CGC ATT TTC CTT G	For <i>ace2⁺</i>
Space2RTright	TGT TGT TGA GAG TCC AGC TCA T	For <i>ace2⁺</i>
Spadg1_273-293F	5'- CCC AAC TAG CGC TAC TAC GAC -3'	For <i>adg1⁺</i>
Spadg1_356-375R	5'- AGT GTC CAC TGG CGG ATT AC -3'	For <i>adg1⁺</i>

Spagn1RTF1.0	CCAGCGTTTCCTCAGACTTC	For <i>agn1</i> ⁺
Spagn1RTR1.0	GCCCGTGCTAACATCAGAAT	For <i>agn1</i> ⁺
Spcf _h _857-875F	CGG AGG TAG CAT CTC AGC A	For <i>cfh4</i> ⁺
Spcf _h _R945-965R	GCC AAC ATG TAC AAG GGA AGA	For <i>cfh4</i> ⁺
Spsce3RTa	GGCGCTCAGAGGATTCAC	For <i>sce3</i> ⁺
Spsce3RTs	GTGACTGGGTTCGTCGTG	For <i>sce3</i> ⁺

^aRecognition sites for restriction endonucleases are underlined

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Table 3Identification numbers of orthologues of *Sch. pombe* Ace2p and Sep1p

Species	Orthologue of	
	Ace2p	Sep1p
<i>Sch. octosporus</i>	SOCG_05516.5	SOCG_05578.5
<i>Sch. cryophilus</i>	SPOG_04824.3	SPOG_02771.3
<i>Sch. japonicus</i>	SJAG_02252.4	SJAG_03143.4
<i>Ajellomyces dermatitidis</i> (<i>Blastomyces dermatitidis</i>)	EEQ76326.1	EEQ90210.1
<i>Arthroderma benhamiae</i>	EFE35352	EFR01121
<i>Aspergillus nidulans</i> (<i>Emericella nidulans</i>)	AN4873 (EAA60951)	AN8858 (EAA60146)
<i>Botrytis cinerea</i> (<i>Botryotinia fuckeliana</i>)	BC1G_10340.1	BC1G_06590.1
<i>Candida albicans</i>	orf19.6124	orf19.4853
<i>Candida glabrata</i>	CAGL0E01331g	CAGL0J03608g
<i>Coccidioides immitis</i>	CIRG_07744.1	CIRG_03169.1
<i>Debaromyces hansenii</i>	DEHA2E03850g	DEHA2F11308g
<i>Eremothecium gossypii</i> (<i>Ashbya gossypii</i>)	ERGO0G03916g	ERGO0A05654g
<i>Histoplasma capsulatum</i>	HCEG_02984.2	HCBG_02171.2
<i>Kluyveromyces lactis</i>	KLLA0D16456g	KLLA0C08778g
<i>Magnaporthe grisea</i> (<i>Magnaporthe oryzae</i>)	MGG_04328.6	MGG_06422.6
<i>Neosartorya fischeri</i> (<i>Aspergillus fischerianus</i>)	XP_001263337.1	XP_001266068.1
<i>Neurospora crassa</i>	NCU06907.5	NCU06173.4
<i>Penicillium chrysogenum</i>	XP_002559373.1 (CAP92019)	XP_002562627.1 (CAP85394)
<i>Rhizopus oryzae</i>	RO3G_14665.3	RO3G_09342.3
<i>Saccharomyces cerevisiae</i>	YLR131C	YCR065W
<i>Sclerotinia sclerotiorum</i>	SS1G_11942.1	SS1G_00580.1
<i>Sordaria macrospora</i>	CBI57998.1	CBI57326.1
<i>Talaromyces stipitatus</i>	XP_002480363.1	XP_002482427.1
<i>Yarrowia lipolytica</i>	YALI0E16973g	YALI0C01463g
<i>Verticillium albo-atrum</i>	XP_003006956.1 (CAP85394)	XP_003003314.1 (EEY20766)

(Fig. 1)

(Fig. 2)

(Fig. 3)

(Fig. 4)

A

(Fig. 4)

B

(Fig. 5)

(Fig. 6)

(Fig. 7)

(Fig. 8)

(Fig. 9)