Deletion of *dhc1* to investigate mating-dependent nuclear migration in the basidiomycete *Schizophyllum commune*

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### Abbreviations

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<th>Description</th>
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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AAA</td>
<td>ATPase associated diverse cellular activities</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulfate</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>CTAB</td>
<td>cetyl trimethylammonium bromide</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>desoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>desoxynucleoside triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>HCCA</td>
<td>alpha-cyano-4-hydroxy-cinnamic acid</td>
</tr>
<tr>
<td>IPG</td>
<td>immobilized pH gradient</td>
</tr>
<tr>
<td>Kb</td>
<td>kilo base pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KOG</td>
<td>EuKaryotic Orthologous Groups</td>
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<tr>
<td>MAPK</td>
<td>Mitogen-Activated protein kinase</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule/s</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organized centers</td>
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<tr>
<td>Ncbi</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>NLS</td>
<td>Nuclear localization sequence</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>PEG</td>
<td>polyethylene gluol</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescent protein</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RPKM</td>
<td>reads per kilobase of exon model per million mapped reads</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecy sulfate</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>tetramethylethylenediamine</td>
</tr>
<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<tr>
<td>V</td>
<td>volumes</td>
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<tr>
<td>Vhr</td>
<td>volt hours</td>
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<tr>
<td>vs.</td>
<td>versus</td>
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<tr>
<td>wt</td>
<td>wild type</td>
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1.) Introduction

1.1. The fungal kingdom
Fungi are described as eukaryotic organisms with filamentous growth forms, as hyphae or mycelium as well as yeast forms. Fungi are ubiquitous saprobes or degraders of dead organic material, symbiotic partners for other organisms and pathogens in all kingdoms (Redecker, 2002).

![Fungal tree of life](image)

**Figure 1: Fungal tree of life (Stajich et al., 2009)**

True fungi are summarized in three groups (Fig 1). The evolutionary oldest group contains the simple, very basal fungi, like Chytridiomycota. Also Mucoromycotina (former Zygomycetes) belong to this domain. The higher developed Ascomycetes form the second large domain. Basidiomycota, containing mushrooms, jelly fungi and basidiomycetous yeasts form the third group which encompasses Pucciniomycotina, Ustilaginomycotina and Agaricomycotina. Molecular biology studies have shown that the Agaricomycotina are divided into Tremellomycetes, Dacrymycetes and Agaricomycetes (Hibbett, 2006; Stajich et al., 2009).
1.2. *Schizophyllum commune* as a model organism

The split gill fungus *Schizophyllum commune*, a mushroom forming agaricomycete, is a cosmopolitan wood decaying fungus causing white rot on hard wood. It is characterized by conspicuous shell-shaped, 2-3 cm large fruiting bodies (Fig. 2). The fungus is used as a model organism in basic molecular biology research to understand the regulation of sexual development, hyphal growth or the lignolytical capability. It completes its entire life cycle within approximately 14 days under laboratory conditions and a well established transformation and gene deletion protocol can be applied for genetical manipulation. Additionally, the published genome of *S. commune* allows investigation of the transcriptome or proteome at molecular levels (Cooke, 1961; Kothe, 1996; Palmer and Horton, 2006; Ohm et al., 2010).

![Figure 2: Fruiting bodies of *S. commune*: A) on well defined media under laboratory conditions 10 days past inoculation; B/C) under natural conditions on beech (biosphere reserve Berchtesgadener Land, Königssee, peninsula St. Bartholomä, Germany)](image)

The German botanist Hans Kniep characterized the mating system of basidiomycetes, especially of *S. commune*, already in the early 1920s. He detected two independent mating type loci, called *A* and *B*. In the 1950s, Papazian completed the knowledge of the mating type genes and found out that there are two linked multiallelic subloci, α and β, in every mating type locus. For *Aα*, *Bα* and *Bβ*, each with 9 allelic specificities, and 32 allelic specificities in *Aβ*, a total of more than 23,000 mating types can be found in nature. To form a fertile mating,
both partners have to be mating type compatible. In detail, that means that both need a different genetic background, or specificity, in their mating type genes. Only if both partners differ in either or both \( \alpha \) and \( \beta \) specificities in both \( A \) and \( B \) genes, a compatible mating reaction can take place. After the fusion of the hyphae, the nuclei migrate to build a pair – one nucleus from each mating partner – per tip cell. Then, both nuclei divide synchronously. The tip cell forms a so called hook cell, where one nucleus of the first pair migrates to the hook cell. Then, septa are laid down at the places of both mitotic spindles separating the daughter nuclei. Thus, one nucleus is trapped in the hook cell until the hook fuses with the subterminal cell and the trapped nucleus migrates to join its partner in the subterminal cell. This assures that each cell of the growing mycelium has two different nuclei, one of each mating partner. The fused hook cell – then called clamp cell – is the typical structure in dikaryons of basidiomycetes, although not all basidiomycetes show such nice and constant clamp formation as is observed with \textit{S. commune} (Fig. 3). Under promoting environmental conditions, the mycelium of the dikaryon starts to aggregate and form primordia. These structures are pre-stages of fruiting bodies. The fertile fruiting bodies release haploid basidiospores which are able to germinate again to from haploid, monokaryotic strains (Fig 4.; Niederpruem and Wessels, 1969; Raudaskoski and Koltin, 1973; Palmer and Horton, 2006; Schubert \textit{et al.}, 2006).

\textbf{Figure 3: Sexual development in \textit{S. commune}.} A) Process of dikaryon formation B) SEM-image of a clamp cell of \textit{S. commune}.\hfill
If both mating partners share a common $A$ factor, nuclear migration can take place. The nuclei do not undergo the pairing and the conjugated nuclear division, and also the hook cell cannot be formed. The phenotype of this mating reaction is called “flat”. This phenotype is characterized by less aerial mycelium and hyperbranched hyphae with highly variable numbers of nuclei in every cell, as well as a decrease of septation. A second semi-compatible reaction occurs when both mating partner share a common $B$ factor. The nuclei do not migrate after cell fusion; instead they form pairs and one nucleus migrates to the formed hook cell. The hook cell is not able to fuse with the cell behind the tip and the nucleus stays trapped in the hook. This cell is called “pseudoclamp”. The phenotype of this reaction is called “Barrage”, because it is characterized by a separation zone of mycelium that is formed between the two mating partners. If both mating loci are of the same specificity, mating fails to appear (Fig. 5; Raper and Miles, 1958; Koltin et al., 1979; Kothe, 1999; Raudaskoski and Kothe, 2010). Because of these four possible mating interactions, the mating system has been termed tetrapolar (Kniep, 1920, 1922).
1.3. The cytoskeleton of filamentous fungi

In filamentous fungi, the cytoskeleton is responsible for polarized growth and hyphae formation. Two main structures play an essential role – microtubules and actin. Microtubules appear as tracks in hyphae; they are organized from MTOCs (microtubule organizing centers) which contain γ-tubulin at the minus-end of microtubules. Growth of microtubules starts at MTOCs by extension of α/β-tubulin dimers starting from γ-tubulin. Depolymerization of tubulin dimers at the plus-end leads to shortening of microtubules (Xiang and Fischer, 2004; Steinberg, 2007a).

Microtubules (MTs) are necessary for formation and stabilization of the mitotic spindle during the cell cycle in filamentous fungi. Additionally, MTs are involved in vesicle transport from the central parts of the cell to the tip. Vesicles containing cell wall material, which is needed at the hyphal tip to synthesize the growing cell wall, are transported this way. The Spitzenkörper or vesicle supply center is located at the hyphal tip, where the vesicles are accumulated (Girbhardt, 1957; Wessels, 1986; Bartnicki-Garcia et al., 2000; Steinberg, 2007b; Fischer et al., 2008).

Actin is a globular protein (G-actin), which can polymerize to filaments (F-actin). Actin is responsible for flexibility of hyphae, morphological changes, cell division and intracellular trafficking (Hennessey et al., 1993). It exists in different forms in hyphae – as patches located at the growing tip, at cables at the cell periphery and as ring-like structures at septa.

Figure 5: A) Cycle of sexual development influenced by mating-type loci $A$ and $B$; B) tetrapolar mating-reactions in $S.\ commune$ (pictures modified after E.-M. Jung)
Therefore, it is assumed that actin is required for cell wall assembly at the growing tip (Xiang and Plamann, 2003).

Actin and microtubules are tracks, on which motor proteins can move cargo to different locations in the cell. Three types of molecular motors are known: kinesins and dynein, which ride along microtubules, and myosin, which transports cargo along actin. They translate chemical to mechanical energy. In this pathway, ATP is hydrolyzed at the globular head domain of motor proteins. This results in a confirmation change of the molecule and makes movement along microtubules or actin possible (Schliwa and Woehlke, 2003).

Myosin consists of one to two heavy chains each with one or several light chains (Korn, 2000). The heavy chains form the head domain that interacts with actin, the neck region links the heavy chain to light chains. The tail region is formed by light chains that carry the particular cargo (Foth et al., 2006). Myosins are necessary for contractions at the cell surface, for vesicle transport and intracellular streaming (Hoyt et al., 1997).

Fungal cells contain 10 to 11 different kinesins. They are plus-end directed, microtubule-dependent motor proteins formed by two identical heavy chains and two identical light chains (Lakämper et al., 2003). Kinesins are involved in all plus-end directed transport at microtubules, including spindle formation and spindle function. They are essential for long distance transport of cell organelles and vesicles (Xiang, 2003; Fig 6).

**Figure 6: Organization of the cytoskeleton in tip cells of filamentous fungi.** Dynein and Kinesin move their cargo along microtubules in opposite directions, myosin is connected to actin filaments.
1.4. The motor protein dynein

1.4.1. Molecular composition and functions of dynein molecules

Dynein is required for vesicle transport, perinuclear localization of the Golgi apparatus, nuclear and spindle positioning, as well as spindle assembly during mitosis and meiosis. In fungi, dynein is also required for nuclear migration (Karki and Holzbaur, 1999; Yamamoto and Hiraoka, 2003). Dynein accumulates at the hyphal tip, where – in addition to MTOCs at the nuclear envelope - the minus-end of MTs is located. This ensures that vesicles reach the hyphal tip and polarized growth of hyphae can take place (Schuster et al., 2011).

Dynein is a macromolecular complex composed of different protein subunits that interact with microtubules. This complex consists of two heavy chains (~500 kDa), several intermediate (~70 kDa) and light intermediate chains (53 – 59 kDa) and light chains (8 – 23 kDa). The dynein heavy chain forms the globular head of the molecule which associates with microtubules. A neck region links the heavy chain to light and intermediate chains which bind the cargo for fast track transportation from the plus- to the minus-end of microtubules (Fig. 7; Holzbaur and Vallee, 1994; Plamann et al., 1994; Hirakawa et al., 2000; Pfister et al., 2006).

Figure 7: Structure of the dynein complex. Shown are the different subunit chains of the dynein complex. The heavy chain consists of 3 domains which are described in the text.
To exploit their entire functionality, dynein molecules need several interacting proteins. Dynactin is a protein complex consisting of two important structures: an actin-like filament composed of the actin-related protein Arp1, and a side chain. This structure is formed by the dimer p150\textsuperscript{Glued} which contains binding sites for microtubules, dynein intermediate chains and Arp1. Dynactin acts as a regulator for dynein motility and connects dynein molecules to other cell components (Yamamoto and Hiraoka, 2003; King et al., 2003).

Further proteins interacting with dynein are Lis1 and Clip-170. Lis1 was first identified as the protein causing lissencephaly in humans (Markus et al., 2011). The C-terminus of Lis1 binds at dynein molecules and is therefore required for nuclear and spindle positioning in fungi (Xiang, 2003; Torisawa et al., 2011). Clip-170, in contrast, is necessary for accumulation of dynactin at the plus-end of microtubules and for nuclear migration in fungi (Yamamoto and Hiraoka 2003).

1.4.2. The dynein heavy chain

The dynein heavy chain is a 4600 aa large protein subunit of the dynein complex. It consists of a 1300 aa large N-terminal region which contains the dimerization domain of the protein, needed to dimerize with the second heavy chain of the molecule. The 3300 aa large C-terminal region contains the motor machinery, where chemical energy is converted into mechanical energy. The motor domain is formed by six AAA-modules (ATPase associated diverse cellular activities). AAA1 to AAA4 each contain a Walker A (P-loop) and a Walker B motif. Walker A motifs are known to function as nucleotide binding site. AAA1 and AAA3 have a strong binding activity, whereas AAA2 and AAA4 have a decreased activity which leads to the assumption of a regulatory role of these modules. AAA5 and AAA6 have lost nucleotide binding sites. Between AAA4 and AAA5, the microtubule binding site (or B-link, a 10-12 nm long stalk) is located (Carter et al. 2011).

ATP can bind directly to the first AAA modules. This results in a re-orientation of the dynein heavy chain. In this process, the modules move closer to each other and the microtubule binding site moves towards the microtubule. Hydrolysis of ATP at AAA1 leads to movement of the neck domain, which is linked to AAA1. The microtubule binding site is lifted upwards and connects to the microtubule. The hydrolyzed products at AAA1 to AAA4 are discharged from the AAA modules and the neck domain swings to the left, while the dynein molecule moves to the right (Fig. 8; Asai and Koonce, 2001).
In contrast to other eukaryotes, the dynein heavy chain of the rust fungus *Ustilago maydis* (Ustilaginomycotina) is encoded by two separate proteins. Both proteins cover the entire length of approximately 4600 aa together. The dynein heavy chain is spilt in *U. maydis* between the fourth AAA module and the microtubule binding domain. The deletion of either *dyn1* or *dyn2* in *U. maydis* is lethal, which proofs the essential functionality of the dynein heavy chain in eukaryotes (Straube *et al.*, 2001).

In *S. commune*, also two separate genes encoding the dynein heavy chain, were found (D. Schubert, personal communication).

*Figure 8: Functionality of dynein.* Detailed description is to be found in the text. (brown = microtubule, light green ring = AAA modules, bright green = microtubule binding site, dark green = neck, violet = bound nucleotides, purple = hydrolysed nucleotides). (modified after Asai and Koonce, 2001)
1.5. Aim of this study

The dynein heavy chain of *S. commune* is encoded by two genes – *dhc1* and *dhc2*. A detailed phylogenetic investigation of the dynein heavy chain has to be performed to provide information on the split event during basidiomycete evolution and the organization of the dynein heavy chain encoding gene(s) in other fungi.

Via immunofluorescence microscopy, the localization of both gene products in the cell has to be demonstrated. Also cellular localization of other cytoskeleton components should be investigated through antibody staining methods to complete the knowledge about their organization in the cell. It is expected, that a co-localization of both gene products can be detected. As dynein is involved in the cell cycle of *S. commune*, *dhc1* and *dhc2* could form a complex to fulfill its function in mitosis.

The deletion of *dhc2* is not lethal in *S. commune*. As this gene contains the important motor machinery, which is essential for the functionality of the dynein complex, other genes or complexes must provide the transport of cargo to the minus-end of microtubules. Transcriptome analysis of the ∆dhc2 strain and its parental strains should be performed to identify cytoskeletal components or motor proteins which can fulfill the function of dynein. With the current RNA sequencing method, differences in the transcriptome of all strains can be demonstrated.

To complete the knowledge of the function of the dynein heavy chain genes in *S. commune*, a knock-out of *dhc1* needs to be accomplished. As the knock-out of *dhc2* is viable, it is assumed that also ∆dhc1 strains are able to survive. To compare these strains to the already existing ∆dhc2 strains, detailed microscopic investigations according to cell morphology, nuclear migration and mating behavior should be performed.

To simplify proteomic studies, a protein map of cytosolic proteins of *S. commune* should be developed. Via 2-dimensional gelelectrophoresis the reference gel of a monokaryotic strain of *S. commune* shall be produced. Exclusive protein spots on the reference gel should be identified with mass spectrometry. Possible posttranslational modifications could be shown. A second monokaryotic strain shall be adducted to demonstrate strain specific differences in *S. commune*. Additionally, a dikaryotic proteome shall be created via 2-dimensional gelelectrophoresis to identify dikaryon specific proteins.
2.) Material and methods

2.1. Chemicals

Chemicals, used in this study, were purchased from Merck KGaA (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), Sigma-Aldrich Laborchemikalien GmbH (Seelze, Germany) and Serva Electrophoresis GmbH (Heidelberg, Germany). Special chemicals are mentioned separately. Endonucleases were purchased from New England Biolabs GmbH (Frankfurt am Main, Germany) and Jena Bioscience GmbH (Jena, Germany).

2.2. Organisms, oligonucleotides, plasmids and antibodies

Organisms, oligonucleotides and plasmids used in this study are listed in table 1, 2 and 3.

Table 1: Organisms used in this study. * Strains were obtained from the JMRC (Jena Microbial Resource Collection)

<table>
<thead>
<tr>
<th>Strain description</th>
<th>Species</th>
<th>genotyp</th>
<th>source</th>
</tr>
</thead>
<tbody>
<tr>
<td>12-43</td>
<td>S. commune</td>
<td>$A_{3,5}B_{2,2}$</td>
<td>*</td>
</tr>
<tr>
<td>Δku80</td>
<td>S. commune</td>
<td>$A_{4,3}B_{4,1}$</td>
<td>L. Lugones, Utrecht, Netherlands; modified by S. Madhavan, Microbial Communication, FSU Jena, Germany</td>
</tr>
<tr>
<td>E6</td>
<td>S. commune</td>
<td>$A_{4,6}B_{2,1}$</td>
<td>*</td>
</tr>
<tr>
<td>4-39</td>
<td>S. commune</td>
<td>$A_{1,1}B_{3,2}$</td>
<td>*</td>
</tr>
<tr>
<td>W22</td>
<td>S. commune</td>
<td>$A_{4,6}B_{3,2}$</td>
<td>*</td>
</tr>
<tr>
<td>T41</td>
<td>S. commune</td>
<td>$A_{4,7}B_{8,4}$</td>
<td>*</td>
</tr>
<tr>
<td>Δdhc1</td>
<td>S. commune</td>
<td>$A_{4,3}B_{4,1}$</td>
<td>this study</td>
</tr>
<tr>
<td>Δdhc2_5</td>
<td>S. commune</td>
<td>$A_{3,5}B_{2,2}$</td>
<td>Strain collection FSU Jena, Microbial Communication, Germany</td>
</tr>
<tr>
<td>K12 DH5α</td>
<td>Escherichia coli</td>
<td>/</td>
<td>Gibco Life Technologies, Karlsruhe, Germany</td>
</tr>
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Table 2: Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>5’</th>
<th>3’</th>
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</thead>
<tbody>
<tr>
<td>dhc1AaXbal</td>
<td>AGT CTC TAG AGC CAC ACC GAA TCG CCA GCA GAT C</td>
<td></td>
</tr>
<tr>
<td>dhc1AbXbal</td>
<td>CCA TTC TAG AGG GGG AAG CAG CGC GAG CAG GTA</td>
<td></td>
</tr>
<tr>
<td>dhc1Ba</td>
<td>ACG CGC ATA GGG GCA CCG ACA A</td>
<td></td>
</tr>
<tr>
<td>dhc1Bb</td>
<td>GCA AGC GCC ATC CCC AGC AGT</td>
<td></td>
</tr>
<tr>
<td>Dhc1for</td>
<td>TCG AGC TAT CGC TGC TGC AT</td>
<td></td>
</tr>
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</table>
Table 3: Plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Characterization</th>
<th>Source</th>
</tr>
</thead>
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<tr>
<td>pDrive</td>
<td>TA cloning vector, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Qiagen GmbH, Hilden, Germany</td>
</tr>
<tr>
<td>pChi</td>
<td>pBlueskript, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>K. Lengeler, former Microbial Communication, FSU Jena (now Heinrich-Heine-Universität, Düsseldorf, Germany)</td>
</tr>
<tr>
<td>pBluescript II SK</td>
<td>cloning vector</td>
<td>Stratagene GmbH, Heidelberg, Germany</td>
</tr>
<tr>
<td>pΔdhc1</td>
<td>pBlueskript, Amp&lt;sup&gt;R&lt;/sup&gt; deletion cassette for dhc1</td>
<td>this study</td>
</tr>
</tbody>
</table>

First antibodies ScDhc1 and ScDhc2 were produced by BioGenes GmbH (Berlin, Germany). All second antibodies were purchased from Sigma-Aldrich Chemie GmbH, Schnelldorf, Germany. Antibodies are listed in table 4.

Table 4: Antibodies used in this study

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Label</th>
<th>Sequence</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>ScDHC1</td>
<td>Anti-Dhc1 (from rabbit)</td>
<td>TRITC</td>
<td>C-ALPPLRDITNKRRD</td>
<td>1:50</td>
</tr>
<tr>
<td>ScDHC2</td>
<td>Anti-Dhc2 (from rat)</td>
<td>TRITC</td>
<td>C-GILKRERMQQARED</td>
<td>1:50</td>
</tr>
<tr>
<td>T6778</td>
<td>Anti-rabbit (IgG)</td>
<td>TRITC</td>
<td></td>
<td>1:100</td>
</tr>
<tr>
<td>T4280</td>
<td>Anti-rat (IgG)</td>
<td>TRITC</td>
<td></td>
<td>1:100</td>
</tr>
<tr>
<td>F6258</td>
<td>Anti-rat (IgG)</td>
<td>FITC</td>
<td></td>
<td>1:100</td>
</tr>
<tr>
<td>T9026</td>
<td>Anti-tubulin (from mouse)</td>
<td>FITC</td>
<td></td>
<td>1:500</td>
</tr>
<tr>
<td>F4018</td>
<td>Anti-mouse (IgG)</td>
<td>FITC</td>
<td></td>
<td>1:1000</td>
</tr>
</tbody>
</table>
2.3. Media and solutions

2.3.1. Growth media for *S. commune*

Media were sterilized at 120 °C for 30 minutes in an autoclave.

**Complex yeast medium (CYM):**
- 2 g peptone
- 2 g yeast extract
- 20 g glucose
- 0.5 g MgSO$_4$
- 0.5 g KH$_2$PO$_4$
- 1 g K$_2$HPO$_4$
- 18 g Agar-Agar for solid media

**CYM-T**
- CYM + 4mM tryptohane

**Minimal Media (MM)**
- 20 g glucose
- 2 g aspartic acid
- 0.5 g MgSO$_4$
- 0.5 g KH$_2$PO$_4$
- 120 µg Thiaminiumdichloride
- pH 6.3
- 15 g Agar-Agar for solid media

2.3.2. Solutions for immunofluorescence

**Fixation solution**
- 50 mM PIPES, pH 6.7
- 25 mM EGTA, pH 8
- 5 mM MgSO$_4$
- 3.7% formaldehyde

**PME**
- 50 mM PIPES, pH 6.7
- 25 mM EGTA, pH 8
- 5 mM MgSO$_4$

**PBS**
- 137 mM NaCl
- 2.68 mM KCl
- 8.45 mM Na$_2$HPO$_4$
- 1.47 mM KH$_2$PO$_4$

**Extraction solution**
- 100 mM PIPES, pH 6.7
- 25 mM EGTA, pH 8
- 0.1 % Igepal

**Embedding media**
- 0.1 M Tris/Cl, pH 8
- 50% glycerin
- 1 mg/ml phenyldiamine
- 0.1 – 1 µg/ml DAPI
2.3.3. Solutions for DNA-Isolation

DNA-Extractionbuffer
- 100 mM Tris/HCl, pH 8.0
- 50 mM EDTA, pH 8.0
- 2 % SDS

CTAB/NaCl
- 0.7 M NaCl
- 10 % CTAB

1xTE
- 10 mM Tris/HCl
- 1 mM EDTA, pH 8.0

2.3.4. Solutions for plasmid preparation

SolA
- 25 mM Tris, pH 8.5
- 10 mM EDTA, pH 8.0
- 50 mM glucose

SolB
- 200 mM NaOH
- 1 % SDS

SolC
- 2.55 M potassium acetate, pH 5.8

2.3.5. Solutions for transformation of *S. commune*

0.5M MgSO$_4$
- 0.5 M MgSO$_4$
- 20 mM MES, pH 6.3

1M MgSO$_4$
- 1 M MgSO$_4$
- 20 mM MES, pH 6.3

1M Sorbitol
- 1 M Sorbitol
- 20 mM MES, pH 6.3

PEG
- 50 % PEG 4000
- 10 mM MES, pH 6.3

Rescue-media
- MM
- 0.5 M Sucrose

Top-agar
- MM
- 0.8 % Agar

Bottom-agar
- MM
- 1.5 % Agar

2.3.6. Solutions for protein isolation

Solution A
- 80 mM Tris-HCl, pH 7.4
- 1.2 M NaCl

Solution B
- 40 mM Tris-HCl, pH 7.4
- 600 mM NaCl
- 4 % Triton X-114

Precipitation buffer
- 20 % TCA
- 50 % acetone

Rehydration buffer
- 8 M urea
- 2 M thiourea
- 4 % CHAPS
2.3.7. Solutions for 2-D gelelectrophoresis

Solution 1
6 M urea
2 % SDS
30 % glycerin
75 mM Tris-HCl pH 8.8
1 % DTT

Solution 2
6 M urea
2% SDS
30 % glycerin
75 mM Tris-HCl pH 8.8
4 % iodacetamide

Polyacrylamid gel (for 6 gels)
189 ml polyacrylamid
1.5 M Tris-HCl pH 8.8
101 ml *Aqua dest.*
38 ml Rhinohide
4.5 ml 10 % SDS
62 µl TEMED
4.5 ml 10 % APS

Fixing solution
40 % methanol
7 % acetic acid

Staining solution
20 % methanol
2 % o-phosphoric acid (85 %)
10 % ammonium sulfate
0.1 % Coomassie Brilliant Blue G-250

Neutralization solution
12 g Tris-base pH 6.5 (with o-phosphoric acid (85 %))

Destaining solution
25 % methanol

2.3.8. Solutions for tryptic digestion

Enzyme solution
20 µg/µl trypsin (Promega, Mannheim, Germany)
25 mM NH₄HCO₃

Extraction buffer
0.1 % TFA/acetonitrile, 1:1
2.4. Growth conditions

*S. commune* was grown on CYM/CYM-T at 30 °C for five days on solid media.

For DNA- and protein isolation, liquid media was used. *S. commune* was macerated in 50 ml of the adequate media with blender and incubated on a shaker at 30 °C and 150 rpm for two days. The pre-culture was mazerated again, filled up with media to 200 ml and incubated for two days at 30 °C and 150 rpm on a shaker. The well grown mycelium was filtered through a nylon membrane, washed with *Aqua dest.* and, if necessary, stored at -20 °C.

For immunofluorescence microscopy, solid media was covered with sterile cover slides. *S. commune* was inoculated in the gaps between two glass slides and was grown at 30 °C for three to four days.

For RNA-isolation *S. commune* was grown on solid media which was covered with sterile cellophane (Wilhelm Isermann KG, Walsrode, Germany). After seven days of incubation at 30 °C, mycelium was harvested and stored at -20 °C.

*E. coli* was grown on Standard I media (Carl Roth, Germany) at 37 °C. If necessary, antibiotics (100 µg/ml Ampicillin) were added. For cultivation on solid media, 1.8 g agar-agar was attached to the medium. For blue/white screening of cultures, transformed with a plasmid containing the *lacZ* gene, the indicator 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (40 mg/ml solved in DMSO) was added to solid media.

2.5. Microscopical investigations

2.5.1. General microscopy

Microscopic samples were investigated with Axioplan 2 (Carl Zeiss AG, Jena). Images were analyzed with Spot Advanced (Version 4.6, Diagnostic Instruments, Sterling Heights).

2.5.2. Immunofluorescence

The immunofluorescence protocol was modified from Raju and Dahl (1982) and Fischer and Timberlake (1995). Overgrown cover slides were fixed with fixation solution for 90 minutes. After that, hyphae were washed with PME three times. Cell wall degradation was performed with 30 mg/ml lysing enzyme (*Trichoderma harzianum*, Glucanex, Sigma Aldrich, Munich, Germany), lysed in 500 µl egg white + 500 µl PME for 20 minutes. For permeabilization of the cells, extraction solution was added for five minutes. After five minutes of blocking with milk powder, the first antibody (Tab.4) was added and incubated over night at 4 °C. The first
antibody was removed by washing the hyphae with PBS. After the incubation with the second antibody for 1 h at 37 °C and washing with PBS, the cover slide was incorporated in embedding medium containing DAPI fluorescence dye (1 µg/ml).

Microscopic samples were investigated with LSM 5 Axio Observer (Carl Zeiss AG, Jena). Images were analyzed with Zen2009 (Carl Zeiss AG, Jena).

### 2.5.3. Scanning electron microscopy

Mycelium of *S. commune* colonies was air dried for one week on a sterile glass slide and additionally incubated in an exsiccat for three days. Samples were fixed on a sample holder sputtered with a thin layer of gold to receive a conductive surface of the sample (EMI Tech K500) Samples were analysed with SEM, Philips XL 30 ESEM. Images were recorded with Scandium software (version 5.0 analySIS Image Processing Soft Imaging System GmbH, Münster).

### 2.6. Phylogenetic studies

Amino acid sequences were downloaded from databases mentioned in Appendix 1.

Amino acid sequences were aligned with MAFFT v6 using the BLOSSUM80 matrix and the E-INS-i option. For alignment quality control and adjustment, BIOEDIT v 7.0.9.0 was used (Hall, 1999; Katoh and Toh, 2008).

Phylogenetic reconstruction was conducted using MrBayes v. 3.1.2. For validation of data, RAxML Blackbox v. 7.2.6 was used. Phylogenetic trees were evaluated with FigTree v 1.2.3 (http://tree.bio.ed.ac.uk/software/figtree/) and modified with CorelDraw9 (Version 9.337, Corel Corporation) (Huelsenbeck and Ronquist, 2001; Stamatakis et al., 2008).

### 2.7. Construction of the deletion-cassette for *dhc1*

#### 2.7.1. DNA-Isolation

Mycelium of a 200 ml liquid culture of *S. commune* 12-43 was harvested and ground in liquid nitrogen. 3 g of ground mycelium was mixed with 10 ml DNA-Extractionbuffer and incubated at 65 °C for one hour. Adjacent the sample was centrifuged at 11,000 rpm and 4 °C for ten minutes; the supernatant was transferred into a new reaction tube.
M NaCl and 0.1 V CTAB/NaCl was added and the sample was incubated at 65 °C for 20 minutes and adjacent cooled down on ice. The sample was mixed with 1 V chloroform and centrifuged at 11,000 rpm and 4 °C for ten minutes; the upper phase of the sample was transferred into a new reaction tube. This step was repeated three times. Afterwards 0.7 V isopropanol was added and the sample was centrifuged at 11,000 rpm and 4 °C for 30 minutes. The supernatant was removed, the DNA-pellet was cleaned with 70 % ethanol and dried in a vacuum centrifuge. The pellet was resuspended in 750 µl TE and incubated at 65 °C for five minutes. The sample was centrifuged at 11,000 rpm and 4 °C for ten minutes and the supernatant was collected in a new reaction tube. This step was repeated three times. DNA was precipitated with 2 V 98 % ethanol for 20 minutes. Adjacent, the sample was centrifuged at 11,000 rpm and 4 °C for 30 minutes. The supernatant was removed, the DNA-pellet was cleaned with 70 % ethanol and dried in a vacuum centrifuge. Pellet was resuspended in 1 ml 1 x TE and stored at -20°C.

2.7.2. Polymerase chain reaction (PCR) and agarose gel electrophoresis

A routinely used 20 µl PCR-reaction consists of 9.4 µl Aqua dest., 4 µl polymerase buffer, 2 µl of each primer, 0.5 µl dNTPs, 2 µl DNA, 0.1 µl Taq-polymerase (GoTaq, Promega GmbH, Mannheim, Germany). PCR-run started with a denaturation of DNA for three minutes at 94 °C. This step was followed by 30 cycles of denaturation (30 seconds at 94 °C), annealing of primers (45 seconds at annealing temperature) and an elongation step at 72 °C for 1 min/kb. Cycles are followed by a final elongation step at 72 °C for one hour. PCR-samples were loaded on a 1 % agarose gel. After the run, the gel was incubated in ethidiumbromide (1 µl/ 10 ml) and visualized under UV light. If necessary, DNA bands with the expected size were extracted from the gel with Jetsorb (Genomed, Bad Oeynhausen, Germany) according to the protocol provided with the kit.

2.7.3. Cloning of PCR fragments

Amplificated fragments were cloned in the cloning vector pDrive (Qiagen, Hilden, Germany). Therefore, 2 µl amplified DNA, 2.5 µl ligation mix and 0.5 µl pDrive were incubated at 16 °C over night. Afterwards ligation reaction was stopped at 70 ºC for ten minutes. 2 µl ligated sample were mixed to 100 µl electrocompetent cells of E. coli DH5α and added to an electroporation cuvette (Peqlab, Erlangen, Germany). In a gene pulser (E_C Apparatus
Corporation, Holbrook, USA) a voltage of 2.8 kV at 25 µF and 220 Ω was applied to the cuvette. Adjacent the cells were resuspended in 900 µl Standard I media and incubated at 37 °C for 30 minutes for cell reconstitution. At the end, 200 µl of the sample were plated on Standard I plates containing Ampicillin and X-Gal. Plates were incubated at 37 °C overnight.

2.7.4. Plasmid preparation

White colonies of transformed E.coli cells were picked and transferred into 2 ml Standard I media containing Ampicillin. Samples were incubated at 37 °C overnight. Adjacent cells were pelleted in a centrifuge for one minute at high speed. Pellet was resuspended in 200 µl SolA. Afterwards 400 µl SolB were added and sample was incubated at room temperature for three minutes. 300 µl cold SolC were pipetted to the mix and the sample was incubated on ice for 5 minutes. At 13,000 rpm, the sample was centrifuged for 20 minutes and the DNA containing supernatant was transferred into a new reaction tube. Subsequently, 0.7 V isopropanol was mixed to the sample to precipitate the plasmid DNA and centrifuged for 30 minutes at 13,000 rpm. Supernatant was removed and pellet was cleaned with 70 % ethanol and afterwards dried in a vacuum centrifuge. Pellet was resuspended in 40 µl Aqua. dest. and stored at 4 °C.

To investigate the isolated plasmids a digestion with an appropriate restriction endonuclease followed (12,6 µl Aqua. dest., 2 µl enzyme buffer, 0.2 µl RNAse, 0.2 µl restriction enzyme, 5 µl DNA) overnight at 37 °C. Samples were loaded on a 1 % agarose gel. For DNA sequencing, or if especially purified DNA was needed, plasmid preparation was done with Fermentas mini (Fermentas, St. Leon-Rot, Germany) kit according to instruction manual. Sequencing was done at GATC Biotech (Konstanz, Germany) and for sequence analysis Vector NTI Advance 11.0 (Invitrogen Corporation) was used.

Plasmid-DNA was digested with XbaI (12,8 µl Aqua. dest., 2 µl B2 buffer, 0.2 µl XbaI, 5 µl DNA) for flank A and EcoRI (12,8 µl Aqua. dest., 2 µl EcoRI buffer, 0.2 µl EcoRI, 5 µl DNA) for flank B in an over-night restriction at 37 °C. Both flanks were cloned successively in the restriction sites XbaI and EcoRI of plasmid pChi, respectively pBluescript II SK.
2.8. Transformation of *S. commune* strain after Specht *et al.*, 1988

2.8.1. Protoplast preparation

A 400 ml liquid culture of *S. commune* was allocated into sterile 50 ml reaction tubes and pelleted for five minutes at 4,000 rpm. The supernatant was removed, the mycelium was resuspended in 40 ml 0.5 M MgSO₄ and centrifuged for ten minutes at 4,000 rpm. The supernatant was removed, the pellet resuspendend in 1 M MgSO₄ and again centrifuged as mentioned before. The supernatant was removed and the approximately 10 ml mycelium was solved in 10 ml 1 M MgSO₄. 100 mg/ml Caylase C3 (Cayla, France) from *Tolypocladium geodes* (0.1 g solved in 1 ml 1 M MgSO₄) was added to the solution. For transformation of stain 12-43, 100 mg/ml Novozyme 234 (InterSpex Products Inc, Foster City, USA) was used instead of Caylase C3. Mycelium was incubated at 30 °C for four hours and mixed every 30 minutes. After the digestion of the cell wall, 1 V cold *Aqua dest.* was added to mycelium to release an osmolaric shock to release protoplasts. The solution was centrifuged at 4 °C and 1,800 rpm for five minutes and the supernatant with protoplasts was collected in a new reaction tube. The mycelium was mixed with 20 ml 0.5 M MgSO₄ and again centrifuged for ten minutes at 1,800 rpm and 4 °C. Supernatant was collected again. This step was repeated three times. The protoplast solution was divided into new reaction tubes (20 ml per tube) and filled up with 1 V 1 M Sorbitol. Protoplasts were centrifuged for ten minutes at 1,100 rpm and 4 °C. The supernatant was removed and approximately 5 ml solution was left in the tube. Protoplasts were affiliated in one tube and centrifuged as mentioned above. The supernatant was removed and the protoplasts were mixed with 1 ml 0.5 M Sorbitol + 50 mM CaCl₂. Protoplasts were stored on ice at 4 °C over night.

2.8.2. Transfection of protoplasts

For transfection of protoplasts, 120 µl DNA-solution (6 µl 1M CaCl₂ and 5 µg DNA filled up with 1xTE to120 µl) was prepared on ice. To each DNA-solution 200 µl protoplasts were pipetted. The mix was incubated on ice for 30 minutes. Afterwards 320 µl 50 % PEG-solution was added to the protoplasts and the samples were incubated on ice for one minute. Adjacent the sample was carefully mixed with the pipette and transferred into 5 ml rescue-media. Protoplasts were incubated at RT over night.
2.8.3. Inoculation of protoplasts

After melting and cooling down 5ml top-agar per protoplast sample, top agar and protoplasts were mixed and plated on MM. After an incubation of 5 to 8 days, transformants were transferred to new plates. Adjacent growing transformants were inoculated on full media plates containing bottom-agar.

2.8.4. Verification of successful transformation

Transformants were analyzed via PCR on the existence of the deletion cassette, respectively of the wild type gene, with the oligonucleotides dhc1for and dhc1rev. PCR-run started with a denaturation of DNA for two minutes at 95 °C. This step is followed by 30 cycles of denaturation (30 seconds at 95 °C), annealing of primers (45 seconds at 55 °C) and an elongation step (3 minutes at 72 °C). Cycles are followed by a final elongation step at 72 °C for five minutes.

2.9. Transcriptome Analysis

2.9.1. Sample preparation

Mycelium of S. commune was harvested and ground in liquid nitrogen. RNA was isolated according to the protocol delivered with the kit (RNeasy Plant Mini Kit, Qiagen, Hilden, Germany). RNA was stored at -80 °C.

2.9.2. Next generation sequencing via Illumina HiSeq 2000

RNA-sequencing was performed by LGC Genomics, Berlin (Germany). Therefore, a mRNA-based cDNA-library was created. Sequencing adaptors were ligated to cDNA fragments to result in 50 bp single reads of the expressed genes. After the run, adaptors were cut from the resulted reads.

2.9.3. Analysis of Raw Data

Raw data of RNA sequences were mapped against the genome of S. commune using the splice junction mapper TopHat (release 1.4.1). The number of reads mapped within each gene was defined with Htseq. Expression values (RPKM) for every gene was calculated using the statistical software R. For expression differences, fold changes of mean value (condition A) : mean-value (condition B) were determined. For different statistical tests, DeSeq, EdgeR,
BaySeq, and Noiseq were used to verify expression differences (Kvam et al., 2012; Tarazona et al., 2011). A gene was defined to be differentially expressed if each method reported differential expression (false discovery rate adjusted p-value cutoff 0.01).

2.10. Proteome analysis via 2-dimensional gelelectrophoresis

2.10.1. Sample preparation of cytoplasmatic protein

Mycelium of *S. commune* was harvested and ground in liquid nitrogen. Performance of protein isolation was modified after Bordier, 1981. 3 – 5 g mycelium was transferred into a 50 ml reaction tube, filled up to 25 ml with solution A and mixed with 10 ml solution B. The sample was incubated on ice for one hour and afterwards centrifuged at 11,000 rpm and 4 °C for 15 minutes. Supernatant was transferred into a new reaction tube. The sample was incubated at 30 °C for three minutes and adjacent centrifuged at 1,600 rpm and RT for ten minutes. The aqueous phase was transferred into a new reaction tube and filled up with 1 V precipitation buffer. The sample was incubated at 4 °C over night. After incubation sample was centrifuged at 11,000 rpm and 4 °C for ten minutes. Pellet was separated in 1.5 ml reaction tubes and washed with acetone for four times. Afterwards pellet was dried in a vacuum centrifuge and adjacent resuspended in rehydration buffer. Sample was centrifuged for ten minutes at 13,000 rpm and 4 °C. Supernatant was transferred into new reaction tube and stored at -20 °C.

Amount of protein in the sample was defined *via* Bradford assay (1976).

2.10.2. Sample loading

To apply proteins onto an IPG-strip (Immobiline DryStrip pH 3 - 7 NL; pH 3 - 11 NL, 24 cm, GE Healthcare, Uppsala, Sweden), 250 µg protein were filled up with rehydration buffer up to a volume of 450 µl. To visualize proteins, 3 µl Coomassie Brilliant Blue was added to the mix. Protein sample was transferred into an IPG Box (GE, Healthcare, Uppsala, Sweden) and the IPG-strip was applied with gel downwards in the sample. Rehydration took place over night at RT.
2.10.3. First dimension of 2-D gelelectrophoresis

During isoelectric focusing, proteins were separated according to their isoelectric point on the IPG-strip. Therefore, strips were transferred into an Ettan™ IGPhor II™ focusing apparatus (GE Healthcare, Uppsala, Sweden). Program for isoelectric focusing was elected according to its pH-range (Tab. 5).

**Table 5: Program for isoelectric focussing of IPG-strips**

<table>
<thead>
<tr>
<th>Step</th>
<th>Immobiline DryStrip pH 3 - 11 NL, 24 cm, GE Healthcare</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4 h 300 V (gradient)</td>
</tr>
<tr>
<td>2</td>
<td>4 h 600 V (gradient)</td>
</tr>
<tr>
<td>3</td>
<td>4 h 1000 V (gradient)</td>
</tr>
<tr>
<td>4</td>
<td>4 h 8000 V (gradient)</td>
</tr>
<tr>
<td>5</td>
<td>24000 Vhr 8000 V (step)</td>
</tr>
</tbody>
</table>

2.10.4. Second dimension of 2-D gelelectrophoresis

The focused stripes were equilibrated for 20 minutes with solution 1 and adjacent 20 minutes with solution 2. Stripes were loaded on 10 % polyacrylamide gels. The gels were running in an Ettan™ DALTtwelve chamber (GE Healthcare, Uppsala, Sweden) filled with 1x running buffer in the lower part and 2x running buffer in the upper part of the chamber. Gels were running at 1 W per gel for one hour and afterwards at 15 W per gel for four hours.

Gels were fixed for 30 minutes and stained with staining solution over night. Afterwards, gels were neutralized with Tris-base pH 6.5 for 10 minutes and destained with 25 % methanol.

Gels were scanned with an Epson Bio Step ViewPix scanner (Tokyo, Japan) and analyzed with Delta2D software v. 4.3 (Decodon, Greifswald, Germany).
2.11. Protein analysis

2.11.1. Tryptic digestion

Preparation of proteins was performed after method of Shevchenko, 1996. After washing the gels with *Aqua dest.* two times for 20 minutes, spots were picked and incubated in 200 µl 50 mM NH₄HCO₃/acetonitril 1:1 for 15 minutes. After repeating this step, protein spots were shrunk and rehydrated for four times. The spots were air dried and adjacent digested with 10 µl enzyme solution for 35 minutes on ice. After removal of the enzyme, 3 µl 25 mM NH₄HCO₃ was added and spots were incubated at 37 °C over night. Closing, the spots were resuspended in 10 µl extraction buffer.

2.11.2. Protein analysis

For analysis of proteins, 1 µl matrix was transferred to an Anchor Chip Target and mixed with 1 µl protein sample. After crystallization of the samples, proteins were analyzed on a ultrafleXtreme™ MALDI-TOF/TOF (Bruker Daltonics, Germany).

Measurement of samples was set up with flexControl software followed by analysis with flexAnalysis (both Bruker Compass 1.3). Protein peaks were assigned to software ProteinScape 2.1, which was connected to a *Schizophyllum commune*-database based on the genome sequence of *S. commune*. 
3.) Results

3.1. Organization of dynein heavy chain encoding genes in *S. commune*

The dynein heavy chain is a large protein of the dynein complex, a motor protein which transports cargo along microtubules. In *S. commune*, the dynein heavy chain is encoded by two separate genes. The gene dhc1 encodes the N-terminal region of the dynein heavy chain with a length of approximately 4 kb and is interrupted by 9 introns. It contains the dimerization domain (1731 – 2427 bp) of dynein heavy chain, which enables the interaction with the second dynein heavy chain.

![Diagram of dhc1 and dhc2 genes](image)

**Figure 9: Organization of dhc1 and dhc2 in S. commune**

The gene dhc2 codes for the C-terminus of the normal dynein heavy chain and is approximately 11 kb large. Dhc2 is interrupted by 16 introns. This larger gene contains the important motor machinery of the dynein molecule which converts chemical to mechanical energy. This complex is formed by six AAA-modules. Leaning against the known dynein sequences, in *S. commune* the first four AAA-modules contain a Walker A motif (GXXXXXGKT/S) (Tab. 6), which is able to bind nucleotides (AAA1 = 2124 – 2799 bp; AAA2 = 3024 – 3789 bp; AAA3 = 4143 – 4890 bp; AAA4 = 5172 – 5979 bp). Between the
fourth and the fifth AAA-module, the microtubule binding site is located at 6339 – 6714 bp. The fifth AAA-module (7137 – 7824 bp) and the sixth AAA-module (8478 – 9120 bp) do not show any Walker A structure (Fig. 9). Both genes are located on chromosome I with a distance of approximately 413 kb.

**Table 6**: Amino acid sequence of the first four AAA-modules of the dynein heavy chain in different species

<table>
<thead>
<tr>
<th>species</th>
<th>p-loop in AAA1</th>
<th>p-loop in AAA2</th>
<th>p-loop in AAA3</th>
<th>p-loop in AAA4</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. commune</em></td>
<td>GPAGTGKT</td>
<td>GPSGSGKT</td>
<td>GPPGSGKT</td>
<td>GVSAGGT</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>GPAGTGKT</td>
<td>GKSRSKGS</td>
<td>GPPGSGKT</td>
<td>GVSAGGT</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>GPAGTGKT</td>
<td>GKSRSKGS</td>
<td>GPPGSGKT</td>
<td>GVSAGGT</td>
</tr>
<tr>
<td><em>H. sapiens</em></td>
<td>GPAGTGKT</td>
<td>GPSGSGKT</td>
<td>GPPGSGKT</td>
<td>GVSAGGT</td>
</tr>
</tbody>
</table>

According to the phylogenetic tree (Fig. 11), the dynein heavy chain is highly conserved in all eukaryotic domains. Merely, the dynein heavy chain of Ascomycete yeasts is affected by a higher mutation rate, which can be deduced from long branches in the phylogenetic tree.

A split dynein heavy chain is a unique phenomenon in higher Basidiomycota. Within this group, three split events took place during evolution. The first split occurred in the group of Ustilaginomycotina, which contain usually dimorphic plant pathogens. The split point within this group is conserved between the fourth and fifth AAA module in all three species in the phylogenetic tree, where all three are smut fungi. A different split point can be seen in Wallemiomycetes, where the split is located between the first and the second AAA module. Wallemiomycetes contain only few species which are basidiomycetous molds. In Agaricomycetes and Dacrymycetes, the motor machinery is separated completely from the N-terminal region. This organization is highly conserved within this group. Both belong to the subdivision Agaricomycotina. This subdivision contains mychorrizal fungi, wood rotting fungi and parasites which are well known because of their eye-catching fruiting bodies. In the very basal Basidiomycota shown in the phylogenetic tree, the dynein heavy chain is unsplit. Species in the phylogenetic tree belong either to rustfungi (Pucciniomycotina), which are parasites for plants, animals or fungi. The other basal group is Tremellomycetes containing the yeastlike basidiomycetes belonging to the genus *Cryptococcus*.

The organization of the distribution of *dhc1* and *dhc2* varies within the group of Agaricomycetes. Location of both genes on one scaffold indicates the location of both on one
Results

chromosome as it is proven for *S. commune*. For some species a location on two different scaffolds occurs (Fig. 10).

In table 7, the composition of amino acids at the split point of the dynein heavy chain is shown. The last 10 aa of Dhc1 are highly conserved within Agaricomycetes, but are missing in all other investigated species in the phylogenetic tree. The first 22 aa of Dhc2 are also shown in the table. A conserved motif consisting of polar and nonpolar amino acids can be found. This motif can be found in Ustilaginomycotina, too, but at a different location within the sequence of the dynein heavy chain proteins. Additionally, other fungal domains, like filamentous ascomycetes, feature this motif less conserved in their dynein heavy chain sequence.

![Figure 10: Gene distribution of *dhc1* and *dhc2* in elected Agaricomycetes. Arrows indicate the reading direction of the genes. Location on different scaffolds is illustrated by a split scaffold.](image)

*Figure 10: Gene distribution of *dhc1* and *dhc2* in elected Agaricomycetes.* Arrows indicate the reading direction of the genes. Location on different scaffolds is illustrated by a split scaffold.
Table 7: Sequence of aa and the split point of Dhc1 and Dhc2. Identical aa are bold, differences in red.

<table>
<thead>
<tr>
<th>Species</th>
<th>Dhc1</th>
<th>Dhc2</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. bisporus</td>
<td>EGLVGAPRRF</td>
<td>MEIFTNSSTAAAVTFITFVQ</td>
</tr>
<tr>
<td>A. delicata</td>
<td>EGLVGAPRRF</td>
<td>MEIAPNSSTAAAVTFITFVQ</td>
</tr>
<tr>
<td>D. squalens</td>
<td>EGLVGPPRRF</td>
<td>MELLAPSNSSTAAAVTFITFVQ</td>
</tr>
<tr>
<td>F. mediterranea</td>
<td>EGLQGAPRRF</td>
<td>MEVRLEVAFASNSTAAAVTFITFVQ</td>
</tr>
<tr>
<td>Ganoderma spec.</td>
<td>EGLVGAPRRF</td>
<td>MELLAPSNSSTAAAVTFITFVQ</td>
</tr>
<tr>
<td>P. chrysosporium</td>
<td>EGLVGAPRRF</td>
<td>SMEVLAPSNSSTAAAVQFITFVQ</td>
</tr>
<tr>
<td>P. ostreatus</td>
<td>EGLVGQVRRF</td>
<td>MEAFASNSSTAAAVQFITFVQ</td>
</tr>
<tr>
<td>S. commune</td>
<td>EGLVGAPRRF</td>
<td>MPQATASPSNSSTAAAVTFITFVQ</td>
</tr>
<tr>
<td>S. lacrymans</td>
<td>EGLLGTRRF</td>
<td>MEAFSPSTSTAAAVTFITFVQ</td>
</tr>
<tr>
<td>W. cocos</td>
<td>EGLVGAPRRF</td>
<td>MELLTSSNSSTAAAVTFITFVQ</td>
</tr>
<tr>
<td>U. maydis</td>
<td>RVELEQHSIEGSSTAQAVTFITFVQ</td>
<td></td>
</tr>
<tr>
<td>W. sebi</td>
<td>RYELETQIEGSSTVQAVSFITFVQ</td>
<td></td>
</tr>
<tr>
<td>S. roseus</td>
<td>RHDLEQHSIETSSTAAVTFITFVQ</td>
<td></td>
</tr>
<tr>
<td>N. crassa</td>
<td>RKDLEGQAMTANSTAEAVRFITIVQ</td>
<td></td>
</tr>
<tr>
<td>A. fumigatus</td>
<td>RHDLEGKSLDASSTAHAVSFITIVQ</td>
<td></td>
</tr>
</tbody>
</table>

3.2. Localization of Dhc1 and Dhc2 in hyphae

The proteins Dhc1 and Dhc2 are cytoplasmic proteins. With specific antibodies for Dhc1 and Dhc2, both proteins were visualized in the cell. A higher abundance of Dhc1 compared to Dhc2 can be observed. Co-localization of both proteins appears at some places in the cell and in association to nuclei (Fig. 12, 13). Co-localization is an important hint, that both proteins could form a complex to migrate into the nucleus to fulfill their function during the cell cycle.
Figure 11: Phylogeny of the dynein heavy chain. Amino acid sequence of the dynein heavy chain of all organisms present in the phylogenetic tree, were aligned and used for calculation of their phylogeny.
Figure 12: Threedimensional image of a hyphae of *S. commune*. Dhc1 TRITC-labeled (Filter BP 560-615; Laser 543 nm 22.0 %), Dhc2 FITC-labeled (Filter BP 505-530; Laser 488 nm 7.0 %). A) dikaryotic hyphae with a co-localization of Dhc1 and Dhc2 (arrows). B) detailed view on a co-localized Dhc1-Dhc2-“complex” in a hypha of *S. commune* (arrow).
3.3. Deletion of \( \text{dhc1} \)

To create a dynein heavy chain 1 knock-out strain, a deletion cassette was constructed to replace the \( \text{dhc1} \) gene by the autotrophy marker gene \( \text{ura1} \) of \( S. \text{commune} \). Flanks upstream and downstream of \( \text{dhc1} \) were amplified via PCR. The 1585 bp large Flank A contains 585 bp of \( \text{dhc1} \) and was amplified with primers \( \text{dhc1AaXbaI} \) and \( \text{dhc1AbXbaI} \). The product was restricted with \( \text{XbaI} \) and cloned into the \( \text{XbaI} \) restriction site of vector \( \text{pChi} \), which already contained the \( \text{ura1} \) marker gene of \( S. \text{commune} \). Construct FlankA-ura1 was subsequently cloned into \( \text{BamHI} \) and \( \text{NotI} \) restriction sites of vector \( \text{pBluescript II SK} \). Flank B (1.699 kb), which contained 699 bp of \( \text{dhc1} \) was amplified with primers \( \text{dhc1Ba} \) and \( \text{dhc1Bb} \). It was cloned into the \( \text{EcoRI} \) restriction site of \( \text{pBluescript II SK} \). The final deletion plasmid \( \text{p\Deltadhc1} \) was used for transformation of \( S. \text{commune} \) strain 12-43 and \( \Delta\text{ku80} \) (Fig. 14).
Results

Figure 14: Deletion plasmid p\(\Delta\text{dhc1}\). A) Wildtype gene \(\text{dhc1}\) with flanking regions upstream and downstream; B) deletion cassette in p\(\Delta\text{dhc1}\).

Through protoplast transformation of strains \(\Delta\text{ku80}\) and \(12\text{-}43\), 8 possible knock-out mutant strains could be generated. Transformant 1-7 were derived from strain \(\Delta\text{ku80}\) and transformant 8 from 12-43. With the first PCR using primers dhc1f or and dhc1r, the amplification of either the wildtype gene (2.9 kb) or the integrated deletion cassette (1.5 kb) was proven (Fig. 15). An ectopic integration of the deletion cassette results in 2 bands in the agarose gel, whereas only one band at 1.5 kb indicates a knock-out event (Fig. 16).

Figure 15: PCR strategy to prove the integration of the deletion cassette in the genome of \(S.\text{commune}\)

The second strategy was composed of testing the replacement of \(\text{dhc1}\) by \(\text{ura1}\) based on a nested PCR. First, a 6 kb and a 7 kb fragment, containing either the wildtype gene or the deletion cassette for \(\text{dhc1}\), were amplified with primers dhc1up and dhc1down. This fragment was used as a template for the downstream PCR. For this second PCR, oligonucleotides urafor and B-test amplifying a 905 bp large fragment of the deletion cassette were used (Fig.17).
Results

Figure 16: Illustration of PCR results to amplify the deletion cassette fragment. In the wildtype, the 2.9 kb band was amplified (red). In the transformants, the 1.5 kb fragment of the deletion cassette was amplified (green). PCR results were obtained from several runs.

Figure 17: PCR strategy to show the replacement of the wildtype gene with the marker gene ura1 A) first PCR sample to amplify the first target for the nested PCR; B) second PCR sample to amplify a fragment of the deletion cassette within the target DNA

The first PCR gave a result at the expected size for every tested strain. In the second PCR, a fragment with the expected size was amplified in all tested mutant strains. These mutant strains contain the deletion cassette at the correct position in their genome and are therefore dhc1 knock-out strains. In the two wildtype strains, no DNA-fragment was amplified, which shows that dhc1 is still intact (Fig. A1).

Via two independent PCR experiments with different strategies and in Southern hybridization (data shown in Mai, 2012), it could be shown that the deletion cassette was integrated in the genome of S. commune after transformation and replaced the gene dhc1.
3.4. Characterization of Δdhc1 knock-out strains

In total, 8 Δdhc1 strains were obtained, 7 from the transformation of the strain Δku80 and one from the transformation of wildtype strain 12-43. Transformants, which have Δku80 as parental strain, are named Δdhc1_1, Δdhc1_2, Δdhc1_3, Δdhc1_4 Δdhc1_5, Δdhc1_6 Δdhc1_7. The transformant which resulted from the transformation of strain 12-43 is named Δdhc1_59.

By comparing the colony shape of the mutant strains with the parental strain, no differences can be seen, except a slightly reduced formation of aerial mycelium in strains Δdhc1_4 and Δdhc1_5, a feature often associated with transformation or protoplasting and regeneration. Additionally, hyphal growth is not differing in mutants and the parental strain. The growth rate, as well, is similar to the progenitor (Fig. 18). A production of an indole-dye as a circle at the colony edge was observed in all mutants after long-term storage. This phenomenon did not occur in the wildtypes.

![Image of colony morphology](image)

**Figure 18: Colony morphology of transformants obtained from Δku80.** In the second and third row, hyphae of the investigated strains are shown, with a higher magnification in the third row.

Strain 12-43 has a fluffy colony structure and hyphae grow straight on the medium-surface. In contrast, Δdhc1_59 produces a high amount of aerial mycelium before it starts growing on the circumfluent medium (Fig. 19). After five days of incubation at 30 °C, the wildtype colony had covered an area of $9.5 \pm 0.6 \text{ cm}^2$, while knock-out mutants only reached $3.27 \pm 0.29 \text{ cm}^2$. Thus, growth was reduced 2.91-fold.
Figure 19: Colony morphology of transformant obtained from 12-43. In row A the wildtype colony and hyphal structure is shown. In row B strain Δdhc1_59 colony and hyphal structure can be seen. In picture C a detailed view on hypha of Δdhc1_59 show their curled growth, which is not present in strain 12-43.

3.4.1. Cell length is reduced in Δdhc1 strains

For analysis of cell sizes, 100 tip cells and 100 hyphal cells were measured for length. Three different strains were chosen according to their macroscopic shape: strain Δdhc1_1 and Δdhc1_2 show a similar shape, while Δdhc1_5 differs slightly from the other obtained mutants. In all mutant strains, a reduction of cell length was observed. While in the mutants, the cell length hardly reaches more than 200 µm, tip cells in the wildtype Δku80 can reach a length up to 450 µm and more (Fig. 20).

Figure 20: Diagram showing the cell length of tip cells (wildtype = blue, Δdhc1_1 = red, Δdhc1_2 = green, Δdhc1_5 = violet)
This effect can be seen also in hyphal cells. In the mutant strains, cells are not larger than 150 µm. In the wildtype, a few cells larger than 150 µm were documented (Fig. 21).

**Figure 21:** Diagram showing the cell length of hyphal cells (wildtype = blue, ∆dhc1_1 = red, ∆dhc1_2 = green, ∆dhc1_5 = violet)

In the ∆dhc1_59 a reduction of cell length was observed. Most cells are between 1 and 150 µm large. Cell length hardly reaches more than 300 µm. Tip cells in the wildtype 12-43 can reach a length up to 450 µm and more (Fig. 22).

**Figure 22:** Diagram showing the cell length of tip cells (wildtype = black, ∆dhc1_59 = grey).
This effect can be seen also in hyphal cells. In the mutant strains, cells are not larger than 150 µm, which was seen only for a small amount of cells. Most cells are short – only between 1 and 100 µm. In the wildtype strain cells can reach a length up to 350 µm with the highest amount of cells between 50 and 200 µm in length (Fig. 23).

![length of hyphal cells](image)

Figure 23: Diagram showing the cell length of hyphal cells (wildtype = black, Δdhc1_59 = grey).

3.4.2. Nuclear position changes in deletion mutants lacking *dhc1*

The distribution of nuclei was observed in 100 tip and 100 hyphal cells of the wildtype Δku80 and the Δdhc1 strains 1, 2 and 5. Nuclei in wildtype cells are located directly in the center of the cell, with only very few deviations. In the Δdhc1 strains, the differences in nuclei distribution are obvious (Fig. 24).

![Nuclear position in tip cells and hyphal cells](image)
Results

Figure 24: Diagram showing the nuclear position in the investigated strains. In tip cells, measurements were taken from the septa towards the tip, in hyphal cells from septa to septa. Position 1 shows the location of the nucleus close the old septa. Position 3 indicates a location at the tip, respectively at the new septa. (wildtype = blue, Δdhc1_1 = red, Δdhc1_2 = green, Δdhc1_5 = violet)

The distribution of nuclei was observed in 100 tip and 100 hyphal cells of the wildtype 12-43 and the Δdhc1_59. Nuclei in wildtype cells are located directly in the center of the cell with only very few meanderings. In Δdhc1_59 nuclei are distributed all over the cell, although the majority of cells contain the nucleus also in the center (Fig. 25).

Figure 25: Diagram showing the nuclear position in the investigated strains. In tip cells, measurements were taken from the septa towards the tip, in hyphal cells from septa to septa. Position 1 shows the location of the nucleus close the old septa. Position 3 indicates a location at the tip, respectively at the new septa. (wildtype = black, Δdhc1_59 = grey).

3.4.3. Additional anomalies in the Δdhc1 strains

In microscopic investigations, several other characteristics were documented: a) Δdhc1 strains show a spherical, hyphal growth (Fig. 26); b) hyphae grow in coils in all investigated strains (Fig. 27); c) all strains show an increased branching rate (Fig. 28) and d) all investigated strains produce cells which contain more than one nucleus (Fig. 29).
Figure 26: Protuberances at hyphae of Δdhc1 strains (arrow). A) strain Δdhc1_59 with a strong effect of spherical, hyphal growth. B) strain Δdhc1_5 showing less protuberances. For strain Δdhc1_1 see figure 29 A.

Figure 27: Coil formation in Δdhc1 strains. A) Δdhc1_1; B) Δdhc1_2; C) Δdhc1_5; D) Δdhc1_59.
Figure 28: Hyper branching effects in Δdhc1 strains. A) Δdhc1_1; B) Δdhc1_2; C) Δdhc1_59.

Figure 29: Bi – and Multinuclear cells in Δdhc1 strains. A) Δdhc1_1; B) Δdhc1_2; C) Δdhc1_5; D) Δdhc1_59.
3.4.4. Mating and sexual development

In a confrontation assay of a *dhcl* deletion strain with a compatible wildtype, strain T41, it was possible to observe fruiting bodies on both sides. The Δdhc1 strains are therefore able to accept and donate nuclei. On the wildtype side, typical *S. commune* fruiting bodies can be seen, which are unstiped and show a normal gill development. Fruiting bodies on the mutant side have a stem of approximately 1 cm length and lower gill formation (Fig. 30). Spores were produced from both fruiting body groups.

Strain Δdhc1_59 is also able to donate and accept nuclei. A stable dikaryon can be formed with a compatible mating partner. A higher amount of clamps was investigated in the mutant strain (Fig. 31 A). Under optimal conditions also fruiting bodies can be formed. These fruiting bodies are very small and not fully opened after 10 days after primordial appearance. No gills were seen in these fruiting bodies (Fig. 31 B).

![Image of fruiting bodies](image)

Figure 30: Fruiting body formation of the compatible mating interaction between wildtype T41 and Δdhc1_2.
Figure 31: Mating experiments between a compatible wildtype and Δdhc1_59. A) Formation of a dikaryon after 3 days of growth. Arrows are indicating clamp cell formation in both mating partners. After dikaryotization, strain Δdhc1_59 shows the phenotype of the wildtype B) Fruiting bodies in an early stage after 14 days of growth.
3.4.5. Comparison of Δdhc1 strains with Δdhc2

The phenotype of Δdhc1 strains can be compared to the already existing Δdhc2 strains. Similarities and differences are summarized in Table 8.

Table 8: Similarities and differences in dynein heavy chain deletion mutants

<table>
<thead>
<tr>
<th></th>
<th>Δdhc1 obtained from Δku80</th>
<th>Δdhc1 obtained form 12-43</th>
<th>Δdhc2</th>
</tr>
</thead>
<tbody>
<tr>
<td>strains viable?</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>colony</td>
<td>- growth rate is not reduced</td>
<td>- 3 fold reduced growth rate</td>
<td>- 4 fold reduced growth rate</td>
</tr>
<tr>
<td></td>
<td>- colony shape is varying between the single transformants</td>
<td>- increase in aerial mycelium</td>
<td>- decrease in aerial mycelium</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- fluffy colony structure</td>
<td>- dense colony structure</td>
</tr>
<tr>
<td>hyphae</td>
<td>- curled hyphae with non polarized growth</td>
<td>- curled hyphae with non polarized growth</td>
<td>- curled hyphae with non polarized growth</td>
</tr>
<tr>
<td></td>
<td>- blistered</td>
<td>- blistered</td>
<td></td>
</tr>
<tr>
<td>cell length</td>
<td>- reduced compared to wild type</td>
<td>- reduced compared to wild type</td>
<td>- reduced compared to wild type</td>
</tr>
<tr>
<td>nucleus</td>
<td>- position varies in every cell</td>
<td>- position varies in every cell</td>
<td>- position varies in every cell</td>
</tr>
<tr>
<td>mating behaviour</td>
<td>- formation of a full dikaryon with a compatible mating partner</td>
<td>- formation of a full dikaryon with a compatible mating partner</td>
<td>- only donation of nuclei to mating partner</td>
</tr>
<tr>
<td>fruiting bodies</td>
<td>- fruiting bodies form a stem and less gills</td>
<td>- fruiting bodies are very small and not fully developed under given conditions</td>
<td>- normal fruiting body production on the mating partner side</td>
</tr>
<tr>
<td>spore production</td>
<td>- both mating partner produce spores</td>
<td>- no spore production observed under given conditions</td>
<td>- spore production only by mating partner</td>
</tr>
</tbody>
</table>

3.5. Transcriptome analysis via RNA-sequencing

For transcriptome studies, the two wildtype strains 12-43 and E6 and the deletion mutant Δdhc2 were used in RNA-sequencing experiments. For strain 12-43, a total mapping of 68.9 % was reached with a spliced mapping of 7.3 %. For strain E6, total mapping of 69 % and spliced mapping of 7.2 % were observed. In the mutant, total mapping reached 73.3 % and spliced mapping of 7.6 %.

The three investigated strains 12-43, E6 and Δdhc2 of S. commune were used to analyze differentially expressed genes. Approximately 14650 genes could be identified via RNA sequencing in every tested strain. Differential expression of genes in the different conditions compared (see table 9), was determined for approximately 13600 genes. A gene was accepted...
as differentially expressed, if all four statistical tests (DeSeq, EdgeR, BaySeq, and Noiseq) confirm the fact. In a comparison of two wildtypes, 642 genes show differences in expression. 12-43 compared to Δdhc2 features 734 differentially expressed genes, while E6 vs. Δdhc2 shows 726 genes with an up- or down-regulated expression in the mutant (Tab. 9).

Table 9: Number of genes identified to be differentially expressed

<table>
<thead>
<tr>
<th></th>
<th>12-43 vs. E6</th>
<th>12-43 vs. Δdhc2</th>
<th>E6 vs. Δdhc2</th>
</tr>
</thead>
<tbody>
<tr>
<td>differentially</td>
<td>13596</td>
<td>13545</td>
<td>13580</td>
</tr>
<tr>
<td>expressed genes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>identified in total</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>4 statistical tests</td>
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<td>734</td>
<td>726</td>
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<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>3 statistical tests</td>
<td>1221</td>
<td>1267</td>
<td>1775</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 statistical tests</td>
<td>2377</td>
<td>2500</td>
<td>3839</td>
</tr>
<tr>
<td>TRUE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 statistical test</td>
<td>1419</td>
<td>1234</td>
<td>1244</td>
</tr>
<tr>
<td>TRUE</td>
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<tr>
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<td>7937</td>
<td>7810</td>
<td>5996</td>
</tr>
<tr>
<td>TRUE</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 32: Venn diagram of RNA-Sequencing. Differentially regulated genes in the comparison 12-43 vs. E6 are in red, 12-43 vs. Δdhc2 are in green and E6 vs. Δdhc2 are marked in blue. The 22 genes in the center of the diagram indicate the genes which are differentially regulated in all comparisons, respectively in all 3 strains.
The Venn diagram (Fig. 32) shows 22 genes which are differentially regulated in all three investigated conditions, with nine of them not yet having been annotated in S. commune. For four genes, annotations in other basidiomycetes were found. The two identified WSC-domain-containing genes gave hits in several basidiomycetes, e.g. in C. cinerea, that one can emanate from the sameness in S. commune. The annotated genes are coding mainly for extracellular proteins or are involved in pathways of protein secretion or secretion of organic matter (Tab. 10).

**Table 10: 22 differentially regulated genes in all three strains.** Non-annotated genes are indicated with / . For details see discussion and table A2.

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Gene</th>
<th>NCBI blast</th>
</tr>
</thead>
<tbody>
<tr>
<td>1037942</td>
<td>/</td>
<td>no result found in other fungi</td>
</tr>
<tr>
<td>1038458</td>
<td>Major facilitator superfamily MFS-1</td>
<td>Proteophosphoglycan ppg4 (R. glutinis)</td>
</tr>
<tr>
<td>1039406</td>
<td>/</td>
<td>Actin-like ATPase domain-containing gene (A. delicata)</td>
</tr>
<tr>
<td>1081190</td>
<td>/</td>
<td></td>
</tr>
<tr>
<td>1082687</td>
<td>Cutinase</td>
<td></td>
</tr>
<tr>
<td>1105422</td>
<td>Glycoside hydrolase, family 61</td>
<td></td>
</tr>
<tr>
<td>1135605</td>
<td>/</td>
<td>no result found in other fungi</td>
</tr>
<tr>
<td>1151728</td>
<td>NmrA-like</td>
<td></td>
</tr>
<tr>
<td>1187228</td>
<td>/</td>
<td>WSC-domain-containing gene, identified in several fungi</td>
</tr>
<tr>
<td>1188048</td>
<td>/</td>
<td>no result found in other fungi</td>
</tr>
<tr>
<td>1189960</td>
<td>/</td>
<td>no result found in other fungi</td>
</tr>
<tr>
<td>1191755</td>
<td>/</td>
<td>no result found in other fungi</td>
</tr>
<tr>
<td>1212538</td>
<td>Cytochrome P450</td>
<td></td>
</tr>
<tr>
<td>1213381</td>
<td>Heat shock protein Hsp20</td>
<td></td>
</tr>
<tr>
<td>1215940</td>
<td>Thaumatin, pathogenesis-related</td>
<td></td>
</tr>
<tr>
<td>1340620</td>
<td>Glutathione S-transferase, C-terminal</td>
<td></td>
</tr>
<tr>
<td>1359387</td>
<td>/</td>
<td>WSC-domain-containing gene, identified in several fungi</td>
</tr>
<tr>
<td>235431</td>
<td>Glycosyl hydrolase, family 13, catalytic region</td>
<td></td>
</tr>
<tr>
<td>54466</td>
<td>Glycoside hydrolase, family 61</td>
<td></td>
</tr>
<tr>
<td>56366</td>
<td>Glycoside hydrolase, family 16</td>
<td></td>
</tr>
</tbody>
</table>
3.5.1. Detailed investigation of ∆dhc2

Protoplasts of strain 12-43 were transformed with the deletion plasmid p∆dhc2. To avoid possible lethality of that knock-out, a stable dikaryon was produced by adding macerated mycelium of the compatible strain E6 to the transfected protoplasts. The resulting dikaryon produced fruiting bodies and spores, which were plate on selective medium.

*S. commune* strains with a deletion in *dhc2* showed different phenotypes. The fluffy colony structure of wildtype colonies was altered to yield a very dense and granular structure with less aerial mycelium. The growth rate of the mutant strain is 4 fold reduced; hyphae spread sinuously and undirected on the media. Cells in ∆dhc2 are truncated, and a defect in nuclear positioning was observed. Additionally, the acceptance of nuclei from a mating partner failed.

250 genes have a different expression in ∆dhc2 (Fig. 33). Many genes are not annotated or do not have a classification on the *S. commune* homepage. Most differentially expressed genes with a clear function are localized in metabolic pathways.

From the 250 genes, 163 genes are annotated. 110 of these genes can be assigned to a pathway by KOG (Fig. 34), while 53 genes have no classification for KOG. A high amount of genes show a predicted function only. Also in metabolic pathways, a high amount of genes were differentially expressed.

However, the deletion of *dhc2* is not lethal, as has been described for other basidiomycetes. *Via* RNA-sequencing of 12-43, E6 and ∆dhc2, an up-regulation of motor proteins kinesin-2 (4.6 fold upregulated in ∆dhc2) and kinesin-14 (9.2 fold upregulated in ∆dhc2) was detected in strain ∆dhc2 compared to 12-43. Both kinesin genes are microtubule-associated motor proteins.
Results

Figure 33: Distribution of 250 differentially regulated genes in ∆dhc2 to the defined KOG groups adopted from http://genome.jgi.doe.gov/cgi-bin/kogBrowser?db=Schco2. 1 = Cellular processing and signaling; 2 = Information storage and processing; 3 = Metabolism; 4 = Poorly characterized; 5 = not annotated; 6 = no classification

Figure 34: Distribution of 110 annotated, differentially regulated genes in ∆dhc2 to the defined KOG groups adopted from http://genome.jgi.doe.gov/cgi-bin/kogBrowser?db=Schco2.
3.5.2. Transcriptome differences between the wildtypes 12-43 and E6

642 genes show a differential expression in strain E6 compared to 12-43. This is a difference of 4.7% of the total transcriptome of both strains. A log2 (fold change) ± 2.0 was observed for 564 genes. A log2(fold change) ± 5.0 was calculated for 93 genes (Fig. 35). The 10 lowest and the 10 highest expressed genes were used for illustration (Fig. 36).

![Figure 35: Distribution of 93 differentially regulated genes in 12-43 compared to E6 to the defined KOG groups adopted from http://genome.jgi.doe.gov/cgi-bin/kogBrowser?db=Schco2. 1 = Cellular processing and signaling; 2 = Information storage and processing; 3 = Metabolism; 4 = Poorly characterized; 5 = not annotated](image)

Gene regulation in 12-43

![Gene regulation in 12-43](image)
Figure 36: Gene regulation in 12-43 compared to E6. Illustrated are the 10 highest and lowest regulated, annotated genes from the comparison 12-43 vs. E6. 1 = Predicted E3 ubiquitin ligase; 2 = Splicing coactivator SRm160/300, subunit SRm300; 3 = Nuclear localization sequence binding protein; 4 = SAM-dependent methyltransferases; 5 = Large RNA-binding protein (RRM superfamily); 6 = von Willebrand factor and related coagulation proteins; 7 = Nuclear GTPase/ATPase p130; 8 = Protein kinase PITSLRE and related kinases; 9 = Molecular chaperone (small heat-shock protein Hsp26/Hsp42); 10 = Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies; 11 = mating pheromone activity; 12 = Putative transcriptional regulator DJ-1; 13 = L-kynurenine hydrolase; 14 = Molecular chaperone (DnaJ superfamily); 15 = O-methyltransferase; 16 = O-methyltransferase; 17 = MYND Zn-finger and ankyrin repeat protein; 18 = mating pheromone activity; 19 = Splicing coactivator SRm160/300, subunit SRm300; 20 = mating pheromone activity.

3.6. The proteome of S. commune

To simplify the work on proteomic studies, a protein map of S. commune was established. The reference strain 12-43 was used to create the master-gel of cytosolic proteins for this fungus. A second monokaryotic strain, 4-39, was used to compare two monokaryotic strains. A dikaryon, 12-43 x W22, was used to compare different developmental stages.

The protein map of strain 12-43 cytosolic proteome of S. commune could differentiate 417 proteins with identification, with additional 14 spots not being identified. The Mascot Score of at least 54.0 was not reached by 95 analyzed spots (Fig. 37, 38).

Figure 37: Distribution of analyzed proteins of the mastergel from strain 12-43.

For a comparison of two different monokaryotic strains, 4-39 was analyzed with 91 proteins showing a different abundance to 12-43. A higher abundance can be seen for 29 proteins, while 41 proteins had a lower abundance (Fig. 39, 40).
Figure 38: Protein map of cytosolic proteins of strain 12-43 of *S. commune*. All 527 selected spots are labeled.
Figure 39: Differential expression between two monokarytic strains. 12-43 image in blue, 4-39 image in orange. Black spots indicate equal amounts of protein in both strains. For pH range, see master gel (Fig. 38).
The dikaryon established after mating interaction is a developmental state with the capacity to form fruiting bodies. Thus, the two different mycelia of monokaryon and dikaryon were compared for their proteome profiles. A different abundance in the dikaryon can be seen for 121 proteins. An increase of abundance was observed for 51 proteins, a decrease for 37 spots (Fig. 41). Proteins with a 5-fold regulation are shown in Fig. 42.
Figure 41: Comparison of cytosolic proteins of the mastergel with the dikaryon. 12-43 image in blue, dikaryon image in orange. Black spots indicate equal proteins in both gels. Orientation of pH range is equal to mastergel (Fig. 38).
**Figure 42:** Diagram showing the different abundance of proteins in the dikaryon compared to 12-43. 

1 = Protein kinase, core, 2 = BTB/POZ fold (SKP1 component, dimerisation), 3 = Haloacid dehalogenase-like hydrolase, 4 = D-isomer specific 2-hydroxyacid dehydrogenase, catalytic region, 5 = Translation elongation factor EF1B, gamma chain, conserved, 6 = Phosphoglycerate kinase*, 7 = Cyclin-like F-Box, 8 = Glyceraldehyde 3-phosphate dehydrogenase*, 9 = Protein synthesis factor, GTP-binding*, 10 = ATP-citrate lyase/succinyl-CoA ligase, 11 = not annotated, 12 = Alpha-D-phosphohexomutase, C-terminal, 13 = Glyceraldehyde 3-phosphate dehydrogenase, 14 = Peroxisome membrane protein, Pex16*, 15 = Glyceraldehyde 3-phosphate dehydrogenase*, 16 = Protein synthesis factor, GTP-binding, 17 = Glyceraldehyde 3-phosphate dehydrogenase*; * indicates spots present only in dikaryon.
4. Discussion

Sexual development and hyphal growth in *S. commune* is dependent on transport processes such as nuclear migration and vesicle transport, as well as on the precise distribution of nuclei after mitosis in the cell cycle. Thereby, the motor protein dynein plays an essential role. The dynein complex transports its cargo to the minus-end of microtubules. Energy for this movement is gained from the conversion of chemical to mechanical energy at the dynein heavy chain protein. In *S. commune*, the dynein heavy chain is encoded by two separate proteins. Dhc1 forms the N-terminal region of the heavy chain encoding the dimerization domain. The larger protein Dhc2 encodes the motor machinery and the microtubule binding domain.

4.1. Phylogeny of the dynein heavy chain

The phylogenetic tree based on the current phylogeny of fungi, shows dynein heavy chain gene structure in different phyla. An exception can be seen only with Saccharomycotina and Taphrinomycotina, which cluster, in contrast to the fungal phylogeny, as a monophyletic clade. This could be caused by a higher mutation rate within this groups resulting in long-branch effects in the phylogenetic tree (Matheny et al., 2006; Hibbett et al., 2007; Ebersberger et al., 2012).

Phylogenetic studies have shown three independent splits of the dynein heavy chain in higher Basidiomycota. In the basal Pucciniomycota and Tremellomycetes, the dynein heavy chain is encoded by a single, full-length heavy chain of cytoplasmatic dynein. The organization and the type of split of the dynein heavy chain are highly conserved in every group.

The three splits of the dynein heavy chain within the Basidiomycota lead to the assumption of a unique function of the gene among higher Basidiomycetes, because in all other Eukaryotes the dynein heavy chain is unsplit. The dynein heavy chain is a very large protein (~ 4500 aa), which is responsible for spindle organization and elongation during mitosis in fungi (Steinberg, 2000). In Ascomycetes and Zygomycetes the nuclear envelope stays intact during mitosis, so that the nuclear pore complex is required for contact between the chromosomes and the cytoplasm. In this “closed mitosis”, all required proteins need to pass the nuclear envelope to fulfill their function inside the nucleus (Zickler, 1970; Butt and Humber, 1989; Theisen et al., 2008). The protein, encoding the dynein heavy chain, responsible for the right process of mitosis, could be too large to pass the nuclear pore complex. Therefore, a split dynein heavy chain would be beneficial to avoid this problem. In other basidiomycetes, like *U.*
maydis, the nuclear envelope breaks down during mitosis. This process is called “open mitosis” (Straube et al., 2005). In S. commune, a late “open mitosis“ takes place, which also leads to the break-down of the nuclear envelope. Therefore, the dynein heavy chain protein easily migrates into the nucleus, at least during anaphase. The hypothesis, that the size of the dynein heavy chain protein could be the reason for its split in higher basidiomycetes, thus, cannot be confirmed.

It could be shown that Dhc1 and Dhc2 are localized in the cytoplasm. Both proteins are distributed all over the cell, in dikaryotic strains both can be found also in the clamp cells. Co-localization was observed between both proteins, specifically close to nuclei, but also at other positions within the cell. In detailed fluorescence images, no gap between Dhc1 and Dhc2 exists. It is thus possible that both proteins form a complex. With cNLS Mapper (cut-off > 5), no signal sequence for the localization of Dhc1 in the nucleus was observed. In contrast, for Dhc2, two sequences were found at 1581-1610 aa and 3850-3879 aa, which indicate an uptake into the nucleus, at least during specific stages in the cell cycle. That leads to the suggestion that Dhc1 has to form a connection to Dhc2, or other protein(s) are needed for migration into the nucleus during mitosis (Kosugi et al., 2009). This hypothesis is supported by the analysis with cello v. 2.5, where a localisation in the cytoplasm is predicted for Dhc1, while Dhc2 can occur in the cytoplasm and in the nucleus. By the fused sequences of both proteins, a localization of the complex in the cytoplasm and in the nucleus is predicted (Yu et al., 2006). Additionally, the software WoLF PSORT puts out the same result for the localization of Dhc1 and Dhc2 (Horton et al., 2007). Co-immunoprecipitation experiments for Dhc1 and Dhc2 failed so far and no complex formation could be observed. On one hand, it is possible that no complex formation is necessary for the full functionality of both proteins. On the other hand, a connection of ligands to either Dhc1, Dhc2, or both, is possible, which might lead to a conformation change.

The split point between Dhc1 and Dhc2 is conserved in all Agaricomycetes showing the same split pattern like S. commune. The last 8 aa of Dhc1 cannot be found in Agaricomycetes with an unsplit dynein heavy chain. Also the aa upstream of the NSSTAAAVTFITFVQ-motif in Dhc2 are missing in the unsplit protein. That leads to a reorganization which could result in the split of the dynein heavy chain. By analyzing the sequence of Dhc1 upstream of the split point, a possible dimerization domain to connect Dhc1 to Dhc2 was not found so far.

Taken together, a reason of the split of the dynein heavy chain remains unanswered.
4.2. The deletion of either \textit{dhc1} or \textit{dhc2} is viable

In the filamentous Ascomycetes \textit{N. crassa} and \textit{A. nidulans}, mutants with a deletion for the dynein heavy chain gene have been described. In \textit{N. crassa}, colonies of dynein heavy chain deletion mutants show a reduced growth rate. Hyphae grow sinuously and show an irregular nuclear distribution of nuclei in cells (Bruno et al., 1996). In \textit{A. nidulans}, nuclei divide in conidia but they do not migrate into the germ tube. Colonies of \textit{A. nidulans} mutants form small colonies (Xiang et al., 1994). In summary, both dynein heavy chain mutants show a growth change and a defect in nuclear migration and distribution.

In \textit{Candida albicans}, the deletion of the dynein heavy chain gene leads to an extended cell cycle time as well as defects in spindle assembly during mitosis. Cells of the fungus grow larger and show a reduced number of bi- or multinucleate cells. During the filamentous growth stage, hypha formation and hyphal growth show defects (Martin et al., 2004).

In the corn-smut fungus \textit{Ustilago maydis} (Basidiomycota – Ustilaginomycetes) the first dynein heavy chain mutants for basidiomycetes were described. In this dimorphic fungus, the dynein heavy chain is encoded by two genes, \textit{dyn1} and \textit{dyn2}. The knock-out of one of these genes in \textit{U. maydis} is lethal, which was overcome by the generation of knock-down mutants. Cells in the mutant strains grow larger, and the cell shape was spherical. A defect in nuclear migration was also observed (Straube et al., 2001). In contrast to \textit{U. maydis}, the knock-out of either \textit{dhc1} or \textit{dhc2} is not lethal in \textit{S. commune}.

4.2.1 $\Delta$\textit{dhc1} strains show a different phenotype

In strain $\Delta$\textit{dhc1}-59 a reduced growth rate was measured. Also a change in the colony morphology was observed, resulting in a higher amount of aerial mycelium. For the mutants obtained from $\Delta$\textit{ku80}, no reduction in growth rate or morphological changes in the colony shape were documented. Only $\Delta$\textit{dhc1}_5 shows a phenotype similar to the “flat” phenotype of semicompatible mating interactions of \textit{S. commune}. A thin mutation, which can occur in context with transformation experiments in \textit{S. commune}, where one characteristic is a reduced formation of aerial mycelium, can be excluded for this strain (Fowler and Mitton, 2000).

Cells in $\Delta$\textit{dhc1} strains are considerably shorter, which is different from the \textit{U. maydis} mutants. A similarity in phenotypes was the spherical shape of the cells in $\Delta$\textit{dhc1} strains, which hints at a function in cell shape for Dhc1. Supporting this feature, cells in $\Delta$\textit{dhc1} strains with two
nuclei in a monokaryotic strain were documented. A possible defect in septae formation on the precise position of the mitotic spindle, or a defect in nuclear distribution is likely.

A defect in nuclear positioning was also observed. Nuclei usually are located at the cell center. In ∆dhc1 strains, the nucleus can take any position in the cell, even close to a septum. Nuclei can be donated to a mating partner, and can be accepted. Thus, for Dhc1 a defect in nuclear migration could not be shown. In accordance with this, fruiting body formation and sporulation could take place. In contrast to ∆dhc1 strains, ∆dhc2 strains can donate nuclei to a mating partner, but fail to accept nuclei. Therefore, fruiting bodies can be formed only at the side of the mating partner. In contrast to dhp1, dhp2 functions in nuclear migration during the life cycle of S. commune. The deletion of dhc1 has no influence on the migration during dikaryon formation itself.

In ∆dhc2 strains, a reduction in aerial mycelium formation and a 4 fold reduced growth rate of colonies were observed. Hyphae grow sinuously in every direction in contrast to directed growth of wildtype hyphae. The knock-out of dhc2 leads to truncated cells and to a defect in nuclear distribution and migration. The knock-out of dhc1 leads to phenotypic changes similar to the knock-out of dhc2. Both genes thus seem to cooperate with a common function in cell cycle with three major activities: (i) maintaining cell shape; (ii) distribution of nuclei and (iii) positioning of the nucleus in every cell (Brunsch et al., 2012).

The increased formation of aerial mycelium in ∆dhc1_59 strain gives a hint, that hydrophobins could be affected to the deletion of dhc1. Hydrophobins are secreted proteins which coat the hyphal tip as a monolayer at hydrophobic-hydrophilic interfaces. Thus, hyphae are able to grow straight to the air to assure fruiting body formation and consequently spore production for distribution of the fungus (Bayry et al., 2012). Especially the Sc3 hydrophobin, which is active in mono- and dikaryons of S. commune could show a higher regulation in the mutant obtained from 12-43. In the mutants obtained from Δku80 this effect is not as distinct as in the one from 12-43. Possibly, both wildtype strains have a different expression in Sc3 (Schuurs et al., 1997).

4.2.2. Phenotypes in ∆dhc1 strains are dependent on the parental background

Genetical manipulation in S. commune was performed by transfecting protoplasts with a deletionplasmid. In these experiments an ectopic integration caused by nonhomologous end-joining (NHEJ) is much more probable than the designated replacement of the gene of interest with the deletion cassette. Latter is caused by homologous recombination. To increase the
homologous recombination rate, either one of the important genes, ∆ku70 or ∆ku80, which form a dimer in the first step of NHEJ, needs to be inactivated. The NHEJ-pathway is interrupted and so, DNA-repair takes place by homologous recombination (Choquer et al., 2008; Wang et al., 2011).

For *S. commune*, a ∆ku80 strain exists. In this strain, the transformation rate was dramatically decreased, with only 15 transformants, but 7 (47 %) showing the desired deletion of *dhc1*. In the transformation experiment with the wildtype strain 12-43, only 1 % of 105 transormants contained the deletion cassette at the correct position in the genome. Although the transformation rate in ∆ku80 is much lower than in other wildtypes of *S. commune*, the efficiency of homologous recombination is increased.

In the transformants obtained in this study, an influence of the genetic background on the phenotype was observed. In transformants of the wildtype strain 12-43, e.g. with ∆dhc1_59, cell shape and nuclear positioning defects are stronger as compared to mutants derived from ∆ku80. As *ku80* functions in DNA-repair during cell metabolism, mitosis and meiosis, these processes are affected by the deletion of *ku80* (Koike et al., 1999). Phenotypical changes caused by the missing DNA-repair process as well as an influence of the deletion of *ku80* on further gene deletions in this strain, cannot be excluded.

4.2.3. Kinesin-5 and Kinesin-14 can substitute for the function of *dhc2*

RNA-sequencing was used to identify genes that can take over the function of *dhc2*. For fungi, a kinesin-14 is described, which is a minus-end directed kinesin (Steinberg, 2007). In our investigation, we found a 9-fold upregulation of kinesin-14 in the ∆dhc2 strain compared to the wildtype 12-43. In the two investigated wildtypes used to generate the ∆dhc2 strain, also a 9-fold upregulation of kinesin-14 was found in the wildtype strain E6 compared to 12-43. For strain 12-43, less reads for this gene were found in the transcriptome, while for E6 and ∆dhc2 an equal amount of reads was found. This was the initial evidence that strain 12-43 lacks either the gene for kinesin-14 or has no sufficient sequence similarity in *kin14*. In Hanisch (2012), this phenomenon was proven with different PCR experiments. A general PCR with primers at the beginning and the end of the gene gave no result in 12-43. Different primer sets for qRT-PCR gave a result in strain E6, but again yielded no bands for 12-43. This leads to the hypothesis, that ∆dhc2 strains are only viable, if the kinesin-14 of strain E6 is present in their genome. However, investigation of additional ∆dhc2 strains on the presence of kinesin-14 in their genome is necessary.
Kinesin-5 also can provide minus-end directed transport along microtubules, when it interacts with dynactin (Kardon and Vale, 2009). An up-regulation of kinesin-2, the *S. commune* homolog to kinesin-5 of *A. nidulans* was found in strain Δdhc2. Thus, this motor also can take over the function of *dhc2*.

In strain Δdhc1_59, which has also the genetical background of strain 12-43, kinesin-14 cannot have an influence on the viability of the strain, as the kinesin-14 copy is missing or not conserved in the genome of 12-43.

### 4.2.4. The transcriptome of Δdhc2 shows differences in 250 genes

The genetic background of Δdhc2 consists of two *S. commune* wildtypes. The regulation of a gene may not only depend on the deletion of *dhc2* in this strain, but also on the genomic composition in the Δdhc2 genome, depending from the parental origin of every specific gene. In the resulting *dhc2* deletion mutants, phenotypic differences cannot be seen. All seven Δdhc2 strains have an identical morphology, colony shape and cell shape.

In Δdhc2, 110 annotated genes with a proposed function show different regulation. More than the half of these genes is involved in metabolic pathways, including the transport of different substrates, organic compounds and metabolic products. Significant in this group are genes for transporters for several substrates. Twelve major facilitator superfamilies (MFS) are differentially regulated in the mutant strain: with only three exceptions, all the differentially expressed MFS genes are upregulated in the two wildtypes compared to Δdhc2. Several general substrate transporters and three ABC (ATP-binding cassette) transporter-like genes are upregulated in Δdhc2. The active and passive transport over membranes is realized by these genes, which contribute to multidrug resistance, e.g. in *S. cerevisiae*, where the genes are involved in cell protection and biogenesis of fatty acids (Kovalchuk and Driessen, 2010). Furthermore, three specialized transporters are upregulated in Δdhc2: two sulphate transporters and a malic acid transporter. Possibly, the Δdhc2 strain produces more metabolic products, which need to be secreted.

In addition, the high number of differentially expressed genes involved in metabolic pathways could be a hint for an increase of cell metabolism. Transporters need to work more efficiently to introduce substrates for the pathways, which could lead to an up-regulation of transporter genes in the mutant strain. This would coincide with increased cell stress, as seen with an up-regulation of two heat-shock proteins in Δdhc2. The expression of these genes increases with rising stress in the cell. This could be underlined by several Cytochrome P450 genes, which
show an up-regulation in $\Delta$dhc2. Up-regulation of these genes means high oxidative stress in the mutant which correlates with the up-regulation of heat-shock proteins (Lewis, 2002).

A high number of genes involved in secretion or in producing secreted proteins were identified. This correlates with the differentially expressed genes according to metabolism and cellular transport. A changed metabolism could lead to different secretion products in $\Delta$dhc2.

Fungal mating-type pheromones are also differentially regulated in $\Delta$dhc2. All four identified genes are up-regulated in the mutant. The mutant has the parental background of both wildtypes. It should have the mating specificity of either 12-43 or E6 or a mix of both. As the mating-type of every strain of *S. commune* is specific, also pheromones and pheromone-receptors are strain specific. Thus, genes according to mating-types are always identified as differentially expressed, because every strain has different transcripts for its mating-type genes.

The *dhc1* gene was never identified as a differentially expressed gene in $\Delta$dhc2. This leads to the hypothesis, that the knock-out of *dhc2* has no effect on the expression of *dhc1*. A down-regulation of *dhc1* would mean that this gene is dependend on *dhc2* in transcript regulation, which is not likely. An up-regulation, in contrast, would show a compensatory transcriptional response. Both situations can be excluded with RNA-sequencing data, which underlines the autonomy of the two genes encoding the dynein heavy chain in *S. commune*.

4.3. Transcriptome differences can be found in all three investigated strains

4.3.1. High genomic diversity between different strains of *S. commune* wildtypes

In the comparison of *S. commune* 12-43 to *S. commune* E6, 642 differentially regulated genes were identified. With an up- or down-regulation of more than $2^5$, still 93 genes were identified. In this study, the 10 lowest and the 10 highest expressed genes were used to show dramatic differences between two monokaryotic wildtypes of *S. commune*. It was shown that nearly 7% nucleotide differences occur between two strains of *S. commune* in the housekeeping gene *ura1*. This underlines the divergence within the genus *S. commune* (Lengeler and Kothe, 1994). Also the high number of possible mating-types in nature results in constant genetic exchange during dikaryon formation and following spore distribution in *S. commune*.

The 10 down-regulated genes have postulated functions in cellular processes and metabolism. The lowest expressed gene in 12-43, with a down-regulation of 2700 fold, is a predicted E3
ubiquitin ligase. This gene is responsible for degradation of destroyed, misfolded or unnecessary proteins in the cell. Together with the E2 ubiquitin-conjugating enzyme, an attachment of ubiquitin to lysine residues in the targeted protein is realized. This ubiquination is a well known posttranslational modification (PMS) targeting proteins to the proteasome. Another PMS causing gene did show a significant down-regulation of 147 fold in strain 12-43 – the molecular chaperone coding for the small heat-shock protein Hsp26/Hsp42. They play an important role in protein-protein-interactions, as well as in protein folding and unfolding. Additionally, they avoid protein aggregation.

With a downregulation between 1300 fold (nuclear localization sequence binding protein) and 2400 fold (splicing co-activator SRm160/300) two genes involved in transcription and RNA processing and modification were identified in 12-43. The SRm160/300 was also identified as nuclear matrix antigen (McCracken et al., 2005). The different expression in two wildtypes could be due to the strain specific nuclear antigens. These antigens could play a role in nuclear-recognition during the mating process. An S-adenyl-L-methionine- dependent methyltransferase was seen with a downregulation of 240 fold in 12-43. This gene is responsible for protein trafficking and sorting. Additionally, it functions in biosynthesis and gene expression. Thus, considerable differences between two strains of S. commune in general cellular functions have been observed.

The 10 highest up-regulated genes in strain 12-43 compared to E6 belong mainly to mate recognition, with an up-regulation between 57 and 388 fold for pheromone response genes. Since Strains 12-43 and E6 are two fully compatible strains and would form a functional dikaryon. This is due to their different mating-type genes. A significant upregulation, respectively downregulation of these genes is expected in the comparison of two compatible strains. Strain 12-43 has different pheromone receptors to recognize compatible mating partners than strain E6.

Three metabolic genes were identified with a high up-regulation: a kynureninase and two O-methyltransferases. O-methyltransferases act as degraders of lignin and possibly are necessary for detoxification in white-rot fungi (Jeffers et al., 1997). Kynureninases are involved in the catabolism of tryptophane. Strain 12-43 is able to degrade tryptophane out of the substrate by itself. Strain E6 in contrast needs an addition of tryptophane in the media for optimal growth and therefore has a defective tryptophane catabolism. This could explain the higher expression of a gene involved in tryptophane catabolism in strain 12-43 compared to E6 (Phillips, 2011).
While one molecular chaperone was downregulated in strain 12-43, another chaperone (encoding the small heat-shock protein Hsp40) was up-regulated. This could mean that not every heat-shock protein is either present or active in every monokaryotic strain of *S. commune*. Both phenomenons could be due to possible divergence in the gene sequences. Additionally, as mentioned for *kin-14*, the composition of the genome is strain specific in *S. commune*.

The up-regulated splicing co-activator SRm130/300 in strain 12-43 could be the complementary gene to the up-regulated copy in E6. As mentioned before, this gene functions as a nuclear antigen. It is identified as differentially expressed in both strains because both strains should have a special nuclear antigen with different genomic sequence.

Two genes involved in transcription are upregulated in strain 12-43. A putative transcription regulator of the DJ-1 family was identified. A real function in transcription of DJ-1 is mostly reported in humans (Wilson, 2011). In plants this gene functions in several processes like protecting the organism against oxidative stress and assures cell survival (Lin *et al.*, 2011). A function in fungi is unclear but an overlapping function to the one in plants seems likely. The second gene identified was a MYND Zinc fingers containing the MYND domain which is necessary for protein-protein interaction of transcriptional co-repressor proteins (Wang *et al.*, 2010).

In summary, differentially expressed genes in a wildtype comparison fulfill a wide range of functions and are present in all pathways in *S. commune*. A network for strain specific characterizations cannot be confirmed. As a nucleotide difference of 7 – 9% is common between monokaryotic strains in *S. commune*, differences in amount of reads per transcript of a gene can occur.

4.3.2. Between all three strains 22 genes show differential regulation

Only 22 of more than 14,000 genes are differentially regulated in all 3 investigated strains. 9 of them are still not annotated. The other genes are extracellular proteins itself or are involved in secretory processes in *S. commune*. Proteins of the glycosyl hydrolase family are already identified in secretome studies of *S. commune*. Members of this family extract cellulases and hemicellulases, as well as amylases (Ring, 2012). One major facilitator superfamily (MFS) was identified. This gene belongs to a uniporter-symporter-antiporter family. They transport only small molecules in response to chemiosmotic ion gradients (Pao *et al.*, 1998). This gene has the highest regulation in strain E6. This strain has no copy of tryptophane in its genome.
Tryptophane needs to be added to the growth media and needs to be taken up by the fungus. Possibly, the MFS is responsible for this uptake and therefore, this gene is higher expressed in E6. A cutinase is also differentially expressed in all strains. This gene is common in plant pathogenic fungi, which secrete the cutinase to degrade the cuticle of plants for easy penetration of their host (Dutta et al., 2009). Differentially regulated glutathione-s-transferase is involved in detoxification. Glutathione is bound to organic matter and is secreted or degraded in vacuoles (Morel et al., 2009). Other genes are expressed under stress conditions, like thaumatin and heat shock proteins. Thaumatin is used for plant transformation to increase the resistance of plants against fungal pathogens (Popowich et al., 2007). The highest expression of the majority of identified genes in all three strains can be found in 12-43. This leads to the conclusion, that this strain has an increased pathogenicity compared to the other strains, as cutinase and thaumatin are highly expressed. The glycoside hydrolases are both regulated in the two wildtypes dependent from the saccharide they need or used as a carbon source.

4.3.3. Correlation between the transcriptome and proteome in monokaryons

As mentioned before, the composition of the genetic information varies from strain to strain in S. commune. Also on the proteomic level differentially abundant proteins were found. Again a few proteins stay unclear because of the missing annotation.

The identified proteins with a varying abundance higher or lower than 5 fold, are involved in different metabolic pathways. Again, the known strains of S. commune seem to have a different cell metabolism.

Conspicuous is the protein kinase found with a higher abundance in strain 4-39. Protein kinases are essential for signal transduction, metabolism, movement, etc. Two main groups of protein kinases are serine/threonine and tyrosine kinases are known. They regulate protein phosphorylation and dephosphorylation, which lead to PMS. Protein kinases influence a high number of cellular functions in metabolism. The higher abundance of this protein in 4-39 could also accompany the different abundances of metabolic proteins in this strain (Park et al., 2011).

The transcriptome and proteome of S. commune monokaryons show differences in metabolic pathways. Identified genes and proteins do not correlate in detail. On the one hand it is not possible to synchronize transcripts and proteins. On the other hand the growth conditions of S. commune strains for both analyses differ in culture design and age of the cultures. For
proteome analysis, four days old liquid culture was used while for RNA-sequencing cultures grew on solid media for seven days. Differences between the biological replicates in both experiments were minimal. Thus, the number of replicates should not have an influence on the comparability of transcriptome and proteome of *S. commune*.

### 4.4. A protein map of *S. commune* will allow future analyses

The proteome of *S. commune* was analyzed on 2-dimensional protein gels. A wide pH-range was used to illustrate the cytosolic proteome. More than 900 protein spots were recognized on a gel of a monokaryotic strain of *S. commune*, but not all of them could be used for mass spectrometry because of their weak abundance in the gel. In total, 526 protein spots were analyzed, most of which were distributed at isoelectric point between 3 and 7. Alkaline proteins with their isoelectric point between 8 and 12 were rare.

A number of proteins with the same molecular weight, but differences in their isoelectric point, likely are caused by posttranslational modifications (PMS). Aside from glycosilation, acetylation and ubiquination, phosphorylation is the most common PMS (Glinski and Weckwerth, 2006). Phosphate groups are bound to serine or threonin residues (Leach and Brown, 2012). Indeed, mass spectrometry could show that these protein chains are formed by several copies of the same protein.

Not every selected protein was identified. Almost 1/5 of the analyzed proteins did not reach the threshold of 54.0. Nevertheless, the mascot score of these proteins was close to the threshold. Even after 4 repetitions of the mass spectrometry, there was no clear result for these spots. The reason rather is a bioinformatics problem related to the high sequence divergence between different strains discussed above. For creating the sequenced genome of *S. commune*, the monokaryotic strain H4-8 was used, which is also the basis for the database for identifying peptides (Ohm *et al*., 2010). The strain used for the proteomic studies is 12-43.

Only 14 spots gave no result. This could be the case, if the analyzed protein had a low concentration. Also, the genomic variation between strain 12-43 and H4-8 could lead to no result for some spots.

### 4.5. Differences associated with the dikaryotic proteome

During the change from a monokaryotic life phase to a dikaryotic stage in *S. commune*, the MAPK pathway has to be passed through. By pheromone recognition of a compatible mating-partner, a signal cascade is activated. In this process, the pheromone receptor interacts with
the pheromone and the G-protein, linked to the receptor, dissociates into subunits Ga and Gβγ. Consecutively, the MAPK is induced, where several protein kinases are involved. The expression of the transcription factor Ste12 induces the mating response (Raudaskoski and Kothe, 2010). The protein kinase is upregulated until the dikaryon formation takes place. After dikaryotization, the expression of the protein kinase decreases. The protein kinase found on the 2D-gel of the dikaryotic strain is approximately 28 fold decreased. A detailed annotation which protein kinase is annotated with the protein ID 1029853 is not given in the genome sequence of S. commune. Additionally, a cyclin-like F-box is downregulated in the dikaryon. This protein could possibly be a part of the transcription factor needed for the initiation of the mating response.

Again, several proteins of metabolic pathways show different abundance in the dikaryon compared to strain 12-43. This may lead to the assumption that dikaryotic strains have a changed metabolism, especially in glycolysis. For four glyceraldehyde-3-phosphate dehydrogenases (GPD), a higher abundance in the dikaryon was observed (Harmsen et al., 1992). Three spots were identified as GPDs which are located at the same molecular weight on the 2dimensional gel with only small switches of their position concerning their isoelectric point. PMS could cause these additional spots coding a GPD present in the dikaryotic proteom.

Possible hints on more differences on the genetic composition of a dikaryon could be delivered by transcriptome analysis. 2d-gelectrophoresis illustrates only a fraction of the genomic background of S. commune and is maybe not sensitive enough to capture all differences between two compared strains.

4.6. Outlook

With the present work, it was shown that the dynein heavy chain in the basidiomycete S. commune is encoded by two separate genes. The knock-out of either dhc1 or dhc2 is viable. For complementation of the investigations, a double knock-out mutant is necessary. Furthermore, other components of the cytoskeleton within the ∆dhc1, ∆dhc2 and ∆dhc1dhc2 strains should be investigated in more detail with microscopical techniques concerning possible changes in their cellular organization, structure or position.

Additionally, the deletion of kin-5 and/or kin-14 in the dynein heavy chain mutants could be interesting to show that viability of the dynein strains is dependent from other microtubule-associated minus-end transporters.
The co-localization of both genes in the cell was proven via immunofluorescence of *dhc1* or *dhc2*. To show a possible interaction, co-immunoprecipitation or FRET experiments should be performed. Also, possible interactions with other proteins could be shown this way. By labeling each gene with either GFP or RFP, life images of the dynein molecule can be taken. Possible co-localization of Dhc1 and Dhc2 can potentially also be seen also with this method.

RNA-sequencing data for three different wildtype strains (12-43, E6, T26) of the fungus are available now, allowing for comparison of differential expression of genes in new mutant strains. At the same time, differences in gene structure, intron distribution or intron abundance in other wildtype strains will be possible, as well as an optimized gene annotation with the existing genome sequence. Detailed analysis of the RNA-sequencing results should be done to find other interesting or strain specific genes of *S. commune*. To date, expression studies in *S. commune* strains were performed using RNA microarrays. The available data could be compared to RNA-sequencing to find either overlapping expression of elected genes, or differences and limitations of both methods.

Strain specific differences were shown for *S. commune* wildtypes on the proteome level, as well as an initial investigation of developmental stages. Proteome studies should be completed by analyzing the proteome at different stages of fruiting body formation, like primordia and fully developed fruiting bodies. Additionally, investigation of the *S. commune* proteome under different growth conditions and substrates including wood, or a comparison to the proteome of new mutant strains has now become possible with the proteome map.

The transcriptome-analysis gives hints that also the secretome of *S. commune* varies between monokaryotic strains. In addition to the first experiments on secretory proteins, more strains of *S. commune* should be included in the experimental design as well as growth of the strains on different media.

In general, the base for further analysis on the transcriptome and proteome of *S. commune* was established with the present work. This is helpful for new research approaches and the corresponding experimental designs within the work field of *S. commune*. 
**5.) Summary**

The split gill fungus *Schizophyllum commune* (*Agaricomycetes*) is a model organism in microbiological research. It is characterized by a tetrapolar mating-system leading to intensive studies on fungal development. The mating system is formed by two independent loci, *A* and *B*, each of which is composed of different subloci. Thus, more than 23,000 different mating types occur in nature. Furthermore, an efficient transformation protocol was developed for *S. commune*, allowing the molecular manipulation of the genome.

During the formation of the characteristic, dikaryotic, clamped mycelium of Basidiomycetes, a fast nuclear transport and exchange is necessary. This is assured by the motor protein dynein. Dynein molecules transport their cargo along microtubules, a component of the cytoskeleton. Crucial for the full function of the molecule is the dynein heavy chain, which converts chemical to mechanical energy and therefore enables the movement of dynein along its microtubule tracks. In higher basidiomycetes, dynein heavy chain is encoded by two genes – one N-terminal and one C-terminal part of the generally unipartite proteins in other fungi or throughout the eukaryote domain. In phylogenetic analyses, it could be shown that within the group of Basidiomycetes, three independent splits of the dynein heavy chain took place. In each split, a structural rearrangement of both genes occurred. The third split appeared within the Agaricomycetes, where the gene structure is highly conserved in all investigated fungi. In *S. commune*, a member of the Agaricomycetes, both genes could be isolated and were named *dhc1* and *dhc2*. Knock-out strains for both genes were created. The knock-out of *dhc1* is viable and shows different phenotypes, like truncated, spherical cells and a defect in nuclear migration and positioning. A knock-out of *dhc2* in *S. commune* is also viable, and the mutant shows phenotypical changes with a clear function in nuclear migration and nuclei positioning, which is overlapping to the function of *dhc1*. The viability of ∆*dhc2* is ensured by a higher expression of kinesin-14, a minus-end directed kinesin in fungi, which was proven via RNA-sequencing. With this method, transcriptome differences between two wildtypes were shown which were verified by proteome studies. Also differences between monokaryotic strains and a dikaryon could be shown.
6.) Zusammenfassung


7.) References


**Korn, E. D., 2000.** Coevolution of head, neck, and tail domains of myosin heavy chains. Proc Natl Acad Sci U S A. 97, 12559-64.


**Kothe, E., 1996.** Tetrapolar fungal mating types: sexes by the thousands. FEMS Microbiol Rev. 18, 65-87.


# Appendix

Table A1: Dataset of phylogenetic tree (representatives of Agaricomycotina are indicated as classes, for Wallemiomycetes no subphylum is assigned)

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Figure A1: Proof of the successful deletion of *dhc1* via nested PCR A) first PCR with the amplified target DNA band at approximately 7 kb; B) second PCR with the amplified 905 bp fragment of the deletion cassette. PCR was run several times, to get results for every strain.
Table A2: Detailed list of 22 genes which are differentially regulated in all 3 investigated strains.

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Table A3: 250 genes, differentially expressed in Δdhc2. Genes, which have a different expression in all 3 strains, are highlighted in grey.

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Table A4: 93 differentially regulated genes in 12-43 compared to E6. Listed are all genes with a log2(fold change) $<> 5$.

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**Beta-N-acetylhexosaminidase**
Carbohydrate transport and metabolism

**von Willebrand factor and related coagulation proteins**
Extracellular structures

**Methylase**
General function prediction only

**Myosin phosphatase, regulatory subunit**
Posttranslational modification, protein turnover, chaperones

**Predicted transporter (major facilitator superfamily)**
General function prediction only

**Transcription factor of the Forkhead/HNF3 family**
Transcription

**O-methyltransferase**
Secondary metabolites biosynthesis, transport and catabolism

**Predicted transporter (major facilitator superfamily)**
General function prediction only

**Multidrug resistance-associated protein/mitoxantrone resistance protein, ABC superfamily**
Secondary metabolites biosynthesis, transport and catabolism

**Nucleolar GTPase**
General function prediction only
| 1247302 | 5,687646979 | Mating pheromone activity |
| 17250 | 5,8543118 |
| 1105778 | 5,917275334 |
| 1093152 | 5,971440214 |
| 1149020 | 6,280503832 | Putative transcriptional regulator DJ-1 |
| 1210979 | 6,552858907 |
| 1081190 | 6,58651433 |
| 1030522 | 6,770785519 | L-kynurenine hydrolase |
| 49803 | 6,941659415 | Molecular chaperone (DnaJ superfamily) |
| 1124671 | 6,946614174 | O-methyltransferase |
| 1193121 | 7,052414769 |
| 1161353 | 7,097822803 |
| 1205547 | 7,258371076 | O-methyltransferase |
| 1161129 | 7,354812577 |
| 1314520 | 7,414224981 |
| 1163888 | 7,429931009 |
| 238931 | 7,478065869 |
| 1164524 | 7,512903021 | MYND Zn-finger and ankyrin repeat protein |
| 1257055 | 7,555379202 |
| 1194165 | 7,610052785 |
| 1108741 | 7,675688765 |
| 1163891 | 7,723253728 | Splicing coactivator SRm160/300, subunit SRm300 |
| 60526 | 7,777467936 | Mating pheromone activity |
| 1340215 | 7,789446875 |
| 83333 | 8,131854928 |
| 1238578 | 8,154026417 | Splicing coactivator SRm160/300, subunit SRm300 |
| 1176386 | 8,360473041 |
| 1098759 | 8,366547511 |
| 1136981 | 8,415377001 |
| 1260454 | 8,553534897 |
Mating pheromone activity
**Table A5:** List of proteins identified on the mastergel of strain 12-43. Spots number 58, 60, 97, 169, 181, 258, 363, 377, 425, 441, 471, 479, 481, 505 were not identified.

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35 1029475  Aminotransferase, class I and II
36 1034798  Zinc finger, CCCH-type
37 1053097  Aminotransferase, class I and II
38 1061621  GroES-related
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44 1182045  D-isomer specific 2-hydroxyacid dehydrogenase, catalytic region
45 1033906  D-isomer specific 2-hydroxyacid dehydrogenase, catalytic region
46 1033906  D-isomer specific 2-hydroxyacid dehydrogenase, catalytic region
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51 1030706  not annotated
52 1090946  Ketose-bisphosphate aldolase, class-II
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56 1034625  NAD(P)-binding
59 1036393  Aldo/keto reductase  Poorly characterized
61 1034625  NAD(P)-binding
62 1192436  Thiamine pyrophosphate enzyme, central region  metabolism
63 1037126  Protein synthesis factor, GTP-binding
65 1033509  Aldo/keto reductase  Poorly characterized
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Fumarate lyase
Citrate synthase-like
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Phosphoglycerate kinase
Citrate synthase-like
Citrate synthase, eukaryotic
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BTB/POZ fold (SKP1 component, dimerisation)
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Table A7: Different abundance of protein spots in the compared gels of strain 12-43 and 4-39. Green labeled spots have a lower abundance in strain 4-39, red labeled spots have a higher abundance compared to 12-43. If a protein has no spot number, they do not appear on the mastergel.

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Table A8: Different abundance of protein spots in the compared gels of strain 12-43 and the dikaryon. Green spots have a lower abundance in dikaryon, red spots have a higher abundance compared to 12-43. If a protein has no spotnumber, they do not appear on the mastergel.

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Selbständigkeitserklärung

Die vorliegende Dissertation habe ich selbständig verfasst und keine anderen als die von mir angegebenen Quellen, persönliche Mitteilungen und Hilfsmittel benutzt.


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Melanie Brunsch

Jena, den 09.10.2012