Membrane localization study of *Schizosaccharomyces pombe* proteins

Sdd1p, Sad1p and Cut11p.

Thesis for Bachelor’s Degree

Supervisor Tiina Tamm, Ph.D
ABBREVIATIONS
AP – alkaline phosphatase
BCIP – 5-bromo-4-chloro-3-indolyl phosphate
cMT – cytoplasmic microtubules
CP – central plaque
DMSO – dimethyl sulfoxide
DMF – dimethylformamide
DTT – dithiothreitol
ECL – enhanced chemiluminescence
EM – electron microscopy
EMM – Edinburgh minimal medium
GFP – green fluorescence protein
HA – hemagglutinin epitope
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMMT – hidden Markov model transmembrane analysis
HRP – horse radish peroxidase
IL – inner layer
IP – inner plaque
MT – microtubules
MTOC – microtubule-organizing center
NBT – nitro blue tetrazolium
NPD – non-parental ditype of tetrad
NE – nuclear envelope
nMT – nuclear microtubules
NPC – nuclear pore complex
OP – outer plaque
PBS – phosphate buffered saline
Pk – Pk epitope of SV5 P protein
PD – parental ditype of tetrad
PMSF – phenylmethanesulfonyl fluoride
SDS – sodium dodecyl sulphate
SPAS – sporulation agar with supplements
SPB – spindle pole body
SUN – Sad1-UNC-84 homology
TCA – trichloroacetic acid
TM – transmembrane
Triton X-100 – polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
TT – tetratype of tetrad
YES – yeast extract medium with supplements
INTRODUCTION

Spindle pole body (SPB) is microtubule-organizing center (MTOC) in yeast, which regulates the arrangements of microtubule filaments in the cell. The SPB is required for a variety of functions, including chromosome segregation during mitosis and meiosis, cytokinesis, cellular morphogenesis and intracellular trafficking. At presence, the best characterized SPB is from the budding yeast *Saccharomyces cerevisiae*. Many *S. cerevisiae* SPB components have homologues in fission yeast *Schizosaccharomyces pombe*, popular system for studying cell cycle and chromosome dynamics. The SPB functions and duplication pathway were studied using electron microscopy (EM) and various yeast mutants. Despite the abundance of information, a lot about SPB connection with membranes in both fission and budding yeasts is still not well understood.

Sdd1p, Sad1p and Cut11p are essential *S. pombe* proteins required for SPB functioning (Tamm *et al.*, 2007; Hagan and Yanagida, 1995; West *et al.*, 1998). They all have been predicted to be integral membrane proteins, but no biochemical studies were carried to confirm these predictions. Therefore the aim of this study was to confirm membrane localization of Sdd1p, Sad1p and Cut11p using biochemical approach and compare received data with known budding yeast homologues.

The author of this work is very grateful to supervisor, Tiina Tamm, for sharing her experience and knowledge. I would also like to thank all other colleagues for their support, warm atmosphere and general help.
1. REVIEW OF LITERATURE

1.1. Microtubule-organizing center (MTOC)

The MTOC is the organelle that manages the distribution of microtubule filaments in the cell. It includes such centers as basal bodies in algae, SPB in yeasts and centrosomes in higher eukaryotes. Nucleation of microtubules by eukaryotic MTOCs is required for a variety of functions, including chromosome segregation during mitosis and meiosis, cytokinesis, fertilization, cellular morphogenesis, cell motility, and intracellular trafficking.

1.2. Spindle pole body (SPB)

Because of the complex structure of the centrosome, many subsequent researchers have focused their studies on the functional equivalent of the centrosome in yeast, namely the SPB. At presence, the best characterized MTOC is the SPB from the budding yeast *S. cerevisiae*. Many components of this organelle have been identified using biochemical and genetic approaches and their arrangement within the SPB is determined (Jaspersen and Winey, 2004; Helfant *et al.*, 2002). Popular system for studying cell cycle and chromosome dynamics is fission yeast *S. pombe*. The tools and methods for fission yeast are very similar to those used in budding yeast. Both species of yeast have a “closed” mitosis, what means, that nuclear envelope (NE) doesn’t break down during cell division (De Souza and Osmani, 2007). Spindle formation and subsequent chromosome segregation occur within the nucleus. The SPB provides an opportunity to study an MTOC in which microtubules (MTs) are directionally ordered.

1.2.1. SPB structure in *S. cerevisiae*

The structure of SPB complex will be characterized on the example of *S. cerevisiae*, because its components and architecture at the moment are better understood. The EM (Byers and Goetsch, 1974), cryo-EM (Bullitt *et al.*, 1997) and electron tomography (O’Toole *et al.*, 1999) studies show, that the SPB is cylindrical multi-layered organelle (Figure 1). The first external layer is an outer plaque (OP), which connects to the cytoplasmic microtubules (cMT). Then the first intermediate layer (IL1) comes, followed by the second intermediate layer (IL2); a central plaque (CP), which is at the level of the NE and is connected to it by hook-like structures, an ill-defined inner plaque (IP); and a layer of the inner plaque that contains capped nuclear microtubules (nMT) ends (Bullitt *et al.*, 1997). The CP and IL2 appeared as
distinct but highly ordered layers (Bullitt et al., 1997). The other layers (MT ends, IP, IL1, and OP) do not show ordered packing (O’Toole et al., 1999).

One side of the central plaque is associated with a region of the NE termed the half-bridge (Figure 2). The half-bridge is also a multi-layered structure consisting of two continuous layers that mirror the membrane bilayers and an additional layer that lies on the cytoplasmic side of the half-bridge and is associated with the CP and IL2 at the edge of the SPB (O’Toole et al., 1999). Both sides of the half bridge are not equivalent.

**Figure 1.** An image of an isolated budding yeast SPB in amorphous ice. The layers of the SPB are indicated: OP, ILs, IL1, IL2, CP, IP1, IP2. The distal end of a broken MT is indicated as MTd. Inset: thin section of a yeast SPB after detergent extraction. Bar, 500Å. (from Bullitt et al., 1997)

### 1.2.2. SPB components

The molecular mass of a diploid SPB in budding yeast, including microtubules and microtubule-associated proteins is ca 1 – 1.5 GDa, a core SPB is 0.3 – 0.5 GDa (Adams and Kilmartin, 1999; Bullitt et al., 1997). Approximately 17 protein components have been identified and studied to date (Jaspersen and Winey, 2004). All SPB proteins can be divided into free groups: core components, half-bridge components and components needed for connection with NE (Figure 2).

There is no definite motif or structure, that makes a protein belong to SPB, but analysis of known SPB proteins and their genes shows several common features. The core contains mostly proteins with coiled-coil motifs, that allow to form dimmers, either with themselves or with others proteins (Wigge et al., 1998) and maintain regular structures (e.g. CP, IL2). Many SPB genes contain MluI cell cycle boxes in their promoter elements that lead to G1 specific
gene transcription (McIntosh et al., 1991). The primary sequence of SPB components should contain consensus phosphorylation sites for mitotic kinases, because the SPB is highly phosphorylated (Wigge et al., 1998).

*S. cerevisiae* SPB components Ndc1p and Mps3p and their *S. pombe* homologues Cut11p and Sad1p, respectively, will be characterized in more details in sections 1.3.1, 1.3.2, 1.3.3 and 1.3.4. Also budding yeast proteins Brr6p, Brl1p and their predicted homologue in fission yeast Sdd1p will be described in section 1.3.5.

**Figure 2.** Protein composition of *S. cerevisiae* SPB. The positions of SPB components within the organelle are illustrated along with nuclear and cytoplasmic microtubules (nMTs and cMTs) and the NE (from Jaspersen and Winey, 2004).

1.2.3. SPB duplication

*S. cerevisiae* SPBs are embedded in the NE throughout the yeast life cycle and are able to nucleate both nuclear and cytoplasmic microtubules (Buyers and Goetsch, 1974, 1975). Duplication of the SPB once, and only once, during each cell cycle is essential for formation of a bipolar mitotic spindle and accurate chromosome segregation. The SPB duplication pathway has been studied using EM and various SPB duplication mutants (Buyers and Goetsch, 1974, 1975). Despite that the duplication in budding yeast occurs in G1 phase of the
cell cycle, defects in SPB duplication aren’t detected until mitosis when cells fail to form a functional bipolar spindle.

SPB duplication in *S. cerevisiae* can be divided into several steps (Figure 3). The first step occurs early in G1, when satellite material forms on cytoplasmic tip of half-bridge (Byers and Goetsch, 1974, 1975). During the second step half-bridge elongates and completes its nuclear and cytoplasmic faces fusion (Adams and Kilmartin, 1999). In the same time satellite forms duplication plaque, a layered structure that is similar to the cytoplasmic half of a mature SPB (Adams and Kilmartin, 1999; Byers and Goetsch, 1974, 1975). The last step of SPB duplication is insertion of the duplication plaque into the NE and assembly of nuclear SPB components (Byers and Goetsch, 1974, 1975). At the end of G1 yeast cells contain two duplicated side-by-side SPBs connected by a complete bridge (Byers and Goetsch, 1974, 1975). Then bridge separates and SPB nucleates bipolar spindle. SPB continues to grow until mitosis, so protein components are able to incorporate into both SPBs throughout the cell cycle (Bullitt *et al.*, 1997).

The SPB duplication mechanisms in fission and budding yeast have a lot in common, but there are several different features, needed to be mentioned. The most important is that the SPB of *S. pombe* spends most of interphase in the cytoplasm, closely apposed to the NE (Tanaka and Kanabe, 1986). The SPB duplicates in the cytoplasm and the two resulting structures are connected by a bridge until the mitotic spindle forms (Figure 3). Information about time, when duplication appears, is controversial. Earlier reports suggest that *S. pombe* SPB duplication takes place at late G2 (Ding *et al.*, 1997). Later data disprove this and shows that SPB is actually duplicated before on G1/S boundary (Uzawa *et al.*, 2004). After duplication the SPB is associated with the cytoplasmic face of the NE. Different to budding yeast no spindle formation occurs during S-phase in fission yeast. As the cell enters mitosis, the NE invaginates beneath the SPB and forms an opening, or fenestra, into which the duplicated SPB settles (Uzawa *et al.*, 2004). Each part of the double SPB initiates intranuclear MTs. Then the two daughter SPBs separate to lie in distinct fenestrae, bound to the polar ends of the spindle MTs. As anaphase proceeds, the nuclear fenestrae closes, and the SPBs are extruded back into the cytoplasm. The movement of the SPB in and out of the NE during the cell cycle needs the presence of a membrane-anchoring system, but the mechanism for attaching the SPB to the NE has not yet described.

Duplication is a complicated process with many involved factors. Despite the abundance of information, our knowledge about duplication mechanism and regulation is limited. For
example, it is not known, how duplication plaque is inserted into nuclear membrane, how only single new SPB formation is controlled during cell division, how SPB growth is maintained and what kind of connections hold SPB embedded in NE.

Figure 3. Diagram of SPB duplication in budding yeast (a), fission yeast (b) (modified from Adams and Kilmartin, 2000).
1.3. *S. pombe* proteins studied during this work and their *S. cerevisiae* homologues

1.3.1. Ndc1p is a shared component of SPB and nuclear pore complex (NPC) in *S. cerevisiae*

*NDC1* is essential *S. cerevisiae* gene that encodes a protein with molecular weight of 74 kDa. *ndc1* conditional mutant strains segregate their entire chromosomal DNA to one spindle pole at the nonpermissive temperature, yielding one cell with double genome and another cell that contains no DNA (Winey *et al*., 1993). The large budded cell contains one SPB of normal appearance and a second one that is defective. The normal functional SPB is capable to nucleate microtubules on both its nuclear and cytoplasmic faces. The defective SPB is separated from normal SPB and has microtubules only on the cytoplasmic face. This phenotype suggests that *NDC1* product is required for insertion of newly formed SPB into its normal position in the NE after the satellite-bearing stage of SPB duplication. It also demonstrates that Ndc1p (for nuclear division cycle no. 1 protein) is not required either for separation of the SPBs or the subsequent formation of the mitotic spindle. Further mutant studies showed, that overexpression of *NDC1* also leads to SPB duplication defects indistinguishable from those observed on conditional mutants (Chial *et al*., 1999). Experiments with synchronized cells showed that *NDC1* gene activity is required during G1 for SPB duplication (Winey *et al*., 1993).

Fluorescence and immuno-EM studies confirmed Ndc1p localisation near SPBs during the whole cell cycle (Chial *et al*., 1998). Immunofluorescence microscopy staining showed for Ndc1p similar staining as for NPC component such as Nup49 (nucleoporin). Immunogold labeling was most often seen at the periphery of the NPCs and at the edges of the central plaque of the SPB (Chial *et al*., 1998). The localization of Ndc1p to both of these organelles implies a common mechanism for the assembly of NPCs and SPBs into the NE. These results suggest that NPC and SPB may be more similar than previously thought.

To determine the topology of Ndc1p two different strategies were chosen: sequence analysis and biochemical analysis.

Ndc1p structure (Figure 4) was predicted using hydropathy plots (Winey *et al*., 1993, Lau *et al*., 2006). The Hidden Markov Model Transmembrane (HMMT) analysis found six transmembrane (TM) domains in the N-terminal half (Lau *et al*., 2006). Although a plausible seventh TM domain was predicted previously from a Kyte-Doolittle hydropathy plot (Winey
Ndc1p was classified as a multipass signal anchor type II membrane protein with both N- and C-termini exposed to the cytoplasm (Lau et al., 2006).

Biochemical analysis included carbonate extraction of NE fraction and limited proteolysis (Chial et al., 1998; Lau et al., 2006). Samples from the NE fraction were extracted with carbonate followed by centrifugation to determine whether Ndc1p was found in the supernatant or in the membrane-containing pellet. Further Western blot analysis suggested that it is an integral membrane protein of the NE, because Ndc1p wasn’t extracted from membrane fraction. Using a strain containing a C-terminal myc-tagged Ndc1p, showed that the C-terminus was not protected after trypsin digestion (Lau et al., 2006). If the C-terminus was lumenal, it should have given an expected fragment of 45 kDa, which was not observed. This result suggests that the C-terminus is exposed to the cytoplasm. The orientation of the N-terminus wasn’t investigated, because epitope-tagging of Ndc1p at the N-terminus renders the cells nonviable (Lau et al., 2006).

Deletion mutations analysis showed that most of Ndc1p appears to be essential for its proper localization and functioning, except the region between amino acids 368 and 466 (Lau et al., 2004). Deleted region does not contain any of the predicted TM domains. One possible explanation is that most of the truncated proteins lack one or two of the predicted TM domains that are likely required for proper localization and orientation in the NE.

The mechanism by which Ndc1p anchors the SPB and NPCs during their assembly is currently not well understood. It is speculated that Ndc1p can interact with their components via its own cytoplasmic domain or TM domains (Lau et al., 2006). Ndc1p can also be

Figure 4. Predicted Ndc1p membrane topology, drawn not to scale. Numbers indicate amino acid positions (modified from Stavru et al., 2006).
important during outer and inner membrane fusion or stabilization, this step is required for both NPC and SPB insertion.

1.3.2. Cut11p is Ndc1p homologue in *S. pombe*

Cut11p (for cells untimely torn no.11 protein) is predicted to be Ndc1p homolog in *S. pombe* with molecular weight of 69 kDa. Similar to Ndc1p Cut11p is known to localise at NPC and SPB (West *et al.*, 1998). Cut11p plays an important role in assembly and insertion of both complexes. Cut11p has very similar topology to Ndc1p predicted by HMMT, the only difference is additional seventh TM domain (Lau *et al.*, 2006, West *et al.*, 1998). There are no regions of particularly high or low similarity between these two proteins, but their overall charge profiles are very similar. It is speculated, that additional TM domain is not inserted in membrane because charge analysis found positive charges on both sides of the potential seventh TM region of Cut11p (Lau *et al.*, 2006). It has been experimentally demonstrated that when a TM domain is spanned by charged regions, the potential TM domain is often not inserted into the membrane but is instead left out (Gafvelin *et al.*, 1997). Such proteins have been referred to as “frustrated” multi-pass proteins. If Cut11p is so called “frustrated” protein, then it will have six actual TM domains with C-termi exposed to the cytoplasm. Up to date any biochemical analysis data to confirm membrane localisation and N-, C-terminus arrangement is absent.

The cut11 mutant cells had some similarities in phenotype with ndc1 mutants. Temperature sensitive cut11 mutants failed in chromosome segregation (West *et al.*, 1998). These actions lead to aneuploidy and cell death. Lethality occurred as a result of an aberrant mitosis. SPBs either failed to complete normal duplication or were free floating in the nucleoplasm. These observations suggest that SPBs in these cells fail to anchor properly in the NE and at least one SPB fails to mature properly. The data also imply that nuclear MTs growth can be initiated in the absence of embedded SPBs.

Localization of Cut11p tagged with the GFP showed punctuate NE staining throughout the cell cycle and localization to SPBs from early prophase to mid anaphase (West *et al.*, 1998). This SPB localization correlates with the time in the cell cycle when SPBs are inserted into the NE (Ding *et al.*, 1997). Conjugating cells and mature asci lacked Cut11p staining at the SPBs, but cells in meiotic divisions showed SPB staining. Immuno-EM verified the localization of Cut11p to mitotic SPBs and NPCs (West *et al.*, 1998). This data suggests that Cut11p is an essential component of the mitotic SPB as well as a component of the NPC.
The SPB insertion in into NE during mitosis and SPB association with NE during interphase in *S. pombe* suggest that fission yeast needs two mechanisms to bind SPB to NE. Cut11p may be essential in one of them. How exactly Cut11p binds SPB components is not well understood. It is possible that binding occurs via C-terminus of protein and specially its seventh TM domain, which can interact with SPB or NPC components. A cell cycle-dependent change in Cut11p SPB binding also has a lot of questions.

1.3.3. Mps3p is a protein required for SPB duplication in *S. cerevisiae*

*MPS3* is an essential gene that encodes 79 kDa protein. *mps3-1* mutants arrest at the nonpermissive temperature in mitosis as large budded cells that contain a single, unduplicated SPB lacking any recognizable half-bridge structure (Jaspersen *et al.*, 2002, Nishikawa *et al.*, 2003). At the permissive temperature frequently was observed a mitotic delay, the core SPB was intact, but the half-bridge was smaller and indistinct, and no satellite structure could be detected (Jaspersen *et al.*, 2002, Nishikawa *et al.*, 2003). Based on this information it was concluded that *MPS3* is required for SPB duplication.

![Figure 5. The alignment between Mps3 and Sad1p. Acidic indicates acidic N-terminus, cc – coiled-coil domains, tm – transmembrane domains, pQ – poly-glutamine region, SUN – Sad1-UNC-84 homology) domains (from Jaspersen *et al.*, 2006).](image)

Analysis of Mps3p-GFP (for monopolar spindle no. 3 protein) localization in living and fixed cells showed perinuclear staining of GFP fluorescence with one or two bright dots at the ends of the spindle microtubule (Nishikawa *et al.*, 2003). Staining was observed during all cell cycle stages (Jaspersen *et al.*, 2002). Mps3p localization was also confirmed by immuno-EM (Jaspersen *et al.*, 2002). These results suggest that Mps3p is a NE protein and is enriched in the SPB. Unchanged staining with NPC clustered mutants suggested that Mps3p is not a component of the NPC (Nishikawa *et al.*, 2003). Experimentally is shown that *MPS3* function is required for the first step of SPB duplication in early G1 (Jaspersen *et al.*, 2002).

Mps3p has several domains (Figure 5): an acidic N-terminus, a TM segment, two coiled-coil domains, a poly-glutamine region, and a SUN (Sad1-UNC-84 homology) domain (Jaspersen...
et al., 2002; Nishikawa et al., 2003). SUN domain-containing proteins are a family of inner NE proteins (Hagan and Yanagida, 1995; Malone et al., 1999). SUN proteins are anchored in the inner NE by their TM segment. The C-terminal SUN domain is located in the space between the inner and outer nuclear membranes (Starr and Fischer, 2005). Protease protection and protein binding experiments demonstrated that the N-terminus of Mps3 is exposed to the cytoplasm/nucleoplasm, whereas the C-terminus, including the SUN domain, is located in the space between the inner and outer nuclear membranes (Nishikawa et al., 2003; Jaspersen et al., 2006). Flow cytometric analysis of DNA content and budding index confirmed that all of the Mps3p SUN domain mutants arrest in mitosis with a monopolar spindle (Jaspersen et al., 2006). The nucleoplasmic N-terminal acidic domain of Mps3 is not essential for viability (Nishikawa et al., 2003, Bupp et al., 2007).

Biochemical studies showed that Mps3p is extracted from membranes only in buffers containing detergent (Jaspersen et al., 2002; Nishikawa et al., 2003). Mps3p was detected in the pellet fraction that contains the cellular membranes. The fact, that Mps3p could only be extracted from the pellet in the presence of the detergent, indicates that Mps3p is an integral membrane protein.

1.3.4. Sad1p is Mps3p homologue in S. pombe

The sad+ (for spindle architecture disrupted) is an essential gene that encodes a protein with a molecular mass of 58 kDa (Hagan and Yanagida, 1995). Sequence analysis predicted a potential membrane-spanning domain and an acidic N-terminus (Figure 5). It also contains Sad1-UNC-like C-terminal and coiled-coil domain. Unfortunately, there is no biochemical data that can confirm or disprove computational predictions and clarify protein topology.

Both temperature sensitive and deletion mutants were unable to form a normal mitotic spindle or others formed spindles on which chromosomes failed to separate (Hagan and Yanagida, 1995). Some chromosomes fail to associate with the spindle. Due to the absence of EM studies the exact mutant defects are uncharacterized.

Immunofluorescence microscopy studies showed, that Sad1p exclusively associated with the SPB throughout the mitotic and meiotic cycles (Hagan and Yanagida, 1995). Dependent upon cell cycle stage, one or two dots were seen on the nuclear periphery. Generally in interphase, when microtubules are entirely cytoplasmic, a single dot was always associated with a cytoplasmic microtubule, while mitotic cells always contained two dots, one at either end of the spindle, the location of the SPB (Hagan and Yanagida, 1995). Moderate overexpression of
Sadlp resulted in its localization to the nuclear periphery (Hagan and Yanagida, 1995), what is typical to nuclear membrane proteins. All this data taken together indicates that Sadlp is NE protein associated with the SPB. Sadlp may play a role in SPB structure, such as maintaining a functional interface with the nuclear membrane or in providing an anchor for the attachment of microtubule motor proteins.

1.3.5. Brr6p, Br11p - membrane proteins required for nuclear transport and their homolog in fission yeast, Sdd1p

*S. cerevisiae* has two essential genes *BRR6* and *BRL1*, mutations in both cause accumulation of mRNA and proteins containing nuclear export signal in the nucleus. *BRR6* encodes a protein with molecular mass of 22.8 kDa. The Brr6p (for bad response to refrigeration protein) was predicted to have two putative TM domains (Figure 6) (de Bruyn Kops and Guthrie, 2001). *BRL1* encodes a protein with estimated molecular mass of 53.4 kDa. Br11p (for Br6 like protein no. 1) is homologous with Brr6p at the C-terminal domain, which is well conserved in the Brr6/Br11 proteins family and in eukaryotes that undergo a closed mitosis (Saitoh *et al.*, 2005). Br11p also contains two TM domains (Figure 6). Series of temperature-sensitive mutants of Br11p showed that all brl1 mutations were localized to the conserved C-terminal domain that is essential for a function of Br11p. In contrast to the Brr6/Br11 domain, the N-terminal region is not conserved (Saitoh *et al.*, 2005). The experiments with truncated *BRL1* fragments, which were generated by stepwise deletion of the *BRL1* gene from the N-terminus, showed that deletion till 193rd amino acid in *brl1-193* still can complement lethal phenotype of the *brl1A* strain (Saitoh *et al.*, 2005). Visualization by fluorescence microscopy of GFP-tagged Brr6p (de Bruyn Kops and Guthrie, 2001) and Br11-GFP (Saitoh *et al.*, 2005) reveals a predominantly nuclear rim distribution. The Brr6-GFP rim staining pattern was punctuate, similar to that seen with NPC components suggesting that Brr6p and Br11p may be located at or near pores.

Brr6p membrane localization and topology were confirmed biochemically. Brr6-GFP remained associated with the cell pellets in samples extracted under low salt, high salt or high pH conditions but was efficiently extracted in low salt buffer containing detergent (de Bruyn Kops and Guthrie, 2001). Br11p biochemical studies showed the similar results (Saitoh *et al.*, 2005). This is typical for integral membrane proteins. The membrane topology of Brr6p was established using trypsin digestion in presence or absence of detergent. The C-terminal Brr6p fragment was protected from trypsin digestion in the absence but not in the presence of detergent (de Bruyn Kops and Guthrie, 2001). This data suggest that Brr6p is anchored in the
membrane via a predicted TM domain identified at the C-terminus. The C-terminal part is extending into NE lumen and N-terminus is located outside. Attempts to determine the orientation of Br1lp in the NE lumen were unsuccessful (Saitoh et al., 2005). Although it is not clear yet whether Br1lp is localized in the outer or inner nuclear membrane, the idea that the Brr6p/Br1lp complex functions in accelerating nuclear export is likely. The genetic analysis suggested that Br1lp functionally interacts with Br6r6p. An interaction of Br1lp with Br6r6p was confirmed by the two-hybrid method (Saitoh et al., 2005). One of possibilities is that Br1lp and Br6r6p make a single complex that corresponds to SpBr1l.

**Figure 6.** The alignment between Br1lp, Sdd1p and Br6r6p (from Saitoh et al., 2005). The gray regions indicate a highly homologous Br6r6/Br1lp domain at C-terminus. The black regions shows putative TM domains.

Sequence analysis showed that while *S. cerevisiae* has two Br6r6/Br1lp family proteins, both of which are essential for growth, *S. pombe* has a single Br6r6/Br1lp family protein, called SpBr1lp. Highly related molecules exist in all fungal genomes that have been sequenced to date (Tamm et al., 2007, Presti et al., 2007). It is noticeable that the size of the protein in br1lp-193 mutant is similar to that of SpBr1lp (Saitoh et al., 2005).

To test whether SpBr1lp fulfils the functions of both, Br1lp and Br6r6p, the *SpBRL1* gene was introduced into either the *br1lA* or the *brr6A* strain (Saitoh et al., 2005). The *SpBRL1* gene rescued the lethality of both *br1lA* and *brr6A* strains. This finding is remarkable and suggests that both Br1lp and Br6r6p might comprise a complex that corresponds to SpBr1lp. In addition SpBr1lp also showed interaction with itself in two-hybrid method (Saitoh et al., 2005).

Lately was found that SpBr1lp have an additional functions in *S. pombe*. *BRL1* gene was isolated during functional screen in *S. pombe* to isolate dimethyl sulfoxide (DMSO) sensitive mutants showing the defects in spindle formation and the mutant was called *sdd1-ds1* (Tamm et al., 2007). The aim of this screen was to identify new genes involved in the control of
mitotic spindle function. *sddl-ds1* mutants was unable to form a spindle in rich media in the presence of 4% DMSO.

Sdd1p (for spindle DMSO defect no. 1 protein) is an essential molecule that is required for the SPB insertion into the NE (Tamm et al., 2007, Presti et al., 2007). Sdd1p is a protein with molecular weight of 33.5 kDa, containing two putative TM domains at the C-terminal part of the protein. Sdd1p localises to the NE throughout the cell cycle and associates with the SPB from early prophase to mid-anaphase (Tamm et al., 2007). The Sdd1p function in SPB insertion into the NE is unknown. The localization study results indicate that Sdd1p is most likely a membrane component concentrated at the NE. As such, Sdd1p could be either a peripheral membrane protein, associated with the envelope via interactions with nucleoporins or other envelope-associated proteins, or an integral membrane protein. To date the predicted topology isn’t studied by any biochemical experiments.
2. EXPERIMENTAL PART

2.1. MATERIALS AND METHODS

2.1.1. Used strains, the media and growth-conditions

Used *S. pombe* strains are listed in table 1.

**Table 1. *S. pombe* strains**

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<th>Strain name</th>
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<td>h-</td>
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<td>sdd1PkN:ura cut11.6HA:kan^R ade6.M216 leu1.32 ura4.d18</td>
<td>Sdd1p and Cut11p extraction profile</td>
<td>This study</td>
</tr>
<tr>
<td>TYSP 146</td>
<td>h+</td>
<td>sdd1PkN:ura cut11.6HA:kan^R ade6.M216 leu1.32 ura4.d18</td>
<td>Sdd1p and Cut11p extraction profile</td>
<td>This study</td>
</tr>
<tr>
<td>TYSP 157</td>
<td>h-</td>
<td>nda3.KM311 leu1.32</td>
<td>Tubulin extraction profile</td>
<td>Hiraoka et al., 1984</td>
</tr>
</tbody>
</table>

*S. pombe* cells were grown aerobically at 25°C or 30°C using yeast extract medium with supplements (YES) (Table S1). TYSP 157 strain was grown at 32°C in YES and then shifted to 18°C for six hours to obtain the phenotype. Edinburgh minimal medium (EMM) (Table S2, S3) was prepared for auxotrophic growth. Appropriate supplements, amino acids, adenine, and uracil, were added to a final concentration of 75 mg/l. For mating the sporulation agar with supplements (SPAS) was used (Table S4). Leucine, uracile, and adenine were added as supplements to final concentration 45 mg/l.
2.1.2. The construction of *S. pombe* strains: crosses and tetrad analysis

*S. pombe* strains (TYSP 43, TYSP 44, TYSP 109, TYSP 110, TYSP 112, TYSP 115) were grown on solid YES medium for 2 – 3 days at 30°C.

The following crosses were made:

TYSP 43 x TYSP 109  
TYSP 43 x TYSP 115  
TYSP 44 x TYSP 110  
TYSP 44 x TYSP 112

A toothpick full of cells from the first strain from a pair (*e.g.* TYSP 43) was taken and a small patch on a mating plate was made. An equivalent amount of the second strain cells (*e.g.* TYSP 109) was taken and added to the previous patch, mixed well. A drop of sterile distilled water was added and the cells were mixed thoroughly on the agar plate. Mating plates were incubated at 25°C for 2 – 3 days. After 3 days plates were monitored microscopically for formation of asci.

Asci were placed in a line apart on a YES plate, using a micromanipulator (MSM systems, Singer instruments). The ascus walls were then left to break down at 36°C for about 4 – 5 hours. Each ascus was then micromanipulated to give a line of four isolated spores, separated by about 5 – 10 mm. The spores were incubated until colonies formed at 30°C. Then each plate with crosses was replica plated to YES, YES with geniticin (200 mg/l, G-418 sulphate, PAA Laboratories GmbH), EMM without uracil, leucine or adenine plates for selective growth at 30°C for 2 days. After 2 days, the colonies with appropriate genotype were selected. For making glycerol stocks the picked strains were grown in liquid YES medium at 30°C till the stationary growth phase. Precipitated cells were resuspended in YES with 30% glycerol. Strains were stored at -80°C until use.

2.1.3. Cell counting

Instead of optical density, *S. pombe* cells were counted by haemocytometer during all procedures. Neubauer-chamber (0.100 mm N 8105, 1/25 mm²) and an Olympus CX 31 microscope at 10x40 magnification were used for counting cells. Per each sample, 6 big squares were counted and an average value of X was calculated. For calculation of cell number the following equation was used: $X \cdot 25 \cdot 10^6 \cdot 100 = \text{cells/ml}$. 
2.1.4. TCA protein precipitation

$5 \cdot 10^7$ cells per sample in mid-exponential growth phase were harvested by centrifugation at 3,200 rpm for 3 minutes at room temperature (Hettich zentrifugen, Universal 32R, 1617). Supernatant was removed and cells were resuspended in distilled water to wash out the media. After that, the cells were centrifuged for 15 seconds at room temperature, at 13,400 rpm (Eppendorf minispin, F45-12-11). Supernatant was discarded. All the following procedures were carried either on ice or at 4°C. 200 µl of 20% TCA and 500 µl of glass beads were added. Cells were broken for 2 minutes by the „Disruptor genie” vortex mixer. Then 500 µl of 5% TCA were added. Cell suspension was centrifuged for 2 minutes at 2,100 rpm (Hettich zentrifugen, Universal 32R, 1617) to separate glass beads. Suspension was centrifuged at 14,000 rpm for 5 minutes (Hettich zentrifugen, Micro 200R, 2424), supernatant was removed. The sample was suspended in 70 µl 1xSDS (12 mM Tris-HCl (pH 6.8), 4.8% glycerol, 0.4% SDS, 2.88 mM DTT, 0.2% bromophenol blue) and 30 µl 1M Tris-HCl pH 8.0. Samples were stored at -80°C until use.

2.1.5. Protein extraction

A protocol for membrane protein extraction was taken from de Bruyn Kops et al. (2001) and Saitoh et al. (2005) and optimized during this study. *S. pombe* cells were grown in YES medium to obtain mid-exponential growth phase and harvested ($10^8$ cells per sample) by centrifugation at 3,200 rpm for 3 minutes at room temperature (Hettich zentrifugen, Universal 32R, 1617). Medium was removed, pelleted cells were resuspended in 1 ml of lysis-extraction buffer (20 mM HEPES pH 7.9, 150 mM NaCl, 1 mM EDTA) to wash out the media. To each extraction buffer protease inhibitors were added: aprotinin (1.8 µg/ml), leupeptin (5 µg/ml), pepstatin (5 µg/ml), PMSF (10 µM). Cells were centrifuged for 15 seconds at 13,400 rpm (Eppendorf minispin, F45-12-11) at room temperature. Supernatant was eliminated. 500 µl of glass beads and 250 µl of buffers with different extraction conditions were added (Table 2). For each buffer 2 samples were prepared and finally combined together. The following procedures were conducted under different temperature conditions that depended on buffer characteristic (Table 2). Cells were disrupted for 8 minutes with “Disruptor genie” vortex mixer in order to obtain the maximal break of cells. Then, to separate glass beads, suspension was centrifuged for 2 minutes at 2100 rpm (Hettich zentrifugen, Universal 32R, 1617). Cells were newly resuspended and centrifuged for 5 minutes at 1,600 rpm 5 minutes (Hettich zentrifugen, Micro 200R, 2424) to remove the unbroken ones. Supernatants were transferred to fresh tube. Samples were incubated for 20 minutes. Afterwards, samples (400 – 450 µl)
were pelleted in an ultracentrifuge (Beckman coulter, Optima L-90K, 70.1 Ti) for 1 hour at 32,000 rpm. Pellet and supernatant were resuspended with 5xSDS buffer in an appropriate ratio, stored at -80°C. All samples were analysed by Western blot analysis, TCA protein denaturation sample (see section 2.1.4.) of the same cell culture was used as a positive control.

Table 2. The composition of extraction buffers

<table>
<thead>
<tr>
<th>Buffer composition</th>
<th>Final concentration in lysis buffer</th>
<th>Purpose</th>
<th>Procedure temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis buffer</td>
<td>-</td>
<td>Isolation of soluble proteins</td>
<td>4°C</td>
</tr>
<tr>
<td>Lysis buffer + NaCl</td>
<td>1 M</td>
<td>Solubilizes peripheral membrane proteins</td>
<td>4°C</td>
</tr>
<tr>
<td>Lysis buffer + Na₂CO₃</td>
<td>0.1 M</td>
<td>Solubilizes intracellular vesicles</td>
<td>4°C</td>
</tr>
<tr>
<td>Lysis buffer + Triton X-100</td>
<td>4%</td>
<td>Non-ionic detergent, solubilizes most membrane proteins</td>
<td>room temperature</td>
</tr>
<tr>
<td>Lysis buffer + SDS</td>
<td>2%</td>
<td>Ionic detergent, solubilizes all membrane proteins</td>
<td>room temperature</td>
</tr>
<tr>
<td>Lysis buffer + Urea</td>
<td>1.6 M</td>
<td>Solubilizes peripheral membrane protein</td>
<td>4°C</td>
</tr>
</tbody>
</table>

2.1.6. Western blot analysis

The protein samples were separated by mass, using 10% polyacrylamide gel (Simpson, 2003). The protein samples were denatured for 5 minutes at 95°C. Samples were centrifuged for 1 minute at 13,400 rpm (Eppendorf minispin, F45-12-11). 12 – 18 µl of protein samples were loaded. 3 µl of protein prestained marker (Prestained Protein Marker Molecular Weight, Fermentas) were used to control mass. Gel was run for 3 hours at room temperature with 90 V. The gel was placed in protein transfer buffer (25 mM Tris, 193 mM glycine, 0,037% SDS with 10% methanol). The transfer to the nitrocellulose membrane (Hybond ECL nitrocellulose membrane, GE Healthcare) was carried out for 45 minutes at 15 V, using Fastblot 44B semi-dry blotter (Biometra).

The blocking of a non-specific binding was achieved by placing the membrane in a blocking solution (3% low-fat milk powder in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.3) for 1 hour at room temperature. The membrane was incubated with primary antibody overnight at 4°C.
Table shows a list of used antibodies and their dilutions. The membrane was properly washed during 1 hour with 6 changes of the washing buffer (PBS) at room temperature. If secondary antibody was used, the membrane was incubated for 2 hours with it at room temperature. The membrane was washed with PBS. Afterwards, proteins were visualised using alkaline phosphatase (AP) or enhanced chemiluminescence (ECL) detection systems.

For protein visualization with ECL commercial (Super Signal West Pico Chemoluminescent Substrate, Pierce) or a self-made substrate was used. For the self-made substrate two solutions were mixed on total volume of 3 ml: solution A (30 µl of luminol stock, 13.2 µl of p-coumaric acid stock, 300 µl of 1M Tris-HCl pH 8.5) and solution B (300 µl of 1 M Tris-HCl pH 8.5, 1.83 µl of 30% H2O2). The membrane was incubated in solution for 4 minutes. The membrane was exposed in darkness with sensitive surface of Fuji Medical X-ray Film.

Table 3. List of antibodies used for detection

<table>
<thead>
<tr>
<th>Protein</th>
<th>Primary antibody</th>
<th>Secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pk-Sdd1</td>
<td>Anti-Pk (1:100 in 3% low-fat milk in PBS), Southern et al., 1991, Hanke et al., 1992</td>
<td>The goat anti-mouse IgG (whole molecule) - alkaline phosphatase, (dilution 1:15,000 in PBS), Sigma</td>
</tr>
<tr>
<td>Cut11-HA</td>
<td>Anti-HA-HRP (1:2,000 dilution in 3% low-fat milk in PBS), Santa Cruz Biotechnology</td>
<td>-</td>
</tr>
<tr>
<td>12-CA5 Anti-HA (1:200 in 1% low-fat milk in PBS), Field et al., 1988</td>
<td>The goat anti-mouse IgG (whole molecule) - alkaline phosphatase (dilution 1:15,000 in PBS), Sigma</td>
<td></td>
</tr>
<tr>
<td>12-CA5 Anti-HA (1:200 in 1% low-fat milk in PBS), Field et al., 1988</td>
<td>The goat polyclonal antibody (IgG-HRP) to mouse IgG – peroxidase(1:5,000 in PBS), Quattromed</td>
<td></td>
</tr>
<tr>
<td>Sad1-HA</td>
<td>12-CA5 Anti-HA (1:200 in 1% low-fat milk in PBS), Field et al., 1988</td>
<td>The goat anti-mouse IgG (whole molecule) - alkaline phosphatase, (dilution 1:15,000 in PBS), Sigma</td>
</tr>
<tr>
<td>12-CA5 Anti-HA (1:200 in 1% low-fat milk in PBS), Field et al., 1988</td>
<td>The goat polyclonal antibody (IgG-HRP) to mouse IgG – peroxidase(1:5,000 in PBS), Quattromed</td>
<td></td>
</tr>
<tr>
<td>Alpha-tubulin</td>
<td>TAT1 (1:250 in 3% low-fat milk in PBS), from Dr. Keith Gull</td>
<td>The goat anti-mouse IgG (whole molecule) - alkaline phosphatase, (dilution 1:15,000 in PBS), Sigma</td>
</tr>
</tbody>
</table>
For AP detection 33 µl of NBT (4-nitro blue tetrazolium chloride, 50 mg/ml stock in 70% DMF) and 66 µl of BCIP (5-bromo-4-chloro-3-indolyl-phosphate, 50 mg/ml stock in 100% DMF) were mixed in 10 ml AP buffer (100 mM NaCl, 5 mM MgCl₂, 100 mM Tris-HCl pH 9.5). The membrane was immediately placed in the mixture. The reaction was carried in darkness for several minutes, until protein bands were clearly distinguished. The reaction was stopped with distilled water.

2.1.7. Images and processing

Images were captured by Olympus C-765 camera, Microtech ScanMaker 8700 scanner and processed with the use of Abode Photoshop (Adobe Systems Incorporated).
2.2. RESULTS AND DISCUSSION

2.2.1. The construction of *S. pombe* strains

New strains were constructed with purpose to use same protein extraction samples for 2 protein analysis. This approach gives more reliable data, because it can be seen how two different proteins act during identical experimental conditions.

Fission yeast is generally haploid, but under starvation conditions strains with different mating types conjugate. The mating cells will form transient diploids and then proceed directly into meiosis. Three types of tetrad are normally produced: the parental ditype (PD) if there is no recombination, the tetratype (TT) if there is single crossing-over (*i.e.* two recombinants are produced per ascus) and the non-parental ditype (NPD) if there is double crossing-over (*i.e.* four recombinants are produced) (Figure 7). Using two strains of interest with different marker genes, new one with characteristic from both parents can be produced.

![Figure 7. The example of tetrad analysis from TYSP44 x TYSP112 cross. Rows with letters a – d indicate colonies formed from one ascus. Numbers 1 – 4 indicate recombinants ordinal numbers. Right row shows type of produced tetrad: PD – parental ditype, NPD – non-parental ditype. YES, YES + G418 (geneticin), EMM –URA are media used for selection. Colonies 1a, 1b and 2d can be chosen as strains containing both Pk-Sdd1 and Cut11-HA.](image)

TYSP43 and TYSP44 strains contain Sdd1p with N-terminal Pk-tag linked with *ura4*+ auxotrophic marker gene. According to strains genotype, they will grow on EMM without uracil. TYSP109 and TYSP110 strains contain Sad1p with C-terminal HA-tag linked with geneticin resistance gene (*kan*^R^). TYSP112 and TYSP115 strains contain Cut11p with C-terminal HA-tag linked with geneticin resistance gene (*kan*^R^). TYSP1109, TYSP1110, TYSP1112 and TYSP1115 will grow on YES with geneticin and were not able to grow on EMM without uracil. All proteins are expressed under endogenous promoter.
Strains with different mating type were crossed and tetrad analysis was carried out as described in section 2.1.2. Grown colonies were replica plated on following plates: YES, YES with geniticin, EMM without uracil, EMM without adenine and EMM without leucine.

Only colonies with ura4+ auxotrophic marker gene will grow on EMM without uracil. If cells do so, they contain Pk-Sdd1 because it is linked with ura4+ auxotrophic marker gene. Only colonies with geniticin resistance gene will grow on YES with geniticin. Cells growing on this medium will contain Cut11-HA or Sad1-HA, because these genes are linked with geniticin resistance gene. If the same colony grows on both YES with geniticin and EMM without uracil plates, then it contains both genes of interest.

After replica plating the colonies growing both on YES with geniticin and EMM without uracil, but not growing on EMM without adenine and leucine, were selected (Figure 7). As a result the following S. pombe strains were isolated: TYSP139 and TYSP140, containing both Pk-Sdd1 and Sad1-HA; TYSP 141 and TYSP146, containing both Pk-Sdd1p and Cut11p-HA.

2.2.2. Optimization of protein detection with antibodies

The optimization of protein detection was needed for control of protein expression in created S. pombe strains and for reliable analysis of protein extraction procedures. TCA protein precipitation samples of strains TYSP 139, TYSP 140, TYSP 141, TYSP 146, TYSP 157 were separated on SDS-PAGE and then transferred to nitrocellulose membrane (Section 2.1.6.). For α-tubulin detection monoclonal anti-α-tubulin TAT1 antibody was used, which recognizes both α-tubulins, Nda2p and Atb2p (Figure 8). The goat anti-mouse antibody conjugated with AP was used as secondary antibody. Staining gave well distinguished bands that closely migrates at 55 kDa for Atb2p and 57 kDa for Nda2p as reported in Radcliffe et al. (1998) (Figure 8). Thus this method was chosen for further α-tubulin analysis.

For Pk-Sdd1p detection anti-Pk primary monoclonal antibody was used, which gave well distinguished bands, if secondary antibody was the goat anti-mouse antibody conjugated with AP (Figure 9.). Cut11p and Sad1p have HA-tag on their C-terminus therefore 12-CA5 anti-HA was used as primary antibody, goat anti-mouse antibodies conjugated with AP or peroxidase were tested as secondary antibodies. Sad1p-HA was well detected with both secondary antibodies and for further analysis was chosen AP method and TYSP 140 strain. Cut11p-HA was weakly detectable with AP system, antibody conjugated with peroxidase gave no staining at all. Therefore for Cut11p-HA analysis another anti-HA antibody conjugated with horse radish peroxidase (HRP) was tested (Figure 8). The better sensitivity of
this method will allow further analysis of Cut11p in TYSP146 strain. Sad1p-HA and Cut11-HA are seen on gel with greater molecular weight that predicted. This observation for Sad1p was expected because the altered mobility on SDS-PAGE was previously reported (Hagan and Yanagida, 1995). This phenomenon may be caused by protein phosphorylation.

![Figure 8. The result of Western blot analysis of TCA precipitation samples. (A) TCA precipitation samples from strains TYSP140, TYSP141 and TYSP146 cell lysate. Anti-HA-HRP antibody and ECL detection system was used for recognition. (B) TCA precipitation samples from strain TYSP157 cell lysate. TAT1 antibody and AP detection system was used for recognition.](image)

2.2.3. Protein extraction

The localization study results indicate that Sdd1p and Sad1p are most likely a membrane component concentrated at the NE (Tamm et al., 2007; Hagan and Yanagida, 1995). As such, Sdd1p and Sad1p could be either a peripheral membrane protein, associated with the NE via interactions with membrane proteins, or integral membrane proteins.

One of the characteristics that distinguish integral from peripheral membrane proteins is their relative solubility under different extraction conditions. While peripheral membrane proteins can be readily removed by treatment with high ionic strength or high pH, solubilization of integral membrane proteins requires detergent to disrupt lipid-lipid and lipid-protein interactions (Table 2). Therefore the ability of Na$_2$CO$_3$, urea, Triton X-100, SDS and NaCl to solubilize Sdd1p and Sad1p was tested (Section 2.1.5.).
The extraction profile of Pk-Sdd1 and Sad1-HA from TYSP140 cell lysate. Buffer conditions are indicated in upper row. Pellet (P) and supernatant (S) fractions are shown in different columns. Nitrocellulose membrane was horizontally cut and incubated with different antibodies. Anti-Pk antibody for Pk-Sdd1 recognition and 12-CA5 anti-HA for Sad1-HA recognition was used. Visualized with AP system.

The result of the extraction experiment is presented in figure 9. Sdd1p as well as Sad1p aren’t extracted from membrane fraction by lysis-extraction buffer solution, Na$_2$CO$_3$, urea, NaCl and even Triton X-100. Sdd1p and Sad1p require ionic detergent, such as SDS, to disrupt interactions with membrane. These data come to an agreement with biochemical studies results with S. cerevisiae homologues Brr6p, Brl1p and Mps3p. Mps3p was extracted from membranes only in buffers containing 1% Triton X-100 or 1% Triton X-100 with 1 M NaCl (Jaspersen et al., 2002; Nishikawa et al., 2003).

The extraction profile of α-tubulin from TYSP157 cell lysate. Buffer conditions are indicated in upper row. Pellet (P) and supernatant (S) fractions are shown in different columns. TAT1 antibody and AP system was used.
Brr6-GFP was efficiently extracted only in low salt buffer containing 4% Triton X-100 (de Bruyn Kops and Guthrie, 2001). Brl1p was extracted from membranes only in buffer with 150 mM NaCl and 4% Triton X-100 (Saitoh et al., 2005). All this data agree with sequence analysis predictions and suggest that Sdd1p and Sad1p integral membrane proteins.

As a control, for this experiment was chosen α-tubulin. α-tubulin with β-tubulin together form microtubule filaments. Despite that cold sensitive mutant TYSP 157 was used, where microtubules are depolymerised after 6 hour at 18°C, Western blot analysis showed similar extraction profile as for Sdd1p and Sad1p. Therefore control protein wasn’t chosen properly. In the future investigation other control protein is needed. It may be a confirmed soluble cytoplasmic protein, that doesn’t polymerise and form any complex structures. In future, the extraction of Cut11p is needed to be optimized. We also plan to study Sdd1p, Sad1p and Cut11p N- and C-terminus topology using trypsin digestion procedure. The unanswered question is: do Sdd1p, Sad1p and Cut11p interact with each other? If they interact, then in which way? It can be studied using yeast two-hybrid system optimized for detection of interactions between membrane proteins.
SUMMARY

During this study new *S. pombe* strains were constructed using crosses and tetrad analysis. These strains contain pairs of tagged proteins: Pk-Sdd1 and Cut11-HA, Pk-Sdd1 and Sad1-HA, respectively. The optimization of Sdd1p, Cut11p, Sad1p and α-tubulin detection using different antibodies and visualization by ECL and AP systems was carried out.

The aim of this study was to confirm predicted membrane localization of Sdd1p, Sad1p and Cut11p using biochemical approach. Therefore the ability of Na$_2$CO$_3$, urea, Triton X-100, SDS and NaCl to solubilizes Sdd1p and Sad1p from membrane fraction was tested. Given proteins were able to solubilize only in presence of high ionic detergent. This biochemical data agrees with sequence analysis predictions and confirms that Sdd1p and Sad1p are integral membrane proteins.
**Schizosaccharomyces pombe** valkude Sdd1p, Sad1p ja Cut11p membraanides paiknemise uurimine.

Olga Jasnovidova

**RESÜMEE**

Mikrotorukesi organiseeriv keskus (MTOC) on organell, mis reguleerib mikrotorukeste jaotust rakus. Pärmdes on selliseks MTOC-kse pärmi tsentrosoom, mida kutsutakse käävi polaarsuse kehaks e. *spindle pole body* (SPB).


Eksperimentaalne töö käigus konstrueeriti uued *S. pombe* tüved kasutades ristamist ja tetraadide analüüsi. Uued tüved sisaldavad kahte epitoopidega märgistatud valku: Sdd1, mille N-terminuses paikneb Pk epitoop koos Cut11, mille C-terminuses asub HA epitoop (Pk-Sdd1, Cut11-HA); Sdd1, mille N-terminuses paikneb Pk epitoop koos Sad1, mille C-termuses on HA epitoop (Pk-Sdd1, Sad1-HA). Kõik need valgud ekspresseruvad endogeensetel promootoritel. Töö käigus optimiseeriti tingimusi, et analüüsida Pk-Sdd1, Cut11-HA, Sad1-HA ja α-tubuliini Western blot analüüsilit. Selleks testiti erinevate antiekahade kombinatsioone ning alusel fosfataasid või ECL-il põhinevad detekteerimise süsteeme. Valkude membraanis paiknemise uurimiseks valmistati eksponentsiaalses kasvukaasas olevatest pärmi kultuuridest rakulúsaadid kasutades erineva koostisega lüüsipuhvrideid. Tsentrifugeerimise abil eraldati lüsaatide lahustuv ning sademe fraktsioonid ning analüüsti Pk-Sdd1, Sad1-HA ja α-tubuliini paiknemist. Tulemused näitasid, et uuritavad valgud asusid lahustuvas fraktsioonis vaid körget ioonset detergendi (SDS) sisaldava puhvriga ekstraheerimisel, mis on iseloomulik just integraalsetele membraanivalkudele.

Kokkuvõtteks, läbiviidud biokemilised katsed koos valkude primaarstruktuuri analüüsiga näitavad, et Sdd1 ja Sad1 on membraanivalgud, mis paiknevad membraanis integraalselt.
REFERENCES


Ding, R., West, R. R., Morphew, M., Oakley, B. R. and McIntosh R. J. (1997). The spindle pole body of *Schizosaccharomyces pombe* enters and leaves the nuclear envelope as the cell cycle proceeds. Mol Biol Cell. 8: 1461-79.


**SUPPLEMENTARY INFORMATION**

Table S1. Components of yeast extract medium with supplements (YES)

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Difco yeast extract</td>
<td>0.5% (w/v)</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.0% (w/v)</td>
</tr>
<tr>
<td>Adenine</td>
<td>225 mg/l</td>
</tr>
<tr>
<td>Histidine</td>
<td>225 mg/l</td>
</tr>
<tr>
<td>Leucine</td>
<td>225 mg/l</td>
</tr>
<tr>
<td>Uracil</td>
<td>225 mg/l</td>
</tr>
<tr>
<td>Lysine hydrochloride</td>
<td>225 mg/l</td>
</tr>
</tbody>
</table>

For solid medium 20 g/l of agar (Difco Bacto) was added. (Moreno *et al.*, 1991)
Table S2. Components of Edinburgh minimal medium (EMM)

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH phthalate</td>
<td>14.7 mM</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>15.5 mM</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>93.5 mM</td>
</tr>
<tr>
<td>Glucose</td>
<td>111 mM</td>
</tr>
<tr>
<td>Salts stock (50x)</td>
<td>20 ml/l</td>
</tr>
<tr>
<td>Minerals stock (10000x)</td>
<td>0.1 ml/l</td>
</tr>
<tr>
<td>Vitamins stock (1000x)</td>
<td>1 ml/l</td>
</tr>
</tbody>
</table>

For solid medium 20 g/l of agar (Difco Bacto) was added.

Table S3. Composition of stock solutions for EMM

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Salts</strong></td>
<td></td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>5.2 mM</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>13.4 mM</td>
</tr>
<tr>
<td>Na₂SO₄</td>
<td>0.28 mM</td>
</tr>
<tr>
<td><strong>Minerals</strong></td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>8.1 µM</td>
</tr>
<tr>
<td>MnSO₄</td>
<td>2.37 µM</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>1.39 µM</td>
</tr>
<tr>
<td>FeCl₃·6H₂O</td>
<td>0.74 µM</td>
</tr>
<tr>
<td>MoO₄·2H₂O</td>
<td>0.25 µM</td>
</tr>
<tr>
<td>KI</td>
<td>0.6 µM</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>0.16 µM</td>
</tr>
<tr>
<td>Citric acid</td>
<td>4.76 µM</td>
</tr>
<tr>
<td><strong>Vitamins</strong></td>
<td></td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>81.2 µM</td>
</tr>
<tr>
<td>Inositol</td>
<td>55.5 µM</td>
</tr>
<tr>
<td>Biotin</td>
<td>40.8 µM</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>4.2 µM</td>
</tr>
</tbody>
</table>

All stock solutions were filter-sterilized and stored frozen or at 4°C.
Table S4. Components of sporulation agar with supplements (SPAS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>1% (w/v)</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>7.3 mM</td>
</tr>
<tr>
<td>Vitamins stock (1000x)</td>
<td>1 ml/l</td>
</tr>
</tbody>
</table>

Supplements: 45 mg/l adenine, leucine, uracil and lysine hydrochloride (1/5 normal). Supplements were added according to strain genotype. For solid medium 30 g/l of agar (Difco Bacto) was added.