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# The yeast Ski complex is a hetero-tetramer

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## Abstract

The yeast Ski complex assists the exosome in the degradation of mRNA. The Ski complex consists of three components; Ski2, Ski3, and Ski8, believed to be present in a 1:1:1 stoichiometry. Measuring the mass of intact isolated endogenously expressed Ski complexes by native mass spectrometry we unambiguously demonstrate that the Ski complex has a hetero-tetrameric stoichiometry consisting of one copy of Ski2 and Ski3 and two copies of Ski8. To validate the stoichiometry of the Ski complex, we performed tandem mass spectrometry. In these experiments one Ski8 subunit was ejected concomitant with the formation of a Ski2/Ski3/Ski8 fragment, confirming the proposed stoichiometry. To probe the topology of the Ski complex we disrupted the complex and mass analyzed the thus formed subcomplexes, detecting Ski8–Ski8, Ski2–Ski3, Ski8–Ski2, and Ski8–Ski8–Ski2. Combining all data we construct an improved structural model of the Ski complex.

**Keywords:** Ski complex; exosome; native mass spectrometry; protein complex structure and topology

The recently introduced combination of tandem affinity purification (TAP) (Rigaut et al. 1999) and peptide mass spectrometry has added substantially more information to large databases of protein–protein interaction networks, in particular in yeast (Gavin et al. 2002; Krogan et al. 2004; Collins et al. 2007). Although they are very powerful approaches, unfortunately, detailed information on each of the identified protein complexes, such as complex stoichiometry, topology of protein–protein interactions, and overall structure, is not easily addressed. Advances in mass spectrometry have enabled the investigation of non-covalent protein interactions of heterogeneous large macromolecular complexes, providing a new complementary

tool in structural biology (Ilag et al. 2004; van den Heuvel et al. 2006; van Duijn et al. 2006; Sharon et al. 2007). The potential of macromolecular mass spectrometry to also probe endogenously expressed heterogeneous protein complexes has been recently demonstrated (Hernandez et al. 2006; Synowsky et al. 2006) whereby the endogenously expressed TAP-purified yeast exosome was probed for complex stoichiometry, overall topology of the protein complex, and individual protein post-translational modifications. In this work we focused on the exosome related Ski complex endogenously expressed and purified from yeast using the TAP tagging technique.

The Ski complex is involved in exosome mediated 3' → 5' mRNA degradation. It is hypothesized that the Ski complex recruits the exosome to its substrate, where it is subsequently degraded (Anderson and Parker 1998). Next to its involvement in exosome directed degradation of RNA, the Ski complex has been shown to protect the cell from viral replication by blocking synthesis of extrinsic mRNA transcripts (Widner and Wickner 1993).

The Ski complex consists of three subunits. The largest subunit, Ski3, has a mass of 163 kDa and contains a tetratricopeptide (TPR) motif (Rhee et al. 1989). Next, Ski2 has a mass of 146 kDa and is a putative RNA

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**Abbreviations:** TAP, tandem affinity purification; LTQ, linear ion trap mass spectrometer; Q-ToF, quadrupole time-of-flight mass spectrometer; LC MS/MS, liquid chromatography tandem mass spectrometry; ESI-MS, electrospray ionization mass spectrometry.

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helicase (Widner and Wickner 1993). Finally, Ski8 is a protein with a mass of 44 kDa, containing a WD-repeat domain (Matsumoto et al. 1993). The crystal structure of Ski8 shows a seven-bladed  $\beta$  propeller and may therefore function as a scaffolding protein in the Ski complex (Cheng et al. 2004; Madrona and Wilson 2004). Ski3 and Ski8 were found to copurify by using Myc-tagged Ski2, and by using [ $^{35}\text{S}$ ]-methionine labeled cells and semi-quantitative Western blotting a 1:1:1 stoichiometry for Ski2, Ski3, and Ski8 in the Ski complex has been proposed. Ski deletion strains have suggested association of Ski2 to Ski3 and Ski2 to Ski8 in the presence of Ski8 and Ski3, respectively. Additionally, a weak interaction between Ski3 and Ski8 is proposed (Brown et al. 2000). Based on suitable structural templates the structure of the intact Ski complex was recently modeled using computational structure predictions (Aloy et al. 2004). This model predicted a nonlinear Ski8–Ski2–Ski3 conformation of the complex for direct interactions between Ski8 and Ski2 and between Ski2 and Ski3.

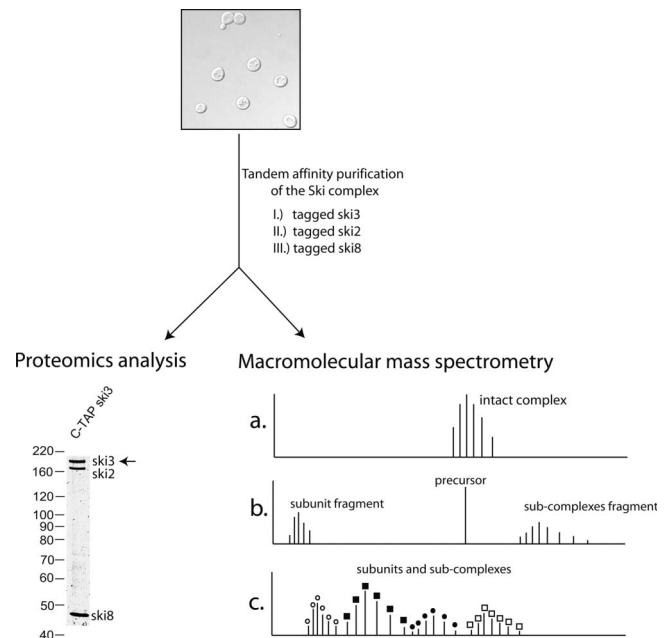
Here we analyze the endogenously expressed Ski complex from *Saccharomyces cerevisiae* using macromolecular mass spectrometry. We retrieve the Ski complex using the tandem affinity purification technique, taking each of the three proteins subsequently as an entry point. Our data allow us to construct a new structural model for the yeast Ski complex and unambiguously show for the first time that the stoichiometry of the Ski complex is such that it contains one copy of Ski2 and Ski3, but two copies of Ski8.

## Results

In Figure 1 we have summarized the experimental approach to investigate the stoichiometry and topology of the Ski complex by standard proteomics methods and macromolecular mass spectrometry. The Ski complex was endogenously expressed in *S. cerevisiae*, and purified using the tandem TAP-TAG affinity approach. Ski2, Ski3, and Ski8 as the known constituents of the Ski complex were independently used as tagged entry points.

### Proteomics analysis of the yeast Ski complex

To evaluate the quality of the Ski complex purification we analyzed the samples on a 1D gel. In Figure 1 the gel for the complex with Ski3 as the entry point is shown. MS analysis clearly showed that the three bands originated from Ski2, Ski3, and Ski8, with no other significant hits. Similar results were obtained from purifications with the Ski2 and Ski8 entry points (data not shown). In a high-throughput proteomic analysis, it was reported that with any tagged Ski protein several other proteins copurified (Krogan et al. 2006). In contrast, our purifications were very pure, which may be surprising, as at least Ski8 plays a role in another protein complex. Ski8 relocates from the cytoplasm to

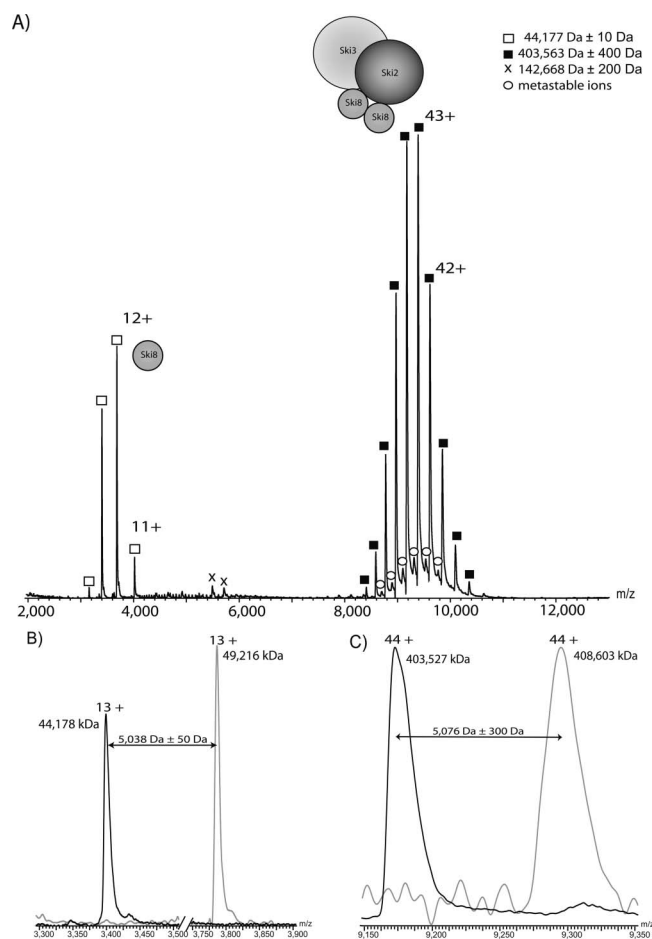


**Figure 1.** Schematic overview of the experimental approach. The Ski complex was tandem affinity purified from yeast cell using either Ski3, Ski2, or Ski8 as bait protein. Proteins of these purified complexes were first separated by 1D gel SDS-PAGE and identified by standard proteomics analyses. The 1D gel shown was obtained using Ski3 as bait (as indicated with the arrow) and revealed only three bands, identified as indicated. Then, the purified protein complexes were buffer exchanged into a mass spectrometry amenable buffer. Next, the masses of the intact Ski complexes were measured by macromolecular mass spectrometry (A) and tandem mass spectrometry was used to confirm the stoichiometry of the complexes (B). To probe the subunit topology of the Ski complex, the buffer solution was acidified, disrupting the Ski complex leading to the formation and mass detection of Ski subunits and Ski sub-complexes (C).

the nucleus, where it is involved in double-strand breaks (DSBs) formation catalyzed by Spo11 (Arora et al. 2004). However, we did not detect spo11 when Ski8 was used as bait. Since our purifications were performed on whole-cell lysates, nuclear proteins may be of relatively low abundance. The isolation of solely the Ski complex may be due to our more stringent and careful washing and purification procedures but generally point at the lower specificity of TAP pulldowns in high-throughput experiments.

### Macromolecular mass spectrometry on intact Ski complexes

In Figure 2A the mass spectrum is shown of the purified Ski complex, using Ski3 as the entry point. The spectrum reveals two charge distributions, originating from two species. The ion series at  $m/z$  values between 8500 to 11,000 (■) corresponds to a mass of  $403,527 \pm 500$  Da. By adding the theoretical masses of tagged-Ski3 (168,803 Da), Ski2 (146,059 Da), and Ski8 (44,232 Da) assuming a 1:1:1 stoichiometry (Brown et al. 2000), the expected mass



**Figure 2.** Macromolecular mass spectrometry on intact Ski complexes. (A) Mass spectrum of the purified Ski Complex using Ski3 as the entry point. The spectrum shows two main ion series (■ and □) originating from species with a mass of 403,527 kDa and 44,178 kDa, representing the full intact Ski complex and monomeric Ski8, respectively. The lower abundant charge distributions are due to metastable ion formation (○) and a protein contamination (x) always observed in our purifications. (B, C) Zoomed-in and overlaid parts of the mass spectra of the intact Ski complex purifications using Ski3 and Ski8 as TAP-tagged proteins, respectively. The black trace shows the ion signal of Ski3 as TAP-tagged protein and the gray trace of TAP-tagged Ski8. In B the 13+ ions of Ski8 are depicted, whereby the ion signal of monomeric Ski8 is shifted to higher m/z values in the tagged Ski8 purification, caused by the presence of the TAP tag of 5038 Da. In C the 44+ ions of the intact Ski complex are depicted. The Ski complex purified with Ski8 shows a shift of 5076 Da to higher m/z values, clearly indicating that a second TAP tag was present in this complex.

should be 359,094 Da. The discrepancy between the expected and the actual mass of the Ski complex is 44,433 Da, close to the mass of Ski8, suggesting an unexpected stoichiometry of 1:1:2 with Ski8 present in two copies. Another charge distribution is present at values between 3000 and 4000 m/z, originating from species with a mass of  $44,178 \pm 25$  Da, which is in agreement with the expected mass of Ski8, indicating that some free Ski8 is present in our purified samples. Importantly, we detect no

other ion signals, such as for a possible 1:1:1 Ski complex, indicating that the observed 1:1:2 stoichiometry is the exclusive stoichiometry present in the yeast Ski complex.

The obtained mass spectra using Ski2 as the entry point looked very similar to those presented in Figure 2A (data not shown). The obtained mass of the major species detected in this purification was  $403,561 \pm 1000$  Da (see Table 1), thus again hinting at the 1:1:2 stoichiometry. If our suggested 1:1:2 stoichiometry is correct, we should, with the mass accuracy of our method, be able to distinguish the expected mass difference between the Ski complex purified from either the Ski2/Ski3 entry points and the Ski8 entry point. Using the tagged-Ski8 for the Ski complex purification we introduce two TAP-TAGs into the complex, if there are indeed two copies of Ski8 present. After purification this extra TAG leads to mass increase of 5077 Da, corresponding to the second remaining calmodulin binding peptide (CBP) of the Tag of the total complex compared to Ski2 and Ski3 as entry points. The mass spectrum using Ski8 as the entry point shows a species of  $408,603 \pm 400$  Da (see Table 1), again confirming the 1:1:2 stoichiometry. We also detected minor signals for free Ski8, now having a mass of  $49,216 \pm 15$  Da. For illustrative purposes, we overlaid in Figure 2B the detected 13+ charged ions of Ski8 from the Ski complex isolated with TAP-tagged Ski3 and TAP-tagged Ski8, directly showing the expected mass shift of  $5038 \pm 50$  Da originating from the tagged Ski8. Similarly, we overlaid the ion signals of the 44+ ions of the Ski complex, isolated with TAP-tagged Ski3 and TAP-tagged Ski8, as depicted in Figure 2C. The ion signals are shifted, corresponding to a shift in mass of  $5077 \pm 300$  Da. This unambiguously proves that two Ski8 molecules are present in the yeast Ski complex. Our results are in contradiction to a 1:1:1 stoichiometry for Ski2, Ski3, and Ski8 in the Ski complex on the basis of semiquantitative Western blotting (Brown et al. 2000).

#### Tandem mass spectrometry on intact Ski complexes

To further probe the identity and stoichiometry of the purified protein complexes we explored tandem mass

**Table 1.** Masses of the intact yeast Ski complex using different entry points

TAP-TAG bait protein	Measured mass of Ski complex (Da) <sup>a</sup>	Complex assignment
Ski2	$403,561 \pm 1000$	(CBP-Ski2)-Ski3-Ski8-Ski8
Ski3	$403,527 \pm 500$	Ski2-(CBP-Ski3)-Ski8-Ski8
Ski8	$408,603 \pm 400$	Ski2-Ski3-(CBP-Ski8-CBP-Ski8)

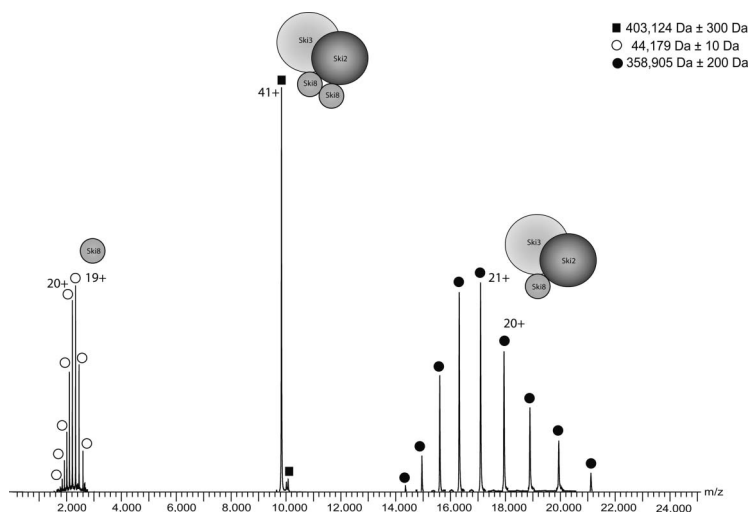
<sup>a</sup>The indicated error range was estimated from the standard deviations in the measured masses as measured by mass spectrometry following several different purifications.

spectrometry on the intact Ski complexes. In such tandem mass spectrometry experiments precursor ions are mass selected and subjected to collision-induced dissociation (Sobott et al. 2002; van den Heuvel et al. 2006; van Duijn et al. 2006; Sharon and Robinson 2007). Figure 3 shows a typical tandem mass spectrum whereby the Ski complex precursor ions having 41+ charges were mass selected (■). The intact Ski complex fragmented into two products. One of these products, appearing in between  $m/z$  1700 and 2700 (○) originates from a Ski8 with a mass of  $44,179 \pm 25$  Da. The second concomitant product is detected between  $m/z$  14,500 and 21,200 (●) and corresponds to CBP-Ski3-Ski2-Ski8 with a mass of  $358,905 \pm 200$  Da. Indeed, summing up the detected masses of the two fragment ions leads to a precursor mass of  $403,124 \pm 500$  Da, which is in agreement with the expected mass for the intact complex (see Table 1). Tandem mass spectrometry experiments on different charge states of the Ski3-tagged Ski complex revealed identical fragmentation patterns, as did similar experiments on the complexes obtained from the two different entry points (data not shown). These tandem mass spectrometry results therefore confirm our hypothesis that the Ski complex is a hetero-tetramer consisting of a single copy of Ski2 and Ski3 and two copies of Ski8.

#### Topology of the Ski complex

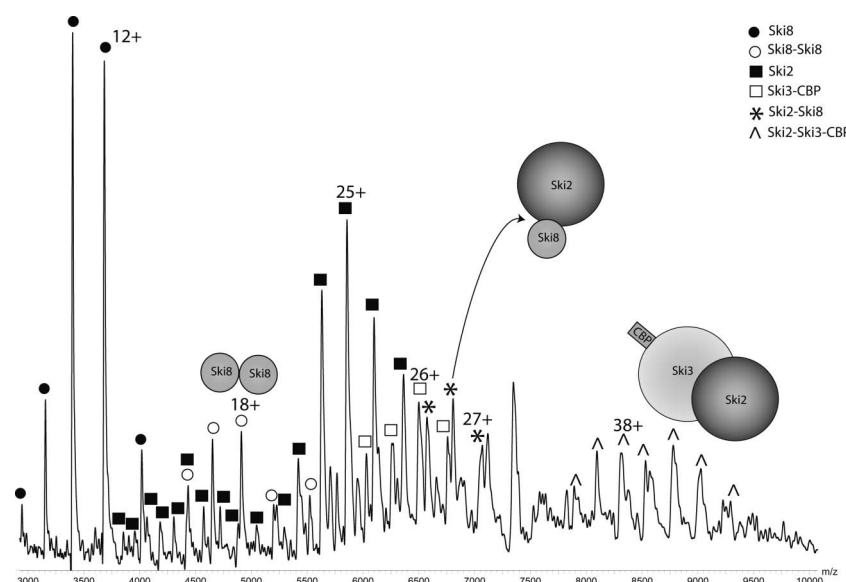
Here we probe the overall topology of the Ski complex by a gentle disruption of the complex induced by the addition of 0.5% formic acid (v/v) prior to mass spectrometric analysis. The spectrum shown in Figure 4 reveals a multitude of charge state distributions, from which the masses of several

species could be derived. First of all, no charge distribution was observed to correspond to the intact Ski complex, indicating that the complex was completely dissociated. Between  $m/z$  3000 and 4000 a charge envelope from 11+ to 15+ as denoted with (●) matches to a mass of  $44,165 \pm 25$  Da or Ski8. More interestingly, a second charge distribution between  $m/z$  4300 and 5600 with charges from 16+ to 20+ is present (○) originating from species with a mass of  $88,388 \pm 56$  Da, matching perfectly to a dimer of Ski8. Furthermore, we detected monomeric Ski2 (between  $m/z$  3700 and 6400 with charge states from 23+ to 38+ [■]) with a measured mass of  $146,214 \pm 150$  Da, and monomeric tagged-Ski3 with a mass of  $168,909 \pm 150$  Da (the charge envelope observed between  $m/z$  6000 and 6600 with charges from 25+ to 28+ [□]). Two additional subcomplexes were identified. As denoted with (\*) at  $m/z$  6500 to 7100 covering charges from 27+ to 29+ and corresponding to a mass of  $190,484 \pm 100$  Da, a dimeric subspecies is detected. Taking the experimental mass as determined for monomeric Ski8 and Ski2, a mass of 190,392 Da was expected, clearly assigning this subspecies to Ski2-Ski8. Moreover another charge envelope at  $m/z$  7700 to 9300 with charge states of 34+ to 40+ (△) results in a mass of  $315,730 \pm 610$  Da, suggesting a dimeric subcomplex consisting of TAP-Ski3-Ski2. The expected mass of TAP-Ski3-Ski2 is 315,123 Da. Due to the complexity of the spectrum it is not possible to further annotate more charge distributions with a high confidence. However, in an analysis of another Ski complex purification with Ski3 as bait we detected clear signals of a species corresponding to a mass of  $234,499 \pm 75$  Da, suggesting a trimeric subcomplex of Ski2-Ski8-Ski8. A theoretical calculation



**Figure 3.** Tandem mass spectrometry on intact Ski complexes. Tandem mass spectrum of the Ski3 TAP-tagged Ski complex. The 41+ charge state of the total Ski complex (■) was subjected to collision-induced dissociation using a collision voltage of 175 V. The CID process resulted in the formation of two concomitant fragment ion series, corresponding to a species consisting of Ski3-Ski2-Ski8 (●) with a mass of 358,905 Da and Ski8 (○) with a mass of 44,179 Da, respectively.





**Figure 4.** Topology of the Ski complex. Mass spectra acquired of the Ski3 TAP-tagged Ski complex following acid-induced disruption. The mass spectrum reveals ion signals of several subunits and subcomplexes, corresponding to Ski8 (●), Ski2 (■), Ski3 (□), Ski8-Ski8 (○), Ski2-Ski8 (\*), and Ski3-Ski2 (^).

based on experimental masses suggests 234,570 Da. The experimental measured masses of monomeric proteins and subcomplexes are summarized in Table 2 and Table 3. Co-immunoprecipitation experiments in yeast deletion strains have already shown that Ski2-Ski3, Ski2-Ski8, and Ski3-Ski8 are associated with each other (Brown et al. 2000). Computational modeling based on suitable structural templates of the individual Ski proteins described the interactions between Ski8-Ski2 and Ski2-Ski3 (Aloy et al. 2004).

Our mass spectrometric analyses of the resultant subcomplexes confirm most previously identified interactions, but provide even more data to propose a new overall topology. By using each protein of the Ski complex as bait and isolating the Ski complex under stringent conditions we were able to consistently confirm the hetero-tetrameric stoichiometry of the Ski complex. Our data reveal that two

copies of Ski8, forming a dimer, are present in the Ski complex. This Ski8 dimer could be further placed into the Ski complex by detecting a trimer consisting of Ski2 and the dimer of Ski8, indicating that at least one of the Ski8 proteins is in contact with Ski2. Our data also revealed that Ski2 and Ski3 are connected to each other, which was expected. We did not detect any subcomplexes confirming directly the existence of a direct link between Ski3 and Ski8, but we cannot exclude that such a connection exists.

Taking all our data together we propose a new structural model for the yeast Ski complex, which is depicted in Figure 5. In Figure 5 three alike albeit different structural topologies are depicted, based on the fact that the two Ski8s are interconnected, Ski2 and Ski3 are linked, and at least one Ski8 binds to Ski2. Our data do not allow us to distinguish whether just one (model I) or two Ski8

**Table 2.** Masses of the Ski subunits compared with predicted masses as extracted from the gene sequences

Ski proteins	Measured mass (Da) <sup>a</sup>	Theoretical mass (Da)
Ski8	44,178 ± 25	44,232
Ski2	146,214 ± 150	146,059
Ski3	163,827 ± 30	163,726
Ski8-CBP	49,216 ± 15	49,309
Ski2-CBP	—	151,136
Ski3-CBP	168,909 ± 150	168,803

CBP with a mass of 5077 Da indicates that the TAP-tag was linked to that subunit.

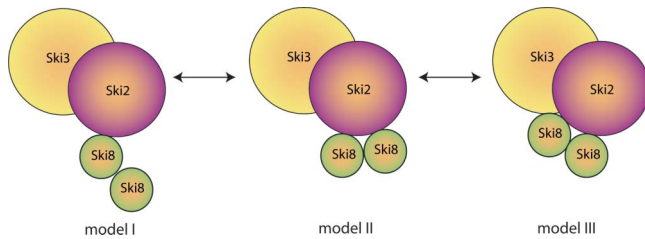
<sup>a</sup>The indicated error range was estimated from the standard deviations in the measured masses as measured by mass spectrometry following several different purifications.

**Table 3.** Measured masses of detected subcomplexes compared to the mass of these subcomplexes obtained by summing up the mass of each subunit present

Subcomplex	Measured mass (Da) <sup>a</sup>	Theoretical mass (Da)
Ski8-Ski8	88,388 ± 56	88,356
Ski8-CBP-Ski8-CBP	98,404 ± 85	98,432
Ski2-Ski8	190,484 ± 100	190,392
Ski2-Ski3-CBP	315,730 ± 610	315,123
Ski8-Ski3-CBP	—	213,087
Ski2-Ski8-Ski8	234,499 ± 75	234,570

CBP indicates the TAP-tag on the protein.

<sup>a</sup>The indicated error range was estimated from the standard deviations in the measured masses as measured by mass spectrometry following several different purifications.



**Figure 5.** Structural model of the Ski complex. Ski3 is in direct contact with Ski2. Ski2 is connected to at least one Ski8. The second Ski8 is bound to the first Ski8. The direct interaction of the second Ski8 with Ski2 and Ski3 could not be unambiguously established, leading to three similar, albeit slightly different predicted overall topologies.

(models II and III) subunits interact with Ski2, individually, and could therefore be symmetric in the overall structure. Interestingly, in models I and III both Ski8 proteins are not identical, and may also fulfill different functions in the Ski complex, but this requires further experimental evidence. Model III allows the previously proposed interaction between Ski8 and Ski3.

## Discussion

Structure determination on endogenously expressed heterogeneous protein complexes is still a challenging task. Due to minute amounts of purified protein complex, well-established techniques such as X-ray crystallography and NMR often fail. In the approach presented here we use the powerful combination of tandem affinity purification combined with standard proteomics methods and macromolecular mass spectrometry to structurally investigate the yeast Ski complex in detail.

We propose a new stoichiometry and topology for the yeast Ski complex. The Ski complex is not a heterotrimer with 1:1:1 stoichiometry but a heterotetramer with a 1:1:2 stoichiometry, whereby one copy of Ski3 and Ski2 and two proteins of Ski8 are assembled per Ski complex. Our mass spectrometric data corroborate the proposed direct interaction between Ski3 and Ski2 as well as between Ski2 and Ski8. The second molecule of Ski8 forms a dimeric structure with the other Ski8 subunit, but its exact topology is difficult to pinpoint in the overall assembly. Although, our data leave some remaining questions about the Ski complex topology (Fig. 5), it will provide a useful template for further, more in-depth structural and functional biology approaches into the structure of the Ski complex.

## Materials and Methods

### Yeast strain, cultivation, and protein purification

The *S. cerevisiae* strain MGD35313D, BSY17 containing Ski2, Ski3, or Ski8 as the C-terminal tagged entry point was

purchased at Euroscarf. Cell cultivation and protein purification with Ski2, Ski3, and Ski8 were essentially performed as described previously (Synowsky et al. 2006). All purifications of the Ski complex, using separately Ski3, Ski2, and Ski8 as bait, were performed using a salt concentration of 300 mM NaCl in the lysis buffer.

### Proteomic analysis of the Ski complex

The purified Ski proteins were separated on a 10% SDS gel and stained using 0.1% (v/v) Coomassie Brilliant Blue G250. Peptide preparation and LC-MS/MS analyses using an LTQ Mass Spectrometer (ThermoFisher) were performed as described previously (Synowsky et al. 2006). The peptide tolerance was fixed to 0.5 Da and the MS/MS tolerance to 0.9 Da.

### Analysis of the Ski complex by macromolecular mass spectrometry

For the mass spectrometric analysis of the intact protein complexes, the elution buffer of the TAP-purified Ski complex was exchanged to 150 mM NH<sub>4</sub>Ac-buffer (pH 6.8). The mass spectrometer was externally calibrated using aqueous CsI (100 mg/mL) solutions. Analysis of the intact protein complex and subcomplexes thereof was performed using a LCT (Waters). Typical spraying conditions were capillary voltage 1200 V and sample cone voltage 150 V. To investigate the topology of the Ski complex it was disrupted by adding 0.5% formic acid (v/v) to the sample solution prior to mass spectrometric analysis. Tandem mass spectrometry experiments were performed essentially as described previously (van den Heuvel et al. 2006). The collision energy of the ions was gradually increased from 50 V to 200 V using a xenon gas pressure of  $\sim 2 \times 10^{-2}$  mbar.

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