5 Discussion

5.1 Organization of the mammalian TRAPP complex

According to a single-particle EM study [76], the nine subunits of yeast TRAPP II form a core complex that dimerizes through Trs65p (see 1.3.3.2 and Fig 5.1.1 a). The dimeric state of yeast TRAPP II was also supported by earlier gel filtration studies, which showed that TRAPP II eluted at retention time corresponding to a molecular weight of at least 1000 kDa (about twice the value of the summed molecular weights of all the individual components) [56]. In mammalian cells, where no Trs65p ortholog is found, a Trs65p-mediated dimerization would not be possible. Furthermore, in gel filtration studies, human TRAPP eluted at a peak corresponding to a molecular weight of about 670 kDa [63], which approximately equals the value of the summed molecular weights of all human TRAPP subunits, including Trs85. Therefore, it is reasonable to suspect that, unlike yeast TRAPP II, the mammalian TRAPP complex is predominantly monomeric.

With a CoIP experiment, an interaction between an Ehoc-1 N-terminal fragment (Ehoc-1 N, 1-276) and an NIBP C-terminal conserved domain (NIBP C, 941-1086) was detected (4.2.3). Based on this result and the yeast TRAPP II model, a preliminary model could be derived for the mammalian TRAPP complex (Fig 5.1.1).

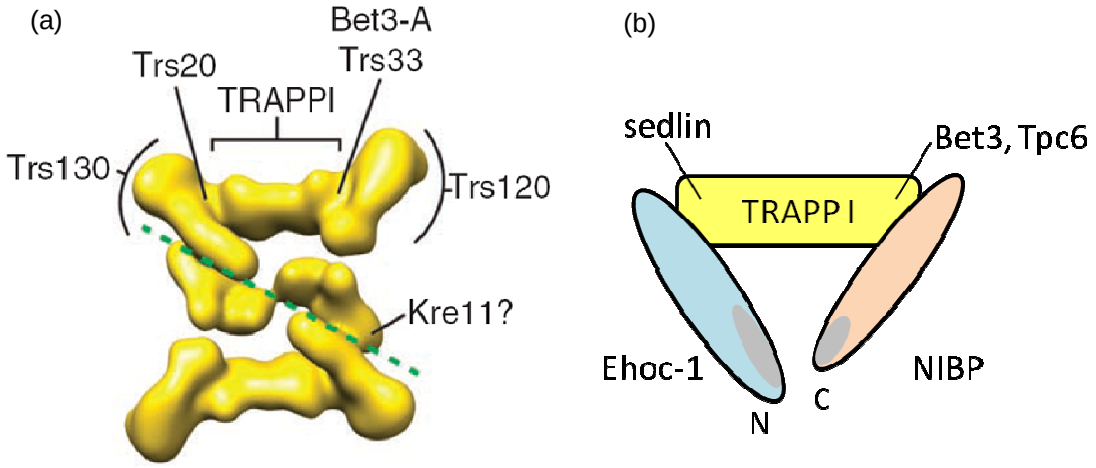


Fig 5.1.1 Models for yeast TRAPP II and mammalian TRAPP complex
(a) Yeast TRAPP II model, taken from [76].
(b) Preliminary model for the mammalian TRAPP complex, the subunit Trs85 not included. The fragments involved in the interaction, Ehoc-1 N (1-276) and NIBP C (941-1086), are colored grey.

Firstly, the six small TRAPP subunits of the Bet3 and Bet5 subfamilies can form a flat complex similar to yeast TRAPP I. Then Ehoc-1 and NIBP cap the TRAPP I – like complex on both ends and form a triangular structure. This structure is further stabilized by interactions between the Ehoc-1 N-terminal and the NIBP C-terminal conserved segments (Fig 5.1.1 b). This is also in agreement with the experiments by Yip *et al.* [76], who found that the presence of Trs120p with a C-terminal GFP tag would disturb the dimerization of yeast TRAPP II, while Trs130 with the same tag would not. This suggested that in yeast TRAPP II, the C-terminus of Trs120p is near the dimer interface, while the C-terminus of Trs130p is not.

5.2 Characterization of Tca17

5.2.1 Oligomerization state of Tca17

In the crystal structure of Tca17, a crystallographic dimer was found, which was mainly stabilized by a disulfide bond between two Cys121 residues from two symmetry-related molecules. In order to study the oligomerization state of Tca17 in solution, several biophysical experiments were performed, including analytical ultra-centrifugation, static light scattering and non-reducing SDS-PAGE. From these experiments, Tca17 is predominantly monomeric in the presence of reducing agent. In a non-reducing environment, most of Tca17 still remains monomeric, while a small portion (< 5%) dimerizes via formation of disulfide bonds (see 4.4.3). Since the dimeric species is only favored in a non-reducing buffer, an unbound Tca17 molecule in the reducing environment of yeast cytosol should only be found as a monomer.

5.2.2 Sequence conservation of Tca17/TRAPPC2L

The protein pair of TRAPPC2 and TRAPPC2L are found in varieties of eukaryotic cells, suggesting a conserved and distinct function for either protein. To understand the function of Tca17 on the basis of sequence conservation, 18 TRAPPC2L orthologs from different species, including the better characterized orthologs in metazoans and several hypothetical proteins from yeast cells, were aligned using ClustalW [117]. The sequence conservation was then evaluated by the ConSurf webserver [118], which gives a normalized ConSurf score to each residue (for multiple sequence alignment, colored according to ConSurf scores, see Appendix C). The scores are a relative measure of evolutionary conservation, with the lowest score representing the

most conserved position in a protein. Tca17 structure representations, colored according to sequence conservation, are shown in Fig 5.2.1.

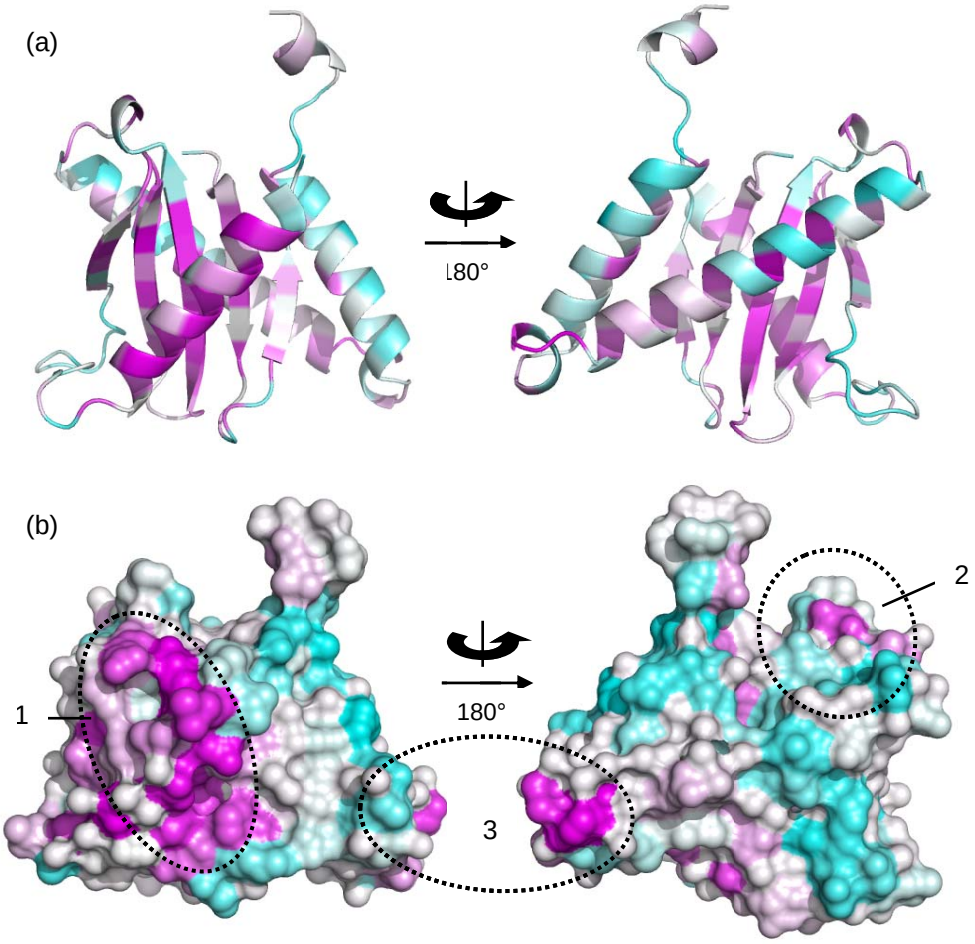


Fig 5.2.1 Tca17 crystal structure colored by sequence conservation score, calculated by ConSurf [118].
(a) Cartoon representation. (b) Surface representation.
Magenta, conserved residues, corresponding to negative ConSurf scores.
White, average residues.
Cyan, variable residues, corresponding to positive ConSurf scores.
The three conserved patches on the surface of Tca17 were marked out with circles and numbers 1-3.

In general, Tca17/TRAPPC2L is less conserved than the TRAPP subunit TRAPPC2 across the species, as expected for a protein which is not directly involved in the function of TRAPP, but might serve as a regulator for its assembly and functions. Many of the most conserved residues lie in the hydrophobic core of the lower part (Fig 5.2.1 a, colored in magenta). From the mainchain temperature factors (see Fig 4.4.4), the lower part is also suggested to have less internal flexibility. These two results suggest that maintaining a stable fold in this region is important for the function of Tca17.

More interestingly, in the surface representation (Fig 5.2.1 b), much surface area of Tca17 is rather variable (colored in cyan), whereas three distinct conserved patches (colored in magenta, marked by circles and numbers) can be identified. The function of these patches will be examined further in the following discussions.

5.3 Electrostatic potential distribution

Since a regulatory function of Tca17 can only be realized by protein-protein interactions, an electrostatic potential distribution on the surface of Tca17, calculated from the crystal structure, would provide useful information for studying its functions.

All electrostatic potential representation images shown in this work were calculated with the program DELPHI [119], and visualised by Pymol [114]. Positive and negative potential was colored blue and red, respectively, at the 10 kT level.

5.3.1 Comparison among Bet5 subfamily members

In a previous tandem affinity purification and size exclusion chromatography experiment by Scrivens *et al*. [83], Tca17 was shown to physically interact with TRAPP and preferentially with yeast TRAPP II. However, the exact nature of this interaction is still not clear.

As a member of the Bet5 subfamily of TRAPP subunits, Tca17 shares a similar longin fold as sedlin, Bet5 and Trs23 (see Fig 4.4.1 and 1.3.4). This raised the possibility that Tca17 could be integrated into the TRAPP complex in place of any of the three TRAPP subunits. To study this possibility, the electrostatic potential distributions of four Bet5 subfamily members are calculated and compared (Fig 5.3.1).

Though sharing a similar central longin fold, the lengths and arrangements of the loops are quite different in these four proteins. As a result, the outlines of their surface representations show little resemblance to each other. However, it is still possible to compare the nature of their surface when these four proteins are shown in the same orientation. Fig 5.3.1 shows that the electrostatic potential distribution of Tca17 is qualitatively similar to that of sedlin, but clearly different from Bet5p or Trs23p, especially on the one-helix side. Structurally, judging from the r.m.s.d values calculated by FATCAT (Table 4.4.1), Tca17 is also slightly more similar to sedlin than

to Bet5p and Trs23p. As a result, if Tca17 can be transiently integrated into the TRAPP complex in place of any Bet5-subfamily protein, it is more likely to substitute Trs20p, the yeast ortholog of sedlin, than Bet5p or Trs23p.

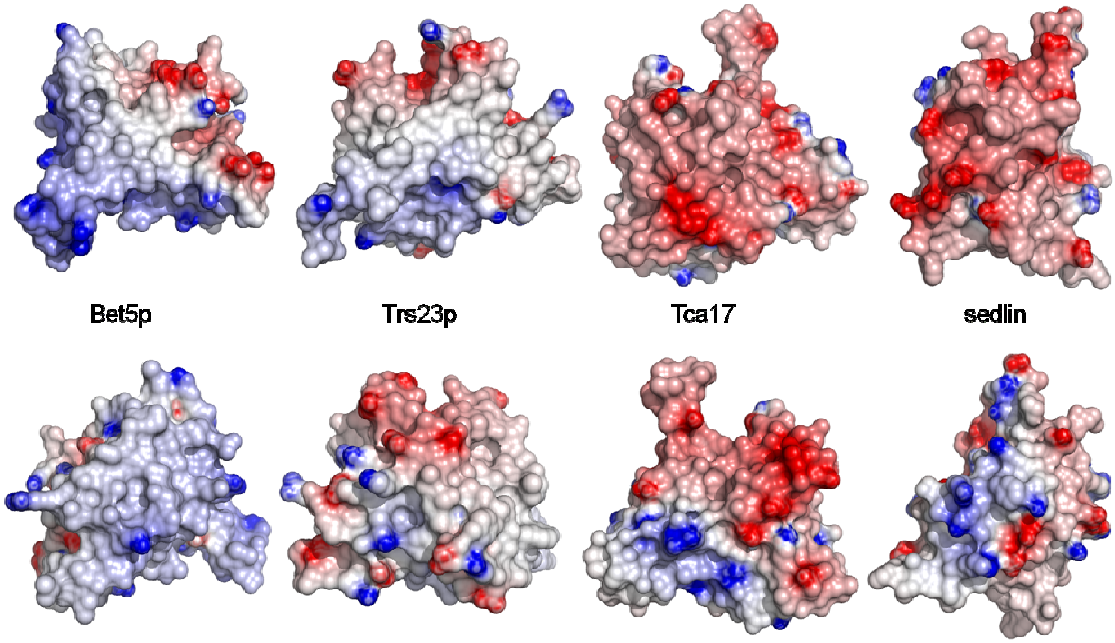


Fig 5.3.1 Comparing the surfaces of Bet5 subfamily members Bet5p (PDB id: 3CUE, chain C), Trs23p (PDB id: 3CUE, chain A), Tca17 and sedlin (PDB id: 1H3Q).
Upper panel: one-helix side. Lower panel: two-helix side. All four proteins are visualised in the same orientation.
The electrostatic potential was calculated by DELPHI [119]. Positive and negative potential was colored blue and red, respectively, at the 10 kT level.

5.3.2 Electrostatic potential on Tca17 surface

To understand its possible protein interacting behavior, the electrostatic potential distribution on the surface of Tca17 was studied in detail, in comparison with the structure of mouse sedlin. On the one-helix side, both proteins show a widely spread negative charge (Fig 5.3.2 a&b). Especially important is a distinct negatively charged patch present on both proteins. In sedlin, this patch is positioned on the presumably cytosolic side of the TRAPP complex, and free for protein interactions (for instance with SNAREs). The SEDT disease linked residue Asp47 of sedlin [120] is also located in this area. Interestingly, this area is highly conserved among Tca17/ TRAPPC2L proteins from various species (conserved patch 1 in Fig 5.2.1 b), suggesting that it might also be an important interaction site for Tca17. Furthermore, both the residue Asp45, which corresponds to Asp47 in sedlin, and its neighboring

residue Leu44, are 100% conserved among all TRAPPC2 and TRAPPC2L proteins (marked out with red box in Appendix C). When the structures of Tca17 and sedlin are superimposed, it is observed that the two conserved residues are positioned at similar positions on the surface of the protein (Fig 5.3.2 c). If studied more closely, the orientation of the side chains of these two residues is also quite similar (Fig 5.3.2 d). It is possible that these two residues serve as a specific protein binding site in both TRAPPC2 and TRAPPC2L proteins.

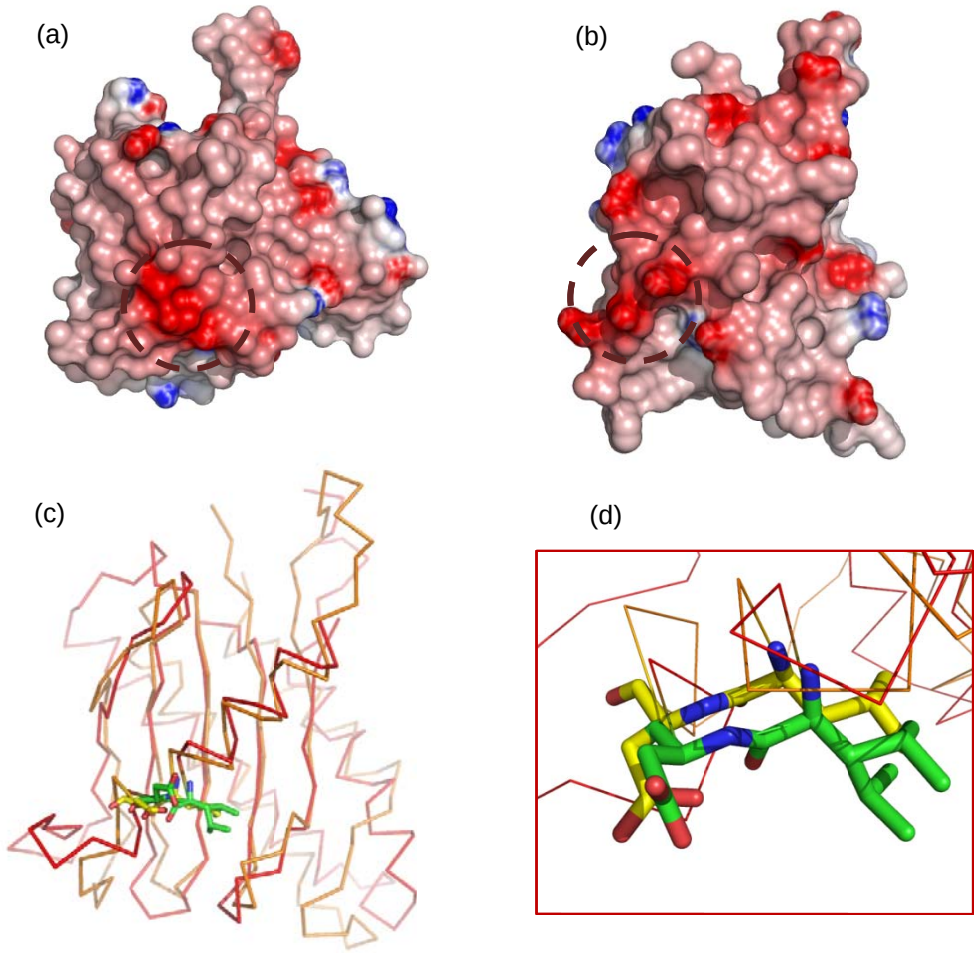
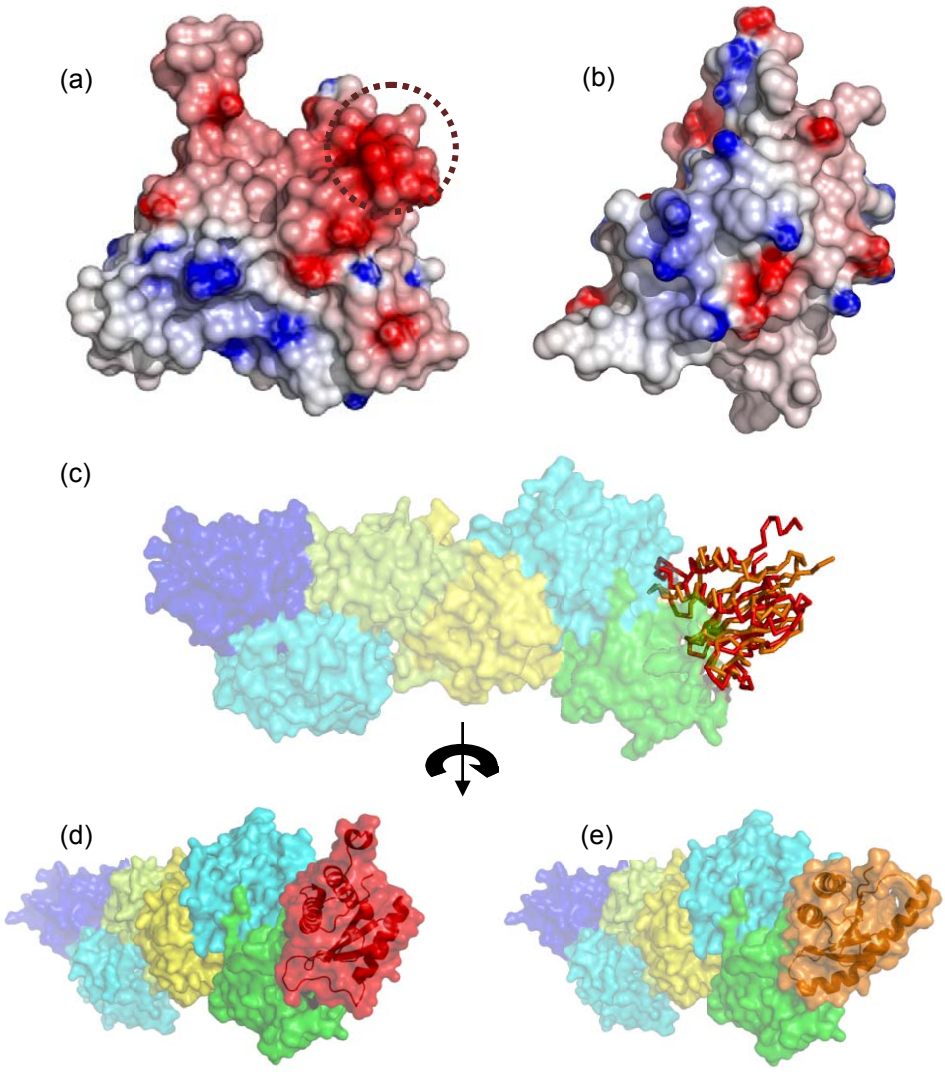


Fig 5.3.2 Comparing Tca17 and sedlin viewed from the one-helix side
(a) Surface of Tca17 on the one-helix side.
(b) Surface of sedlin on the one-helix side.
(c) Structures of Tca17 (red) and sedlin (orange) are superimposed, shown as ribbons. The two conserved residues Leu and Asp from Tca17 (in green) and from sedlin (in yellow) are shown as sticks.
(d) Part of (c) viewed in detail.
The electrostatic potential was calculated by DELPHI [119]. Positive and negative potential was colored blue and red, respectively, at the 10 kT level. The negative charged patches on either protein are marked by circles.

On the two-helix side, Tca17 looks more different from sedlin. While the surface of sedlin is mostly neutral, with only a few positive spots (Fig 5.3.3 b), Tca17 shows an obvious negative patch, composed of the loop between β2-α2 and the beginning of α2 (Fig 5.3.3 a, marked with circle). In order to investigate the possible function of this patch, Tca17 was superimposed with sedlin as present in the TRAPP I model (Fig 5.3.3 c). The TRAPP I model was shown from the membrane associating side. In order to observe the surfaces on Tca17 and sedlin corresponding to this negative patch, the TRAPP I model with Tca17 or sedlin was turned 60° clockwise (Fig 5.3.3 d&e, respectively).

Comparing the surfaces of Tca17 and sedlin in this orientation (Fig 5.3.3 f&g), it is clear that, if Tca17 is indeed integrated into TRAPP I in place of sedlin/Trs20p, a strongly negative charged area will be introduced into the membrane facing surface of TRAPP I, which might weaken the membrane association of TRAPP I.



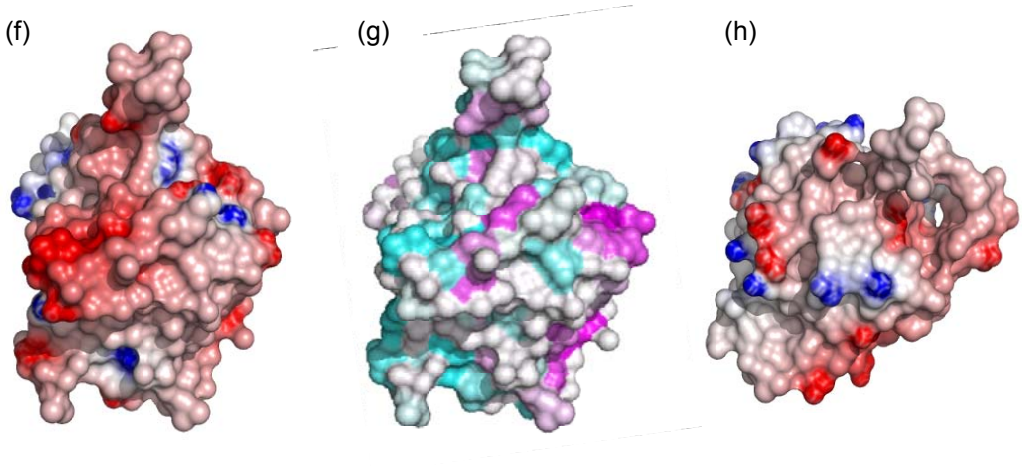


Fig 5.3.3 Comparing Tca17 and sedlin from the two-helix side
(a) Surface of Tca17 on the two-helix side. The negatively charged patch is marked by a circle.
(b) Surface of sedlin on the two-helix side.
(c) Tca17 (red) superimposed onto sedlin (orange) in the TRAPP I model (shown from the putative membrane facing side). Tca17 and sedlin were shown as ribbons, while other TRAPP I subunits were shown in surface representations. Green, Trs31; cyan, Bet3; yellow, Trs23; lemon, Bet5; blue, Trs33.
(d) TRAPP I model from (c) turned 60° clockwise. Tca17 shown in red.
(e) TRAPP I model from (c) turned 60° clockwise. Sedlin shown in orange.
(f) Electrostatic surface of Tca17, shown as the orientation in (d).
(g) Surface of Tca17, colored by sequence conservation score, shown as the orientation in (d).
(h) Electrostatic surface of sedlin, shown as the orientation in (e).
The electrostatic potential was calculated by DELPHI [119]. Positive and negative potential was colored blue and red, respectively, at the 10 kT level.
Sequence conservation score was calculated by ConSurf [118]. Magenta, conserved residues; white, average residues; cyan, variable residues.

Besides the putative membrane-facing surface (left part in Fig 5.3.3 f), the negative patch on the Tca17 surface extends further to the side of the protein which is facing away from TRAPP I (central and right part in Fig 5.3.3 f). A weak sequence conservation was also detected in this area (magenta area in Fig 5.3.3 g), corresponding to the conserved patch 2 in Fig 5.2.1 b. This suggests that this area might be used a binding site for some unknown binding partners, which is part of Tca17 regulatory functions.

According to the yeast TRAPP II model (Fig 5.1.1 a), this end of TRAPP I would be capped by the TRAPP II specific subunit Trs130p. Since an interaction between Tca17 and Trs130p was identified earlier in a Co-IP experiment by Montpetit *et al.* [82], it is possible to propose that this conserved patch on Tca17 surface might be important to link Tca17 with the TRAPP II complex.

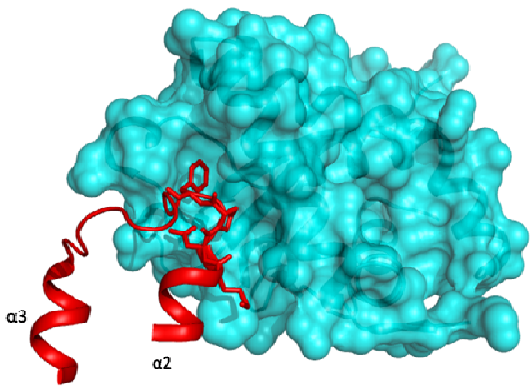
5.4 Possible interaction of Tca17 and TRAPP I subunits

According to the current model of the TRAPP I complex (see 1.3.3), sedlin/Trs20p is integrated into the complex through interactions with Trs31 and Bet3 (Fig 1.6 a). With the Tca17 structure available, it is possible to compare the interaction interface areas of Tca17 and its TRAPP homolog, sedlin, (sedlin interactions were calculated by Pisa [116]) and examine whether both interactions are still possible. Also, an interaction with Trs33p will also be examined.

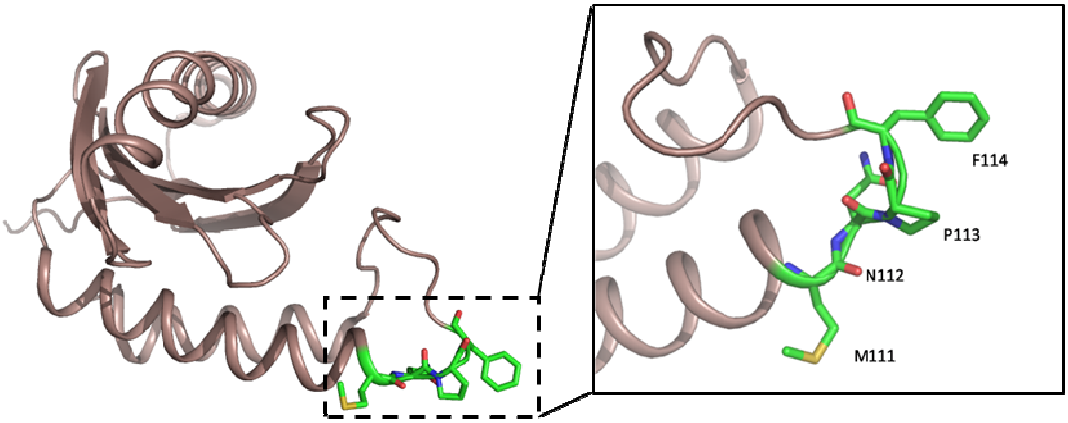
5.4.1 Possible interaction with Bet3

The interaction between mammalian sedlin and Bet3 is shown in Fig 5.4.1 a. Bet3 is shown in surface representation, while the binding loop of sedlin is shown as cartoon, with the most important residues appearing as sticks. This Bet3 interacting motif of four AAs (^{111}MNPF) is shown in more detail in Fig 5.4.1 b. In the structure of Tca17, this motif is rather well maintained (^{121}CNPL, Fig 5.4.1 c), corresponding to the conserved patch 3 in the surface representation of sequence conservation (Fig 5.2.1 b). Like the motif in sedlin, it is positioned between α2 and α3, protrudes from the Tca17 structure and is ready for binding. In the crystal structure of Tca17, the Cys121 from this motif is involved in disulfide bond formation (4.4.3.1). As discussed above (4.4.3.2 and 5.2.1), this disulfide bond is very unlikely to be formed in reducing buffers or inside cells, which leaves Cys121 free for interactions. A very weak interaction of Tca17 and Bet3p was detected by GST pull-down experiments (Fig 4.5.1). It thus seems possible that Tca17 uses a similar binding mode as its homolog sedlin in Bet3 binding. However, some local rearrangement might be necessary to remove hindrance from nearby helices, which might weaken the Bet3 interaction.

(a)



(b)



(c)

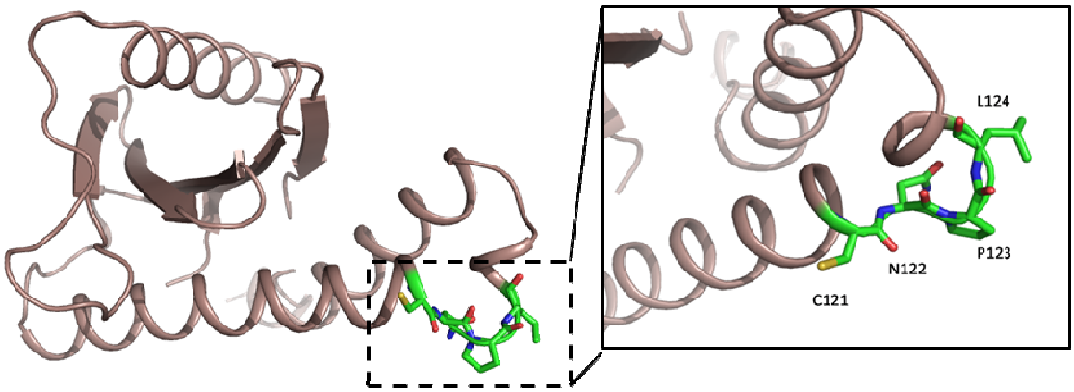


Fig 5.4.1 Bet3 interacting motif of sedlin and Tca17
(a) The interface between sedlin and Bet3 (PDB id: 2J3W). Bet3 is shown in surface representation, while the binding loop of sedlin is shown as carton, with the directly interacting residues as sticks.
(b) Bet3 interaction motif in sedlin.
(c) Hypothetical Bet3 interaction motif in Tca17.

5.4.2 Possible interaction with Trs31

In an earlier Co-IP experiment by Montpetit *et al.* [82], Trs31p was shown to interact with Tca17. It was also shown that Bet3p, Trs31p and Tca17 could be co-expressed and co-purified [83], supporting the possibility that Tca17 can interact with Bet3p and Trs31p in place of Trs20p/sedlin. With the Tca17 structure, it is possible to study its putative interaction with Trs31p in more detail.

Compared with the interaction surface between sedlin and Bet3, the interaction of sedlin and Trs31 involves a larger area (Fig 5.4.2 a). The interface of this interaction was analyzed by Pisa [116]. The residues of sedlin involved in contacts were marked on the surface of sedlin (Fig 5.4.2 b), and the residues involved in H-bonds were colored red in Fig 5.4.2 a. However, when the surface of this area (Fig 5.4.2 c) was

compared with its corresponding area on Tca17 (Fig 5.4.2 d), no similar charge pattern could be identified. Also, no obvious sequence conservation was found in the corresponding area on Tca17 (Fig 5.4.2 e). Therefore, it was not possible to deduce the binding mode of Tca17 with Trs31p from comparison with the sedlin – Trs31 interaction.

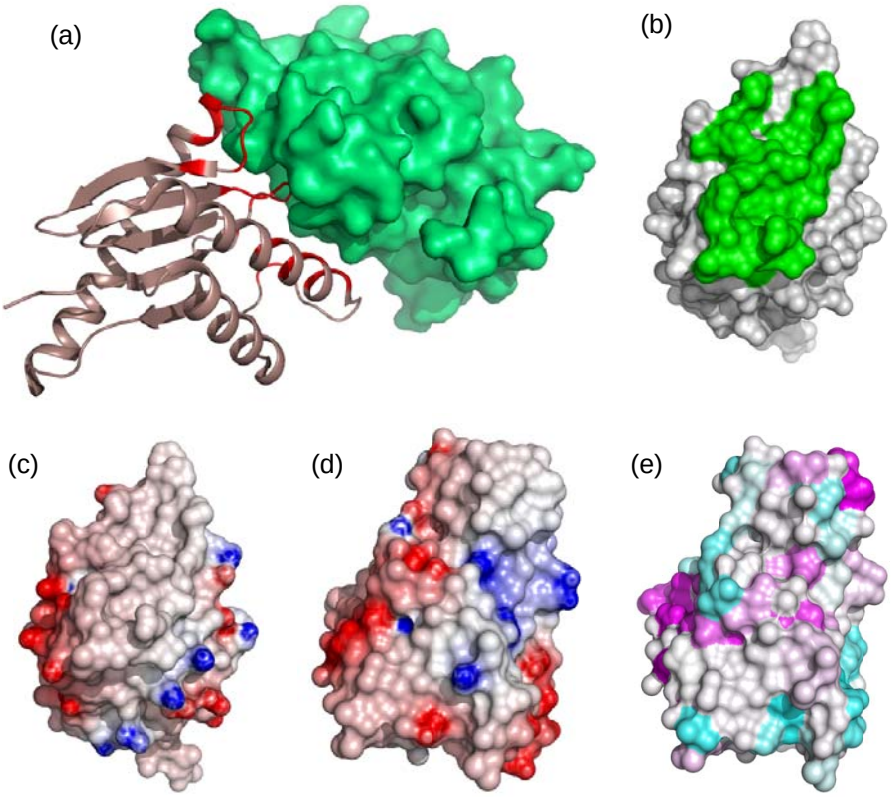


Fig 5.4.2 Trs31 interaction with sedlin and Tca17
(a) The interface between sedlin and Trs31 (PDB id: 2J3W). Trs31 is shown as surface presentation, while sedlin is shown as cartoon. The residues in sedlin, which are involved in hydrogen-bonds with Trs31, are colored red.
(b) The surface area on sedlin which is directly contacting Trs31 is colored green.
(c) Electrostatic potential on the surface of sedlin. The same surface as in (b) is shown.
(d) Putative Trs31-interacting surface on Tca17. Electrostatic potential is shown on surface representation.
(e) Same surface as in (d), colored by sequence conservation score.
The electrostatic potential was calculated by DELPHI [119]. Positive and negative potential was colored blue and red, respectively, at the 10 kT level.
Sequence conservation score was calculated by ConSurf [118]. Magenta, conserved residues; white, average residues; cyan, variable residues.

5.4.3 Possible interaction with Trs33p

The interaction of Tca17 and Trs33p, though not detected by GST pull-down experiments, was suggested by earlier finding that Tca17 needs Tr33p and Trs65p for its interaction with TRAPP [82]. The nature of this interaction is still not understood. However, taking a closer look at the mammalian Bet5-Trs33 interaction, it is found that the Trs33-binding motif in Bet5 (Fig 5.4.3) is comparable with the putative Bet3-binding motif in Tca17 (Fig 5.4.1 c). This suggested the possibility that with some local rearrangement, Tca17 might use the same motif to transiently bind free Trs33. The possibility of this interaction needs to be further investigated.

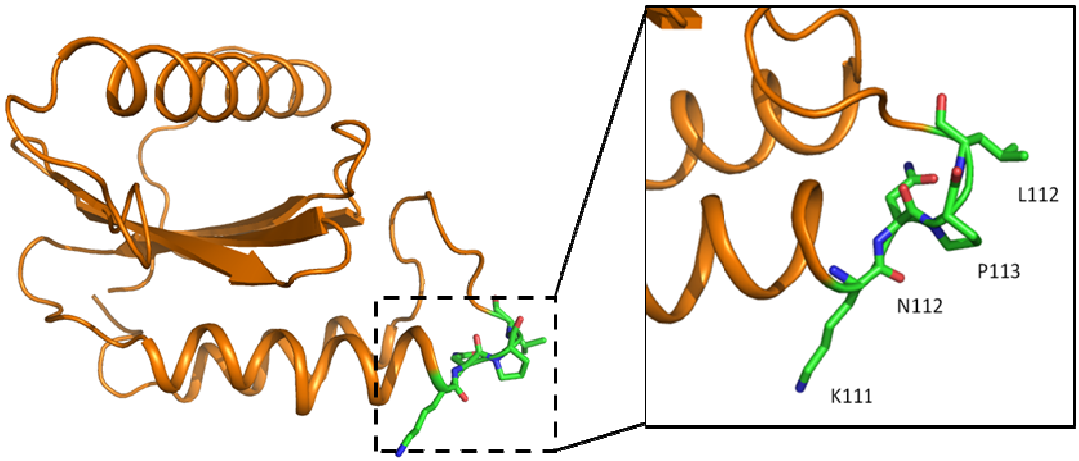


Fig 5.4.3 Trs33 interacting motif in Bet5

5.5 Conclusion

In this work, considerable effort was first invested to study the structure of the two large human TRAPP subunits NIBP and Ehoc-1. A fragment of Ehoc-1 could be produced as recombinant protein in *E. coli*, but could not be studied further with X-ray crystallography. Based on a Co-IP experiments on NIBP and Ehoc-1 fragments and the yeast TRAPP II model, a preliminary model could be proposed for the mammalian TRAPP complex, where the central TRAPP-I like subcomplex is capped on both ends by NIBP and Ehoc-1 and form a triangular structure. This structure may be further stabilized by interactions between conserved peptide segments located near the Ehoc-1 N-terminus and the NIBP C-terminus.

In the second part of this work, a formerly little known yeast TRAPP-associated protein, Tca17, was studied using biochemical and biophysical methods, as well as X-ray crystallography. The crystal structure at 1.8 Å resolution shows that Tca17 adopts the longin fold characteristic for the Bet5 subfamily of small TRAPP subunits. This fold is comprised of a central β-sheet formed by five antiparallel β-strands, and flanked by one α-helix on one side (α1) and two α-helices on the other side (α2, α3).

On the sequence and structure level, Tca17 is most closely related to the TRAPP subunit Trs20p/sedlin. It can bind the TRAPP subunits Bet3p and Trs33p *in vitro*, and might regulate the function of TRAPP by transiently integrating into TRAPP. It remains unclear how the integration of Tca17 into TRAPP might be controlled and promoted. If Tca17 were integrated into TRAPP in place of sedlin/Trs20p, the membrane association of TRAPP might be expected to be weakened, since Tca17 would introduce a negatively charged patch into the presumed membrane association interface of TRAPP.