Final report

1. Introduction

Trip8b is a TPR-domain containing regulatory protein in the brain that is responsible for the modulation of gating and able to regulate the surface expression of the so-called HCN (hyperpolarization-activated cycling nucleotide gated) channels. Trip8b shares fundamental structural properties with the peroxisomal import receptor PEX5. The project aimed to uncover interactions that are important in the role of Trip8b, and also to reveal the most prominent structural and functional properties that are shared between Trip8b and PEX5.

2. Trip8b interactions

a. Trip8b, HCN3 and Rab8b expression and purification

Many constructs for large scale production of Trip8b, HCN1, HCN3 and Rab8b were prepared and tested for protein expression. Since investigation with structure biology methods of these proteins and their interactions required highly pure protein samples, I used various expression tags and E. coli hosts to find the best possible combination for large scale protein expression.

The tested constructs were the following:

HCN1(FL), HCN1(452-910), HCN1(452-591) – pETM20 clones (His-TRX tag)

HCN1(488-910), HCN1(660-910), HCN1(701-910), HCN1(452-591), HCN1(452-788), HCN1(660-788), HCN3 (660-779) – pETM11 clones (His-tag)

Trip8b (FL), Trip8b(166-567), Trip8b(194-567), Trip8b(225-567) – these constructs are prepared in pETM20 (His-TRX tags) and pCDF11 (His-tag) vectors.

Since single expression of the HCN proteins resulted in low expression yields, I decided to perform co-expression experiments:

HCN3 (452-910) pETM20 – Tripb (FL) (pCDF11)

HCN1 (660-779)pETM20 – Trip8b (225-567) pCDF11

Rab8b (1-176), Rab8b (6-176), Rab8b (1-207), Rab8b (6-207), all in petM11 (His-tag)

After many rounds of protein expression and purification tests I have chosen the Trip8b construct 166-567 to use as the main protein sample for further investigations. From the different HCN3 constructs I got the best results with the protein that contains only the C-terminal region, 660-779. Expression of the mouse Rab8b protein resulted in low yields after cleaving off the His-tag, and until now I have not managed to increase the total protein amount in the final product. The other constructs, including all of the tested HCN1 proteins, resulted in either poor expression or no protein at all. Cloning was failed with the AP-2 construct and therefore this protein was omitted from the experiments. All successful protein expression were done with either BL21 (DE3) RIL or Rosetta cell lines.

Proper folding of the expressed proteins were checked by CD measurements.

The final, optimized protocol for the protein production is the following:

Cultures were grown in Terrific Broth medium and induced during the mid-log phase with 0.5 mM isopropyl-β-D-thiogalactopyranosid overnight at 21˚C (or 30 ˚C in the case of Rab8b). The 6xHis, or 6xHIS-TRX tags are cleavable with tobacco etch virus (TEV) protease. The cleared lysate was loaded onto a nickel nitrilotriacetic acid (Ni-NTA) agarose matrix gravity flow column, washed with 10 column volumes of 50mM Tris (pH 8.0), 150 mM NaCl, 2 mM ß-mercaptoethanol buffer and eluted with the same buffer containing 400 mM imidazole. Fusion proteins were cleaved with TEV protease overnight (1mg/50mg protein) at 4˚C and dialyzed against 50mM Tris (pH 8.0), 150 mM NaCl, 2 mM ß-mercaptoethanol, and 20 mM imidazole. The samples were then applied to a Ni-NTA column and the flow-through was collected. As a final purification step, gel filtration was performed using a Superdex 75 (16/60) column.

b. Interaction studies

The interaction of Trip8b and HCN3 was first discovered by yeast-two-hybrid assays. To test the interaction in vitro, I have performed a pull-down experiment to get an overview about the interaction of Trip8b and selected partners.

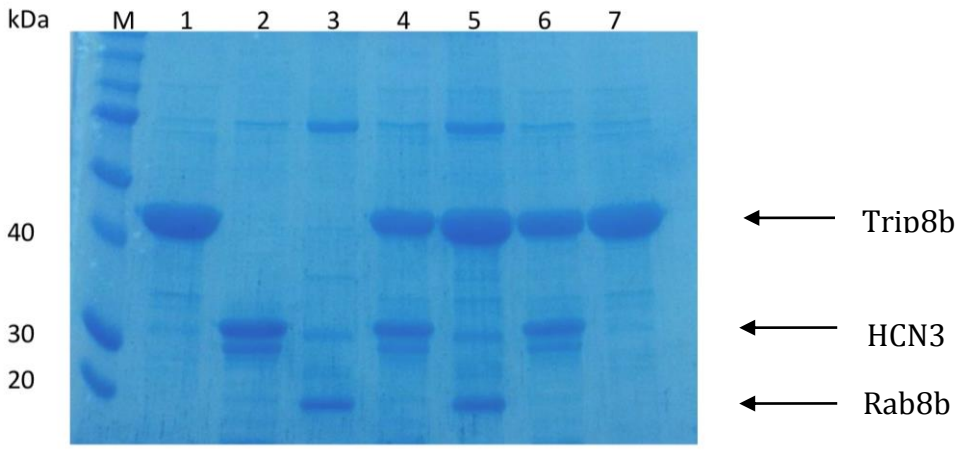

Figure 1 Pull-down experiment of Trip8b complexes. M: marker, 1: Trip8b (166-567) alone, 2: HCN3 (660-779) alone, 3: Rab8b (1-176) alone, 4: Trip8b (166-567) and HCN3 (660-779), 5: Trip8b (166-567) and Rab8b (1-176), 6: Trip8b (166-567) , HCN3 (660-779) and Rab8b (1-176), 7: negative control, Trip8b and Pex19p

According to the experiment, Trip8b interacts with both the tested HCN3 and Rab8b constructs. Interestingly, when both proteins were added together to Trip8b, only the HCN3 construct bound. This implies that either the two partners compete for a (partially) overlapping binding site on Trip8b, or the Rab8b interaction is only an artifact. According to literature, the interaction of Trip8b and Rab8b is involved in regulated secretory pathways in AtT20 cells, and they likely play a role in cAMP-induced secretion of adrenocorticotropic hormone (ACTH) (REF Chen).

c. Quantification of Trip8b – HCN peptides interaction

Trip8b interacts in the brain with four different HCNs. I was curious whether the different C-terminal tripeptides of the four HCNs would lead to different binding affinities when they interact with Trip8b. To test this, I performed ITC measurements to quantify the binding thermodynamics of all four HCNs when bound to Trip8b. The following peptides were used for the measurements:

HCN1 YPRFASNL

HCN2 YSRLSSNL

HCN3 YPQISANM

HCN4 YSKLPSNL

HCN peptide	**K_d (µM)**	**N**	**ΔH (kJ/mol)**	**TΔS (kJ/mol)**
HCN1	1.0±0.13	0.94±0.05	-36.6±0.3	-0.25
HCN2	1.1±0.14	0.93±0.03	-29.1±0.6	5.0
HCN3	16.5±8.1	0.65±0.2	-29.9±2.7	-2.5
HCN4	3.0±0.8	0.99±0.07	-22.1±0.9	8.9

TABLE 1. ITC measurements of HCN peptides

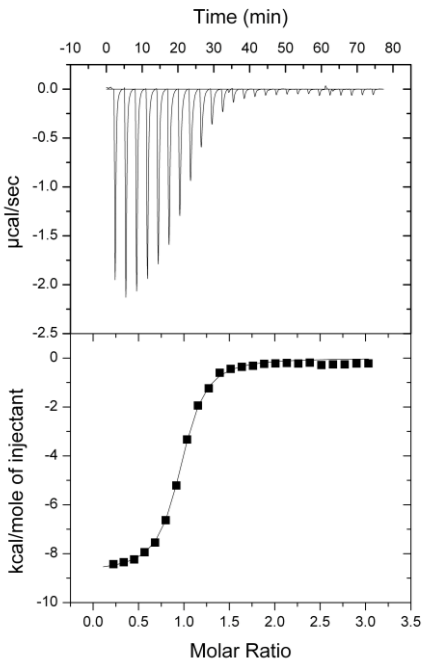

Figure 2. Representative ITC measurement (Trip8b-HCN1)

The HCN1 peptide was found to be the strongest binder, and there are no major differences between peptides 1,2 and 4 in terms of binding affinities (Table 1). HCN3, which has a somewhat special targeting signal (-ANM), was found to have the lowest affinity. For peroxisomal proteins the binding affinities fall between the 10 nM to the 100 μM range (Ghosh et al, 2010). Similarly to the HCN proteins, the primary structure of the targeting signal seems to be the major determinant of binding affinity, and often the non-consensus type signals are the weaker binders. It is important to note, that full lengths proteins are expected to have higher binding affinities because of the formation of interactions outside the canonical binding site.

d. Crystallization trials

Initial crystallization trials were performed using available proteins and protein complexes (Trip8b (166-567) – HCN3 (660-779), Trip8b-peptide complexes, Trip8b alone). The screening was done using a sparse matrix screen in a 24 well plate format. While in some of the drops crystalline material could be observed, no crystals suitable for testing were found. The most promising results were obtained from a drop containing the Trip8b (166-567) – HCN3 (660-779) complex and sodium malonate, in which spherulites grew.

3. Interaction mechanism of TPR domain containing proteins

Interaction studies with Trip8b have revealed that binding characteristics of the TPR domain containing proteins Trip8b and PEX5 are very similar. To further investigate the binding mechanism of TPR domains with their interaction partners, I have determined the structure of PEX5 in complex with mutated versions of a model protein, alanine-glyoxylate aminotransferase (Figure 3). (Expression constructs were already available.) Complex structure determination was done using the CCP4 software package (MOSFLM, Scala, Phaser, Refmac5) and Coot. Structure quality was assessed with Molprobity (PDB codes: 4KYO and 4KXK). The new structures can serve as suitable models to study the mechanism of Trip8b's HCN binding based on the structural similarities.

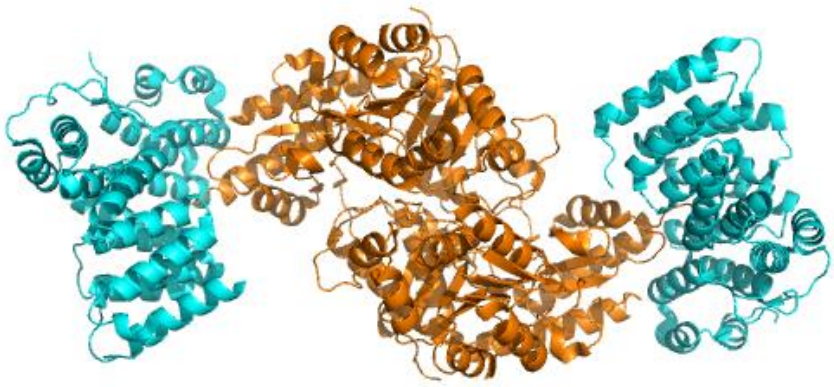

Figure 3. In the PEX5-AGT(K390A) complex one AGT dimer is bound to two PEX5 molecules. PEX5: cyan; AGT dimer: orange.

Tetratricopeptide repeat (TPR-) domain containing proteins serve as protein interaction platforms in several molecular systems (Allan and Ratajczak, 2011). Peroxisomal proteins are recognized and translocated to their target organelle by the cytosolic receptor PEX5, which is able to recognize specific C-terminal sequence motifs called peroxisomal targeting signals (PTS), and an identical type of signal sequence drives the interaction of HCNs with Trip8b. Recent crystal structures of both TRIP8b and PEX5 TPR domains reveal very high structural similarity (Figure 4). (Stanley et al., 2006, Fodor et al., 2012, Bankston et al., 2012)

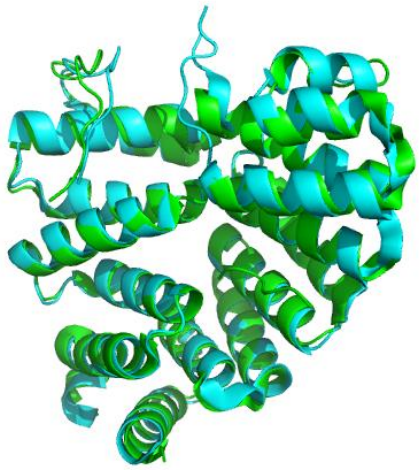

Figure 4. Structural alignment of the TPR domain of PEX5 (cyan) and Trip8b (green).

The available crystal structures of TPR-domain containing protein complexes reveal that upon binding the C-terminal region of the cargo deeply penetrates into the binding cavity of the TPR, and in the same time the receptor undergoes a substantial conformational change.

Initially it was believed that the strength of the receptor-cargo binding simply correlates with the number of residues in the signal peptide that are capable of forming specific interactions. However, structural and biochemical analysis of

recent structures suggested that prediction of binding affinity is not possible based on simply the presence of such residues (Stanley et al., 2006, Fodor et al., 2012, Bankston et al., 2012).

The newly determined structures contain the TPR domain of Pex5 and AGT (K390A) or AGT (K390A/K391A), respectively. The mutated residues are located in the PTS1 region, in the -2 position (K390A) or in the -2 and -1 positions (K390A/K391A) (when the very C-terminal leucine is considered to be in position 0). The overall structure of both complexes is very similar to the wild-type complex (Figure 3). However, there are minor, but substantial differences in the conformation of the TPR domain (Figure 5). The PTS1 segments in both mutant complexes form an extensive interaction network. The core of the interaction network is represented by three asparagines of PEX5 (415, 534, 561) that connect either main- or sidechain atoms of the signal tripeptide. Upon cargo binding, the conformational change of the C-terminal domain of PEX5 seems to be dependent on the cargo bound: in case of the single mutant, the TPR repeats come closer to each other.

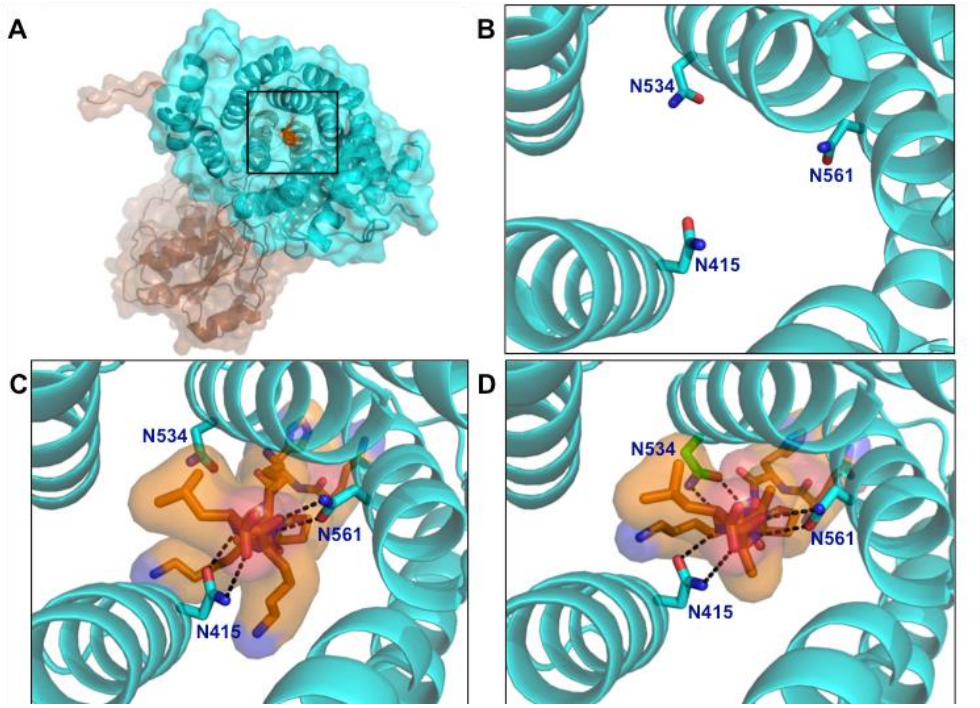

Figure 5. Conformational changes of the PTS1-binding pocket of PEX5 upon protein cargo binding. **A) Overview of the structure of the PEX5(C)–AGT complex, with the area of the PTS1-binding cavity boxed. PTS1 interaction area of B) PEX5(C) in the absence of protein cargo (Stanley et al., 2006); C) PEX5(C), upon binding to AGT (wt) (Fodor et al., 2012); D) PEX5(C), upon binding to AGT (K390A). In PEX5(C)–AGT (K390A), all three PTS1 0, -1, and -2 positions are bound to Asn415 (TPR-2), Asn534 (TPR-6) and Asn561 (TPR-7) from PEX5(C) in a triangular arrangement. In the PEX5(C)–AGT (wt) complex, the interaction of the PTS1 -2 position with Asn534 from PEX5(C) is impeded because of the presence of a bulky lysine side chain in position 390. Colors: AGT, brown/orange; PEX5(C), cyan. Residue Asn534 of PEX5(C) is highlighted in green.**

Table 2. Crystallographic data

	PEX5(C)-AGT(K390A)	PEX5(C)-AGT(K390A/K391A)
Data collection		
Space group	P1	P1
Cell dimensions		
a, *b*, *c* (Å)	57.7, 74.8, 91.2	57.6, 74.7, 91.2
a, b, g (°)	87.5, 83.7, 89.6	87.5, 83.6, 89.8
Resolution (Å)	51.0-2.2 (2.32-2.20)	19.8-2.9 (3.06-2.90)
R_{merge}	11.4 (46.6)	13.4 (46.2)
I / sI	8.7 (2.0)	7.3 (2.1)
Completeness (%)	95.2 (90.9)	96.8 (96.8)
Redundancy	2.6 (2.6)	2.2 (2.2)
Refinement		
Resolution (Å)	51.0-2.2	19.8-2.9
No. reflections	71036	30756
R_{work} / R_{free}	17.7 / 22.3	20.8 / 25.0
No. atoms		
Protein	10494	10516
Water	732	2
Sulphate	10	10
B-factors		
Protein	29.4	34.3
Water	32.1	8.3
Sulphate	21.3	36.9
R.m.s. deviations		
Bond lengths (Å)	0.011	0.006
Bond angles (°)	1.263	0.918

To further investigate the observed structural changes, binding affinities were measured for the mutant proteins. Interestingly, the K390A mutant bound the TPR domain of PEX5 with 10 times higher affinity, than the wild type, and this substantial difference is coupled with the most pronounced change of the TPR domain conformation. The single mutant K391A (no crystal structure is available) lost the ability to bind the TPR domain, while the double mutation (K390A/K391A) rescued the interaction, with an affinity close to wild-type and a moderate conformational change (Table 3.).

Table 3. PEX5 and TRIP8b TPR domain properties

TPR containing molecule	Interacting partner	Cavity volume (Å^3)	Binding affinity (μM)
PEX5 (C)	-	904	-
PEX5 (C)	AGT (wt)	468	3.5 ± 0.4[1]
PEX5 (C)	AGT (K390A)	277	0.36 ± 0.03
PEX5 (C)	AGT (K391A/K391A)	318	5.0 ± 0.8
PEX5 (C)	SCP2	252	0.11± 0.03[2]
TRIP8b-1aΔ1-205	HCN2 peptide	294	0.16 ± 0.03[3,4]
TRIP8b-1aΔ1-165	HCN3 (660-779)	To be determined	2.0 ± 0.5
TRIP8b-1aΔ1-165	HCN1 peptide	To be determined	1.0±0.13
TRIP8b-1aΔ1-165	HCN2 peptide	To be determined	1.1±0.14
TRIP8b-1aΔ1-165	HCN3 peptide	To be determined	16.5±8.1
TRIP8b-1aΔ1-165	HCN4 peptide	To be determined	3.0±0.8

1) Fodor, K. et al. (2012) Requirements for Peroxisomal Targeting of Alanine-Glyoxylate Aminotransferase as an Essential Determinant in Primary Hyperoxaluria Type 1. PLoS Biol *10*, e1001309.
2) Stanley, W. et al. (2006) Recognition of a Functional Peroxisome Type 1 Target by the Dynamic Import Receptor PEX5. Mol. Cell. 24 (5)) 653-663.
3) Banski, J. R. et al. (2012) Structure and stoichiometry of an accessory subunit TRIP8b interaction with hyperpolarization-activated cyclic nucleotide-gated channels. *PNAS 109, 7899-7904.*
4) There is a 10 fold difference of binding affinities in the case of an HCN2 peptide which can be a result of the different length Trip8b constructs.

Based on the data available for Pex5 interactions, HCN binding affinities can also be analyzed. Regarding HCN binding affinities to TRIP8b, a 7-mer HCN2 peptide with a more "consensus-like" interaction signal (-SNL) has a Kd of 0.16 μM (Bankston et al., 2012), while HCN3 with a less optimal C-terminal sequence (-ANM) has a Kd of 2 μM. Similar results were obtained by using a different Trip8b construct (see Table 3). Therefore it can be proposed that higher binding affinity is mirrored by the receptor structure: depending on the amino acid composition of the targeting signal, the TPR domain is able to adapt to different signals by slightly repositioning the TPR repeats. This mechanism would allow specific binding of cargo molecules in a semi-specific binding pocket and could be a

general property of TPR domains. In an attempt to quantify the structural change upon binding I measured the binding cavity volumes making use of the crystals structures (Figure 6). Cavity volume of the TPR domain in its apo form, in the presence of low- to high-affinity cargos were determined. The results show that the binding cavity can shrink to 1/3 of its original volume when a higher affinity cargo is bound, while intermediate values were obtained for proteins with lower affinities.

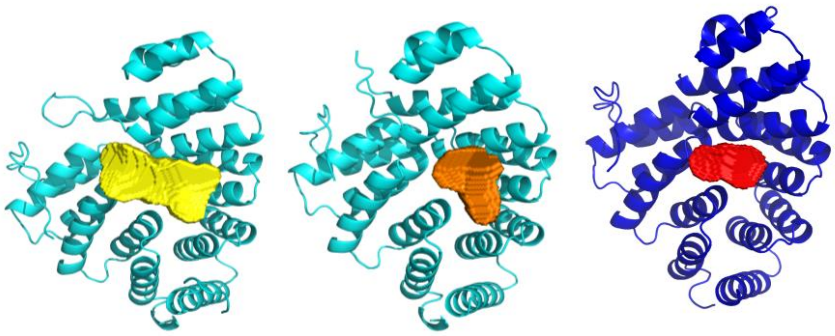

Figure 6. Volume representation of the binding cavities in TPR domains. 1) Apo Pex5 TPR 2) AGT (low affinity cargo) bound Pex5 TPR) 3) HCN 2 peptide (moderate affinity cargo) bound Trip8b TPR

In conclusion, analysis of the specific interactions formed by residues in the signal peptide and comparison of binding affinities between various cargo molecules and the TPR domains suggest that the rate of conformational change of Trip8b (or PEX5) upon interaction is a major determinant of binding affinity and thus is a regulating factor. Given that HCN3 seems to be the only HCN that is not modulated by intracellular cAMP, its lower Trip8b binding affinity may have a functional role. High-resolution crystal structures of further TRIP8b complexes in the future will allow us to elucidate the TPR interaction mechanism across different molecular systems. (A manuscript with more details is available, which requires minor revisions and will be sent back to the journal Traffic within weeks.)

4. PEX5-Pcs60p modeling

As part of a new collaboration project with Ralf Erdmann's group from the University of Bochum, Germany, I tried to further evaluate the cargo binding mechanism of TPR domains. The modeling studies were initiated by recent results of mass spectrometry analysis on complexes of the C-terminal domain of Saccharomyces cerevisiae Pex5 and the extended PTS1 of the peroxisomal protein Pcs60p. Octapeptides were created from the PTS1 of Pcs60p using pBenzophenone labeling and the resulted covalent complexes (Pex5-peptide complexes) were studied with MALDI-TOF. The experiment aimed to prove that pBenzophenone labeling is a useful tool to study PEX5 interactions.

To confirm the MS data, first I have created a model of the S.c. Pex5 molecule with the Pcs60p peptide. Homolgy model of ScPex5 (301-612) was made using the RaptorX server. The final model using the template 2J9Q chain A gave an overall score of 312 and a P value of 9.2×10^{-7}. To create an initial model for the

Pcs60p C-terminal octapeptide I used the human PEX5-SCP2 complex (PDB code: 2C0L) as a template. The C-terminal peptide sequence of SCP2 was mutated to that of Pcs60p and merged with the ScPex5 (301-612) model into one single pdb file with Coot. This new file was uploaded to the FlexPepDock server to create a model of the ScPex5 (301-612) in complex with the extended eight-residue long PTS1 of Pcs60p. The first ten docking solutions, of which the best one gave an overall Rosetta score of -56.584, were visually analysed using PyMol. The model well agrees with the MS data and suggests that the combination of covalent labeling and mass spectrometry can be a powerfull tool in interaction mapping. A manuscript is under preparation.

As a second step, I have created a similar model, this time using the C-terminal domain of Trip8b and the peptides from the four HCNs. All four peptides were able to dock into the cavity of Trip8b (Figure 7). To my surprise, the peptide backbones adopted the same conformation (except the N-termini), their position within the cavity was also idential and no difference could be observed for the low-affinity binder HCN3. This would suggest (although indirectly) the importance of the TPR domain conformational change in the different binding affinities.

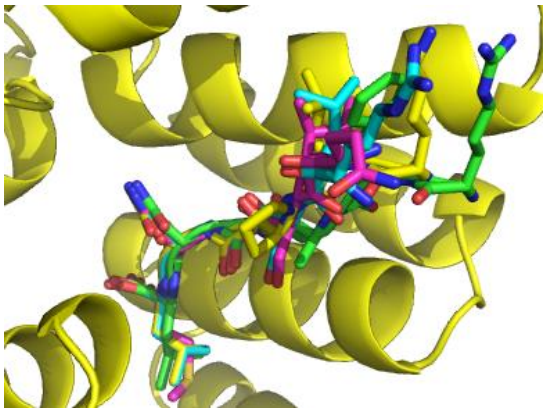

Figure 7. Alignment of the four HCN peptides when they are bound to Trip8b (yellow).

5. Conclusions

Trip8b is a key molecule in the regulation of hyperpolarization-activated cycling nucleotide gated channels. It contains a TPR domain that is used as a binding platform to bind its partners via a C-terminal signal peptide. Other molecules, like Rab8b, also bind Trip8b that suggests that Trip8b is involved in several molecular processes in the brain.

It has been described for various molecular systems that enhanced ligand binding is achieved by the aid of secondary interactions, co-factors or co-regulators. Notable examples are G-protein coupled receptors, integrins or PDZ-domain containing proteins. (Lane et al., 2013; Springer and Dustin, 2012; Ye and Zhang, 2013) However, TPR-domain mediated interactions are somewhat special, since the removal of the specificity of the signal peptide facilitates interaction through conformational changes in the receptor. The available data

demonstrate that as a consequence the gain in binding affinity is permitted by an unimpeded induced fit mechanism.

Trip8b can bind both HCNs and Rab8b, most probably in a sequential manner. The mechanism of HCN binding seems to be very similar to the interaction mechanism of the peroxisomal receptor PEX5 that may suggest a common evolutionary origin of the two. Similarly to many peroxisomal proteins, correct localization of HCNs is a prerequisite for their proper function. An important property of TPR domains that they are able to differentiate between the various cargo molecules and adapt to different signals by slightly repositioning the TPR repeats.

6. Additional activities:

6.1 Networking, new collaborations

A new collaboration was established with the group of Ralf Erdmann at the Ruhr University, Bochum to co-operate on structural investigation of PTS1 protein binding to Pex5.

6.2 Conference

I attended the annual meeting of the Biophysical Society and presented a poster titled „The highly dynamic protein binding property of TPR domains in Trip8b and PEX5". The meeting was also a great opportunity to meet leading researchers from the ion channel field.

6.3 Teaching

I gave an „Introduction to Biochemistry" course at the university.

7. Other

I would like to note that until the last moments of my employment at ELTE I had to use my own personal laptop for work because I was not allowed to order a new computer despite the approved research budget. In addition to this, extreme bureaucracy and the lack of proper administrative assistance made certain tasks very complicated (ordering from abroad, travel arrangement etc.).

8. Publication

Fodor, K., Wolf, J., Reglinski, K., Passon, D.M., Lou, Y., Schliebs, W., Erdmann, R., Wilmanns, M. Ligand-induced compaction of the PEX5 receptor-binding cavity regulates cargo import efficiency into peroxisomes. *Submitted to Traffic and reviewed, minor revision is required (IF: 4.919).*

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