FINAL REPORT

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Identification and characterisation of novel targets of the evolutionarily conserved Protein phosphatase 4

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The major aims of the OTKA-PD proposal were to identify and characterise specific substrate proteins of the evolutionarily conserved Protein Phosphatase 4, PP4, and to dissect PP4's function in regulating centromere integrity via CENP-C (Lipinszki et al., 2015). We wished to understand the mechanism that PP4 uses to bind its substrates by identifying the proteins interacting with Falafel, the substrate-binding regulatory 3 (R3) subunit of the heterotrimeric PP4.

Major achievements:

1. Pull-down. According to the plan, during the 1st year of the grant we established GFP- or ProteinA-tagged Falafel expressing transgenic fruit flies and D.Mel-2 cultured cells, respectively, and performed affinity-purification coupled to mass-spectrometry (AP-MS) experiments. Unexpectedly, only few novel partners of Falafel were identified in this screen, therefore we concluded that full-length Falafel as part of the holoenzyme complex would either transiently recognize its putative binding partners or the activity/surface of the substrate-binding domains/motifs are strictly regulated or hidden. To overcome this problem, during the 1st and 2nd years of the grant we generated and used in AP-MS experiments the following Falafel (Flfl) pieces expressed in transgenic flies (ProteinA-fusion) or bacteria (GST-fusion), respectively:

1. Flfl-**EVH1** [aa 1-168]
2. Flfl-**SMK-1** [aa 169-361]
3. Flfl-**N** [aa 1-361]
4. Flfl-**M** [aa 362-665]
5. Flfl-**C** [aa 666-973]

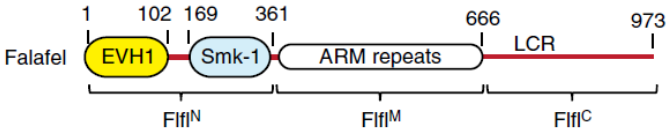

Figure 1. Schematic representation of the common structural elements of Falafel.

EVH1. Proteins expressed in bacteria were immobilised onto GSH-sepharose beads and mixed with wild-type *Drosophila* embryo extract (age: 0-10 h), a rich source of mitotic proteins. ProteinA-tagged pieces of Falafel expressed in transgenic flies were purified from syncytial embryos according to (Lipinszki et al., 2014). MS analyses and bioinformatics of the bound proteins have led to the identification of circa **60 Falafel-interacting proteins**, putative PP4 substrates (40 in the 1st and 20 in the 2nd year). We found that most of these proteins specifically bind to the conserved, N-terminally localized EVH1 domain of Falafel (Figure 1)

that has been shown to play a role in substrate recognition and recruitment (Lipinszki et al., 2015; Sousa-Nunes et al., 2009). We then classified the EVH1-interacting proteins into groups based on their subcellular location and function:

1. Centromeric proteins (e.g. CENP-C)
2. Kinetochore components (e.g. Spc105R) that provide a platform for the Spindle Assembly Checkpoint, SAC.
3. All 4 subunits of the Chromosome Passenger Complex (CPC), including Incenp, Deterin, Borealin and AuroraB kinase.
4. Proteins involved in mitotic spindle formation (e.g. Megator).
5. Components of the centrosome, including proteins having a role in centrosome conversion, maturation and duplication (e.g. Ana1, Ana2, Cep135).

SMK-1. Besides EVH1, Falafel contains an Smk-1/DUF625 *(Domain with Unknown Function 625, which is present in Smk-1 protein, a component of the IIs longevity pathway that regulates aging in C. elegans (Wolff et al., 2006))* domain occupying the amino-terminal region of the protein (Figure 1). Smk-1 is well-conserved in Falafel and its orthologues, however nothing is known about its function. Indeed, very few interactors were found in association with Falafel-SMK1 domain in our screen. However, this includes the entire RZZ complex (Rough Deal-Zwilch-ZW10), the dynein motor protein and a kinase, all which have a critical role in regulation of SAC activity. SAC prevents chromosome separation until all chromosomes are properly attached to the mitotic spindle. This finding was intriguing, because we saw earlier that Falafel depletion from cells by RNAi (or other genetic methods) abrogates the SAC, consequently PP4 regulates the activity of this checkpoint in mitosis. This finding clearly suggests that the EVH1 domain of Falafel/R3 orthologues is not solely responsible for substrate recognition, and it had opened new research avenues in the field of PP4 study. We took this opportunity and started to carefully investigate the function and structure of SMK-1 in relation to PP4 activity.

2. *In vitro* interaction. Our research proved that individual domains of Falafel efficiently pull-down entire protein complexes. It is not clear, however, which subunit of such a complex forms an interaction with PP4. In order to clarify this as well as to test which of the 60 putative Falafel-interacting proteins are physical partners of Falafel (EVH1 or SMK-1 domains), we aimed to perform classical *in vitro* protein-protein interaction assay. During the 2nd year we cloned almost all 60 identified proteins to be used in a rabbit reticulocyte lysate-based (RLL) coupled *in vitro* transcription/translation reaction (IVTT) at 30 °C or in a wheat-germ IVTT system at 25 °C. In IVTT reactions either ^{35}S-Met or biotinylated-Lys was used to label the prey proteins for subsequent detection by autoradiography or immunoblotting. As proposed we used GST-tagged Falafel-EVH1 or SMK-1, as bait, and mixed with the candidate proteins, prey, to test physical interaction as described in (Lipinszki et al., 2015). We have almost finished this screen (considering that the grant was shortened to 2.5 years) and already validated circa 10 important mitotic proteins from the list as direct interactors of Falafel (EVH1 or SMK-1). Below, the CPC component INCENP is shown to physically interact with the EVH1 domain of Falafel (Figure 2 left panels), while ZW-10, component of the heterotrimeric RZZ complex that regulates SAC activity binds directly to the SMK-1 domain of Falafel (Figure 2 right panels).

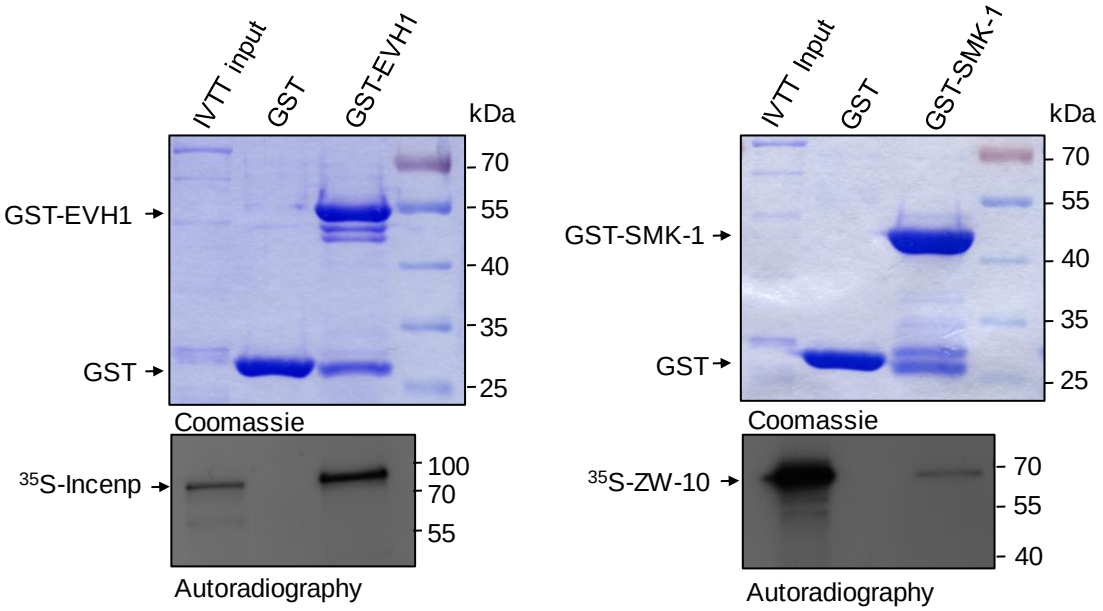

Figure 2. An example of the *in vitro* binding assay demonstrates that GST-EVH1 (left panels) specifically bind to ^{35}S-Met-labelled INCENP, a component of the chromosome passenger complex (CPC) regulating chromosome alignment in mitosis, sister chromatid cohesion, spindle assembly and cytokinesis; while GST-SMK-1 (right panels) specifically interact with ^{35}S-Met-labelled ZW-10, component of the Rod-Zwilch-ZW-10 (RZZ) complex that is essential in governing the spindle assembly checkpoint.

Moreover, we started to narrow down the interacting surfaces between EVH1 or SMK-1 and the putative partners for two reasons:

1) To identify EVH1- or SMK-1-specific consensus binding motifs in PP4 substrates.
2) To determine the mode of substrate binding of SMK-1 (similarly to EVH1 (Lipinszki et al., 2015) by resolving the crystal structure of the domain in complex with the interacting motif. This is in progress.

To do this we chopped candidate proteins into 2-4 overlapping pieces and used in the above describes *in vitro* binding assay. For example, this has revealed that the third part (very C-terminus) of INCENP possess a putative Falafel-interacting motif (FFTP), that is similar to the CENP-C Falafel-interacting motif FKKP described in (Lipinszki et al., 2015). However, sequence analysis of the identified (and published) Falafel interactors have not revealed any well-defined consensus binding motif. Therefore, further, more precise narrowing-down experiments are under progress in all candidates.

2. We also found that the centrosomal protein Ana1 directly binds to Falafel and co-localizes with PP4 within the centriole (confirmed by super-resolution microscopy in collaboration with David Glover (University of Cambridge)). This was an important finding, since previously it was shown that Ana1 is required for centriole-to-centrosome conversion (Fu and Lipinszki et al., 2016), a prerequisite for centrosome maturation and duplication as well as spindle formation. Moreover, all these processes are regulated by protein phosphorylation. 20 years ago Patricia Cohen's lab in Dundee showed that PP4 is essential for centrosome maturation (Helps et al., 1998), but the mechanism had not been clarified. Based on our new findings we believe that PP4 is recruited to the centriole by Ana1 to antagonize the activity of the centrosomal protein kinases, such as Plk4. In collaboration with the Glover lab in Cambridge we

demonstrated that Plk4 phosphorylates Ana2, which then gets loaded onto the site of pro-centriole formation ((Dzhindzhev et al., 2017) and recruits Sas6, the component of the centriole cartwheel, a prerequisite for pro-centriole formation (Dzhindzhev et al., 2014). It is therefore an interesting future challenge to uncover the centrosomal substrates and centrosomal roles of the PP4 phosphatase.

3. We also started to map (de)phosphorylation sites on the key centromeric protein CENP-C, which has been shown to be a direct interactor and presumed mitotic substrate of PP4. This would tell us whether phosphoregulation of CENP-C by PP4 is essential in maintaining the integrity of the mitotic kinetochore or the major role of CENP-C is to recruit PP4 at the centromere that dephosphorylates other proteins within this area. We focused on the modified residues adjacent to (or within) the Falafel-Binding-Domain of CENP-C and generated phospho-null (Ser/Thr-to-Ala substitutions) and phospho-mimicking (Ser/Thr-to-Asp/Glu substitutions) mutant variants of CENP-C, all resistant to RNAi. In transgenic flies we replaced endogenous CENP-C with these mutant forms and found that both phospho-mimicking and null bound to PP4, but only phospho-null was able to localize to the centromere and maintain the integrity of the kinetochore. This suggests that PP4 is involved in the constant dephosphorylation of CENP-C, a prerequisite for proper centromere function.

4. Another interesting hit from the screen was the conserved and essential heterochromatin-associated protein, Barrier of Autointegration Factor or BAF. It is well established that BAF regulates the formation of the nuclear membrane after mitosis. Our preliminary results suggested, however, that BAF does not directly interact with Falafel, but via CENP-C (thus CENP-C is also a recruiting platform for PP4). This observation has initiated a collaboration with the labs of Ferran Azorín (IRB Barcelona) and Marcin Przewloka (University of Southampton) who found that BAF co-localises with CENP-C at the centromere, which seems to be PP4 activity-dependent. Moreover, this specific localisation of BAF has different function than that of the heterochromatin-associated BAF. We (in Szeged) have generated a series of recombinant plasmids and transgenic flies, e.g. those expressing RNAi-resistant CENP-C, wild type or PP4-binding deficient, respectively, that has been used to replace endogenous CENP-C. With these constructs we could selectively eliminate PP4 from the centromere to understand the exact function of BAF in regulation of mitosis.

5. During the last 2.5 years we also focused on the reconstitution of PP4 holoenzyme from recombinant proteins. In cooperation with György Pósfai's lab (BRC) we generated and thoroughly tested an *E. coli* strain that is more efficient in expressing Eukaryotic proteins (manuscript under submission). With this strain we managed to express good amount of Falafel and could reconstitute the entire PP4 complex. The work is still in progress. In addition to this, we discovered and characterized a conserved motif, which when fused to transgenic proteins significantly increases their stability by preventing their degradation by the proteasome (another manuscript is under preparation). This provides us the possibility to overexpress PP4 subunits in *Drosophila* embryos for subsequent purification and in vitro analysis.

Closing remarks

During the 2.5 year post-doctoral OTKA (PD115404) project the grant holder (PI) got a permanent senior research associate position at the Institute of Biochemistry (BRC) and became the group leader of the Momentum (Lendület) Cell Cycle Regulation Group supported by the Hungarian Academy of Sciences. Most of the above detailed experiments were performed by the PI and a PhD student (as part of his thesis) under his supervision. In addition to the above

mentioned 2 manuscripts that are under preparation/submission, 2 reviews in Hungarian (Biokémia 2017.) and 2 research articles in peer-reviewed journals were published (Cell Report, just accepted; Open Biology 2017) in relation to this project.

References

Dzhindzhev, N.S., G. Tzolovsky, Z. **Lipinszki**, M. Abdelaziz, J. Debski, M. Dadlez, and D.M. Glover. 2017. Two-step phosphorylation of Ana2 by Plk4 is required for the sequential loading of Ana2 and Sas6 to initiate procentriole formation. *Open biology*. 7.

Dzhindzhev, N.S., G. Tzolovsky, Z. **Lipinszki**, S. Schneider, R. Lattao, J. Fu, J. Debski, M. Dadlez, and D.M. Glover. 2014. Plk4 phosphorylates Ana2 to trigger Sas6 recruitment and procentriole formation. *Current biology : CB*. 24:2526-2532.

Fu, J., Z. **Lipinszki**, H. Rangone, M. Min, C. Mykura, J. Chao-Chu, S. Schneider, N.S. Dzhindzhev, M. Gottardo, M.G. Riparbelli, G. Callaini, and D.M. Glover. 2016. Conserved molecular interactions in centriole-to-centrosome conversion. *Nature cell biology*. 18:87-99.

Helps, N.R., N.D. Brewis, K. Lineruth, T. Davis, K. Kaiser, and P.T.W. Cohen. 1998. Protein phosphatase 4 is an essential enzyme required for organisation of microtubules at centrosomes in Drosophila embryos. *Journal of cell science*. 111:1331-1340.

Lipinszki, Z., S. Lefevre, M.S. Savoian, M.R. Singleton, D.M. Glover, and M.R. Przewloka. 2015. Centromeric binding and activity of Protein Phosphatase 4. *Nat Commun*. 6:5894.

Lipinszki, Z., P. Wang, R. Grant, C. Lindon, N.S. Dzhindzhev, P.P. D'Avino, M.R. Przewloka, D.M. Glover, and V. Archambault. 2014. Affinity purification of protein complexes from Drosophila embryos in cell cycle studies. *Methods in molecular biology*. 1170:571-588.

Sousa-Nunes, R., W. Chia, and W.G. Somers. 2009. Protein phosphatase 4 mediates localization of the Miranda complex during Drosophila neuroblast asymmetric divisions. *Genes & development*. 23:359-372.

Wolff, S., H. Ma, D. Burch, G.A. Maciel, T. Hunter, and A. Dillin. 2006. SMK-1, an essential regulator of DAF-16-mediated longevity. *Cell*. 124:1039-1053.

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