Systems level profiling of cell growth and death promoting MAP kinase networks

My major goal with the Frontline proposal was to secure funding for the group to move into bigger systems compared to the classical biochemical and structural biology focused work that we had been doing earlier, and thus to be able to answer systems level questions in cellular signaling. I believe that this goal was accomplished and we developed the capacity to work with a great number of proteins in parallel in vitro, to carry out higher level studies (*e.g.*, computational modeling or interactomics), and to use different cell-based assays in higher throughput as earlier. Thematically my goal with this proposal was to set the stage for the development of molecules that can re-establish the balance between cell growth and cell death promoting MAPK signaling activities in pathological states. This latter goal was somewhat even exceeded compared to how I had planned it since we identified a new set of compounds about halfway of the project which were novel and could be used to perturb MAPK based signaling networks in the cell. After this discovery the focus started to shift towards chemical biology. This was one of the reasons why I "moved" into a new institute and established the Biomolecular Interaction Group in the Institute of Organic Chemistry, Research Center for the Natural Sciences. Research personnel fluctuation on the project, partly due to uncertainties/job insecurities because of several restructuring phases experienced at RCNS lately, was fairly large, particularly regarding newly recruited postdocs, for example Krisztina Paál, Péter Egri and Eszter Szarka who left the project early for more secure academic or industry positions. Despite all this, 5 PhD (Gergő Gógl, Klára Kirsch, Neha Singh, Péter Sok, and Ádám Levente Póti), and 8 MSc (Sarolt Magyary, Evelin Németh, Tamás Takács, Orsolya Ember, Bettina Balázs, Kinga Papp, Dóra Földesi-Nagy, Laura Dénes) students finished his/her thesis work in the lab related to the project during the grant period.

We published 3 major papers (Nature Communications), 4 other papers (Structure, Front Mol Bios, 2 Int J Mol Sci), 1 review (TIBS), two book chapters (Adv Exp Med Biol and Protein Interactions: The Molecular Basis of Interactomics) and contributed to 6 additional papers (see public list of publications). We submitted 2 patent applications, a PCT application, and two manuscripts related to the latter which are currently under revision at *Nature Communications*.

In the next few pages I will briefly summarize the work that we did related to those published papers where we had a major contribution and then I will describe the highlights of the two manuscripts currently at the revision stage in more detail. These latter two stories are inherently tied to the patent applications that we concluded at the end of last year, and unfortunately my original publication schedule got greatly delayed because of this, therefore these results are still unpublished, albeit I am hopeful that they will be available soon.

We explored the interactions of MAPKs with their substrate kinases (MAPK activated protein kinases, MAPKAPK). We systematically examined MAPK-MAPKAPK binding and activation in vitro with purified proteins, and explored the solution structure of four different MAPK-MAPKAPK pairs (ERK2-RSK1, ERK2-MK2, ERK5-MK2 and p38-MK2) in the unphosphorylated as well as in the phosphorylated forms by small X-ray angle scattering (SAXS), and also determined the crystal structure of some of the MAPK-MAPKAPK pairs. This analysis allowed the modeling of the different kinase heterodimers and we found that the cell death promoting p38-MK2 heterodimer has fundamentally different quaternary structure compared to the cell growth promoting ERK2-RSK1

heterodimer in its inactive state, which can be exploited by a new class of small molecules that bind to MAPKs in a binary complex specific manner. In addition to this, we characterized MAPK-MAPKAPK binding in cells as well as signaling from endogenous MAPKs to MAPKAPKs. For the latter, we used engineered HEK293T cell lines which we developed so that MAPKs could be activated by their upstream activators only. This approach allows very specific activation of individual MAPKs and these cell lines are great tools to explore downstream responses elicited upon MAPK activation. These results were published in *Structure* (Sok et al, 2020).

We also explored the MAPK based regulation of the ATF2 transcription factor. It has been known from the 90's that MAPKs regulate the transcriptional activity of this protein, but it was unknown how this was mechanistically done. We showed that p38 and JNK co-regulate this transcription factor by binding to distinct sites in the disordered ATF2 transactivation domain. The two enzymes collectively control the phosphorylation of the same phospho-switch region involved in transcriptional cofactor binding in a non-linear fashion. By combining structural, biochemical and cell-based approaches we showed that kinase binding motifs, or also called docking motifs, and phosphorylation sites line up to maximize MAPK based co-regulation. We demonstrated for the first time how the activity of an ancient transcription controlling phosphoswitch became dependent on the relative flux of upstream signals. Here we had to use systems level modeling of protein sub-networks to show how two parallel MAPK pathways converge on the same phosphoswitch and "work together" to control gene expression in a novel way, which appears to be a vertebrate specific trait. This work was published in *Nature Communications* and got a Faculty 1000 recommendation.

We published a study on the use of MAPK docking motif containing cell-penetrating peptides to interfere with ERK1/2- and p38-mediated signaling in cells (Alexa et al, 2021). We concluded that these peptides could have only limited use because their entry into cells and their half-life is limited. Therefore, we put more effort into the development of small molecules that could interfere with the protein-protein interactions of MAPK-based complexes and started a collaboration with chemists and established a unique molecular collection that was designed to be able to specifically target the MAPK docking groove, which is the major protein-protein interaction surface on MAPKs (see later).

Related to our efforts on finding molecules modulating protein kinase networks, we came up with a new kinase assay concept that we originally developed to identify compounds that bind into the MAPK docking groove (Póti el al 2023). We wanted to screen chemically very diverse academic compound collections in a fast, reliable, and economical way. We used a dynamic protein fragment complementation based concept and created constructs for the Phosphorylation Assisted Luciferase Complementation Assay (PhALC). Apart from the small molecule luciferase substrate the assay does not require any commercially expensive reagents and all the components can be self-made in a molecular biology lab. We used this assay to identify and characterize small compounds that bind and affect the activity of cell survival and death controlling kinases (MAPKs and RSK). The modular design of the constructs allows the implementation of the assay to monitor the activity of any protein kinase in principle and we believe that it provides a good alternative for quantitative characterization of kinase activities compared to classical methods (e.g., radiometric techniques or phosho-western blots)

We also identified important kinase-controlled functional regions in key signaling proteins important in tyrosine kinase signaling and addressed how ser/thr phosphorylation may directly affect the

dephosphorylation of nearby lying tyrosine phosphorylation sites in insulin receptor kinase substrate 1 (IRS1) by the SHP2 tyrosine phosphatase, which is an important enzyme involved in cell growth regulation. We described a nice example on how ser/thr phosphorylation pathways mediated by MAPKs may modulate signaling via evolutionarily and functionally clearly distinct tyrosine kinase signaling pathways (Zeke et al, 2022).

In our proteomic studies on MAPK partner proteins we noticed that several MAPK phosphorylation target sites fall within a region that is often affected by cancer-associated mutations (from COSMIC) and that these motifs have sequences that resemble to some known FBXW7 ubiquitin ligase binding sites. These latter bind to the WD40 domain containing F-box protein upon bis-phosphorylation. Phospho-degrons are biochemically interesting examples of domain-linear motif type protein-protein binding. Apart from that the phosphorylated motif needs to be able to bind into the shallow WD40 domain binding slot, the motif needs to be compatible with protein kinases that phosphorylate them. Because of the specific topology of the phospho-peptide binding slot on the WD40 domain, extracellular signal or stress regulated proline-directed kinases such as MAPKs could potentially play an important role in this. We identified more than a dozen motifs located in different proteins that bind as bis-phosphorylated motifs to FBXW7 with high affinity and showed that MAPKs can indeed affect the level of these proteins in cells (Singh et al, 2022). We posited that MAPKs may partner up with FBXW7 to set the level of ubiquitine ligase clients depending on MAPK signaling pathway activity. Conversely, pathological up-regulation of MAPK pathways which is a general hallmark in cancers may contribute to the disease. For example, when one of the newly identified degron motif is taken out from its natural regulatory context, for example in the case of an endometrial specific *JAZF1-SUZ12* gene fusion, the gain-of-function effect of the degron could be detrimental under specific regulatory conditions (namely upon up-regulation of p38 due to hypoxia), since it will alter the degradation rate of otherwise stable proteins (Singh et al, 2022).

We also worked on some viral proteins that were known to modulate ERK signaling and thereby affecting cell growth. We wanted to understand how the Kaposi's sarcoma herpes viral protein ORF45 upregulates ERK phosphorylation in infected host cells. We found that the viral protein hijacks the ERK signaling network by tapping into the kinase docking groove mediated protein-protein interactions of the MKK-ERK-RSK signaling cascade. The systems level consequence of ORF45 binding to the ERK-RSK binary complex is a net increase of ERK phosphorylation due to an off-set in the dynamic equilibrium between kinase mediated upregulation and phosphatase mediated inactivation. This work was important because it led us to discover a new linear motif/kinase docking groove system determining the substrate binding specificity of RSK, an AGC kinase directly regulated by ERK1/2. We expanded the work on this new system and provided experimental evidence that linear motif based docking plays a major role not only for MAPKs but for AGC kinases too (Alexa et al, 2022). We will continue this work with the focus now on the exploration of the intact signaling network involving the new AGC kinase docking surface on RSK, and possibly on other AGC kinases. This work on ERK-related signaling in combination with our first *Nature Communications* paper fom 2020 on JNK- and p38-based complexes highlight the important regulatory roles of different linear motif binding surfaces on serine/threonine kinases. These studies set the stage for MAPK-based signaling output modulation via a new strategy: by directly interfering with the system's key protein-protein binding interactions (namely kinase docking) instead of the classical way of blocking kinase activity directly by ATP competitive drugs.

We submitted two manuscripts to *Nature Communications* last year (the abstracts are appended at the end of the report; both manuscript have gone through a major revision based on reviewers' comments and were sent back to the editor):

1) Targeting a key protein-protein interaction surface on mitogen-activated protein kinases by a precision-guided warhead scaffold with cyclic and chiral structure

and

2) Reversible covalent c-Jun N-terminal kinase (JNK) inhibitors targeting a specific cysteine by precision-guided Michael-acceptor warheads

These were the outcome of a collaborative project between organic chemists (Organocatalyis Research Group, RCNS, dr Tibor Soós) interested in the total-synthesis of complex molecules such as terpenoids and protein biochemists (my group) focusing on kinases involved in cellular signaling. We identified a novel cyclohexenone scaffold that can be used as an effective warhead to covalently target surface cysteines on proteins. In contrast to acrylamides, the contemporary gold standard in drug development, these cyclic enones form not only reversible covalent bonds to nucleophilic cysteines, but also to histidines. Additionally, the unique chemical property of these electrophiles could be exploited for enthalpic anchoring to bind into shallow protein-protein interaction surfaces or attenuating various off-target effects (e.g., with other protein -SH or GSH). We decided to submit two manuscripts to be able to focus on the new warheads from these two different aspects. In the first manuscript we show how we identified electronically and sterically fine-tuned cyclohexenone derivatives that can selectively bind into the so-called docking groove of mitogen-activated protein kinases, and thus interfere with the protein-protein interactions of these important enzymes. We also demonstrate that this structurally complex cyclic Michael acceptor scaffold provides an alternative to conventional ATP-competitive drugs and thus sets the stage for fundamentally new modulation of signaling pathways involved in regulating cell growth or cell death. Additionally, thanks to the complexity and chirality of these Michael acceptors, we recognized that the asymmetric centers could be used to govern and reroute cysteine vs histidine binding in the MAP kinase docking groove. In the second manuscript we show that the new cyclohexenone/pentenone scaffold can be engineered for binding affinity, residence time and specificity by fine-tuning steric and electronic properties of the warhead. Here we analyzed new covalent inhibitors in another test system. JNKs have a unique cysteine that previously was targeted by an acrylamide warhead, and we carried out comparative studies on potency with ATP competitive composite drugs comprised of irreversible or reversible covalent bond forming warheads. Additionally, we demonstrate further useful properties of cyclic enones: they could be exploited to increase specificity and residence time and thus limit off-target effects and/or improve pharmaceutical properties.

Targeting a key protein-protein interaction surface on mitogen-activated protein kinases by a precision-guided warhead scaffold with cyclic and chiral structure

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For mitogen-activated protein kinases (MAPKs) a shallow surface – distinct from the substrate binding pocket – called the D(ocking)-groove governs partner protein binding. Screening of broad range of Michael acceptor compounds identified a sterically crowded cyclohexenone moiety as a promising scaffold. We show that compounds bearing this structurally complex chiral warhead are able to target the conserved MAPK D-groove cysteine via reversible covalent modification and interfere with the protein-protein interactions of MAPKs. The electronic and steric properties of the Michael acceptor can be tailored via different substitution patterns. The inversion of the chiral center of the warhead can reroute chemical bond formation with the targeted cysteine towards the neighboring, but less nucleophilic histidine. Compounds bind to the shallow MAPK D-groove with low micromolar affinity in vitro and perturb MAPK signaling networks in the cell. This class of chiral, cyclic and enhanced 3D shaped Michael acceptor scaffolds offers an alternative to conventional ATP-competitive drugs modulating MAPK signaling pathways.

Reversible covalent c-Jun N-terminal kinase (JNK) inhibitors targeting a specific cysteine by precision-guided Michael-acceptor warheads

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There has been a surge of interest in covalent inhibitors for protein kinases in recent years. Despite success in oncology, the off-target reactivity of these molecules is still hampering the use of covalent warhead-based strategies. Herein, we disclose the development of precision-guided warheads to mitigate the off-target challenge. These reversible warheads have a complex and cyclic structure with optional chirality center and tailored steric and electronic properties. To validate our proof-of-concept, we modified acrylamide-based covalent inhibitors of c-Jun N-terminal kinases (JNKs). We show that the cyclic warheads have high resilience against off-target thiols. Additionally, the binding affinity, residence time, and even JNK isoform specificity can be fine-tuned by adjusting the substitution pattern or using divergent and orthogonal synthetic elaboration of the warhead. Taken together, the cyclic warheads presented in this study will be a useful tool for medicinal chemists for the deliberate design of safer and functionally fine-tuned covalent inhibitors.