Exploration of JNK mediated protein-protein interactions: from structures to cellular functions

Research Output

This project was an OTKA international cooperation grant and in accordance with this we worked together with 8 different research groups from three different continents (3 from Germany, 2 from France, 2 from Australia, and 1 from the US). There were several visits to these collaborating groups involving several young group members and one of the PhD students was able to spend 3 month in a foreign collaborating lab and obtain special skills in live-cell imaging techniques.

5 of the 9 reported research papers are directly linked to JNK and its protein-protein interactions (papers in bold type indicate corresponding authorship):
- **Zeke et al, 2015, Molecular Systems Biology**
- **Zeke et al. 2016, Microbiology Molecular Biology Reviews**
- Gouw et al, 2018, Nucleic Acids Research
- Latré De Laté, 2019, Cellular Microbiology
- **Kirsch et al, 2020, Nature Communications**

4 other papers are only indirectly linked to the topic of this JNK focused grant: they report on or apply an important technique/technology that was developed in this project and then was used to tackle other protein-protein interaction or MAPK signaling based problems:

- **Kirsch et al, 2016, Advanced Experimental Medical Biology**: a book chapter in which we discussed protein machinery which influences extracellular signal reception, intracellular pathway activity, and cytoskeletal or transcriptional activity.

- **Gógl et al, 2018, FEBS Journal**: we applied the luciferase complementation based assay (NanoBit) to study the dynamics of ERK-RSK protein complex association-dissociation in live cells upon extracellular stimulation. This technique was crucial to study JNK mediated protein-protein interactions under extracellular stimulation in other studies.

- Gógl et al, 2019, Journal of Molecular Biology: we used the luciferase complementation based in-cell assay to characterize RSK-PDZ domain interactions. Here the assay was used to validate a dozen of protein-protein interactions that were formerly characterized biochemically in the collaborating lab.

- **Sok et al, 2020, Structure**: we characterized the structural assembly of the p38-MK2 signaling complex in this paper. This study is listed because it introduced an important technical advance, which we later used to study JNK-based signaling, too. We established a Tet-on inducible expression system with which MAPK-selective activation became possible with the addition of a synthetic inducer. This, in contrary to stimulation of cells with hormones or cytokines eliciting promiscuous kinase activation, allowed controlled and specific activation of one MAPK at a time.

Scientific Achievements

The project addressed the protein-protein interactions of JNK. JNK, similarly to other MAPKs such ERK1/2 or p38, are structurally compact protein kinases with a surprisingly broad signaling spectra: they mediate the phosphorylation of hundreds of downstream substrates and thus control almost all aspects of cellular life. We postulated that in order to understand the functional roles of JNK, we need to understand the specificity of its protein-protein interactions and gain insights into what distinguishes it from other MAPK paralogs (i.e. ERK and p38 kinases). We found that depending on the linear sequence motif JNK binding can be highly specific. Moreover, we identified dozens of potential phosphorylation sites next to validated JNK-binding sites. This work on JNK was published early and set the stage for further work later (**Zeke et al, 2015, MSB**). The specificity of MAPK binding linear motifs which occur in hundreds of known and putative MAPK substrates had been long enigmatic and the curators of the Eukaryotic Linear Motif (ELM) database invited us to participate in an update on the ELM database regarding these motifs (Gouw et al, 2018; NAR; **Figure 1**).

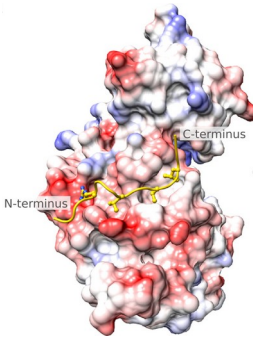

Figure 1. The structure of JNK binding to he MAPK docking motif shown for the DOC_MAPK_NFAT4_5 ELM motif (in yellow; with regular expression: [RK][∧P][∧P][LIM].L.[LIVMF]). Here, charged amino acids [RK] are followed by hydrophobic residues in a pattern that is compatible with JNK binding only (PDB:2XS0). Source: Gouw et al 2018, NAR

We wrote a highly cited review on this topic with one of the collaborating Australian groups **(Zeke at al, 2016: MMBR**), where we collected all known JNK substrates and found that most of the known JNK partners have sequence motifs that correspond to JNK-docking peptide crystal structures that we determined earlier (JIP-1 type or NFAT4-type; **Figure 2**).

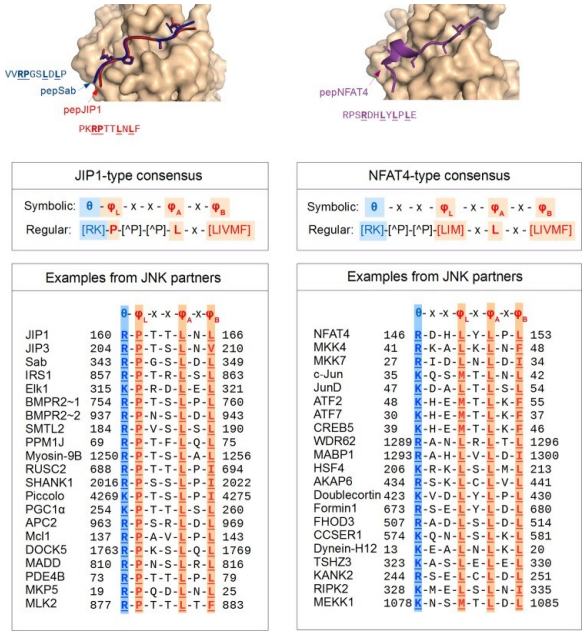

Figure 2: Different types of JNK-binding motifs (JIP1-type and NFAT4-type). These two different motif classes, which use different conformational solutions to bind the so-called JNK docking groove, were originally identified in the JNK binding scaffold protein, JIP1, and in the JNK-controlled transcription factor, NFAT4. The lower panels list dozens of JNK partners with their corresponding sequence patterns. Source: Zeke et al., 2016, MMBR

We also collaborated with a French group who worked on a macroshizont mammalian parasite (Theleria) and found that one of the parasite's surface proteins (p104) specifically recruits JNK and uses this protein kinase to manipulate signaling in macrophages (Latré De Laté et al, 2019; Cell. Microb.) We contributed to this work by validating the physical interaction between human JNK and the parasite's gp104 protein and identified the binding motif responsible for JNK recruitment and for its cytoplasmic sequestration, resulting in immunosuppression, in the host cell.

According to the original work plan we wanted to examine JNK-based protein-protein interactions in the cell - in the context of an intact signaling network since this had been missing from earlier studies. This required some technology capable of monitoring protein-protein binding in live cells and specific labeling of the MAPK and its interaction partner were also needed. We planned to use FRET on fluorescently labeled proteins. However, this approach failed; mostly because of difficulties in the logistics of getting reliable access to a high-end fluorescent confocal microscope. Therefore, we used a dynamic version of the luciferase complementation assay concept (NanoBit) that enabled us to follow JNK-based interactions with their selected full-length partner protein in real time and in live cells, for example after applying an extracellular stimulus (**Gógl et al, 2018, FEBS J**; Gógl et al, 2019 JMB). Another approach that failed was the selectively labeling of JNK with unnatural amino acids at critical positions via amber codon suppression (e.g. with cyclooctyne containing bioorthogonal groups for azide- or tetrazine dye based selective labeling or with photocaged lysine (PCK) at important lysine positions). We worked out how to label some JNK substrates in bacteria and produced some recombinant labeled proteins with this approach, but the use of this was limited, as our functional studies would have naturally required unnatural amino acid incorporation and specific labeling in live mammalian cells. We obtained the necessary constructs from Jason Chin's lab as it had been originally planned, but apart from the test protein we were able to suppress amber codons neither in JNK nor in its substrates. Later, we were told that amber suppression only rarely works in mammalian cells. This was a big setback as we wanted to induce JNK signaling activity based on this strategy. The original plan was to replace a critical lysine residue in the JNK docking groove with PCK by amber codon suppression and initiate protein-protein binding selectively by irradiation with light. Because of this, we searched for a more feasible alternative. We managed to establish a Tet-on based doxycyclin inducible system where each MAPK is activated by selective up-regulation of a constitutively active version of its upstream kinase. This approach was published and used for the first time in a paper on p38 (**Sok et al, 2020, Structure**). Acknowledgedly, this was not as elegant as originally planned, but it did the trick.

The original work plan proposed to examine JNK/importin and JNK/NF-Y complexes implicated in nuclear transport and general transcription regulation, respectively, based on some functional studies from the other collaborating foreign groups. We set out to test if these interactions indeed directly happen in vitro as well as in cells. Unfortunately, we were not able to validate these interactions and concluded that JNK does not interact with these important cellular regulator proteins directly. The interactions are indirect and likely involve some other proteins and the identification of these would have required substantial further work. Instead of this, we decided to focus on two complexes that we validated both in vitro as well as in cells: JNK-DCX and JNK-ATF2, involved in microtubule formation and gene expression control, respectively.

DCX binds to microtubule (MT) protofilaments and is expressed only in developing neurons. It binds to MTs with its doublecourtin (DC) domains and is also known to bind JNK which regulates its activity through phosphorylation. Originally, it was suggested that one of the DC domains mediates JNK binding. We were intrigued by this but we found that it is only the disordered C-terminal region of DCX which is needed for JNK recruitment. The minimal interacting region can be mapped to a 23-amino-acid long C-terminal disordered region. We crystallized this complex and collected X-ray diffraction data to 3.5 Å resolution, but for publication we would like to collect higher resolution data in the future, possibly in the spring of

2021. The electrondensity map at this limited resolution suggests a new binding conformation for this unusually long peptide (since JNK-binding docking peptides are normally only 8-10 aa long). The preliminary structural model shows that in addition to the MAPK docking groove the peptide contacts an additional surface on JNK, albeit we need to collect better quality data on a synchroton beamline to show this unambiguously, which, fortunately, seem to be more of a technical rather than a conceptual challenge.

We had been suspecting for a long time that there are additional protein-protein interaction (PPI) surface areas/grooves on the compact MAPK domain beyond the well-characterized MAPK docking groove (see Figure 1). Intriguingly, some of the known JNK substrates had a small structured domain next to their JNK binding docking motif. The best example for this is ATF2 (see Figure 2), which is a transcription factor known to be phosphorylated by JNK at two threonines in its transactivation domain (TAD) turning on its transcriptional activity. We found that JNK binding to ATF2 requires a Zn-finger domain preceding the MAPK-binding docking motif. We determined the crystal structure of the JNK-ATF2(TAD) complex that revealed how the Zn-finger contributes to binding. When we were validating this new structure in cell-based assays we noticed that the ATF2 TAD may also be phosphorylated by p38 but this was diminished when JNK was also turned on. This was not only a serendipitous discovery, though, as we used the highly selective Tet-on inducible system to activate only MAPK at a time or in combination deliberately, moreover, we were also able to monitor MAPK-ATF2 binary complex formation with the new dynamic luciferase complementation assay in live cells under various physiological conditions. To cut a long story short - where we also had to elucidate how p38 binds to the ATF2 TAD, which then resulted in the discovery of a novel PPI surface, the FENEF-motif binding F-site forming only on activated p38, which is different from the earlier known docking groove - we found that the transcription controlling phosphoswitch in the ATF2 TAD is under the control of two functionally distinct signaling pathways, JNK and p38. The organization of the TAD (*i.e.* the Zn-finger, MAPK binding linear motifs and phosphorylation sites) is different in vertebrates and invertebrates. Vertebrates acquired a unique capacity to limit p38 mediated flux depending on the strength of JNK pathway activity. The mechanistic basis for this seemingly complex regulation is simple: the new p38 binding motif has an evolutionarily more recent but conserved phosphorylation site (SP) in all vertebrate ATF2 orthologs and phosphorylation at this site by JNK interferes with ATF2-p38 binding (**Figure 3**). This study has been published recently (**Kirsch et al, 2020, Nat. Comm.**).

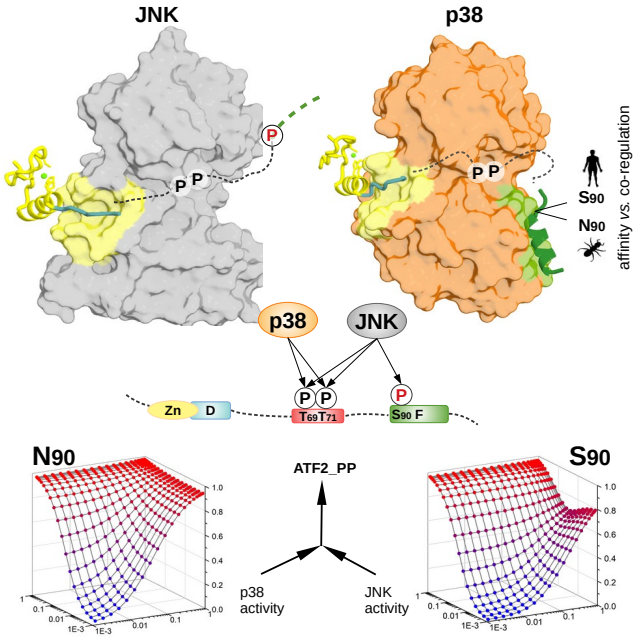

Figure 3. Complex regulation of the ATF2 TAD by two MAPKs, JNK and p38. The schematic architecture of the ATF2 is shown in the middle: Zn, Zn-finger: D, Docking motif; T69T71, transcription controlling phosphoswitch; F, FENEF-motif; and S90, evolutionarily more recent JNK phosphorylation site. The structural panels on top show the crystal structure of JNK1 bound to the Zn+D region or the crystal structure of activated p38 binding to the FENEF motif (P indicates phosphorylation sites; unstructured TAD regions are shown with dashed lines.) Panels below show cellular ATF2 phosphorylation (N90: invetebrate TAD and S90: vertebrate TAD) under different JNK or p38 activation levels. Plots were calculated by using a mechanistic model. Source: Kirsch et al, 2020, Nat. Comm.

Outlook

Concepts developed and technical progress achieved within this JNK project greatly helped us to produce solid preliminary data for the OTKA Elvonal project that started in 2018. We currently use many of the tools developed here to address MAPK mediated signaling more broadly and we also aim to harness the wealth of structural information obtained on JNK in MAPK based rational drug development.