WP2.2. Inhibition of intramembrane catalysis and protein-protein interactions for novel targeting of inflammatory and cancer pathologies. (Stříšovský)

Research aims

- To characterise iRhom structurally and mechanistically.
- To identify and characterise cellular proteins key for the signalling and trafficking function of iRhom and define their interaction sites with iRhom.
- To develop small molecules and peptide macrocycles blocking these interactions and inhibiting iRhom function.

Research plan and methodology

Novel transmembrane proteins called iRhoms (non-protease members of the rhomboid intramembrane superfamily) play an essential role in the biogenesis and post-translational activation of a membrane anchored metalloprotease ADAM17/TACE, which has a key role in inflammatory pathologies and cancers and has been a difficult-to-exploit therapeutic target. Based on our demonstrated experience in the mechanistic, structural and cell biological analysis of rhomboid intramembrane proteases and iRhoms, we propose to combine these approaches to investigate the structure and mechanism of iRhoms and evaluate the ways of their pharmacological targeting. Using advanced medicinal chemistry approaches including biological selection, we will exploit this information in designing small molecules blocking iRhom function. This would represent a novel and highly specific anti-inflammatory strategy targeting the biogenesis of TNFα, and a novel anti-cancer strategy targeting EGF receptor signalling.

The mechanisms of rhomboid family proteins will generally involve intramembrane and extramembrane protein-protein interactions and their interplay with the lipid environment. In iRhoms, the rhomboid-fold transmembrane core probably binds the transmembrane domains of the client proteins, and two highly conserved globular domains, the N-terminal cytoplasmic domain (NTD) and the luminal iRhom homology domain (IRHD), are key for iRhom function. IRHD probably participates in client protein binding while NTD binds protein partners from the cytosol. None have been published yet, but our preliminary collaborative proteomics screen has yielded several candidates that are now being tested. Since deletions in NTD abrogate iRhom function, an obvious strategy to modulate iRhom function is to design ligands binding and disable the NTD or interaction with its key protein partners. The project will be organized into the following two work packages that are partially independent, but also synergistic.

Structure and mechanism of iRhom

The iRhom is an integral membrane protein, which is generally a challenging object for structural investigation. It will be essential to first establish a robust expression system. We will overexpress iRhom in human cells in suspension culture (HEK293T-6E[1]), purify it after detergent extraction from cellular membranes and determine its functionality by the interaction with recombinant ADAM17 and absence of interaction with recombinant ADAM10 constructs. We will then subject the full-length iRhom to electron microscopic characterization in collaboration with cryoEM expert Dr. Vinothkumar Kutti Ragunath at the Laboratory of Molecular Biology in Cambridge, UK. Given the size of iRhom we are expecting to get at least a low-resolution structure. The iRhom transmembrane core is anticipated to have a similar architecture as a rhomboid protease, and the main structural difference from the canonical rhomboid fold will be the globular domains, the N-terminal domain and the IRHD. These domains are important for iRhom function, and it will be important to characterise them structurally at high-resolution. We will perform an expression screen of NTD and IRHD construct using bacterial and mammalian expression systems, and characterize them by X-ray crystallography and/or NMR. Ideally, these high-resolution structures will be fit into the low resolution maps from electron microscopy or into small-angle X-ray scattering (SAXS) data yielding a hybrid structure. An alternative (low-resolution) method of structural characterization of iRhom and its constituent domains will be hydrogen-deuterium exchange mass-spectrometry and crosslinking mass-spectrometry (XL-MS).

After we generate recombinant iRhom, we will map the interaction site of iRhom with its client protein ADAM17 using XL-MS and verify the results using microscale thermophoresis and site-directed

mutagenesis of iRhom. To identify any new interactors of iRhom, being client proteins or regulatory interactors of NTD, we will use state-of-the-art proximity proteomics approach exploiting an engineered biotin ligase fused to iRhom (the BioID method[2]). We will express the ligase-iRhom fusion from a regulatable promotor at near-endogenous level in stably transfected iRhom-deficient immortalized mouse embryonic fibroblasts (available from the collaborating Adrain laboratory). The hits will be validated first in cells by heterologous expression of epitope-tagged forms and at endogenous levels by immunoprecipitation. We will then test if they interact directly with iRhom using purified recombinant iRhom or its constituent domains and individual candidates in vitro, and splitGFP system[3] in cells. At the cell-biological level, we will investigate the trafficking of iRhom and its interaction with ADAM17 and any newly found interactors using the SunTag[4] and splitGFP system in live cells.

Finally, the binding interfaces of newly identified iRhom interactors (from our proteomics experiments or from literature) with iRhom will be mapped and complexes characterized structurally, by deletion analysis, XL-MS, X-ray crystallography or NMR, as appropriate and feasible. This work package will constitute the bulk of this project.

Inhibition of iRhom

We will first validate which of the newly identified interactors are important for the function of iRhom by depleting individual endogenous proteins by RNA interference or SRISPR/Cas9 in murine embryonic fibroblasts or immortalized macrophages, and by using detection of bioactive ADAM17 at the cell surface, shedding of ADAM17 substrates, and EGFR transactivation assay as readouts. The best validated interactor(s) that will be essential for the function of iRhom will be prepared in recombinant purified form, and an interaction assay(s) with the appropriate binding domain of iRhom suitable for high-throughput screening (based on microscale thermophoresis or alpha-screen) will be established. A similar assay will be established for iRhom and its client protein ADAM17. We will carry out high-throughput screens to identify compounds that prevent/disrupt interaction of ADAM17 or any newly identified interactors with iRhom. For this, we will need access to compound libraries (possible within the Open Innovation programme of Astra Zeneca or at European Lead Factory https://www.europeanleadfactory.eu/). As an alternative to high throughput screening for an inhibitor of iRhom-client/binder interactions, we will use the new and powerful technology of phage display of bicyclic peptides[5] to select binders to iRhom NTD, IRHD or ADAM17 itself (collaboration with Prof. Christian Heinis, EPFL, Switzerland). Bicyclic peptides form a two-ring structure that is conformationally constrained, and they combine the power of biological selection of polypeptides with the rigidity of small-molecule scaffolds. They have been successfully used to generate extremely selective and potent ligands of enzymes and receptors, and peptide macrocycles in general are promising for targeting protein-protein interfaces and other targets previously deemed undruggable[6]. In all cases, the hits will be further validated using biophysical methods, and their complexes with iRhom will be structurally characterized, if possible. Compounds will be resynthesized and iteratively improved by medicinal chemistry at the IOCB (Dr. Majer's group), aided by modelling and cheminformatic approaches (Prof. Hobza's group). The most promising inhibitors of protein-protein interactions will be tested in cells using the iRhom function assay described above, and the best compounds will be tested in mice in a TNFα secretion assay and LPS stimulation assay[7].

On the technical side, we have established membrane protein expression systems in *E.coli* and mammalian cells in suspension, and are experienced in membrane protein extraction, purification and crystallization. We are technically equipped for protein purification and characterisation (FPLC, SEC MALLS, microscale thermophoresis, calorimetry and surface plasmone resonance). We are equipped with all the instrumentation and skills necessary for basic molecular cell biology (PCR cycler, near infrared scanner for quantitative western blotting, tissue culture facility, confocal microscope in the context of a shared facility). In collaboration with the IOCB proteomics facility (Dr. Hubálek, Mgr. Březinová), we have established and are routinely using several quantitative proteomics techniques including but not limited to stable isotope labelling in cell culture (SILAC). The purchase of the new Orbitrap mass spectrometer will greatly benefit this work package, namely the proteomic identification of the interactors and post-translational modifications of iRhom NTD.

References:

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Research schedule

2018-2020

- Preparation of recombinant full-length iRhom, NTD, IRHD, and ADAM17.
- Bicyclic phage display of NTD, IRHD and ADAM17 to identify their ligands.

2018-2022

- Structural characterization of full-length iRhom, NTD, and IRHD.
- Identification of iRhom interactors using BioID.
- Cell biological and structural characterization of iRhom interactors.
- Development of high-throughput assays for the disruptors of iRhom-client interactions.
- HTS, hit validation and development.
- Hit validation in cells and testing in mice.

Publications and patents

Publications

	Jimp
2017	0
2018	1
2019	1
2020	1
2021	1
2022	1
Total	5

Molecular Cell
Nature Structural & Molecular Biology
PNAS
EMBO Journal
EMBO Reports
Structure

Patents and patent applications

	Patents (granted)	International patent applications (filed)
2017	0	0
2018	0	0
2019	0	1
2020	0	0
2021	0	0
2022	1	0
Total	1	1

We expect IP protection in the following area: Small molecules blocking the interaction of iRhom with its protein binding partners.

Cooperation with foreign institutions

- Laboratory of membrane trafficking, Instituto Gulbenkian de Ciência, Lisbon, Portugal: iRhom biology, mouse genetics, source of immortalised primary cells (macrophages) from iRhom knock-out animals, in vivo tests of iRhom inhibitors in disease models.
- Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom: cryoelectron microscopy of iRhom.