



Investigating the Role of miR-26b in Vascular Calcification using Single-Cell RNA sequencing

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#### **Background and Aims of the Project**

Vascular calcification (VC) is a cell-mediated process whose central role in its pathogenesis is the acquisition of osteoblastlike phenotype by healthy vascular cells in response to procalcific. They are likely dependent on the cell type, the vascular bed, the VC type and the presence or absence of underlying diseases. Calcifying the internal part of the vessels (intima) is partly driven by a phenotypic transition in endothelial cells (ECs). In contrast, medial calcification is caused by a breakdown of the extracellular matrix and phenotypic change of the vascular smooth muscle cells (VSMCs). Despite recent advances, the mechanisms driving the early aspects of this vascular-to-osteoblast-like cell transition still need to be fully understood. Caporali's lab found new evidence implicating microRNa-26 (miR-26) causal role in regulating endothelial cell phenotype and contributing to vascular calcification. Following pilot data in vitro, the lab also generated miR26 global knockout mice (miR26KO), spontaneously developing a calcific phenotype in the aorta. The main aim is to explore how the loss of miR-26b promotes the deregulation of signalling to evoke vascular calcification in the aorta and the contribution of different cell types to this process.

During my summer placement, the main aim of my project was to identify the signalling network and the cell contribution during vascular calcification in miR26KO using the data analysis platform Cellenics, an open-source analytics tool for single-cell RNA sequencing.

#### Methods Used

<u>Data Processing:</u> Using the data obtained from the dissection of the aorta of 3 male wild-type mice and 3 male miR-26 knockout, single-cell sequencing was analysed using the software Cellenics, as observed in Figure 1. To do so, the data was first processed by filtering it by class, mitochondrial content, and the number of genes vs. unique molecular identifiers (UMIs). Moreover, it was also filtered by doublet, the data was integrated, and the embedding was configured.



Figure 1- Summary of the Single-cell RNA sequencing process, from dissection of the tissue to cell type identification

- <u>Cell identification by clusters</u>: Cells were identified and organised by custom clusters using automatic Seq-type (for both mice Heart and mice Immune genes) and literature search-based marker genes.

- Identification of Subgroups of Immune Cells: Using literature research, subgroups within the 3 main groups of immune cells were identified to observe how the subgroups would behave in the knockout model. This was done through the previous identification of marker genes for each sub-group.
- <u>Potential target identification and analysis</u>: Previously identified targets by the lab and targets identified overlapping the genes from Cellenics and the original RNAseq bulk were analysed using the "gene expression" section of the software Cellenics.



Figure 2- Summary of the Methods used for clustering and cell type identification

#### Results and outcomes

- Initially, 6 main clusters were identified: Smooth Muscle Cells (SMC), Endothelial Cells (EC), Fibroblasts, T cells, B cells and Macrophages, as seen in Figure 3A. When compared between wild-type and Knockout models, it was found that B and T cell populations decreased in the KO, while Macrophages and EC were increased, as can be observed in Figure 3B.
- Identification of Immune cell subgroups was performed. Using the markers genes specified in Figure 4A, 7 immune cell subgroups were identified: M1 and M2-like macrophages, Cytotoxic T cells, Th1, Th2, Th17 and Treg T cells. As observed in Figure 4B, the groups that presented the most significant change between samples were macrophages, both M1 and M2, which presented an increase of proportion in KO compared to WT. Moreover, B cells and CD8+ groups were significantly decreased in KO.
- Although an overlap between the bulk data and the data obtained from the software was done to find specific downregulated targets, no significant targets were found.



Figure 3- (A) Clustering of cells by the 6 main groups identified. (B) Proportion of the cell groups in Wildtype and miR26KO mice. (C) Marker genes expression of the cell groups.



Figure 4- Final classification of the Immune Cells with the indication of the main marker genes (in black) for each type



Figure 5- Proportion of the final immune cell groups in Wildtype and miR26KO mice

**Future Directions in which the project could be taken** The next possible steps could be validating the identified targets by immunohistochemistry in the aorta of WT and miR26KO mice and analysing the images using ImageJ. Moreover, using R studio, a further overlapping the data obtained from single-cell sequencing and the original RNA-seq bulk of raw data could be done to identify more targets.

#### Departures from original project plan

To find further targets, an overlap between the genes obtained from the single-cell sequencing using the software and the original RNA-seq bulk was done. Although this was not on the original plan, the group suggested having more targets to compare to see how this would behave in the different cells' groups could be interesting.

# Value of studentship to the student and to the research group

Through my work on this project, I gained a range of transferable and technical skills that will be useful for my future career in research as I plan to progress into a Master's Programme of Research, hopefully. Specifically, I acquired technical skills in bioinformatics, particularly in analysing scRNA-seq data using the Cellenics platform. This is an essential skill for any researcher working in genomics or transcriptomics. My work was incredibly valuable for the research group because, for the first time, we identified and characterized changes in the immune cell populations in this mouse model. This can open a new line of research never explored before.

Furthermore, I improved my presentation and writing skills thanks to the feedback and guidance from my supervisor and other members of the Caporali lab. Effective communication is a crucial skill for any researcher, and I believe that the opportunity to present my work and receive constructive feedback will be invaluable in improving my ability to communicate my research findings effectively.

Overall, I believe that the skills I gained through this project will be highly transferable and useful for my future career in research.



Figure 6- Photograph of the student Rosa Bauzà Sansó and the supervisor Andrea Caporali in front of the Queen Medical Research Institute in Edinburgh





# Investigating the Interaction between Clathrin and ESCRT Machinery at the Endosome

<u>Student</u>: Iva Organdjieva <u>Supervisors</u>: Dr. Kit Briant and Prof. Frances Brodsky

#### Introduction

Endocytic cargo protein degradation is a pathway which involves the formation of intraluminal vesicles (ILVs) at the endosome. This process is mediated by the endosomal sorting complex required for transport (ESCRT) machinery consisting of four protein subcomplexes (ESCRT 0-III). Charged multivesicular body protein 4b (CHMP4B) is an ESCRT-III component and is responsible for membrane deformation and scission together with the ATPase VPS4. CHMP4B is recruited after ESCRT-0 to -II and shows fast and transient dynamics at the endosome<sup>1</sup>. The membrane coat protein clathrin is required in this pathway for cargo accumulation at endosomes during endocytic cargo degradation. Importantly, clathrin is recruited to the endosome by the interaction between clathrin heavy chain and ESCRT-0 component HRS and is required for ESCRT-0 dissociation. Wenzel, E.M et al have shown that in HRS mutants, the ESCRT-0 component is hyperstabilised at the endosomal membrane and alters CHMP4B dynamics, recruitment, and strength of interaction<sup>1</sup>. Recently, CC2D1A, a master regulator of signalling pathways, was identified by the Brodsky lab as a specific interacting protein of clathrin light chain isoform CLCa (unpublished). CC2D1A also directly interacts with CHMP4B, providing a possible new link between clathrin and ESCRTs. Mice knockout studies have shown that genetic defects in CC2D1A are implicated in autism spectrum disorder (ASD)<sup>2</sup>. Therefore, we hypothesise that the interaction between CLCa and CC2D1A regulates ESCRT assembly at the endosome by indirectly interacting and recruiting the ESCRT-III component CHMP4B. Consequently, the hypothesis explores how the CLCa-CC2D1A interaction at the endosome can affect cargo degradation and normal neuronal differentiation, which may be affected in ASD.

#### Aims of the project

The overall aim of the project is to explore the potential link between the ESCRT and clathrin pathways at the endosome by investigating the CLCa-CC2D1A and CLCa-CHMP4B interaction and dynamics in the formation of ILVs during receptor and cargo degradation.

Aims:

- 1. Generate stable Hela cell lines expressing fluorescentlytagged CLCa, CHMP4B, and/or CC2D1A proteins by viral transfection
- Perform immunofluorescence to confirm protein expression and localisation at the endosome of GFP-CC2D1A or CHMP4B-L-GFP relative to mCherry-CLCa and the endosomal marker EEA1
- 3. Perform real time live-cell imaging to determine the kinetics of CHMP4B and CC2D1A recruitment to the

endosome after EGF-647 treatment by tracking GFP-CC2D1A or CHMP4B-L-GFP relative to mCherry-CLCa

## **Description of work**

<u>Molecular biology</u>: PCR-based plasmid construction and Hi-Fi assembly was used to ligate the fluorescently-tagged protein sequences into a pNG72 backbone obtained from the Martin-Serrano Lab. The PCR products were transformed into *E.coli* DH5a cells. The plasmids were isolated and sent for DNA sequencing to confirm the ligation.

<u>Cell culture</u>: Gag-Pol, VSVG and tagged protein plasmids were transfected into HEK293 cells for viral production. The viral particles were used to generate stable HeLa cell lines (mCherry-CLCa, CHMP4B-L-GFP, GFP-CC2D1A). Stable mCherry-CLCa cells were transfected with CHMP4B-L-GFP and GFP-CC2D1A separately. The cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in a humidified incubator at 37°C with 5% CO2.

<u>Western blotting:</u> Cell lysates were separated by SDS-PAGE and analysed by immunoblotting. The proteins were immunodetected using anti-GFP and anti-mCherry antibodies before and after FACS.

Immunofluorescence: Cells were fixed with 3% PFA for immunostaining. The cells were stained before and after FACS for the endosomal marker EEA1 and the CHC antibody X22. Before FACS, the cells were co-stained for anti-GFP and anti-CC2D1A antibodies. The images were processed using ImageJ. <u>FACS</u>: the single-transfected cell lines were sorted into medium and high expressing batches. The double-transfected cell lines were sorted into a high-expressing batch. The cells were sorted using the Sony MA900 Cell Sorter.

<u>Live cell imaging</u> was done using the sorted cell cultures by two different methods:

1. Cells were cooled on ice and cell surface EGF receptors labelled with cold EGF media (200ng/ul). After 20 minutes on ice, the EGF was swapped with warm Fluorobrite media to initiate endocytosis. The cells were imaged live by confocal microscopy, with 1 image recorded every 3 seconds for 25 minutes. 2. Pre-warmed EGF media was added to the cells and incubated at  $37^{\circ}$ C for 2 minutes. The EGF media was swapped with Fluorobrite and the cells were recorded by confocal microscope for 25 minutes as previously.

#### **Results and outcomes**



Figure 1. Immunoblotting for GFP and mCherry proteins in the stable HeLa cell lines before FACS. 3, 5) Very low expression of CHMP4B-L-GFP in both single-transfected and double-transfected cells. 4) Low expression of GFP-

CC2D1A in double-transfected cells.  $\ensuremath{\textbf{4-5}}\xspace$  Low expression of mCherry-CLCa in double-transfected cells



*Figure 2.* Immunoblotting for GFP and mCherry proteins in stable HeLa cell lines after FACS. 1-2, 7-8) Increased expression of mCherry-CLCa in all sorted cells. 5-6) Relatively increased expression of CHMP4B-L-GFP in single-transfected cells. 7-8) Expression of GFP-tagged proteins in double-transfected cells remained low.



Figure 3. Immunofluorescence images of mCherry-CLCa + GFP-CC2D1A and mCherry-CLCa + CHMP4B-L-GFP cells stained with anti-CC2D1A and anti-GFP



Figure 4. Immunofluorescence images of mCherry-CLCa + GFP-CC2D1A and mCherry-CLCa + CHMP4B-L-GFP cells stained with endosomal marker EEA1

Immunofluorescence of fixed cells confirmed the localisation of CLCa, CC2D1A and CHMP4B at the endosome. Colocalisation between the tagged proteins was more challenging to determine due to the varying expression between the GFP and mCherry-tagged proteins. Staining with anti-CC2D1A and anti-GFP enabled us to amplify the GFP signal and confirm the localisation of the weakly expressed CC2D1A. The use of FACS enabled us to obtain cells with higher expression of the proteins. The images in Figure 4 show several puncta of colocalisation between CLCa with CC2D1A and CHMP4B at the endosome (EEA1).

By tracking EGF-647, we obtained videos from live cell imaging that show several points of interaction between CHMP4B and CC2D1A with CLCa (data not shown). These results confirmed that our methods for tracking the interaction in live cells were successful. For analysis, we would require higher quality videos on more specialised live-cell imaging microscopes to accurately quantify the dynamics of CHMP4B and CC2D1A, which is the most challenging step.

#### Departures from the original aim

We were not able to knockdown CLCa in the stable cell lines and perform live cell imaging on these cells due to time limitation.

#### Future directions for the project

The obtained results provide a solid starting point for this project and have covered our major aims. The project is still ongoing and Dr. Briant is currently focusing on improving live cell imaging with these cells. Knockdown of CLCa will also be performed. These cells will be recorded using a specialized confocal microscope for live cell imaging, which allows easier and more accurate quantification of protein dynamics.

#### Value of the studentship

<u>To the student:</u> The studentship provided me with the opportunity to gain first-hand experience of biomedical research in academia. I gained skills in molecular cloning, HiFi plasmid assembly, PCR, transfection of plasmid DNA, bioinformatical skills in primer design, and cell biology skills in culturing mammalian cell lines, Western blotting, and FACS. Additionally, performing confocal microscopy is an extremely valuable skill, which I can apply to my final year project. In the Brodsky Lab, my confidence was boosted and I improved my collaborative and organizational skills. The technical and transferable skills I was equipped with during this studentship are invaluable to my future studies and potential career in research. My hope is that this research will also contribute to new discoveries related to neuronal development and ASD.

<u>To the lab:</u> The cell lines developed during this project will be of great use to the lab to determine the role of the CC2D1A – CLCa on CHMP4B dynamics, which is a key question for our group. Following this studentship, Dr. Briant has given these cells to collaborators with a specialism in live cell microscopy who will test the cells on several microscopes to assess which will be the best system for quantifiable live cell microscopy.



Iva Organdjieva, Dr. Kit Briant, and Prof. Frances Brodsky; Iva presenting her work to the lab members

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**Biochemical Society Summer** 

Vacation Studentship Report 2023



# Cloning and Overexpression of *Haloarcula hispanica* Head-Tailed HHTV-1 Virus Proteins in *Escherichia coli* for cryo-EM Analysis

Student: Aitor Mateo Supervisor: Fred Antson

# Introduction:

There are an estimated 10<sup>31</sup> virus particles on earth (1) and they are responsible for a number of diseases including human disorders caused by COVID-19, flu and Herpesviruses. Despite this, many aspects of virion assembly remain unknown, and in particular, very little research has been conducted into archaeal viruses compared to bacterial and eukaryotic organisms. One unresolved aspect is the precise mechanism by which dsDNA viruses fill their capsids with DNA. Uncovering this mechanism could enhance our grasp of viral pathology, aid in viral disease treatment, and allow us to explore novel research directions, such as using the virus as a vector for gene transformation.

# Aims:

The goal of this project was to study four *Haloarcula hispanica* archeal virus HHTV-1 proteins involved in DNA packaging, referred to as gp6, gp7, gp8 and gp9. Of these proteins, gp9 was known to be the large terminase while one of the other three was thought to be the small terminase. Both the large and small terminase proteins play a crucial role during viral DNA packaging. Specific aims were:

- 1. Produce recombinant plasmids containing gp6, gp7, gp8 or gp9.
- 2. Transform each of these plasmids into an expression strain of *E. coli*.
- 3. Grow large cultures of transformed cells to produce large quantities of target proteins.
- 4. Purify expressed proteins.

# Methods and description of work:

Cloning PCR was first performed on the viral genome using primers specific to each gene and Phusion polymerase to obtain a PCR product containing amplified gp gene(confirmed by 1% agarose gel electrophoresis). Next, pET-28a transformation vector was linearised with restriction enzymes and an infusion reaction was performed using Takara Infusion mix(2) to insert the PCR product. The primers used during the initial PCR were designed such that the ends of the product would have a 15bp overlap with the ends of the linearised vector, which the In-fusion mix recognises to fuse them together, reforming the plasmid now with the gene introduced(confirmed by agarose gel).

External sequencing verified the correct gene sequence before transformation into Stellar E. Coli competent cells via heat shock. Transformants were plated on 1mM kanamycin plates and further screened via colony PCR and agarose gel for successful transformation.

A small-scale expression test was then performed to determine whether the protein could be expressed successfully as well as whether it grew better at high(37°C) or low(16°C) temperature. For this, cultures of successful transformants were grown in LB media until reaching an optical density of 0.6, after which its expression was induced. The gene of interest was inserted into a lac operon within the transformation vector, which allowed for control of its expression using IPTG, a molecule very similar in structure to lactose.

IPTG (1mM final concentration) was added, and the culture grew until reaching stationary phase (12-16hr). Fractions were collected: total, soluble, insoluble, and nickel resin-bound (via engineered His tag). Expression was confirmed through SDS-PAGE gel analysis (Coomassie blue staining) and western blot using the engineered His tag. This procedure was repeated on a large scale using 1 L flasks and collected proteins were purified by ion chromatography.



**Figure 1**. Side by side view of SDS-Page gel and western blot demonstrating protein expression. The "S" fraction refers to the "soluble" protein in the sample while the "B" fraction refers to the protein "bound" to the nickel resin. An intense band approximately within the expected size region indicates successful expression. This test indicates that gp6,7, and 8 were successfully expressed but gp9 expression is inconclusive.

# Results and adjustments to the original plan

Transforming gp9 into E. Coli proved challenging, prompting us to experiment with different strains and create custom competent cells, deviating from the original plan. Eventually, all four genes were successfully transformed into an expression strain. While gp6 and gp7 were successfully expressed, purified, and collected, gp8 had limited yield due to low expression, and gp9 remained unattainable. This suggests potential toxicity to E. Coli, requiring an alternative method for acquisition.

#### Future directions for the project

The logical next step to perform would be to purify the remaining proteins and complete cryo-EM analysis to perform a structural analysis of each protein. A further step to be taken would be to produce empty viral capsids for further study of the DNA packaging mechanism, as well as the interactions between the proteins I produced.

#### Value of studentship

#### <u>To student</u>

Working on this project I have learnt a wide range of biochemical techniques related to genetic engineering and protein production as well as transferable skills such as time management and autonomy. I believe these skills will be extremely valuable in any future research projects I might undertake. I was also able to experience first-hand what the life of a researcher is like, which will help me make informed decisions about what career paths to pursue in the future. To lab

The vacation studentship allowed our laboratory to introduce Aitor to the research environment, enabling him to collaborate closely with colleagues, engage in lab activities/seminars and achieve significant results that establish a solid foundation for determining the structures of several proteins from the archaeal virus using cryo-EM.

#### To Biochemistry society

This project helped the biochemistry society to achieve its goal of supporting career development and lifelong engagement by providing the opportunity for me to develop key laboratory and professional skills and providing insight into the life of a researcher.



*Figure 2. Aitor Mateo(left) and Professor Fred Antson(right) in the protein purification lab.* 

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# Evaluation of different copolymers for purification of Glucose transporter 4 (GLUT4) from adipose cells.

**Student:** Emily Strickland, **Supervisors:** Dr Francoise Koumanov & Dr Paul Whitley, The University of Bath, Department for Health, and Department of life sciences.

#### Background and aims of the project:

Amphipathic co-polymers such as Styrene-maleic acid (SMA) have been utilised for extraction of endogenous integral membrane proteins in nanodiscs known as SMALPS[1]. This mode of extraction has been successfully used in structural and functional studies of membrane proteins including high resolution Cryo-EM[2]. The Koumanov/ Whitley labs are developing a pipeline combining SMA extraction with affinity purification using

amylose/MaltoseBindingProtein-proteinA (MBP-pA) method, with the immediate aim of pulling down the integral membrane protein Glucose transporter 4 (GLUT4) from adipocyte (3T3-L1) cells for downstream functional studies (Fig.1). However, the MBP-pA fusion protein dissociates from amylose resin in the presence of even low concentrations of SMA, making the pipeline unworkable. The goal of this 6-week studentship was to screen a range of other co-



#### Materials and Methods:

**Materials.** Co-polymers including DIBMA screening kit (HEPES) & Ultrasolute Amphipol-17[3] were purchased from CubeBiotech. SMA-2000 was purchased from Cray valley and hydrolysed to form SMA following the protocol described by [4]. MBP-pA/amylose beads were prepared following protocol described in [5].

Assay to test MBP-pA binding to Amylose beads. 700µl of co-polymer solution in phosphate buffered saline (PBS) was incubated with 100µl of MBP-pA/amylose beads for 1 hour with rotation at room temperature. Beads were pelleted by centrifugation at 3000xg for 2 min and the supernatant was removed. Beads were washed in column buffer (10mM Tris, 150 mM NaCl, pH 7.4). The amount of MBP-pA remaining associated with the beads was assessed by SDS-PAGE, immunoblotting and densitometry.

Polymer extraction of proteins from mammalian cells. Confluent 3T3-L1 fibroblast were washed with PBS and 300µl co-polymer solutions containing protease inhibitors was applied to each well. Cells/debris were scraped from the dishes, placed in microfuge tubes, and incubated for 1 h at room temperature. Samples were separated into 'soluble' and 'insoluble fractions' by ultracentrifugation at 540,000xg for 20 mins. Fractions were further analysed by SDS-PAGE and immunoblotting to identify proteins present in the different fractions. The same protocol was used to extract proteins from insulin-stimulated fully differentiated adipocytes (10 day differentiation protocol followed as per[6]) Immunoprecipitation of GLUT4. MBP-pA Amylose beads were loaded with anti-GLUT4 antibody and washed in column buffer prior to incubation with co-polymer extracts (soluble fractions). Beads were washed to remove unbound material and samples eluted with 40 mM maltose in PBS. Note: These are very mild elution conditions in order to maintain the structural integrity of eluted proteins.

**Transmission electron microscopy TEM** - Samples were placed onto chromium coater-glow discharged, 200 Mesh copper grids. Samples were washed with distilled water and incubated with 4% Uranyl acetate before transmission electron microscopy was carried out.

**Results and outcomes of studentship, including any relevant figures** Initial experiments confirmed that SMA-2000 significantly interferes with binding of MBP-pA to amylose resin (Fig2 and data not shown). As our immunoprecipitation pipeline requires MBP-pA to remain bound to resin different co-polymers were screened (Fig2).



Co-polymer (0.1875% concentration)

Co-polymer (1.5% concentration)

Figure 2- Co-polymer amylose/ MBP-Pa interference assays. Relative binding of MBP-pA to amylose beads incubated with 0.1875% or 1.5% co-polymer dissolved in PBS. MBP-pA bound to the amylose beads was quantified after SDS-PAGE and immunoblotting. Results were expressed as relative binding compared to the control condition (PBS). Mean  $\pm$  SD (n=3). Comparisons between conditions with two-way Anova \*\* p < 0.01.

The results revealed that all the tested co-polymers perform more favourably than SMA in maintaining interaction between MBP-pA and the amylose resin at low concentrations. DIBMA 12, DIBMA 10 and Amphipol-17 allowed significant binding even at higher (1.5%) concentrations. We therefore concluded that DIBMA 12, DIBMA 10 and Amphipol-17 were worthy of further investigation. For our pipeline to work the polymer should not only allow the MBPpA/Amylose interaction but should be efficient at extracting membrane proteins from mammalian cells. We therefore tested efficiency of extraction of selected membrane proteins from 3T3-L1 cells using the chosen polymers (Fig3).



Figure 3. Testing the extraction efficiency of different polymers on fibroblasts. 3T3-L1 fibroblasts were treated with indicated polymers, detergent (RIPA) or PBS controls . Soluble and insoluble cell contents were separated by ultracentrifugation. These fractions were separated by SDS-PAGE and immunoblotted for selected proteins (see key) and quantified. Relative abundance of the protein of interest in the soluble fraction was calculated as a percentage of the total amount in the extract.

It is apparent that DIBMA10 and DIBMA12 have relatively low efficiency of membrane protein extraction as shown by the low amounts of GLUT1, Na<sup>+</sup>/ K<sup>+</sup> ATPase and PDGFR- $\alpha$  in the supernatants

following centrifugation. Amphipol-17 extracted GLUT1 and Na<sup>+</sup>/ K<sup>+</sup> ATPase with similar efficiency to RIPA (detergent control) although PDGFR- $\alpha$  extraction was quite low.

As our initial aim was to develop a pipeline for purification of GLUT4 we performed extraction from 3T3L1 cells that had been differentiated into adipocytes (Fig.4). 3T3-L1 fibroblasts do not express GLUT4 whereas adipocytes do.



Figure 4. Testing the extraction efficiency of amphipol 17 on adipocytes. 3T3-L1 adipocytes were treated with 0.5% amphipol-17 or detergent (RIPA).soluble (S) and insoluble cell contents (P) were prepared by ultracentrifugation. These fractions were separated by SDS-PAGE and immunoblotted for selected proteins (see key) and quantified. Proportion (%) of selected protein present in the supernatants (soluble fraction) and pellets (insoluble fraction) is plotted on graph n=2).

Evidently, Amphipol-17 extracts GLUT4, our target with similar efficiency as RIPA detergent, (Fig4). Therefore, we concluded that amphipol-17 solubilised cell fractions are suitable for immunoprecipitation experiments (Fig5).



Figure 5. Figure 5. Affinity isolation of GLUT4 solubilised with Amphipol-17. Adipocytes were treated with Amphipol-17 and soluble cell fraction was prepared (lane 7). This fraction was incubated with amylose MBP-pA anti-GLUT4 beads. Beads were washed and elution was performed using maltose. Eluate (lane 1) and proteins remaining on the beads post elution (lane 2) were analysed. Lanes 3 to 6 – irrelevant samples.

The results (Fig5) indicate that GLUT4 has been pulled down/enriched on the amylose beads. Encouragingly there is no evidence of Na<sup>+</sup>/K<sup>+</sup> ATPase bound to the beads suggesting that there is selectivity for GLUT4 in the immunoprecipitation. However, the amount of GLUT4

eluted from the beads with maltose was very low.

Despite the low yield of GLUT4 eluted from the beads we decided to image the eluate using TEM (Fig6) (along with other fractions from the extraction and controls (not shown).



Figure 6- Transmission electron microscopy micrograph of amphipol-17 adipocyte extraction. Scale bar 100 nm. Yellow circle represents single nanodisc

#### While the results of

the TEM are very preliminary, we were encouraged that we could see dense particles of a size consistent with being nanodiscs in the sample similar to those seen in [2].

#### Future directions in which the project could be taken

Purification of proteins needs to be scaled up. Elution from the amylose column needs to be optimised, this may be through the use of column rather than batch purification, as immunoblotting shows this recovers the highest abundance of GLUT4 (data not shown). Mass spectrometry proteomics would then be carried out on the purified material to assess the purity of the GLUT4 preparation prior to any cryo-EM. This may identify known GLUT4 binding proteins such as TUG [7], which will encourage us to develop methodology to study membrane protein interactions that are disrupted during conventional detergent extraction.

#### Departures from original project plan

As the lab had pre-existing stocks of recombinant MBP-pA the planned bacterial expression for week 1 was not necessary and I started testing the co-polymer interference in week 1 and 2. This allowed me to go much further in the characterisation of new co-polymers. I carried out polymer extraction in fibroblast cells (3T3-L1) as well as in adipocytes, this allowed more repeats of successful polymers to be carried out once 10-day differentiation of adipocytes was complete. This efficient use of my time early in the project meant that I could learn and perform TEM imaging of nanodiscs, and maltose elution's extracted from adipocytes and fibroblasts, which was not originally planned.

# Value of studentship to the student, focusing on skills developed (technical and transferable) and contribution to future career plans

During this unique and exciting opportunity, I have gained invaluable transferable skills such as organisation, time keeping, experimental design, and critical analysis which has allowed me to shape the direction of the project and achieve beyond the proposed timescale of the project. These skills alongside the numerous new technical skills such as SDS-PAGE, western blotting, transmission electron microscopy and cell culture will be greatly beneficial to take into my last two years at university in my M. Biochemistry degree and beyond, hopefully into a research career, which I realise now is something I wish to pursue as a result of this fantastic experience.

#### Value of studentship to the research group

From our perspective as supervisors Emily's summer studentship was invaluable. Without Emily this work would not have been performed so promptly. Emily has really pushed the project along and achieved more than we had anticipated in a 6-week period. Amphipol-17 has been identified as a polymer that efficiently solubilises membrane proteins in mammalian cells and is compatible with our pipeline for the affinity purification of GLUT4. When examined using TEM the affinity purified GLUT4 sample looks to contain particles of a size and shape consistent with nanodiscs. We will definitely be exploring the use of Amphipol-17 in a continuation of this, and other studies and we are considering including these results in a research article.

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Pictures- Top left- Emily in cell biology lab, Top right-Emily observing Transmission electron microscopy of eluate samples, Bottom image- lab group photo (L to R- Jariya Buniam, Francoise Koumanov, Paul Whitley, Linston Stanley, Emily Strickland, Alisha Spencer)

# Introduction

Every year, there are approximately 9,000 new cases of Small Cell Lung Cancer (SCLC) in the UK and despite standard treatment, there is a low overall survival, approximately 7% at 5 years ("Lung Cancer...", 2023). This is due to the development of drug resistance in the malignant cells which results from phosphorylated hnRNPA1 binding to XIAP and Bcl-xl leading to anti-apoptotic, survival signals. UP1, the N-terminal DNA/RNA binding domain of hnRNPA1, is one therapeutic target that has been identified. Locating compounds that can bind to UP1 and selectively inhibit the binding of UP1 to XIAP and Bcl-xl is significant in overcoming mechanisms of drug resistance. It is expected that when utilised together with standard chemotherapy treatments, antitumor responses should be attained and overall survival should increase.

# Aims of the project

The project is intended to optimise the conditions for small molecule soaking in UP1 microcrystals. UP1 is a model system for drug screening in microcrystals. It has been shown that X-ray diffraction can be recorded with similar quality from UP1 crystals and microcrystals. Soaking in microcrystals also comes with many advantages compared to large crystals, as it allows lower concentrations of ligand and shorter incubation times, therefore minimizing damage to the target protein.

# Methods

# UP1 protein production

Competent *E. coli* BL21 cells were transformed using UP1 wildtype plasmids. The *E. coli*, now containing a lac operon, was then incubated before being signalled using IPTG to express proteins. The cells were then incubated overnight for optimal protein production.

# Immobilized metal affinity chromatography

To purify the expressed protein, it was concentrated and then purified using a nickel column. The recombinant protein contained a His-tag which was bound to the nickel through electrostatic attraction. Purification was then completed through elution with imidazole.

# Ion exchange chromatography

Purifying the protein with further specificity was conducted through an ion exchange column. To solve the issue of slight protein contamination, an ion exchange purification was done with two MES-based buffers, one without NaCl and one containing NaCl.

# Crystallisation of UP1 microcrystals

There were numerous attempts to crystallise UP1 into microcrystals to try and optimise small molecule soaking conditions. This consisted of using buffers with different ratios of UP1 to crystallisation buffers, using buffers with varying concentrations of Polyethylene glycol 4000 (PEG) and 2-Methyl-2,4-pentanediol (MPD), using different pHs and using varying concentrations of protein.

# Fixed target serial crystallography

This technique is the collection of data from several microcrystals and the merging of that data. It utilises a silicon chip that allows 25,600 potential positions to record data from in 10 minutes (Diamond Light Source).

# **Results and Discussions**

During the summer studentship, the crystallisation of UP1 into microcrystals was successful. However, when attempting to perform small molecule soaking in the microcrystals at the Diamond Light Source synchrotron, we were unable to properly recreate UP1 microcrystals which resulted in an excess of

protein precipitate. While at the I24 beamline, we failed to obtain any results of significance regarding the soaking of small molecules as the x-ray crystallography of the microcrystals had no relevant hits.

Although we were not able to complete our overall aim, we did manage to optimise the process of protein expression and crystallisation. The conditions that we found were optimal for the production of microcrystals were either the 10ul of UP1 with 10ul of 20% (PEG and MPD) buffer or the 16.7ul UP1 with 13.3 ul 18% (PEG and MPD) buffer, both with Tris at pH8.5 and 10mg/ml UP1 concentration. We also found that 22% (PEG and MPD) buffer also worked, however, made crystals that were too big for our purposes.

# **Future directions**

The research done could be taken further if the crystallisation is successful when performing X-ray crystallography, as the optimisation of small molecule soaking could be done and researched further. Along with this, it would aid in discovering and optimising a therapeutic target for Small Cell Lung Cancer by reducing the role that hnRNPA1 plays in the illness as we could see what small molecules can bind to it with high affinity.

# Departures from original plan

When purifying the protein, it didn't purify as we planned and as such we had to express the proteins again from the start midway through the project. Along with this, we had numerous attempts of crystallising the protein resulting in a clear solution without any crystals. We eventually managed to crystalise our protein, realising that something in the crystallisation buffer was at fault as we used an older buffer that managed to work. We had to constantly adapt and adjust our conditions as we faced various setbacks with crystallising. However, there was not enough time to redo the final part of our project and complete the overarching goal.

# Value of studentship

Working with Dr. Prischi, I learned many skills in the lab, such as cell transformations, culturing, protein expression, SDS-PAGE electrophoresis, chemically competent cell creation and chromatography. I have also improved other skills that can be applied to other areas of my life, including time management (from having many time-dependent steps in our experiments) and communication from working with my supervisor. By participating in this project and completing it, I have been able to experience what the field of research would be like for myself. This has further motivated and strengthened my resolve to pursue a career as an academic. The studentship also optimised crystallisation conditions and provided information for future research.

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# The Purification and Substrate Specificity of a Soluble Squalene-Hopene Cyclase Enzyme.

Student: Soren Bentley; Supervisors: Jennifer Littlechild, Simone De Rose, Hayley Blaber, Michail Isupov, Harley Worthy. Biocatalysis Centre, University of Exeter.

# Introduction

This project was carried out within the Exeter Biocatalysis Centre to investigate the substrate specificity of a squalenehopene cyclase (SHC) enzyme. SHCs are a family of enzymes that catalyse the production of hopene from a linear precursor, squalene (figure 1). This single-step biotransformation results in the formation of five ring structures, thirteen covalent bonds and nine stereocentres, making it one of the most complex enzymatic reactions in nature[1]. The product of the reaction, hopene, is an important precursor in the synthesis of several fragrance and flavour molecules.

Typically, SHCs exist as membrane-bound proteins, however, the Littlechild group have been able to identify a novel bacterial variant that lacks a membrane binding region and can be cloned and overexpressed in *Escherichia coli* in soluble form (see figure **2**). The crystallographic structure of this new SHC has been determined (PDB code: 8PAK[2]). Characterising its activity towards squalene as a substrate is of great interest, as it has the potential to be used for the sustainable synthesis of hopene in the health care and fragrance industries.

# Aims of the project

- Overexpress the enzyme in *E. coli* cells that have been transformed with an appropriate recombinant plasmid
- Purify the enzyme using fast protein liquid chromatography on gel filtration and anion-exchange columns.
- Use sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) to evaluate the level of protein over-expression and final purity of the enzyme.
- Assay the enzyme's activity towards squalene as a substrate
- Crystallise the enzyme with a known inhibitor. The crystals will be used to solve the structure of the enzyme-inhibitor complex which will provide further information regarding the binding of substrates within the active site.

# Methods used

# Overexpression and purification

A pET11a+ plasmid containing the gene encoding for the SHC enzyme was transformed into *E. coli* BL21 (DE3) cells via heat shock. These cells were then grown in LB broth at  $37^{\circ}$ C up to an optical density at 600nm of 0.6. Expression of the gene was induced by adding 1mM isopropyl  $\beta$ -D-thiogalactopyranoside (IPTG). After incubating overnight at  $20^{\circ}$ C, the cells were harvested by centrifugation and resuspended in 50mM MOPS, pH 7.0, 100mM NaCl. Sonication



Figure 1: Adapted from [3]: Schematic showing the conversion of squalene into hopene with the numbered arrows indicating the sequence of intermediate electron transfers. Within the active site of SHC enzymes, a highly-conserved aspartic acid residue (labelled here as "D") carries out the proton transfer that induces the polycyclisation of squalene. Once this has occured, a non-specific basic residue (labelled as "B") will accept a proton to form the final product, hopene.

was then used to disrupt the cells and the resulting lysate was clarified by centrifugation. Size-exclusion chromatography (SEC) and anion-exchange chromatography columns were then used to purify the protein from the crude supernatant. Samples from the cell lysate and the subsequent purification steps were run on SDS-PAGE to confirm the level overexpression and purity of the SHC enzyme.

#### Assay

The enzyme's activity towards squalene was assayed using a recently published method[4]. Here, 20mM squalene was added to 0.284mg/mL enzyme with 0.5%w/v Tween 80 or Triton X-100 detergent being used to solubilise the squalene. The reaction was then incubated at 30°C for 17 hours. All reactions were carried out in triplicate with negative controls that contained 20mM squalene and 0.5%w/v detergent, without any enzyme present. This procedure was repeated using a temperature of 40°C. The samples were then analysed by HPLC to confirm the presence of hopene.

# Crystallography

SHC enzyme was concentrated to 40 mg/mL and mixed with  $45 \mu \text{M}$  of a known inhibitor that was dissolved in dimethylsulfoxide (DMSO) and methyl acetate. Microbatch crystallisation trials were then set up with Morpheus<sup>®</sup>Fusion protein crystallisation screens using an Oryx8 crystallization robot. Crystals appeared within one week in several conditions, these were harvested and sent to the Diamond Light Source Synchrotron to obtain high resolution diffraction data.



Figure 2: Cartoon representation of the SHC enzyme under investigation.



Figure 3: Diagram showing the structure of the enzyme's active site solved from the crystal diffraction data. The molecule shown in green and red is 2-methyl-2,4-pentanediol (MPD) which is a chemical additive in the Morpheus<sup>®</sup>Fusion crystallisation screen. Presence of MPD in the active site confirms that binding did not occur between the enzyme and inhibitor during crystallisation.

# **Results and discussion**

## Assay

Analysis of the assay samples is still in progress. This is due to an unexpected delay in the purchase of the specific column required for HPLC, as per the method in [4].

# Crystallography

X-ray diffraction datasets were collected and processed through an automatic pipeline based on a software called XDS[5]. Using these data, the structure of the enzyme was determined to a resolution of 1.4 Å. This was achieved through molecular replacement, using another recently-determined SHC structure as a search model. This structural resolution is the highest that the Exeter Biocatalysis Centre have been able to obtain for this enzyme and will therefore be useful for modelling in future crystallographic studies.

Upon inspection of the structure, it is clear that a molecule is present within the active site (see figure **3**). The contours of this molecule's electron density match the structure of 2-methyl-2,4-pentanediol (MPD), a chemical component of the Morpheus<sup>®</sup>Fusion crystallisation screen. The presence of this molecule within the enzyme's active site indicates that enzyme-inhibitor binding did not occur during crystallisation.

# Future directions in which the project could be taken

The assay samples will be stored at  $4^{\circ}$ C until they can be analysed by HPLC. The results from this analysis will largely determine the future directions of the project. If conversion from squalene to hopene is observed, the next objectives will focus on optimising the reaction conditions (e.g. pH, temperature) and scaling up the reaction for hopene production. If there is no reaction, different parameters of the assay method, such as reaction time and detergent concentration will be altered to try and elicit enzyme activity towards the substrate.

It is also important that more crystallisation trials are set up to gain insight into the binding of substrates within the enzyme's active site. Cocrystallising the enzyme and inhibitor will be repeated, with detergents being added to promote binding between the two species. The method of soaking native enzyme crystals in a solution of the inhibitor will also be carried out to try and successfully capture complexation.

# Departures from original project plan

There were no departures from the original project plan.

# Value of the studentship

*Research group*: Soren been hands-on with all the techniques described and worked alongside other members of the Littlechild group. He has obtained new results that have contributed to the SHC project that was originally funded by Innovate UK Grant No. 104457.

Student: This studentship has allowed me to gain excellent experience in the field of biocatalysis. I have enjoyed gaining valuable insight into the importance of chemistry within biological systems and how this can be used to improve the sustainability of industrial processes. I have been able to improve my adaptability and flexibility through proactive learning whilst also enhancing my communication proficiency and ability to work as part of a team. Alongside these transferable skills, I have developed competence in numerous biochemical techniques which has helped to increase my lab confidence immensely. Ultimately, the experience has intensified my passion for research and my desire to pursue PhD programmes relating to biotechnology and sustainable chemistry.

# Acknowledgements

Funding of the studentship by the Biochemical Society is gratefully acknowledged. The success of this programme has contributed to achieving their strategic objectives which focus on promoting the importance of molecular bioscience as well as providing career and lifelong development to all its members. Thank you for your generosity.

Thanks must also be given to the Exeter Biocatalysis Centre for hosting the studentship; they consistently provided exceptional supervision and fascinating insight into their work throughout this enriching experience.



 $Figure \ 4: \ {\rm Image \ of \ Soren \ and \ Simone \ setting \ up \ crystallisation \ trials \ using \ the \ Oryx8 \ robot.$ 

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# Investigating the potential inter-membrane phospholipid transport function of paraquat-inducible ABC (PqiABC) with fluorescent proteoliposomes

Student: Henry Box Supervisor: Dr Timothy Knowles





Left to right: Dr Timothy Knowles, Henry Box, Hannah Johnston

# Introduction

For more successful antibacterial drug development, a more thorough understanding of the cell envelope is required as it maintains the integrity and shape of the cell. In gram negative bacteria (which

constitute 9 of 12 priority pathogens identified by the World Health Organisation [1]), the envelope is an essential structure composed of two membranes sandwiching a peptidoglycan layer. Although this structure has been known for some time, its assembly mechanisms have only recently begun to emerge. Components of the outer membrane (which makes contact with the host) are delivered by complex protein machinery: proteins via the Bam complex, lipoproteins via the Lol pathway and lipopolysaccharides via the Lpt pathway. Mechanisms for phospholipid transport remain elusive, though.

The first pathway correlated with phospholipid transport was the Mla pathway but has since been postulated not to be responsible for the bulk of phospholipid transport and rather more responsible for maintaining outer membrane asymmetry [2, 3]. However, a protein complex with structural similarity to Mla is believed to span the entire intermembrane space with a hydrophobic tunnel and could be responsible for the bulk movement of phospholipids. This machinery is called paraquat-inducible ABC (PqiABC).

Understanding the mechanisms of phospholipid transport, and so potentially the mechanism PqiABC, could provide a new avenue for antimicrobials development. If such mechanisms are conserved across gram-negative bacterial species, then such knowledge could contribute to the fight against a significant number of diseases caused by bacterial infection. This aligns with the strategy set out by the Biochemical Society as they aim to highlight the importance of gathering biochemical knowledge in relation to global health and fighting disease, as was demonstrated recently during the COVID-19 pandemic.

# Aims

This project aimed to confirm preliminary evidence gathered by the Knowles laboratory at The University of Birmingham which suggests that PqiABC is a phospholipid pump. Doing so will require an assay which can routinely assay the system in real time. Therefore, the first aim was to design and collect data from a FRET-based transport assay making use of proteoliposomes containing PqiABC and fluorescently-tagged phospholipids (Figure 1). We predicted that when the PqiABC system was complete, there would be a change in fluorescence signal as the fluorescently-tagged phospholipids were transported from one proteoliposome to the other. We also aimed to find the functional impact of the presence of metal chelator versus supplemented zinc ions as analysis of the PqiA structure with an AlphaFold model implies zinc coordination (Figure 2).





# **Departures from original project plan**

I began work on an alternative assay for the routine measurement of phospholipid transport between proteoliposomes. This would rely on the activity of the cardiolipin synthase A (ClsA) to convert POPE into cardiolipin after they were transported from a proteoliposome without ClsA to one with ClsA. A thin-layer chromatograph would reveal cardiolipin presence, and therefore PqiABC transport. The main aim for me was to purify this protein successfully as the most recent protocol was published in the 1990's [6].

# Methods

**PqiAB:** Production of strep-tagged PqiAB involved transformation of plasmid pET26b and growth in C43 *E. Coli*, then affinity-based and size exclusion chromatography. **Proteoliposome preparation:** Liposomes containing set proportions of un-tagged POPC and NBD-tagged or rhodamine tagged POPC were prepared in a fume cupboard then sonicated before adding either PqiAB or PqiC to each. This was done in duplicate – with EDTA or zinc chloride supplemented within the buffers. **FRET assay:** Baseline fluorescence was measured with the NBD-tagged PqiAB or PqiC proteoliposomes. Fluorescence was measured over time upon addition of the rhodamine-tagged proteoliposomes with the corresponding PqiAB or PqiC component. **C-terminal His-tagged ClsA purification and construct modification:** 



Protocol inspired by Hiraoka *et.* al [6]. Having found that C43 *E. coli* expressed the protein better than BL21 in post-induction Western blots, the protein was unfortunately absent after Ni-NTA column chromatography. An AlphaFold model suggests that the His-tag would be better situated on the N-terminal (Figure 3), though, so primers were designed to remove the C-terminal tag and add it to N-terminal on the previous DNA construct.

# Results

The overarching result of the FRET-based transport assay is largely inconclusive with regards to the phospholipid-transporting function of PqiABC and its functional dependence on zinc. When chelator (EDTA) was present, there was no significant sign of transport because the levels of FRET due to NBD quenching are insignificant as indicated by the difference between the assays and the sonicated controls (representing maximal NBD-rhodamine mixing and so maximal NBD quenching). The same was true for the assays containing zinc (Figure 4). Therefore, this assay design has not shown PqiABC phospholipid transport between proteoliposomes.



# **Future directions**

There are many reasons why this assay may not have worked. Most fundamentally, PqiABC might not be a phospholipid transporter. Alternatively, the fluorescent tags could be sterically interfering with transport through the protein. There might even be an energetic requirement not factored into this design by proton motive force (PMF). The ClsA-based proteoliposome assay could help clarify these possibilities by removing the requirement for fluorescent tags. However, a method to ensure that the proteoliposomes do not burst and mix due to increasing pH while testing PMF will need to be considered carefully. It is uncertain, though, whether the Knowles lab will return to the FRETbased assay due to these complexities. Therefore, the group will continue developing a ClsA purification protocol and perhaps find success monitoring transport this way.

# Value of studentship To the student

As I move into the lab-based fourth year of an MSci course, these six weeks have been an invaluable introduction to extended research projects. From participating in lab meetings to contributing to high level conversations about the design of the assays, there have been many transferrable skills developed in a new context such as project management and problem solving. Additionally, I can now convincingly evidence my enjoyment for scientific research, strengthening my desire to pursue a PhD in structural biology.

# To the research group

"Henry's research has been hugely beneficial to the research within the Knowles lab. His excellent approach to experimental design, identifying the correct controls to use and careful consideration of normalising the data, has irrefutably confirmed that our approach to study the PqiABC complex, using a FRET based assay, was not the right direction to take. Furthermore, through discussions with him and his valuable input we have devised an alternative approach utilising a ClsA based assay, which we are currently investigating. Overall, his research has led to a far deeper understanding of working with PqiABC, that has fundamentally changed the direction of research within the Knowles group." – *Dr Knowles.* 

# Acknowledgements

All PqiC used in this project was previously prepared by Knowles lab PhD student Hannah Johnston, who also supervised me daily.

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# BAYLISS LAB



# Investigating the effect of adapter proteins, SHC and SHP2, on biomolecular condensate formation and MAPK/ERK pathway progression in EML4-ALK V1 cell lines.

Student: Jonathan Hunt Supervisors: Dr Josephina Sampson, Prof. Richard Bayliss

#### **Background:**

In 2-9% of Non-small-cell lung cancer cases, the oncogenic fusion between EML4 and ALK generates biomolecular condensates(1). Inhibitors such as Ceritinib were developed to block the ATP binding site of ALK to reduce signalling, whilst resulting in the condensates' dissolution. However, the inhibitor Alectinib, whilst abolishing the kinase activity, also increased the prevalence of condensates. The formation of a Lys-Glu salt bridge within the activation loop was then confirmed to be the specific driver of condensate formation. Other mechanisms of condensate inhibition other than the ATP site are yet to be explored thoroughly.

#### Aims:

The project was intended to gauge a deeper understanding of potential other methods of inhibition of EML4-ALK signalling through the role of adapter proteins in condensate formation. Specifically, the aims of the project were:

- Visualize the effects of SHC & SHP2 knockdown on condensate formation using Immunofluorescence staining.
- Investigate the SHC & SHP2 knockdown effect on ALK activity and progression of the MAPK/ERK pathway using western blotting.
- Model ALK protein interactions using modelling software such as AlphaFold to identify potential key sites involved in condensate formation.

#### Work undertaken:

Immunofluorescence: RNA interference was executed on the EML4-ALK, doxycycline-inducible BEAS2B cell line. Using lipofectamine, SHC, SHP2 and GAPDH oligos were transfected into the cells. Cells were fixed and permeabilised before immunofluorescent staining. (Figure 1)

<u>Western blotting:</u> Lysates from an NSCLC patient-derived cell line (H3122 V1) were separated through SDS PAGE and transferred onto PVDF membranes. Cell lysates were RNAi transfected with GAPDH, SHC and SHP2 oligonucleotides. Primary and secondary antibodies conjugated with HRP were used to detect the presence of proteins in Fig.2 using a chemiluminescent substrate. (Figure 2)

<u>Modelling:</u> AlK phosphorylation site peptides were modelled on Alphafold against SH2 domains of SHC and SHP2. Pymod3 was used to strengthen the models through comparison to crystal structures of other SH2 interactions. (Figure 3)

# **Results and Discussion:**



In the Dox+ control, cells express both ALK and SHC. The orange signals on the merged image indicate the two proteins colocalise in condensates. However, under Dox- ALK expression is not induced, and SHC signals are dispersed within the cytoplasm. After RNAi with SHC oligo 8 and 9, ALK signals are dispersed within the cytoplasm, compared to the foci seen in the + control. B represents the significant drop in condensate formation from DOX+ to SHC RNAi knockdown p<0.05. ALK foci are present in C compared to shp2 RNAi, where ALK unusually localizes to the microtubules, rather than dispersing to the cytoplasm. In D, only SHP2i 9 had no significant difference in the number of foci per cell compared to GAPDHi, p<0.05. The data suggests SHC and SHP2 play a key role in EML4-ALK condensate formation.



Figure 2: Western blot analysis of H3122 V1 cell lysates after transfection of SHP2 RNAi oligonucleotides. DMSO was used as a control. Lysates were analysed for the relative presence/phosphorylation of the proteins indicated above, using complementary antibodies. GAPDH was used as a loading control.

The predicted binding site for SHP2 is Phosphotyrosine 1283, the third of three phosphotyrosine sites on the ALK peptide, DIyRASyyRKGGCAM. Looking at 3A, the

Looking at Figure 2, the success of the RNAi is evidenced by the reduction in the intensity of anti-SHP2 bands across all four oligos compared to the DMSO control and the anti-GAPDH under GAPDHi. There is a reduction in the intensity of anti-pALK bands in the oligo 9-12 lanes compared to the control and a relative reduction compared to the total ALK. Downstream in the MAPK pathway, there is a reduction in the intensity of anti-pERK bands across SHP2i 9, 10 and 12, compared to the control and total ERK. Anti-SHC signals are less intense with oligos 10-12 compared to the controls, with no significant changes in GRB2. Phosphorylated ALK and ERK in the DMSO control indicates EML4-ALK kinase activity. However, during SHP2 RNAi knockdown, ALK phosphorylation is reduced, along with ERK phosphorylation under oligos 9,10 and 12. This implies that the adapter SHP2 plays a significant role in maintaining the activity of the EML4-ALK fusion and thus MAPK pathway progression. Similarly, the reduction in SHC suggests its binding is affected by the knockdown of SHP2.



used the template: SHP2 interaction with GAB1 peptide. B- AlphaFold prediction (phosphotyrosine independent) of SHP2 (E17L point mutation) and the ALK Y1283 peptide.

phosphorylated tyrosine interacts with the peptide backbone of SHP2, but a Glut-Val interaction may hold the phosphotyrosine in place. Using AlphaFold to substitute Glut for Leu (blocking the interaction), the prediction favours the second tyrosine in the peptide instead, implying the interaction is key for the Y1283 SHP2 binding.

# What next?

A significant direction the project step can go is to understand the regulation of ALK activity through SHC and SHP binding. Binding affinity assays can help understand which key sites play a role in adapter protein binding and condensate formation, such as E17. This brings possibilities for alternative ALK inhibition to ATP site blockers. On a cellular level, differences between SHP2 and SHC knockdown can be further analysed such as microtubule localisation seen in Fig 2C.

#### Impact

EML4-ALK NSCLC is a new area of research, with unique subcellular activity involving condensate formation. Research into the fusion protein has the potential to provide novel treatment options to aid cancer resistance. Moreover, it acts as a gateway to an advanced understanding of biomolecular condensates, applicable to all cellular research worldwide. The project has aligned well with the biochemical society strategy, providing me with crucial skills for the future and the work reaching collaborators internationally.

#### Skills and future career plans

I have gained a range of skills from specific lab techniques to transferable skills such as effective notetaking, problem-solving and presentation skills. When undertaking techniques such as western blotting, RNA interference and cell culture, as well as learning the skills involved, I was able to diagnose issues to improve for repeats and find innovative ways around them. Presenting my results at the end of the six weeks helped to identify areas to improve on, such as specific audience targeting. I look forward to being able to use everything I have learned for the remainder of my studies, and I hope to carry them with me through a research career.

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Generating a Fob1-mScarletl fusion protein to image ribosomal DNA using fluorescence microscopy by Zara Meaby



# Introduction:

The yeast ribosomal DNA (rDNA) locus has about 150 tandem repeats of identical sequences<sup>1</sup>. Each repeat contains an RFB site<sup>2</sup> that allows binding of a protein called Fob1 once per individual rDNA repeat. The number of rDNA repeats is linked with fitness with higher number of repeats indicative of higher fitness while lower number of repeats indicate lower fitness and ageing. Hence, estimating the number of rDNA repeats in an organism is important to estimate its fitness. Owing to the highly identical nature of rDNA repeat sequences, it is impossible to estimate the number of repeats using modern high-throughput sequencing methods. Traditionally, more laborious electrophoresis methods have been used to estimate the numbers of these repeats<sup>3</sup>. In the current project, we aimed to develop an in vivo Fob1-based imaging method to estimate the number of rDNA repeats in Saccharomyces cerevisiae. Since the Fob1 protein binds just once per rDNA repeat it is possible to estimate the number of repeats by "counting" the frequency of Fob1 binding in yeast using fluorescent microscopy.

# Aims and objectives:

The aim of this project was to estimate the number of rDNA repeats using Slimfield fluorescent microscopy<sup>4</sup>. To achieve this, we set out to tag the Fob1 protein with a fluorescent protein mScarletl. We aimed to introduce the resulting fluorescent fusion into a suite of yeast strains carrying varying number of rDNA repeats and estimate the frequency of Fob1 binding to DNA by observing the relative fluorescence intensities of molecules bound to DNA. These intensities would give a readout of the frequency of Fob1 protein binding to rDNA.

The following were the objectives of the project:

- 1) Generation of Fob1-mScarletl fusion by infusion cloning: Cloning of Fob1 into an mScarlet-I plasmid and characterisation of clones
- 2) Introduction of positive clones into yeast and imaging the resulting strains.

# Methods:

The methods used in this project were largely molecular biology related. I generated DNA fragments from yeast genomic DNA and cloned them using standard techniques such as PCR, Infusion cloning, transformation and colony screen using EcoRI digestion. I was able to generate and screen the recombinant clones myself, and also had the opportunity to image cells from the existing stocks using

Single-molecule Slimfield fluorescence microscopy.

# **Results:**

To generate the Fob1-mScarlet fusion, a pair of PCR primers with homologies to the rDNA binding protein Fob1 were designed to amplify a plasmid carrying the gene encoding mScarletl fluorescent protein. Another pair of primers were designed to amplify Fob1 such that Infusion cloning (Takara Biosciences) of the two PCR products generated an inframe mScarletI-fob1 fusion on the resulting plasmid. Clones were selected by transforming the infusion



Figure 1: Restriction digestion of mScarletI-Fob1 clones to screen for positive clones. Plasmid clones were digested with EcoRI enzyme and analysed on a 0.8% agarose gel.

Legend: lanes 2-12: uncut plasmids: 14-24: plasmids digested with EcoRI; lanes 1, 13 and 25: 1Kb ladder. Lanes 1, 2, 3, 6, 8, 9 and 10 show bands corresponding to the expected 5.3 and 3.8 Kb, indicating that these are the correct clones.







cloning mix into commercially available competent E. coli cells and screening by restriction digestion with EcoRI enzyme (figure 1). Positive clones were then confirmed by sequencing.

Four of the positive clones were sequenced to confirm that the fusions in-frame. All clones carried the expected in-frame Fob1-mScarletI fusion. Representative results from one clone sequence are illustrated in Figure 2.

# **Conclusions:**

I successfully generated Fob1-mScarlet-I clones. The resulting clones can be used to quantify the number of rDNA repeats using Slimfield microscopy. I also successfully visualised the Fob1-mGFP clones using the Slimfield microscope.

# **Future directions:**

Due to the short time frame of the project, I was unable to transform the verified Fob1-mScarletI clone into yeast test strains. However, I was able to generate preliminary images from the existing laboratory stocks using Slimfield microscopy. Yeast cells carrying an existing Fob1-mGFP fusion were imaged under a Slimfield microscope using 561nm laser at 5 ms exposure time to estimate the number of rDNA repeats that are bound by Fob1-GFP in yeast strains. I was able to visualise Fob1-mGFP foci in these cells (figure 3).

The next step of the project would be to introduce the Fob1-mScarlet-I plasmid into yeast laboratory strains carrying varying numbers of rDNA repeats and estimating rDNA copy number using Slimfield

fluorescence microscopy. This would inform us of the feasibility of this approach to estimate rDNA copy number.

# Value of studentship:

Student: Overall, it was an extremely enjoyable and informative experience. I have gained many technical lab skills including handling and measuring nucleic acid products as well as transforming them to host vectors. I encountered equipment such as microscopes that I have not used before. Over the course of the studentship I have become much more confident in the lab. I have been able to get to experience what life could be like after graduating. The studentship has prepared me to start my final year project at university.

Supervisor: I really enjoyed supervising Zara over the summer. She is enthusiastic, has a keen eye for detail and picked up techniques quickly. Despite being on a short duration of only six weeks she made significant progress and successfully cloned Fob1-mScarlet-I and characterised the resulting clones. Thanks to Zara's efforts, I now have a construct that I can use to generate preliminary data for my fellowship applications.

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The effect of lithium on Frontotemporal Dementia



<u>Student</u>: Tanisha Lohia; <u>Supervisor</u>: Dr Teresa Niccoli; Institute of Healthy Aging, University College London, UK

# Introduction:

Frontotemporal Dementia (FTD) is a neurodegenerative disease highly prevalent in those under 65. The most common genetic cause of FTD is a hexanucleotide repeat expansion in the 72<sup>nd</sup> ORF region on chromosome 9, known as C9orf72. The resultant toxicity is due to the production of dipeptide repeat proteins, which are synthesised by the non-canonical non-ATG initiated translation. The non-ATG initiation site is introduced within the hexanucleotide expansion in C9orf72. A model with 36 hexanucleotide repeats was developed in *Drosophila melanogaster*, which could be inducibly expressed using the Gal4-UAS system. Previous research has shown that lithium, which can ameliorate Aβ toxicity in Alzheimer's, may also mitigate toxicity observed in C9-expressing flies. Two key genes, Cdk5 and Sgg, are vital in normal neuronal function, cell signalling, and gene expression. The downregulation of these contribute to the pathophysiology of FTD, which affects the lifespan of flies expressing downregulated Cdk5 and Sgg. Their protein products, kinase enzymes, are also known targets for lithium, making them ideal for this study.

# Aims of the project

The overall aim of this project is to investigate the effect of lithium on the lifespan of C9orf72-expressing *Drosophila melanogaster.* Specifically,

- To set up stocks of three genotypes of flies: downregulated Cdk5, downregulated Sgg and "wildtype", and to induce C9-linked FTD.
- To set up, run and analyse lifespan assays of these flies, both with and without lithium

# Methods used

To achieve these aims, 100 female virgins from each relevant stock were collected. These were crossed with C9-expressing males in a 4:1 ratio; 25 males were placed into each mating cage. To practise the techniques, in my first week I collected virgins and set up a mating cage from the v-w+ stock only. In the second week, I collected and set up cages for all three stocks (to generate comparable results). The cages were tipped on the next day, and I collected eggs on the 2 subsequent days (day 2 and 3). I collected the eggs by adding phosphate-buffered saline and drawing up the suspension using a cut-tip pipette. I deposited these eggs into bottles containing food and incubated at 25C for approximately 10 days, until the flies had hatched and mated. At this point, I collected females from the bottles, taking care to select against flies expressing TM6b, a balancer chromosome. If selected, these would be unaffected by the C9 gene, and thus impact results. From each genotype, I wanted to split flies into 10 vials of 15 flies (150 in total) for each of four conditions, either exposed to RU-486 (which induced the C9 gene), exposed to Lithium, both, or neither. In other words, I required 600 flies from each of v-w+, cdk5 downregulated and Sgg downregulated. These vials were then placed into Drosoflipper frames, to allow for scoring and tipping of lifespans. I scored the number of dead flies and tipped the lifespan assays for each condition every two days, putting my results into a pre-formatted Excel sheet.



# The effect of lithium on Frontotemporal Dementia



# Results and outcomes



In my hands, I found that lithium made a statistically significant improvement to the lifespan of C9 cdk5 flies (P=0.012). However, the other conditions did not experience a significant difference under lithium. This could be due to a number of reasons, such as experimental design and the inherent variability expected when using living organisms as model systems. There could also have been flaws in the execution; for example, we had issues with collecting an adequate number of flies for certain conditions (see below).

# Future directions

In the future it may be possible to further studies to determine why lithium did not have the intended effect, as previous studies have shown its efficacy in similar scenarios.

# Departures from original plan

Despite collecting seemingly enough eggs, I was unable to fulfil the required 600 flies from each genotype (see breakdown above) when collecting from the cages I set up in my second week. I was able to get 10 vials of 15 flies for each of my RU- conditions (C9 cdk5 Li-, C9 cdk5 Li+, C9 Sgg Li-, C9 Sgg Li+) except for C9 v-w+. I also got 10 x 15 flies for the following conditions on RU+: C9 cdk5 Li-, C9 cdk5 Li+, C9 Sgg Li-, C9 Sgg Li+ and C9 v-w+. To supplement the data I would obtain for C9 v-w+ flies on RU+, I used flies I had collected from my first cage, which I had split evenly into an RU+Li- and an RU-Li- condition. The ones I had previously on RU+Li-, I split between RU+Li-, and RU+Li+, giving me 5x15 flies for each, and reserved ~100 for a western blot. The ones I had previously on RU-Li-, I split evenly for RU+Li- and RU+Li+. This gave me flies that had been on RU+ since splitting (and some put on lithium late) and flies that were put on RU+ late (with and without Lithium).

# Value of studentship

This experience was incredibly valuable to me. Not only did I learn advanced laboratory techniques such as western blotting and qPCR, but I developed a new-found appreciation for the lab environment, further confirming my future goals. I have gained important experience in *Drosophila* genetics and husbandry procedures and put my theoretical knowledge to practical use. I felt honoured to have contributed to research in this field, despite an inconclusive outcome.



Figure 2 Tanisha tipping lifespans.



# **Biochemical Society Summer Vacation Studentship 2023**

# Analysis of the interaction between dematin and the adaptor protein 14-3-3

## Student: Areeba Tariq

Supervisor: Dr Jürgen Müller, Mr. João Tomás

#### Background

Obesity and diabetes are associated with high blood sugar, high fat levels, and inflammation; these conditions impact around 4.3 million individuals who are diagnosed in the UK and modulate a range of complex cellular signalling pathways inside the cell (Diabetes UK, 2023). The dematin protein has been originally found within red blood cells, but is also present in skeletal muscle, a key tissue involved in the regulation of blood sugar levels.

Dematin binds to a specific glucose transporter as well as to the structural elements of the cell, i.e., the cytoskeleton. Mice in which dematin cannot bind to the cytoskeleton are obese, implying that functional dematin may protect mice (and possibly humans) from obesity and its complications. However, the molecular mechanisms that regulate the association of dematin with the cytoskeleton and sugar metabolism are currently unknown.

## **Aims and Objectives**

The overall aim of this project was to analyse whether previously identified dematin modifications regulate its function in terms of protein:protein interactions and actin binding. This project will specifically investigate the binding of dematin to the small adaptor molecule 14-3-3 and its localisation inside of the cell.

#### Methods

Dematin proteins as well as the adaptor protein 14-3-3 will be expressed in HEK393 cells. The expression of the proteins will be evaluated by western blotting and their interaction using co-immunoprecipitation and western blotting. In addition, dematin proteins will be expressed in C2C12-muscle cells and the localisation of dematin will be analysed in relation to the cellular actin network using immunocytochemistry and microscopic analysis.

#### **Results and Analysis**

**Binding of 14-3-3 to dematin**: Dematin and 14-3-3 were transfected into HEK293 cells and their expression was

validated using western blotting with specific antibodies. Figure 1A shows that the proteins are well expressed. Furthermore, co-immunoprecipitation analyses were performed using a specific antibody against dematin. Copurified 14-3-3 was detected using western blotting with an anti-14-3-3 antibody, confirming the interaction of both proteins (Figure 1B).



**Figure 1**: Co-immunoprecipitation of dematin with 14-3-3. Dematin and 14-3-3 proteins were transfected into HEK293 cells. Co-immunoprecipitation was carried out using an antibody against dematin. Western blots were performed to confirm protein expression (A) and the co-purification of 14-3-3 (B).

Localisation of dematin in muscle cells: C2C12 cells from the mouse myoblast cell line were cultured and

transfected with dematin. The dematin protein was labelled using a specific antibody. In addition, the actin cytoskeleton was labelled with an antibody against  $\beta$ -actin. The nucleus was stained with DAPI. The results demonstrate efficient expression of dematin in the transfected cells, which exhibits a similar staining pattern as cellular actin.



**Figure 2**: Expression of dematin in muscle cells. Dematin was transfected into C2C12 cells. Dematin and actin were detected using specific antibodies and secondary antibodies coupled to Alexa488 (green) and Alexa568 (red), respectively. Cell nuclei were also stained using DAPI (blue).

#### **Future Directions**

The binding of dematin could be analysed with mutations in specific posttranslational modification sites (amino acids) to further understand the binding and function of 14-3-3 binding to dematin. Furthermore, the role of this interaction could be assessed in actin function and obesity.

# Value of Studentship to Student

The studentship was an enjoyable experience that provided me with invaluable lab experience which would have otherwise been inaccessible, and further sparked my interests in pursuing a future career within biomedical research. The opportunity allowed me to develop crucial transferable skills such as time management, problem-solving, accurate recordkeeping, and data collection, which I can apply not only as a student but also within my future career. Additionally, I gained proficiency in various techniques including cell culture, cell transfection, and cell lysis, western blotting, among others, and was able to independently conduct experiments by the end of the project.

Overall, this experience has had a significant impact on my personal and professional growth, fully immersing me in the life of a researcher.

# Value of Studentship to Lab

It was great to have Areeba in the laboratory over the summer. She learned the techniques well and contributed significant data to the research project that will be used in future studies. Areeba was very enthusiastic and a great member of the laboratory team.



Figure 3: Areeba Tariq and Dr Jürgen Müller in the Lab.

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# Investigating the Role of Dynamic Ubiquitin Signaling in Orchestrating Plant Immunity

Student: Lavanya Yogesvaren; Supervisor: Professor Steven Spoel, Spoel Research Group, Institute of Molecular Plant Sciences

# **Introduction**

As the world's population is expected to exceed 9.7 billion in the year 2050, agricultural yields must increase significantly to accommodate a growing population, while overcoming the challenge of limited arable land, and the persistence of P&P's (pathogens and pests). Therefore scientists are looking into improving crops genetically. Over the course of evolution, plants and P&Ps have developed genetic strategies against each other. Today, P&Ps are known to reduce crop yields by up to 40% (Sevary et al., 2019). Upon recognition of pathogen associated molecular patterns (PAMPs), an immune response is triggered. A key player in this response is the highly-conserved, small protein, ubiquitin, which attaches to a variety of substrates via the ubiquitination pathway consisting of: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3). Once attached, ubiquitin acts as a signaling molecule, affecting the fate of the substrate. It has the ability to form at least eight different chain topologies, enabled by its seven lysine residues (K6, K11, K27, K29, K33, K48, K63) or its N-terminal methionine (M1). Although much is known about the role of K48 topologies in targeting substrates for proteasome-mediated degradation, little is known about the remaining seven topologies. Spoel research group recently identified an increase in different ubiquitin chain topologies upon pathogen infection, suggesting they are important drivers of plant immunity. We sought to learn more about the substrates affected by ubiquitination, and discover if there is a relationship between substrate type and chain topology.

## <u>Aims</u>

To identify the substrates of various ubiquitin chain topologies by designing mutant ubiquitins which contain a single lysine residue, therefore can only be incorporated in single chain types. To do this, we

i) Clone the gene in E.coli to express and purify our mutant proteins.

ii) Add the purified mutant ubiquitin to plant cell extract for in vitro chain formation.

# **Method/description of work**

Expressing ubiquitin in E. coli-

- Mutant ubiquitin was produced by my supervisor earlier on. We use Wild Type (WT) Ubiquitin which has all linkage sites in-tact, and 7KR ubiquitin which has all seven lysine (K) (K) residues changed to arginine (R). Using this 7KR ubiquitin we would be able to observe and identify M1 linked ubiquitin chains since it can only be incorporated in that specific chain type. Both constructs contain an internal STREP II tag for purification. (Fig 1).
- We inserted the sequences of WT and 7KR ubiquitin into pET-28a plasmid backbones, then transformed those plasmids into *E. coli* strain BL21(DE3)pLysS for protein expression.
- We standardized protein induction by varying the temperature and concentration of the inducer (IPTG).

Optimisation of an *in vitro* ubiquitination reaction-To identify the substrates of different ubiquitin chain topologies, we aim to see the incorporation of mutant ubiquitin by the plant cell extract (PCE). We hypothesize that the present

WT ubqatg cag atc ttc gtc aag act ttg acc gga aag acc atc acc ctt gaa gtg gaa agc tcc gac acc att gac aac gtc aag gcc aag atc cag gac aag gaa ggt att cct ccg gac cag cag cgt ctc atc ttc gct gga aag cag ctt gag gat gga tcggcctggtcgcatccgcagttcgagaagtcggccact ttg gcc gac tac aac atc cag aag gag tct act ctt cac ttg gtc ctg cgt ctt cgt ggt ggt ttc taa 7KR ubqatg cag atc ttc gct gga aGg act ttg acc gga aGg acc atc acc ctt gaa gtg gaa agc tcc gac acc att gac aac gtc aGg gc aGg atc cag gac gga tga tcgcctggtcgcatccgcagttcgagaagtcggcc act att gc gag aGg act ttg acc gga aGg acc atc acc ctt gaa gtg gaa agc tcc gac acc att gc gag aGg acg ctt gag gat gga tgga tcgcctggtcgcatccgcagttcgagaagtcggcc act ttg gcc gac tac aac atc cag aGg gag tct act ctt cac ttg gtc ctg cgt ctt cgt ggt ggt ttc taa These are the sequences of our ubiquitin inserts: Wild type (WT) and mutant (7KR). Mutations are bolded in both WT and

7KR, showing the seven lysine's that were changed to arginine's. The strep tag (area of sequence with no spaces) is bolded in 7KR's sequence, and red in color for both sequences.

#### **Reference**

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N. and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution*, 3(3), pp.430–439. doi:https://doi.org/10.1038/s41559-018-0793-y.





# THE UNIVERSITY of EDINBURGH



ubiquitination machinery in the PCE will be able to incorporate the added ubiquitin to make ubiquitin chains *in vitro*. First we had to check the feasibility of this idea and optimize the experiment conditions.

- We added FLAG- tagged WT ubiquitin to PCE in three different conditions, containing no added enzymes, or additional E1 enzyme, or additional E1 and E2 enzymes..
- We tested the incorporation of FLAG-Ubiquitin by doing an  $\alpha$ -FLAG immunoblot.
- In subsequent reactions, we would then put STREP-tagged ubiquitin (WT and mutants) into PCE to replace the commercial FLAG-Ubiquitin..)

# Assessment of results and outcomes of the studentship

We saw the incorporation of ubiquitin increase with the addition of both E1 and E2 enzymes (Fig 2). Although we see the most incorporation with the addition of both E1 and E2 enzymes, we observed that PCE on its own can drive ubiquitination without the addition of enzymes.

We found that it was difficult to detect STREP-tagged ubiquitin in *E. coli* to confirm expression, due to the presence of ubiquitin-like proteins and endogenous sequences that exhibited similarity to the STREP tag. We tried two antibodies (FK2 and P4D1), and saw nonspecific binding in both cases (Fig 3.) Upon a BLAST search, we discovered significant similarity between our insert and endogenous *E. coli* proteins. Overall, we discovered that this experiment had potential, however we would need to improve our method of detecting ubiquitin. Once that is made possible, we will be able to implement it.

# **Further steps**

The research done this summer provides a good basis for further development of this project. We were able to transform our constructs into our plasmids and our plasmids into *E. coli*, and were able to successfully carry out an *in vitro* ubiquitination reaction using plant cell extract.

# **Departures from the original proposal**

As the STREP-tag proved unsuccessful for this experiment, we discussed using a different tag, such as polyhistidine, to visualize ubiquitin. This tag would not show too much similarity to *E. coli* endogenous sequences, allowing us to clearly observe ubiquitin, and we would be able to cleave it off later- leaving ubiquitin intact.

# Value of studentship

As this was my first lab experience, it was equally as informative as it was fun. I had a lovely time working with the people in the lab and learning about their research. I learned a lot about PCR (a lot!) and western blots, and how you can change the conditions to suit your

experiment. Not only did I learn techniques relevant to my project, I was also able to participate in other experiments of interest. I looked into the effects of DNA damage on *Arabidopsis* phenotypes in different growing conditions, and learned how to take and represent quantitative data. I got to peek into various other projects going on in the lab, and meet some incredible people. I learned how to do qPCR, agroinfiltration, seed sterilization, primer design, and so much more. Furthermore, my supervisor provided informative explanations that aided me throughout the project. My knowledge of ubiquitin and plant science has grown immensely. I miss being in the lab, but I am excited to see what I can do with everything I learned this summer! The work performed supported an ongoing PhD project. The generated clones of a protein of interest for the lab will be used in further experimentation to discover the functions of various ubiquitin chain topologies.

Savary, S., Willocquet, L., Pethybridge, S.J., Esker, P., McRoberts, N. and Nelson, A. (2019). The global burden of pathogens and pests on major food crops. *Nature Ecology & Evolution*, 3(3), pp.430–439. doi:https://doi.org/10.1038/s41559-018-0793-y.





Western blot showing the incorporation of ubiquitin in 4 different conditions: negative control (no ub added), Ub (no added enzyme), Ub+E1 (added E1 to PCE), Ub+E1+E2 (added E1 and E2). Blue arrows denote ladder. Ub+E1+E2 showed the highest binding capacity



Fig 3. Western blots

Western blots of induced (Ind) and uninduced (UI) cultures. We took samples from supernatants (SN) and pellets (PEL). Although P4D1 showed less nonspecific binding than FK2, we were not able to identify ubiquitino in these blots.



# Targeting a Bioactive lipid in PI3K-induced Oncogenicity

Student: Patricia Jean Eror Barnes



Patricia Jean Eror Barnes and Dr. George Poulogiannis

#### **Introduction and Aims**

LPA, a mitogenic lipid species overproduced in cancers, is important for wound healing and chemoattraction and has been associated with all ten hallmarks of cancer<sup>1,2</sup>. LPA has been claimed to activate oncogenic PI3K signalling, however due to its complex synthesis and metabolism, it has been difficult to target therapeutically (Fig. 1). Mutations in the PI3K pathway are found in over 35% of cancer cases and mainly target the p110 $\alpha$  isoform of PI3K, PTEN or AKT<sup>1</sup>. While it has been an appealing therapeutic target, pharmacological inhibition of this pathway has been constrained due to acquired drug resistance and toxicity<sup>3</sup>. In certain cases, synergistic treatments with diet and drugs can be potentially more effective than drugs alone <sup>4</sup>. The aim of this project was to investigate the relationship between these two pathways and capture metabolic vulnerabilities that could be utilised in a therapeutic setting to combat PI3K pathway mutant cell lines.

#### **Material and Methods**

**Cell culture:** MCF-10A breast epithelial cells with either wild-type (WT) or mutant (MUT) *PIK3CA* (E545K or H1047R) and/or WT or MUT AKT (E17K) were maintained in Dulbecco's Modified Eagle's Medium/F12 prepared following previously established protocols<sup>5</sup>. 24-hour post-seeding, media was changed to Fatty Acid-Free (FAF) media.

ELISA: Following seeding of MCF-10A cells in culture medium for 24 hours, the cell monolayer was rinsed with PBS and the media was replaced with fatty acid free (FAF) media containing  $\omega$  - 6:3 fatty acids (FAs) in ratios of 1:1, 1:2, and 10:1. using docosahexaenoic acid (Sigma eicosapentoic acid (Sigma #E2011) and #53171), arachidonic acid (Sigma #10931) Quantification of LPA was carried out 24 hours post-media change with an ELISA Kit (Cloud Clone Corp, CEK623Ge) according to the protocol. manufacturer's Statistical analysis was performed using two-way ANOVA in GraphPad and Excel.

• Supervisor: George Poulogiannis

**Spheroids:** 1000 cells were seeded in low-adherence 96well plates and centrifuged for 5 min at 1,200 rpm. Spheroids formed at 37°C over a period of 3 days. On day 3, Matrigel (Corning, 354230) containing 23 $\mu$ M LPA (Sigma, L7260) was placed on top of the spheroid. Photographs were taken every 24 hours. ImageJ software was used to quantify invasion. Data were analysed in Excel.



Figure 1. Overview of LPA synthesis. PC: phosphatidylcholine, LPC: lysophosphatidylcholine, MAG: monoacylglycerol, PS: phosphatidylserine, PI: phosphatidylinositol, PE: phosphatidylethanolamine, G3P: glycerol-3-phosphate, PA: phosphatidic acid, DAG: diacylglycerol. Enzymes include ATX: autotaxin, PLA1/2: phospholipase A1 and A2, sPLA2: secretory phospholipase A2, PLC: phospholipase C, DAGK: diacylglycerol kinase, PAP: phosphatidic acid phosphatase, cPLA2: cytosolic phospholipase A2, iPLA2: independent phospholipase A2, AGPAT: acyl-glycerol-3-phosphate acetyltransferase, LPA1: 1-acyl-glycerol-3-phosphate acetyltransferase, LPP1-3: lipid phosphate acetyltransferase. Image generated in BioRender.

#### Results

A 2:1 ratio of  $\omega$ -3 to  $\omega$ -6 fatty acids reduces LPA levels in *PIK3CA* mutant cells. PLA<sub>2</sub> enzymes in the LPA synthesis pathway show phospholipid substrate specificity-dependent composition of the sn-2 position of the phospholipid<sup>6</sup>. To measure LPA levels in response to fatty acid availability, *PIK3CA* isogenic mutant cells were grown in FAF media containing ratios of 1:1, 1:2 and 10:1  $\omega$ -6 to  $\omega$ -3 fatty acids. Interestingly, *PIK3CA* mutant cells exposed to media with a ratio of 1:2  $\omega$ -6 to  $\omega$ -3 FAs showed a reduction in extracellular LPA levels equivalent to the levels observed in WT cells (Fig. 2).



Figure 2. 1:2 ratio of omega-3 to omega-6 shows a reduction of LPA levels. Extracellular levels of LPA in MCF-10 mutagenic cell lines \* = P < 0.05, \*\*\* = P  $\leq$  0.001, \*\*\*\* = P  $\leq$  0.001 The data above was analysed using two-way ANOVA and multiple comparisons test.

**LPA enhances invasion** To investigate the effect of LPA on cell invasion, matrigel-embedded spheroids of *PIK3CA* isogenic MUT cells were treated with LPA. Interestingly, LPA treatment enhanced the invaded surface area of the cells, with the difference being more prominent in *PIK3CA* H1047R- and AKT E17K- MUT cells (Fig. 3A-B).



**Figure 3. LPA enhances matrigel invasion of MCF-10A isogenic cells**. (A) MCF-10A *PIK3CA WT*, E545K H1047R, AKT WT and E17K spheroid growth 5 days after LPA treatment. (B) Representative images of spheroids at day 0 and day 5.

## Discussion

In this study, we showed that elevated levels of LPA observed in the context of hyperactivated PI3K/AKT signalling can be rescued when cells are cultured in media with a 1:2  $\omega$ -6:3 FA ratio. The tested ratios align with profiles of modern diets representing balanced (1:1), healthy (1:2) and a conservative representation of the current Western (10:1) diet<sup>7</sup>. This suggests that LPA synthesis in *PIK3CA* MUT cells has a dependency on the profile of FAs available to the cell (Fig. 2).

To investigate if elevated LPA influenced cells phenotypically in the context of PI3K/AKT signalling, we performed an invasion assay. All cell lines tested exhibited greater invasion capacity into the matrigel following LPA treatment with a more prominent increase observed in *PIK3CA* MUT lines (Fig. 3).

This experiment falls in agreement with the established role of LPA as a potent oncometabolite that induces tumour cell aggressiveness. Further experiments are required to verify the functional significance of our findings *in vivo*.

Overall, it has been shown that LPA levels in *PIK3CA* MUT cells are influenced by the omega 6:3 FA ratio, and increased LPA abundance promotes oncogenic invasion. Targeting oncogene-induced overgrowth is challenging, but it is tempting to speculate that pharmacological inhibition of the PI3K/AKT pathway with simultaneous deprivation of LPA precursors could be the key to improving therapeutic outcomes in PI3K/AKT-mutant cancers.

# Departure from original Proposal

Phosphatidylcholine, a precursor of LPA, was originally to be tested in the context of *PIK3CA* mutation. Instead, experiments focused on the role of omega 6:3 fats to align with ongoing work within the lab.

# **Future Directions**

Further investigation of LPA synthesis in response to changes in signalling and diet will continue to determine the mechanistic relationship between oncogenic PI3K/AKT signalling and LPA metabolism and help to identify novel metabolic vulnerabilities for PI3K/AKT-mutant cancers.

# Value of Studentship

**To the student:** I am grateful for this studentship for the invaluable opportunity it gave me to work in the highly collaborative Poulogiannis' Signalling and Cancer Metabolism lab at the ICR. It was an honour to work in this world-class facility with experts in their field. I look forward to using the biochemical and molecular techniques I learned here in my future research career.

**To the lab**: Patricia has been an exceptional student and it was great to see her enthusiasm and drive for research. The data that she generated will undoubtedly help with further elucidating the functional significance of PI3K pathway-induced metabolic reprogramming.

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# Investigating interactions between the BRCT Domain of BRCA1 & Affimers

Student: Tharushi Wijesiriwardena Supervisor: Dr Qian Wu, Mr William Wilson

Hereditary breast and ovarian cancer (HBOC) occurs as a result of germline mutations to the tumour suppressor gene BRCA1 (Breast Cancer Suscpetibility Gene 1). In DNA damage response pathways, BRCA1 maintains genomic integrity through its interactions with mediators and effector proteins, thus facilitating the recognition and repair of DNA damage (Leung and Glover, 2011). Such interactions occur via the BRCT domains, present on the C-terminal of the



Fig.1 **Structure of BRCA1** The tandem C-terminal BRCT domain (the region of interest for this project) depicted has several tumour suppressive functions. Image obtained from Werner (2022).

protein. Characterising these interactions has become increasingly important in understanding overall BRCA1 function. Binding to BRCT may provide a novel therapeutic approach that can not only contribute towards modulating the physiological roles of BRCA1, and thereby improving patient outcomes, but also improving understanding of the protein's interactions and functions outside of HBOC. In this project, small, highly-specific stabilising molecules, called Affimers (~12 kDa) were used to target various regions of the BRCA1 BRCT domain, in order to characterise interactions. In doing so, this research will advance current understandings of the function of BRCA1.

# AIMS

- 1) Express the target proteins, BRCA1-BRCT and selected Affimers from bacterial cells.
- 2) Purify samples using nickel affinity columns and size exclusion chromatography.
- 3) Characterise interactions of the BRCA1-BRCT and selected Affimer using a biophyscial assay (isothermal titration calorimetry, ITC)

# METHODS\_

Previous research conducted by the lab confirmed the association of BRCA1-BRCT with 14 Affimers. One of these Affimers was selected for investigation due to its promising results in preliminary studies. Pet11 plasmid containing the selected Affimer and pHAT2 plasmid containing His-BRCT DNA was transformed into BL21(DE3) cells. IPTG (isopropyl  $\beta$ -d-1-thiogalactopyranoside) was used to induce expression. Cells then underwent lysis and nickel purification. BRCT was concentrated using a Vivaspin column and both samples then underwent S75 Size Exclusion Chromatography (SEC) and dialysis. ITC was conducted on a MicroCal ITC200, testing Affimer samples pre- and post-gel filtration. Alongside this, primers were designed for mutant BRCT and then used to transform pHAT2 mutated His-BRCT into DH5 $\alpha$ .

# RESULTS



Fig.2 SDS-PAGE of Flow through & Elutions post-Nickel purification. (1) 1kB DNA ladder, (2) Affimer flowthrough, (3) Affimer Wash, (4-6) Affimer Elutions 1,2,3, (7) ladder, (8) BRCT flowthrough, (9) BRCT Wash, (10-12) Elutions 1-3. Following Ni-affinity, an SDS-PAGE gel was conducted to confirm whether the proteins of interest had been eluted (Fig.2). Using SEC chromatograms, chosen samples were dialysed for ITC (Fig.3). The pre-Affimer+BRCT had aggregated, and thus could not confirm protein-protein interactions. Similarly, the post-Affimer+BRCT sample had an endothermic signal, suggesting multimerisation or a change in conformation. When mapping these results to the preliminary results conducted by a previous student, there was a distinct difference in binding interactions, suggesting that further repeats should be conducted to better characterise the interactions between BRCT and the selected Affimer.

The designed primers were used in a gradient PCR, with ranging annealing temperatures. Proteins showed multiple banding and low expression (Fig.4) resulting in no colony formation. Therefore primers were redesigned (elongated by several bases either side) for future use.



Fig.4 Agarose Gel of PCR products. From left to right: P+ ladder, (1-7) PCR products with varying annealing

# OUTCOMES OF THE STUDENTSHIP

These findings contradict the previously demonstrated interaction between the selected Affimer and the BRCT-BRCA1 domain. Due to low sample size, the previous experiment was conducted only once, and the additional data gained during this project will enable future researchers working on BRCT-BRCA1 and Affimer interactions to gain a better awareness of the range of possible interactions. Due to these unexpected outcomes, I have developed my ability to troubleshoot experiments, learing how to critically analyse methodologies, identify potential isses and systematically explore alternative approaches.

# DEPARTURES FROM ORIGINAL PROPOSAL

Due to the unexpected outcomes observed during this project, many experiments were conducted to repeat and confirm unexpected results and to investigate why such results occurred. As we were unable to characterise interactions between the BRCA1-BRCT and selected Affimer through the ITC, I did not continue the project to characterise the interactions of BRCA1-BRCT with the 13 other Affimers. Furthermore, I was not able to learn X-Ray crystallography due to insufficient quantities of protein sample.

# VALUE OF THE STUDENTSHIP TO THE STUDENT AND THE LAB\_

The studentship significantly contributed to the expansion of my technical and transferable skills. I became proficient in various wet lab techniques, such as protein expression and purification, PCR, and training on how to use an AKTA and ITC. Furthermore, I gained a comprehensive understanding of experimental design and execution, teaching me the importance of planning and understanding protocols. The challenging nature of the project also taught me perseverance and adaptability, both skills that I wish to develop further during a PhD. I was well-integrated into both the Wu lab and The Astbury Centre for Structural Molecular Biology, which the Wu lab is part of, enabling me to form meaningful working relationships with both members within and outside of the lab. Through this, I was able to shadow cryo-EM sample preparation and given the opportunity to attend weekly electron-microscopy based seminars delivered by PhD students. Also, I attended the 2023 Astbury Away Day, where I listened to numerous talks and poster presentations related to structural biology, furthering my interest in the research area, and diversifying my understanding of the range of projects available to pursue through a PhD.

# FUTURE DIRECTIONS OF THE PROJECT

This project will be taken over by the new PhD student in the lab, who will have a much longer period with which to characterise interactions of BRCA1-BRCT with a range of Affimers, using multiple biophysical techniques. Additionally, the newly designed primers will aid them to produce mutant protiens, to compare against interactions of the wildtype. Ultimately, such research will further the understanding of BRCA1 and its role in cancer proliferation.

Fig.5 Lab Work and Members The first panel is the student conduct transformations, the second panel shows the student operating the AKTA during size exclusion chromatography and the third panel shows the members of the lab (from left to right: Rouyi Guo (the previous masters student), Tharushi Wijesiriwardena (summer student), and William Wilson (PhD Supervisor).



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Investigating the effect of extracellular pH on the mechano-response of cardiac fibroblasts



Student: Ria Hunt Supervisor: Dr Hamish Gilbert

# Introduction

Cardiovascular diseases are the primary cause of death worldwide, causing 17.9 million fatalities per year. (World Health Organisation, 2022) Cardiac fibroblasts are a crucial component of the heart, as they oversee myocardial development and maintain homeostasis. When cardiac fibroblasts malfunction and differentiate into myofibroblasts, they trigger a condition called cardiac fibrosis. Cardiac fibrosis refers to the scarring of the heart muscle, which can lead to heart failure and cardiac dysfunction. There are no known treatments for cardiac fibrosis currently, emphasising the need to discover novel therapeutic options to treat this condition.

Studies have shown that as cardiac fibroblasts differentiate into myofibroblasts, the stiffness of the extracellular matrix increases due to an elevated deposition of collagen type I. (Hinderer and Schenke-Layland, 2019) Mechanical properties of cardiac tissue alter in response to changes in the chemical microenvironment during ischemic heart disease. One component known to vary is tissue pH, which falls from 7.4 in healthy tissue to 6.8 in diseased tissue. However, it remains to be determined how this pH decrease affects the ability of cardiac fibroblasts to adapt to changes in tissue stiffness.

# Aims

The overall aim of this project was to investigate how mouse cardiac fibroblasts respond to shifts in extracellular pH, which leads towards discovering a method to revert the diseased tissue back into healthy tissue.

# Method

Cardiac fibroblasts were extracted from mice and cultivated on six plates of type I coated polyacrylamide gels at stiffness levels of 8kPa and 50kPa, replicating healthy and diseased tissue. The cells were cultured in pH modified medium between pHs 6.3 and 7.3 for several days. Two plates were fixed in situ for confocal microscopy, while the remaining four plates were used for RNA extraction. The RNA was extracted from the cells and used to obtain cDNA via reverse transcriptase. The cDNA was subsequently diluted to 5ng/µl and combined with fibrotic markers to make a reaction mix for real-time PCR, using the SYBR Green assay. Gene expression of the fibrotic markers was quantified, with PPIA functioning as a housekeeping gene for comparative analysis. The two plates used for confocal microscopy were first stained with antibodies so that a-SMA, DNA and the F-actin cytoskeleton, could be visualised.

# Outcomes of the project



**Figure 1. Gene expression of MMP2 and MMP9 relative to PPIA and normalised to pH 7.3.** Once the Ct values were obtained from real-time PCR, they were converted into 2-ddCt results and logged for gene expression comparisons. Both MMP2 and MMP9 had an increased expression at pH 6.7 compared to the control of pH 7.3. MMP2 was expressed less at pH 6.3 than pH 7.3.

MMP2 and MMP9 are associated with collagen degradation, which rises during cardiac fibrosis. At the lower pH 6.7, both MMP2 and MMP9 have a higher expression compared to pH 7.3, indicating that they are activated during fibrosis to respond to the increased collagen levels. Conversely, MMP2 exhibits reduced expression at pH 6.3, suggesting that further pH reductions result in MMP2 having lower activity.



Figure 2. Gene expression of TCF21 and DLK1 relative to PPIA and normalised to pH 7.3. DLK1 was expressed higher at pH 6.7 than pH 7.3. TCF21 had a significantly lower expression at pH 6.3 compared to pH 7.3.

TCF21 regulates the development of cardiac fibroblasts, but its expression decreases as the cardiac fibroblasts differentiate into myofibroblasts at lower pH levels. DLK1 showed a higher expression at pH 6.7, implying that it is expressed more in myofibroblasts. However, contrasting studies suggest DLK1 inhibits cardiac fibroblasts from differentiating into myofibroblasts. (Troncone et al., 2017)



Figure 3. Gene expression of TNC, FN1 and PXN relative to PPIA and normalised to pH 7.3. Compared to the control of pH 7.3, TNC had a lower expression at pH 6.7 and higher expression at pH 6.3. Both FN1 and PXN had lower expressions at pH 6.3. PXN was not expressed at pH 7.3 so was normalised to pH 6.7.

TNC is a regulator for controlling inflammation and tissue repair, so is expressed highly in myofibroblasts. Although expression for TNC was lower for pH 6.7 compared to the control, it increased for pH 6.3. FN1 is crucial for collagen deposition and contributes to the differentiation of cardiac fibroblasts to myofibroblasts. Despite its higher expression in myofibroblasts, the data found that FN1 decreased in expression at pH 6.3. PXN functions as a focal adhesion adaptor protein responsible for recruiting signalling proteins and was only exhibited at lower pH levels. This indicates PXN is upregulated in myofibroblasts, implicating its involvement in one of the signalling pathways during cardiac fibrosis.



Figure 4. Expression of  $\alpha$ -SMA from cells cultured on soft (8kPa) and stiff (50kPa) plates in pH 6.3 and 7.3 modified media. The plates were stained with antibodies and viewed under a confocal microscope under the TXRED filter. The CTCF value was calculated for each well and used to compare the expression of  $\alpha$ -SMA, with the results normalised to pH 7.3 on the soft gel.

The protein  $\alpha$ -SMA is found in myofibroblasts but not cardiac fibroblasts. The expression was highest in cells cultured on stiff gel at pH 6.3, mimicking diseased tissue. Surprisingly,  $\alpha$ -SMA was also expressed highly in cells cultured on soft gel at pH 7.3, which emulates healthy tissue. Despite this, the data does indicate that pH influences the stiffness response.

# Impacts of the work

The group will continue to work on understanding the mechano-biology of cardiac fibroblasts. The expression of additional fibrotic markers will be investigated at different pHs in order to develop a further understanding of how extracellular pH impacts cardiac fibroblasts. The outcomes from this project will contribute towards the ongoing pursuit of a treatment for cardiac fibrosis.

Furthermore, research from this studentship contributes to the broader interests of the molecular bioscience community, aligning with the Biochemical Society's strategy. Over the last two decades. mechanotransduction has been a topic of interest to researchers and is involved in numerous biological responses. Less is known of how chemical pathways influence mechanotransduction, called mechanochemical signalling. We investigated how both extracellular pH and substrate stiffness influence the mechano-response of cardiac fibroblasts, utilising a combination of qPCR and confocal microscopy to assess cellular changes and measure gene expression. This enables further research into how different chemical pathways affect mechanotransduction. The results of this experiment are applicable beyond cardiac biology because factors such as ageing and disease can alter the pH and mechanical properties of numerous tissues.

# Subject-specific and transferable skills gained and contribution to future career plans

Working on this project has provided me with invaluable work experience and allowed me to learn and develop different skills. Among the new laboratory techniques that I learnt were RNA extraction, RNA reverse transcription, qPCR, preparing pH modified culture media and confocal microscopy, which has significantly prepared me for my final year project at university. The experience has helped me to develop my critical thinking and data analysis abilities, particularly in the context of interpreting real-time PCR results and drawing meaningful conclusions. Working in a group has boosted my confidence and taught me how to effectively communicate within a research team. Additionally, I learnt how to manage my time effectively and how to maintain organisation when independently culturing cells in the lab. I really enjoyed the experience gained from this studentship and this has reinforced my desire to pursue a PhD in biochemistry.

#### Acknowledgements

I would like to thank Dr Hamish Gilbert and the team for this valuable opportunity. I would also like to thank the Biochemical Society for their support over the 6 weeks.

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# **Biochemical Society Student Report**

# Aims of the project

Over the summer, I completed my Internship at the University of Bristol's Griffiths Lab. The overarching aim of this research group was to answer how soil biota diversity and the interaction of below and above ground organisms influences forest functioning and regeneration in Old-growth European forests. Within this expansive project, my aim was to aid with their investigation by mapping the interactions in soil biotic communities within the scale of the microclimate in their field plots; as well as looking at the link Carbon sequestration. This was done with the purpose of establishing a higher resolution insight into what factors influence Carbon sequestration in microclimates, which is on a smaller scale than previous work done. This was to address gaps in knowledge that underpin forest restoration strategies.

# Summary of the work undertaken

To help investigate this question, I first undertook a variety of laboratory techniques to capture the heterogeneity in the soil biota. From each field plot, five eDNA samples were taken from each randomly allocated microclimate measurement stations. I helped to freeze dry these samples to homogenize the soil before eDNA sequencing was used to find what species were present and to construct the soil food web within the microclimate. This method was used because it captured the DNA from all organisms that had been to the site and left DNA. However, the drawback of this technique is that the biomass of each trophic level cannot be found, so we used three other techniques to determine this.

To uncover the biomass of the microfauna, mesofauna and macrofauna present in each of the plot's microclimates, I helped to do perform Nematode extractions, Tullgren's and looking at species collected in pitfall traps. This allowed us to compare the eDNA results and gave us insight into the biomass of the species collected.

I also helped to take a host of Abiotic measurements to determine C stocks in the soil, which could then be used later in data analysis to determine links between the C stocks and soil biotic diversity. I also determined the amount of microbial carbon stored in the samples by performing chloroform extraction techniques in the lab, which bursts the microbial cells so you can measure the carbon in the soil before and after the technique. Soil moisture content was measured by drying the soil to see the change in weight. I was also able to witness demonstrations on taking the pH of the soil samples.

Another aspect that was investigated was characterizing the understory vegetation present. This was done to investigate if the biomass or species of leaf litter collecting on the ground was influencing decomposition processes and carbon sequestration. To do this, we collected leaf litter falling from the canopy in each plot was collected in nets. The biomass of each species of tree species leaves was then determined. This was done to aid the lab in assessing the relative importance of soil biotic, abiotic factors and vegetation composition in determining diversity-productivity relationships.

# Description of results/ outcome of the project and how this will be taken forward by the research group.

The work I did will be used in analysis by other members of the research group to test their hypothesis that: the community composition of soil organisms explains a substantial proportion of the unexplained variation in forest diversity-productivity relationships and is positively associated with carbon storage. This analysis is being undertaken once they finish the rest of their lab-based experiments later this month, as they also desire to measure Nitrogen, Phosphorus, soil bulk density and organic matter content of their samples as factors, which was not possible to do in the period of my project. However, the work I did will be an integral part in their study and will contribute to their findings at the end of their project. They will use the information I have collected alongside these other factors to determine how species interactions in the microclimate scale can influence carbon stocks.

# Consideration of the Impact of the work/results

One of the primary aims of this research group is to address gaps in knowledge regarding mechanisms that underpin successful forest restoration. This group's work will help produce more effective policies and implement forest restoration projects of old-growth European forests. The group has support from the groups Woodland Trust and Natural England, demonstrating its relevance. This is in line with the Biochemistry societies ethos, as it promotes sharing new knowledge and research that will be used with other groups to promote effective policies. It also will encourage a wider dialogue in the field as this work collaborates with many other research labs.

# Discussion of the subject specific transferable skills gained and its contribution to further career goals

The support offered by this grant has allowed me the opportunity to engage with experienced scientists, in a research group with a demonstrated record of high quality and significant research on the scientific community. The studentship has also enabled me to make long-lasting connections in the biochemical field and has given me unique and rare insight into the process of research. Working in an environment that was previously unfamiliar for me has improved my resilience and adaptability to new events and has challenged me to become a more pro-active learner to face many different tasks. I have gained many transferable skills to take forward into my future career, such as working within a team to achieve required targets and conducting myself in the most professional and appropriate manner. I would like to thank the Biochemistry society for the chance to gain experience in biochemical research and I hope this experience will enhance my eligibility for jobs on the graduate market.

# Acknowledgements

I would like to express my gratitude towards my PI, Hannah Griffiths as well as the other members of the Lab: Holly Langridge, Lais Ferreria Maia and Emily Bingham.



# Investigating growth factor-specific PI3K signalling activation in a PIK3CA-H1047R HeLa cell disease model

Student: Oliwia Mruk; School of Life Sciences, University of Dundee

Supervisor: Dr Ralitsa Madsen; MRC-PPU, School of Life Sciences, University of Dundee

# Introduction

The phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) pathway is crucial for many cellular processes. Although the hyperactivation of this pathway is the cause of many cancers and developmental disorders, known as PIK3CA-related overgrowth spectrum (PROS), the underlying mechanisms are not well understood. Development of a more comprehensive PI3K signalling map is key to addressing these gaps and, in the future, allow for the development of less toxic drug alternatives, improving patient outcomes.

This project investigated the pathway dynamics of the PIK3CA-H1047R mutation. HeLa cells containing varying doses of the mutation (WT (wild-type), 1x H1047R, 2x H1047R and inactive (introduced frameshift)) expressing the FOXO-based KTR reporter (biosensor of AKT activity downstream of PI3K) were engineered by Dr Madsen for the purposes of investigating the dose-dependent effects of the mutation and growth factor stimulation.

# Aims of the project

The project's aim was to explore the PI3K signalling pathway activation through investigating:

- if/how the identity of the signalling pathway in response to GFs (growth factors) changes in disease-causing mutations; as compared through WT (wild-type) versus CRISPR-engineered PIK3CA-H1047R HeLa cells,
- 2. what the differences between the growth factor (IGF-1 and insulin) responses involved in the pathway are.

Additionally, this project provided an opportunity to examine a new experimental setup and, thus, verify the reproducibility of the live-cell imaging workflows previously performed.

# Methods used

The methods used during this project relied heavily on tissue culture of CRISPR-engineered HeLa cell line clones, stably expressing a FOXO-based KTR reporter. All cell lines were cultured in complete medium (DMEM, with the addition of 10% Fetal Bovine Serum and L-glutamine) at 37°C and 5%  $CO_2$ . The expression of the reporter was verified using a fluorescence microscope. A genotyping protocol for phenotype verification was performed as well. Preparation for imaging experiments included:

**Day 1**: Seeding of clones (WT, 1x H1047R, 2x H1047R, inactive) 24h beforehand at 30,000 or 40,000 cells/ml in a 35mm μ-Dish (Ibidi), complete medium,

**Day 2**: Starvation of cells 2h before experiment commencement using FluoroBrite<sup>™</sup> DMEM (with the addition of L-glutamine and Penicillin-Streptomycin).

The computational methods relied on the use of Fiji, Matlab, Python and R; pipelines previously developed by Dr Madsen and collaborator Dr Alix Le Marois (Francis Crick Institute).

# Results and outcomes of studentship

By the end of the studentship, 3 datasets, comparing IGF-1 and insulin, using widefield fluorescence microscopy were generated. The results were quantified using the aforementioned analysis pipeline and the generated results revealed:

- 1. differences in signalling responses to IGF-1 and insulin,
- 2. observed blurring of response in H1047R clones,
- 3. the experimental design is reproducible across different microscopy setups.



Figure 1. Images of WT HeLa cell responses to IGF-1 at A. time point 1 (baseline), B. time point 16 (60 minutes after the addition of IGF-1), C. time point 36 (60 minutes after the addition of alpelisib, alpha-specific PI3K inhibitor), experimental run two

# Departures from original plan

While the original plan was the measurement of PIP3/PI(3,4)P2 upon growth factor stimulation using total internal reflection fluorescence (TIRF) microscopy, we experienced some technical issues with the setup. Although some images were produced, they were not sufficient for analysis. All subsequent experiments were shifted to single-cell measurements of AKT activity through the use of a KTR reporter, mentioned in the Methods section. The comparison of growth factors, due to time limitations, included IGF-1 and insulin, instead of the previously proposed EGF versus epigen.

# Future directions in which the project could be taken

My project focused on comparisons of signalling dynamics downstream of the receptor tyrosine kinases' (RTKs) ligands, IGF-1 and insulin. The next, crucial step in understanding the complex spatiotemporal dynamics of PI3K signalling is the investigation of PI3K activation upon concentration changes of these ligands. In order to construct a comprehensive PI3K signalling map, the engineering and testing of further disease models is necessary; an example being the engineering of the E545K mutation in HeLa cells. Further cell models better mimicking the conditions present *in vivo* can also be considered for future work.

# Value of studentship

Working under the supervision of Dr Madsen has been an unforgettable educational experience. I got to practice my existing skills in the lab and learn entirely new procedures, including tissue culture with HeLa cells and iPSCs (induced pluripotent stem cells), genotyping and running microscopy experiments, following best practice. The studentship was also a unique opportunity for me to improve computational biology skills alongside my work in the lab, allowing me to better prepare for my final year Honours project and further opportunities. Additionally, Dr Madsen's hands-on approach gave me an insight into the work of a researcher; this included management of the lab, meetings with collaborators, research paper preparations and PROS community engagement. The studentship proved very beneficial to the Madsen Lab as well; the acquired data will ultimately contribute to the generation of PI3K dynamic signalling maps required for the development of predictive models of the pathway input-output relationships. My PI3K/AKT signalling diagram will also be featured on the lab's interactive website.



Figure 2. The Madsen Lab members; Grey Yin (helping visiting student), Dr Ralitsa Madsen (supervisor) and Oliwia Mruk. From left to right





Figure 3. Oliwia's achievements after the 6-week Biochemical Society research studentship, generated by Dr Madsen




SerpinA12 (vaspin) inhibits enzyme Kallikrein 7, which degrades growth factor Midkine in melanoma

**Student:** Adomas Liugaila; **Supervisor**: Dr Chris Morris

#### Introduction

SerpinA12 (vaspin) was identified as a new biomarker in melanoma during a phage display screen against metastatic melanoma cells by the Morris lab. The group has since worked on elaborating the role of vaspin and has identified the key cell signalling pathway. Recent *in vivo* findings have supported the role of vaspin in promoting metastasis through a canonical serpin mechanism that involves the blockade of kallikrein 7, which is known to degrade the recently identified pro-metastatic factor, midkine (Fig. 1).



**Figure 1.** *Hypothesized pathway in melanoma*. Increased levels of vaspin inhibit KLK7, which usually degrades MDK. Nondegraded MDK then induces MTOR pathway, which induces VEGFR. As a result of VEGFR, metastasis in lymphatic endothelia increases.

#### Aims

- 1. Confirm that Kallikrein 7 (KLK7) degrades Midkine (MDK);
- 2. Identify if SerpinA12 (vaspin) inhibits Kallikrein 7 (KLK7);
- 3. Discover whether the inhibitory serpin activity of vaspin on KLK7 results in the subsequent increases in the levels of pro-metastatic MDK.

#### Methods

- 1. *SDS-PAGE* used to test MDK degradation by KLK7 and KLK7 inhibition by vaspin. *Coomassie stain* and *Silver staining* techniques used;
- 2. Western blot used after 1014 melanoma cells transfection in order to overexpress KLK7 to show MDK degradation
- 3. Fluorogenic enzyme kinetics assay used to determine whether KLK7 activation by Thermolysin was successful;
- 4. Mammalian cell culture and transfection;
- 5. Bacterial cell culture and plasmid transformation in order to insert KLK7 and MDK sequences into E. coli;
- 6. *Electrophoresis* used to determine *E. coli* colonies that had the most successful transformation;
- 7. BCA assay used to determine correct protein concentrations of lysed transfected cells.

#### Outcomes

- 1. Successfully showed that KLK7 degrades MDK by SDS-PAGE
- 2. Successfully completed transformation of MDK and KLK7 in *E. coli*
- 3. Successful activation of KLK7, approved by fluorogenic enzyme kinetics assay
- 4. Successfully determined protein concentration using BCA assay later on used for SDS-PAGE and Western Blot
- 5. Maintained mammalian HaCaT and 1014 cells and completed transfection

#### Results

KLK7 degrades MDK (Fig. 2) but it was challenging to prove vaspin-KLK7 interactions because commercially sourced rh vaspin was undetectable. Complaints were made to the manufacturer and replacements tested. Regrettably, the SDS-PAGE results failed to show a clear result. In the last experiment of the placement, see that vaspin might inhibit KLK7 (Fig. 3), although the quality of vaspin seems to be quite low. While waiting for replacement rhVaspin, I transfected 1014 murine melanoma cells with a pcDNA 3.1 construct overexpressing KLK7 and MDK. The term aim of the experiment was to stable develop lines that overexpress these genes. Agarose gel was used to confirm plasmid DNA integrity before transfection. Western Blotting of KLK7 and MDK was undertaken, but results were not recorded before the end of the placement.





**Figure 2.** *MDK degradation by KLK7* at different time points from 0 to 15 min at 37 °C shown by SDS-PAGE. Degradation starts at 0 min (lane 4) and is already complete after 3 min (lane 6).



**Figure 3.** *KLK7 inhibition by vaspin* at different time points from 0 to 60 min at 37 °C shown by SDS-PAGE. Two different batches of vaspin tested: lanes 4-7 and lanes 8-10. In control lanes 3 and 8 no vaspin observed, however in lanes 4 and 9 vaspin is observed. Only in lane 9 there is a faint band of possibly KLK7 and vaspin complex at around 110 kDa.

#### **Future directions**

- 1. Confirming hypothesis of vaspin inhibition on KLK7 using quality vaspin product.
- 2. Overexpression of MDK, vaspin and KLK7 in melanoma cells and downstream analysis of cell signalling pathways in lymphatic endothelial cells e.g., mTOR pathway

#### Departures from original project plan

Hypothesis regarding inhibitory activity of vaspin could not be completely evaluated because of the quality of the protein that was delivered. I undertook some *in silico* analysis of the Cancer Genome Atlas, with the aim of identifying RNA expression patterns in clinical melanoma samples.

#### Value of studentship to the student

- 1. Laboratory techniques consolidated: SDS-PAGE, Western Blot, Fluorogenic enzymes kinetic assay, handling mammalian cell cultures, bacterial cell cultures, electrophoresis.
- 2. Data handling skills using The Cancer Genome Atlas (TCGA) Program, looking at mRNA expression regarding vaspin, MDK and KLK7 genes and correlation between these genes.
- 3. Learned to design experiments, come to my own conclusions, solve arising problems from various experiments troubleshooting.
- 4. Teamworking skills, as well as data presentation skills during meetings with the supervisor.
- 5. All of the above mentioned skills are highly beneficial in the career as a scientist and prepared me for my future PhD studies or work in the laboratory in general.

#### Value of the studentship to the research group

- 1. Proof of MDK degradation by KLK7.
- 2. Parathion of MDK/KLK7 plasmids for future transfection studies.
- 3. Preliminary findings from TCGA data mining.

The aim of this research project was to understand the interactions between ribosome-nascent chain complexes (RNCs), specifically an FLN5 RNC with point mutation Y719E and a +47 nascent chain attached, and a Y719E nanobody, NB5. The project used 1H-15N HMQC NMR spectroscopy to look at the interaction between the nanobody and a 15N-labelled isolated nascent chain protein, and later between the nanobody and the +47 Y719E RNC. This was carried out to understand whether the nanobody had any effect on the folding of the nascent chain. The majority of my time at the lab was spent carrying out preparations of the RNCs, nanobodies and isolated Y719E proteins. This involved growing the proteins in cultures under different expression systems until the desired pellet could be extracted, and then purifying the resulting cell pellet in order to obtain the pure protein samples. Purification steps involved running metal affinity chromatography, size exclusion chromatography, butyl columns, sucrose cushions and physical cell lysis.

I spent the entirety of the seven weeks in the lab, doing practical work everyday. I began by recapping key molecular biology techniques such as PCR and gel electrophoresis. I was then introduced to my research project and began by focusing on purifying the Y719E FLN5 nanobody. This involved learning purification techniques including metal affinity chromatography on a nickel column and size exclusion chromatography on a Superdex 75 column. I was then supervised on my first growth prep, following a protocol for the +47 Y719E RNCs and isolated Y719E proteins. This provided lots of practice in essential microbiology techniques such as maintaining a sterile environment and preventing cross-contamination, and the process increased my confidence using different types of centrifuges independently. I then purified the RNCs and isolated proteins which allowed me to practice many techniques which I had been earlier introduced to, this time without direct supervision, and to learn some more purification techniques. These included lysis using a french press, centrifugation using sucrose cushions, nickel affinity chromatography and butyl columns, using an AKTA system, for the RNC cells and further practice with the nickel column affinity chromatography and S75 column size exclusion chromatography, also using the AKTA system, for the isolated protein purification. Once all of the required proteins had been purified, and the purification had been checked by running gels, such as SDS-PAGE and BisTris, and western blots, I analysed the interaction between the isolated Y719E protein samples and the nanobody using HMQC 2D NMR. I learnt how to prepare samples for NMR and then had the chance to observe and help with running the equipment. I was also shown how to use the TopSpin software to visualise the results. I had planned to also introduce the nanobody to the +47 Y719E RNCs but there was not enough of the RNC or of the nanobody from the purification in order to do so. Therefore, I began another round of RNC and nanobody growth and purification. However, this was a very useful opportunity to follow the protocol almost completely independently and allowed me to further practice using the aforementioned equipment and techniques.

After all of the samples had been purified, 3 samples were prepared for NMR, one with only Y719E isolated protein, one with a 2:1 ratio of isolated Y719E:nanobody and one with a 1:1 ratio of isolated Y719E:nanobody. The results from the NMR did not show any significant changes in protein folding with or without the nanobody, or between the two different nanobody concentrations. After analysing the samples with NMR, I ran SDS-PAGE gels and western blots with all the samples in order to confirm the presence and concentration of the nanobodies. These confirmed that there was no nanobody present in the isolated Y719E only sample, and that the 1:1 Y719E:Nb sample had double the amount of nanobody to the 2:1 Y719E:Nb sample. Therefore it is likely that the nanobody was present as expected but did not interact with the RNC to any significant effect.

While there were no statistically significant impacts on protein folding in this pilot study, in the future further research on the interactions between nanobodies and RNCs and their potential impact on protein folding could be carried out with other nanobodies which maybe target different parts of the RNC. Any significant impact on protein folding could then be manipulated to help prevent misfolding and aggregation and therefore could have therapeutic uses for disorders such as Huntingtons or Parkinsons. Therefore, this field of research has an important medical impact, whilst also improving the understanding of protein-folding processes, particularly whilst the nascent chain is still attached to the ribosome.

Throughout the project I was well supported by the whole group, particularly by my supervisor, and where possible I also learnt about the different research projects being carried by other members of the group. I even had the chance to observe and help with RNC growth for my supervisor's project looking at the HTT protein causing Huntington's disease. This interaction with different members of the team was an invaluable experience, allowing me to learn techniques in different ways. Being involved in such an important lab, with autonomy and independence over my own project, while still being supported and supervised throughout, allowed me to gain confidence and improve my awareness of current research being carried out and my understanding of how the research process works. This experience has improved my organisational skills, since I was often running multiple preps at the same time, and it has improved my analytical skills and time-management. This is an opportunity which I know has put me in a much better position going into the third year of my degree and especially for my Master's, and will improve my prospects when working towards a postgraduate degree and a career in research. Furthermore, it has given me a real-world perspective on the topics of proteinfolding and ribosome biochemistry, deepening my appreciation and understanding for the topic and inspiring me to continue specialising in this field.

One of the fundamental aims of this summer project was familiarizing myself with some popular molecular modelling and molecular viewing software such as Pymol and ChimeraX. Browsing through different protein structures obtained from the Protein Data Bank (PDB), I was able to view these structures primarily on ChimeraX (https://www.cgl.ucsf.edu/chimerax/) and also Pymol https://pymol.org/2/. The proper version of the latter was not available for free usage, hence I mainly decided to familiarize myself with Chimera X which remains completely free for academic use. With repetition, I was able to learn about a range of available tools and features implemented on Chimera X. This involved overlaying one protein 3D structure onto another using the matchmaker, highlighting the different domains and structures of interest and looking at specific residues for which different interactions were taking place. Earlier in the summer internship, I was also introduced to Uniprot (https://www.uniprot.org/)- a database of protein sequences, the PDB (https://www.rcsb.org/) - Protein Data Bank, as well as different databases such as the STRING and the STITCH database. Once accustomed to this as well as ChimeraX, I began looking at protein structure modelling using AlphaFold2.0. A major aim of my work was to learn the basic protein structure viewing, some relevant databases mentioned above and then apply the skill and knowledge gain to model some protein-protein interaction that is relevant from physiological and pathophysiological point of view. For this, we chose to predict the interaction between Neuropilin-1 (NRP-1) and some mutant forms of glycyl-transfer RNA (tRNA) synthetase (GlyRS) that underlie Charcot–Marie–Tooth (CMT) type 2 Diseases (GlyRS<sup>CMT2D</sup>) .

As NRP-1 binding with GLyRS<sup>CMT2D</sup> interferes with VEGF binding to NRP-1, it was essential to look at this binding in further detail. VEGF-A binds with NRP1'S B1 domain and to see this interaction we focused on the B1 and B2 domain as a comparison to see the boundaries. Uniprot sequences and previous structures of B1B2 (of NRP-1 protein) interactions with VEGFA from the PDB (2QQI,1KEX) were first gathered. Next, the sequences were inserted into Alphafold and the session was run. From this we were able to conclude that the B1B2 structure is structurally similar to the 2QQI complex. Furthermore, the B1 domain generated by alphafold was structurally similar to the 1KEX complex on the Protein data bank, this was done by matchmaker tool on chimeraX.

The first main problem was seen as with my VEGFA165 + NRP1B1B2 structure. The CRCDKPRR peptide should be into the B1 domain as seen in another model. Therefore, it was possible that alphafold needed a VEGF receptor to get the model. The B2 structure was accurate but as the peptide needed to be into the B1 domain it was not structurally accurate. We then tried Cluspro (https://cluspro.bu.edu/login.php)instead of AlphaFold. The same result was gathered with Cluspro balance as once again, the CRCDKPRR peptide was not into the B1 domain. Cluspro Vdw+et worked however as the peptide was into the B1 domain.

To investigate more into detail about NRPB1 domain with VEGFA, the VEGFA protein was truncated and tested MiniVEGFA (the peptide ERTCRCDKPRR) with B1 binding using Rosettafold, EsmFold and Alphafold. Out of Rosettafold, EsmFold and Alphafold, Alphafold was the most accurate in modelling mini VEGFA with NRP1B1 domain. In a minimalistic matter, if NRP1 is modelled with mini VEGFA, then Alphafold can make a successful model and prediction for the complex. The 7P5U structure from the PDB was used as the DRAATPHHRPQPR peptide showed to interact with the B1 domain. From this the structure was constantly remodeled adding 1 more residue each time (PQR to RPQPR to HRPQPR) to determine the specific point in which it stops working. From these last 2 experiments we can conclude that Alphafold was unable to predict a suitable structure for the smaller peptide compared with the B1 domain, however Alphafold was able to successfully predict the complex of mini VEGFA with the B1 domain.

Another task was to predict NRP1-B1 complexes with MiniWARS, T2-WARS and human tryptophan – Trna ligase using ColabFold based AlphaFold multimer. MiniWARS binding with NRPB1 stops VEGFA binding with B1 as shown by Alphafold miniwars B1 domain. NRP1 B1 interacts differently with mini WARS than with full WARS.

Finally I predicted the surface electrostatics of neuropilin 1 using APBS and predicted the conformational flexibility of proteins using Md simulation – CABSflex.

This project was important for me as I was provided with an opportunity to learn the basics of molecular modelling and molecular viewing using ChimeraX and Pymol. I was further able to learn how to predict different protein protein complexes and do protein structure modelling using Alpha Fold, Rosetta Fold, ESM fold. Finally, I was shown protein-protein docking using Cluspro and ZDOCK. This project provides societal benefit as different types of drugs such as peptidomimetic drugs could be developed to stop the unwanted binding and interaction of NRP-1 with mutant GlyRS<sup>CMT2D</sup> and therefore not preventing the binding of VEGF to NRP-1. This will, in turn, prevent peripheral neuronal degenerations associated with CMT2D. Finally, this summer internship helps the biochemical society achieve its strategies by offering flexible working models, this allowed me to complete a lot of the summer work from my remote work location at home. Furthermore, the internship provides inclusivity and diversity and finally an engagement experience with the community.

With the skills generated through this internship, I am able to create and predict protein protein interactions and generate different models for them which could be used in a scientific research paper or journal. Through this internship, I am currently working with the BMPR2 protein and trying to look at the interaction between BMPR2 and other receptors and proteins to potentially work on a research paper. Furthermore, I can potentially work with Dr Rahman to publish a paper based on our findings in the summer internship which will further elevate my research experience and lead to more opportunities in the future.



#### Student: Enzo Zanchet; Supervisor: Josh Bennett, Mark Banfield; Banfield Lab, John Innes Centre, Norwich, UK.

#### Introduction

Plants and plant pathogens live in a constant molecular arm's race. Secreted virulence proteins, named effectors, are used to manipulate the host to promote successful colonisation. By contrast, plants have cell surface and intracellular immune receptors to mount a defence against pathogen attack. The most common of which, NLRs (nucleotide-binding, leucine-rich repeat receptors) are able to detect and elicit an immune response upon detection of pathogen effectors. The effector repertoire of pathogens is highly adaptive, with mutations and deletions allowing pathogens to evade immune recognition, while NLRs also adapt to new effector variants. This area of study is important as plant pathogens cause widespread disease in certain staple crops (e.g., wheat, rice, maize, potato) resulting in significant yield loss and if left unchecked serious scarcity in food supply chains. With a growing global population to feed, it is crucial to understand plant disease so as to better engineer resistance and avoid food shortages. During this project we studied an effector from the fungal plant pathogen *Magnaporthe oryzae* that can infect a wide range of cereal crops. Intriguingly, this effector is recognised by a novel configuration of geneticially paired immune receptors in the host.

#### Aims of the project

The project aims to understand the specific effector-receptor dynamics of one of *M. oryzae* virulence proteins. To do so the project will 1) Investigate whether the effector is recognised by the paired immune receptors by observing levels of plant cell death in the model plant *Nicotiana benthamiana*. A product of successful plant immune receptor response is cell death, which prevents the spread of infection of the biotrophic pathogens that require living tissue for their life cycle. 2) Model and then experimentally determine the effector protein structure. For the latter, we express and purify the effector protein from *E. coli*, crystallise the protein, and obtain *X*-ray diffraction data.

#### Description of work

For the cell death assays, *Agrobacterium tumefaciens* was transformed with DNA constructs to enable gene delivery into plant cells (*via* agroinfiltration) where the effector and immune receptors would then be expressed. Following agroinfiltration, *N. benthamiana* leaves were left for 5 days prior to imaging cell death areas with UV light and scoring using a discrete scoring index (Maqpool *et al.*, 2015). For the protein structure studies, AlphaFold2 was used to predict the structure of the effector, but it is important to confirm any model using experimental data that can be obtained with *X*-ray crystallography. To express the effector, *E. coli* 'SHuffle' cells were transformed with the appropriate plasmid. The protein was produced in *E. coli* culture and then purified using affinity and gel chromatography. The integrity of the purified effector was confirmed with mass spectrometry. Purified, concentrated protein sample was then subjected to crystallisation experiments, set-up using a Douglas Instruments Oryx8 robot. Crystallisation experiments were monitored over the period of 1 week to assess crystal growth.

#### Assessment of results and outcome

Protein structure: the effector was successfully purified and confirmed as the protein of interest (figure 1A). The protein readily crystallised under multiple conditions over the course of 24 hours (figure 1B). The crystals formed were harvested and shipped to Diamond Light Source synchrotron and observed to diffract X-rays to a resolution of 1.2 Angstroms. However, current attempts to "solve" the structure by molecular replacement methods, using the AlphaFold2 model, were unsuccessful. Interestingly, the effector structure model shares some similarity with proteins of known structure (figure 1C), but confirmation of this requires further study.



Figure 1. The effector was purified to homogeneity and crystallised readily. A) Mass spectrometry of the purified sample shows a high peak at the effector's expected mass. B) Effector crystals from an optimisation plate following successful early crystallisation tests. C) Alphafold2 prediction of the effector aligned with another protein.

In planta cell death studies: only when paired with both immune receptors, did the effector elicit a significant level of cell death as compared to a positive control for cell death (figure 2) whose mechanism of recognition has been prepreviously established to also require these paired immune receptors.



Figure 2. The studied effector (highlighted group) produced similar cell death levels (first condition group) only when expressed together with both immune receptors.

#### Future directions in which the project could be taken

A significant next step will be to focus on solving the effector structure through experimental phasing, for example using crystal soaks with heavy atoms. Once the effector's structure is determined, comparisons can be made to other effector families to see whether this effector adopts shared fold or is novel. It is hoped that this information, through the use of additional in planta cell death assays, can inform understanding of the mechanism of recognition. Ultimately, such information may help engineer new disease resistance in crops.

#### Departures from original proposal

While not strictly a departure, I was lucky enough to be present and involved in remote X-ray data collection from the protein crystals I obtained.

#### Value of studentship to student and supervisor

*Student*: I have learned a range of lab/machine/bioinformatic techniques (agrobacteria preparation + leaf agroinfiltration, protein crystallisation, spectrophotometry, Western blotting, protein purification through affinity and gel chromatography, DNA extraction, modelling predictive protein structure with AlphaFold2, plasmid assembly in Benchling, etc) through first-hand experience, and through observing experts in the laboratory. Additionally, I got to refine my pipetting skills and revisit techniques introduced at university such as Golden Gate cloning as well as programming with R during data analysis. What I found most invigorating was the ability to perform the complete cycle of research. That is; from preparing my lab space, data collection/analysis and lastly presentation; as opposed to the "pre-digested", truncated steps I had been used to – this studentship gave me a tangible insight into the life of a wet lab scientist and inspiration to seek a plant biology module in my third year studying BSc Molecular Biology & Genetics.

*Supervisor*: The studentship with Enzo was a valuable experience for me as the direct day-to-day supervisor in the laboratory. As an early career scientist myself, this was my first time directly mentoring a student. From this experience I was able to develop my leadership and organisational skills to train Enzo to eventually become an independent and productive summer student in a short space of time to advance the project along very well. There are few short-term project students who manage to produce diffracting protein crystals in such a short time!



Figure 3. Josh Bennett and Enzo with in planta cell death assays ready to be imaged.

**Reference:** A Maqbool, H Saitoh, M Franceschetti, CEM Stevenson, A Uemura, H Kanzaki, S Kamoun, R Terauchi, MJ Banfield (2015) 'Structural basis of pathogen recognition by an integrated HMA domain in a plant NLR immune receptor', *eLife* 4:e08709.

# Investigating the effect of a novel mitophagy activator on mitochondrial function and mutant load in cybrid cell lines





Student: Alim Devecioglu; Supervisor: Professor Michael Duchen, UCL

#### Background and Aims

Approximately 1 in 4300 individuals suffer from mitochondrial DNA (mtDNA) mutations that impair functioning of the respiratory chain and present debilitating consequences, with no currently known treatments [1]. Patients carrying mtDNA mutations commonly display heteroplasmy, where they possess both mutated and wild-type mtDNA, with relative proportions defined by the mutant load. Symptoms are known to manifest once heteroplasmy levels surpass a certain biochemical threshold [2], and reducing mutant load is therefore an attractive possible treatment strategy. Notably, recent evidence has indicated an upregulation of PI3K-Akt-mTORC1 signalling in cells harbouring the m.3243A>G mutation, which was suggested to downregulate mitophagy, enabling maintenance of dysfunctional mitochondria [3,4]. Inhibition of mTORC1 signalling was shown to restore bioenergetic function and decrease mutant load [3]. This same effect of mTORC1 inhibition was not observed in m.8993T>G cybrids, suggesting that altered signalling pathways are likely mutation-dependent [3]. Therefore, compounds stimulating mitophagy may hold therapeutic potential for certain mitochondrial disorders, by mediating clearance of mutant mtDNA to a level where patients become asymptomatic.

Accordingly, we have been provided with a novel mitophagy activator, CAP-1902 from Capacity Bio. This project aims to treat cybrid cell lines displaying heteroplasmy with 3 different concentrations of this drug, verify that mitophagy has been activated, and subsequently determine whether mutant load has decreased and bioenergetic function has been rescued.

#### <u>Methods</u>

**Cell Lines:** A549 cybrids carrying the m.3243A>G (MT-TL1) mutation, and 143B cybrids (N50 and N80) harbouring the m.8993T>G (MT-ATP6) mutation were cultured in high glucose DMEM (Gibco) supplemented with 10% FBS (Gibco) and 1% (v/v) penicillin–streptomycin (Gibco), in an incubator at 37°C and 5% CO<sub>2</sub>.

**Cell Culture:** Cells were passaged every 5-7 days at 70-80% confluence, using 0.25% trypsin-EDTA (Gibco). Media was changed 3-4 times a week. A 100  $\mu$ M stock solution of CAP-1902 in DMSO was diluted in media to achieve concentrations of 20 nM, 100 nM and 1  $\mu$ M. Untreated cells were cultured in media containing a respective volume of DMSO.

**ARMS-qPCR:** DNA was extracted from generated cell pellets with DNeasy Blood and Tissue Kit (Qiagen) and samples standardized to 0.4 ng/µl for ARMS-qPCR. Master mixes were prepared with 1 µL each of forward and reverse ARMS primers (5 µM working solution) and 5 µL of SYBR Green. 7 µL of master mix and 3 µL of DNA sample was added per well in a 96-well PCR plate, with triplicates for each sample. The CFX96 Touch Real-Time PCR Detection System (Bio-Rad) was used for PCR amplification.

**Respirometry:** ECAR and OCR was measured using the XF Cell MitoStress Kit and Seahorse Bioscience XFe96 bioanalyzer. Cells were seeded in a Seahorse XF Cell Culture Microplate for 2 days. Prior to measurements, media was removed and replaced with 175  $\mu$ L Seahorse XF Base Medium (with added 10mM glucose, 1 mM pyruvate and 2 mM glutamine), then incubated for 1hr at 37°C (without CO<sub>2</sub>). A BCA assay was performed to quantify protein levels for normalisation.

**Fluorescence Microscopy:** Cells were seeded in 35 mm FluoroDishes and incubated for 48hrs at 37°C and 5% CO<sub>2</sub>. Media was replaced with 1 mL phenol red-free high glucose DMEM prior to imaging with an LSM 880 (Carl Zeiss) confocal microscope. **Image Analysis:**  $F_{543}/F_{458}$  ratiometric images were generated on ImageJ following subtraction of mean background signal.

#### <u>Results</u>

## Result 1: Upregulated mitophagy in 20 nM drug-treated m.3243A>G cybrids

A statistically significant increase in mean  $F_{543}/F_{458}$  ratio from 3.14 ± 1.68 in untreated to 4.19 ± 2.36 in 20 nMtreated m.3243A>G cybrids was observed, indicating higher number of mitochondria engulfed in lysosomes for degradation (Fig. 1). Though imaging was performed on 100 nM and 1  $\mu$ M-treated cybrids, no signal was visualized, likely due to unsuccessful transduction with mt-Keima.

#### Result 2: Increased respiratory capacity in 20 nM drugtreated m.3243A>G cybrids after 2 weeks

100 nM and 1  $\mu$ M treatments did not yield observable changes in OCR in relation to untreated (Fig. 2). Conversely, 20 nM-treated m.3243A>G cybrids displayed a significant increase in maximal respiratory capacity and respiratory reserve compared to untreated (Fig. 2f-g), indicating possible improvements in bioenergetic capability.



**Fig 1. Activation of mitophagy in m.3243A>G cybrids treated for 2 weeks. (a)** Representative images of mt-Keima emission at 458 nm and 543 nm following subtraction of mean background signal. Ratiometric images were generated on ImageJ with addition of a median filter for visualization purposes. (b)  $F_{543}/F_{458}$  ratios for ROIs were plotted and represented as mean ± SD. Data underwent two-tail *t*-test analysis,  $n \ge 27$  (\*p < 0.05).



**Fig 2. Mitochondrial respiration parameters of m.3243A>G cybrids.** Seahorse XFe96 Analyser was used to measure (**a**) oxygen respiratory capacity (OCR) and (**e**) extracellular acidification rate (ECAR) of m.3243A>G cybrids treated for 2-weeks with mitophagy activator (20 nM, 100 nM or 1  $\mu$ M) or untreated (*n* = 3 culture wells). (**b**) Basal respiration, (**c**) ATP-linked respiration, (**d**) non-mitochondrial respiration, (**f**) maximal respiratory capacity, (**g**) respiratory reserve and (**h**) proton leak values calculated are plotted, represented as mean ± SD. All data underwent one-way ANOVA analysis with Tukey's multiple comparison test, *n* = 3 culture wells, (\**p* < 0.05).

#### Result 3: Unchanged mutant load following treatment with mitophagy activator

There were no observable changes in mutant load subsequent to a 19-day treatment for m.3243A>G cybrids, and 15-day treatment for N50 and N80 cybrids (Fig. 3).



**Fig 3. ARMS-qPCR results indicating no change in mutant load following sustained treatment.** (a) m.3243A>G cybrids were treated for 19 days with mitophagy activator (20 nM, 100 nM or 1 μM) or untreated (*n* = 2 biological repeats). (b) N50 (*n* = 1) and (c) N80 (*n* = 1) cybrids underwent 15-day treatment.

#### **Discussion and Future Directions**

A sustained 2–3-week treatment with a mitophagy activator (CAP-1902) was not shown to reduce mutant load in cybrids harbouring m.3243A>G or m.8993T>G mutations. Results in the latter case are in line with previous findings that inhibiting PI3K-Akt-mTORC1 signalling in m.8993T>G cybrids does not decrease mutant load [3]. Therefore, pharmacological interventions for mitochondrial disorders will likely require targeting of mutation-specific signalling pathways. Alternatively, this could indicate that mitophagy activation alone is insufficient to clear mutant mtDNA. Interestingly, improvements in respiratory capacity were observed in 20nm-treated m.3243A>G cybrids, possibly suggesting that more prolonged exposure to CAP-1902 may be needed to see concomitant changes in mutant load. Future experiments should further investigate the potential for CAP-1902 to rescue bioenergetic function.



Fig 4. Anitta Chacko (PhD student), Michael Duchen (project supervisor), Alim Devecioglu and Gabriel Valdebenito (PhD student). From left to right.

Fig 5. Alim standardizing DNA

samples for ARMS-qPCR.

#### **Departures from Original Project**

In the first 4 weeks we treated patient-derived fibroblasts which had to be discarded, as we discovered original cell culture flasks were cross-contaminated. We moved to using cybrids due to their faster proliferation, for data collection in a shorter time span.

#### Studentship Value

**Student:** I have gained experience in performing numerous molecular biology techniques, mammalian cell culture and image analysis, which has allowed me to develop confidence in a laboratory setting that will be vital for my future studies and 3<sup>rd</sup> year project. I also cultivated transferable skills such as organisation and time management – by conducting experiments in a timely manner – and problem-solving from several cases of troubleshooting. **Lab:** The laboratory has generated preliminary data assessing the viability of a mitophagy activator as a therapeutic strategy for treating mitochondrial disorders. The improved respiratory parameters are encouraging and offer direction for future studies.



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3. Chung et al., (2021), Nat Commun. **12**(1):6409.

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#### Student: Marc Leonardo Lagraña Santamaria; Supervisor: Dr Clare F. Megarity

Introduction: The electrochemical leaf (e-Leaf) is a new electrochemical platform to drive and control enzymes using electricity in a nanoconfined space that is more like their natural environment. This is due to two key factors, the use of a photosynthetic enzyme, ferredoxin NADP<sup>+</sup> reductase (FNR) and the use of a highly porous metal oxide electrode (Scheme 1). In photosynthesis, FNR receives light-excited electrons (e<sup>-</sup>) from ferredoxin and uses them to reduce NADP<sup>+</sup> to NADPH. The NADPH is then used by the Calvin cycle, the biosynthetic enzyme cascade that makes glucose in plants. In the e-Leaf, FNR is loaded into a porous electrode and e<sup>-</sup> are provided directly to FNR's active site flavin cofactor from the electrode, by applying a potential (voltage); FNR then uses the electrons to catalyse the interconversion of NADP<sup>+</sup>/NADPH. Extended enzyme cascades are also trapped in the pores which, like the Calvin cycle use the NADPH. The cascade must contain one enzyme that needs NADP(H) to allow it to couple to FNR. In this project, first, a reductive aminase (RedAM) enzyme was coupled to FNR in the e-Leaf as an exemplar system.<sup>1</sup> Then, the bacterial enzyme, dihydrofolate reductase (DHFR), from the folate pathway was monitored in the e-Leaf. DHFR catalyses the reduction of dihydrofolic acid (DHFA) to tetrahydrofolate (THFA) using NADPH (Scheme 1).



**Scheme 1.** DHFR coupled to FNR in the e-Leaf: pore (white) surrounding bulk solution (blue).

<u>Aims of the project</u>: The aim of this project is to monitor DHFR in the e-Leaf. Preliminary work by Dr Megarity showed that DHFR behaved differently when confined in the e-Leaf electrode compared to dilute solution. My objective then was to find out why that was happening. This will help achieve the overall aim of studying the bacterial folate pathway in the e-Leaf for antibiotic resistance research.

#### **Methods**

**Expression and purification of FNR:** FNR was overexpressed in *Escherichia coli* and purified by nickel-affinity chromatography as described.<sup>2</sup> Bacterial DHFR had been prepared previously and its concentration was measured using a nanodrop spectrophotometer (280 nm).

#### Electrode Fabrication by Electrophoretic Deposition:

Conductive supports were used (indium tin oxide glass, titanium, and graphite disc electrodes), onto which particles of indium tin oxide (ITO) were deposited by electrophoretic deposition<sup>3</sup> to form the porous layer of the working electrodes.

**Enzyme Loading:** FNR and DHFR were loaded into the electrode pores by either drop casting a concentrated droplet of enzymes onto the surface and incubating for 30 minutes at 4°C or by sinking an electrode into a continuously stirred solution containing the enzymes for 30 minutes at 4°C. In both cases, before use in an experiment, the excess enzyme molecules were rinsed off so that only those in the pores remained.

**Cyclic Voltammetry:** A three-electrode system (working (ITO), counter (Pt) and reference electrode (Ag/AgCl)) was used. In cyclic voltammetry, the potential applied to the working electrode is swept linearly between two limits and the enzyme rate is directly measured as electrical current as a function of the potential, which is the driving force.

#### **Results and outcomes**

#### **Expression and Purification of FNR**

Figure 1 shows the purification gel for FNR. Lane 1 shows the overexpressed enzyme in cells and purified elutions are indicated. The enzyme was bright yellow due to its flavin cofactor, and its concentration was 0.47 mM after desalting and concentration.



**Figure 1.** Expression and purification of FNR monitored by SDS gel electrophoresis.

#### A model enzyme in the e-Leaf: Reductive aminase

Before starting the experiments with DHFR, we evaluated a reductive aminase (RedAm) enzyme as our model system since it is active in the e-Leaf and is well characterised. Figure 2 shows catalysis by RedAm coupled to FNR measured by CV. RedAm and FNR were coloaded in the pores with only the cyclopropylamine and NADP<sup>+</sup> present in the solution from the start. The black trace corresponds to the interconversion of NADP<sup>+</sup>/NADPH by FNR. The red trace shows the coupled activity of RedAm upon addition of cyclohexanone (reaction scheme shown above CV).



Figure 2. Reductive amination catalysed by RedAm coupled to FNR in the e-Leaf (red trace). 20  $\mu$ M NADP<sup>+</sup>, 20 mM cyclohexanone and 50 mM propylamine, scan rate 1 mVs<sup>-1</sup>

#### DHFR: UV-VIS and the e-Leaf

The activity of DHFR was first tested by UV-VIS spectroscopy by measuring the decrease in absorbance at 340 nm due to the depletion of NADPH. The enzyme was active, and the reaction went to completion. Different conditions were tested for DHFR in the e-Leaf including pH, enzyme loading and substrate concentration. The best activity of DHFR in the e-Leaf is shown in Figure 3 (red trace). As it can be observed, the catalytic current due to DHFR-FNR coupling is significantly lower than that for RedAm. Our current hypothesis to explain this is that DHFR's product release, which is the rate limiting step, is even more limiting in the pore environment because, once released, it stays in the pore and can bind again with DHFR.

**Future directions:** The next step will be to include another enzyme that uses the product of DHFR as substrate to prevent it from binding again to DHFR. Ultimately, the whole bacterial folate pathway will need to be studied.



Figure 3. Reduction of DHFA catalysed by DHFR coupled to FNR in the e-Leaf (red trace). 100  $\mu$ M NADP<sup>+</sup>, 0.2 mM DHFA.

**Departures from original project plan:** We did not complete the dilute solution kinetic analysis for DHFR beyond initial activity tests because we focused our work in the e-Leaf to achieve a promising result. We added the work on RedAm as a parallel study.

Value of the studentship to the student and the research group: In Dr Megarity's group, I have developed many lab skills, such as, cell culture, electrochemistry, and anaerobic glovebox work. I have also enriched many transferable skills, such as, time management, an essential skill in the lab due to the timed experiments I conducted. I also learnt how to keep detailed lab books which is essential as a scientist. As time passed, I felt more comfortable and secure in the lab. I also improved my scientific communication skills as we analysed our experiments. I also met a lot of other PI and Ph.D. students, which gave me an insight into their experience. This reassured me of my decision to end up in academic research. All the data I collected from my experiments is of significant help to Dr Megarity's group. I will help them to understand a little bit the odd behaviour of DHFR in the e-Leaf.



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Student: Marta Alonso Martínez Supervisor: Iría Medraño-Fernandez



#### Aims of the project

The main objective of the study was to characterize the internalization of the second messenger  $H_2O_2$  in the keratinocytelike HaCaT cell line.  $H_2O_2$  exerts a primordial role in proliferation, migration, and differentiation in all types of cells. However, the molecule is produced extracellularly and must then be transported back to the cytosol to reach its intracellular targets. The process is mediated by a family of channels called peroxiporins. As the work carried out in this project is framed in a wider project that aims to engineer lab-grown skin substitutes accurately mimicking the original tissue, it is paramount to characterize the redox processes that lead to the formation of a functional epithelium.

#### Methods used

Analysis of  $H_2O_2$  transport in HaCaT cells was performed by using cells stably expressing the specific and ratiometric fluorescent HyPer7 probe in its cytosol. HyPer7 has two excitation peaks at different wavelengths (488 and 405) whose ratio of emission changes depending on its state of oxidation. Thus, when expressed intracellularly HyPer7 is a reliable indicator of the kinetics of intake after exposing the cells to a controlled challenge with  $H_2O_2$ . For conducting these measurements, HaCaT-HyPer7 cells were cultured in 6-well plates onto coverslips and then analysed with live-imaging. HyPer7 fluorescent variations were followed exposing the cells to 4 different concentrations to elaborate titrated response curves:  $5\mu$ M,  $10\mu$ M,  $25\mu$ M, and  $50\mu$ M. The process was recorded in a time span of 5 minutes, where the corresponding concentration of  $H_2O_2$  was introduced in the culture at the 0-minute mark of the resulting plots (see Fig. 1). As control for probe oxidation, a pulse of 5mM of the reducing agent DTT was introduced at the 3-minute mark in the resulting plots (see Fig. 1). The response of the probe against time was obtained by computing the mean intensity of either fluorescence with ImageJ to obtain the ratio and plotted with GraphPad Prism. The percentage of probe activation that corresponds to the highest level of sensor activation under each condition was also calculated, using the highest concentration as reference value.

The identification of the peroxiporin isoforms responsible for the transport of  $H_2O_2$  in HaCaT cells was performed through silencing experiments by incubation with specific siRNAs to downregulate the production of aquaporin-3 (AQP3) and 8 (AQP8), the main representants of the functional family. In this subset of experiments there was a control group (no siRNA-treated), a group treated with the siRNA for AQP3, a group treated with the siRNA for AQP8, and a final group treated with both as control for potential summatory transport efforts. All the samples were cultured as specified before and subjected to the 50 $\mu$ M H<sub>2</sub>O<sub>2</sub> challenge while acquiring with live-imaging. The results were analysed with ImageJ and plotted with GraphPad Prism, as described above.

#### Results and outcomes

1. Definition of  $H_2O_2$  internalization curves in keratinocytes stably expressing a  $H_2O_2$ -specific ratiometric probe in the cytosol. As shown in Fig. 1, the fold change of the HyPer7 ratio (488/405nm) increased following the concentration of  $H_2O_2$  the cells were treated with. In every challenge applied we can discern a common behaviour. Once the  $H_2O_2$  is introduced in the live-imaging chamber there is a sudden and rather quick spike in the HyPer7 response, which represents the increasing uptake of the molecule by HaCaT cells. Eventually, this curve hits a plateau, arriving to a maximum. As control for probe oxidation, DTT is added at the end of the course, and as expected, the ratio abruptly decreases revealing the sharp reduction of HyPer7.



The kinetical 488/405nm ratio signal of each curve can be translated into a column graph reflecting the percentage of transport of  $H_2O_2$  into cells (Fig.2), which allows for better visualization. The highest concentration (50µM) was set as reference value. The scaled concentrations are now translated into scaled columns. Thus, we have been able to construct doseresponse plots that truly reflect the capacity of transport of our cellular model. These results will be the bases for future experiments in which physiological  $H_2O_2$  concentrations participating in fundamental cellular processes in HaCaT cells will be graduated.

Fig 2. Percentage of H<sub>2</sub>O<sub>2</sub> transport in different concentrations



2. Determination of the channel isoform(s) controlling  $H_2O_2$ fluxes in keratinocytes by silencing experiments. The results of the silencing experiments shown in Fig. 3 demonstrate that both AQP3 and AQP8 isoforms participate in the transport of  $H_2O_2$  in HaCaT cells, though with different relevance. Compared with the reference value (dark red column, labelled as 50uM in the graph), in both cases the silencing of the expression of the protein reduces the intake of  $H_2O_2$  when applying an exogenous 50µM challenge. The siRNA for AQP8 decreased  $H_2O_2$  transport by 30%, while the one for AQP3 had a higher impact decreasing intake by 60%. The differential capacity of transport reflects that the leading aquaporin in  $H_2O_2$ transport in HaCaT cells would be AQP3. The double silencing



Figure 3. Percentage of  $H_2O_2$  transport with different silencing treatments

that should have shown the summatory effect of both siRNAs was not informative in this subset of experiments, as in all cases only AQP3 was targeted by the silencing, while AQP8 was still expressed despite of addition of its specific siRNA (data not shown).

3. *Is AQP3 redox-regulated*? Previous research of our group has shown that AQP8 can be redox-regulated resulting in a complete abrogation of  $H_2O_2$  internalization (Medraño-Fernandez et al, 2016). The results reported were performed in HeLa a cellular model in which only AQP8 is a functional peroxiporin, and produced characteristic kinetic plots in which despite of the exogenous  $H_2O_2$  bolus applied there was no HyPer activation. Being an oxidative modification the



Fig. Examples of fold change ratio of HyPer? in stressed cells

culprit of channel closure, the DTT that was added at the end of the course, instead of causing a decrease in the ratio, lead to a transient spike on the probe response due to AQP8 re-opening. Interestingly, during our experiments, we have observed a similar phenomenon arising in some of our samples (Fig. 4). As in HaCaT both AQP3 and AQP8 are functional H2O2 transporters this suggests that also AQP3 could be targeted by a similar gating mechanism.

#### Future directions

Although the results presented here are promising, more replicates should be included to reduce the error bars for statistically significant results and to firmly state the role of each peroxiporin in  $H_2O_2$  internalization in HaCaT. However, the fact that both AQP3 and AQP8 are functional indicates that separated processes will be driven by each isoform. Investigating which signalling cascade is associated to each  $H_2O_2$  transporter will be of undoubted interest.

#### Value of studentship to the student

This studentship has allowed me to transfer my knowledge obtained in the classroom and apply it to the experiments and their results. It has improved my precision on performing experiments, while increasing my resilience, patience and determination when facing failures. I have gained the ability to operate complex laboratory equipment, like a Leica Dmi8 high-velocity microscope, and I have learned to predict outcomes, analyse results, and explain anomalies using biochemical logic and scientific understanding of the topic. I have thoroughly expanded my understanding on cell culturing techniques and treatments, like silencing; detection of fluorescent probes, like HyPer7; and I have learned the importance of redox signalling, and H<sub>2</sub>O<sub>2</sub> specifically, in epithelial cells. Furthermore, working as part of a research group I have been able to learn responsibility, teamwork, time management and professionalism. In this setting I have also been allowed to explore other research being carried out in the laboratory and by shadowing other team members I have been exposed to different experiments and techniques that have broaden my knowledge significantly.

#### Value of studentship to the research group

This project has aided the research group in their efforts to characterize the cellular and molecular processes involved skin tissue, which is paramount in order to engineer a functional epithelial tissue. It will hopefully be a step towards their end goal that is to provide adequate care to patients suffering from damaging skin conditions. This research also aligns with the Biochemical Society's strategy, by sharing the results with the scientific community promoting further research that will quicken the achievement and providing me with support and professional training as an early bioscientist.



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#### The Role of the SAGA Histone Acetyltransferase and Core Structural Module in DNA Damage Repair



#### Student: Ethan Sip Zi Cheng

Supervisor: Dr. Adam Wilkinson and Dr. Leonid Olender, MRC Weatherall Institute of Molecular Medicine, University of Oxford

#### Introduction

The Spt-Ada-Gcn5 acetyltransferase (SAGA) complex is a highly-conserved transcriptional coactivator, with its multiple modules each serving a distinct function. Alongside a core structural module, SAGA contains a histone deubiquitinase (DUB) module and histone acetyltransferase (HAT) module that modify chromatin and contribute to transcriptional regulation. Recent findings from the Wilkinson lab identified the SAGA subunits Tada2b (member of the HAT module) and Taf5I (member of the core structural module) as key regulators of lineage commitment and ageing in haematopoietic stem cells (HSCs) (Haney et al., bioRxiv, 2022). However, the mechanism remains unclear.

Recent findings have also shown that the SAGA DUB module affects DNA double-strand break (DSB) damage repair signalling (Ramachandran et al., 2016). When key subunits are knocked out, the loss of H2BK12Oub deubiquitylation activity will interfere with proper vH2AX formation and DSB repair. Given the high cooperation and coordination between the SAGA modules, we hypothesised that the other modules also play a role in DSB repair. Here, assays utilising immunoglobulin class switch recombination, which utilises NHEJ, act as a proxy to test for defects in DSB repair.

#### <u>Aims</u>

- Ensure that γH2AX is formed when DSBs are present in murine haematopoietic stem and progenitor cells (HSPCs).
- 2. Observe the effects of CRISPR knockout of key subunits in the SAGA HAT and core structural module on class switch recombination.

#### **Methods**

#### • DNA damage induction and HSC kill curve

Mouse bone marrow cKit+ HSPCs were extracted and cultured. The topoisomerase I inhibitor camptothecin and the topoisomerase II inhibitor etoposide were then titrated to identify the optimum concentration to cause DSBs without being lethal.

#### Intracellular Staining for yH2AX

50nM of camptothecin and 100nM of etoposide were separately added to extracted HSCs. The cells were then fixed and permeabilized before anti-yH2AX antibody was added. Flow cytometry was used to identify the population of yH2AX+ cells.

#### CRISPR knockout of SAGA complex subunits

Ribonucleoproteins (RNPs) of sgRNAs with Cas9 were introduced into CH12 (murine B-cell lymphoma) cells via electroporation. The resulting cells were serially diluted to grow single-cell clones from which we selected those with a homozygous KO genotype.

#### • PCR and Sanger sequencing for identification of homozygous CH12 knockout (KO) clones

Samples from the clonal cultures were lysed for PCR with primers to amplify the edited region. The PCR product was purified and sent for Sanger sequencing. The results were analysed using the CRISPR ICE analysis tool (Synthego). • Class Switch Recombination Assay (CSA)

Homozygous KO CH12 clones were stimulated by anti-CD40 antibody, TGFβ and IL4 to induce class switching from the isotype IgM to IgA. After ~48h, the cells were stained with anti-IgA and assessed by flow cytometry to determine the frequency of IgA+ cells.

#### **Results and Outcomes**

The yH2AX intracellular staining does show a population of yH2AX+ cells which is larger for cells exposed to 50nM camptothecin, which was initially identified as the more lethal treatment. This shows that yH2AX formation does follow induction of DSBs in HSPCs (*Fig. 1*).



Figure 1: Flow cytometry plot of FITC (conj. to anti- vH2AX) fluorescence against BV421 (conj. to anti-cKit) fluorescence. Cells untreated with DNA damaging agents did not show any vH2AX, while cells treated with camptothecin at 50nM (centre) and etoposide at 100nM (left) both showed a clear population of vH2AX+ cells.



Figure 2: CSA result which includes all obtained clones. Some clones (Rosa26 18, Tada2b 11b, Taf5l 410) show an anomalous phenotype and are excluded from analysis.



Figure 3: Final CSA results which is normalised to the control clones for Rosa26. Clones of the same gene knockout were grouped together. Anomalous results were excluded from the calculations. One-way ANOVA was run to test for significance.

CRISPR KOs were performed using sgRNAs targeting genes for Rosa26 (a genomic safe harbour, acts as a control), Tada2b (from HAT module), Taf5I and Taf6I (from core structural module). Altogether, we managed to obtain four homozygous KO clones for each gene except Taf6I, which we only derived one. Multiple CSA experiments suggested that while Tada2b KO does not show an effect on class switching, Taf5I KO likely does (*Fig. 3*). While the limited data collected for Taf6I KO does suggest a potential effect, it is currently inconclusive.

Unfortunately, after continuous passages, the CH12 cells started showing abnormal phenotypes and behaviour in culture. These clones were excluded from the data analysis.

#### **Discussion and Future Directions**

We found that the SAGA core structural module might be involved in DNA damage repair as Taf5I KO significantly impairs class switch recombination (*Fig. 3*). Considering the role of the core structural module in mediating interactions and coordination between the other modules, along with the findings from Ramachandran et al., 2016, the effect may be due to interference in the activity of the DUB module. Better understanding of the involvement of the SAGA complex in the DSB repair in HSCs will help improve the current perspective on the processes underlying HSC ageing and the development of haematological malignancies.

The emergence of abnormal phenotypes in culture was an unfortunate circumstance most likely due to a bad batch of CH12 cells that was used to generate the KO clones. The inherent genomic instability of cancer cells coupled with the harsh conditions required to form KO clones likely caused unwanted

mutations that altered the phenotype. Repeats of this experiment with a batch of early passage CH12 cells while avoiding prolonged culture will likely solve this problem.

The future of this project likely involves targeting different SAGA subunits for CRISPR KO, particularly the DUB and HAT catalytic subunits Usp22 and Kat2a. CRISPR KOs can also be done in primary HSCs, with DSB repair reporter plasmids being used in lieu of class switching. Expanding on the role of yH2AX, intracellular staining for yH2AX and H2BK120ub after CRISPR KO of key SAGA subunits would help elucidate the mechanism behind the observed defect on DSB repair.

#### Value of Studentship

**To student:** Through this studentship, I have not only learnt an immense array of experimental techniques (e.g. mammalian cell culture, flow cytometry, PCR etc.) but also the bioinformatics which precede and follow them. Furthermore, having first-hand experience in scientific research with all its ups and downs helped me hone my critical thinking to solve problems and form hypotheses. The lab was also very friendly and helped me through discussions and some great advice.

**To lab:** Hosting Ethan via this studentship was a great opportunity for our lab to contribute to the training of a new generation of scientists. It was also excellent for the



In order from left to right: Ms. Grace Meaker, Ethan Sip, Dr. Leonid Olender and Dr. Adam Wilkinson

development of leadership and mentorship skills. Ethan took responsibility for driving forward this research project, performed experiments independently and to a high standard, and made impressive progress over a short period of time. His work has helped to shape the future direction of our research into the role of SAGA in HSCs.

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Student: Karim Abdelhamid Supervisor: Claudio Raimondi

Biochemical society report

## Introduction to the Project's Aims and Objectives:

The scope of our project evolved from a broad exploration of ideas to a more specific focus due to the mounting evidence pointing to a significant association between the detrimental effect of iron accumulation on endothelial cell function and cardiovascular and neurodegenerative diseases. The hosting lab has shown that a transmembrane protein called Neuropilin-1 (NRP1) suppresses iron-dependent oxidative stress and senescence in endothelial cells. Thus, our primary aims were two: firstly, to gain further evidence on the role of exogenous iron in endothelial cell senescence in view to understand the contribution of iron overload in endothelial cells to the development of cardiovascular disease and neurodegeneration. This direction was guided by previous research that highlighted elevated iron levels as a common feature in both cardiovascular and neurodegenerative diseases, by a recent study from the hosting lab showing that NRP1 has osteoprotective and anti-inflammatory function, and by preliminary data showing that iron modulates NRP1 expression. Secondly, we sought to investigate the mechanism on how iron modulates NRP1 expression. This investigation aimed to shed light on whether Neuropilin-1 could serve as biomarker or a potential therapeutic target for diseases linked to iron accumulation.

## Summary of the Work Undertaken:

Over the course of my six-week internship in the laboratory, I acquired a wealth of valuable techniques and skills that will be pivotal for my future endeavours, especially as I aspire to pursue my own research. Coming from a background with limited prior laboratory experience, I leave this internship equipped with both knowledge and practical expertise. My learning journey encompassed several key areas:

1. Culturing and Splitting Primary Endothelial Cells: I gained proficiency in culturing and accurately splitting primary endothelial cells. This encompassed tasks such as the precise handling of cell cultures media preparation, dilution calculations, preparation of cell lysates for protein and RNA studies, and immunostaining. Importantly, these skills will allow me to confidently work with any cell type and to perform cell-based in vitro assays in future projects.

2. Experimental Planning and Design:

Collaborating with Dr Raimondi, I actively participated in the planning and design of our experiments. We introduced dose-dependent treatments by subjecting a group of cultured cells to iron treatment in growth media while maintaining another group in control, untreated growth media.

3. Immunostaining Techniques: I performed immunostaining procedures, whereby cells were labelled with specific antibodies to detect Neuropilin-1 and other proteins used as controls. This allowed us to assess the impact of iron levels on treated cells.

4. Reverse Transcription and qPCR: I acquired proficiency in RNA purification, reverse transcription to generate cDNA and in operating a Real Time qPCR operation. This included interpreting quantitative data and deriving meaningful conclusion from the results.

### **Description of Results/Outcome of the Project**:

Our research showed compelling results with significant meaning. The most noteworthy discovery was that cells subjected to iron treatment exhibited heightened expression levels of NRP1. This finding raises intriguing questions about the potential for therapeutic interventions targeting Neuropilin-1 to mitigate the incidence of cardiovascular and neurodegenerative diseases.

During my internship, I not only achieved significant milestones but also confronted challenges and learned from my experiences. One such challenge was the realization of the toxic nature of DMSO when towing the cells, which should be quickly removed or diluted with sufficient media to avoid cellular stress or cell death.

## Impact of the Work/Results:

The work we conducted is important for the vascular field as it can open for new research into Neuropilin-1 and new in vitro and in vivo testing for ways it can affect human cells and what diseases it can be associated with. The potential societal benefit of our research is substantial. As the global burden of dementia and cardiovascular diseases continues to grow, identifying modifiable risk factors becomes paramount. Our work suggests that targeting iron accumulation in endothelial cells may offer a novel therapeutic approach and that NRP1 could be a biomarker and a therapeutic target. If successful, this could translate into interventions that reduce the incidence and severity of both cardiovascular diseases and neurodegenerative disorders. Ultimately, our findings have the potential to enhance the quality of life for individuals at risk of these debilitating conditions.

## **Contribution of the Skills/Studentship to Future Career Plans/Goals:**

The laboratory skills I acquired during my internship hold significant value for my future research aspirations, particularly in senescence and immunology. Proficiency in cell handling, immunostaining, PCR and cell culture techniques equips me with essential tools for my Ph.D. studies, facilitating in-depth exploration of cellular processes. These skills are complemented by transferable attributes, such as attention to detail and effective time management, which enhance my ability to contribute meaningfully to scientific research and to multitasking, collaborate effectively, and address complex challenges in a dynamic research landscape. This research has expanded my horizons, and I am eager to continue my journey in scientific inquiry and contribute to the pursuit of solutions for complex health issues.



Figure 1: one of my attempts in culturing HUVEC cells





INSTITUTE OF CHILD HEALTH



### **Biochemical Society Summer Vacation Studentship Report 2023**

#### Student: Thea Jakobi

Supervisors: Elizabeth C Rosser, Diana Matei and Persephone Jenkins, Centre for Adolescent Rheumatology Versus Arthritis at University College London, University College London and Great Ormond Street Hospitals and the Division of Medicine, University College London, UK

#### Introduction

Juvenile Idiopathic Arthritis (JIA) is the most prevalent chronic inflammatory condition of childhood (Martini et al., 2022). JIA encompasses the following groups of childhood arthritides, systemic arthritis, enthesis-related arthritis (ERA), rheumatoid factor positive (RF+), rheumatoid factor negative (RF-) oligo/polyarticular arthritis (OA/PO) and undifferentiated arthritis, according to the International League Against Rheumatism classification (Martini et al., 2022). Each of these JIA subtypes have differences associated with their clinical characterisation but all share similarities including pain, warmth and swelling in at least one joint. Pathogenic changes to the gut-site, such as dysbiosis of the gut-microbiota, are central to the pathogenic process in both adult-onset rheumatoid arthritis (RA) and JIA. However, the cellular and molecular mechanisms underpinning the associations between the gut and joint inflammation remain unknown. Previously it has been shown that serum levels of intestinal permeability biomarkers, including lipopolysaccharide binding protein (LBP) and intestinal-fatty acid binding protein (I-FABP) are higher in RA patients compared to healthy controls (HCs) (Matei et al., 2021). A paper that focussed on paediatric JIA patients that were systemic treatment-naïve, found that patients with PO JIA, OA JIA and spondyloarthropathies had significantly greater circulating LBP concentrations than HCs (Fotis et al., 2017).

#### Aims and Objectives

In this studentship, I set out to investigate whether the biomarkers of intestinal permeability, namely LBP and I-FABP, are increased in the serum of our cohort of adolescent JIA patients (n=61) compared to age-matched HCs (n=29). The age range of our cohort was from 13 to 23.

#### Summary of work undertaken

I first curated a collection of serum samples from a bioresource of peripheral blood samples taken from JIA patients. The different JIA subtypes that we chose to investigate were ERA, RF+ OA/PO and RF- OA/PO. These groups can be subdivided into whether their disease was active (at least one joint inflamed) or inactive (no current joint inflammation). Importantly, a similar number of samples for each subtype within each JIA group was selected.

After ELISA practice, I ran three ELISA plates for LBP and one for I-FABP following the protocols given by the manufacturer that came with the kits ordered; DuoSet ELISA LBP and Millipore I-FABP. Consistency across different plates and dates was achieved by using four samples as references across the three LBP plates. To make sure that all groups were represented on each plate to allow integration of the data, there was at least one of each JIA subtype (ERA, RF+ OA/PO, RF- OA/PO) as well as a HC. The given concentration of the standard was plotted against the optical density to generate a standard curve which was used to interpolate the unknown

concentrations of LBP and I-FABP in the serum samples. To standardise the concentration of LBP across plates I divided each concentration by the average of the two chosen references on the plate and multiplied it by the average of the average of the three pairs of references. The standard error of the mean and an unpaired t-test or one-way ANOVA were completed to calculate the error bars and p-value, respectively.

#### Description of results and forward directions

First, I compared LBP concentrations in the HCs to the whole cohort of JIA patients (see Figure 1A). I found that the LBP levels in JIA patients on average were not significantly increased compared to HCs. However, when splitting our JIA cohort into different JIA subtypes - ERA, RF+ and RF- OA/PO subtypes - there was a statistically significant differences in the level of serum LBP between the HC and ERA, as well as between ERA and RF- OA/PO (see Figure 1B). To investigate whether this was associated with disease activity or treatment, ERA was split further, but neither disease activity nor biologics showed any significant differences in LBP concentration (see Figure 1C and D). The increased LBP concentration in ERA indicates that patients within the ERA subtype may experience heightened intestinal permeability. Of interest, it is well-known that ERA has a strong association with subclinical gut inflammation and patients often have swelling in joints concurrently with symptoms of inflammatory bowel disease (Aggarwal et al., 2016).



**Figure 1. There is increased LBP in the serum of JIA patients with ERA.** Standardised concentrations of LBP (ng/mL). A) LBP concentrations (ng/mL) in HC compared to JIA. B) LBP concentrations in HC compared to JIA subtypes. C) LBP concentrations in HC compared to ERA active and ERA inactive. D) LBP concentrations in HC compared to ERA biologics (ERA YES) or ERA no biologics (ERA NO). Error bars were calculated using the standard error of the mean. The lack of p-values indicates non-significance.

Due to time limitations, it was not possible to perform the I-FABP ELISA on enough samples. It would be necessary to complete at least one more ELISA plate. A future direction is to perform these necessary analyses and to investigate the levels of other biomarkers of 'gut-health' such as serotonin, D-Lactate, zonulin and retinoic acid in JIA patients compared to controls. The Rosser group is now planning to build on the work from my summer studentship and carry out these proposed experiments.

#### <u>Impact</u>

The research I carried out contributes to the increased understanding of the role of gut inflammation in the pathogenesis of JIA subtypes. With continued research in this field, LBP or other intestinal permeability markers could be used as a diagnostic tool for JIA patients, particularly ERA patients, to allow the application of personalised drug strategies such as those that target gut permeability. This is important since early treatment of JIA is associated with better long-term disease outcomes and some patients still do not respond to current treatment regimens (Garner et al., 2021). The Biochemical Society has supported me, an early career molecular bioscientist, which is in line with their strategy. I may have helped build the Biochemical Society's international reputation by doing weekly updates tagging BiochemSoc via Twitter.

#### Contribution to future goals and transferrable skills

I enjoyed how multifaceted working as a researcher is, from the manual aspects, data analysis, giving presentations, reading papers to writing. I have also confirmed my passion for Immunology and thus learned that I would like to work in Immunology research. Learning how to perform ELISAs is therefore a valuable skill. Moreover, I had such a great time that I requested to carry out my BSc laboratory project in the Rosser lab. Lastly, I have greatly improved my teamwork and communication skills. More specifically, I recognised the importance of attending weekly meetings to ensure everyone works well as a team and is well integrated.



**Figure 2. The Rosser group.** I am on the left, Persephone Jenkins fourth from the left, Elizabeth Rosser fifth from the left and Diana Matei seventh from the left. It was great working with the whole team!

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Student: Alvina Imran Supervisor: Matthew Jones

#### Post programme report

Cancer is a major global health burden associated with millions of deaths annually. Whilst current chemotherapeutic treatments for cancer are effective, they possess substantial off-target effects (Hepatotoxicity, Neurotoxicity and Cardiotoxicity) which all have a substantial impact on long-term survival. These off-target effects of chemotherapeutics are significantly more severe in children when used to treat childhood cancers such as medulloblastoma. It is therefore imperative to evaluate new therapeutics for their efficacy and potential off-target effects.

A potential rich and diverse source of these new therapeutics is those derived from plants termed phytopharmaceuticals. Phytopharmaceuticals have been used for millennia to treat various disorders during traditional medicinal practices worldwide, however several traditional compounds are now routinely used clinically. Numerous lead compounds have been highlighted for the development of clinically utilised agents. One of these being Celastrol, a pentacyclic triterpene isolated from *Tripterygium wilfordii*, which is shown to possess anti-inflammatory and antioxidant properties in vitro. Investigations also reveal that Celastrol possesses anti-cancer properties against various cancer types.

Following a recent collaboration with Kidscan Children's Cancer Research, a numerous phytochemicals have been identified as producing potent cytotoxicity against medulloblastoma cells, with the most potent of these being celastrol. Celastrol was shown to produce  $EC_{50}$  values of less than 500nM against the group 3 medulloblastoma cell line HD-MB03 through the induction of apoptotic cell death highlighting its potential as a therapeutic agent for medulloblastoma.

The objectives of the project revolved around the evaluation of celastrol on medulloblastoma subtypes, something that has never been characterised, and explore its cardiotoxic potential. My project focussed to see if previously described activity was also the case in other medulloblastoma subtypes including the sonic hedgehog subtype cell line, DAOY. I preliminarily investigated the potential cardiac implications of celastrol utilising an in vitro model of cardiotoxicity (H9c2 cells). To evaluate these effects, technical research methodologies including flow cytometry, live cell microscopy and fluorescent microscopy were utilised to determine the mechanism of action observed in group 3 medulloblastoma cells is mimicked in DAOY cells.

The results of this studentship showed celastrol to be effective as a chemotherapeutic agent against both HD-MB03 and DAOY cell lines in a time and concentration dependent manner (Figure 1A&B), with  $EC_{50}$  values ranging between 0.24–0.51µM and 0.21–0.64µM respectively. It was also found that cytotoxicity of celastrol was irreversible after treatment for 24 hours when treated with concentration of >500nM before removal and washout of the celastrol (Figure 1C&D). Using time-lapse live-cell microscopy we identified that celastrol produced no effect on cellular migration (Figure 2). Fluorescent microscopy revealed that celastrol significantly increased the activation of Caspase 3/7 (Figure 3), providing further evidence of apoptotic cell death and providing preliminary information on the mechanism of cell death induced by celastrol.

Treatment of terminally differentiated cardiac myoblasts (H9c2 cells) with celastrol, to screen for cardiotoxicity, revealed that celastrol induced cardiotoxicity at concentrations >0.5 $\mu$ M following treatment for 72 hours (Figure 4) with no toxicity observed at lower treatment timepoints (Data not shown).

These data highlight celastrol as a relatively safe and effective therapeutic lead for the treatment of medulloblastoma. The research group will now seek to continue this study by exploring changes in key apoptosis and cell cycle regulatory gene and protein expression by qRT-PCR and Western Blotting in medulloblastoma cells following treatment with celastrol. The group is also seeking to develop an internal collaboration with nano-medicine specialists, to evaluate if celastrol can be nano-encapsulated to increase its potency and minimise off-target effects.

This project may evoke other scientists to think about exploring traditional medicine-derived compounds for their wider therapeutic potential and develop an understanding of their molecular mechanisms of action. In traditional medicine, thousands of compounds/extracts are used to treat numerous ailments, however, there remains limited exploration of the molecules within these extracts and their mechanisms of action. The development of an understanding of the mechanisms may make them more desired for translation to in-vivo study or clinical trials, as well as for scientists to have a basis for the generation of modified derivatives which possess more potent effects or less off target toxicity.

Throughout this project we continued to develop our in vitro model of cardiotoxicity to screen novel compounds as cardiotoxicity is a common side effect of chemotherapies. As phytomedicines traditionally have fewer off-target effects than synthetic compounds, by simultaneously screening compounds for their therapeutic and cardiotoxic potential, will highlight promising lead compounds to take forward for molecular evaluation. This could be of interest to the wider bioscience community as a tool for developing novel compounds of therapeutic interest.

This project has provided me with a diverse set of highly sought skills, both practical and transferable, in the research field. This studentship has enabled me to build a comprehensive portfolio of laboratory skills, enhancing my post-graduation employability and preparing me for potential postgraduate research pursuits such as MRes, or a PhD programmes. These skills range from fundamental laboratory techniques like cell culture and cell viability assays to more advanced methodologies such as time-lapse live-cell microscopy, fluorescence microscopy, and flow cytometry.

During instances when my experiments encountered setbacks I showed resilience, honed my problem-solving abilities to pinpoint sources of experimental error and gained proficiency in troubleshooting technical issues by collaborating with fellow research students, academics, and technical staff. Furthermore, I had the opportunity to participate in workshops, which significantly contributed to the development of my analytical and communication skills. This encompassed areas like academic writing, data presentation, and statistical analysis of biological data. Consequently, I was able to deliver an oral presentation summarising the findings and conclusions of my studentship to fellow members of the research laboratory and a broader audience of academic staff. The results of my research are promising and may lead

to the creation of a conference abstract for potential presentation at scientific research conferences in the future. Most notably, this experience afforded me a deep sight into the day-to-day responsibilities of a research scientist engaged in interdisciplinary research, something I could not experience without this studentship.



Figure 1 - The cytotoxic effects of celastrol against medulloblastoma subtypes. (A & B) Dose response curves comparing the cytotoxicity of celastrol and against HD-MB03 (Panel A) and DAOY (Panel B) cell lines following treatment for 24 (Red), 48 (Blue) and 72 hours (Black). (C) Representative crystal violet staining images showing HD-MB03 colonies arising after celastrol wash-out over 10 days recovery. Pre-treatments with celastrol were carried out over 48 hours before wash-out. (D) Corresponding Mean ± SD crystal violet absorbance derived from 6 independent repeats. All data compared to vehicle alone (0.5 % v/v DMSO). Statistical significance (P < 0.05) was determined following a one-way ANOVA with a Tukey's post-hoc test \*\*P < 0.001; \*\*\*\* P < 0.0001. n = 6 independent repeats for all experiments.



#### Project background and aims

Perturbed cellular signalling and resistance to apoptotic cell death can lead to cancer development. My host lab is investigating whether lysosomal-mediated cell death can be used to kill breast cancer cells resistant to apoptosis. To support this goal, my summer project aimed to characterise the autophagic-lysosomal phenotype of different breast cancer subtype-specific cell lines.

#### Summary of work undertaken, results obtained and future directions

I compared the autophagic environments in 1 normal (MCF10A) and 4 breast cancer cell lines representing the main breast cancer subtypes: luminal A/B (MCF7), HER2+ (SKBR3), and Triple Negative Breast Cancer (TNBC: MDA-MB-231 and MDA-MB-468). To investigate autophagic activity (flux), I exposed the different cell lines to nutrient starvation and/or an autophagy inhibitor (bafilomycin A) and assessed autophagosome accumulation by performing immunofluorescence staining and confocal microscopy for the autophagosome marker, LC3B (see Figure 1A for example images). Under normal conditions, the number of LC3-positive puncta increases with starvation and bafilomycin A exposure. I evaluated autophagic flux by counting the number of LC3B puncta per cell using a modified image analysis pipeline (Schüssele et al., 2023). For MDA-MB-468 and MCF7 cells, LC3B puncta count increased with bafilomycin treatment under non-starved and starvation conditions (Figure 1B) – showing autophagic flux. MCF10A cells however possessed high basal autophagic activity, with few differences observed between conditions (Figure 1A-B). In SKBR3 cells, LC3B puncta number did not appear to change with bafilomycin A and/or starvation, suggesting low levels of autophagic flux. Overall, MDA-MB-231 had fewer LC3B puncta under basal conditions, which did not increase in response to combined starvation and bafilomycin treatment, suggesting potential defects in autophagic induction and/or lysosomal degradation (Figure 1B). In MDA-MB-468 cells, control levels were similar to starved, suggesting reliance on autophagy even under fed conditions. However, the close proximity of many autophagosomes makes accurate image quantification challenging, therefore these results should be interpreted with caution before additional independent repeat experiments.

To investigate this further, I transfected cell lines with a GFP-mCherry-LC3B construct which allows autophagosomes (GFP and mCherry) and autolysosomes (mCherry only) to be distinguished due to the pH sensitivity of GFP, thus allowing monitoring of autophagy activity by live fluorescence imaging. In SKBR3, MCF10A and MDA-MB-468 cell lines, there is a clear increase in yellow puncta and red puncta in starved cells compared to control (Figure 1C). This increase in autophagosomes and autolysosomes shows autophagy has been induced by starvation, which had been difficult to detect when immunostaining for LC3 in fixed cells. SKBR3 cells had the lowest basal level of autophagosomes and autolysosomes, followed by MCF10A and MDA-MB-468 cells, with starvation showing the same trend (Figure 1C). Unfortunately, transfection issues prevented the analysis of MDA-MB-231 and MCF7 cell lines, which will require optimisation in follow-up studies.

Finally, I treated cell lines with the lysosomotropic agent hydrochloroquine (HCQ) to explore their sensitivities to lysosomal permeabilisation. In MCF10A, MDA-MB-231, MDA-MB-468 and SKBR3 cell lines, HCQ treatment resulted in increased lipidated LC3B-II levels (Figure 1D), indicating their sensitivities to HCQ-induced autophagy inhibition. However, relative basal LC3B-II levels differed between these lines, with SKBR3 and TNBC lines possessing the lowest and highest levels respectively, further demonstrating their different autophagic phenotypes as suggested by my imaging experiments. Alongside, I assessed the protein levels of the key autophagy receptor p62, which is expected to accumulate in response to autophagy inhibition, as observed in the SKBR3 cell line. Changes in p62 levels in other cell lines, however, were subtler; perhaps due to transcriptional

regulation and/or potential proteasome degradation. Future studies should therefore assess p62 transcript levels.

In summary, I sought to characterise the autophagic phenotypes of distinct breast cancer cell lines. My initial findings suggest that HER2+ SKBR3 cells possess lower basal autophagy levels, and that these cells are relatively insensitive to lysosomotropic agents. My results also suggest that TNBC cell lines are more dependent on autophagy and sensitive to inhibition. In future work, my host lab will repeat these experiments and use other methods to induce lysosomal permeabilisation to assess the different sensitivities of each cell line to autophagy-lysosomal perturbations. This will further our understanding of the contribution of autophagic-lysosomal pathways in distinct breast cancer subtypes.

#### Project impact

Breast cancer is the most common female cancer, and multidrug resistance is a serious problem to patient prognosis. This work will help inform our understanding of the role of autophagy-lysosomal pathways in different breast cancer subtypes, and whether some subtypes might be susceptible to autophagy inhibition and lysosome dysfunction. Ultimately, this could help develop lysosome-targeting treatments for therapy-resistant cancers. This is especially beneficial for TNBC patients, who have a worse prognosis and no targeted therapy options.

This studentship helped the Biochemical Society achieve its strategy as I had the opportunity to share my results with my host department's Epithelial Tumour Biology group, and I gained knowledge from presentations during group meetings. As a future bioscientist, this experience has provided me with invaluable support for my career development.

#### Skills gained and contribution to future career plans/goals:

I have gained new laboratory skills and I can now independently perform techniques including mammalian cell culture, western blotting and fixed and live-cell confocal microscopy. Additionally, I assisted my fellow lab members with generating novel organoid cultures and preparing samples for flow cytometry.

Alongside lab-based skills, I have refined a variety of transferable skills. Analysing and interpreting experimental results has improved my data handling, and troubleshooting has developed my problem-solving abilities. Moreover, giving presentations in lab and multi-group meetings has improved my oral communication skills. Finally, performing background research prepared me for future literature reviews and research projects.

This experience has opened my eyes to the world of scientific research and the work – both successes and failures – behind academic papers. As a result of my studentship, I am inspired to pursue postgraduate studies and lab work. The connections I made in my six weeks will be a valuable resource for the next steps in my academic journey.

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#### **Figures**:





Figure 1: Breast cell lines have different levels of autophagic flux/different responses. A-B: MCF10A, MDA-MB-468, MDA-MB-231, MCF7 and SKBR3 cells were subjected to the following treatments: control (complete media and buffer); baf (complete media and 50nM bafilomycin A); starve (HBSS media and buffer); starve + baf (HBSS media and 50nM bafilomycin A) for 2 hours before being fixed. Samples were stained using antibodies for LC3 and DAPI and imaged using confocal microscopy. A) Example images for MCF10A and MDA-MB-468 cells after CellProfiler analysis, LC3B puncta shown as green dots. B) Graph shows the average LC3 puncta count for each cell line under the different conditions. Image analysis was performed in CellProfiler using representative images to determine the number of LC3 puncta per cell. C= control, B = baf, S = starve, S+B = starve+ baf C) MCF10A, MDA-MB-468 and SKBR3 cells were transfected with tandem mCherry-GFP-LC3 construct using lipofectamine 2000 for 5 hours, then subjected to starvation (HBSS media) or control (complete mediam) for 3 hours before live spinning disk confocal fluorescence microscopy. Representative images were taken of the cells. Red puncta represent autophagosomes. Graphs show the average number of yellow and red puncta per cell for both conditions. Puncta were manually counted using limageJ. \* = p<0.05 \*\* = p<0.001 \*\*\* = p<0.001 vs. control, independent samples t-test. Data are mean ± S.D. D) The effects of hydroxychloroquine on autophagy across representative breast cancer cell lines. Cells from MCF10A, MDA-MB-468 and SKBR3 cell lines were treated with 10ug/ml HCQ (+) or DMSO (-) for 1 hour. LC3B and p62 protein expression levels were analysed by western blot to determine autophagic flux. GAPDH was used as a loading control.



Figure 2: Group photo. Left to right – Dr Bethan Lloyd-Lewis, Eloise Lines, Neve Prowting (PhD student)



Report by Aleksandra Teriosina



#### Introduction and aims.

Bacterial microcompartments (BMCs) are selectively permeable protein shells which encapsulate an enzymatic core (Hagen et al., 2018). Their formation provides a competitive advantage as it allows for the separation of toxic intermediates formed during catabolic reactions from the cytosol, contributing to a larger metabolic versatility (Planamente & Frank, 2019). It is therefore unsurprising that operons encoding for BMCs are found amongst a myriad of bacterial species (Kerfeld et al., 2018), including members of the gut microbiome. A 1,2-propanediol-utilization (PDU) BMC encoding operon has been identified in *Ruminococcus gnavus*. The PDU genes within the BMC operon are vitamin B12 independent, however the PDU CDE genes elsewhere in the genome are predicted to be vitamin B12 dependant through bioinformatic analysis. This project aimed to establish the association of *R. gnavus* PDU CDE genes with the BMC and determine whether their action is vitamin B12 dependent.

#### Methods.

#### Anaerobic growth assay and propionate production

An anaerobic growth assay was set up in order to measure and compare the growth of *R. gnavus* ATCC29149 in the presence of glucose, fucose, rhamnose and 1,2-propanediol in YCFA media. In order to analyse the vitamin B12 dependency of *R. gnavus* growth, three different YCFA medias were used, which consisted of: normal YCFA; YCFA with vitamin B12 removed through BTUG2 protein affinity chromatography; YCFA with vitamin B12 added. The optical density was measured every 30 minutes over a 24-hour period, and the data was analysed on Microsoft excel, where growth curves were generated. This was repeated with samples taken at 0, 3 and 8 hours, for analysis of propionate production.

#### Heterologous expression of R. gnavus genes

Several *R. gnavus* genes encoding for PDU and BMC proteins were cloned and transformed into *E. coli* BL21 cells. Target genes were cloned through infusion cloning, utilising primers which were designed using SnapGene. The target inserts were amplified through a PCR reaction, before subsequent transformation into *E. coli* BL21 competent cells through heat shock.

Following incubation at 37° for 1 hour, 100 µl of cells were spread on LB plates containing 50 µg/ml kanamycin and incubated at 37° overnight. Six of the resulting colonies from each transformation were analysed through colony PCR and DNA agarose gel electrophoresis, to confirm the correct plasmid was present.

In total, we expressed 9 different PHISTEV plasmids which consisted of: the PDU CDE genes 189, 190 and 191, each with and without the BMC proteins; a cargo protein with the BMC proteins; just the BMC proteins; and a cluster containing putative shell proteins aside from the main PDU BMC genes.

#### Protein expression and purification

Following the successful transformation of *R. gnavus* genes into *E. coli* BL21 cells, the proteins were expressed in autoinduction media (AIM). A 1% innocula of AIM containing 50  $\mu$ g/ml kanamycin was

prepared for each transformation. These were incubated at 37<sup>o</sup> for 5 hours, before the temperature was lowered to 22<sup>o</sup> and incubated for 72 hours.

The proteins were subsequently extracted from each AIM. The cell lysates which were thought to contain the PDU CDE genes without the BMC proteins, were purified through nickel affinity chromatography on an ÄKTA Pure protein purification system. As we were unable to isolate any protein from our samples, SDS-PAGE analysis of the insoluble cell pellet revealed that the PDU CDE proteins 189, 190 and 191 were insoluble.

The cell lysates extracted from the cells consisting of plasmids containing the BMC proteins were subjected to ultracentrifugation in a 35-65% sucrose density gradient at 130,000 G for 16 hours. Following this 1 ml fractions were taken from each of the samples for SDS-PAGE analysis.

#### Results and outcomes of studentship.

Analysis of *R. gnavus* anaerobic growth assay data indicated the utilisation of glucose, rhamnose and fucose, however not 1,2- propanediol. The addition of vitamin B12 to YCFA did not significantly improve growth.



*Figure 1:* Growth curves from anaerobic growth assay on *R. Gnavus. Ruminococcus gnavus* was grown in four different sugars, and no sugar as a control, with three different YCFA media's, which differed in vitamin B12 content. A was YCFA with vitamin B12 removed through BTUG2 protein affinity chromatography. **B** was normal YCFA. **C** was YCFA with vitamin B12 added.

Heterologous expression of *R. gnavus* genes into *E.coli* BL21 cells was successful and the resulting colonies were verified through colony PCR.



Figure 2: DNA gel electrophoresis following colony PCR. Six colonies were selected for colony PCR following transformation. A shows the plasmid containing the cargo protein with the BMC. B shows the putative shell proteins. C, D and E show the PDU CDE 189, 190 and 191 genes without the BMC.

#### Future directions.

Future goals include the heterologous expression of *R. gnavus* bacterial microcompartments in *E. coli.* This will be performed in presence and absence of known cargo proteins and putative cargo proteins, to determine the full extent of BMC utilisation in *R. gnavus.* Furthermore, the impact of expression of this BMC will be investigated in relation to the production of SCFAs, in particular propionate.

#### Departures from original project plan.

Due to the difficulties in purifying the proteins, we were unable to do vitamin B12 binding assays or bioimaging of BMCs. Samples taken during the anaerobic growth assay on *R. gnavus* will be analysed at a later date for propionate production, to determine if addition of vitamin B12 had an effect. As the PDU CDE genes are insoluble they will require solubilisation through denaturing, followed by refolding and subsequent purification through chromatographic techniques.

#### Value of studentship to the student.

Woking in the Juge lab has allowed me to develop a myriad of lab and interpersonal skills which will be invaluable to me when completing a PhD in a closely related area of research. I was able to gain experience in microbiological techniques such as cloning and subsequent culturing and heterologous expression, as well as the use of the anaerobic chamber. I was able to extract plasmids for analysis through DNA agarose gel electrophoresis and verify proteins through SDS-PAGE. Through shadowing of my supervisor, I was able to gain some experience in the use of the AKTA pure protein purification system and the ultracentrifuge. In addition to lab skills, I was able to expand a range of interpersonal skills such as my oral presentation skills by presenting the progress I had made at fortnightly lab meetings. Within the Norwich research park there are several weekly seminars which I was able to attend, allowing me to expand my scientific curiosity and knowledge in other areas of research.

#### Value of studentship to the research group.

The studentship gave valuable teaching and supervisory experience to a PDRA which they can take forward in future roles. The student provided the group with valuable support in pushing forward the research project, successfully cloning multiple constructs, and testing expression of different constructs. Furthermore, the student performed numerous growth curves which progressed the project and generated data which will form a basis of future publications, determining the roles on BMCs in *R. gnavus*.

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## **Generation of Biased Ligand Screening Prediction Platform**

<u>Supervisors</u>: Dr David A Sykes & Dr Dmitry B Veprintsev, University of Nottingham, UK <u>Student</u>: N T Ngan Phan, University of Nottingham, UK

## Introduction:

G protein-coupled receptors (GPCRs) are essential drug targets, constituting 30% of clinically used drugs. Biased agonism as a drug discovery paradigm, wherein GPCRs signal through specific pathways, is of significant interest. However, the current understanding of effector driven GPCR conformational changes is limited due to standard binding assay constraints, which routinely measure only low-affinity receptor forms. Nanobodies provide an alternative measure but are expensive, receptor-specific, and time-consuming to produce.

Our research aims to bridge this knowledge gap by developing a universal system for studying drug affinity for specific receptor-effector conformations. We propose a fusion protein model system that combines GPCRs and modified G protein to recapitulate native receptor-effector conformations, eliminating the need for nanobodies.

## Aims of the project:

- (1) Identify candidate ligands capable of inducing biased agonism, either towards  $G_s$  protein or arrestin signalling pathways, specific to the  $\beta_2$  adrenergic receptor ( $\beta_2 AR$ ).
- (2) Investigate the kinetics of drugs binding to different receptor-effector combinations.
- (3) Explore the potential impact of temperature and salt levels on experimental outcomes.

## Methods:

**Saturation binding assays:** Fluorescent propranolol affinity was determined through saturation binding assays employing terbium-labelled fusion protein within an assay buffer composed of Hank's Balanced Salt Solution (HBSS), 5 mM HEPES, 0.1% BSA, and 0.02% F-127 detergent. Total binding was assessed with a DMSO solution, while nonspecific binding was assessed with 1  $\mu$ M alprenolol.

The dissociation kinetics of F-propranolol were assessed by introducing a high saturation concentration of 1  $\mu$ M alprenolol to F-propranolol (40nM) which was prebound to the receptors.

**Competition binding assays:** This assay contained a fixed concentration of green propranolol (10nM), a serial dilution of competitor, and terbium-labelled fusion protein in assay buffer. Assays were conducted at room temperature. Subsequently, data were obtained using a PHERAstar FSX plate reader, processed with MARS software, and subjected to further analysis within GraphPad PRISM 10.

<b>Result:</b>	
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β₂AR	Saturation Binding		Association	Dissociation	
	K <sub>d</sub> (nM)	B <sub>max</sub>	K <sub>on</sub> (M⁻¹min⁻¹)	K (min⁻¹)	Half-life (0.693/k)
WT	4.40 ± 0.67	2243.5 ± 552.86	11620540 ± 2893259	0.08 ± 0.005	8.48 ± 0.53
mG₅	4.40 ± 0.95	1942 ± 127.04	10563274 ± 6824183	0.03 ± 0.001	21.39 ± 0.81
Arrestin	3.69 ± 0.44	1791.6 ± 120.22	12943659 ± 2165306	0.06 ± 0.005	$11.00 \pm 0.90$

**Table 1:** Dissociation constants ( $K_d$ ) and maximum receptor densities ( $B_{max}$ ) in  $\beta_2AR$ -based binding assays for both the wild-type (WT) and miniG-coupled (mG) forms, alongside with ligand binding association and dissociation rates.

**Result 1:** F-propranolol has similar receptor affinity for WT, mG<sub>s</sub> and arrestin fusions.

**Result 2:** The differences observed in fluorescent propranolol's binding kinetics. The association rates across the different membrane preparations were similar but dissociation rates of F-propranolol differed for different receptor-effector combinations, suggestive of different receptor conformations.



Figure 1: Concentrationresponse curves showing half maximal inhibitory concentration (IC<sub>50</sub>) values of (A) S- propanolol; (B) Formoterol; (C) Isoprenaline; (D) BI167-107, competing with Fpropranolol for different receptor conformations (native WT, mG and arrestin bound). Measurements were taken at the 1-hour time point. Data are mean ± S.E.M.

Compounds	WT	mG	Arrestin
S – Propanolol	9.18 ± 0.19	9.62 ± 0.18	9.35 ± 0.08
Formoterol	7.51 ± 0.11	8.61 ± 0.17	8.09 ± 0.12
Isoprenaline	5.58 ± 0.14	7.94 ± 0.09	6.07 ± 0.11
BI167-107	9.03 ± 0.14	8.76 ± 0.11	9.29 ± 0.11

**Table 2:** Experimental  $pK_i$  values for the competitors shown in Figure 1, which bind to the  $\beta_2AR$ based WT, mG-coupled and arrestin bound forms, are presented. Values are presented as the mean  $\pm$  S.E.M. with data from at least three independent experiments.
## Result 3: A clear and distinct shift is observed in favor of mG compared to arrestin for certain competitors.

S-propanolol, formoterol, and isoprenaline display bias behaviour, characterised by their higher potency and stronger affinity for mG compared to arrestin and WT receptors.

Competitor BI167-107 presents a neutral response for binding to either  $\beta_2$ AR-based WT or mG forms. These findings are supported by the IC<sub>50</sub> curves and the pKi values presented in Figure 1 and Table 2 respectively.

## **Outcomes:**

In this study, using the  $\beta_2AR$  as a model system, we demonstrate that our fusion proteins effectively measure high affinity binding compared to the wild-type receptor, offering a promising avenue for studying bias agonism at GPCRs. This innovative approach eliminates the need for nanobodies, ensuring native receptoreffector conformations. Our methodology provides an efficient and cost-effective means of investigating GPCRs, and drug induced conformational changes, with the potential to discover effective biased agonists. This research has the potential to lead to more effective treatments for a variety of diseases.

## **Future directions:**

This methodology can be extended to explore the interactions with other  $\beta_2AR$ -coupled proteins, such as G<sub>i</sub>, thereby expanding our knowledge of GPCR signalling pathways. Furthermore, its versatility can be leveraged for studying a wide range of GPCR receptors, enabling a comprehensive understanding of their functional characteristics and bias agonisms. These insights have the potential to drive drug discovery efforts, potentially leading to the development of novel therapies for a variety of diseases, ultimately improving healthcare options and patient outcomes.

## Values of studentship to student and the lab:

During my time in Veprintsev lab, I developed proficiency in conducting binding assays and utilising Prism software for data analysis. I have also enhanced my critical thinking and scientific communication skills. This period also ignited my passion for GPCR research and reinforced my commitment to pursuing a biochemistry-related Ph.D.

Looking back, I deeply appreciate the unwaving support from my supervisors and lab colleagues, and the generous funding provided by the Biochemical Society, which were instrumental in my growth and achievements.

The studentship also contributed to the ongoing Veprintsev lab research and will be featured in upcoming publications.



*Figure 3:* Image of Prof. Dmitry Veprintsev, Ngan Phan and Dr David Sykes. From left to right.