### **Title: Is palmitoylation an important regulator of vGLUT1 and vGLUT2? By Adriana Bizior** Introduction and aims:

Specialised connections known as synapses are the site of neuron-neuron communication in the nervous system. This communication involves release of chemical neurotransmitters from the pre-synaptic neuron, which bind to receptors on the post-synaptic neuron, initiating an appropriate response. Palmitoylation, the only reversible lipid modification of proteins, has emerged recently as a novel regulator of post-synaptic physiology, where it modulates the localisation and function of several key proteins, including AMPA and NMDA glutamate receptors. However, relatively little is known about the role of this modification in regulating pre-synaptic proteins. This project, therefore was focusing on the vesicular glutamate transporters vGLUT1 and vGLUT2, known to mediate the uptake of the excitatory neurotransmitter-glutamate into synaptic vesicles, recently found to be modified by palmitoylation. These transporter proteins are not only essential for normal neuronal function and communication but have also been linked to neurological disorders such as Alzheimer's disease and Parkinson's disease. The aim of this studentship was to explore whether palmitoylation is an important regulator of vGLUT1/2. To do this, I mutated known palmitoylation sites in these proteins, used click chemistry to assess the effect of these mutations on palmitoylation, and examined the localisation of cysteine mutants in HEK293 cells.

### My work and results:

Palmitoylation occurs on cysteine residues, hence PCR was used with specific oligonucleotide primers to generate site-directed mutants of an epitope (HA)-tagged vGLUT1 plasmid, where each of the two cysteines codons were mutated to codons for alanine. The samples were sequenced to confirm mutation of the cysteine codons.



**Fig. 1 (A)** Gel electrophoresis showing purified plasmids encoding HA-vGLUT1 WT (control) and constructs with two cysteine codons (TGC) mutated to alanine codons (GCC) (HA-vGLUT1+P1 and HA-vGLUT1+P2) (B) vGLUT1 DNA sequence confirming the sites of cysteine to alanine codon changes shown in red boxes.

Unfortunately, I was not able to confirm expression of the HA-vGLUT1 mutant protein in HEK293 cells and therefore I focused on previously-generated EGFP-vGLUT2 constructs; the 3CA constructs indicates a mutant in which three cysteine codons are changed to alanine codons.



**Fig. 2** Effect of different temperatures on migration of EGFP-vGLUT2 on SDS gels. HEK293T cells were transfected for 24 hours with wild type or 3CA mutant EGFP-vGLUT2. Cells were then lysed, heated at 37°C or 95°C and examined by immunoblotting with anti-HA antibody. The position of molecular marker is shown on the left.

As some transmembrane proteins aggregate upon boiling, before analysing palmitoylation of the vGLUT2 mutants, different temperatures were examined (in duplicate) to estimate the optimum temperature for EGFP-vGLUT2 protein. Levels of monomeric EGFP-vGLUT2 were higher following high temperature treatment of the samples, as visualised in Fig.2, and therefore samples were treated in this way prior to analysis.

A click chemistry approach was used to check whether the introduced mutation affects palmitoylation. Kidney HEK293T cells were transfected with 1µg EGFP-VGLUT2 WT/3CA mutants together with 1.5µg PEFBOS (negative control) or 1.5µg HA-DHHC3 enzyme (a highly active palmitoylation enzyme) in the presence of PEI or Lipofectamine 2000. "4 hours post-transfection, cells were incubated with 100µM unlabelled palmitic acid (n=1) or palmitic acid azide (n=2) for 6 hours at 37°C, lysed, clicked with 2.5µM of alkyne-PEG (5kDa) and examined by immunoblotting with anti-mouse 680 antibody (for protein detection) anti-rat 680antibody (for DHHC3 enzyme detection). Results are shown in Fig. 3.



**Fig. 3** Palmitoylation of EGFP- tagged vGLUT2 WT and 3CA mutant. HEK293T cells were transfected for 24 hours with wild type or 3CA mutant. Cells were then lysed, clicked with alkyne-PEG (5kDa) and examined by immunoblotting with anti-GFP (A) or anti-HA antibody (B). The position of molecular marker is shown on the left; molecular weight of EGFP-VGLUT2 is ~80kDa and DHHC3 ~30kDa.

The basis of the click assay is that proteins should be increased by 5kDa for every palmitic acid azide incorporated. Overall, the results of the click assay were inconclusive, and we were not able to confirm loss of palmitoylation with cysteine mutant of EGFP-vGLUT2 ("3CA").

Finally, to check whether cellular localisation of VGLUT protein is affected by palmitoylation 1µg WT and 3CA mutants of EGFP-vGLU2 were expressed in HEK293T cells in presence of Lipofectamine 2000 (2:1 ratio to 1µg of DNA) and visualised using confocal microscopy. As a vesicular protein, vGLUT2 was expected to have a punctate distribution with possible staining of the plasma membrane. This pattern is seen with the WT vGLUT2 protein (Fig. 4A). On the other hand, the 3CA mutated EGFP-vGLUT2 plasmid showed enrichment near the Golgi apparatus indicating that palmitoylation may influence cellular trafficking of this protein.



**Fig.4** Confocal microscopy analysis of EGFP-vGLUT2 WT (A) and 3CA mutant (B) in HEK293T. A- shows EGFP-vGLUT2 WT localised mainly near the plasma membrane; B shows EGFPvGLUT2 3CA mutant with disturbed intracellular localisation

### Future directions:

This experimental analysis provided a unique insight into the role of palmitoylation in targeting of pre-synaptic proteins. Future work should focus on confirming palmitoylation sites in vGLUT1/2 and undertaking further analysis of cysteine mutant vGLUT1/2 localisation, in particular, examining the distribution of the protein in neurons. It will be important to confirm the specific localisation of vGLUT2 3CA mutant intracellularly, by co-staining internal organelles, like the Golgi apparatus, with antibodies detectable by confocal microscopy. It will also be interesting to explore how the function of vGLUT1 and vGLUT2 is affected by blocking palmitoylation and if defects in vGLUT1 or vGLUT2 palmitoylation are associated with disorders such as Alzheimer's and Parkinson's disease. Note that original project was aiming to examine vGLUT1 protein only. However, due to difficulties with expression of HA-vGLUT1 cysteine mutant, and limited time, the project mainly focused on EGFP-vGLUT2. In the future however, I would like to try different approaches to increase the expression of vGLUT1, through increasing the amount of DNA or prolonged incubation time.

### How the grant has contributed to or shaped my career aspirations

This studentship allowed me to develop many important skills, including time management, good organisation and record keeping. I received training in several important techniques, including click chemistry, confocal microscopy, cell culture and transfection. In addition, I consolidated skills that I have learned in my current degree programme, including gel electrophoresis and immunoblotting, and polymerase chain reaction. I also benefited from working in a dynamic laboratory environment where I realised that being a scientist/researcher is not easy. It requires a lot of patience and determination, as things often do not go as planned or expected. Biology-related sciences are unexpected, hence so fascinating. Apart from having exciting time and getting a lot of experience in the lab, I also became confident about my career goal which is to finish my course with a first-class degree and further develop my scientific expertise through undertaking a PhD focusing on biochemical and cell processes linked to disease.

### Value of the studentship

The studentship was very valuable to me as it allowed me to experience and develop important laboratory skills, consolidate on other practical skills and experience the different roles played by lab members. The work I undertook also contributed to the lab by providing interesting pilot data on the role of palmitoylation in vGLUT2 localisation- the effects of palmitoylation on vGLUT2 trafficking will be followed-up by others.

## **Biochemical Society Studentship 2018**

### Study of Hex-D Kinetic Mechanisms & Interactions

(Protein expression and purification, kinetic studies and crystallisation)



University of St Andrews

Under Supervision of Dr. Tracey Gloster University of St. Andrews

Submitted by Aishvi Jain Bachelor of Technology, Biochemical Engineering and Biotechnology Indian Institute of Technology, Delhi, India



Mammalian  $\beta$ -hexosaminidases have been shown to play essential roles in cellular physiology and health. These enzymes are responsible for the cleavage of the monosaccharides N-acetylglucosamine (GlcNAc) and N-acetylgalactosamine (GalNAc) from cellular substrates. No mechanistic studies have focused on the role of one of these nucleocytoplasmically localized  $\beta$ -hexosaminidase, and its cellular function remains unknown. Using a series of kinetic and mechanistic investigations into HexD, we are trying to define the mechanism and interaction with binding sites. (Reference)

### <u>Aim:</u>

Using a series of kinetic and mechanistic investigations into HexD, to define the mechanism and interaction of binding sites of HexD with various amino acids, peptides, and buffers.

### **Description of work and results:**

### **Purification**

Over-expressed the gene encoding HexD in *Escherichia coli* and undertook purification of HexD using nickel affinity chromatography and gel filtration chromatography using PBS buffer (for kinetics) and HEPES buffer (for crystallisation).

- Purified with PBS buffer to produce 1.7mg and 1.3mg HexD
- Purified with HEPES buffer to produce 9.35mg/ml (1.5ml) and 10.87mg/ml (1ml) HexD.



### Kinetics:

Kinetic assays and inhibition assay on HexD to determine the Km, Ki and Vmax with 4-nitrophenyl GalNAc substrate in the presence of ~15 amino acids, peptides or 5 buffers selected from thermoflour assay.



Michaelis plot (subs+enz)Micheal plot (subs+enz+inh)1/Vmax vs 1/substrate plot(Vmax vs substrate plot)(Vmax vs substrate plot)(linear lines representing different

ear lines representing different inhibitor tri- glycine conc.)

**Standard plot abs. vs conc.** 

### **Crystallisation**

Crystallisation of HexD in complex with ~15 amino acids, peptides or 5 buffers selected from thermoflour assay was successful. X-ray diffraction data were collected at Diamond Light Source and the structure of the protein was solved using CCP4 programs.

Amino Acid	Crystals	Data
Tri Gly	Y	Y
Leu	Y	Y
Serine	Y	Y
Glutamine	Y	N
Gly	Y	Y
Leu Gly	Y	Y
Ala Gly	Y	Y
ME4G	Y	N
Mes	Y	N
Bicine	Y	N
HEPES	Y	N

Tricine	Y	N
Bistris	Y	N
Phenylalanine	Y	N
Asparagine	Y	N
Aspartic acid	Y	N
Tryptophan	Y	N
4Gly	Y	Y
Tyrosine	Y	N
Arginine	N	N
Glutamic acid	Y	N
Valine	Y	N

### Future directions in which the project can be taken:

- To study interaction with tri-glycine (mainly), isothermal titration calorimetry should be performed.
- Resolve protein structure to find the specific binding site, interaction & behaviour with amino acids.
- Perform Rubic screen (Thermoflour) to analyse the stability with various amino acids.

### **Outcomes of the studentship/Grant contribution:**

I have had the opportunity of working on multiple projects and have learnt a variety of skills from both my project and of those of my colleagues. I also explored many new techniques, which I wouldn't have learnt without the support of the TMG Lab group and that of the Biochemical Society. I am very certain given this wonderful experience that I had, that I want to pursue a career in biochemistry and biotechnology.

### Value of the studentship:

This studentship has left me very comfortable and confident in a lab environment which is not just because of the many new techniques I've learnt rather, but the first-hand experience of the scientific method – including planning, executing and optimising an experiment, collecting and interpreting the data, troubleshooting, and finally presenting the results, was the key. This experience has helped to reinforce my interest in scientific research and motivated me to seriously consider pursuing a Ph.D.

### Acknowledgements:

I would like to thank the Biochemical Society for a Summer Studentship for supporting and Dr. Tracey Gloster, Dr. Stephen McMahon and Dr. Verena Oehler for supervising me.

**Submitted by Aishvi Jain** Indian Institute of Technology, Delhi, India

### Summer Studentship Report: The Notch Signalling Pathway (Alex Davies)

The Notch signalling pathway is a core metazoan signal transduction system with both developmental and tissue homeostasis functions. Over the past 8 weeks I have been looking into the structural and functional importance of membrane binding by the Notch ligand human (h) Jagged 1. This has involved a detailed experimental study of hJagged 1 variants (extracellular NE3 fragment) expressed as His-tag fusion proteins using common biochemical techniques, bioinformatic comparisons between hJagged 1 and the *Drosophila* homologue Serrate and a background literature research into lipid binding properties of Notch ligands. There was also an opportunity to research genetic disorders associated with Notch ligands such as Alagille syndrome and extra hepatic biliary atresia (EHBA) and identify ligand variants found in various cancers (COSMIC database).

Early procedures carried out included transformation of NEB 5-alpha competent *E. coli*, with a wild type hJagged1 plasmid, selection of antibiotic-resistant colonies and making a Maxi prep of this plasmid. The concentration of wild type plasmid was measured and then sent off for DNA sequencing to ensure that this contained the correct DNA sequence for hJagged 1, before using it as a template. The sequence was confirmed by importing data into SNAPGENE. DNA Primers encoding two cancer-associated missense mutations identified in COSMIC were designed and ordered. These mutant DNA primers encoded A58V and D72N substitutions. An overlap extension PCR mutagenesis was then performed with PHL-seq, A58V and D72N forward and reverse primers. Initial cycles of this procedure involved generation of A58V and D72N left and right arm products. Later cycles involved self-priming and exponential amplification of left and right arm PCR products to produce whole insert DNA fragments encoding each variant. Sizes of PCR products at every stage were confirmed using agarose gel electrophoresis and a commercial DNA ladder. Both mutant products were then purified (to remove KOD thermostable polymerase) using gel extraction. A digest using *Eco*R1 and *Kpn*1 restriction enzymes was performed on the PCR products and the pHL-seq expression plasmid and confirmed with agarose gel electrophoresis. Backbone and insertion products then underwent a gel extraction procedure following band excision and were then ligated together using T4 DNA ligase. Mutant plasmids encoding A58V and D72N Jagged1 variants were then transformed into NEB 5-alpha competent *E.coli*. Colonies from each plate were expanded and a midi plasmid prep was then performed for both mutants. The concentrations of plasmid for each colony were then measured using Nanodrop and sent off for DNA sequencing. This was to check that each expression plasmid contained the correct mutation of interest before transfection could take place.

Human embryonic kidney (HEK293T) cells grown in a tissue culture flask were split 1:4 and allowed to grow until they were 80%-90% confluent and ready for transfection. A small scale transfection was performed initially using both mutant plasmids, a wild type positive control (wt Jagged-1) and a negative control with no DNA. Samples of conditioned media and cell lysate were taken after 4 days and analysed by reducing SDS PAGE and Western blotting to confirm wild type and mutant protein were produced. In each case a band of similar size to the wildtype Jagged-1 extracellular fragment was present in the media and was recognised by an anti-His tag HRP-conjugated antibody. A large-scale protein preparation of the D72N Jagged 1 variant was then carried out to obtain purified protein for a series of functional assays. The two purification methods used were metal affinity chromatography and gel

filtration. A Notch activation assay using a split luciferase reporter was then performed to test the activity of purified D72N Jagged1 ligand. The D72N sample was tested against a negative control F207A Jagged1 which does not interact with Notch, a wild type Jagged1 positive control, and further controls (0 cells, 0 ligand).

Good amounts of wild type and D72N and A58V plasmids were obtained (and dissolved to give solutions at ~150-850ng/ul) which showed that transformation and plasmid purification procedures were successfully performed. DNA sequencing of each confirmed the insertion of each mutation by overlap extension PCR was successful. Western blotting results following the small scale transfection of HEK cells with both A58V and D72N plasmids showed good yields of protein in the extracellular media. This demonstrated that each introduced missense mutation had not destabilised the core structure of the C2 domain resulting in misfolding and cellular retention. Due to time constraints only the D72N variant was taken forward and a large scale transfection of HEK cells performed in preparation for protein purification. Reducing and non-reducing SDS-PAGE of pre- and post-purification D72N samples showed that both metal affinity chromatography and gel filtration procedures were successful in obtaining D72N protein of good purity (>90%) for functional assays. I was able to perform the Notch activation assay by coating a 96-well plate with purified D72N mutant protein to which I added Notch reporter cells. In this experiment the D72N showed similar luciferase activity to wild type protein and considerably more activity than negative controls including the F207A variant. These results showed that the Notch activation assay was successfully carried out, and that the D72N variant did not appear to affect Notch activation substantially. Further repeat assays will be required to titrate the Jagged variants with the Notch reporter cells and to allow statistical evaluation. The second variant A58V can be purified using the same protocol and its ability to activate Notch compared with D72N. Further functional assays can be carried out to investigate lipid binding and Notch binding of both A58V and D72N mutants.

Overall I have gained invaluable practical experience during the last 8 weeks with the Handford Lab. Procedures such as overlap extension PCR, plasmid preparation, transformation of *E.coli*, together with an introduction to tissue culture work and transient transfection techniques, protein purification by different types of chromatography and use of a luciferase reporter assay has prepared me well for my final year project at university. I am much more confident making up solutions, and preparing appropriate dilutions of stocks. I understand the value of keeping an up to date account of my day- to day lab work which records exactly the concentrations of reagents used, and the conditions under which the experiment was performed. Furthermore, participating in an active research project has given me an excellent insight into the world of biochemistry research, which will be important when deciding what I wish to do next after finishing my undergraduate course in Cardiff. I would like to say thank you to everyone in the Handford lab, particularly Yao Meng my day to day supervisor, for allowing me to contribute to their research over the last 8 weeks. I would also like to thank the Biochemical Society for their Summer Studentship support.

Alex Davies



### **Developing Ligands to Interact with i-Motif**

Student: Daniela Stoyanova, Supervisor: Dr Zoe Waller, School of Pharmacy, University of East Anglia

### **Background:**

DNA sequences rich in Guanine and Cytosine are able to form alternative secondary structures called G-quadruplex and i-motif. The G-quadruplexes have been shown to exist in human cells and stabilisation of the structure had led to downregulation of gene expression<sup>1</sup>. The i-motif structure is formed in regions of the DNA rich in cytosine. This fourstranded structure is composed of parallelstranded DNA duplexes held together in antiparallel orientation by intercalated C-C base pair interactions. Sequences that have can potentially fold into i-motif structure are found across the whole genome, especially in regions that control gene expression. Molecules which bind i-motif could be used for up- or downregulation of a specific gene, which can have a significant result on the development of a disease. However, there are currently very few ligands that are known to specifically bind i-motifs. This is why the focus of this project is to identify ligands that have affinity and specificity for i-motif structures.

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

- Identifying new ligands that bind the i-Motif structure in different DNA sequences through Thiazole Orange (TO) Fluorescent Intercalator Displacement (FID) assay and FRET based DNA melting.
- Testing the activity of the new ligands against other secondary DNA structures, such as G-quadruplex and Double Stranded, in order to determine their affinity for biding to different structures.

### Methods:

### Thiazole Orange (TO) Fluorescence Intercalator Displacement (FID) Assay:

Thiazole Orange (TO) is not fluorescent on its own, however, upon binding to i-motif its orientation changes which causes it to fluoresce. TO is able to bind and dissociate from this complex easily, hence why it was used in this assay. By adding a ligand that binds i-motif, TO is displaced and a loss of fluorescence is observed. A limitation to this method is the fact that the compounds tested must not be fluorescent in the presence of the buffer as it might give misleading results.

### Fluorescence Resonance Energy Transfer

(FRET) Assay: This assay was used to determine whether the compounds tested are able to stabilise i-motif and to increase the melting temperature ( $T_m$ ) of the DNA. The change in the melting temperature ( $\Delta T_m$ ) was calculated to provide comparison between the different ligands and the different DNA sequences used.

### Results & Discussion:

FID: Four compounds were tested against the i-motif forming sequence called Deathassociated Protein (DAP), using 50 mM Nacaco/100 mM KCl as buffer at pH 6.8. Sodium Cacodylate was used at 50 mM as the ligands tested contained functional groups that could change the pH, making it more basic. As the i-motif becomes less stable under more basic conditions, the functional groups in the ligands could have led to the unfolding of the structure and ultimately to giving false positive results. Mitoxantrone was used as appositive control in this experiment, as it is known to bund i-motif and displace TO. Unfortunately, none of the compounds showed any significant displacement when

they were compared to the positive control, which was able to displace 50% of the TO at 1.88 µM concentration. The experiment was repeated for the same four ligands, using different i-motif sequences (hTelo-C and cMyc-C) and respectively, the appropriate buffer conditions. The ligands did not show any significant displacement, even at high concentrations of 90 µM. The precursors of the four ligands were tested for binding affinity against DAP, as hits from high throughput screening were found to have similar structures. Unfortunately, those compounds did not show any displacement of TO either and the highest concertation at which they were tested was 45  $\mu$ M.

FRET: FRET based DNA melt was performed for 8 compounds in total. They were tested against the DAP i-motif forming sequence at pH 6.8 in 10 mM Sodium Cacodylate buffer. Unfortunately, none of the tested compounds showed any stabilisation of the structure. This could be due to a solubility issue, inappropriate buffer conditions or lack of activity of the compounds. The experiment was repeated for the same compounds using 10% DMSO to improve the solubility of the molecules and it was also done at pH 7. Despite the changes to the experiment conditions, the potential ligands did not show any stabilisation of i-motif and the  $\Delta T_m$ calculated was between 0 and 1, which was within error.

### **Future directions:**

The compounds tested did not show any significant binding to the i-motif structure of different DNA sequences. FID and FRET might not be the most appropriate techniques for identifying their activity, hence why other types of assays can be used in the future. The structures of the compounds can be further modified and new experiments can be conducted. Overall, in order to understand the activity of those compound further more in depth analysis of their properties should be performed.

### Departures from the original proposal:

The primary proposal was to synthesise analogues of a compound that had previously showed i-motif binding properties. However, a few analogues had already been synthesised by a PhD student in Dr Waller's research group, hence why the focus was on testing the activity of the compounds. As they were not showing significant To displacement in the FID assay, FRET was also used.

### Impact of the studentship:

Working in Dr Waller's lab for 6 weeks allowed me to gain a better insight of what it is like to do independent research. Everyone I worked with was extremely helpful and provided me with loads of support whenever I needed it. I am really happy that I took this opportunity because I am now much more confident in my practical skill, which would benefit me when completing my final year project. As a Pharmacy student, I believe I now have a much bigger appreciation for all the work carried out when developing new compounds. Overall, this experience has made much even more motivated to peruse a PhD and a research career after completing my degree!

### Statement by Supervisor:

Daniela really worked hard in this placement. In addition to the data she has described here, she also learned other techniques the group were doing at the time, so she is aware of other techniques as well. Moreover her literature searching skills have also improved, through finding ligands which bind i-motif which have been published in the literature. She was an asset to the group and would really do well in a research environment, if this I the path she chooses on completing her training as a Pharmacist.

### **References:**

 G. Biffi, D. Tannahill; J. McCafferty, S. Balasubramanian, Nat. Chem. 2013, 5, 182.

### The self-assembly of tau proteins into paired helical filaments

Student-Bronwen Foster; Supervisor-Louise Serpell; Mentor- Youssra Al-Hilaly



### Introduction

The pathology of Alzheimer's Disease (AD) is characterised by the accumulation of AB protein into plagues, and the presence of neurofibrillary tangles (NFTs) composed of tau protein assembled into paired helical filaments (PHF) in neural tissue. This, in turn, leads to the neurodegenerative features of the disease which includes oxidative stress, inflammation and compromised axonal transport - eventually resulting in neuronal cell death [1]. Tau protein, a product of the MAPT gene, has a low molecular weight and is found natively unfolded in the normal brain with a natively unfolded structure. Alternative mRNA splicing generates six different isoforms of tau varying in their length. The C-terminus contains three or four imperfect tandem repeats of a 31 or 32 amino acid sequence [2]. Tau plays an important role in microtubule assembly and stabilisation which is vital for effective axonal transport and signal transduction [3]. The PHFs formed from misfolded tau protein are made from two protofilaments exhibiting a ribbon-like structure with a cross- $\beta$  arrangement.

There are two main hypotheses to explain the molecular event(s) which trigger the formation of PHFs; the hyperphosphorylation and the truncation of tau protein [3]. It has been shown that hyperphosphorylated tau in AD models exhibits mislocalisation of tau to the dendrites which interferes with synaptic function by impairing glutamate receptor trafficking [4]. Equally, truncated tau may represent an early neurotoxic form which could act as a central element for the assembly of endogenous tau into NFTs [5]. In vitro forms of full-length tau have been studied extensively to ascertain how they form NFTs. However, often these are made with the anionic cofactor heparin which is thought to template assembly [6]. dGAE, is a truncated form of tau protein comprising of 95 amino acids and corresponds to residues 297-391. dGAE can self-assemble into filaments without the addition of anionic cofactors and these filaments not only share characteristics with amyloid, but also resemble the PHFs found in AD brains [2]. In order to directly compare the PHFs in AD brains with those formed by dGAE, we decided to characterise the dGAE PHFs using transmission electron microscopy (TEM). Images allowed us to measure the width of the twisted segment, the untwisted segment and the distance from one twist to the next (crossover repeat) on the PHF.

These measurements have previously been taken from AD brain PHFs and also other in vitro tau samples [6, 7, 8, 9] which seem to form a consensus for a measurement of ~10nm for the twisted segment, ~20nm for the untwisted segment and between ~65-80nm for the crossover repeat.

### Methods

### Assembly of truncated tau fibrils

310  $\mu$ M of dGAE with 10 mM of DTT was incubated in 10 mM phosphate buffer, pH 7.4 at 37 °C with agitation at 400 oscillations per minute (Eppendorf thermomixer C, Eppendorf, Germany) for 48 hours.

### Negative Stain TEM

Preparation of electron microscopy grids involved placing a sample of 4  $\mu$ l of dGAE sample onto formvar/carboncoated 400-mesh copper grids (Agar Scientific). Excess sample was blotted and was then washed with 4  $\mu$ l of 0.22  $\mu$ M filtered milliQ water. 4  $\mu$ l at 2% w/v of uranyl acetate was added to the grid and left for 1 minute before blotting.

The grid was then allowed to air-dry. Immunogold Labelling of Tissue

Sections for TEM

Minimal cold fixation and embedding protocols for TEM as previously achieved [10] were used for preparing AD hippocampal brain tissue blocks and an established methodology for immunogold



labelling was used [11]. PBS+, a modified phosphatebuffered saline at pH 8.2 was made and used for all following procedures; immunoreagent dilutions, rinsing and all dilutions of antibodies and secondary gold probes. Thin sections were assembled onto TEM support grids which were then incubated with normal goat serum (1.10 dilution) for 30 minutes at room temperature in order to block the binding of non-specific secondary antibody binding. Grids were then labelled with T22 (1.1000 dilution) and incubated overnight at 4°C. The sections were rinsed 3 x 2-minute with PBS+. They were then immunolabelled with GaR10 secondary probes (all 1.10 dilution), for 1 hour at room temperature. After 3 x 10minute PBS+ and 4 x 5-minute distilled water rinses, the grids were then post-stained in 0.22  $\mu$ M-filtered 0.5% (w/v) aqueous uranyl acetate (positive stain) for 1 hour. The grids were then rinsed a further 5 x 2-minutes with water before allowing to dry.

### TEM Imaging

TEM projection images were collected using a JEOL JEM1400-Plus Transmission Electron Microscope operated at 120 kV equipped with a Gatan OneView camera (4kx4k). Images were recorded at 25 fps with drift correction using GMS3. 25 images of AD brain sections were analysed from

### three different cases and one control. Two grids of dGAE were analysed.

### **Results**

Twisted segment (red arrow), untwisted segment (green arrow), crossover repeat (blue bracket)



- Twist comparison –

of difference -1.984 to

1.2 – 6.876, significant

- Crossover repeat

2.646, ns



Figure 1a). (left) Paired helical filament formed from dGAE sample (Tau 15 C3 2)

Figure 1b). (above) Paired helical filament labelled with anti-tau antibody T22 in a section of AD brain tissue.

Analysis of dGAE vs AD PHFs



From this research, we have shown that the truncated tau, dGAE, closely resembles PHFs of tau found in AD brains. This was achieved without the addition of heparin and so this finding is significant as most in vitro tau fibrils have been made with the addition of heparin to replicate PHFs of tau. Due to dGAE forming PHFs without the addition of heparin, it calls into question as to whether the phosphorylation of tau is necessary to precede the formation of PHFs in the disease pathology of AD. The hyperphosphorylation of tau protein seen in AD brains could be explained by a neuroprotective response. Tau

### fibrils in the NFT may be phosphorylated in order to hide the toxic tau species [12], after the truncation of tau has occurred. Our research provides support towards this theory as we have shown that in vitro truncated tau can form into PHFs, similar to AD brain PHFs, without aggregation inducer.

### **Future directions**

Our research project has shown that this truncated tau protein is able to form PHFs without the addition of anionic cofactors and that these are highly comparable to the PHFs found in AD brains. Therefore, this can further support truncated tau as being the trigger for the formation of PHFs. dGAE can also be used in future research to ascertain the effect of tau aggregation and potential drug targets which could act as toxicity inhibitors, potentially reducing the damage caused to brain tissue.

### Departures from original proposal

Straight filaments were not measured due to time constraints. Likewise, more time is required to analyse more PHFs of AD brain sections for statistical analysis.

### Value of studentship

Working amongst fellow students and researchers has taught me key skills necessary for a future career in research. It has enlightened me as to what is necessary to create a robust and thorough research project which is invaluable as I embark on my 3<sup>rd</sup>-year research project in the same lab group. The connections I have made both within the lab and across the School of Life Sciences has allowed me to broaden my network within the scientific community, which will further support me in advancing my research skills. Though challenging, I have greatly appreciated and enjoyed the training provided to use the TEM which gualifies me to use this apparatus in future projects. Having the opportunity to participate in research in an area I am particularly interested in has confirmed my ambition to work in this field.

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### Study of ATP-induced inhibition of cytochrome *c* oxidase Student: Diana Andreea Catana Supervisor: Dr. Amandine Maréchal



In scientific research, all conducted experiments have to exhibit three main features: validity, reliability and replicability, all of which pertain to a precise experimental design. The aim of my project was to study the phosphorylation-dependent allosteric ATP-inhibition of yeast cytochrome c oxidase (CcO) which implied acquiring laboratory skills and learning the basic concepts of experimental design.

### Experimental design, results and discussion

Three major questions arose during my project. Is ATP the sole cause of the reported inhibition of CcO or do artifacts of measurement also contribute? What is the effect of posttranslational modification on this inhibition? Is the inhibition the result of ATP binding to the enzyme and if so, can we get information on its binding site? Different caveats were identified (**Fig. 2**) that highlighted the important controls for the validation of my data.



Enzyme activity was measured with a Clarke-type oxygen electrode in the following conditions: 3.2nMCcO,  $50\mu$ M cytochrome *c* and 1% Tween 20 in a 50mM potassium phosphate buffer at pH 7.4 and 20°C. The effect of ATP was assessed from the lower  $O_2$  consumption rate measured after addition of 5mM ATP to the solution.

Fig. 1- Main questions driving the experiment and their respective caveats

To confirm that the observed inhibition of CcO was solely influenced by the addition of nucleotides, appropriate tests and controls were employed. Firstly, all reagents were prepared in the same buffer solution, any pH changes arising during the preparation of stock solution were carefully corrected to exclude any possible effect of pH.

Additionally, attention was paid to potential changes in ionic strength for it is known to dramatically affect CcO activity simply because the enzyme binds to its substrate (cytochrome *c*) via electrostatic interactions. Hence, control experiments mimicking the ionic strength change induced by addition of nucleotide were performed with potassium chloride (41.7 mM KCl to mimic 5mM ATP).

We also tested two different systems to reduce cytochrome c and induce the O<sub>2</sub> reduction reaction: 18mM sodium ascorbate or a combination of 2mM sodium ascorbate and 40µM TMPD (N,N,N',N'-tetramethyl-p-phenylenediamine). The latter method is the most widely used in the literature that nevertheless has a flaw as it hides the true electron transfer dependency between cytochrome c and CcO as TMPD can donate its electron directly to CcO, bypassing cytochrome c which results in higher turnovers being recorded.<sup>1</sup> We confirmed this behaviour and used the high ascorbate electron donor system in subsequent experiments. However at such a high concentration, ascorbate autooxidation couldn't be neglected and we found a way around this by measuring a baseline of oxygen consumption in presence of sodium ascorbate before inducing the reaction by addition of CcO hence redesigning our experiment workflow.

At that stage, we confidently confirmed that CcO inhibition was solely due to ATP. Next, was to test the effect of phosphorylation or dephosphorylation on this inhibition. Sub-experiments were carried out to test the effect of incubation time on CcO activity as well as the effect of additional reagents required for the generation of two batches of enzyme only differing by their phosphorylation state (cAMP, pyruvate kinase, phosphoenolpyruvate). Through statistical analysis, it was confirmed that there is essentially no difference between the samples that have been incubated the same amount of time with the different reagents used for phosphorylation/dephosphorylation of CcO. However, the activity of CcO slightly decreased with incubation time highlighting the importance of parallel null samples and other controls to measure the true 100% activity of the enzyme as the phosphorylation/dephosphorylation protocols unfold.

My data show that (**Fig. 2A**) the ATP inhibition is conserved throughout the experiments, regardless of the incubation time. Furthermore, the phosphorylated sample displayed higher activity than the native enzyme which can be explain in two ways. Firstly, the phosphorylated yeast CcO could lose the ATP-induced inhibition as the additional phosphate would impede nucleotide binding.<sup>2</sup> Secondly, it is also possible that the high concentrations of cytochrome *c* in the solution (50 $\mu$ M) relieve the allosteric inhibition.<sup>3</sup> A simple repetition of the experiment at a lower concentration of cytochrome *c* would allow definitively to conclude on this. Also, in both incubations incubations it can be seen that the activity of CcO is similar between the native and dephosphorylated state, suggesting that the native state of our yeast is unphosphorylated.



*Fig. 2 – Summary of results. (A) Turnovers of yeast CcO in different phosphorylation states measured in the high ascorbate electron donor system (B) ATR-FTIR spectra of ATP induced changes of bovine CcO in different phosphorylation states* 

Finally, I used Attenuated Total Reflectance-Fourier Transform InfraRed (ATR-FTIR) spectroscopy to study ATP binding to CcO. Three different ATR-ready samples of bovine CcO were prepared: native, phosphorylated and dephosphorylated using a protocol established in the lab and whose description was previously published.<sup>4</sup> All reagents used were complexed with potassium ions in order to minimize the presence of sodium or calcium ions which could induce structural changes in CcO and yield false positives.<sup>5</sup> Furthermore, the ATP binding sites were cleared of cholate used during purification of the enzyme by incubating the enzyme overnight at 4°C with cardiolipin in a molar ratio of 40:1 cardiolipin:heme  $aa_3$ .<sup>6</sup> Also, it is noteworthy that phosphorylations are unstable modifications and hence layer preparation and spectra recording were done on the same day.

The enzyme was kept in its oxidized state by perfusion of a buffer containing 1mM ferricyanide supplemented or not with ATP to record the ATP binding or unbinding IR spectra of CcO, respectively. This procedure was repeated for each sample of CcO (native, phosphorylated, dephosphorylated) (Fig. 2B).No significant differences can be seen when comparing the three IR spectra. This could arise from a lack of posttranslational modification due to a failing protocol including the layer preparation protocol that could be too harsh and remove any phosphorylation of the enzyme. We can nevertheless conclude that in the native condition, no conformational change was associated to ATP binding ruling out binding site for ATP on the enzyme.

### Future directions and departures from original proposal

Multiple mutant yeast strains should be tested using the logic presented above. For example, different subunit KO mutants could be tested or different small molecules can be tested for allostery. Also, the experiment detailed here could be reproduced to generate more than 3 repeats for each activity assay or IR spectra recording to support and verify our conclusions. Due to time constraints, comparative studies between bovine and yeast CcO or between different yeast mutants were not carried out.

### Acknowledgements and value of the studentship

I would like to thank Dr. Amandine Maréchal, Dr. Andrew Hartley, Dr. Nikos Pinotsis, Prof. Peter Rich and William Zhang for all the advice, support, trust in me to pursue a research project nearly from scratch and encouragements to be as independent as possible. I am grateful that I was given the opportunity to design my own plan for the experiment, research different protocols in the literature and face problems that I would not have encountered just by sitting in a lecture theatre or reading more theory. In my opinion, this studentship helped me develop in more than one way: I am handier and more confident while working in the lab, I learned how important proper planning is and how experimental design, if neglected, can lead to a lot of unwanted complications. In this paper, I wanted to share the essence of the science behind the project, which is how to answer a question while trying to rule out as many confounding variables as possible. I would also like to thank the Biochemical Society for supporting me financially to pursue my dream of becoming a scientist.

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### Summer Vacation Project: Control of BK channel trafficking by protein de-acylation

Principal investigator: Prof. Mike Shipston Daily Supervisor: Ms Heather McClafferty Student: Mr Derrick Chi Ho Tang



### Introduction:

S-acylation (i.e. S-palmitoylation) is a process for lipid modifications on membrane proteins, not limited to the membrane surface expression of calcium-activated voltage-gated potassium (BK) channels, in general, Sacylation can also be involved in cellular transport and signalling. S-acylation is a reversible process controlled by enzymes for S-acylation and de-acylation, and these enzymes are referred as acyl thioesterases. Pilot data from Shipston lab suggested that de-acylation enzyme, ABHD17a may have a role in controlling the membrane surface expression of BK channels.

### Aims:

To interrogate the role of ABHD17a enzyme, firstly, we aim to test whether the process of ABHD17a dependent de-acylation influences the surface expression of BK channels. Importantly to clarify, we are interested in determining whether the de-acylation of specific cysteine residue on the BK channel are important for protein surface delivery. Secondly, we aim to define whether ABHD17a primarily controls surface delivery or internalisation of the BK channel.

### Methods:

On-cell Western (OCW) biochemical assay for ABHD17a on BK channel surface expression:

Multiple channel and ABHD17a mutants are co-expressed in HEK293 cells in conjunction with a channel surface expression assay that enables screening (96-well plate format). Signals from the epitope tagged BK channels are scanned on Licor. The ratio of surface (determine by extracellular flag-tag) to total (intracellular – HA tag) channel expression is used for analyses.

### Acyl-resin-assisted capture (RAC) for examining the effect of de-acylation on BK channel:

HEK293 cells co-transfected with channels and ABHD17a enzymes and mutants, are lysed, blocked using MMTS and precipitated. After cleaving of thioester bonds, the precipitated proteins contain free cysteine thiolates will be captured by thiopropyl sepharose beads. Samples are analysed on 10% SDS-PAGE gel and probed with antibodies. Signals are quantified using Licor and measurements are exported into Excel. Background is taken as the reading from the NaCl control pull down lanes and is subtracted from the HA pull down lanes before dividing HA pull down by the input value. This gives an image ratio percentage of pull down to input values and allows comparison across experiments.

### **Results:**





### (Fig 1): Top. ABHD17a-D19aa mutant

increases the Zero channels by 20% while that the WT enzyme increases by 80% and 160% in the case of C53:54:56A (C3A). Also, Strex channel is increased by 30% by the WT enzyme and the effect is markedly reduced in the mutant enzyme, while the StrexC3A is broadly unaffected by the WT enzyme. Interestingly, Strex(C12:13A) channel is increased by 70% by the WT enzyme but brought back to normal in the enzyme mutant. (Fig 2) Bottom. The results show that co-transfection with ABHD17a reduced Sacylation of the Strex channel by 80% compared to its pcDNA3 control, and similar drop was observed in that of the mutant enzyme. For the Zero channels, the WT enzyme slightly reduces the S-acylation by about 15% and 30% in the enzyme mutant when compared to its own pcDNA3 control. However, large error bars are observed across experiments.

### Future direction:

The work has provided new insight into the potential role of ABHD17a thioesterases in the control of BK channel surface delivery and trafficking through both S-acylation dependent and independent mechanisms. A key goal is now to exploit the data in this project to better understand the molecular mechanism controlling surface expression by ABHD17a and potential substrates and pathways involved.

### Value of Studentship to me and the lab

### Student:

Over the 8 weeks, I learnt to apply my knowledge and lab skills and develop further in a professional setting. I would like to give me special thanks to my daily supervisor and my principal investigator, Ms Heather McClafferty and Prof. Mike Shipston for their extremely friendly hospitality and patience throughout. With the full support from both the Biochemical Society and members from Shipston lab, I have equipped myself with the necessary knowledge and techniques and have found my passion in biomedical sciences. I am excited and looking forward to pursuing a career in laboratory-based research after graduation.

### Supervisor:

Derrick was a very enthusiastic, proactive and engaged student in the lab and quickly mastered a range of complex assays. He worked extremely well in the laboratory – both independently as well as seeking advice as required. He generated some interesting and high quality data from a number of screens that have provided new insights into the control of channel trafficking and functions of the ABHD17a family of thioesterases. He wrote up his report independently and showed a talent for laboratory research.

# Defining optimal combinations of an ATM inhibitor with radiotherapy in PTEN-dependent prostate cancer

### Student: Caitlin Hounsell Supervisors: Dr Kelly Redmond and Professor Kevin Prise

### Aims:

Prostate cancer is the most prevalent cancer in men (1), with one in eight men suffering from prostate cancer during their lifetime within the UK (2). Radiotherapy is a commonly administered treatment method for localized and advanced prostate cancers; however, the development of radioresistance is common. This can be due to various mechanisms such as a mutation or inactivation of phosphatase and tensin homolog (PTEN) which leads to a poor prognosis and a higher chance of tumour recurrences post-treatment. Ataxia telangiectasia mutated (ATM) inhibitors have previously been shown to radiosensitize cancer cells to radiation treatments (3) and show potential as a novel treatment method for PTEN-deficient prostate cancers (4). Preliminary work within this group indicates ATM inhibitor, KU-60019, is less toxic in normal prostate cells than in prostate cancer cells. The aim of this project was to determine if there is a PTEN-dependency on the radiosensitivity caused by ATM inhibitor, KU-60019, when used in a combination with radiation treatments.

### Materials and Methods

### Cell lines:

Prostate cancer cell lines NT01, PTEN expressing, and sh11.02, PTEN-deficient, isogenic forms of cell line DU145, were used throughout this project.

Inhibitor:

ATM inhibitor, KU-60019, was used throughout this project.

Clonogenic Cell Survival Assay:

Cells were seeded into 6-well plates to predetermined cell densities. 1  $\mu$ M ATM inhibitor, KU-60019, was administered 1 hour prior to treatment with radiation. Plates were irradiated using the Radiation cabinet at the CCRCB, Queen's University Belfast. Doses of 0, 0.5, 1, 2, 4, and 8 Gy were administered. Cells were left to proliferate for 7-9 days at 37 °C. Colonies were stained using 0.4% Crystal Violet and 95% methanol. Colonies greater than 50 cells were deemed as a surviving colony and were scored manually. Surviving fractions were calculated by dividing the number of surviving colonies at a given radiation dose by the number of surviving colonies at the unirradiated dose.

### Immunofluorescence:

Cells were grown on coverslips inside 6-well plates. Cells were treated with 1 µM ATM inhibitor, KU-60019, 1 hr prior to radiation treatment. At specific time intervals (1hr, 4hr, 24hr) post radiation treatment the media was aspirated, and the cells washed with PBS. Cells were fixed with 50% methanol 50% acetone for 8 minutes at room temperature. Cells were permeabilised with 0.5% Triton X-100 for 8-10 minutes at 4 °C. Cells were then blocked with blocking buffer (0.2% skim milk, 5% serum, 0.1% Triton X-100 in PBS) and left for 1 hr at room temperature. Cells were incubated for 1 hr in y- H2AX and 53BP1 primary antibody, washed and then further incubated in the dark for 1 hr with secondary antibodies. Foci were visualised using a fluorescent microscope (Zeiss).

### Western Blots:

Cells were pelleted by centrifugation at 2000rpm at 4 °C for 5 mins; ensuring that all media is removed. They underwent further ultracentrifugation for 1 min. The samples were frozen, then resuspended in 150  $\mu$ l RIPA + PI. The samples underwent freeze/thaw then were incubated on ice for 30 mins and then spun in a microcentrifuge at 13.2k rpm at 4 °C for 20 mins to pellet cell debris. Predetermined protein quantities were added to 5  $\mu$ l western loading buffer, and the proteins were loaded at 95 °C for 5 minutes. The samples were loaded

onto 10% SDS-PAGE gel and ran for 2 hr, then transferred onto a nitrocellulose membrane and incubated at 4 °C in primary antibody overnight. They were then washed and incubated in secondary antibodies for a further 1 hr. The images were then visualised.

### Assessment of Results:



Fig. 1 A). Curve of clonogenic cell survival of NT01 seeded at different cell densities. B) Curve of clonogenic cell survival in both cell lines, NT01 shown in black and sh11.02 shown in blue, with or without the treatment of the inhibitor KU-60019.

performed using cell line NT01, seeded at different densities to optimize the method for clonogenic seeding of this cell line for subsequent assays involving the ATM inhibitor, KU-60019. The ATM inhibitor, KU-60019, has been shown to radiosensitize both cell lines. This outcome follows from previous work showing that PTEN and ATM are synthetically lethal. At the concentration of inhibitor used, 1  $\mu$ M, there is no significant difference between the two cell lines radiosensitivity.





Fig. 2 A) Mean number of 53BP1 foci counted at specific time points post 1Gy radiation in both cell lines, NT01 and sh11.02. Foci were counted with and without the presence of the ATM inhibitor, KU-60019. B) Mean number of  $\gamma$ -H2AX foci counted at specific time points post 1Gy radiation in both cell lines, NT01 and sh11.02.C) Images taken of foci at 24 hr post-irradiation in both cell lines. Images of treated and untreated foci are shown.

The PTEN- deficient cell line, sh11.02, is more susceptible to DNA damage after treatment with the inhibitor, KU-60019, followed by radiation (Fig.2). There are greater numbers of foci visible for the inhibitor treated cell lines compared to the solely irradiated cell lines, the greater number of foci indicate a greater number of DNA damage points.



Fig 3. Western Blot analysis of DU145 prostate cancer cell lines NT01 and sh11.02 showing PTEN expression. Cell line sh11.02 is a PTEN knockdown cell line showing little PTEN expression compared to NT01 PTEN expression. Western analysis performed for cell line validation. Equal loading was confirmed using B-Actin expression.

### Future Direction:

Following on from this project it is necessary to show a decreased amount of activated ATM in the cells treated with the ATM inhibitor, KU-60019; to prove that the inhibitor is having the desired effect. Currently the data collected shows a similar radiosensitization effect in both cell lines, reducing the dose of inhibitor administered (a high dose of  $1\mu$ M was administered) will give a clear indication if the inhibitor radiosensitizes the PTEN-deficient cell line, sh11.02, more, as previously hypothesized (4).

### Departure from original proposal:

The prostate cell line DU145 was used instead of the originally proposed prostate cell line PC-3, as this enabled the investigation of the PTEN dependency of the ATM inhibitor radiation



combination proposed. The ATM inhibitor KU-60019 was used instead of its newer version, AZD0156, as this was consistent with the previous work carried out by this group. It was also important to ensure that the inhibition effect was seen in the DU145 cell line before using the new inhibitor, AZD0156, on this cell line.

### Value of the studentship:

This project has given me the opportunity to work semi-independently in a research environment learning many new techniques which I wouldn't have otherwise been able to learn. Not only have my laboratory skills vastly improved but I have also gained knowledge into the processes and theory behind cancer research. Throughout this project I have been able to meet other member of the multidisciplinary research group and fellow students, who have been able to give me firsthand insights into research. As part of this project I was able to take part in a poster presentation presenting my work. I will be able to take many of the skills that I have learnt in the lab and apply them to my own degree programme and project.

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#### INSTITUTE OF INTEGRATIVE BIOLOGY

Exploring the role of proteoglycans and glycosaminoglycans in thoracic aortic aneurysm and dissection



Student: Christopher James Steel Supervisor: Dr Hannah Davies

#### Introduction

Thoracic aortic aneurysm (TAA) is defined as a bulge in the aorta greater than 1.5 times its normal diameter. This bulging greatly increases the risk of delamination, dissection and rupture. All of which come with an increased risk of mortality and the significant burden of morbidity (Booher, et al, 2011).

There are multiple congenital syndromes associated with an increased risk of TAA. Conditions such as bicuspid valve syndrome (BAV) greatly increase the chances of an individual developing TAA (Braverman, et al, 2005). The increase in likelihood can be attributed to an interaction of genetic and haemodynamic changes created by the change in valve anatomy (Mathieu, et al, 2015). Furthermore, the biochemical changes which lead to the aortic wall becoming compromised are relatively poorly understood. The medial pooling of glycosaminoglycan's (GAGs) within the aorta have been suggested to result in intramural stress and Donnan swelling pressures. Stress which may be sufficient enough to disrupt aorta homeostasis, thus leading to delamination and/or aneurysm (Roccabianca, et al, 2014). In support of this, a recent study showed an increase in the expression of the chondroitin sulphate rich proteoglycans versican and aggrecan in TAA patients (Cikach, et al, 2018). Currently to our knowledge, there are no studies investigating GAG pooling between the aortopathie patient subsets we plan to investigate.

#### Aims and objectives

Investigate whether GAG/PG pooling differs between two different aortopathy patient subsets and whether this pooling will contribute to altered vessel wall integrity leading to aortic dissection. These subsets will be coined aortopathy A and aortopathy B. Histological techniques will be employed to analyse tissue samples. Furthermore, dot blotting and electrophoresis will be carried using papain digested tissue samples to investigate differences in GAG composition between the patient subsets.

### BIOCHEMICAL SOCIETY

### Methods

### **Tissue samples**

Ascending aortic tissue samples were provided by Liverpool Heart and Chest hospital. The samples were taken during aortareplacement surgeries. Post-surgery the tissues were snap frozen in liquid nitrogen, fixed and paraffin embedded. For each analysis carried out, aortopathy A and aortopathy B patient subsets were compared and analysed.

#### **Histological analysis**

#### Alcian blue GAG analysis

6um paraffin embedded transverse sections of aorta were fixed to slides. Slides were then deparaffinised and hydrated to distilled water. Sections were then bathed in a 1% (v/v) alcian blue, 3% (v/v) acetic acid pH 2.5 solution for 30 minutes. The slides were then washed in running tap water for 2 minutes before counterstaining in a 0.1% (v/v) nuclear fast red, 5% (v/v) aluminium sulphate solution. Following this the samples were further washed in running tap water for one minute. Sections were then dehydrated and cleared in xylene before mounting and cover slipping.

### Fluorescence acrylamide carbohydrate electrophoresis (FACE)

Homogenised papain digested aortic tissue was measured into 25ul aliquots. 25ul of a saturated solution of 4-amino naphthalene 1,3 di-sulphonic acid was added to each aliquot and incubated overnight. Following this, the samples were electrophoresed using GlycoMap acrylamide gel (Jackson, 1998) at 250V at  $4^{\circ}$ C for 3 hours.

#### Dot blots

Homogenised papain digested tissue samples were analysed using glypican I and glypican III primary antibodies separately. In addition, heparinise III digested tissue was analysed using heparin sulphate 3G10 primary antibody. 10ul of sample was blotted onto a nitrocellulose membrane and allowed to dry. Membrane was then incubated in a 5% serum PBS solution for 1 hour. A 1:1000 dilution of primary antibody, PBS-T was then used to incubate the membrane overnight at 4°C. Primary antibody was washed off using 3x15 minutes washes of PBS-T. Star bright 700 secondary antibody was then used at a 1:10000 dilution. The membrane was incubated with this solution for 1 hour in the dark. Dot blot visualisation was carried out using ChemiDoc MP imaging system using the StarBright B700 presetting. Image Lab software was then employed for analysis. The volume tool was used to analyse the intensity of the spots, this produces a "adjusted volume" value which was used to quantify the brightness of the dot.

#### Thresholding

Alcian blue stained sections were imaged using a Nikon Eclipse Ci microscope coupled with DS-Fi2 camera using the 10x objective. Multiple adjacent images were taken across the cross-section of the vessel wall and stitched together using NIS Elements image analysis software (v4.13.03). GAG content was analysed using the

thresholding tool in FIJI. Three stitched images per patient were no variability between patients and thus no differences converted to 32-bit grey scale images. Percentage of dark pixels over a 55120 threshold were recorded, averaged for each patient and compared using Mann Whitney means comparison.

#### Results, discussion and studentship outcomes



Figure 1: Alcian blue staining of 6um section of aorta using a Nikon Eclipse Ci microscope coupled with DS-Fi2 camera microscope using the 10x objective. aortopathy A (right), aortopathy B (left).

Alcian blue staining showed clear patches of blue staining (figure 1), demonstrating and supporting the "pooling" suggested by Roccabianca and colleagues (2014). Interestingly, thresholding analysis and a Mann Whitney tes showed this pooling to be significantly different between patient subsets. This suggests that GAGs may be partly responsible for the pathogenesis of aortopathie A, however, may not be involved in the pathogenesis of aortopathie B.



Figure 2: Papain, heparinise III digested tissue dot blot using the heparin sulphate 3G10 primary antibody, imaged using ChemiDoc MP imaging system.



Figure 3: Papain, heparitiniase digested tissue heparin sulphate 3G10 dot blot image lab analysis boxplot

The dot blot of the heparinise III showed clear differences between patients (figure 2). Dot intensity analysis of the groups showed a higher level of heparin sulphate was present in the aortopathy B subgroup when compared with the A group. There was an extreme outlier within group A, this patient will be investigated further for any confounding factors which may explain this anomaly. The glypican I and III antibodies showed between the two aortopathy groups.

The FACE assay was unsuccessful. The protocol needing optimising for the patient tissue samples. Due to time restraints, unfortunately this wasn't possible.

#### **Future directions**

By identifying that GAG pooling seems to be exclusive to aortopathy A, further exploration should be carried out to identify the composition of the GAGs. Further investigation may reveal specific GAGs or PGs of interest, thus elucidating a potential new target for TAA drug therapy.

#### Value of studentship

Student: The 8-week internship has given me the chance to gain valuable experience in working in a research laboratory. I have competence in multiple gained histological. immunohistochemical and electrophoretic techniques. Furthermore, the placement has allowed me contribute towards producing data aimed to be incorporated into a manuscript for publication. I am very grateful for the opportunity, help and support given to me by Hannah. Additionally, I would also like to thank the Biochemical society for funding the project.

Supervisor: This was a valuable studentship and generated exciting data for the project. We are currently expanding this work to investigate a larger cohort and study the role of specific GAGs and PGs in a variety of aortopathies. We are hoping to include this data in a publication in the near future. Chris has gained important lab experience and a range of skills which he can use in his future career.

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### **Biochemical Society Summer Vacation Studentship 2018**

### Tetraspanin-7: a novel autoantigen in Type I Diabetes

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### Introduction

Type I diabetes (TID) is an autoimmune disease that causes destruction of insulin-producing beta cells in the islets of Langerhans by the immune system. This consequence is observed when autoantibodies against multiple islet autoantigens are present in the blood of patients thus enhancing the risk of developing TID. There are four major autoantigens established - insulin, glutamate decarboxylase, IA-2 and zinc transporter-8. Recently a novel autoantigen was discovered – tetraspanin-7. Tetraspanin-7 is a 38-kDa multipass transmembrane glycoprotein with neuroendocrine expression and was confirmed as an autoantigen by demonstrating binding to autoantibodies in blood serum from TID patients. It consists of four transmembrane helices, three cytoplasmic domains and two extracellular domains. Previous studies carried out within the group using chimeric constructs of tetraspanin-7 and the closely related but non-immunogenic tetraspanin-6 indicated that the major antibody-binding regions are located within the cytoplasmic domains.

### Aims and Experimental Approach

The aim of my project was to confirm the findings that the cytoplasmic domains harbour the major antibody epitopes and to localise the antibody contact residues using sitedirected mutagenesis. We selected candidate amino acids for mutation based on sequence differences between tetraspanin-7 and tetraspanin-6 alongside epitope prediction software. We then performed mutagenesis of the wildtype tetraspanin-7 construct, and expressed the wildtype and mutated protein (bearing a luciferase tag) in mammalian cells. Antibody binding was assessed by luminescence immunoprecipitation assay (LIPS).

### Materials and Methods

### Tetraspanin-7 mutagenesis and expression

Based on differences between tetraspanin-7 and tetraspanin-6 and epitope prediction software, we selected the following amino acids for mutation: ME6,7, L15, T17, G83 and P85. Mutagenesis was performed using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent) with primers which were designed to switch the wildtype tetraspanin-7 amino acid to the corresponding residue expressed in the tetraspanin-6 sequence. DpnItreated PCR products were transformed into XL-10-Gold Ultracompetent cells, plated onto agar plates containing ampicillin, and incubated overnight at 37° C. The following day, colonies were picked into LB broth and cultured overnight. Plasmid DNA was isolated by miniprep, and DNA concentrations were measured. We then checked that the correct mutations were present in each of the colonies picked by sequencing the region of the plasmid where the Tspan7 gene was situated. The six different plasmid DNAs – WT, ME6,7LQ, L15F, T17S, G83A and P85A – were transfected into mammalian Cho-K1 cells in a 6-well plate (2x10<sup>5</sup> cells/well). The transfected cells were lysed after 48 h to harvest the luciferase-tagged antigen and the antigen recovery was determined by measuring the light units in each preparation.

# Measuring the capacity of the mutants to bind antibodies

In a LIPS assay, antigen is incubated with antibody and then precipitated with protein A-sepharose prior to filtration. Bound antigen is proportional to the light units detected/well. We incubated 200,000 LU of antigen in a 20- $\mu$ L volume with 5  $\mu$ L sera in 96-well v-bottomed plates overnight at 4° C. Serum samples included seven patients with TID known to have antibodies against tetraspanin-7 and one healthy control. The following day, we added 10  $\mu$ L of 50% Protein A-Sepharose (PAS) per well and incubated with shaking for 30 mins at room temperature. The immunoprecipitate was then transferred to a white filter plate, previously blocked with 0.5% BSA/PBS, and washed four times in PBS-Tween and twice in dH<sub>2</sub>O. After the addition of 40  $\mu$ L of Nanoluciferase substrate, the plate was read on a luminometer.

### **Results and Discussion**

It was previously shown that the third cytoplasmic region of tetraspanin-7 harbours essential amino acids (A243 and F240) for antibody binding. We wanted to investigate the roles of the first and second cytoplasmic domains in the formation of the epitope. The mutations were tested against seven sera from patients with diabetes and one healthy control. Light units were normalised to percentage antibody binding of mutants relative to wildtype antigen (Figure 1). Mutation P85A had little or no effect on antibody binding across all of the patients. Mutation G83A does not affect antibody binding in most of the patients but a reduction in epitope recognition of around 10-20% can be observed in patients 198 and 302. Mutation L15 has a significant effect on antibody binding in patient 198 where the reduction is 40%. It also affects patients 344 and 230 where the reduction is around 20-30%. Mutation ME6,7LQ affects patients 198,344 and 230 where a decrease of about 2025% is present. Therefore, the data from Figure 1 suggests that amino acids M6, E7 and L15 are part of the epitope or reside in close proximity to it.



**Figure 1** Influence of single amino acid substitutions on binding of human antibodies to tetraspanin-7; 331, 198, 240, 245, 302, 344, 230 – patient's sera positive for tetraspanin-7 antibodies.

In order to further understand which amino acids are key players in antibodies binding we used an *in silico* generated tetraspanin-7 structure and mapped the individual amino acids in the cytoplasmic domains that were tested (Figure 2).



**Figure 2** The figure shows the surface of the three cytoplasmic domains of tetraspanin-7. In orange are the amino acids that are not checked for antibodies binding; in yellow are the amino acids that are not essential for antibodies binding; in blue are the amino acids that are essential for antibody recognition

\*The image was generated by PyMOL and lacks the first 10 amino acids of the protein;

\*\*This is not a crystal structure but an in silico generated model.

The structure of the cytoplasmic domains shows that residues that are not in close proximity to F240 and A243 have little or no effect on Abs binding. This suggests that the epitope could be fairly conserved across different patients.

#### **Future Directions**

Following on from my placement, further study of the residues that are in close proximity to F240 and A243 could prove to be essential. These residues might be vital for the formation of the epitope despite the fact that they are conserved across tetraspanin-7 and tetraspanin-6. Residues that form a cleft with F240 and A243 are V11, I12 and T13, suggesting they might play a critical role in antibody binding.

### Deviation from original project

The original aim of the project was to investigate key residues in the epitope formation of Tetraspanin-7. Apart from the original project we also investigated the function and localisation of tetraspanin-7 by expressing a fusion protein of tetraspanin-7 and GFP. The images suggest that tetraspanin-7 was indeed localised on the cell membrane while the insulin granules (red) are localised in the cytosol together with the nucleus stained with DAPI (blue) (Figure 3)



**Figure 3** The picture shows Ins-1 mammalian cells that are expressing the fusion Tspan7-GFP gene; Green – Tspan7-GFP; Blue – nucleus stained with DAPI; Red – insulin.

#### Value of the studentship to the student

The studentship has provided me with an invaluable opportunity to spend 7 weeks in a laboratory during which I gained real research experience that will be extremely helpful for my final year research project as well as during my PhD. Being able to do the summer project helped me not only to facilitate my knowledge in biochemistry but also allowed me to experience the atmosphere of a research lab and to find out how research is planned, undertaken and concluded. I would therefore like to thank the Biochemical Society for funding my placement.

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 Walther, D., *et al.* Tetraspanin 7 autoantibodies in type 1 diabetes, Diabetologia (2016) 29:1973-1976 This summer I spent 2 months as an intern in the lab of Dr Salvador Tomas. The project I was working on aimed to quantify the binding properties of a certain ligand with its receptor and investigating what effect different conditions and concentrations would have on these properties. We were expecting to find a concentration at which the receptors in the membrane began to cluster together. Once this 'switching point' was found we planned to investigate the effect of sterols (estradiol and progesterone) on the switching point, to possibly be utilised as a sensor for these sterols.

The first step in the lab was to synthesise the ligand, PyCh (a pyridyl cholesterol), and then make a solution of it at known concentration. We also used NMR spectroscopy to assess the quality of the product. The receptor also required synthesis, but this step was carried out by my fellow intern. Once the synthesis was complete my first protocol involved making up serial dilutions of vesicles containing PyCh in the membrane, and titrating against a constant concentration of receptor. From here I used UV/Vis photospectrometry to investigate the level of binding and using various methods I used the gathered data to calculate binding constants. From here I carried out several similar sets of experiments to attempt to identify the 'switching point' where the receptor became clustered. These alterations included: using a membrane-bound receptor in vesicles to look at vesicle adhesion rather than binding of vesicle to receptor, using a different type of lipid in the membrane, using different methods for creating the vesicles and using different ratios of lipid:ligand.

We didn't manage to find a switching point as we encountered an issue with our experiment; the binding (particularly the kinetics) was difficult to investigate since when binding occurred the larger complexes formed then precipitated and sunk to the bottom of the cuvettes, making any UV/Vis data we collected unreliable. We found this by comparing a sample when it had just been vortexed and when it hadn't, and got a big difference in spectrum, indicating an issue with precipitation/solubility. Another possible problem we encountered was that the ligand may have had some issues in binding; a similar ligand with a linker region between cholesterol and pyridine could possibly have been more successful. We did however get some useful preliminary results indicating the rough conditions which led to successful binding.

In the future more research could be done if some of the issues we ran into could be overcome, and the original brief of investigating the effects of sterols on the switching point could be looked at to possibly use the difference in switching point as a sensor for these sterols.

There was departure from the original protocol as it took much longer to try and identify the switching point than we had hoped, and so we had to vary many conditions and parameters which we had hoped not to have to do. We also found that the blanks (made with cholesterol, not ligand) for the experiments showed some binding activity, so we did some investigation into the apparent binding of our blanks, as we hadn't expected any binding activity from them.

The grant has been really useful in shaping my aspirations as it allowed me to develop my experience and lab skills in a real laboratory, looking at interesting problems from a researcher's point of view. I wanted to do the internship to give me experience to allow me to make a more informed decision on whether or not I would like to continue working in science, and specifically in research, once I graduate and I now know that I would like to continue in this area.

### Paragraph about value of internship by Dr Tomas:

Mr Daniel Smaje project involved the synthesis of a family of molecules to be used as model ligands and receptors in the study of membrane adhesion. Experiments where to be carried out using lipid vesicles as a model membranes. During the first weeks of his internship, Mr Smaje successfully synthesised an purified using RP-HPLC the required molecules. In particular, the synthesis of the ligand molecule, which was unprecedented in our group, will enable to further our study of membrane adhesion in the near future. Furthermore, the direction of our research on membrane adhesion has been defined by the preliminary studies that Mr Smaje has carried out during this summer. He has characterized the binding of the membrane anchored ligand to a water soluble version of the receptor using UV-Vis spectroscopy techniques and has analysed the data using the appropriate mathematical binding models. He has also carried out initial studies of membrane adhesion, using the receptors and ligands anchored to different vesicles. These experiments have yielded invaluable data of binding affinity that will enable us to optimize the design of the system. In summary, Mr Smaje work represents the seed of a research project that we are confident will lead to high impact publication(s) and that represents a body of the preliminary data that will allow us to seek further funding to pursue this research project.

### Expression and Purification of BMP2 by Elizaveta Shavandina

### **Background**

Bone Morphogenic Protein 2 (BMP2) (Figure 1) is an important protein for the functioning of a human body. It is involved in the development of bones and cartilage, inducing osteoblast differentiation (Marie et al., 2002). The protein could also be used as a biotherapeutic to aid bone regeneration (Khan and Lane, 2004). BMP2 is glycosylated and the correct glycan pattern is vital for function of the protein (Hang et al., 2013), hence engineering BMP2 with correct glycans is essential for its use as a biotherapeutic.



**Figure 1.** Ribbon structure of Human Bone Morphogenic Protein 2 (PDB code: 3BMP)

To assess the glycan profile of produced BMP2, efficient purification of the protein is vital. This project attempted to optimize a method to purify BMP2

from HEK293T cell lines. It also assessed the BMP2 secretion efficiency of a HEK293T cell line stably transfected with BMP2 (B3 clone cells), compared to the wild type (WT) HEK293T cell line and transiently transfected HEK293T cells.

### Work Carried Out



samples from transiently transfected HEK293T cells with BMP2 gene-containing plasmids and GFP gene-containing plasmid and untransfected B3 clone cells.

### Nickel Affinity Purification

To determine the optimal imidazole concentration for the purification of BMP2 from cell culture media, a number of purifications were carried out on samples from WT HEK293T cells and BMP2 producing (B3 clone) HEK293T cells using Nickel Affinity method, with varied concentrations of imidazole. The outcome was assessed via a Western blot. Either higher (10 mM for loading and 15 mM for washing) or lower (5 mM and 10 mM) imidazole concentrations were used. In both cases, concentration of imidazole used for subsequent elutions was the same (500 mM). The blot showed a band of approximately 80 kDa in lanes with B3 clone purified samples, which could contain BMP2 (data not shown). This band was absent in elutions from wild type samples, which shows that B3 clones of HEK293T cells produce His-tagged BMP2 in contrast to WT HEK293T cells, as expected. The result also showed that lower imidazole concentrations used during the experiment gave higher purification efficiency.

Figure 2 shows results of a purification from HEK293T cells transiently transfected with BMP2 expressing or GFP expressing plasmids, with untransfected B3 as control. The

clear bands just below 75 kDa are assumed to be BMP2 (in the red box), as they are only apparent in the samples purified from BMP2-transfected cultures. BMP2 is predicted to have 44.7 kDa molecular weight, yet it is also post-translationally modified in vivo, which has previously been shown to shift its observed electrophoretic mobility to various values, including 50 kDa, 66 kDa, 80 kDa etc. (Wang et al., 1990; Felin et al., 2010). To reduce ambiguity, a different assay was used to accurately establish whether the B3 clone cells are producing BMP2.

### Luciferase Reporter Assay

This is a sensitive and specific assay to assess the level of active BMP2. In this experiment, Promega BrightGlo Luciferase Assay kit was used. As part of the assay, the BMP2 added to the cells can trigger a signal transduction cascade that results in luciferase expression, which catalyzes luciferin oxidation – a bioluminescent reaction. The

amount of emitted light measured using a luminometer is proportional to the added active BMP2. The results of this assay (Figure 3) clearly show that freshly purified samples contain more functional BMP2 than samples that were frozen after purification, implying that freezing negatively affects functionality of the protein. The assay also confirmed the results of the Western blot (Figure 2), showing that active BMP2 is produced by the cells. However, it is unclear how it compares to the levels of BMP2 from wild type (WT) cells from this assay, as, despite the average BMP2 levels seeming higher in wild type cells, error bars are also larger, suggesting the need

for more repeats to attain a more reliable result.

### Departures from the Original Proposal

Originally, the project was going to be focused on comparisons of the glycan profiles of BMP2 exogenously made in HEK293T and Cog4 knockout HEK293T cell lines, as well as the purification and glycan profiling of BMP2. The work carried out deviated slightly from the suggested plan as it was uncertain whether the HEK293T cells transfected with BMP2 expressing plasmid were generating BMP2 and whether the conditions used for its purification were optimal. Therefore, the focus of the project shifted towards mainly exploring the purification of BMP2.



### **Future Directions**

The experiments carried out can be taken

Figure 3. Luciferase Reporter Assay output, showing relative luciferase activity of each tested sample, which represents activity of BMP2.

further by performing more repeats of the Luciferase assay, which would serve better to reliably compare levels of BMP2 expression by different cell types. The productivity of the project can also be improved by using more reliable and time-efficient methods to detect BMP2 in the purified samples. Once it is certain that the purified samples of cell culture media from B3 clone cells contain sufficient amount of BMP2, they can be glycan-profiled according to the original proposal.

### Value of Studentship

The studentship has contributed significantly to my understanding of the biochemical field and academic research environment. This was a great learning experience that provided me with invaluable transferrable and laboratory skills, which will be extremely useful during my third-year group project, undoubtedly improving its flow and its outcomes. The project has inspired me to enter my third year of a biochemistry degree with confidence and determination, and furthered my interest in a career in research.

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# The Sainsbury Laboratory

### Biochemical investigation of evolutionary effector-target

interactions

Student – Esme Peers Supervisors – Abbas Maqbool and Erin Zess

### Introduction and Aims

A selective autophagy pathway common to both *Mirabilis jalapa* and *Solanum tuberosum* was the focus of this project. These plants are infected by oomycete species *Phytophthora mirabilis* and *Phytophthora infestans* respectively (Raffaele et al., 2010). Here, the pathogens release PexRD54, an effector protein, into the plant hosts. The role of *P. infestans* PexRD54 (PiPexRD54) is well characterised in potato, where it outcompetes the native cargo receptor, Joka2, for binding to the selective autophagy protein StATG8-2.2 through its ATG8 Interaction Motif (AIM) (Dagdas et al., 2016) (Figure 1). Thus the defensive pathway is disrupted, and nutrients are redirected from the plant to the infection site (Dagdas et al., 2018).



Figure 3 – Cartoon showing the binding of P. infestans PiPexRD54 effector protein to the selective autophagy protein, ATG8 in potato and displacing the native cargo receptor, Joka2. The amino acid sequence of the Autophagy Interaction Motif (AIM) is highlighted in the black circle

In contrast, the role of *P. mirabilis* PexRD54 (PmPexRD54) in *M. jalapa* infection is not known and this project aimed to investigate this. PmPexRD54

has a glutamate to lysine amino acid substitution in the AIM binding motif. Such a significant alteration in an otherwise highly homologous protein was intriguing, and subsequent *in planta* co-immunoprecipitation (Co-IP) assays showed no distinguishable interaction between PmPexRD54 and *M. jalapa* ATG8s (MjATG8s).

These Co-IPs form the rationale behind this project, which aimed to quantitatively characterize these interactions *in vitro* using isothermal titration calorimetry (ITC). In order to do this, the two effector proteins (PiPexRD54 and PmPexRD54) and three selective autophagy proteins (StATG8-2.2, MjATG8-I and MjATG8-III) were first purified before ITC experiments were performed. The hypothesis for this project was "PmPexRD54 binds with weaker affinity to MjATG8-I and MjATG8-III when compared to PiPexRD54 binding".

### Methods

<u>Expression of ATG8s and PexRD54s</u> – E.coli BL21 cells containing pOPINF (N-terminal cleavable His tag) constructs expressing the MjATG8-I, MjATG8-III, StATG8-2.2, PmPexRD54, or PiPesRD54 genes, respectively, were inoculated into 200 ml LB media with 1 mM carbenicillin selection and grown overnight (ON) at 37°C with shaking. Large scale 6 x 1L cultures of auto-induction media were inoculated with LB culture to an OD600 = 0.1 and grown for ~4 hours with shaking at 37°C until OD600 = 0.4-0.6, then grown ON at 18°C with shaking.

Cells were centrifuged at 4°C at 4200 rpm for 20 minutes and the pellets were resuspended in 140 mL A1 buffer (50 mM Tris-HCl, 50 mM glycine, 0.5 M NaCl, 20 mM imidazole, 5% (v/v) glycerol, pH 8.0) with EDTA-free protease inhibitor. Cells in suspension were lysed by sonication (40% Amplitude; pulse 1 sec on, 3 sec off; 4 min on-time) (whole cell lysate) then centrifuged (30 min; 18,000 rpm; 4°C) and supernatant retained (clear lysate). A 16% SDS-PAGE gel stained with instant blue was used to verify the protein of interest was expressed and present in the soluble form in both the whole cell lysate and the clear lysate.

<u>Purification of ATG8s and PexRD54s</u> – An on-bench nickel IMAC using a 5 mL HisTrap HP was used to purify His-tagged proteins from the clear lysate. The IMAC column was equilibrated with A1 buffer and the protein was eluted with elution buffer (A1 buffer + 0.5 M imidazole). SDS-PAGE analysis of all fractions was performed to confirm proteins were present in the elution fraction at the expected molecular weight. His tags were cleaved from the protein of interest in the elution fraction using 20 µl commercial 3C protease and left ON at 4°C. Buffer exchange was performed to replace the elution buffer with A4 buffer (20 mM HEPES, 0.15 M NaCl, pH 7.5). A second on-bench nickel IMAC using 5 mL His Trap HP removed the cleaved His tag from solution, and the untagged protein was collected in the flow through fraction. SDS-PAGE analysis of all fractions confirmed that His tag cleavage and removal was successful and untagged protein was present in the flow-through. Flow through fractions were pooled and concentrated to ~5 mL. Size exclusion chromatography using Superdex 75 26/600 gel filtration column pre-equilibrated in A4 buffer removed any remaining contaminants. Fractions containing the ATG8 protein of interest were pooled and concentrated as appropriate, and the final concentration was judged by absorbance at 280 nm (using a calculated molar extinction coefficient of each protein). The purity of proteins was judged by running 16% SDS-PAGE gels and stained with instant blue.

<u>Peptide Preparation</u> – Frozen aliquots of PiPexRD54 peptide sequence (KPLDFDWEIV) and PmPexRD54 peptide (KPLDFDWKIV) were thawed on ice and their concentrations determined using calculated extinction coefficients and a

NanoDrop<sup>™</sup> 2000/2000c spectrophotometer (Thermo Scientific) to measure absorbance at 280 nm. Buffer A4 could then be used to dilute samples to 1 mM and 2 mM peptide aliquots as required.

Isothermal Titration Calorimetry (ITC) – All ITC runs were performed using a MicroCal PEAQ-ITC (Malvern, UK) with a reference power of 5 and in A4 buffer. The calorimetric cell contained 300 μl of ATG8 protein at 90 μM and the syringe titrated in PexRD54 peptides at either 1 mM or 2 mM concentration as specified for an initial injection of 0.5 μL followed by eighteen 2 μL injections spaced 150 seconds apart. A pairwise analysis of the binding of ATG8s and PexRD54 peptides was performed at 25°C with a stir speed of 750 rpm as follows: PiPexRD54 (1 mM) vs StATG8-2.2; PiPexRD54 peptide (2 mM) vs MjATG8-I; PmRD54 peptide (2 mM) vs StATG8-2.2 and PmPexRD54 peptide (1 mM) vs MjATG8-I. All runs were repeated in duplicate and the resulting data integrated and fitted to a one-site binding model using in-built Microcal PEAQ-ITC analysis software to determine each interaction's thermodynamic parameters.

#### **Results and Discussion**

Purification of StATG8-2.2 and MjATG8-I to 90  $\mu$ M was successful, evident in the large clean peak of absorbance at 280 nm in fractions C2-C5 (Figure 2A) and C2-C4 (Figure 2B) respectively, recorded using AKTA after the final purification step with a Superdex 75 26/600 gel filtration column. This is supported by the 16% SDS-PAGE gel image stained with instant blue (Figure 2C) where all protein-containing fractions identified by AKTA yielded strong clean bands corresponding to ~12 KDa as expected for ATG8s.

These purified ATG8 proteins were then used in ITC experiments to compare their binding strength with PiPexRD54 and PmPexRD54 peptide swaps. Raw ITC data was integrated then fitted to a one-site binding model using in-built Microcal PEAQ-ITC analysis software (Figure 3). Full kinetic and thermodynamic data, calculated using Microcal PEAQ-ITC analysis software for all runs, is presented in Table 1.



Figure 4 – Purification of MjATG8-I and StATG8-2.2 by AKTA gel filtration was successful. Elution volume vs A<sub>280 nm</sub> was plotted by AKTA. (A) Peak of blue trace shows eluted fractions containing StATG8-2.2 after final S75 gel filtration. (B) Peak of blue trace shows MjATG8-I was eluted in corresponding fractions after final S75 GF on AKTA. (C) SDS-PAGE gel image of StATG8-2.2 AKTA gel filtration elution fractions C2-C5 in lanes 1-4; lane 5 empty and lanes 6-9 MjATG8-I AKTA elution fractions C1-C4.



Figure 5 –Isothermal Titration Calorimetry (ITC) was used to determine the binding affinities of ATG8 proteins to PexRD54s peptides. In each graph the upper panel shows the heat released on binding of protein to peptide whilst the lower panels gives integrated heats of injection with line of best fit (solid line) according to a single site binding model using MicroCal PEAQ-ITC analysis software. (A) - PiPexRD54 vs StATG8-2.2, (B) -PiPexRD54 peptide vs MjATG8-1, (C) - PmPexRD54 peptide vs StATG8-2.2 and (D) - PmPexRD54 peptide vs MjATG8-1. All ITC runs repeated in duplicate, representative data shown. Refer to Table 1 for thermodynamic parameters.

Initial buffer-buffer controls were conducted to ensure there were no buffer interactions and peptide was injected into buffer to control

for the effect of the heat of peptide dilution on experimental results. Both controls showed no prominent heat changes (data not shown).

The positive control was an ITC run to determine the binding parameters of StATG8-2.2 and PiPexRD54 peptide as published ITC data suggests a K<sub>D</sub> of 220 nM for this interaction (Dagdas et al., 2016). Furthermore, X-ray crystallographic studies show that StATG8-2.2 protein binds PiPexRD54 protein with a stoichiometry (N value) of 1 (Maqbool et al., 2016). N values can be calculated from ITC binding curves therefore N is a good initial indicator of the reliability of new ITC data and by extension the derived kinetic and thermodynamic parameters. Here, N values of  $1\pm0.3$  are considered acceptable. Unfortunately, the N values derived from the ITC run StATG8-2.2 vs PiPexRD54 peptide were too large and K<sub>D</sub> values were 0.001 nM and 98.4 nM in duplicated experiments

(Table 1). Therefore this data is assumed erroneous and the PiRD54 peptide used was likely partially degraded. Despite this, it was decided that the apparatus and technique were functional as heats of binding were clearly detectable using ITC.

Table 1 – Kinetic and thermodynamic data determined for a series of interactions between ATG8 proteins and PexRD54 peptides derived from isothermal titration calorimetry (ITC) data analysed using Microcal PEAQ-ITC analysis software.

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Protein	Peptide	Replicate	KD (nM)	N (sites)	ΔH (kcal/mol)	∆G (kcal/mol)
StATG8-2.2	PiPexRD54	а	0.001	1.43	-1.26	-16.4
StATG8-2.2	PiPexRD54	b	98.4	2.02	-1.63	-9.56
MjATG8-I	PiPexRD54	а	831	2.58	-1.67	-8.3
MjATG8-I	PiPexRD54	b	511	3.51	-1.06	-8.58
StATG8-2.2	PmPexRD54	а	7020	1.27	-2.12	-7.03
StATG8-2.2	PmPexRD54	b	5500	1.67	-1.85	-7.18
MjATG8-I	PmPexRD54	а	7840	1.04	-2.32	-6.97
MjATG8-I	PmPexRD54	b	9990	0.87	-2.79	-6.82

It was expected that PiPexRD54 would bind MjATG8-I with similar  $K_D$  values to StATG8-2.2 as MjATG8-I is a close homologue. The  $K_D$  of this interaction averaged 671 nM (Table 1), comparable to the positive control. However, again, the N values were unacceptable due to the partially degraded PiPexRD54 peptide. Drawing from prior Co-IP experiments, reduced binding affinity between PmPexRD54 peptide and StATG8-2.2 was expected. This is

supported by the ITC data, where K<sub>D</sub> values are in the micro molar range, averaging 6.3 μM (Table 1), thus supplying evidence in support of the PmPexRD54 AIM mutation being a major contributor to the reduced binding affinity observed in planta. Note

that only the N value for replicate 'a' (Table 1) was acceptable therefore these experiments should be repeated with a newly synthesised peptide to confirm the results.

Finally, the binding strength of MjATG8-2.2 and PmPexRD54 peptide averaged 8.9 μM (Table 1), comparable to that of PmPexRD54 peptide binding StATG8-2.2. N values for both replicates were also acceptable. As these K<sub>D</sub> values are within the micromolar range whereas PiPexRD54 binds ATG8s in the nanomolar range, further evidence is given that the single amino acid mutation in PmPexRD54's AIM motif is the likely cause of reduced binding affinity to MjATG8-I observed *in planta*. Therefore this data supports the original hypothesis "PmPexRD54 binds with weaker affinity to MjATG8-I when compared to PiPexRD54 binding".

### Future work

- To complete this project, the ITC work should be repeated with full length PiPexRD54 and PmPexRD54 proteins to obtain
  data of a higher quality with greater biological relevance. Furthermore, repeating this project with MjATG8-III (a clade II *M.
  jalapa* ATG8) in place of MjATG8-I (Clade I)) would give confidence that the hypothesis is supported for MjATG8s in both
  clades, as evidenced by prior Co-IPs.
- Obtaining X-ray crystallography structures for MjATG8-I binding PmPexRD54 would be valuable to identify differences in the mode of binding owing to the AIM amino acid substitution.
- Further *in planta* studies to investigate the ability of PmPexRD54 to out-compete the *M. jalapa* Joka2 homologue for the native MjATG8s would inform on the importance of the effector protein in the disruption of the *M. jalapa* selective autophagy pathway.

### Departures from the original project

I was unable to complete the final gel filtration purification of the full length effector proteins PmPexRD54 and PiPexRD54 due to AKTA unavailability within the time frame. Fortunately there were ample PexRD54 peptide freezer stocks so these were used in place of their full length counterparts.

Additionally, the MjATG8-III I purified was not at a sufficiently high concentration to achieve the 90 µM needed for our experiments. Over multiple attempts we had issues culturing MjATG8-III transformed *E.coli* in AIM media so poor protein yields were expected. I therefore focused on working with MjATG8-I.

### Value of the studentship to the student and the lab

I enjoyed my time in the Sainsbury Laboratory immensely and am grateful to my supervisors, Abbas and Erin and to the Biochemical Society for making it possible. Before being awarded this studentship I was shy in the lab, however now I feel confident to design and conduct my own experiments unsupervised which will prove invaluable for my final year research project. This experience also gave me the opportunity to learn theory and experimental technique beyond the scope of my course, taught me to read the literature with a critical eye and to present my findings to others with confidence, all of which will stand me in good stead throughout my university career.

The results of my project will contribute to the work of an ongoing PhD student's project and will influence the design of their subsequent experiments. I regularly contributed to discussion and asked plenty of questions in the weekly lab meetings to which I also presented my findings at the culmination of my project.

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### INVESTIGATING THE EFFECT OF A SMALL MOLECULE INHIBITOR OF THE EXTRACELLULAR MATRIX ON VEGFR2-DRIVEN SIGNALLING

Student: Grace Martin Supervisors: Stephen J. Briddon, Laura E. Kilpatrick and Chloe J. Peach. COMPARE, School of Life Sciences, University of Nottingham.

### Introduction:

There are many well-established growth promoting signalling pathways in cells, which operate through a complex, intertwined and heavily regulated system. When this is disrupted, cells can enter an uncontrolled state and become cancerous. Signalling cascades promoted by Vascular Endothelial Growth Factor Receptor 2 (VEGFR2), and its conjugate ligand VEGF-A, are important in cell migration, proliferation and angiogenesis (1). Many cancers develop sensitivity to VEGF-A signalling, causing the deregulated growth of dysfunctional vasculature and eventually tumour expansion. Although anti-VEGF treatments are available they have major undesired side effects, fuelling the need for new therapeutics. VEGFR2 is a transmembrane receptor tyrosine kinase that is situated upon the cell surface (2). Due to alternate splicing, its cognate ligand exists as several isoforms (those studied here are shown in Figure 1). It is thought that some ligands containing exons six and seven, for example VEGF<sub>165</sub>a and VEGF<sub>189</sub>a, interact with the extracellular matrix (ECM), or more specifically glycosaminoglycan (GAG) heparan sulphate, with high affinity (1). It is consequently predicted that the ECM can serve as a 'pool' for growth factor reserves, however there is limited understanding of the effect of GAGs on VEGFR2/VEGF-A pharmacology.

The aim of this project was to observe the effect of a small molecule inhibitor of GAGs, surfen (3), on the effect of downstream VEGFR2 signalling. This was quantified using a Nuclear Factor of activated T cells (NFAT) reporter gene assay. VEGFR2 activation by VEGF-A elevates intracellular calcium concentrations leading to the dephosphorylation of NFAT, which can then translocate to the nucleus whereupon it initiates transcription. In this assay, a luciferase gene was inserted downstream of the NFAT promoter allowing VEGFR2 driven NFAT activation to be monitored, though bioluminescence (4). These experiments would provide insight into the effect of the ECM on VEGF/VEGFR2 pharmacology, and in particular if this was VEGF-A isoform specific.



Figure 1: The isoforms of VEGF-A and their different mRNA domains resulting in alternative splicing. Domains 2, 3, 4 and 5 bind to the VEGFR2 receptor and it is thought domains 6 and 7 bind heparin. All isoforms shown here were used in this work.

### Methodology:

All assays used HEK293 cells stably expressing a Firefly luciferase downstream of the NFAT promoter (ReLuc2P, Promega Corporation), which were also stably transfected with VEGFR2. Cells were plated at 20,000 cells/well on 96 well plates for 24hrs, before serum starving with serum free Dulbecco's Modified Eagle Medium (DMEM). Cells were pre-incubated with surfen (10µM, 30mins at 37°C/5% CO2 in serum free DMEM/0.1% BSA) then stimulated with various isoforms of the VEGF-A ligands (0.1-10nM;5hrs at 37°C/5% CO2 in DMEM/0.1% BSA), in triplicate wells. ONE-Glo luciferase substrate (Promega Corporation) was added, and luminescence recorded using a TopCount platereader (Promega Corporation). Data were normalised to vehicle wells (0%) and 10nM VEGF<sub>165</sub>a (100%) and plotted as mean ± S.E.M. (n=5).

### Results and Discussion:

A significant decrease in the potency of VEGF<sub>165</sub>a in stimulating the production of NFAT was observed in the presence of the GAG inhibitor surfen (pEC<sub>50</sub> 9.4 $\pm$ 0.2 to 8.6 $\pm$ 0.2, \*P<0.05; paired t test; Table 1). In contrast surfen had no effect on the potency of VEGF<sub>189</sub>a induction of NFAT production, however a non-significant decrease in efficacy was observed (Emax 111.9 $\pm$ 8.5% to 72.3 $\pm$ 13.0%). No effect of surfen was observed on VEGF<sub>121</sub>a or VEGF<sub>165</sub>b responses respectively, consistent with these isoforms lacking the exons responsible for heparin binding (Figure 1). This is the first study to

VEGF-A	Control		10uM Surfen		
Isoform	pEC50	Emax %	pEC50	Emax %	
VEGF165a	9.4 ± 0.2	100%	8.6 ± 0.2	97.4 ± 13.4%	
VEGF165b	8.5 ± 0.4	96.5 ± 9.6%	8.1 ± 0.3	100.3 ± 9.2%	
VEGF189a	9.2 ± 0.2	96.0 ± 9.0%	9.12 ± 0.4	72.3 ± 13.0%	
VEGF121a	9.2 ± 0.1	111.9 ± 8.5%	8.7 ± 0.2	88.1 ± 9.9%	

Table 1: The potency ( $\pm$  S.E.M) and Emax% ( $\pm$ S.E.M) values of each VEGF-A in respect to stimulating NFAT production in the presence or absence of surfen (10µM). All responses were normalised to those observed for 10nM VEGF165a respectively (00%) and averaged from 4-5 independent experiments.

investigate the effect of GAG inhibition on the potency and efficacy of distinct VEGF-A isoforms at VEGFR2. These results show that the GAGs can influence ligand-VEGFR2 pharmacology in an isoform selective manner. Published observations of the affinity of VEGF-A isoforms for GAGs would suggest a greater effect of the GAG inhibitor surfen would be predicted on VEGF<sub>189</sub>a responses due to this isoform having the highest affinity of all VEGF-A isoforms for the GAGs (5). Although decreased efficacy in respect to NFAT production was observed with VEGF<sub>189</sub>a, no effect on potency was seen. Interestingly, there was a decrease in potency of VEGF<sub>165</sub>a in respect to NFAT production with surfen incubation. The VEGF-A isoforms used in this study were chosen to probe the importance of GAG binding domains within VEGF-A isoforms. The lack of effect of surfen on VEGF<sub>189</sub>a potency here may be due to ECM binding isoforms not being solely governed by the presence of heparin binding domains alone, but that the overall protein structure, length and tertiary shape of the VEGF-A ligands may also influence, ECM binding. In addition, the influence of non-specific effect of the inhibitor needs to be investigated.

### Departure from Original Proposal:

Preliminary project ideas involved investigating the effect of specific mutations of the VEGFR2 on signalling, however initial experiments to create cells expressing these mutations were unsuccessful.

### Future Directions:

The influence of the extracellular matrix upon growth factor signalling is of great therapeutic interest due to the documented changes in the ECM observed within the tumour microenvironment. Although it has been shown that GAGs can increase VEGFR2-/VEGF-A signalling, the mechanisms controlling this are still unknown. The use of other heparan sulphate inhibitors such as heparinase enzyme could be utilised and compared to the results here using a small molecule pan GAG inhibitor (surfen). As I investigated the effects of GAG inhibition on signalling, future work could investigate effects on VEGF isoforms affinities at VEGFR2 and its coreceptor NRP1.

### Value of Studentship- Student:

Undertaking the summer project has been an invaluable experience. I have been able to develop as a biochemist and feel confident working independently. It has provided me with knowledge and practical skills that will help me continue my studies, including cell culture, plate-based experiments using bioluminescence resonance energy transfer (BRET) and the chance to observe confocal microscopy. Through the placement, I have had the opportunity to submit an abstract to the British Pharmacological Society, with the aim to attend the December 2018 meeting and independently present my results in a poster. The placement has continued to enthuse my passions for research in the field of cell signalling. I thank the COMPARE research lab kindly, particularly Dr Stephen Briddon, Dr Laura Kilpatrick and Chloe Peach for their support, time and input throughout the project.

### Value of Studentship- Supervisors:

Grace was an excellent student who worked incredibly hard and produced some very high quality data. These have been accepted as a poster presentation for the

upcoming British Pharmacological Society meeting in December. These experiments provide important pilot data which will contribute to ongoing VEGF studies in the lab, and which would have been difficult to perform without the extra pair of hands Grace provided. Additionally, the project provided important supervisory experience to the post-docs and students involved.



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### Can mass spectrometry reveal the drug binding sites of multidrug transporters?

### Small-scale expression and purification trials

My name is Hannah Mbiwan, I will be starting my third and final year in Biochemistry at the end of September. At the end of my second year, I undertook an eight-week placement in the Kerr group, under the direct supervision of Dr. Ian Kerr.

**Aims**: The aim of this summer studentship was to do small-scale expression and purification of Sav1866 protein; a bacterial multidrug pump homologous to the human multidrug pump; ABCB1/P-glycoprotein. It was a trial and error experiment to determine if the membrane protein could be expressed and purified in large quantities.

**Materials and Methods**: The plasmids used already encoded His-tagged Sav1866 obtained through collaboration with Professor Malcolm East from the University of South Hampton, UK. Two *E. coli* strains; BL21.  $\lambda$ DE3 and BLR, were used to express Sav1866, where both expression systems expressed equal amounts of Sav1866. Hence, for the rest of the project, the BLR strain was used.

Once recombinant Sav1866 was successfully expressed, it was solubilized using styrene maleic acid (SMA), a copolymer. After solubilization, Sav1866 was purified using nickel or cobalt affinity chromatography. To optimize purification, different concentrations of imidazole were used during the washing and elution stages, two independent batches of SMA were used to compare solubilization and different resins were used. After successfully purifying Sav1866, the protein purification was scaled up by a factor of 6. Again, Sav1866 was successfully purified using the modified optimised protocol for protein purification.

**Results and Discussion**: Overall, the results were on track. The two expression systems used to express Sav1866 showed equal expression like figure 1 below. For this reason, the BLR strain was used for the remainder of the project.



Figure 1: Comparing the two expression systems. Membrane preps of BLR and BL21 P2 samples from week 1 and week 2 with varied protein amounts of 2, 5 and 10  $\mu$ g. The gel was stained with Instant blue and there was no different in protein expression.

Furthermore, three different types of resins; Ni-sepharose beads, Ni-NTA resin and Co-IDA resin were used to compare how efficiently they purified crude Sav1866. The Ni-sepharose beads were charged in the lab by incubation in NiSO<sub>4</sub> solution, while the latter two resins were commercially bought. Of the three resins used, Co-IDA was the worse at purifying the Sav1866. In fact, there was no purification, as all the protein that came off during the initial flow-through was unbound protein (figure 2). On the other hand, purification involving Ni-sepharose resin and Ni-NTA resin was more effective and the Ni-charged resins were indistinguishable in performance (figure 3).



*Figure 2: Western blot of Co-IDA purification.* The antibody used was anti-alpha His antibody, which was diluted at a ratio of 1:5000 in PBST/Marvel solution. All of Sav1866, membrane protein (62kDa) came off during the UB FT as unbound protein, hence soluble membrane and UB FT have identical bands.



Figure 3: Western blot of protein purification using Ni-sepharose resin (A) and Ni-NTA resin (B). Unlike the Co-IDA resin, both Ni-charged resins purified Sav1866. For both Ni-charged resins, the UB FT was always proportionally smaller than the band for the soluble membrane. However, the downstream band intensities for Ni-NTA resin were stronger than those for Ni-sepharose resin, corresponding to more protein coming off at those concentrations of imidazole.

**Future work**: It would be beneficial to compare Sav1866 solubilization using SMA versus using detergents to compare yield and purity of the isolated protein. Functional assays can be used to check the function of the protein. Since flow cytometry can be used to analyse the characteristics of drug pumps from mammalian cells, a similar assay could be investigated to characterize Sav1866 in bacterial cultures. And finally, the validation of my purified protein by mass spectrometry analysis and using fluorescence to observe Sav1866's interactions with different drug substrates.

**Evaluation**: My eight-week internship was not long enough, but I'm glad that I didn't deviate from my proposed plan. Overall, I thoroughly enjoyed my studentship in the Kerr lab. This opportunity gave me insight about laboratory work. I didn't feel like I had enough time to fully digest the information given to me during my second-year labs, as there were many students to attend to. However, my supervisor took time to answer my questions and I got many chances to make mistakes and retrace my steps as I moved forward. If anything, this experience has made me realize that I do want to continue working in a lab setting as I was able to polish my experimental skills, improve my calculations and start to plan and design my own experiments.



### Investigating the coding potential of SNHG5 (a lncRNA) in Colorectal cancer cells

Student: Hayley Macleod

Supervisor: Dr. Kellie Dean

School of Cell Biology and Biochemistry, University College Cork, Ireland

### Introduction

RNA has always been portrayed as inferior to coding-DNA due to the diverse functions not well understood but this is beginning to change. Long non-coding RNAs are essential for regulating gene expression in eukaryotes (1) but it is beginning to emerge that they have a role to play in the cancer disease state and display more complex functions than originally hypothesised (2). Small nucleolar RNA host gene 5 (SNHG5) is a long non-coding RNA that has been documented to be upregulated in Colorectal cancer (CRC) cells and promotes cancer cell



survival (3). Sucrose gradient data was closely examined which lead to the belief that there may be translating ribosomes associated with SNHG5 (3). To further investigate this lead, RiboGalazy was used to visualise ribosome profiling data from HCT116 CRC cells and a sORF (small open reading frame) of 35 amino acids was identified (4). The sORF SNHG5 is conserved throughout primates including humans, chimpanzees and gorillas. Using Computational peptide folding, SNHG5 took an alpha-helical structure which gives the impression of functionality to this peptide.

It can be hypothesised that SNHG5 is bifunctional with both a non-coding function as well as a coded, protein function. SNHG5 has been observed to have various functions, it works like a sponge to absorb microRNAs to regulate gene expression. It may also stabilize mRNA transcripts by interacting with SPATS2 (SNHG5 target transcript) and blocking the transcript degradation by STAU1 (RNA binding protein). These stable transcripts help to counteract apoptosis and promote CRC cell survival. The upregulation of SNHG5 allows the cell to bypass cell death and allow constant growth and proliferation resulting in cancer (3). This summer project looks to investigate the presence of a SNHG5 peptide from the previously documented "non-coding" IncRNA.

### Aims

The aim of this project is to investigate the coding potential of SNHG5 by attacking either GFP or a FLAG tag to the SNHG5 gene. If the peptide is translated so will GFP or the FLAG tag and this can then be detected. This would suggest SNHG5 may produce a peptide which could contribute to CRC cell survival.

### Methods

# Cloning (PCR, 2-step PCR, Colony-PCR, Restriction Digestion/ligation,Sequencing, Transformation)

Polymerase chain reaction (PCR) was carried out to isolate the fragments of SNHG5 that would be cloned into the vector. There were two sets of constructs made: SNHG5-EGFP and SNHG5-FLAG tagged vectors. The forward primer was mutated to knock out the start codon while the reverse primer excluded the stop codon to allow translation of GFP or FLAG. The primers also contained a Xhol (forward) and Bam(reverse) restriction site to allow for insertion into the vector. PCR, using these mutated primers allowed for the desired inserts to be created: FLAG tagged inserts: 5'-leader (unmutated start codon-AUG) SNHG5, 5'leader sequence (with mutated start codon ATT) SNHG5 insert, sORF SNHG5 insert. EGFP attached inserts: 5'leader-SNHG5 (AUG) fused with EGFP, 5'leader SNHG5(mutated start codon ATT) fused to EGFP, sORF SNHG5 fused with EGFP, EGFP-only (with start codon mutated to ATT).

Two -step PCR technique was carried out to anneal the SNHG5 segments to the GFP with overhanging primers to create complementary ends. Furthermore, the start codon of all EGFP segments was mutated. All these inserts were digested with Xhol and Bam and ligated into the pcmv-c-Flag vector, which (2µl) was transformed into DH5 $\alpha$  cells. Mini-preps extracted the plasmids and PCR screened for positive clones, pcmv-F1 and pcmv-R1 sequencing primers were used for SNHG5 Flag tagged inserts and pcmv-F1 and EGFP reverse primers for the SNHG5-EGFP inserts. Colony PCR was used along with the tradition PCR method to isolate positive clones. Positive clones were sent for sequencing, the samples with a clean sequence chromatogram with the desired mutations present were transformed into Hela cells.

### Fluorescence microscopy

The SNHG5-EGFP constructs were visualised under the fluorescent microscope to detect the green colour.

### Western blot

The HeLa cells were lysed with 75  $\mu$ l SDS with 30  $\mu$ l of each sample loaded onto a 12% SDS-PAGE gel. Milk was used to block any unspecific binding and anti-GFP primary antibody used for an overnight incubation. Anti-rabbit (donkey) secondary antibody was used to detect the GFP attached SNHG5 peptide.

### **Results and Discussion**

Fig 1: Transformed Hela cells under the fluorescent microscope after 24hrs



Fig2:sORF (SNHG5)- EGFP (left)and AUG-EGFP(right) second transformation visualised after



Fig.2: Shows the sORF(SNHG5)-EGFP (left) and AUG(SNHG5) –EGFP (right) after 48hrs, the green fluorescence was very visible for the sORF(SNHG5)-EGFP but the AUG(SNHG5)-EGFP did not enhance as predicted (the results of the other conditions remained the same as the 24hr incubated cells).

Fig1: GFP fluorescence was visualised after 24hrs incubation of HeLa cells. pEGFP-N1, positive control expressed high levels of GFP. sORF (SNHG5)-EGFP expression of GFP can be seen clearly but AUG(SNHG5)-EGFP expression was detected at an extremely low fluorescence at two different light intensities. Untransfected, ATT(SNHG5)-EGFP and EGFP (mutant start) negative controls gave no intensity.

The Hela cells were transformed twice with the same constructs and conditions but the second time they were incubated for 48 hrs instead of 24hrs before they were visualised. This allowed time for the GFP to enhance in the 5'leader-AUG(SNHG5)-EGFP sample as it was unexpectedly low after the first transfection. It was found that sORF (SNHG5)-EGFP fluorescent intensity was high as green cells were clearly visualised, therefore SNHG5 was translated into a peptide. 5'leader-AUG(SNHG5)-EGFP cells fluoresced green but at a very low intensity relative to the negative controls which gave no fluorescence. The longer incubation slightly increases the fluorescence in the 5'-leader-AUG(SNHG5)-EGFP but not as much as expected. This is interesting because the unmutated 5' leader sequence should aid in translation but seems to inhibit it in comparison with the sORF-SNHG5 result. The mutated start codon of the 5'leader-ATT(SNHG5)-EGFP sample gave no fluorescence proving the SNHG5 is the only functional start codon and if translation occurs both the SNHG5 and the GFP are produced. This validates the sORF-SNHG5 fluorescence as GFP can only be produced along-side SNHG5. The mutated start codon EGFP further indicated that only the SNHG5 start codon allows translation as no fluorescence is observed.

<sup>\*</sup>Different light intensitie

### Western blot

Fig:3 The transformed 24hr Hela cells were visualised on a western blot



Fig3: SDS-PAGE gel was used to visualise the transfected HeLa cells for the presence of GFP. The HeLa cells were lysed with 75  $\mu$ l SDS with 30  $\mu$ l of each sample loaded onto a 12% SDS-PAGE gel. Milk was used to block any unspecific binding and anti-GFP primary antibody used for an overnight incubation. Antirabbit (donkey) secondary antibody was used to detect the GFP attached SNHG5 peptide.

12% SDS page gel

sORF(SNHG5)-EGFP gave a band with the same size as pEGFP-N1 positive control (~27 kDa) showing EGFP was produced. This indicates SNHG5 was translated, as it contained the only functional start codon available. 5'-leader AUG(SNHG5)-EGFP may have been produced but at levels too low to be detected on a Western blot. EGFP fragments had a mutated start codon so GFP could only be produced from the start codon supplied by SNHG5. Both peptides must have been translated to see any green colour and to visualise GFP on a western blot. The intense green fluorescence from the sORF(SNHG5)-EGFP transformed HeLa cells and the strong band of GFP seen on the SDS-PAGE gel indicates SNHG5 was translated and is strong evidence towards a peptide formation. There may be many reasons the 5'leader-AUG (SNHG5)-EGFP did not express well in the HeLa cells, the 5'leader sequence may need some contributing factors to allow sufficient translation that may only be present in CRC cell lines.

### **Future Research**

The FLAG tagged constructs must be visualised on a Western blot to investigate if the large size of the GFP protein obscured the previous results. This would also give more evidence indicating if the peptide is truly translated. Once there is firm, compelling evidence that SNHG5 is bifunctional and produced a peptide as well as its RNA, the function of each must be clearly outlined. The peptide vs the RNA must be quantified in Colorectal cancer cells and the effect each on the cancer state must be researched. IncRNAs are emerging to be quite influential to the cancer disease state and it is essential to understand the role they play to fight harder against this monstrous disease.

### Value of Studentship to student and Dean lab

This Summer Studentship has been outstanding to boast my laboratory skills and confidence. The planning, preparation and execution is immensely beneficial going into my final year project and beyond. It has inspired me to continue onto a Research Master with this same project after my final year, so I can dive deeper into the intriguing nature of SNHG5 and cancer biology. This Studentship has opened many doors for me to follow my curiosity and intrigue to understand the complex nature of the cancerous disease state. The Dean lab has also benefited, as this was a completely novel project and the work completed has provided the first piece of experimental evidence toward the coding potential of SNHG5. This could be the next step to understanding Colorectal cancer clearer and more comprehensively.

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### Exploring Interprotein Electron Transfer by Light-driven Photoreduction



Student: Jack Jones

Supervisor: Professor Julea Butt University of East Anglia

### Background

The protein, small tetraheme cytochrome c (STC) is a periplasmic multiheme cytochrome (MHC) found in the bacterial species *Shewanella oneidensis* MR-1. *Shewanella oneidensis* is able to respire anaerobically by transporting electrons through the periplasm, out of the cell using the redox properties of MHCs such as STC.

STC is structured in a way whereby the longest dimension is a chain of four His-His ligated hemes whereby hemes 1,2 and 3,4 are perpendicular to one another whilst hemes 2, 3 are parallel, the crystal structure is displayed in *Fig.1*.



*Fig. 1: Crystal structure of STC from Shewanella oneidensis.* (PDB ID: 1M1P)

In this project two variants of photosensitised STC

were used. These proteins had a Ruthenium label attached to a cysteine residue near heme 1 at residue 10 or heme 4 at residue producing STC-10-RuMe and STC-87-RuMe respectively. When exposed to blue light and in the presence of a sacrificial electron donor (SED, in this case EDTA was used) the excitation of the RuMe- tag allows reduction of the nearest STC heme. Continuous irradiation will eventually lead to full reduction of the STC summarised in, *Fig. 2*.



# Fig. 2: Reaction scheme of RuMe- labelled STC exposed to blue light in the presence of a sacrificial electron donor.

### Aims

The aim of this project was to use absorbance spectroscopy to determine the rate of reduction for both STC-10-RuMe and STC-87-RuMe at different concentrations and with different concentrations of the sacrificial electron donor (EDTA). Furthermore, another aim was to determine the rate at which electrons can be passed from a RuMe- labelled variant of STC to wild-type (WT) STC.

### **Departures from Project Proposal**

In the proposal emphasis was placed on measuring the effect that differing pH's had upon the rate of photoreduction. Based on previously collected data as well as preliminary findings, the decision was made to instead concentrate on the effect of differing concentrations of protein and the sacrificial electron donor, EDTA.
# **Materials and Methods**

**Anaerobic conditions.** All experiments were carried out under anaerobic conditions in a glove box with nitrogen atmosphere and all solutions were purged with nitrogen before use. Before any experiments took place the oxygen scrubbing columns were fully regenerated prior to use. Oxygen levels inside the box were maintained at <3.5 PPM throughout.

**Standard photoreduction procedure.** Photoreduction experiments were performed using anaerobic solutions of the desired composition prepared by appropriate mixing of 20 mM TRIS, 100 mM NaCl, pH 8.5 and 0.5 M EDTA, 20 mM TRIS, 100 mM NaCl, pH 8.5 to which STC was added to the desired concentration. The spectra were measured using a Biowave II Diode-array UV/Vis spectrophotometer with an Omega Optical 475RB Notch filter fitted to prevent photoreduction of the protein sample from the spectrophotometer. The sample was continuously irradiated from above by a Thorlabs mounted LED ( $\lambda_{max}$  455nm) and spectra measured at the desired time points. At the end of each period of irradiation excess dithionite was added to ensure complete reduction of STC and the spectra measured once more.

**Standard data handling.** Prior to data quantification, the buffer-electrolyte +/- EDTA spectrum was subtracted from each of the protein spectra. To account for variation of absolute absorbance over time, the absorbance at 700 nm of the baseline corrected spectra were defined as zero. The protein concentration was then calculated from the absorbance at 552 nm. The % photoreduction was calculated from the absorbance at 418 and 552 nm by comparison to the corresponding values of the fully oxidised (i.e., prior to irradiation) (0%) and fully dithionite reduced (100%) protein.

# **Summary of Results**

**Rate at different [EDTA].** Quantitative analysis including the use of DynaFit 4 modelling software demonstrated that the initial rate of photoreduction of labelled protein increased with increase of [EDTA] to 500 mM, the highest concentration tested. For 500 mM [EDTA] complete reduction was generally observed after one hour.

*Rate at different [STC].* The initial rate of photoreduction of labelled protein was found to be independent of protein concentration for the range of 0.1 to 0.5  $\mu$ M.

*Electron transfer between STC-10-RuMe (STC-87-RuMe) and WT STC.* Both labelled proteins were shown to transfer electrons to WT STC. For a given concentration of labelled protein, the initial rates of photoreduction increased with increase of WT STC. This is consistent with the rate of WT STC photoreduction being defined by collisional, diffusion controlled encounter with the labelled proteins. Further experiments will be required to establish whether the labelled proteins behave differently as the final experiments proved inconclusive due to photodamage of labelled proteins.

# Value to the student

In making the decision to come to university to study biochemistry I had in my mind that I was going to pursue a career in research upon graduation but, until recently, I had not experienced what it was like working in a research environment. Being awarded a Biochemical Society Summer Studentship has been invaluable to me as it has given me the opportunity to experience this and has helped solidify in my mind that research is what I want to be involved in in my future career. In addition to all of this I really enjoyed my time in the lab and was amazed at the sheer amount of work and experiments that I was able to complete in my short time there.

# Value to the lab

The quantitative analysis of photoreduction rate on EDTA concentration has provided Professor Butt's group new insight into the rate determining steps of this process in ruthenium labelled multiheme cytochromes. I was able to demonstrate light driven protein to protein electron transfer for the first time for these systems. My findings are now being followed up by a more extensive study to inform the group's understanding of electron transfer in the *Shewenella* periplasm.

# Investigating the interaction between Sec-23A and N2-Src

Student: Jonathan Cope, Supervisor: Dr. Gareth Evans

# Introduction:

C-Src is a ubiquitous tyrosine kinase with a diverse set of physiological roles. In the brain, alternative splicing of the C-Src transcript gives rise to two further kinases, N1-Src and N2-Src, the functions of which are largely uncharacterised [1, 2]. Recent research has implicated roles of both N1- and N2-Src in the regulation of neuronal differentiation, by showing that inhibition of N-Src results in changes in neuronal morphology [3]. N-Src is also understood to play a role in neurodevelopmental disease; for example, high N-Src expression is associated with a favourable prognosis in neuroblastoma patients [4]. Therefore, a greater understanding of N-Src function may allow us to optimise how we tackle these diseases. However, the substrates upon which N-Src acts remain unclear.

Phosphoproteomic analysis carried out on N2-Src inducible HeLa cell lines, has enabled the Evans lab to identify a set of potential targets of N2-Src. Several of these targets are components of the COPII coat, involved in vesicular transport between the Endoplasmic Reticulum and the Golgi. Previous research has suggested that COPII transport may have a role in the regulation of neuronal dendrite morphology [5]. This may therefore suggest that N2-Src influences neuronal development through the COPII pathway.

Mutations to one specific subunit of the COPII coat, Sec-23A, have been strongly associated with developmental defects [6]. Furthermore, Sec-23A was one of the key potential targets of N2-Src identified in the phosphoproteomic screen performed by the Evans lab. Therefore, it is important to investigate the interaction and potential phosphorylation of Sec-23A by N2-Src, and whether this regulates COPII transport.

# Aims:

To subclone individual domains of Sec-23A (Gelsolin, Trunk,  $\beta$ -Sandwich) for bacterial expression.

To perform assays on the above domains to confirm that N2-Src binds and phosphorylates Sec-23A, and to establish which domain is phosphorylated.

To observe the effect of N2-Src expression on COPII transport in the presence and absence of Sec-23A knockdown by shRNA.

#### Methods:

#### Cloning and expression of Sec-23A domains:

Potential N2-Src phosphorylation sites of *Sec-23A* were identified using PhosphoSite. Primers were designed to generate PCR products encoding Sec-23A domains flanked by BamHI and Sall restriction sites. PCR products were cloned into a pGEX vector and used to transform BL21 competent cells. Protein expression was induced using IPTG.

#### Cell Culture and Transfection:

N2-Src inducible HeLa (N2-HeLa) cells were cultured in DMEM and split three times a week at a ratio of 1:3. Cells were counted prior to plating using a haemocytometer and were plated at a density of  $5 \times 10^4$  cells per well for a 6-well plate or  $10^5$  cells per well for a 24-well plate. Cells were transfected with Sec-23A shRNAs using PEI 24 h after plating and were treated with doxycycline to induce expression of N2-src.

#### Immunocytochemistry:

Cells were fixed using 4% PFA and were permeabilised in 0.1% Triton and 1% BSA. Cells were stained using primary antibodies (mouse anti-FLAG (1:1000), rabbit anti-GFP (1:1000)) in 1% BSA. Secondary antibodies were applied at a concentration of 1:500 in 1% BSA.

# **Results:**

Using PhosphoSite (a curated phosphoproteomic database), we identified potential N2-Src phosphorylation sites in each of the Sec-23A subdomains (Fig. 1A). Primers were designed and used to clone each subdomain using PCR (Fig. 1B). The PCR products were cloned into a pGEX vector plasmid and were subsequently used to transform BL-21 competent cells. These BL-21s were then induced to express the recombinant protein using IPTG (Fig. 1C). Unfortunately, there was not time to assess the proteins in binding and kinase assays.





To observe the effects of N2-Src expression on the cellular distribution of Sec-23A in the presence or absence of Sec-23A inhibition. N2-src inducible HeLa (N2-Hela) cells were induced to express FLAG-tagged N2-Src using doxycycline (Fig. 2). Cells were subsequently transfected with shRNAs targeting Sec-23A or control non-targeting shRNAs. However, due to the failure of the transfections (Fig. 3), we were unable to draw conclusions regarding the effect of N2-src on Sec-23A distribution. The transfections were believed to have been unsuccessful due to issues with the transfection reagent.



**Fig. 2. N2-HeLa cells show N2-Src expression in response to Doxycycline.** N2-HeLa Cells were incubated for 24 hours in the presence or absence of doxycycline. Anti-FLAG staining was used to detect FLAG-tagged N2-src. Scale bar = 20μm.



**Fig. 3. N2-HeLa cells failed to transfect with Sec-23A shRNAs.** N2-HeLa cells were incubated in the presence of PEI and either Sec-23A targeting shRNAs (Sh4) or non-targeting control shRNAs (Scr). shRNAs contained a GFP tag; anti-GFP staining was used to confirm expression. Scale bar = 20μm.

#### **Future Directions:**

Unfortunately, the original aims of the project were not achieved. However, the successful sub-cloning and expression of the Sec-23A domains will enable the lab to perform kinase assays to confirm the phosphorylation of Sec-23A by N2-Src, and to establish which residue is phosphorylated. The unsuccessful transfection experiments performed on the N2-HeLa cells should be repeated to establish the effect of N2-Src phosphorylation on COPII transport.

# Value of the Studentship:

Following this studentship, I feel confident in my ability to maintain cells in culture and to perform experiments to stain cultured cells for fluorescence microscopy. I am also now familiar with the techniques used for sub-cloning experiments. All of these techniques will be invaluable for any further cell/molecular biology research I undertake, including my final year project for my Integrated Master's degree. This studentship has also given me the opportunity to improve my organisational skills and I have discovered the importance of being thorough when it comes to keeping a lab book. In September, I attended the North of England Cell Biology meeting at the University of Huddersfield with Dr. Evans. It was fantastic to have the opportunity to listen to the PhD students at the meeting present their research, knowing that I may be in their position in a couple of years. I would like to thank the Biochemical Society this fantastic opportunity, as well as Dr. Evans for facilitating me in his lab and supporting me throughout my studentship.

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# **Background:**

When stimulated by physical pressure, plant cells have been shown to form of demonstrate organelle and cytoskeletal rearrangement<sup>1</sup> as during microbial invasion attempts. Cell Wall Appositions provide a physical barrier to penetration and a site for coordinating anti-microbial compounds and other defensive responses to the site of microbial invasion<sup>1</sup>. To systematically study the trafficking and localisation of proteins to sites of invasion, the Deeks laboratory (unpublished) has developed a technique to physically stimulate CWA formation during live cell imaging. Plant cells recognise Pathogen Associated Molecular Patterns (PAMPs) including chitin and Damage Associated Molecular Patterns (DAMPs) such as from enzyme mixtures such as Driselase<sup>2</sup>. Molecular pattern mixtures can elicit increased the response to physical cues (Deeks lab, unpublished). PEN3 is an ATP-binding cassette transporter localised to CWAs during invasion. PEN3 exports anti-microbial metabolites at several stages of penetration and pen3 mutants show reduced penetration resistance<sup>3</sup>. This project revolved around creating new workflows to aid future research, here applied to the localisation of PEN3 with and without molecular pattern elicitation and also to analysis of 'pausing' behaviour of touring particles that appear to be traficking FORMIN4. FORMIN4 is an actin binding protein shown by the Deeks group to modulate actin fillaments around CWAs and is important to penetration resistance<sup>4</sup>. Annecdotally, a small proportion of bodies carrying GFP labelled FORMIN4 move against cytosolic flow and could contribute to FORMIN4 cycling when they pause at existing build-ups of FORMIN4 around CWAs exchanging material. Col-0 is the standard wildtype genotype for Aravidopsis thaliana experiments.

# Aims:

 Determine value of further research into activity of touring particles on FORMIN4-GFP transfer. 2) Quantify rate of PEN3-GFP accumulation at site stimulation while optimising and establishing new workflows for similar future analyses.
 Train to use confocal microscopy to gather new data for analysis.

# Method:

# MP1 vs H2O elicitation response measurement

The Deeks Laboratory provided data for analysis; in hand confocal images of *Arabidopsis thaliana* hypocotyls taken each minute for 30-minute periods. *PEN3-GFP* mutants. Treated with either MP1 (molecular pattern mixture) or H<sub>2</sub>0. This was repeated with LatrunculinB (30 min prior to imaging). Images of Col-0 ecotype samples acted as negative controls. Images were initially processed to create series of maximum projections<sup>a</sup> from the Z-stacks<sup>b</sup> at each time point using batch processing then concatenating the output. Each maximum projection time series created was manually

checked for reaction centres against brightfield images and Regions of Interest (ROIs) were drawn each stimulation site. Slices of the stack that were out of focus were discarded. A series of macros were written in Image-J 1 (IJ1) macro language that measured the area surrounding the site. Similar processes in other areas of the same cells gave control values to compare the rate of fluorescence change against. Further macros measured the average pixel value of the cells for normalising data output. Background signal was established using maximum projections of areas of each image lacking cells and subtracted from output data. Percentage fluorescence increase was calculated for all reaction sites and control ROIs. Sites with significantly different (p=0.05) values to the corresponding controls were used for final comparison of H<sub>2</sub>O and MP1 treatments, the data incorporated into a mean fluorescence change at reaction sites from which the mean change at the controls was subtracted. Comparison of the H<sub>2</sub>O and MP1 treatments showed significantly different (p<0.05) percentage fluorescence changes. The Col-0 control showed no meaningful fluorescence heterogeneity. LatrunculinB controls of H<sub>2</sub>O and MP1 treated samples were not statistically analysed due to sparsity of data. All significance values derived from Mann-Whitney U tests.

Touring particle mediated FORMIN4-GFP) accumulation: TIRF microscopy FRAP images from previous FORMIN4-GFP studies conducted by the Deeks laboratory were provided. In this study, only images taken prior to bleaching were used. In each image, possible pausing events were found and recorded manually. A circular ROI was drawn around the static FORMIN4-GFP accumulation where the suspected pausing event occurred along with ROIs at 5 static FORMIN4-GFP depositions and 5 cell background locations visibly lacking FORMIN4-GFP build-up. For each suspect event, the roving body was tracked with a kymograph. A pausing event was defined when body clearly paused at the site and left with measurable frames before and after the event. A macro was written in IJ1 macro language that would measure the manually drawn ROIs of the event and control regions. In Excel, frames either side of the event and the control points were averaged, and the average cell background values were subtracted for background correction. The normalised change in fluorescence at the event locations was compared to that of the controls and found no significant difference (p=0.05) between the percentage fluorescence change at locations with pausing events and those without, tested with Mann-Whitney U tests.

# **Results and Discussion**

<u>MP1 vs H<sub>2</sub>O Elicitation of PEN3 accumulation</u>: Treatment of Arabidopsis cells with MP1 elicitor caused a greater rate of PEN3-GFP accumulation around the site (Fig. 1), at sites where any response was observed. There is a significant difference (p<0.05) between the two groups. Only sites with a definite reaction were used, as indicated in the method.



Figure 1: Mean greyscale value of maximum projection of PEN3-GFP fluorescence signal, background corrected and normalised against mean cell value. Cells treated with molecular pattern mixture (MP1) or H<sub>2</sub>O show significant (p=0.05) responses

This result aligns with previous findings from the Deeks laboratory (unpublished) that increasing molecular pattern treatment increases CWA related responses to physical stimuli. The negative control using Col-0 ecotype showed negligible fluorescence signal. The Latrunculin B treated cells could not be analysed in the same way as there were few reactive sites, as expected due to the depolymerisation of the actin network. Interestingly, one site appeared to show a spot-like fluorescence increase which could be due to static bodies exchanging material without actin-based localisation. It is suggested that this supports the theory that actin filaments facilitate trafficking of PEN3 carrying vesicles, once localised, material can transfer independently. Due to gaps in the in-hand data quantity it is not possible to quantify the difference between the rates of fluorescence accumulation under the two treatments for comparison with other data. Repeating this experiment with insights from the refining of this workflow will enable the next step, quantitatively establishing comparative effects of different treatments or genotypes. An important outcome of this research was the workflow and the code written that will hasten similar analyses in the future. The code has been annotated and archived for use on data being generated around the time of writing.

<u>Touring Body mediated deposition analysis:</u> There were no significant differences between changes in fluorescence of FORMIN4-GFP puncta where the pausing events occurred and those without. The sample size was small as, after gathering the data, kymographs were used to stringently check for this relatively uncommon event. There were only 4 examples (Fig. 2) analysable within about 20 images each with potentially hundreds of moving bodies. This supports to the conclusion that the possible effect of pausing events on FORMIN4 accumulation at CWAs is negligible. This allows future models and experiments to exclude this potential mechanism of trafficking.



Figure 2: Normalised brightness change before and after a pausing event at FORMIN4-GFP deposition spots where a pausing event has happened (red) and controls with no event (blue). A Mann-Whitney U test of comparing all groups against all controls shows no significant difference (p>0.05)

#### **Further directions:**

The main weakness of this project is quantity of data, more is currently being generated for variants of this experiment to shed light on factors affecting PEN3 localisation applying the new workflows to knockout mutants. The touring bodies section was successfully conducted to tie up loose ends and is unlikely to receive more attention.

**Departures from original proposal:** The programming was done in ImageJ1 Macro to avoid over-running by trying to fully-automate within MATLAB. The student was trained in confocal microscopy techniques however there was not time/personnel to collect new data.

Value of studentship: I gained valuable skills in image analysis, coding, and confocal imaging. It has been enjoyable working with Mike Deeks and other lab members, encouraging my interest in bioinformatics. For the lab, confirming the lack of contribution of pausing events to FORMIN4 deposition allows conclusions from previous and future experiments to be made more securely. Creating a detailed workflow for measuring fluorescence gives future data a common protocol for analysis.

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#### LARP1 regulates invasion and migration in cancer cells

Student: Matthew Jackson Supervisors: Dr James Chettle, Prof Sarah Blagden. Department of Oncology, University of Oxford

#### **Introduction**

RNA-binding proteins (RBPs) determine the translational efficiency and turnover rates of multiple cytosolic mRNAs, and commonly promote oncogenesis by driving dysregulated mRNA translation in cancer cells.<sup>1,2</sup> The Blagden lab has previously shown that the RBP La-related protein 1 (LARP1) complexes with over 3000 mRNAs in HeLa cells (termed the "LARP1 interactome"), with many of these mRNAs encoding cancer-related proteins. It has been speculated that LARP1 promotes the stability of these mRNAs or regulates their translation through binding their 5' and 3'-UTRs.<sup>3</sup> High LARP1 expression correlates with poor prognosis in ovarian, cervical and colorectal cancers and has been implicated in cancer cell invasion and migration, resulting in tumour progression and metastasis.<sup>4</sup>

Epithelial-mesenchymal transition (EMT) plays a central role in driving metastasis, and is characterised by the loss of cell junction integrity, reduced apical-basal polarity, and dramatic cytoskeletal rearrangements resulting in the formation of lamellipodia.<sup>5</sup> The increased cell motility associated with EMT is partly driven by enhanced focal adhesion (FA) turnover and cytoskeletal regulation mediated by FA proteins (e.g. integrins, paxillin and vinculin) and actin-binding proteins. This is coupled with improved coordination of force-induced signals, as cells move through different rigidities of the extracellular matrix.<sup>6</sup> EMT is mediated through specific transcription factors such as  $\beta$ -catenin (in the canonical Wnt signalling pathway), Twist and Snail.<sup>7</sup> Moreover, to effectively metastasise, cells must invade the local tissue by remodelling the extracellular matrix and cell-matrix adhesions. This process is principally mediated by the regulated secretion of matrix metalloproteinases (MMPs) from actinbased protrusions (invadopodia) developing at the leading edge of the migrating cancer cell (Fig. 1).<sup>8</sup>



Figure 6. Major events during epithelial-mesenchymal transition (EMT). Four key stages critical for EMT are depicted. EMT is associated with a switch from epithelial to mesenchymal markers. Adapted from [9].

LARP1 enhances invasive and migratory capabilities in HeLa and PC9 (human lung adenocarcinoma) cells, although it is unknown if LARP1 drives these effects across other cancers and cell lines.<sup>3</sup> Furthermore, the mechanisms through which LARP1 potentiates invasion and migration remain elusive. However, the LARP1 interactome is highly enriched for MMPs and EMT hallmarks (such as N-Cadherin and Twist), which strongly supports a direct role for LARP1 in regulating invasion and migration.<sup>3</sup>

In this study, I investigated invasion and migration rates in U2OS (a highly invasive and motile osteosarcoma cell line) and OVCAR-8 (a relatively non-invasive ovarian cancer cell line).<sup>10,11</sup> I also addressed if LARP1 regulated the expression of any EMT-related proteins by knocking down LARP1 in these cell lines.

#### Project Aims

1.Determine if LARP1 knockdown leads to altered invasion and migration rates using a real-time transwell assay system.

2. Determine the role of LARP1 in regulating ÉMT and invasion by measuring protein and mRNA levels of EMT hallmarks and MMPs following LARP1 knockdown.

#### **Methods**

Cell culture and si-RNA-mediated LARP1 knockdown: U2OS and OVCAR-8 cells were cultured in RPMI supplemented with

10% foetal bovine serum (37°C, 5% CO<sub>2</sub>). Cells were transfected with either a non-targeting control siRNA (si-NT) or LARP1-targeting si-RNA (si-LARP1), using Lipofectamine RNAiMax (Invitrogen). After 36 h, cells were serum starved (1% FBS) for 5 h before harvesting by trypsinisation for proliferation and transwell assays. Remaining cells were lysed for RNA and protein isolation.

**RT-qPCR**: Total RNA was isolated using GenElute Mammalian Genomic RNA Miniprep Kit (Sigma-Aldrich), RNA concentrations were equalised across all samples, and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Resulting cDNA was quantified by qPCR using Fast SYBR Green (Invitrogen), with primer sets (200 nM) against individual targets. Sample RNA levels were normalised against a housekeeping gene (OAZ1) and relative expression levels were calculated using the  $2^{-\Delta\Delta Ct}$  method.<sup>12</sup>

Western blotting: Cell lysates were prepared using RIPA buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0), supplemented with protease inhibitor (Sigma-Aldrich). Protein concentrations were measured and standardised using a bicinchoninic acid assay and denatured in Bolt-LDS buffer (Thermo Fisher) supplemented with DTT (50 mM). Proteins were separated by SDS-PAGE on 4-12% Bis-Tris Plus Gels (Invitrogen) before transferring onto nitrocellulose membranes. Membranes were blocked in non-fat milk or bovine serum albumin and incubated at 4°C overnight in primary antibody followed by incubation with HRP-conjugated secondary antibody (90 min, room temperature), washing in TBST (0.1% Tween-20) between incubations. Proteins were visualised with ECL chemiluminescent substrate, using the ChemiDoc<sup>™</sup> Gel Imaging System (Bio-Rad).

Invasion and migration rate assays: Cell kinetics were in CIM-plate 16 monitored transwell plates (Acea Biosciences), comprising two chambers separated by a microporous membrane interdigitated with electrodes. In response to a chemotactic gradient, cells move from the upper wells through the microporous membrane and



Figure 2. Cross-section of a CIM-plate well. A microporous membrane forms the base of the upper chamber. This membrane is untreated for migration assays or overlaid with Matrigel for invasion assays. Extracted from [13].

adhere to the microelectrodes beneath, generating increased impedance values, which are recorded by the xCELLigence RTCA system and expressed as cell index (Fig. 2). For invasion assays, upper wells were coated in 20  $\mu$ L of 100  $\mu$ g/mL Matrigel (Corning). Viability assays were performed alongside invasion assays with CyQUANT NF (Invitrogen) with fluorescence intensity (Ex/Em 480/520 nm), measured using a Polarstar Omega Plate reader (BMG Labtech).

#### Assessment of Results

LARP1 knockdown reduces invasion and migration in U2OS cells. LARP1 knockdown in U2OS resulted in slower cell index increases with and without Matrigel, indicating reduced rates of both invasion and migration (Fig. 3a, 3b). Moreover, linear regression analysis between t=10 h and t=12 h reveal that rates of invasion and migration significantly differ between control and si-LARP1 treated cells (p<0.0001 in both assays) (Fig. 3e). Cell viability assays revealed no significant difference in viability of U2OS cells 40 h after LARP1 knockdown, though a small difference in OVCAR-8 viability was observed (Fig. 3g).

LARP1 knockdown does not affect invasion but reduces migration in OVCAR-8 cells. OVCAR-8 cells displayed poor invasive capabilities that were not reduced any further upon LARP1 knockdown (Fig. 3c). However, similar to U2OS cells, OVCAR-8 cells displayed a slower rate of migration following LARP1 knockdown compared to the control (Fig. 3d). Linear regression analysis indicated that LARP1 knockdown did not significantly alter invasion rate, but did alter migration rate (p<0.0001) (Fig. 3f).



Figure 3. The effect of LARP1 knockdown on invasion and migration rates in U2OS and OVCAR-8 cells. (a-d) Cell index values representing cell movement across xCELLigence transwell plates coated with Matrigel (a,c) or uncoated (b,d). All cell index values are normalised (set to 1.0) relative to 2-3 h after seeding. (a,b) U2OS invasion and migration rates are reduced following LARP1 knockdown. (c,d) OVCAR-8 invasion rates are unaffected but migration rates are reduced following LARP1 knockdown. Representative of 3 independent assays. (e, f) Linear regression analyses of invasion and migration rates (gradients from graphs a-d) between t=10 h and t=12 h. Error bars indicate SEM (n=3). (g) Cell viability 40 h after seeding. Differences in viability following LARP1 knockdown are too small to account for differences in migration or invasion.

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Figure 4. Effects of LARP1 on EMT hallmark and MMP protein expression.. Western blot analysis of EMT and invasion-related proteins following LARP1 knockdown in OVCAR-8 and U2OS cells. ERK2 was used as the loading control.

LARP1 knockdown caused reduced expression of MT1-MMP and the EMT hallmark vinculin. LARP1 was knocked down in OVCAR-8 and U2OS cells with EMT and invasion-related proteins analysed by Western blot (Fig. 4). Following LARP1 knockdown, vinculin expression was reduced in the invasive U2OS cell line but not in OVCAR-8 cells.

MMP expression upon LARP1 knockdown varied between cell lines. MMP2 was detected in U2OS cells but did not appear to be regulated by LARP1. Moreover, MT1-MMP protein expression was unaffected by LARP1 knockdown in OVCAR-8 cells, yet seemed to be LARP1-dependent in U2OS cells. Additional MMPs (MMP9 and MMP10) were probed for but not detected in either cell line. The cell-specific expression of MMPs upon LARP1 knockdown may be attributed to the varied, context-specific roles adopted by MMPs.  $\beta$ catenin, N-cadherin and vimentin expression remained unchanged by LARP1 knockdown.

mRNA levels of selected EMT and invasion-related targets were analysed by RT-qPCR. Two-fold increases or decreases in mean expression following LARP1 knockdown were considered as cut-offs for further investigation (red dashed lines, Fig. 5). Most targets displayed less than two-fold differences. However, vinculin demonstrated a two-fold decrease in mRNA expression in both cell lines, consistent with the result at the protein level in U2OS cells. MT1-MMP mRNA was upregulated in U2OS cells post LARP1 knockdown, in contrast to the protein level. It is possible that this transcriptional increase is a regulatory response to the decreased MT1-MMP protein levels following LARP1 knockdown.

#### Future directions

Further work will be performed by the lab to validate vinculin and MT1-MMP as direct targets of LARP1 and determine if LARP1 drives translation of their encoding mRNAs. Subsequently, rescue assays following LARP1 knockdown will be performed to determine if restoration of LARP1 expression restores vinculin and MT1-MMP

Н Θ change non-targeting 0.5 0.25 Fold (vs 0.062 MMP11 0.0312 MMP230 B.Catenin MMP2 MMP9 MTAMMP SNAIL Vimentin Figure 5. Effects of LARP1 on EMT hallmark and MMP mRNA expression. RT-qPCR LARP1

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Figure 5. Effects of LARP1 on EMT hallmark and MMP mRNA expression. RT-qPCR following LARP1 knockdown in U2OS and OVCAR-8 cells. Fold changes are relative to non-targeting control (si-NT) normalised to OAZ1 as a reference (n=3).

expression. Although this study showed β-catenin expression was unaltered by LARP1 knockdown, previous work has suggested that LARP1 induces the nuclear localisation of β-catenin, rather than increasing its expression level, (thereby allowing it to drive EMT).<sup>14</sup> Accordingly, immunofluorescence assays to determine subcellular localisation of EMT hallmarks will be performed. This work has demonstrated that LARP1 promotes invasion and migration, and it is hoped that further work will establish the mechanism.

#### **Departures from Original Proposal**

In general, the proposed workflow was closely followed, however cell lines used for xCELLigence assays (U2OS and OVCAR-8) deviated from those originally stated. Immunofluorescent staining was not performed as an effective protocol could not be optimised in time. Thus, we were unable to determine the subcellular localisation of EMT hallmarks in response to LARP1 knockdown. RT-qPCR was also performed in addition to the methods initially stated.

#### Value of Studentship

I relished the opportunity to work alongside like-minded scientists, and the placement has certainly cemented my intention to pursue a scientific research career. I have acquired a strong practical skillset and developed interpersonal skills by collaborating and presenting to both lab members and external specialists.

**Supervisor's comments:** Matt was an outstanding student and presented this work as the first author of a poster at the 2018 LARP Society Meeting in Germany, where he was awarded the F1000 prize for best poster presentation (an unprecedented achievement as an undergraduate student). He contributed intelligently at lab meetings and was tremendous fun to work with. Matt and I are extremely grateful to the Biochemical Society for funding this work.

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# Introduction

Clostridium botulinum expresses neurotoxin serotypes BoNT/A-G (Foran et al., 1996); and more recently, a new serotype has just been discovered labelled BoNT/X (Masuyer et al., 2018). Whilst Clostridium tetani only has the one neurotoxin (TeNT). Clostridial neurotoxins are made up of two subunits, a heavy chain and light chain. The heavy chain is responsible for neuronal targeting; the light chain, for cleavage of SNARE proteins located within the synapse which are the cause of the adverse effects. SNARE proteins allow for the transmission of neurotransmitters from the vesicle, through the synaptic membrane, into the synaptic cleft. Cleavage of any of the SNARE proteins results in an inability for this to occur. There are three distinct neuronal SNARE proteins: Synaptobrevin, Syntaxin-1A and SNAP-25 (Rickman et al., 2005). Each is cleaved by a distinct neurotoxin. However, BoNT/C cleaves both Syntaxin-1A and SNAP-25 (Rickman et al., 2004).



*Figure 1*- Illustration of the topology of the neuronal SNAREs highlighting the cleavage sites on SNARE Proteins by Clostridial neurotoxins(Source-Barr *et al.*, 2005).

The neurotoxins are themselves enzymes which cause cleavage by catalysis.

# <u>Aims</u>

The aim of this project was to alter the primary structure of the neurotoxin to stop the catalytic process but still retain binding to the SNARE proteins. Theoretically, a marker could be attached to the neurotoxin which would allow for very precise mapping of neuronal cells. There are many benefits of using enzymes as markers. For example, they have a very high specificity for their target, are a niche group of compounds, are small and therefore are unlikely to be rejected by cells, and lastly, they are easily mutated.

# Methods employed

# Transformation:

cDNA was ligated into the expression plasmid, which was subsequently inserted into strains of E.Coli (XL10-Gold, LOBSTR, AlphaD) by heat-shock transformation. Each strain exhibits certain desired effects such as proliferation, ease of uptake and in the case of LOBSTR, there are fewer contaminating proteins upon purification. 1 µL of plasmid DNA was added to 50 µL of each strain, as well as a control sample of each strain to which no plasmid DNA was added. This was then incubated over ice for 10 minutes and then placed in a bead bath (42 °C) for 30 seconds and again incubated over ice for 5 minutes. 450 µl of LB media were added and the sample was incubated at 200 rpm in the orbital shaker for 45 minutes, at 37 °C. The transformed bacteria were then plated, under sterile conditions, onto plates containing antibiotic (ampicillin or kanamycin), and subsequently incubated overnight

Protein expression and purification:

Single bacterial colonies were added to flasks containing 2xTY media and incubated in the orbital shaker (6 hours, 37 °C). IPTG is added at a concentration of 1mM and then incubated overnight. This was then centrifuged and the pellet frozen.

All proteins purified contained a 6-His tag to aid in purification. This was completed using nickel chromatography on an AKTA and a range of buffers were used to adhere and dissociate the protein from the His-Trap column (GE healthcare). SDS-PAGE was then used to test the purity of the protein sample. If contaminants were present, gel filtration was used, again using the AKTA. The gel filtration consists of a column containing a bead resin. Tween-20 was also added to minimise aggregation of the proteins.





Figure 2- GFP tagged Synaptobrevin being injected into AKTA

Figure 3- GFP tagged Synaptobrevin moving down the gel filtration

#### SDS PAGE electrophoresis:

Protein samples were screened using SDS-PAGE electrophoresis. A loading buffer was added to the sample and aliquoted out into the wells. From this data, protein size and purity can be defined. Coomassie staining was then used to view the bands. This was then imaged, and the bands analysed.

column

#### Mutagenesis:

To stop the catalysis caused by the neurotoxin, the primary structure must be altered and therefore the peptide sequence must be altered. To alter the sequence, site directed mutagenesis was used.



Specially designed primers were inserted into the PCR machine which altered the wild-type tetanus neurotoxin (TeNT) and caused specific point

Figure 4- PC12 cell treated with mutated BoNT/C. Courtesy Beatrice Vale, Rickman Group

mutations. The mutated protein was then expressed and purified as detailed above.

# **Results and Discussion**

SDS-PAGE electrophoresis indicated that the neurotoxin had successfully formed in high concentration when LOBSTR was used, however, analysis of the gel in figures 4 and 5 shows that impurities, such as larger aggregates could have formed during the protein expression stage. After purification and gel filtration, the fractions were pooled from synaptobrevin and TeNT, and the cleavage tested in a digestion assay as shown in Figure 6. The cleavage by the TeNT wild type Produced 3 bands, the highest band is the TeNT, with the other two bands being those of the cleaved synaptobrevin.



Figure 4 and 5- TeNT before gel filtration step.



Figure 6 and 7- TeNT Neurotoxin and GFP taggedsynaptobrevin digest. Top band in figure 6 is TeNT LC. The remaining two bands composing of the cleaved synaptobrevin.

To conclude, the focus was on purifying TeNT and synaptobrevin to then go on to mutating the TeNT and testing the double mutant on purified synaptobrevin. Due to time constraints, testing the double mutant of the TeNT on PC12 cells was not completed.

#### Future Directions

Addition of the mutated BoNT/C neurotoxins to neuronal cells has since been completed by a PhD student working in the Rickman group. The cells used were PC12, which are advantageous due to the high rate of proliferation and easy management. This would then have to be applied to the TeNT, ATTO labelling dye attached and results viewed under the super resolution microscopes. An example of BoNT/C labelling of a cell is shown in figure 4. Furthermore, there are other serotypes of *Botulinum* toxin that have not yet been tested. This would then allow a comparison for resolution quality, ease of use and labelling efficiency.

#### Value of the Studentship

I would like to thank Dr Colin Rickman, The Rickman Group and the Biochemical Society for this invaluable experience and opportunity in this short 8-week summer project. The skills and techniques acquired during this time have not only helped me develop good laboratory techniques but have also reinforced key skills such as time management, presentation/publicspeaking and vocational skills. Furthermore, the biochemistry summer studentship has given me an insight as to what being a researcher in this broad field is like. This has cemented my desire to do a PhD in the future and go on to have a career involving aspects of what I have learnt in this summer project.



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# **Student: Matthew Stedman**

# Supervisor: Dr Ling Hwang

# Introduction:

While a lot is understood about eukaryotic



chromosome segregation through tubulin binding to centromeres using the mitotic spindle, less can be said for our understanding of bacterial chromosome segregation. During the process of binary fission, it is imperative that both daughter cells receive an equal amount of genetic information, both in the form of chromosomes and plasmids. The proteins ParA, ParB and the DNA sequence parS are essential in this process. The parS sequence is located close to the origin of replication (ori). ParB binds the DNA at the parS site to form the partition complex and the ATPase activity of ParA provides for the energy to move the partition complex to the opposite ends of the cell, prior to cell division. ParA binds to nonspecific DNA and the partition complex in an ATP-dependent manner. The ATPase activity of ParA is stimulated by association with ParB. These molecular interactions generate the dynamics to transport the partition complex across the cell to ensure an even distribution to both daughter cells. Here, we used Vibrio cholerae, a multichromosomal bacteria as a model system to study chromosome segregation. V. cholerae is an important human pathogen and the agent of the severe diarrheal disease, cholera. It was previously observed that ParA2 of chromosome 2 forms a cloud over the bacterial nucleoid and moves dynamically across the cell. We believe that these dynamics mediate chromosome segregation. However, the molecular basis of how ParA2 spatiotemporally patterns the nucleoid is not known.

# Aims:

This project aimed to explore the kinetic interactions between *V. cholerae* ParA2 and nonspecific DNA using single-molecule fluorescence microscopy and microfluidics. This was achieved by coating a flow cell with sonicated salmon sperm DNA and flowing GFP-tagged ParA2 through. ParA2-GFP-DNA interactions were directly visualized by our home-built total-internal-reflection-fluorescence (TIRF) microscope to measure association and dissociation rates.

# Methods:

# Flow cell assembly

Flow cells were assembled by sandwiching a quartz slide and microscope coverslip together using double-sided tape with a flow chamber cut out. The flow cell was heated to 120°C for 2 hours to melt the tape to improve adhesion. The quartz slide had two drilled holes that provided for the inlet and outlet that were then attached to nanoports using optical adhesive to allow sample flow through the flow cell.

# **DNA** carpet

A supported lipid bilayer was formed in the flow cell by flowing in 1% biotinylated SUVs at 1mg/ml and incubated on a heat block at 30° for 1 hour with care taken to ensure there were no bubbles to disrupt the bilayer formation. The flow cell was then washed with Tris buffer and Neutravidin was added and incubated for 1 hour. Biotinylated salmon sperm DNA sonicated to a length of 1 kbp was flowed in and incubated at 4°C overnight. The following day the flow cell was washed with Tris buffer at a rate of 20 ul/min to remove unbound DNA without shearing the DNA carpet.

# **TIRF** microscopy

The DNA-carpeted flow cell was placed on the TIRF microscope and imaged using a 488 nm blue laser. The images were collected with a CMOS camera with filters for GFP emission. Images were acquired at 1 frame per second and the fluorescence intensities were recorded and plotted across both sample flow and wash buffer flow. The relative

change in intensity as a function of time over varying protein concentration values could then be used to calculate the binding and dissociation rates of ParA2-GFP with DNA.

# **Results and Discussion:**

Initially the flow cell contained only a single inlet which meant that we had to manually switch between syringes containing sample and wash buffers (Figure 1A). However, this led to some wash buffer to leak into the flow cell causing the protein to dissociate even before image acquisition. We also had to refocus the flow cell each time the syringe was switched causing time delay. The microfluidic system was revised to include a T-splitter which allowed both sample and wash to be directed into the same inlet. However, this led to a large lag time between the sample and wash flows. In addition to this, the T-splitter itself became contaminated with ParA2 which negated the purpose of the wash. Finally, a different flow cell was used that contained two inlets and one outlet (Figure 1B]. This allowed both sample and wash buffer to be instantaneously switched, providing much cleaner data collection.



GFP at 1 uM were measured by recording the fluorescence intensities observed within the flow cell. Due to TIRF illumination, only DNA-bound ParA2-GFP on the flow cell surface can be imaged. Unbound ParA2-GFP in solution remain dark. Therefore, we observed that ParA2-GFP intensities increase when it binds the DNA carpet. Initially strong ParA2-DNA association was observed but there was no dissociation. After many alterations to the buffer and flow cell conditions, we suspected that the His-tag on ParA2-GFP may be interacting with the negatively charged phosphate backbone. Cleavage of the His-tag produced smooth association and dissociation curves of the ParA2-GFP with the DNA carpet (Figure 2).



Sample

Figure 2: ParA2-GFP (1 uM) in the presence of ATP (1 mM) association and dissociation on DNA carpet. This figure shows how data was collected with intensity given on the Y-axis and frame count on the X. Frame count is equivalent to time at 1 frame per second. This acquisition shows the association of ParA2 as the intensity increases and the subsequent dissociation as it decreases. Sample and wash flow were started at 40 and 500 frames respectively.

#### **Future Directions:**

This work could be continued in a myriad of directions. Expanding the data currently collected across more concentration values would allow for determination of an accurate Kd.

Furthermore, adding ParB, a cognate protein that stimulates ParA ATPase activity and dissociation, to either the sample or wash buffers would allow observation of the effect ParB has on ParA association with the DNA. In addition, use of ADP or the non-hydrolysable ATP $\gamma$ S would enable investigation into whether ParA only associates with the DNA when bound to ATP or whether hydrolysis of the ATP is an essential element of association or dissociation. These data would contribute to our understanding to how these ParA dynamic patterns transport the chromosomal loci across the cell during binary fission.

# Value of the studentship:

This laboratory placement was of enormous value to me personally and I feel as though I helped contribute to the research pursued by Dr Hwang. My time in the laboratory gave me more confidence and experience in performing research which has aided me in fourth year by giving me the confidence to assume more autonomy in my research placement. Furthermore, this placement cemented my desire to pursue a PhD next year and gave me perspective on a wider range of techniques that both strengthened my application and allowed me to look at different fields. As a final point, I made friends and connections in the lab over the course of this project. I fully intend to stay informed of how the research is progressing and maintain the relationships made during my studentship.

# Biochemical Society Summer Vacation Studentship 2018



Student: Nathan Fenwick Supervisor: Dr Clare Towse Synthesis and conformational analysis of host-guest alanine based pentapeptides

# Introduction

Peptide drugs are becoming increasingly important in the world of biochemistry, due to the ever-improving available techniques to rationally design peptides for specific functions. In general, peptide drugs have a high level of both efficacy and safety in the body (1), which gives them an edge over other traditional drug types.

However, the issue of design remains. Since peptides are known for their dynamic nature (folding and unfolding at incredible speeds), how can we ensure the peptide remains in the active conformation so as to be effective when administered? The seemingly most



straightforward answer would be to restrict the number of conformations the peptide backbone can occupy. (2) (3) To make any significant headway towards answering this question, it is necessary to be able to quantify the effect of any amino acid on the possible backbone conformations. The best technique for doing so was developed in 1963 and uses Ramachandran plots to visualise the dihedral angles allowed by any particular amino acid.

This could be modelled computationally, however to show the effect in a real peptide system it is necessary to synthesise the required peptides for measurement. There is little point in using peptides below 5 residues in length, since there is less backbone to be affected. Also, since all

amino acids except glycine are chiral, it would be more realistic to use alanine as the standard for a peptide backbone. All of this information informed the decision to synthesise a series of alanine host-guest pentapeptides, with the general formula: AAXAA (Where X is any amino acid to be tested). Two possible avenues to constricting the backbone conformations are to use large quaternary amino acids (4) or D-amino acids (5), neither of which are commonly found in nature.

# **Project Aims**

The aims of the research project were to synthesise, purify and analyse 5 host-guest

pentapeptides. The analysis was to provide information about how the conformational restrictions of D-amino acids and large guaternary deprotection step. After the entire sequence had novel amino acids would affect a larger peptide. This in turn would allow creation of novel peptide and then cleaved using TFA (9.5 ml), TIPS (250 µl) drugs that are forced into active conformations and are therefore more bioavailable.

# Materials and Methods

#### Solid-Phase Peptide Synthesis (SPPS)

SPPS is a commonly used method of chain growth spectrometry. peptide synthesis. By attaching the first protected amino acid to the resin, then deprotecting it and adding the second, there is an increased certainty that a precise amino acid sequence has been synthesised. The steps are described below:

Rink-amide resin was swelled in DMF and deprotected using piperidine (%50 in DMF) so that the first Fmoc-protected amino acid residue could couple. Then began the SPPS cycle of coupling, Kaiser test, flow wash, deprotect, flow wash, couple next residue.

Coupling: Each Fmoc-protected amino acid residue was dissolved in HBTU solution (0.5M in DMF), with DIEA added immediately prior to coupling. When coupling, reaction vessel was shaken for at least 15 minutes. Pure DMF was used for flow washing between steps.

Kaiser test: A fast method of determining if residue coupling has been successful, a colour change from yellow to dark blue indicates a failed test.

Deprotection: Each peptide was deprotected using piperidine (%50 in DMF, 1 ml) twice at each been synthesised, the peptides were acetylated and deionised water (250 µl) for 2 hours, then precipitated using diethyl ether. For removing the water, the peptides were frozen in liquid nitrogen and then lyophilised. Finally, the peptides were purified using HPLC and analysed using mass

At this point in the research, after 3 peptide chains had been synthesised (AAAAA, AAGAA and AAsAA), a problem was encountered. The novel quaternary amino acid had been synthesised and protected using a Boc protecting group, rather than the Fmoc group required by the orthogonal synthesis that was used. Fmoc protection is essential, due to the group being base labile. This means it can be cleaved without interfering with the acid labile linkage between the peptide and the resin. Upon investigation, the amino acid as synthesised was also the ethyl ester rather than the free amino acid.





Fmoc protecting group (base labile)

Boc protecting group (acid labile)

Deviations from the planned project In order to prepare the novel amino acid for SPPS, a strategy was devised. The removal of the ethyl ester needed to be done first, in such a way as to

leave the Boc group present. It was necessary to keep the amino acid protected because when present as the free acid it could not be isolated from aqueous solution. After this had been accomplished, a method for removing the Boc group and adding the Fmoc group could be devised.

Removing the ethyl ester without removing the Boc protecting group required mild conditions, Several complicated methods were tried, including using a weak lewis base with a microwave (although it failed to produce high enough quantities of amino acid for our purposes). Finally, using CaCl<sub>2</sub> and weak NaOH, the required deesterification was achieved. Afterwards, removing the Boc and adding the Fmoc was done in one step using Fmoc chloride and a weak acid, to ensure we could isolate the amino acid.

Due to the various time constraints in the submitted plan, making alterations to the novel amino acid prevented the analysis work from being completed using NMR as planned. However, working on current research requires such risks and the unique problem was rewarding to solve.

# **Further Research**

Unfortunately, due to the deviations required to prepare the amino acid for SPPS, part of the project remains to be completed. Although the synthesis of the novel amino acid was completed, it has yet to be incorporated into a peptide. After that has been completed, the analysis of the peptides using HNMR and comparisons of dihedral backbone angle sampling will complete the scope of the study. As alluded to in the

introduction, the ultimate aim of the project is to improve peptide drug potential by locking the peptides in an active conformation.

#### Value of the Studentship

This funded placement has allowed me access to many fascinating new techniques and ideas surrounding peptide synthesis. I have personally been preparing novel peptides that have never been made before, and this has helped confirm to me that I do want to pursue further education and research. It has also given me many unique skills I would otherwise lack, such as purifying peptides and the SPPS method for synthesising peptides by chain growth. I would like to thank my supervisor (Dr. Towse) for making this incredible project possible and the Biochemical Society for the funding to make it practical.

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# **Biochemical Society Summer Vacation Studentship 2018**



# **PROJECT:** MECHANISMS OF SPINDLE ACTIN ASSEMBLY IN MAMMALIAN EGGS **Student**: Nina Webb **Supervisor**: Dr. Binyam Mogessie

**Introduction**: Every human life begins when an egg and fertilised by a sperm. In oocytes, progenitors of eggs cells, a specialised form of cell division called **meiosis** segregates the chromosomes. Meiotic chromosome segregation errors are remarkably common and can give rise to aneuploidy, a leading cause of human embryo deaths and genetic disorders such as Down's syndrome. Recently, the *MOGESSIE* lab demonstrated that spindle-associated actin (**spindle actin**) works in concert with the conventional microtubule-based spindle to promote faithful chromosome segregation during meiosis in mammalian oocytes (Mogessie and Schuh, *Science*, 2017). Spindle actin organises microtubules into functional kinetochore-fibres that can efficiently align and segregate chromosomes (Mogessie and Schuh, *Science*, 2017). My project was aimed at identifying some of the proteins that could mediate the interaction between spindle actin and microtubules of the meiotic spindle in mouse oocytes.

<u>Aims</u>: To understand the mechanisms of spindle actin assembly, the lab has developed high-resolution live and immunofluorescence imaging assays of actin filament recruitment into meiotic spindles. The goal of my research project was to use these assays and validate some candidate proteins involved in spindle actin assembly, with time allowing, using a new method of protein degradation called TRIM-Away (Clift *et al.*, *Cell*, 2017). As candidates, we selected proteins that were previously reported to simultaneously interact in living cells with actin filaments and microtubules.

<u>Methods:</u> The TRIM-Away method of protein degradation involves the use of specific antibodies to

Kate, Imogen, Nina and Binyam

degrade target proteins in cells (Clift *et al.*, *Cell*, 2017). To identify TRIM-Away suitable antibodies against selected candidate proteins, I performed high-resolution immunofluorescence microscopy using a range of commercially available antibodies. For this purpose, I isolated prophase-arrested oocytes from mouse ovaries and matured them into eggs *in vitro*. Eggs were then fixed at least 4 hours after completion of meiosis I, marked by the presence of polar bodies, and prepared for immunofluorescence. After immunostaining with antibodies against candidate proteins and secondary antibodies, eggs were imaged in three-dimensions using confocal microscopes (Leica SP8 and Zeiss LSM 800). To validate some candidates in living oocytes, I also cloned GFP fusions of genes that encoded them from mouse cDNA, which I then transcribed *in vitro* into mRNA. mRNAs were then introduced into prophase-arrested oocytes by microinjection and, after expression, the localisation of fluorescently-labelled candidates was monitored using high-resolution live cell imaging of oocyte meiosis.

**Results and outcome:** Using high-resolution as well as super-resolution immunofluorescence microscopy, I tested several commercial antibodies against candidate proteins that may mediate actin-microtubule interactions in oocytes. Out of these, I found a number of antibodies that specifically recognised their targets on meiotic spindles in mouse eggs, indicating their promise for use in TRIM-Away loss-of-function assays. In addition, live imaging of some candidate

proteins expressed from mRNAs that encode their fluorescent variants showed encouraging indication that some of these may indeed mediate actin-microtubule crosstalk. These results enabled us to select antibodies that can be used to efficiently degrade their targets using the TRIM-Away method.

During my project, there was time to perform a preliminary loss-of-function experiment using the TRIM-Away assay against one protein that looked particularly promising. For this purpose, I synthesised mRNA encoding TRIM21, a protein required for performing TRIM-Away, and H2B-RFP (a fluorescent marker of chromosomes). These were microinjected into prophase-arrested oocytes together with an antibody against the actin-microtubule crosslinking candidate protein or Mouse IgG (control). Following expression of TRIM21 and H2B-RFP, microinjected oocytes were released from prophase arrest and imaged overnight during meiotic maturation. To understand the effect of candidate protein degradation, I analysed live imaging datasets for chromosome and alignment segregation accuracy. While this analysis and fluorescent Phalloidin labelling of actin did not reveal any detectable errors in chromosome segregation or spindle actin assembly, the expression of H2B-RFP in control oocytes was very low, indicating that TRIM21 mRNA expression may have been insufficient in both control and SAAP antibody injected oocytes.

**Conclusions and further direction:** My project has mainly provided a library of antibodies against candidate actin-microtubule crosslinkers that are likely to work in TRIM-Away loss-of-function assays. For some of these, data from immunofluorescence experiments are supported by high-resolution live imaging experiments. Identifying antibodies that recognise candidate crosslinkers on meiotic spindles in immunofluorescence experiments is the first major step in this research programme. During these 8 weeks, I have identified multiple promising antibodies. My host lab is now well-placed to take them forward for use in TRIM-Away assays. Importantly, my TRIM-Away experiment has revealed that it is necessary to optimise TRIM21 mRNA expression. The next possible steps in this regard include separate microinjection of mRNAs and antibodies, concentration of antibodies before microinjection as well as using fluorescently-labelled TRIM21 mRNA to directly assess its expression levels in TRIM-Away assays. In conclusion, the data I have generated during my project will serve as springboard for launching mechanistic studies of spindle actin assembly.

<u>Value of the studentship:</u> Gaining skills and confidence in the lab throughout this 8-week placement opportunity has been a great way to prepare for the fourth year of my masters degree, which predominately consists of an independent research project. The studentship gave me confidence and knowledge, not only in specific lab techniques but also the general way in which an academic lab is run, including exposure to lab meetings and collaborations. With the end of my undergraduate degree looming, I was looking to develop my skill set and take advantage of opportunities that would prepare me for a career in biochemical research, and this opportunity has enabled me to do exactly that.

<u>Supervisor:</u> This generous support from the Biochemical Society has allowed my lab to host Nina during the summer and generate important piece of data on functional antibodies that can be used for loss-of-function assays to reveal the mechanisms of spindle actin assembly in mammalian eggs. Nina's excellent performance during her time in the lab was very encouraging and I will be looking to host other promising students in the near future, through this scholarship scheme.

Assessing DRP1 Localisation and Drug Function in *LRRK2* Mutants



Student: Olivia Cracknell Supervisors: Ruby Macdonald & Dr Heather Mortiboys

#### INTRODUCTION

Parkinson's disease (PD), the second most common neurodegenerative disorder <sup>1</sup>, is characterised by the loss of dopaminergic neurons in the pars compacta portion of the substantia nigra <sup>2</sup>, leading to motor dysfunction symptoms including bradykinesia, muscular rigidity, and resting tremor. <sup>2</sup> Over 90% of PD cases are sporadic while the remaining 10% are caused by monogenic mutations. <sup>1</sup>

Mitochondrial dysfunction has been implicated in the pathogenesis of both sporadic and familial PD. <sup>1</sup> Mitochondria supply cells with ATP through oxidative phosphorylation, they also have numerous other roles including those in apoptosis and biosynthetic intermediate production. <sup>3</sup> Mitochondria continually undergo fission and fusion <sup>4</sup> to form a dynamic, interconnected network <sup>3</sup>, a process increasingly being implicated with neurodegenerative disease. <sup>4</sup>

Mitochondrial impairment has been observed in fibroblasts from PD patients with Leucine-rich repeat kinase 2 (*LRRK2*) mutations <sup>1</sup>, the most common cause of genetic PD. LRRK2 is a large multi-domain protein with roles in mitochondrial function, retromer complex modulation, endocytosis <sup>5</sup>, neurite outgrowth, synaptic morphogenesis, membrane trafficking, autophagy, and protein synthesis. <sup>2</sup> The most common LRRK2 mutation occurs in the MAPKKK domain and results in a G2019S substitution <sup>2</sup> <sup>6</sup> which causes an increase in kinase activity. <sup>7</sup> The kinase activity of LRRK2 may be inhibited by the drug GSK2578215A (GSK).

Other research groups have previously found reduced basal oxygen consumption rate, increased autophagy, and enhanced mitochondrial damage in G2019S cells. <sup>1</sup> This group have found that G2019S cells show a reduction in mitochondrial membrane potential (MMP) and ATP levels <sup>8</sup>, and a decrease in respiratory chain complex IV activity. <sup>9</sup>

LRRK2 interacts with Dynamin-related protein 1 (DRP1), a protein which regulates mitochondrial fission. <sup>7</sup> LRRK2 phosphorylates DRP1 and recruits it to the mitochondria <sup>4</sup>, and an increase in DRP1 has been found to increase mitochondrial fragmentation and oxidative stress. <sup>7</sup>

# **PROJECT AIMS**

The first aim was to compare the localisation of DRP1 to mitochondria in fibroblasts grown from skin biopsies from individuals with a G2019S mutation who have PD (manifesting), individuals with the mutation but not PD at the time of the biopsy (non-manifesting), and individuals with neither the mutation nor PD (control). The second aim was to study the effects of the drug GSK on live cells of these three types through both MMP and ATP assays. Finally, I was tasked with optimising the staining procedures for a phospho-DRP1 antibody for use in future research.

#### SitraN Sheffield Institute for Translational Neuroscience

#### METHODS AND MATERIALS

#### **Tissue Culture**

Human fibroblasts were grown in glucose-containing media at 37°C in an atmosphere containing 5% CO<sub>2</sub>. Cell lines were kept within 2 passages of each other. For ATP assays, cells were plated in glucose-containing media at 5000 cells/well, and 2000 cells/well for MMP assays and fixing. 24 hrs later, the media was replaced with new glucose-containing media, untreated galactosecontaining media, or galactose-containing media with either 1µM GSK, or 1µM DMSO. 24 hrs later the assays were performed. Galactose-containing media was used to stimulate oxidative phosphorylation since fibroblasts usually produce ATP via glycolysis in glucose-containing media.

#### Immunocytochemistry (ICC)

Cells fixed with 4% paraformaldehyde were permeabilised with 0.1% Triton and blocked with 5% horse serum before being stained with 0.1% primary antibodies: mouse anti-DRP1 and rabbit anti-TOM20 (mitochondrial outer membrane protein). After overnight incubation at 4°C, the cells were stained with 0.1% secondary antibodies: 488 anti-mouse donkey and 568 anti-rabbit goat. After a 1hr incubation, cells were stained with 1% Hoechst then imaged with an Opera Phoenix High-Content Screening System. Alexa 488 and Alexa 568 were the channels used for DRP1 and TOM20 respectively. Between steps, cells were washed with PBST.

#### ATP Assays

Cells were washed with PBS, then 100µlof PBS and 50µl of lysis buffer were added. Cells were then shaken at 700 rpm for 5 mins before 50µl of substrate buffer was added. The plate was shaken again then dark-adapted for 10 mins before being read on the luminescence mode of the Pherastar plate reader. 50µl 0.11% CyQUANT was then added and the plate was left for a 1-3 hr incubation before being read on the fluorescence mode of the plate reader. ATP values were normalised to the well DNA content as determined by CyQUANT. Blank wells were used to correct values to the background.

#### Mitochondrial Membrane Potential Assays

Phenol red-free media was added to half of the cells, and 0.1% CCCP to the other half, the plate was then incubated for 1 hr at 37°C. CCCP functions as an uncoupler. To the cells without CCCP, a solution with 0.1% TMRM and 1% Hoechst was then added, and the same was added to the other cells, only with an additional 0.1% CCCP. The plate was then incubated for 1 hr at 37°C before being washed and imaged on an In Cell microscope. DAPI and Cy3 were the channels used for the nuclei and mitochondria respectively.

#### Analysis

The Opera images were analysed using Harmony High Content Imaging and Analysis Software. The 'Find Spots' feature was used to find mitochondria and DRP1 so that the co-localisation of DRP1 to the mitochondria could be determined. The In Cell images were analysed using the In Cell Developer Toolbox. For all measurements, the results were normalised to their corresponding control value. All statistical analysis was done by ANOVA two-way with a Tukey correction.

#### RESULTS

For all graphs, 'UN GAL' refers to untreated galactose media; and 'DMSO GAL' and 'GSK GAL' refer to 1 $\mu$ M DMSO in galactose media and 1 $\mu$ M of GSK in galactose media respectively. All error bars represent standard deviation.



**Figure 1. Image Analysis.** a. ICC mitochondrial network (Alexa 568); b. ICC cellular DRP1 localisation (Alexa 488); c. Harmony Software 'Find Spots' feature showing DRP1 (coloured) in mitochondria (white); d. In Cell Developer Toolbox mapping out the MMP assay mitochondrial network.

#### DRP1 in Mitochondria / Number of Nuclei



Mean Mitochondrial Intensity/ Mean Cell area



normalised to the mean well cell area, n=3.

Figure 2. ICC Staining.

Values show the colocalisation of DRP1 to mitochondria as determined by the Harmony 'Find Spots' feature. UN GLU refers to untreated glucose media. Three different cell lines were used in this experiment, and each was tested in triplicate esting (n=9).

> Figure 3. Mitochondrial Membrane Potential Assay. Values shown are the well mean TMRM intensities

There are statistically significant differences in DRP1 colocalisation (figure 2) between the manifesting and nonmanifesting lines grown in galactose-containing media, and the control lines grown in glucose-containing media. This may be a result of the galactose-containing media stressing the fibroblasts by forcing them to use oxidative phosphorylation rather than glycolysis. Stressed cells can show increased mitochondrial fragmentation, which may in this case be a result of the fission activity of increased DRP1 co-localised to the mitochondria. There is no statistically significant difference between the control lines grown in glucose-containing and galactose-containing media, suggesting that G2019S mutants may be more sensitive to this stress.

In each condition, MMP is significantly lower in manifesting cells than in control cells (figure 3), and there are no significant differences between manifesting and non-manifesting lines, supporting research previously conducted by this group that a G2019S mutation is associated with reduced MMP. These results suggest that 1 $\mu$ M GSK had no effect on restoring MMP in any of the cell lines.

Cellular ATP levels (figure not shown, n=3) in manifesting and nonmanifesting lines do not appear to be lower than those in the control line, which is unexpected based on the results from previous research. However, this control line has not been used in experiments before, and the ATP levels may have been affected by the age of the individual from which the cells came, or the individual may have an underlying condition affecting ATP production. Treatment with 1µM GSK has no statistically significant effect on cellular ATP content in any cell lines.

# FUTURE DIRECTIONS

Future work may involve the DRP1 inhibitor mdivi-1 and knocking out *LRRK2*. Additionally, once the phospho-DRP1 staining protocol has been completely optimised, it may be carried out to study cellular localisation of phospho-DRP1 in *LRRK2* mutants.

#### DEPARTURES FROM ORIGINAL PROPOSAL

The part of the project concerned with determining DRP1 localisation remained unchanged. However, rather than investigating the ATP production by fibroblasts grown in different substrates, we instead investigated the effect of the LRRK2 kinase inhibitor, GSK, on ATP production and mitochondrial membrane potential.

#### VALUE OF THE STUDENTSHIP

I have learnt to proficiently carry out all the techniques described in the methods section which were all new to me, and I'm sure I will use throughout my research career. I have also observed several others including western blotting. Additionally, attending lab meetings, journal groups, and seminars have given me a taster of life in a lab and broadened my understanding of the subject area. Finally, I thoroughly enjoyed my time here- it has boosted my confidence significantly, and it has confirmed for me that I would like to pursue a career in research.

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# Identifying molecular interactions occurring at a highly conserved region of the ADAMTS-5 transcript

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INSTITUTE OF AGEING AND CHRONIC DISEASE

# Introduction

Osteoarthritis (OA) is a joint disease characterized by the progressive loss of articular cartilage which results in pain and immobility in affected individuals<sup>[1]</sup>. In healthy cartilage, chondrocytes maintain the balance in the synthesis and degradation of extracellular matrix (ECM) components composed mainly of type II collagen and aggrecan<sup>[2]</sup>. However, dysfunction in the ECM remodelling process can result in net tissue loss and development of OA due to increased expression of matrix-degrading enzymes<sup>[1]</sup>. Aggrecan is the predominant proteoglycan in cartilaginous ECM and its loss from cartilage is mediated by members of the a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family that cleave aggrecan at its core protein<sup>[1]</sup>. Members of the ADAMTS family that cleave aggrecan are given the general name 'aggrecanases' and ADAMTS-5 has been shown to be the dominant aggrecanase responsible for the development of OA in mice<sup>[3]</sup>.

The ADAMTS-5 transcript is 9,680 nucleotides long with a 3' untranslated region (UTR) that is greater than 6kb long <sup>[4] [5]</sup> (Fig 1). The 3'UTR plays a significant role in various aspects of post-transcriptional regulation of gene expression through motifs that interact with miRNAs and RNA binding proteins (RNABPs)<sup>[6]</sup>. There is evidence of ADAMTS-5 regulation through miRNA based mechanisms<sup>[7] [8]</sup>; however, its post-transcriptional regulation by RNABPs is yet to be explored. Sequence analysis identified three conserved regions within the ADAMTS-5 3'UTR. One is immediately downstream of the stop codon, another is 3kb 3' of the stop codon and the third is 6kb 3' of the stop codon and precedes the polyadenylation side. The "3kb" region is particularly interesting because it contains motifs that interact with miRNAs and it contains AUUUA pentamers that commonly act as binding sites for RNABPs. This project sought to investigate the interaction of RNABPs with the "3kb" region by employing RNA-Electrophoretic Mobility shift Assay (EMSA), which is based on the principle that the migration of nucleic acid through a gel is retarded when it is bound to a protein.

# Aims

- 1. To create fluorescent RNA probes for the detection of protein interactions with the "3kb" conserved 3'UTR region of the ADAMTS5 transcript.
- 2. To examine the interaction between chondrocyte proteins and RNA probes by carrying out Electrophoretic Mobility Shift Assay (EMSA).
- 3. To identify specific proteins that interact with the ADAMTS-5 3'UTR



Fig 1: The ADAMTS-5 transcript showing the 6 kb long 3'-UTR with the conserved region 1 (CR1), the "3kb" region, and the conserved region 3 (CR3).

# Methods

# In vitro transcription

Deoxyribonucleic acid (DNA) templates complementary to sequences in the "3kb" region of the ADAMTS-5 transcript were purchased from Eurogentec (Fig 2). A sense T7 promoter sequence also purchased from Eurogentec was annealed to the 3'-end of each antisense DNA template as described by Mcdermot *et al.*, 2014. Next, the HiScribe<sup>™</sup> T7 High Yield RNA Synthesis Kit was used for *in vitro* transcription according to the manufactures protocol. Following incubation, the reaction was treated with DNase I (RNase –free) and the resulting ribonucleic acid (RNA) probes were purified using phenol:chloroform extraction and ethanol precipitation.





Fig 2: (A) Regions of the "3kb" region where RNA probes were synthesized. (B) The three arrows labelled "Partial" represent fragments of probe 1 which were synthesised and used as unlabelled RNA in competition experiments. Competition was also carried out using unlabelled full-length probe 1.

# End-labelling of RNA probes

The purified RNA probes were 5'-labelled with IRDye 800CW Maleimide using the 5' EndTag<sup>™</sup> Nucleic Acid Labelling System (Vector Laboratories) according to the manufacturer's instructions. Labelled probes were purified using phenol:chloroform extraction and ethanol precipitation.

# Preparation of cell lysate

Cultured SW1353 chondrosarcoma cells were incubated in serum free media overnight to prevent build-up in wells. Following overnight incubation, media was removed, and cells were washed twice in PBS. Cell lysate was prepared in extraction buffer and collected using a cell scraper.

# RNA-EMSA

To examine the interaction between chondrocyte proteins and cell lysate, cell lysate in extraction buffer was incubated in 40 ng of labelled RNA probe, binding buffer, and 500  $\mu$ g/ml transfer RNA (tRNA) in a final volume of 20  $\mu$ l. A control template DNA provided in the HiScribe<sup>TM</sup> T7 High Yield RNA Synthesis Kit was used as a negative control while unlabelled probes were used as specificity controls. The binding reactions were loaded onto 2.5% agarose gel<sup>[9]</sup> and imaged using the LICOR infrared imaging system.

# RNA-Pull down Assay

RNA Probes were biotinylated at the 3'-end using the Pierce<sup>™</sup> RNA 3'-end Biotinylation Kit (Thermo Scientific) following the manufacturers protocol. Pull-down was performed using streptavidin agarose beads and eluted proteins were separated by SDS-PAGE. Protein bands were visualized using a silver staining kit (Thermo Scientific).

# **Results and Discussion**

EMSA was performed to investigate the interaction between chondrocyte proteins and the "3kb" region of the ADAMTS-5 transcript. As seen in Fig 3, RNA was successfully labelled with IR-Dye and visualized using the LI-COR infrared imaging system. Four interactions were observed for Probe 1 and three for Probe 2. This method provides a simpler and faster visualization technique compared to past methods that used biotinylated RNA which require blotting onto a membrane, UV-crosslinking, and *chemiluminescence*.

To investigate the specificity of the observed interactions, competition experiments using 1X and 10X concentration of unlabelled probe was performed (Fig 4). For each probe, increasing concentration of its unlabelled form was able to compete out the probe; however, unlabelled non-specific RNA did not compete out the interactions (Fig 4A). This confirms the specificity of the shifted bands.

Furthermore, short fragments of Probe 1 were used to compete out full length labelled Probe 1 in order to narrow down the nucleotide sequence responsible for binding to RNABPs. Fig 4B reveals that all three fragments compete out probe 1 to some extent; however, partial 3 was the strongest competitor. Thus, this 37-nucleotide long sequence may contain motifs with a high affinity for RNABPs.



Fig 3: EMSA using SW1353 chondrosoma cell lysate prepared from cells incubated overnight in serum-free media and treated with 10ng/ml of Interleukin-1 $\beta$  (IL-1 $\beta$ ) for 0, 1,3,6,24 and 48 hours. The use of serum-free media gave more defined bands in comparison to the original lysate. Four shifted bands were observed for Probe 1 and at least three for Probe 2. Treatment with IL-1 $\beta$  had no observable effect on the pattern of shifted bands.



Fig 4: (A) Competition experiment for probe 1 using increasing concentrations of unlabeled probe 1, unlabeled control probe and (B) unlabeled fragments of probe 1.

# **Future Work**

Due to the time constraint, specific chondrocyte proteins that interact with the "3kb" region were not identified. Thus, to further this project RNA-Pull down assay requires optimization. This will result in unique bands, which can then be extracted from the gel, purified, and analysed using mass spectrometry. Identified proteins can be confirmed by western blotting or by carrying out antibody super-shift. With this information, the effect of specific RNABPs on ADAMTS-5 mRNA turnover can be explored leading to a deeper understanding of the regulation of its expression. The degree to which the various RNABPs affect the level of ADAMTS-5 could reveal potential targets for therapeutic intervention. In the last week of the studentship, the interaction between proteins from a variety of cell lines and the RNA probes were examined. Lysate was prepared from human embryonic kidney cells 293 (hek293), corneal epithelial cells (hTCEpi), mouse chondrogenic cell (ATDC5) and macrophage cells. Then the band patterns were compared to those of SW1353 chondrosoma cell lysate. The gel revealed that for Probe 1, SW1353 and macrophage lysate resulted in the same four shifted bands (Fig 5). Meanwhile hek293 and ATDC5 revealed at least five RNA-protein interactions. Additionally, hTCEpi had four interactions, but it seems to deviate most from the other lysates. For Probe 2, each lysate gives a different pattern of RNA-Protein interaction (Fig 5). The variation in the pattern of RNA-protein interaction suggests that the "3kb" region is not just a sticky piece of RNA.



Fig 5. EMSA using lysate prepared from SW1353 chondrosoma cell, human embryonic kidney cells 293 (hek293), corneal epithelial cells (hTCEpi), mouse chondrogenic cell (ATDC5) and macrophage cells for both Probe 1 and Probe 2.

# Value of the Studentship

# To the Student

Through this studentship, I gained a better understanding of life as a researcher. I was in a lab with PhD candidates, post docs and others who are at different levels of their research career, which allowed me to gain insight into life as a researcher. Furthermore, I gained experience designing experiments and writing protocols as well as performing new techniques that are beyond what I have done so far in practical modules. I was given the freedom to perform various experiments and this has greatly improved my lab skills. This studentship also gave me an opportunity to practice scientific writing and critical thinking. I truly enjoyed this experience and I'm very grateful to Dr Simon Tew for the opportunity. This experience has definitely confirmed my passion for research and acquiring knowledge.

# <u>To the Lab</u>

Uju has worked incredibly hard during this studentship and it has really helped us to develop our EMSA protocols by moving toward the use of fluorescently tagged probes. We were able to troubleshoot a number of approaches to probe labelling using both 3' and 5' chemistry's, before settling on the 5' IRDye 800CW Maleimide method. We have also been able to start to examine how protein interactions might vary in a well conserved region of the 3'UTR of the ADAMTS5 mRNA. This work will be very useful in support future publications and grant applications.

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# **UCL**

# Characterisation of TREM2 R47H risk factor for Alzheimer's disease

Student: Peipeng Lin Supervisor: Dr. Dervis Salih & Prof. Frances Edwards

#### **Background and Aims**

Genome-wide association studies (GWAS) using patients with late-onset Alzheimer's disease (AD) identified a specific variant R47H of the Triggering Receptor Expressed on Myeloid cell 2 (*TREM2*) gene, which is preferentially expressed in microglia in the central nervous system. The R47H variant elevates the risk of late onset Alzheimer's disease(AD) by 2- to 3-fold. A *Trem2* R47H knock-in (KI) mouse model was generated by The Jackson Laboratory (Stock number: 027918) to understand the *TREM2* related dysfunctions in AD patients carrying R47H variant. While charactering this mouse model our lab observed two transcripts produced by PCR with a primer pair amplifying the junction of exon 1 to exon 2 of *Trem2* spanning the R47H mutation. *The aim of my project was to characterise the alternative splicing in the mouse Trem2 R47H KI model and determine if the same splicing error takes place in AD patients carrying TREM2 R47H variant.* Due to lack of good antibodies against TREM2, it's difficult to analyse the change in TREM2 protein level upon R47H substitution. Recently, two novel anti-TREM2 mouse antibodies (9D10 and 9A9-3) were generated by Cardiff University (Paul Morgan Lab). Therefore, I also tested the novel antibodies for specificity for TREM2 protein with Western Blotting on cortex protein extracted from *Trem2* knock-out (KO) or wildtype (WT) mice.

#### **Methods**

Primer pairs specific to Mouse *Trem2* or human *TREM2* were designed to span exon 1 and 2 including the R47H mutation. PCR was conducted with mouse *Trem2* R47H cDNA or human cDNA converted from cortical total RNA obtained from the Queen Square Brain Bank (Tammaryn Lashley Lab). PCR products were ran on 3% agarose gel containing ethidium bromide, allowing the separation by size. Two abundant transcripts from mouse sample were cut out and purified from the gel. Mouse PCR products were ligated into the pGEM-T Easy vector as part of the TA-vector cloning protocol. Vectors were then transformed into competent *E. coli* cells. After recovery in LB the bacteria were streaked onto agar plates and followed by further overnight growth of single colonies in LB media with ampicillin. The recombinant plasmid DNA was extracted with a "mini-preparation" protocol and sent for sequencing with the M13F/R forward and reverse oligonucleotides.

Total cortex protein was extracted from *Trem2* KO or WT mouse brain through homogenisation in RIPA buffer with sonication. Western blotting was performed with the SDS-PAGE method. Protein samples were firstly ran on an SDS-PAGE gel with an SDS electrophoresis buffer, and then transferred to nitrocellulose membrane in Tris/glycine transfer buffer overnight. The membrane was then blocked in 5% milk TBS-T for an hour. Primary anti-TREM2 mouse antibody was diluted (1:1000) in 5% BSA TBS-T and used to incubate the membrane overnight. To detect the signal, the membrane was incubated in 5% milk TBS-T containing antimouse secondary antibody followed by addition of enhanced chemiluminescence (ECL) substrate using a "Chemi-doc" system (Bio-rad).

#### **Results**

#### Aberrant splicing is mouse specific and is not observed in human

To study the splicing of Trem2 mRNA across exon 1 and 2, primer pair spanning exon 1 and 2 were used to amplify the junction between these exons, and the splicing pattern of *Trem2* was compared between WT mouse siblings and R47H KI mice. WT *Trem2* gene produces a single prominent transcript at 287bp while both this transcript and an additional transcript were observed at 168bp from the *Trem2* gene containing the R47H variant (Fig 1a). Both transcripts from *Trem2* R47H mice were cloned and sequenced. The sequencing results indicated that the transcript of 287bp from both WT and *Trem2* R47H mice shared the same expected sequence as WT (according to the Ensembl database). The additional PCR products from the *Trem2* R47H homozygous mice showed a loss of 119bp from the 5' end of exon 2, including the R47H mutation, which started directly at the splice site after the full-length exon 1 (Fig 1c). This deletion of 119bp would lead to a frameshift mutation and introduce a premature stop codon in exon 2. This alternate splicing may contribute to the reduction in *Trem2* mRNA level observed in the *Trem2* R47H KI mice.

The orthologous PCR reaction was then carried out with human cDNA from healthy control and patients carrying the TREM2 R47H variant with or without AD. Surprisingly, all samples clearly produced a single transcript at 249bp with a smear which may



Figure 1. Splicing pattern of the R47H KI mice and R47H patient carriers. a) two transcripts produced at 287bp and 168bp in the R47H KI mice., b) no additional transcripts produced in human R47H carriers., c) schematic illustration of aberrant splicing in R47H KI mice

be a result of post-mortem delay (Fig 1b). This suggested that the aberrant splicing only takes place in mice carrying the *Trem2* R47H variant.



# • Novel anti-TREM2 antibodies from Cardiff University did not show specificity against TREM2

No significant difference was observed between the signal at 30kDa in WT and *Trem2* KO mice with both AD10 and A9A-3 anti-Trem2 antibodies (Fig 2a,b). Therefore, both antibodies showed no specificity against TREM2 protein at ~30kDa.

# **Conclusions and future directions**

The sequencing results of cloned PCR products from *Trem2* R47H KI mouse revealed an aberrant splicing at exon 2 of *Trem2* in a mouse-specific manner. In this case, the observed reduction of *Trem2* mRNA and activity are highly possible to be related to this splicing difference. Since a similar splicing pattern was not observed with human *TREM2* R47H carriers, this may imply

Figure 2. Western blotting of TREM2 protein with novel anti-TREM2 antibody AD10 in WT or *Trem2* KO mice. (WT: n=3, HET: n=1, HOM: n=4)

that the experimental evidence produced from *Trem2* R47H KI mouse model is not directly comparable to human. The novel anti-TREM2 antibodies AD10 and A9A-3 from Cardiff University failed to specifically mark TREM2 proteins in Western blotting. Therefore, in future experiment we may test the antibodies by immunohistochemistry using WT and *Trem2* KO mice. In addition, our lab identified 5 genes that are predicted to show DNA sequence variants associated with AD in human, and may be affected by differences in TREM2 activity, and so the next step will focus on characterisation the gene and protein expression of these 5 predicted genes in WT and *Trem2* R47H mice with RT-qPCR and immunohistochemistry.

#### Value of studentship

Student: This summer studentship offers me an invaluable chance to work in the Edwards lab with amazing scientists and learn modern molecular biology techniques. During the 8-week project I was able to generate some data that can contribute to a publication of the lab. Overall, this experience makes me more determined to pursue a career in academia and provides me with great advantages in the following PhD applications.

Supervisor: It was a real pleasure to host Peipeng in our lab during the summer for her vacation research, and we are very grateful to the Biochemical Society for supporting her. Peipeng is a very intelligent young scientist and has a very bright future ahead of her. She is extremely determined, dedicated, reliable, and helpful, and performed a couple of important experiments to help us characterise the importance of the R47H allele in mice and humans. She cloned and sequenced different cDNA transcripts of *Trem2* produced in the hippocampus of R47H KI mice, and performed orthologous experiments in human cortical tissue to show that the R47H KI mice show altered splicing of exon 2 of *Trem2* compared to WT mice and humans. The data collected by Peipeng will form figures in our future publications. We hope that this experience has allowed Peipeng to get a taste of research in a productive lab, and the skills that she learnt will stand her in good stead for her future career.

# Investigating Peptide Inhibition of Alpha-Synuclein as a Potential Therapeutic Option





# Ravina Mistry

Supervisors: Dr Jill Madine and James Torpey

## INTRODUCTION

Neurodegenerative disorders from neuronal cell death is a growing concern in the aging population. Alpha-synuclein (Asyn) is a protein that is implicated in the pathogenesis of Parkinson Disease (PD), Dementia with Lewy bodies and Multiple system atrophy. Asyn can aggregate to form intracellular inclusions of misfolded protein aggregates called Lewy bodies (LBs) often found in patients with these diseases. Parkinson's disease is a result of neurodegeneration of dopaminergic neurons from toxicity to protein aggregates<sup>[1]</sup>. In 2016, an

estimated 127,000 people were affected by PD<sup>[2]</sup>. While the incidence of the disease is low, suffers of the disease may live for many years leading to greater numbers of those affected. Additionally, the prevalence of PD will rise in association with the doubling of the over 85's population predicted to occur in the next 25 years<sup>[3]</sup>, increased awareness and greater diagnostic tools. The disease is usually sporadic, where only a small proportion are diagnosed with "early onset" occurring between the age of 21-40, and "juvenile onset" when diagnosed before 20<sup>[2,4]</sup>. PD causes stiffness, tremors and bradykinesia, the main causes of postural instabilities leading to falls and fractures<sup>[4]</sup>. These incidents can ultimately increase the pressure and financial strain on the NHS and emergency services. Targeting Asyn and its aggregation pathway provides a therapeutic strategy to reduce the burden of these diseases.

Collaborators in Bristol have developed a 10-residue peptide KDGIVNGVKA that disrupts aggregation of Asyn and reduces its toxicity to cells<sup>[5]</sup>. The aim of this project was to investigate binding interactions between the peptide and Asyn to further elucidate the mechanism of action of the peptide inhibitor. Furthermore, there are 6 known pathological mutants of Asyn, the peptide will be tested against mutant forms of Asyn to see if it is also active at preventing aggregation in a similar way to wild-type Asyn. This project will employ a range of biophysical techniques including isothermal titration calorimetry (ITC), Thioflavin T (ThT) fluorescence and nuclear magnetic resonance spectroscopy (NMR).

# METHODS

**Protein Production:** Plasmids of wild-type Asyn and 6 other mutants (A30P, E46K, H50Q, G51D, A53T and A53E) with ampicillin resistance were transformed into *E. coli* BL21 and plated on LB agar with ampicillin. Following growth overnight single colonies were picked to create starter cultures that were grown in SOC broth, then minimal media (pH 7.2). Cultures were incubated (37°C) with agitation until  $OD_{600}$ = 0.6 and induced by IPTG, then incubated (18°C) with agitation overnight. Cultures were centrifuged (5,000g) for 20mins and then flash frozen in liquid N<sub>2</sub>. Cells were then lysed by french press, heated at 85°C for 10mins and centrifuged (18,000g) to pellet bacterial cell debris. Asyn was eluted with a salt buffer through an anion exchange column using an AKTA purification system. Factions were analysed by SDS-PAGE, and factions containing Asyn were pooled and concentrated using 5kDa MWCO centrifuge filters (4500g). Protein aliquots were frozen in liquid N<sub>2</sub> for later use.

*ITC Procedure:* A programme was set-up to titrate 1.5μl of ligand (1mM) every 3 mins into Asyn (50μM) at 25°C using a Microlab iTC200, using degassed samples with stirring at 800rpm. Buffer contained potassium phosphate (10mM), potassium fluoride (100mM) and 0.5% sodium azide.

**NMR Experiment Procedure:** NMR experiments were carried out at 25°C on a Bruker 800MHz AVANCE III spectrometer equipped with a TCI cryoprobe. WaterLOGSY experiments were conducted with 256 scans. Natural abundance HSQCs, NOESYs and 1D experiments were also carried out to check samples. TopSpin was used to analyse the data.

*ThT Fluorescence Assay:* WT Asyn and 6 mutants were combined with the aged and fresh peptide. Aged peptide was produced by incubation (37°C) with agitation for 7 days. The aged peptide and fresh peptide were added at a final concentration of 100 $\mu$ M to all Asyn types (100 $\mu$ M) and a control of PBS alone. The samples were incubated (37°C) with agitation for 7 days where 100 $\mu$ I samples were taken on Days 0,1,3,7 and frozen in liquid N<sub>2</sub>. To measure, 1 $\mu$ I of 20mM ThT dye was added to each 100 $\mu$ I sample and fluorescence read with excitation at 450nm and emission at 490 nm, in triplicate using a Molecular Devices Flex Station 3.





Figure 2. Thioflavin T fluorescence showing following incubation of protein for 7 days alone (black) or in the presence of peptide (red) for Asyn and mutant peptides as shown.

Ligand binding on the isothermal titration calorimetry output is indicated by a change in temperature seen as peaks upon addition of ligand. We observed peaks upon addition of peptide to Asyn (Figure 1) suggesting that an interaction is occurring. Decrease in intensity of the peaks upon further addition of peptide suggests that this binding is becoming saturated. Thioflavin T fluorescence is used as a measure of amyloid formation as fluorescence is only observed upon binding to fibrils. Here we show that in the presence of peptide Thioflavin T fluorescence is reduced suggesting that fibril formation is reduced for

WT Asyn and 2 of the mutant proteins G51D and A53E, whereas the peptide does not affect the Thioflavin T fluorescence for the other mutants (Figure 2). This suggests that the peptide could also be a candidate for further development into a therapeutic for disease caused by Asyn mutations G51D and A53E.

# Future directions of the project

Results obtained during this project suggest that the peptide does interact with Asyn. Further work will be carried out probing residues involved in binding guided by this data. This project also showed the peptide can prevent aggregation of mutant proteins in addition to WT. Further work will now assess whether fibrils produced in the presence of peptide from mutant proteins also display less toxicity as observed with WT Asyn.

# Value of the Studentship

**Student:** I enjoyed working on a project that had real world application and experiencing the culture of a professional lab. I can see myself within a research environment and want to apply the knowledge and techniques I have learnt in this project to my future work. I have been introduced to new ways of thinking and problem solving from attending journal club sessions that analysed published work. I have seen more techniques within structural biology and different areas of amyloid research from interacting with others in the team. I feel more confident to work independently in a lab and I am glad that I could be a part of research that contributes to the collaboration.

**Lab**: Having Ravina working on the project for 8 weeks has enabled a wider range of mutant proteins to be tested and new techniques investigated, than would have been achieved by the PhD student working on the project alone. This has now highlighted future avenues to explore. Ravina interacted well within the lab often providing a different point of view to the project. I wish her well in her future career.

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# **Biochemistry Society Summer Vacation Studentship 2018:**

A yeast model for Treacher Collins syndrome



Student: Bindiya Patel (Newcastle University) Supervisor: Nick Watkins (ICAMB, Newcastle University) Background.

The ribosome is a large RNA-protein machine that is responsible for protein synthesis. Ribosome biogenesis is intimately linked to multiple cellular signalling pathways, and defects in ribosome production lead to genetic diseases like Treacher Collins syndrome and Diamond Blackfan anaemia.<sup>1</sup> Ribosome biogenesis and function is very important in this respect which is partially determined by the extensive modifications of rRNA.<sup>2</sup> The box C/D small nucleolar (sno)RNPs co-transcriptionally add 2'-O-methylations which are essential for correct structure and activity of the ribosome.<sup>3</sup> The gene TCOF1 encodes the protein Treacle which has been suggested to bridge interactions between RNA polymerase I and the box C/D snoRNPs to mediate efficient co-transcriptional 2'-O-methylation of ribosomal RNA.<sup>1</sup> Mutations of the TCOF1 gene causes the inherited craniofacial disorder Treacher Collins syndrome.<sup>2</sup>

Previous unpublished studies in the Watkins lab have suggested that Srp40 is the yeast orthologue of Treacle; the protein is essential for efficient rRNA methylation in yeast and is found to interact with box C/D snoRNPs, and RNA pol I subunit Rpc40. In the absence of Srp40, the methylation of 80% of the sites in the rRNA is largely reduced. Using this knowledge, we have a model system to studying the function of Treacle in yeast.<sup>2</sup>

# Aims

This project aimed to investigate the effects of Srp40 and its interactions during rRNA 2'-O-methylation. We looked at both direct and indirect regulators of rRNA 2'-O-methylation identified in the Watkins lab previously. This was done using a combination of approaches such as drop tests and manipulation of yeast genetics through transformations. **Method** 

- Yeast transformation protocols were used to create several variants of the BY4741 and Srp40 protein.
- Drop tests were used to test the growth of variant strains. Using a spectrophotometer, the yeast cultures were grown in fresh media at an equal starting OD of 0.2 for several hours. Exponentially growing yeast cultures were diluted 1/10 six times in a microtitre plate and this was used for drop tests in various media plates.
- DNA was extracted from yeast strains to validate strains. Overnight cultures were pelleted. Breaking and lysis buffer and zirconia was added to the pellets and then the mixture was vortexed and centrifuged at top speed to shear and extract the DNA. The DNA mixture went through a series of washes with ethanol and then resuspended with water.
- PCR was used to test the yeast strains. Repeats were done with both Phusion and Phire polymerase as well as multiple of combinations of primers. Primers used were specific to the SRP40 and NAT genes. 1.2% agarose gel was used to ensure that the samples ran slowly, and clear separation was observed.

# **Results and Discussion**

- The wild type strain BY4741 and several BY4741 variants with different levels of rRNA methylation, were grown on YPD plates with various concentrations of different antibiotics to see if rRNA methylation impacts antibiotic activity. All the antibiotics used are known to target the ribosome.
  - The strains used were, wild type strain BY4741, ΔSrp40, BY4741Δ72-78, BY4741Δ72-78ΔSrp40. [Δ72-78 are a cluster of functionally important snRNPs that were deleted]. The different antibiotics and concentrations used were, 25µl & 50µl of G418 (kanamycin), 25µl & 50µl/ml cycloheximide, 2 & 3µl/ml paromomycin, 1 & 2µl/ml anisomycin, 8µl/ml homoharringtonine and 50 & 100µl/ml of blasticidine. A YPD plate lacking antibiotic was used as a control.
  - Results showed that on both G418 plates, the BY4741∆72-78 showed a significant growth defect relative to the control, thus the lack of these snoRNAs increases G418\_efficacy.
  - However, the strain BY4741∆Srp40 shows enhanced growth in G418, relative to the control, suggesting that the reduced methylation seen in the absence of Srp40 reduces G418 efficacy.
  - $\circ$   $\;$  Similar results, but with weaker effects, were seen with cycloheximide.
  - The other antibiotics, such as paromomycin, anisomycin, homoharringtonine and blasticidine had little effect on the growth of the antibiotics, indicating that changes in methylation did not affect these antibiotics.
- In another experiment, we inserted three different plasmids (p316, X and Xd) into BY4741 and Srp40. The yeast transformants were then drop tested on various plates such as casamino glucose and galactose and -uracil glucose and galactose plates. Plates were incubated at different temperatures of 20°. 25°, 30° and 37°C. This showed whether different media and temperatures would change methylation levels.

- The p316 is an empty plasmid, and thus acts as a control. The X and Xd plasmid directed methylation of a site that would not normally be methylated, and when methylated cause a growth defect. The X is more efficient at methylation than the Xd, and therefore produces a greater growth defect.
- All the plasmids were controlled by a GAL promotor. The plasmid effect was suppressed by glucose media and this acted as a negative control.
- Plates were put in different temperatures to see if it made a difference to methylation. Casamino glucose
   -Uracil glucose
   -Uracil glucose



- Both casamino and –uracil gal plates showed a markedly reduced growth of all yeast strains expressing X and Xd in comparison to casamino and –uracil glu plates, relative to cells with the empty vector.
- The Srp40 X and Xd yeast strains grew better than the BY4741 transformants X and Xd in both casamino gal and -uracil gal.
- The purpose of using –uracil and casamino plates was to see how the methylation levels would change in different media, with the loss of certain amino acids, and nutrients. Casamino plates is more enriched than – the minimal uracil, which was the absolute minimum that the yeast could grow in, so it was interesting to see any differences in the growth of yeast strains.
- Uracil plates showed a very slight growth defect in the Srp40 yeast strains in comparison to casamino plates.
   This suggest that the Srp40 strain requires specific nutrients that is not found in –cracil plates.
- Temperature did not affect methylation rates of both BY4741 and Srp40.
- BY4741, W303, were transformed with the p316 plasmid (empty vector) and GST Srp40 plasmid, which overexpresses the Srp40 protein. These were then drop tested on Casamino glucose and galactose plates and incubated at 30°.
  - The results for BY4741 showed that overexpression of Srp40, decreases cell growth indicating that overexpressing this protein is toxic for the cell.



# Future work

From looking at my research I suggest that more work could be done on exploring the ribosome binding antibiotics, and the different antibiotics and the concentration of which could cause growth defects. Furthermore, several double deletion strains using Has1 and W303 were made however, there was no time to investigate the effects the strains, and the effect they have on methylation rates. Various tests could be performed on these strains to observe how they are regulated. **Value of the studentship** 

# Student:

The summer studentship gave me a chance to develop a range of practical skills and work independently, and I felt that over the 8 weeks I was became more confident in working in a research lab. It gave me an insight into what a career in research entails and has confirmed that I would definitely like to do a MRes. It has been a great learning experience and I would like to thank Nick Watkins, Claudia Schneider and the lab for supporting me through this placement and making it an enjoyable and comfortable environment to work in.

#### Supervisor:

Bindiya has made great progress on this project and produced some interesting results. I believe that these studentships are invaluable for students to learn more about lab work and scientific research. Bindiya has produced some important results for the project, and also made some important strains that we will use in the lab in future experiments. The data produced will contribute to a paper we aim to publish next year and produced material that a PhD student is now using in her project.

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# Introduction

The Virus-X project (Horizon2020) is a Europe-wide Consortium whose raison d'être is to find novel enzymes from the yet-uncharacterised genome of extremophile bacteriophages. This is done with the aim of discovering biotechnological uses whilst also deepening the understanding of viruses. Enzymes from viral sources have already been found that have biotechnological uses, such as the proteases; tobacco etch virus (TEV)<sup>1</sup> and human rhinovirus type 3C<sup>2</sup>. Durham University, as part of this Consortium, receive proteins which have been harvested from Icelandic bacteriophages, capable of existing in extreme environments, such as hot springs. They have been working on characterising and also ascertaining crystal structures of these proteins, and these, coincidentally, were the aims of this project.

# Method and Results

# Protein production

For Protein A<sup>3</sup> the whole process of expression to purification was carried out. *E.Coli* strains, containing the sequence for Protein A, were incubated and shaken to allow replication before the transfer to litre flasks, in order to reach optimal cell density, which equated to an absorbance of 0.7 on the spectrophotometer. At this point,

expression of Protein A was induced using L-Rhamnose before incubation overnight. The samples were centrifuged to give pellets that were re-suspended in lysis buffer. In order to break up the cells and release the protein this re-suspended solution was sonocated before centrifugation to achieve the supernatant containing the Protein A. The purification of Protein A involved running a column; the protein was engineered in order to contain a His-tag which allows use of the HisTrap column. The percentage of elution buffer, which contains a high concentration of imidazole necessary for forcing the protein off the column, is increased incrementally until the system is completely flushed with imidazole and Protein A is pushed off into certain fractions. This is observed via the chromatogram, where a high peak is observed when protein is present. These fractions were dialysed in Tris buffer before starting characterisation. Protein A was able to be purified to >95%, with the His tag cleaved, and put forward for characterisation.



Figure 7; Gel for purification of Protein A; columns left to right: molecular weight ladder, flowthrough from running the protein onto the column, wash where the column was flushed with lysis buffer, column fraction A3, A4, A5 and after dialysis.

# Thermal Shift Assay

Characterisation of Protein A was attempted with the Durham pH screen using the 5000 x SYPRO orange dye. The TSA was used to show the melting point,  $T_M$ , of the protein in the various conditions available in the Durham screen. This  $T_M$  is an indicator of the stability of the protein and conditions where the  $T_M$  increases are found to be stabilising for the protein. This information can then be used to optimise crystallography. However, it was

<sup>&</sup>lt;sup>1</sup> Kapust, R.B., Waugh, D.S. Controlled intracellular processing of fusion proteins by TEV protease. *Protein Expression and Purification*. **19**(2), 312-318 (2000).

<sup>&</sup>lt;sup>2</sup> Cordingley, M.G., Register, R.B., Callahan, P.L., Garsky, V.M., & Colonno, R.J. Cleavage of small peptides in vitro by human rhinovirus 14 3C protease expressed in *Escherichiac coli*. *Journal of Virology*, **63**(12), 5037-5045 (1989).

<sup>&</sup>lt;sup>3</sup> Due to the nature of this research and the legislation in place, which states the genes belong to the country they were discovered, all information regarding names and amino acid sequences are not disclosed within this report. For the purpose of this report the proteins have been arbitrarily named Protein A, Protein B and Protein C.

found that the concentration of protein was too low to warrant better results from the initial TSA run. Meaning a concentration of 0.4 mg/ ml is insufficient, in the case of Protein A specifically, in achieving usable results.

# Crystallisation and Optimisation

Due to the unknown crystallisability of Protein B, which was expressed in *E.coli* and purified by chromatography in Lund, initial crystallisation screens<sup>4</sup> were carried out using various crystallisation kits, starting with the PACT premier<sup>™</sup>. These kits contain solutions offering a variety of conditions for the protein, some of which will be stabilising, and from this optimisation can be undergone in order to ascertain bigger and better crystals. It was found in the case of Protein B that it was very unstable in any environment and it crashes out of most buffers. For future research an attempt to stabilise the protein first would be beneficial.

For Protein C, which was expressed in *E.coli* and purified by chromatography in Lund, initial crystallisation screens were already completed awarding some knowledge on the conditions in which crystallisation had been successful. Therefore optimisation was completed in the hopes of achieving crystals of a higher quality. This proved to be somewhat successful as numerous cubic crystals were produced. These crystals however, were too small to be of publication standard, see figure 2, but this is a good starting point as the optimisation can be adjusted slightly to see if it helps in producing fewer but bigger crystals.



Figure 8; Crystals of Protein C obtained by optimisation.

# **Conclusion**

The outcome of this project should help inform future research. Conditions for protein C crystallisation have been found which can be optimised to produce the supersaturated state required for X-ray diffraction quality crystals. Protein A can enter the initial crystallisation screening stage. Protein B needs to be, first, stabilised before entering the screening process.

<sup>&</sup>lt;sup>4</sup> Jancarik, J., Kim, S.-H. Sparse matrix sampling: a screening method for crystallisation of proteins. *Journal of Applied Crystallography*, **24**, 409-411 (1991).
# **Biochemical Society Summer Studentship report**

# By Ryan McEwan

# Aims:

The overall aim of this project was to assess whether PAR4 is expressed in multiple sclerosis, by use of an EAE mouse model. This project was undertaken in order to learn new techniques in a research environment, which could be used further down the line in other projects which required similar techniques.

# **Description:**

Week 1- A BCA assay was carried out to obtain suitable standards, which could be used for comparison throughout the rest of the project. Then, Western blotting was carried out on spinal cord tissue of naïve and EAE mice to quantify the expression of pAKT, a known downstream signalling protein of PAR4 activation. The same tissues were reprobed for another downstream signalling protein, pERK. After this, the tissues were again reprobed for tubulin, in order to assess whether the differences in expression of these proteins was due to PAR4 activation, or unequal loading during blotting.

Week 2- An antibody for PAR4 was tested using Western blotting on naïve and EAE spinal cord tissue, along with positive and negative controls. These samples were reprobed for tubulin, to further validate the results. During this time, human microglia cells of the HMC3 cell line were being cultured, allowing practice at feeding cells and splitting cells. A BCA assay was carried out using thrombin- which is known to activate PAR4 as well as the thrombin receptor- and AY, a specific agonist for PAR4. The BCA assay involved stimulating the HMC3 cells with thrombin and AY at regular intervals to find the time interval with the peak protein concentration.

Week 3- The stimulated HMC3 cells from the end of the previous week were used for Western blotting to measure pERK expression, in order to investigate whether there is a difference in the expression of this signalling protein in those cells which have been stimulated by thrombin or AY, to find whether PAR4 is present in HMC3 cells. The membrane used was then reprobed for total ERK to find what proportion of ERK was phosphorylated by activated PAR4 and/or thrombin receptor. At the end of the week RNA was extracted from HMC3 and HMC3 with PAR4, and then, using qPCR, cDNA was made.

Week 4- Repeat of the Western blotting of the thrombin and AY stimulants for pERK and then total ERK, as was done in week three. At the end of this week, the HMC3 cells were stimulated using media, media with thrombin and also using various concentrations of thrombin with the thrombin antagonist, SCH, before being used in a BCA assay. This part of the project was undertaken to assess the levels of pERK when the thrombin receptor was inhibited, so that only PAR4 would be activated.

Week 5- During this time I was also introduced to the clinical scoring of EAE mice to monitor disease severity. I was introduced into using the cytochrome to harvest tissue from organs from EAE mice.

Week 6 and 7- Optimisation of PAR4 antibody by staining the spinal cord and lymph node of EAE mice, as well as the spleen and brain of naïve mice, using different concentrations of the PAR4 antibody and also using different concentrations of the rat secondary antibody. The relevant concentrations of PAR4 antibody were used on the spinal cord of EAE mice at different stages of disease to find whether PAR4 is expressed at any point in the disease. The samples made at the end of week four which contained thrombin inhibitor and different concentrations of thrombin were used in Western blotting to find whether there is an increase in pERK levels due to PAR4 activation.

Week 8- The staining for PAR4 of the spinal cord of EAE mice at different stages of disease was repeated, meanwhile naïve and EAE mouse lymph node and spleen samples were used in Western blotting to find out whether there is a difference in the expression of PAR4 in EAE mice. The blot was repeated to find the expression of tubulin, to find whether any differences in expression of PAR4 according to the first blot was due to an increase in the expression of the receptor and not due to experimental error.

# Results:

There was found to be increased expression of pAKT and pERK in EAE mice, according to the Western blot carried out at the beginning of the first week, suggesting initially that there may be increased PAR4 expression in the spinal cord of EAE mice. However, there was also increased tubulin expression, suggesting these differences in pAKT and pERK expression may be due to experimental errors.

The results of the blotting using the PAR4 antibody suggested no difference in PAR4 expression in the spinal cord of naïve and EAE mice, however the antibody was proven to work due to the use of a positive control.

When the stimulations in week two were used in a Western Blot, it was found that that there was less pERK expression in the AY stimulations than the thrombin stimulations, however, because the five-minute stimulation had a lower expression than the zero-minute stimulation, this experiment had to be repeated.

In the repeat of the blot using the stimulations, there was found to be a relatively high level of expression of pERK in both the fifteen-minute AY stimulation and thrombin stimulation. This thrombin stimulation was used in the BCA assay using thrombin inhibitor and different concentrations of the fifteen-minute thrombin sample. When this was used in Western Blotting, there was found to be an increase in pERK expression as the concentration of the sample increased, with little or no pERK expression in the samples with up to  $10\mu$ M of sample, suggesting the upregulation of pERK was due to thrombin receptor activation and not due to PAR4 activation.

When blotting to determine whether PAR4 was expressed in naïve and EAE mouse spleen and lymph node, there was an error during the experiment which made the results inaccurate, and there was no time to repeat this experiment.

The results of the optimisation of the PAR4 antibody showed very little staining at the 1:1000 dilution, as a result, dilutions of 1:500 and higher were used going forward.

There was found to potentially be positive staining for PAR4 in the spinal cord of EAE mice, particularly those at peak disease severity. However, further repetitions of the experiment suggested there was no expression of PAR4 in the spinal cord of EAE mice.

# Future Work:

If there was more time for this project, the western blot of the lymph node and spleen tissues of EAE and naïve mice would be repeated to obtain valid results. Other potential future avenues to explore include using different EAE mouse models, rather than just the one as used in this project and potentially using a PAR4 antibody with stronger binding to the PAR4 protein.

# **Departures from Original Proposal**

The order of the activities which were undertaken in this project were altered compared to that of the original proposal. It was originally proposed that clinically scoring EAE mice would be undertaken in the first two weeks of the proposal, however, due to the mouse not showing signs of having contracted the disease initially, this activity had to be pushed to the second half of the project.

Due to using a PAR4 antibody which hadn't been used in the lab before, the first step was to test and ensure the antibody would work, which was not stated in the original proposal.

# How This Grant Benefitted me

The grant has allowed me to undertake a project which has given me great practice at many important techniques which can be taken with me in my future career endeavours. The grant has also allowed me to partake in a project which has given me more in-depth knowledge into the research of autoimmune and neuroinflammatory disease, something which I would now be greatly interested in being involved in again in my career. Most importantly it has provided me the chance to increase my overall lab experience and assure me that a career in research is definitely a career I would like to pursue.



# INVESTIGATING THE ROLE OF THE CHAPERONE HSPB8 IN ASTROCYTES IN ALZHEIMER'S DISEASE

Introduction

Student: Sara Carrillo Roas,

Supervisors: Aurelio Vazquez de la Torre and Maria Jimenez-Sanchez

One of the hallmarks of Alzheimer's disease (AD) is the formation of AB and tau aggregates due to protein misfolding (1). Thus, studying protein homeostasis is key to further understand the development and progression of AD. Molecular chaperones are key components of the proteostasis network assisting proteins to adopt their native state (2). A subgroup of chaperones, named small heat shock proteins (sHSPs), are particularly interesting since they are upregulated in ageing and neurodegeneration (3). Moreover, members of this family, including HspB8, have been shown to be specifically upregulated in astrocytes in AD and other neurodegenerative diseases (4–7). However, the role of sHSPs within astrocytes is poorly understood and more research is needed in this direction. Interestingly, the recently discovered role of sHSPs as extracellular proteins (8,9) might be important in AD since preliminary data from the lab shows that Cryab and Hsp27, members of the sHSP family, are expressed in astrocytes and secreted to the extracellular media, suggesting that these chaperones might have an effect in neurons. Thus, the lab focuses on the non-cell autonomous role that these chaperones might have on neurons and how this might be affected in AD.

#### **Project aims**

I studied if HspB8, another protein from the same family, is secreted as the other two proteins previously analysed. Thus, the aims of my project were to determine whether (i) HspB8 is expressed in astrocytes, (ii) HspB8 is secreted to the extracellular media from astrocytes, (iii) the secretion of HspB8 is affected in the presence of amyloid beta oligomers (A $\beta$ Os), an AD-like stress.

#### **Materials and Methods**

*Primary mouse astrocytes culture:* Cultures of primary astrocytes were provided by my supervisor, Aurelio Vazquez. I carried out the splitting, seeding and collection in each experiment. Cells were grown in 10% FBS/DMEM before any treatments and in 1% DMEM/FBS when treated.

*Collection of cells:* Conditioned media was collected and centrifuged for 5 min at 2000 rcf at  $4^{\circ}$ C and supernatant was collected. Cell lysates were collected in lysis buffer 1X and centrifuged at 16.1 rcf for 10 min at at  $4^{\circ}$ C.

Preparation of samples for western blotting: 400  $\mu$ L of conditioned media was concentrated by centrifugation for 30 min at 14,000 rcf at 4°C, using the 3Kd centrifugal filters (Amicon) centrifuge concentration tubes. Gels were loaded with 40  $\mu$ L of the concentrated conditioned media mixed with 20  $\mu$ L of sample buffer 3X or with 10 $\mu$ L of cell lysates with 5  $\mu$ L of sample buffer 3X, and then they were incubated at 95°C for 5 min.

*Western blotting:* 12% SDS-PAGE and PDVF membranes were used for all the western blots carried out. An Odyssey CLx Imaging system was used to scan the membranes and the image studio software was used to do the analysis. Antibodies used: HspB8 (cell signalling #3059), Actin (#2066)

*siRNA transfection:* Primary mouse astrocytes were transfected with 100 nM of siRNA control or HspB8 siRNA in Optimen + Lipofectamine 2000. The cells were collected after 4 days of the transfection.

Synthetic ABOs: Synthetic AB42 and AB40 (Peptanova (#4307-v) were prepared from a stock solution (1mg/1mL). HFIP solvent was removed by evaporation from 60  $\mu$ L of the stock solution, resuspended in 2% DMSO and diluted in PBS. Finally, they were incubated at 37°C for 3 or 24 hours to generate AB42Os and AB40Os respectively. A 1:9 physiological ratio of AB42:AB40 was used when treating astrocytes. The vehicle used was a mixture of DMSO and PBS.

*Tg2576 and wildtype media:* tg2576 media was provided by my supervisor. Briefly, media was obtained from primary cortical neurons from tg2576 mice, which express the APP human gene with the Swedish mutation and thus produce and secrete A $\beta$  oligomers (10). Likewise, wildtype media was obtained from wildtype mouse neurons. Media was diluted to get ~2 nM physiological concentrations of A $\beta$ Os.

LDH activity assay: An LDH activity assay was carried out following the Thermo Fisher #88954 protocol.

#### **Results**

# HspB8 is expressed in astrocytes and it is secreted to the extracellular media.

To determine whether astrocytes express HspB8 and if it is secreted to the extracellular media, I analysed cell lysates and conditioned media from primary mouse astrocytes by western blotting. HspB8 was detected in both fractions (Figure 1). Next, I knocked down HspB8 using siRNA to validate the specificity of the HspB8 antibody. The results show a reduction of HspB8 levels extra- and intracellularly when transfected with HspB8 siRNA compared to siRNA control (*Figure 1*). Also, no HspB8 was detected in 1%FBS media. Thus, we can conclude that HspB8 not only is expressed by astrocytes, it is also secreted to the extracellular media in basal conditions.



**Figure 1.** Intracellular (A) and extracellular (B) levels of HspB8 were detected by western blotting. A single experiment was performed. *A*. The graph shows the relative levels of HspB8 normalised to actin as a loading control. *B*. Total levels of HspB8 are shown since no loading control can be used.

#### HspB8 secretion is not affected by synthetic A6Os.

We next tested the effect of synthetic A $\beta$ Os on the levels of HspB8. Primary mouse astrocytes were treated with different concentrations of A $\beta$ Os, ranging from 250 nM to 2  $\mu$ M, and cells were collected after 8 and 24 hours. The results seem to indicate that the presence of synthetic A $\beta$ Os does not affect the intracellular levels of HspB8 (*Figure 2a and 2b*). Interestingly, the extracellular levels increase after treatment with A $\beta$ Os for 8h (*Figure 2c*). Furthermore, no extracellular levels of HspB8 were detected when exposed

to ABOs for 24 hours (*Figure 2d*). Thus, synthetic ABOs did not affect intracellular levels of HspB8 in astrocytes. While the secretion was slightly increased after 8 hours, our results



Figure 2. Intracellular levels of HspB8 after 8 hours (A) and 24 hours (B) of exposure to A $\beta$ Os were detected by western blotting. Likewise, the extracellular levels of HspB8 after 8 hours (C) and 24 hours (D) are shown. All the graphs show the mean of the three independent experiments.

#### were not conclusive.

# HspB8 secretion is increased when exposed to A6Os from tg2576 neuronal media after 24 hours of exposure.

We then treated astrocytes with media from tg2576 neurons, which contains more physiological-like A $\beta$ Os (10). Primary mouse astrocytes were treated with media only (neurobasal) or media from wildtype or tg2576 neurons and then collected after 8 and 24 hours.

Intracellular levels of HspB8 slightly increase when treated with tg2756 media compared to wildtype, both at 8 and 24h, while extracellular levels only increase after 24 hours (*Figure 3*). Importantly, an LDH activity assay was performed to analyse cell toxicity. Since LDH activity in media from A $\beta$ Ostreated astrocytes was similar to control, we can conclude that the increase in extracellular levels of HspB8 come from healthy astrocytes that secrete it to the extracellular media (*data not shown*).

While the data presented here is preliminary and further repeats and experiments are required to elucidate the secretion of HspB8 under the conditions studied, a few conclusions can be drawn: (i) Primary mouse astrocytes secrete HspB8 to the extracellular media. (ii) When astrocytes are exposed to media from neurons, secretion of HspB8 is increased. (iii) The presence of A $\beta$ Os seems to increase the expression of HspB8 and its secretion, though the response does not seem to be fast.

#### **Future directions**

Since astrocytes secrete HspB8, it would be necessary to investigate what triggers this response and how the

presence of AβOs affects its secretion. Ultimately, to determine the effect that secreted HspB8 has on neurons and how this is affected during AD, could help identifying potential therapeutic targets.

#### **Departures from original proposal**

The project only departed slightly from its original proposal. It was stated in the proposal that A $\beta$ Os would be obtained from CHO cells that had been engineered to secrete nanomolar concentrations of disease relevant A $\beta$ Os (11). However, synthetic A $\beta$ Os were used instead. Moreover, since the treatment with synthetic A $\beta$ Os did not show any changes, we used the media from tg2576 neurons as a source of A $\beta$ Os, a more relevant model.



Figure 3. Intracellular levels of HspB8 after 8 hours (A) and 24 hours (B) of exposure to A $\beta$ Os were detected by western blotting. Likewise, the extracellular levels of HspB8 after 8 hours (C) and 24 hours (D) are shown. All the graphs show the mean of the three independent experiments.

#### Value of studentship

This studentship has been extremely beneficial and enjoyable. It has showed me what a research career would be like, helping me to decide on my future path. Moreover, while gaining laboratory skills that are going to be useful for future research projects, I have also learnt to work and think independently. Importantly, these 8 weeks, learning about chaperones, AD and astrocytes, has made me realise that I want to pursue a research career on AD. I am very pleased to have spent my summer here and I thank the Biochemical Society for this valuable experience.

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# Using detergent-free purification methods to study

# the mechanism of LeuT

Shona Hepworth, University of East Anglia

## INTRODUCTION

LeuT is a small amino acid transporter from Aquifex aeolicus. It is a bacterial homologue of neurotransmitter: sodium symporters (NSS) such as the solute carrier 6 family (SLC6)<sup>1</sup>. It was the first bacterial homologue of SLC6 family to be crystallised, as a homodimeric structure, and has remained the main reference transporter since. The SLC6 family plays a vital role in termination of synaptic transmission. Dysfunction of such proteins can lead to severe neurological conditions such as depression and anxiety<sup>1</sup>. There inhibitor complexes are several and conformational changes which have been suggested.

Electron paramagnetic resonance (EPR) is a key biophysical technique used to probe for conformational changes and structural dynamics of membrane proteins such as LeuT. However, the presence of a paramagnetic species is essential. Site directed spin labelling (SDSL) is used to attach small nitroxide spin labels to specific sites of the protein, allowing the use of advanced EPR techniques. EPR scans the magnetic field at a fixed frequency. When the energy gap between two energy levels of electrons resonates with that of the frequency, a transition is seen and recorded as the first derivative. Spectra obtained can then be analysed to obtain information about the protein. Styrene maleic acid lipid particles (SMALPs) are a novel detergent free method of purification. The membrane protein is encapsulated using styrene maleic acid, along with its native lipid environment to yield the SMA particles. These are very stable easv and hence allow downstream characterisation of proteins in more native environments.

#### **PROJECT AIMS**

• Express and purify LeuT, selectively attach EPR spin labels to selected Cys-variants



Figure 9 Leucine transporter (LeuT) homodimeric structure and the result of the detergent-free purification, LeuT-SMALP

- Develop practical techniques of protein purification using novel detergent free approaches, and in the future compare results to detergent purification and reconstitution into liposomes
- Develop efficient spin labelling methods

## METHODS

# <u>Plasmid preparation and Expression of wt-LeuT</u> and A9C variant

Wt-LeuT and A9C variant were expressed in XL1 blue E.Coli cells. A small overnight culture was grown for each, containing 100 mg/mL ampicillin. A QIAprep kit was used to purify the plasmid DNA. Nanodrop of the plasmid yielded a concentration ca. 30µg/mL. After successful plasmid sequencing, the plasmid DNA was then transformed in C41(DE3) competent cells. From this a pre-culture was grown, followed by an overnight culture at 37°C. Four 1L cultures were incubated at 37°C and 190rpm until an OD<sub>600</sub> of 0.6 was reached. This was followed by induction using IPTG, and subsequent incubation overnight at 18°C. Centrifugation at 7000 rpm, 12 minutes gave cell pellets. The cells were resuspended and lysed using sonication and centrifugation to give pellets at concentrations membrane ca. 30µg/mL.

# Detergent-free purification, SMALPs

Styrene maleic acid (SMA) co-polymer was prepared according to standard protocol<sup>2</sup>. LeuT was solubilised using this standard protocol<sup>2</sup>, added to Ni-NTA resin and left overnight shaking at 4°C. LeuT-SMALPs bound to Ni-NTA resin was poured into gravity flow columns, washed and finally eluted with 200mM imidazole. The fractions were analysed using SDS-gel, pure LeuT eluate fractions were pooled and concentrated using Vivaspin spin concentrators

<sup>1</sup> C. Colas et al, SLC transporters: Structure, Function and Drug Discovery, Medchemcomm. 7 (6), 1069-1081 (2016)

S. Lee T et al. A method for detergent-free isolation of membrane proteins in their local lipid environment. Nature Protocols. 11 (7). 1149 (2016)

(100kDa MWCO). Concentrations were estimated by BSA standard SDS gels and subsequent analysis using Image Lab (BioRad). Imidazole was removed by PD10 Desalting Columns.



Figure 10 The visual process of solubilising membrane proteins with styrene maleic acid copolymer<sup>2</sup>

## Spin labelling of LeuT-SMALPs

Two methods of spin labelling were conducted. The first used a 10:1 molar ratio of MTSL to cysteine. Dithiothreitol (DTT) was added to the LeuT-SMALPs to reduce cysteine residues and removed after 4h incubation shaking at 4 °C. The reductant was removed by ZebaSpin column. A comparison without prior reduction with DTT was made. Both were incubated with MTSL rotating at 4 °C overnight, while kept away from light. In the second method of labelling, 1mM MTSL was added to the LeuT-SMALPs bound to,—Ni-NDTA resin. This was left shaking overnight at 4°C, wrapped in foil while kept away from light.

#### RESULTS

I successfully produced LeuT (wild type and A9C variant). The cell pellets obtained were around 5.5g/L cell culture. The average membrane pellet weight was 2.5g/L cell culture. The membrane proteins were successfully encapsulated by SMA co-polymer to form LeuT SMALPs. Upon purification, a clear band in an SDS-page gel was seen, showing the eluate fractions containing LeuT SMALPs.

Spin labelling attempts were unsuccessful as shown by cw-EPR as no nitroxide spin label signals were



ladder<sup>FT</sup><sub>conc</sub> E1 E2 E3 E4 E5 E6 E7 E8 FT ladder 0.25 0.5 0.75 1.0 1.25 <sup>LeuT-</sup><sub>SMALP</sub> Figure 11 Two SDS-page gels. Left: The eluate fractions after single step affinity purification. Right: BSA standards ranging from 0.25 – 1.25  $\mu$ g. The LeuT SMALP sample is the fractions combined and concentrated from single step affinity purification

seen. Further development is needed to find a suitable technique. A first assumption is that SMALPs introduce a rigidity into the system that, although increasing protein stability, might prevent successful attachment of the spin label to the target sites.

#### **FUTURE DIRECTION**

In the future, a successful and efficient spin labelling technique needs to be developed for LeuT-SMALPs to allow conformational studies using EPR. Once this has been determined, distance measurements can be made using a pulsed EPR technique called PELDOR. Detergent purification would allow comparison to detergent-free methods, in addition to reconstitution into liposomes. Going forward from this, the effect of substrate, ions, and inhibitor binding on conformation could also be investigated.

#### DEPARTURE FROM ORIGINAL PROPOSAL

There was no departure from the original proposal.

#### VALUE

I would like to take this opportunity to thank Biochemical Society and Dr Fraser MacMillan's lab for providing me with the chance to complete such an interesting project. I have found it particularly useful by introducing me to biochemical techniques such as protein expression and purification, which I haven't covered in my undergraduate degree. The 8 week placement has confirmed my desire to complete a PhD, and increased my confidence in completing a masters project. In addition to my internship I was given the opportunity to attend 'Experimental techniques for studying proteins and lipids in biological membranes' which helped me improve my networking skills and knowledge,

particularly surrounding SMALPs, which I have successfully utilised within my internship. I was also fortunate enough to be able to present my research in the form of a short talk and poster at the 14<sup>th</sup> international school of Biophysics in Split, Croatia.



# The role of C9orf72 Dipeptide Repeats in neurone morphology

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Amyotrophic lateral sclerosis (ALS) is the most common form of motor neurone disease and frontotemporal dementia (FTD) is the second most common form of dementia. Both are

neurodegenerative disorders with few effective treatments and no known cures. The two disorders share clinical and genetic features and an underlying pathogenesis (1) with patients of both exhibiting symptoms of motor neurone dysfunction and neurodegeneration. A mutation in the form of a repeated GGGGCC hexanucleotide expansion in the non-coding region of C9orf72 is the cause of most familial forms (2). The intronic GGGGCC repeat has been proven to be the cause, but the mechanisms by which it causes neurodegeneration are yet unknown. Possible mechanisms are as follows; the expansion affects C9orf72 expression and leads to loss of function, repeat mRNA leads to alteration in RNA processing, and translation of the repeat leads to dipeptide repeat proteins(DPRs). There are five DPR constructs translated from the expansion; -GA, -GP, -GR, -PA and -PR. Of the five species, GP-, GA-, and GR- have been linked to increased neurotoxicity (3). Whether DPR expression contributes to neurodegeneration is unknown. Poly-GA was found to aggregate and cause toxicity in primary hippocampal and cortical neurones (4) and poly-GR forms neurotoxic aggregates in rodent cortical neurones (3). Despite the presence of these inclusions, their effects on neurone morphology are yet to be studied. Alterations to neurone morphology have been linked to neurodegenerative disorders, therefore this studentship investigated the role DPR constructs play in this precursor to neurodegeneration in ALS/FTD.

#### Aims

To investigate:

- the impact of DPRs on morphology of rat cortical neurones
- the neurotoxic effects of DPR inclusions via Lactose Dehydrogenase Assay
- DPR inclusion localisation using nuclear and cytosolic markers
- differences between computational programs Neurolucida 360 and Neuronstudio in analysis of synaptic/neurone morphology
- whether levels of DPR neurotoxicity are dependent on how long DPR-mediated cells are incubated with toxin

#### Methods

<u>Cell culture:</u> Mature rat cortical neurons were seeded at 120,000 cells per well. Neurons(DIV7) were transfected with control EGFP vector or EGFP-125 repeat Poly-GA, -GP, -GR, -PA or –PR and Red Fluorescent Protein (RFP- cytosolic marker) for 24h and 48h in a medium of OPTI-MEM (1X) and Lipofectamine 2000. Toxicity of DPR-mediated cells was observed and compared with EGFP control group. Separate tests were carried out for 24h and 48h to judge if DPR neurotoxicity is time-dependent.

Lactase Dehydrogenase (LDH) Assay: Respective supernatants of DPR-mediated cells at 24h and 48h post-transfection were put through a LDH Assay, indicating levels of excitotoxicity caused by DPR constructs and how the levels vary between 24h and 48h neuron incubation in toxin.

Immunostaining: Cells at 24h and 48h post-transfection were fixed with 4% PFA with sucrose, permeabilised with 0.5% Triton,

incubated with DAPI 1X (nuclear marker) and mounted onto slides with DAKO mounting medium for immunofluorescent imaging. Inoculation of an overnight E. coli bacterial culture: Green florescent protein (GFP) plasmid construct was created by inoculating a glycerol stock solution of GFP (100mg/ml) with Kanamycin antibiotic(100mg/L) in LB Broth (20g/L). This was incubated for 18h in a bacteriological shaker at 37°C. DPR plasmid constructs were inoculated with Ampicillin antibiotic (50mg/L) in a pre-inoculum at 37°C for 4-6h. This was to guarantee the growth of a bacterial culture in presence of DPR toxin. Pre-inoculum was then incubated for a total for 18 hours at 37°C. Glycerol stock preparation: 500 µl of each respective inoculated bacterial culture was stored at -80 °C for future glycerol plasmid stock. Purifying DNA plasmids: GFP/DPR constructs were purified using the PureLink<sup>™</sup> HiPure Plasmid Midiprep Kit. Resulting protein concentrations were measured using Nano drop software. Neurone analysis programmes: Sholl and spine analyses were carried out using Neurolucida 360, Neuronstudio, and ImageJ/FIJI. Microscope imaging acquisition: Slides were observed under an immunofluorescence microscope and pictures were taken using LAS Advanced florescence software.

#### **Results and discussion**

The following results and discussion are derived from two sets of data; only two experiments were successful due to the unpredictability and sensitivity of cortical neurones in cell culture. Further tests must be run to conclude a statistical analysis and generate replicable conclusions.

# Poly-GP, -GR, -PA and –PR showed a decrease in soma area and perimeter after 48h

All DPR-mediated cells except for cells incubated with Poly-GA showed a significant decrease in soma area and perimeter 48h post-transfection compared to the control EGFP group(fig1B). Soma size is impacted by the presence of DPR inclusions, suggesting that DPRs are neurotoxic. The reduction in soma size could be the cells preparing for apoptosis.

#### Poly –GR, -PA and –PR showed a decrease in soma area and perimeter when comparing 24h and 48h post-transfection. Poly- GA showed an increase and poly-GP showed no change (fig1)

DPR-mediated cells 48h post-transfection showing a decrease in soma size in comparison to 24 hours post-transfection suggests that DPR neurotoxicity levels increase the longer inclusions are in vitro. Poly-GP showing no change suggests that GP neurotoxicity levels are not time dependant.

#### DPR mediated cells revealed localised DPR inclusions

Poly –GA inclusions localised in the soma as dots; inclusions were purely cytosolic.

Poly –GP, -GR and –PA showed cytosolic and nucleolar inclusions, with faint aggregates in the neurites. Poly –GR inclusions were smaller in size than Poly –GP and –PA, which both aggregate within the entire nucleus. Poly –PR inclusions were somatic and nucleolar; all –PR aggregates localised to the cell body(fig2). Poly –GA, -GP, -GR and –PA all show cytoplasmic aggregates that are similar to those in C9orf72 mutation brains (4). This specific localisation of DPRs in C9orf72 neurones could be linked to a potential mechanism underlying C9orf72 linked ALS/FTD.



Figure 1: mean soma area( $\mu$ M) and perimeter ( $\mu$ M) of mature rat cortical neurons transfected with GFP/DPR constructs

(A) 24h post transfection

(B) 48h post-transfection

#### DPRs alter neurone morphology

Via analysis on Image J plugins FIJI and Neurite Tracer, neurone morphology analyses were carried out on DPR-mediated cells 24h post-transfection. The total number of neurites, number of primary and tertiary neurites, and dendritic branching (6) were analysed. Analysis of neurite morphology using Neurite tracer showed that the number of neurites was lower in DPR-mediated cells compared to EGFP cells. The number of primary neurites in DPR mediated cells was higher compared to EGFP cells whereas the number of tertiary neurites and neurites with a length longer than 50  $\mu$ M were higher in EGFP cells (fig3).

DPR-mediated cells also showed a significant decrease in maximum radius when compared to EGFP cells.

Dendritic complexity was measured by counting the number of dendritic crossings within each 12.5  $\mu$ M radius in concentric circles around the soma for each neurone image (6).

Results show that the number of dendritic crossings reduced with every 12.5  $\mu$ M away from the soma, and was also significantly lower in DPR-mediated cells than EGFP cells(fig4).

The results of the neurone morphology analyses suggest that DPR mediated toxicity may prevent neurones from creating a complex dendritic network. The lack of this network results in less communication with neighbouring neurones and may affect synaptic function, in turn leading to neurodegeneration.

#### Computational analysis

As part of my project, I analysed synaptic morphology for my supervisor. I used two neurone analysis programmes to analyse the number and type of dendritic spines, Neurolucida 360 and Neuronstudio, the former of which had not been used before by my host laboratory. I found that Neurolucida was faster and more efficient in analysing dendritic branches and spines than Neuronstudio, and I was also able to use the program to carry out sholl analysis on my own data; Neuronstudio did not allow this. The results for the sholl analysis were the same as the results I obtained from sholl analysis via ImageJ plugin FIJI. My supervisor and her colleagues will therefore be using Neurolucida 360 for all future neurone analysis.



Figure 2: EGFP control and DPR-mediated cells were imaged under an immunofluorescence microscope at 40X magnification using LAS Advanced Fluorescence software. Analysed cells met 4 prerequisite criteria; neurone was in the region of interest, neurone and neurites were easily identifiable from others, neurites were non-truncated, and neuron showed full RFP staining throughout structure (5).



Figure 3: Neuron morphology analysis; total number of neurites, total number of primary and tertiary neurites, and neurite length for DPR-mediated and EGFP cells.



<u>Figure 5:</u> Neuron morphology analysis; dendritic branching analyzed by counting the number of dendritic crossings per  $12.5 \mu$ M radii away from soma.

The results of the neurone morphology analyses suggest that DPR mediated toxicity may prevent neurones from creating a complex dendritic network. The lack of this network results in less communication with neighbouring neurones and may affect synaptic function, in turn leading to neurodegeneration

#### **Deviation from original project**

Originally, I was to focus on DPR impact on synaptic function, with a focus on synaptic morphology via analyses of types and quantities of dendritic spines. When running the first test, I found that DIV10 neurones showed death as early as 24h post transfection with DPRs. To rectify this, I transfected the neurones with DPRs on DIV7, and the cells lived several weeks post transfection. However, cortical neuron spines can only be seen under a microscope after DIV10, and therefore I was unable to carry out spine analysis.

#### Value of studentship

During my studentship, I acquired a variety of lab skills that will prove useful when carrying out the rest of my degree. I was able to work alongside a team of scientists and partake in lab meetings, alongside learning how to manage my own scientific projects and problem solve. I had access to neuron analyses programmes I would not otherwise been able to use, and learnt useful computational skills. All this gave me an insight into working in a lab, and further cemented my plans in pursuing a Masters in neuroscience.

Most importantly, thanks to this project, I was able to gather significant preliminary data that will aid in future further studies on C9orf72 mutations. I would like to thank Dr Patricia Gomez-Suaga, Dr Chris Miller and the Biochemical Society for giving me this great opportunity.

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# Synthesis and binding analysis of potential antiviral inhibitors of calnexin/calreticulin pathway

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# Introduction:

Enveloped viruses, like hepatitis C virus and dengue virus, have a capsid which is composed of lipids from host membranes as well as viral glycoproteins. Viruses must produce a functional N-glycosylated envelope in order to replicate, and they must undergo processing by host endoplasmic reticulum proteinfolding machinery. This involves entry into the endoplasmic reticulum quality control (ERQC) pathway as a path of glycosylation process. When ERQC is partially or completely blocked, a misfolded envelope is produced and virus infectivity and morphogenesis can be compromised. One possible way to block and regulate entry into ERQC is by iminosugars, nitrogen-containing compounds that mimic monosaccharides, which enables them to act as competitive inhibitors of the endoplasmic reticulum  $\alpha$ glucosidase I and II (GluI and GluII). The first step in N-glycosylation involves an attachment of 14sugar oligosaccharide, which is then eventually processed, involving removal of two terminal glucose residues by GluI and GluII. The monoglucosylated moiety serves as a recognition signal for calnexin and calreticulin, which influence the interaction between the protein and various chaperones for proper folding. Therefore, by acting as inhibitors of glucosidases, iminosugars inhibit the calnexin/calreticulin pathway. Our lab has developed more compounds based on the chemistry of iminosugars to produce a similar effect in inhibition of glucosidases, especially GluII. Iminosugars and their derivatives provide an advantage compared to other antiviral drugs since they target conserved host machinery rather than viral machinery, which is highly variable. In addition to iminosugars, we pursued novel classes of inihibitors based on initial hits from fragment-based screening.

# Aims of the project:

The aim of the project was to synthesize and test novel potential inhibitors of calnexin/calreticulin pathway, focusing on GluII.

#### Work carried out:

We started off by testing some of the potential GluII inhibitors (ligands), which had been identified by crystallography, for binding to GluII in solution, using the saturation transfer difference (STD) NMR approach. STD NMR is commonly used to study protein-ligand interactions. Our method involved subtracting the 1D <sup>1</sup>H NMR spectrum of ligand in the presence of saturated GluII (on-resonance) from one recorded without protein saturation (off-resonance spectrum). Therefore, it can be deduced if the

binding occurs, based on the changed free induction decay (FID) signal of protons on the ligand. Beyond simply answering whether each compound binds, we used STD-NMR to develop epitope maps for ligands by comparing the magnitude of STD effects for individual <sup>1</sup>Hs. A proton that is closer to GluII (e.g. deeper binding) produces a larger STD value. We validated the method by comparing the epitope maps to the orientation of the ligands in previously obtained X-ray crystal structures. In other cases, we were able to use the STD-based epitope maps to select the more likely orientation of ligands in the crystal structures, when more than one possibility matched the density. We also used STD-NMR to determine the Kd values of ligands showing



binding. By obtaining NMR spectra at different ligand concentration with constant concentration of GluII, a Scatchard plot was drawn and Kd was fit to the data.

The second part of my research involved organic synthesis of new potential GluII inhibitors. I have performed many standard organic reactions including the Mitsunobu reaction and heterocycle synthesis. Most of the synthetic targets were multiple-step reactions, and hence, I carried out a lot of thin-layer chromatography (TLC) plates, to check reaction progress, and rotary evaporation under pressure, to evaporate solvents from the synthesized compounds. In addition, newly synthesized compounds were purified by liquid flash chromatography and their identity confirmed with NMR and mass spectrometry.

# **Results:**

In total during my internship, I tested more than 40 compounds for binding by STD-NMR, including the development of 7 epitope maps and 15 Kd determinations. 6 novel compounds are prepared, and the lab is continuing to test them now.



# **Future directions:**

The binding data that has been determined by NMR-STD approach can be used to design the next group of potential binders and inhibitors. The newly synthesized molecules can also serve as a 'next generation' of potential inhibitors of GluII. Once inhibitors have been optimised enough *in vitro*, they will have to be tested for their affinity, specificity and selectivity towards GluII *in vivo*. Hopefully, the new inhibitors will have fewer side effects, by avoiding inhibition of intestinal glucosidases, such as maltase.

# **Outcome of the studentship:**

I strongly believe that during my studentship I have gained many experiences regarding wet lab work, and I have grown confidence to work on procedures that as a biochemist I have never carried out before. That involves performing NMR experiments, dealing with raw data and analysing them, as well as performing organic synthesis, which is given less emphasis during my degree. I look forward to use this

Determination of Kd by STD amplification factor



knowledge for my Part II project and further on in life. It is highly valuable that I had an opportunity to work with some of the leading people in the area of antiviral drug development. Further to this, the studentship gave me the opportunity to work under conditions similar to PhD programs and in academia in general, and it made me realize that a career in research is something I am willing to dedicate my time to.

# **Supervisor Statement:**

Zala was a great asset to the lab during her studentship this summer. Her problem-solving ability and positive attitude helped to make substantial progress in our understanding of how well these initial ligands of GluII bind, as well as providing new ligands to be tested through her synthesis. These compounds and data Zala obtained have been instrumental as we continue the project, and we are looking forward to including Zala in the publication process when we reach that stage.

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