Summer Vacation Studentship Report

To date, numerous genes have been discovered to be responsible for familial amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD), with one of the most prominent ones being OPTN (Renton *et al.*, 2011). Through the genome-wide association study (GWAS) approach, it has been revealed that the mutation locating on chromosome 9 open reading frame 72 (C9orf72) contributes to nearly half of familial ALS cases within the tested cohorts. The human C9orf72 gene possesses two non-coding exons followed by 10 coding exons, resulting in 8 different alternative-splicing derived transcripts. In its pathological state, an excessive GGGGCC hexanucleotide repeat expansion (HRE) is observed within the first intron situated between two non-coding exons. One of the proposed pathogenic mechanisms suggests that these repeats can form a G-quadruplex that interferes the interaction between RNA and its respective RNA-binding proteins (Smeyers, Banchi and Latouche, 2021). Additionally, the presence of the HRE hampers transcription, resulting in reduced levels of the C9orf72 protein (Smeyers, Banchi and Latouche, 2021).

Despite the established connection between pathological C9orf72 and ALS/FTD, the precise molecular mechanism remains elusive. To address this, a stable human embryonic kidney (HEK) 293 cell line expressing FLAG-tagged C9orf72 was generated. Subsequent co-immunoprecipitation and mass spectrometry analysis revealed two significant interactors, SMCR8 and WDR41, forming what is referred to as the CSW complex in direct association with C9orf72 (Yang *et al.*, 2016) . Strikingly, the C9orf72-SMCR8 component within the CSW complex shares a high structural homology with another GAP complex known as FLCN-FNIP. Moreover, the CSW complex has been proven to interact with a variety of RAB proteins (members of Ras superfamily of small G proteins), including RAB1A, RAB11, RAB13, and RAB39B (Smeyers, Banchi and Latouche, 2021), adding an additional level of potential for the CSW complex to possess GAP activity. By elucidating the GAP/GEF activity towards GTPases and establishing links between the functionality of C9orf72 with specific signaling cascades in which GTPases are involved, we aim to gain insights into the potential function of C9orf72. This lays down the fundamental basis of my project, which is to explore the molecular basis of C9orf72 complex interactions and identify candidate GTPase substrates and their roles in autophagy-dependent selective uptake of mitochondria (mitophagy).

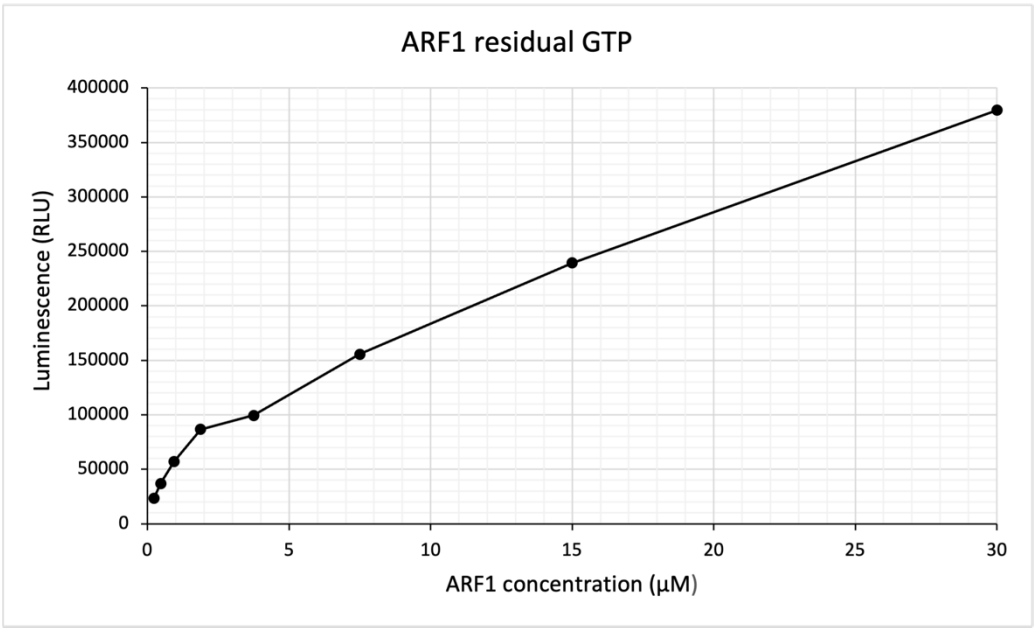
Results

In vitro GTPase-Glo Assay optimisation

The GTPase-Glo assay relies on the ability of GTPase to convert GTP into GDP by hydrolysing the γ-phosphate bond. This hydrolysis is catalysed by the presence of GTPase activating protein (GAP). Regaining GTP requires another protein known as GTP exchange factor (GEF), which stimulate the release of GDP and the reload of GTP to the GTPase so that the GTPase can restore its activity. In this assay, the reaction between a candidate GTPase and the CSW complex in a GTP stock is terminated by addition of GTPase-Glo reagent that converts any unconsumed GTP into ATP, which is subsequently rendered to emit luminescence that can be recorded. It is important to note that the higher the luminescence is, the less the GTP is consumed by GTPases. Prior to the test assay, several parameters need to be optimised, including the GTP stock concentration, reaction time, the GTPase concentration and the CSW concentration. Hence, we replicated the assay using GTPase ARF1, a well-

known GTPase substrate for CSW complex. Regarding the experimental data, we observed that the ARF1 GTPase activity increases with higher concentrations of ARF1, while keeping the CSW concentration constant. This increase in activity is substantial up to a concentration of 10μM, after which it reaches a more moderate rate of increase (*Fig 1a*). Additionally, we noted a linear relationship between the ARF1 concentration and the pre-loaded GTP on ARF1 (*Fig 1b*), indicating that the GTP carried by the GTPase itself is ineligible that must be considered in subsequent assays.

(a)



(b)

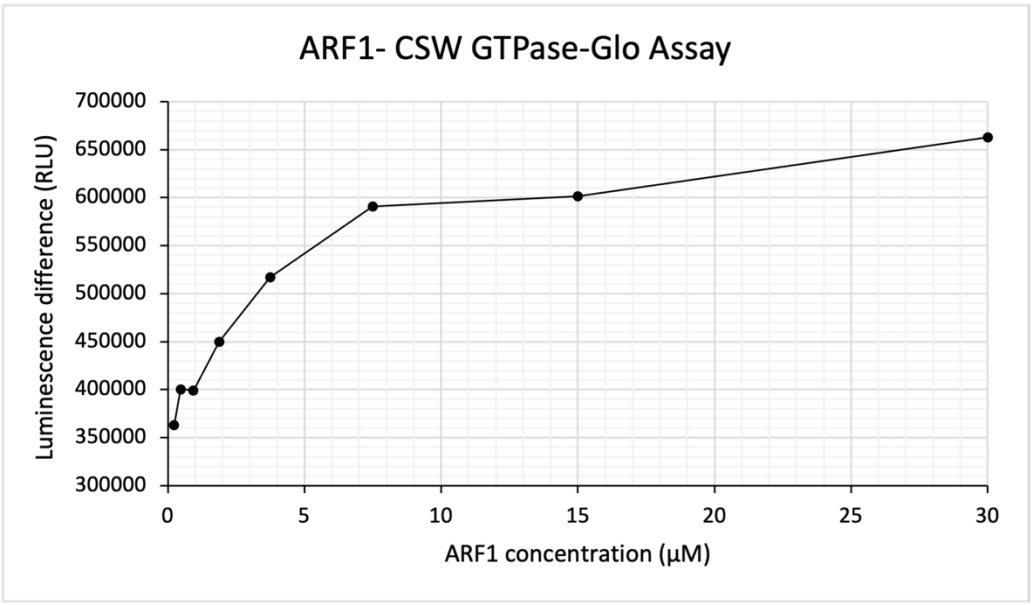
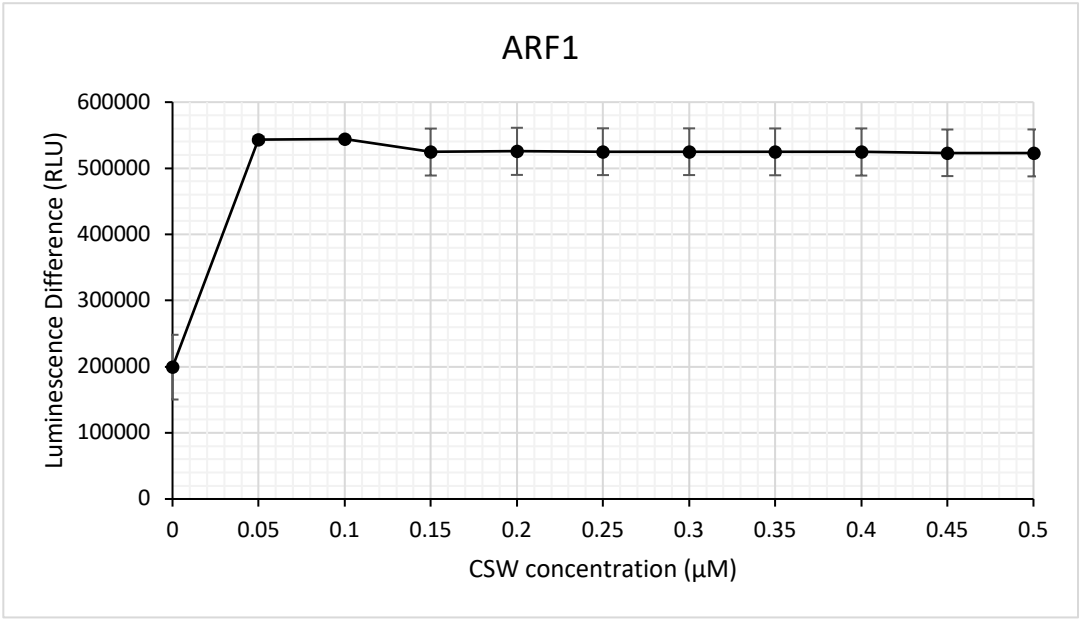


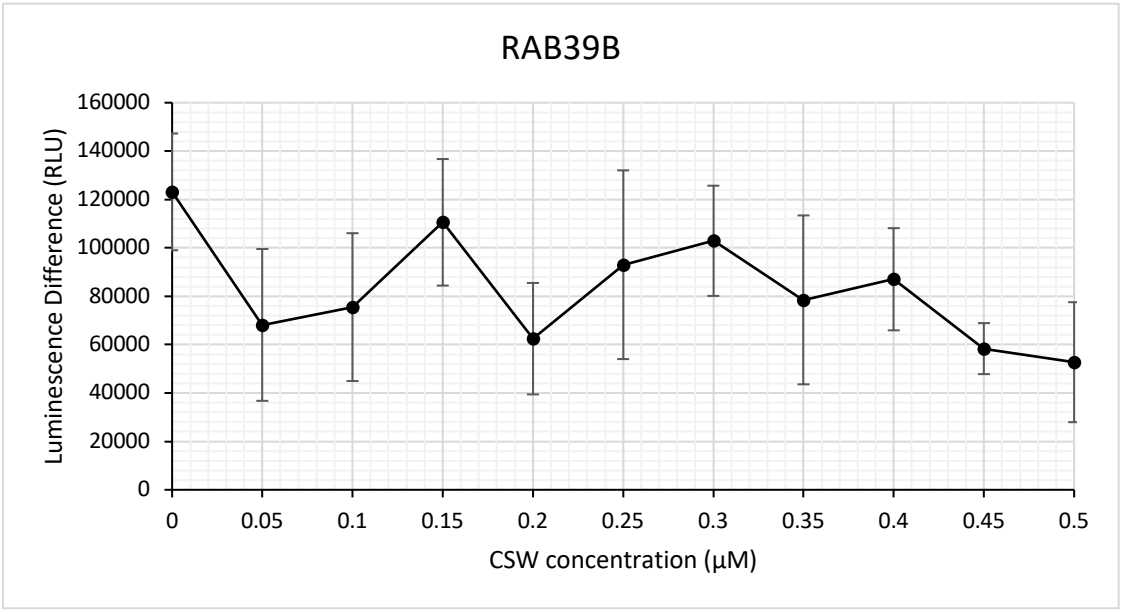
Figure 1. **GTPase-Glo assay optimisation results. (a)** The measurement of ARF1 residual GTP, which was obtained in the absence of GTP stock. **(b)** The GAP assay of ARF1 at various concentrations (0-30μM) with fixed CSW concentration (0.3μM). Luminescence was measured by Clariostar.

CSW is suspected to exhibit GEF activity towards GTPases Rab39A and Rab1A
Following the optimisation process, GTPase-Glo assays targeting Rab39A and Rab1A were conducted. Rab39A and Rab1A are two GTPases that were identified in the mass spectrometry data obtained from the CSW immunoprecipitation sample. Additionally, ARF1 was included as a positive control (*fig 2a*). Notably, GTP consumption was completed even when the CSW concentration was as low as 0.05μM, indicating a need in reducing GTPase concentration in future assays. Surprisingly, Rab39A and Rab1A exhibited distinct luminescence pattern compared to ARF1(*fig 2b,c*), while their luminescence patterns display certain extent of similarity, suggesting that the CSW complex does not serve as a GAP for these two GTPases. Whether the CSW complex exerts GEF activity on Rab39A and Rab1A requires further investigation, which will involve the use of a GEF buffer provided by Promega.

(a)



(b)



(c)

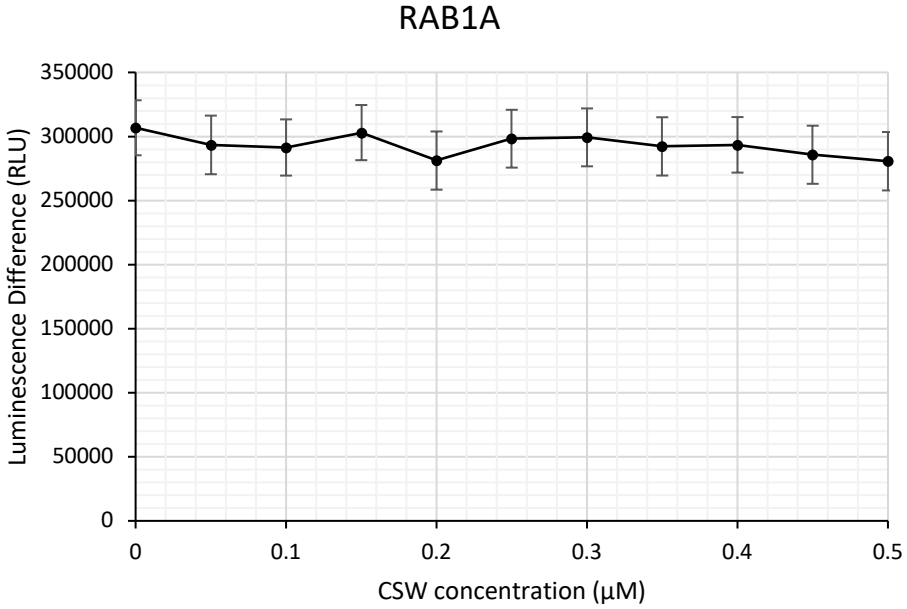


Figure 2. **GTPase-Glo assay results. (a)** The GAP assay of ARF1 at fixed concentration 30μM with varied CSW complex concentration (0-0.5μM, twofold serial dilution). **(b)** The GAP assay of Rab39B at fixed concentration 30μM with varied CSW complex concentrations (0-0.5μM, twofold serial dilution). **(c)** The GAP assay of Rab1A at fixed concentration 30μM with varied CSW complex concentration (0-0.5μM, twofold serial dilution). Luminescence was measured by Clariostar.

Regarding future actions, it is crucial to investigate the GEF activity of the CSW complex against short-listed GTPase candidates. Owing to the limited usage of buffer, multi-channel pipette cannot be employed during the experiment, posing a significant challenge for reaction time control. To address this issue, all GTPase assays were conducted on ice to slow down the reaction as much as possible. Further, a mutant version of CSW that 147 arginine finger of SMCR8 is mutated to alanine should be purified and serve as a negative control for such GTPase assay. Given the nature of this type of assay, only the endpoint of the reaction can be measured. Thus, incorporating alternative GTPase assays, such as Mant-GDP fluorescence, would significantly complement the results by enabling real-time measurements.

Throughout my project, we encountered various challenges, with one notable example being the high background noise in our western blots. By comparing our existing protocol with publications that exhibited clearer blots for our protein of interest and seeking advice from senior members in the lab, I found that the concentration of TWEEN-20 utilised in the washing buffer (0.05%) was lower than in other protocols (0.1%). After making this improvement, we successfully obtained quantifiable blots and confirmed the appropriate concentration for the assay. Besides, I extensively analyse my experimental results using a range of software packages, including Adobe Illustrator, Fiji and Flowjo, which are widely applied in the research field. Furthermore, I presented my results during our lab meetings, which allowed me to not only practice my public speaking skills but also gain a broader perspective on what a career in a lab would be. Lastly, actively participating in wet-lab experiments and collaborating with more experienced members of the team has been a true pleasure. I have experienced the spirit of knowledge transfer, witnessing how science is passed down through generations. I look forward to doing the same when I reach their stage and sharing what I have

learned with future generations, as a way of expressing my gratitude to everyone who has helped me along the way.

References

Renton, A.E. *et al.* (2011) ‘A Hexanucleotide Repeat Expansion in C9ORF72 Is the Cause of Chromosome 9p21-Linked ALS-FTD’, *Neuron*, 72(2), pp. 257–268. Available at: https://doi.org/10.1016/j.neuron.2011.09.010.

Smeyers, J., Banchi, E.-G. and Latouche, M. (2021) ‘C9ORF72: What It Is, What It Does, and Why It Matters’, *Frontiers in Cellular Neuroscience*, 15, p. 661447. Available at: https://doi.org/10.3389/fncel.2021.661447.

Yang, M. *et al.* (2016) ‘A C9ORF72/SMCR8-containing complex regulates ULK1 and plays a dual role in autophagy’, *Science Advances*, 2(9), p. e1601167. Available at: https://doi.org/10.1126/sciadv.1601167.