To investigate the role of IRE1β's loop in IRE1β-AGR2 complex formation

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Word count: 1095 (the word counter counts IRE1β^{LD}/α_{loop} as three words, so the actual count is smaller)

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Introduction

The endoplasmic reticulum (ER) controls the quality of folding of secreted proteins in eukaryotes. Accumulation of misfolded proteins causes ER stress resulting in the induction of an unfolded protein response (UPR) to restore proteostasis [6].

Inositol-Requiring 1 (IRE1) is a UPR regulatory membrane protein with two paralogues in humans: IRE1α and IRE1β. In the basal conditions, both paralogues are inhibited by corresponding chaperones, BiP and AGR2, IRE1α and IRE1β respectively [1, 5]. However, regulation of IRE1β remains enigmatic.

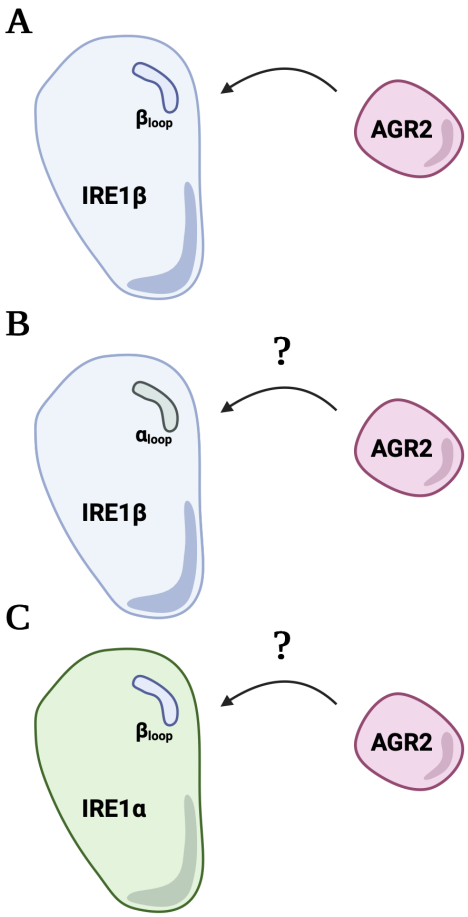

Figure 1: Experimental design.
A: To confirm that our AGR2 engages IRE1β^{LD}. **B**: To express IRE1β^{LD}/α_{loop} chimera and test whether it is engaged by AGR2. **C**: And to express IRE1α^{LD}/β_{loop} chimera and test whether it is engaged by AGR2.

Recent studies proposed a flexible IRE1β's loop region that is engaged by AGR2 [3] and a similar region in IRE1α which does not show significant binding with AGR2 [5]. Here, we investigate the importance of the loop region on the IRE1β-AGR2 complex formation.

To simplify the study, only the luminal domain (LD) of IRE1 was chosen. We began by confirming correct AGR2-IRE1β^{LD} (Fig. 1, A) and AGR2-IRE1β_{loop} associations, with the latter one confirming previous unpublished finding from the group which was carried out at different conditions. Following, we synthesised and tested IRE1β^{LD}/α_{loop} (Fig. 1, B) and IRE1α^{LD}/β_{loop} (Fig. 1, C) chimeras. The study confirms that the IRE1β_{loop} is indeed recognised by AGR2, however in context of IRE1α, the loop is not engaged, signifying a deeper underlying binding mechanism.

Aims and Departures From Original Plan

The initial goal of the project was to investigate which AGR2 conformation assumes in solutions. However, since March, when the project was submitted, the group managed to isolate and synthesise IRE1β_{loop} peptide and successfully prepare IRE chimera plasmids.

Therefore we decided to shift the scope of this investigation. The new project aim was to investigate whether the IRE1β_{loop} is responsible for binding with AGR2. We set the following milestones. To confirm the previous finding that AGR2 engages the loop peptide, and if successful express and purify both IRE1α^{LD}/β_{loop} and IRE1β^{LD}/α_{loop} chimeras, followed by investigating their ability to bind AGR2 with biolayer interferometry (BLI) (Fig. 1).

Results

Protein purification and purity confirmation

AGR2 and both chimeras were expressed in bacteria and purified according to their tags. Additionally, size exclusion chromatography (SEC) was used in IRE1β^{LD}/α_{loop} and IRE1α^{LD}/β_{loop} purification to remove aggregates (Fig. 2, A & B).

Furthermore, we tested the purity of all the used proteins in our investigation on an SDS-PAGE gel. Each sample was loaded at 3 and 9 μL (Fig. 2, C) with protein concentration being constant across all wells. Overall, all samples had the correct weights and good purity.

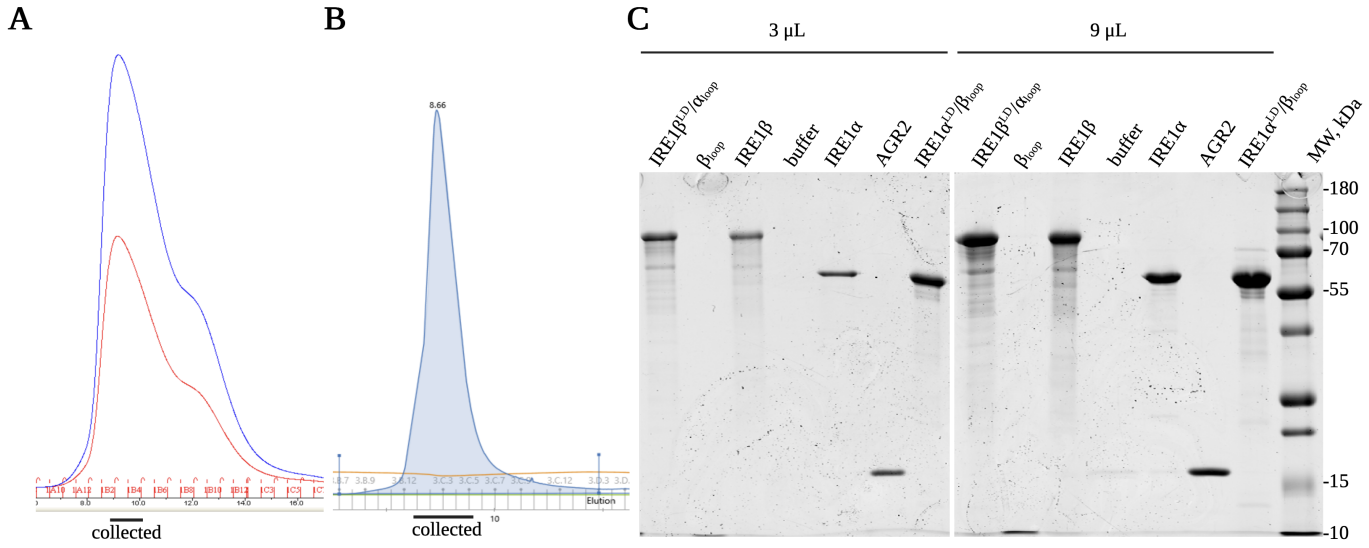

Figure 2: Protein purification
A: SEC purification of IRE1β^{LD}/α_{loop}, column Superdex™ S200. **B**: SEC purification of IRE1α^{LD}/β_{loop}, column Superdex™ S75 increase. **C**: Confirmation of protein purity on a 15% SDS-PAGE gel. Note, BLI ligands (every protein except AGR2) have slightly higher molecular weights (MWs) due to an *in vitro* biotinylation.

Buffer electrolyte concentration affects AGR2's binding affinity

During the first experiments, we observed unexpected trace behaviour at the highest AGR2 concentrations which made the results unusable (Fig. 3, A). To investigate, we experimented with different reaction buffers and electrolyte concentrations. Based on those, we believed that high electrolyte concentration contributed to breaking the dimeric state of AGR2 which is important for binding with IRE1β [2, 3].

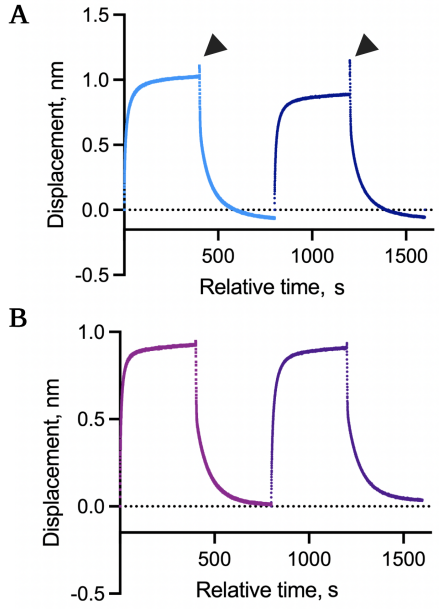

Figure 3: The buffer effect
A: Buffer effect (arrows) was prominent in all the runs at the highest AGR2 concentrations. The peaks appear when the sensor returns to a well with low electrolyte concentration. Ligand: IRE1β^{LD}, relative time from the end of dissociation at [AGR2] = $125\mu M$. **B**: No buffer effect was observed after an exchange of the AGR2 buffer composition. Ligand: IRE1β^{LD}, relative time from the end of dissociation at [AGR2] = $125\mu M$.

Therefore, we prepared a new batch of AGR2 with a lower salt concentration and used dialysis to exchange the buffer composition to one similar to the other protein solutions used in this study, successfully eliminating the trace behaviour (Fig. 3, B).

AGR2 engages IRE1β^{LD} and IRE1β_{loop} as predicted

Once the AGR2 buffer issue was resolved, we set up three replicates to investigate AGR2-IRE1β^{LD} complex formation in new conditions. Each repeat showed the predicted behaviour (Fig. 4, A(i)). To confirm, we plotted the displacement values against AGR2 concentration (Fig. 4, A(ii)) and modelled binding with one site-specific binding (Eqn. 1), where B_{max} is the maximum specific binding and K_d the equilibrium dissociation constant. The obtained constants agreed with previous findings by Neidhardt and colleagues [5].

$$y = \frac{B_{max} \times x}{K_d + x} \tag{1}$$

We then proceeded to test binding with the loop peptide alone (Fig. 4, B(i)). Like before, we ran $n = 3$ replicates (Fig. 4, B(ii)), and observed similar interactions as a different group member with other buffer and experimental conditions.

The difference in experimental B_{max} values (Table 1) is caused by the ligand size. IRE1β^{LD} is larger than the loop peptide, therefore more peptide molecules get attached to sensors, resulting in larger B_{max}. IRE1β^{LD} has smaller K_d than IRE1β_{loop} (Table 1), which indicates that although the loop is responsible for most of the binding, some LD, non-loop, residues are likely to stabilise the complex.

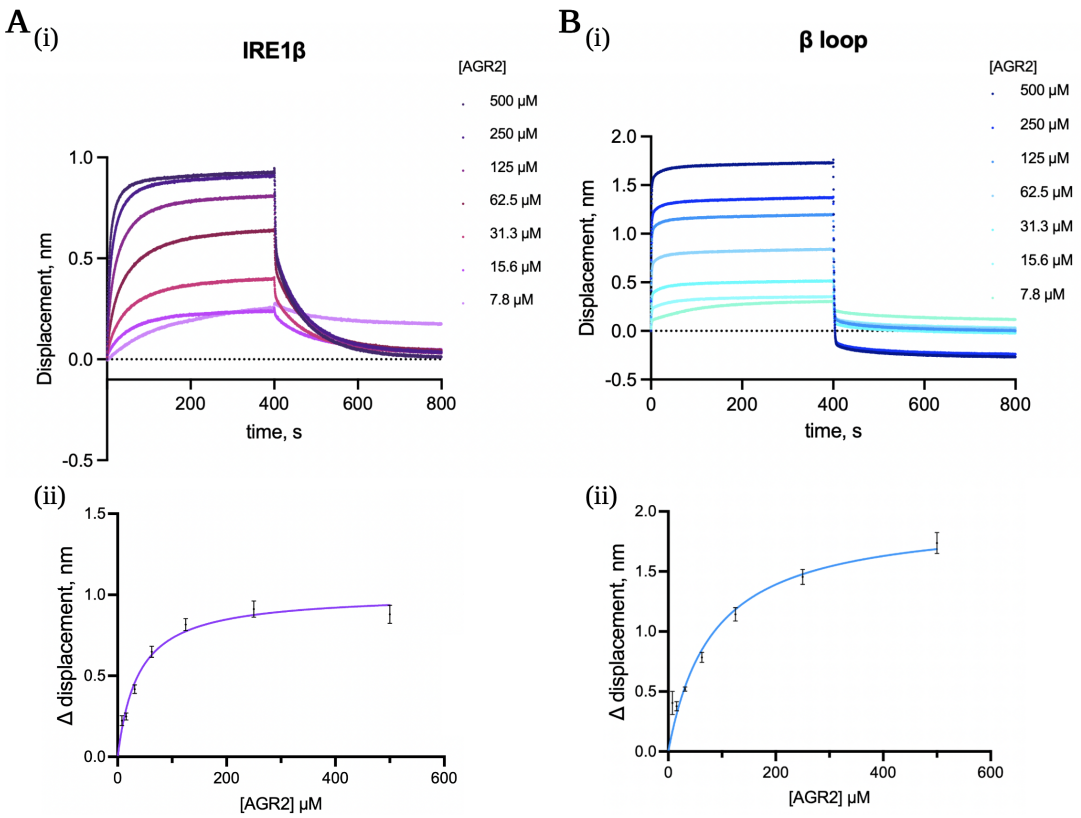

Figure 4: Investigation of AGR2's affinity for the β_{loop}
A: (i) Association and dissociation phases of a selected IRE1β^{LD} replicate across the AGR2 titration, (ii) one site-specific binding fitting for all three replicates. **B**: (i) Association and dissociation phases of a selected IRE1β_{loop} replicate across the AGR2 titration, the phase profiles indicate less specific, more transient complex. (ii) One site-specific binding fitting for all three replicates.

Table 1: Fitting constants for IRE1β^{LD} and IRE1β_{loop}

	IRE1β^{LD}	IRE1β_{loop}
B_{max}, nm	1.007	1.965
K_d, s	37.55	82.46
R squared	0.9763	0.9644

Table 2: Fitting constants for IRE1α^{LD}, IRE1β^{LD}/α_{loop}, and IRE1α^{LD}/β_{loop}

	IRE1α^{LD}	IRE1β^{LD}/α_{loop}	IRE1α^{LD}/β_{loop}
B_{max}, nm	0.2585	0.2490	0.4482
K_d, s	23.57	22.54	230.0
R squared	0.6306	0.7292	0.8604

AGR2 does not significantly engage IRE1α^{LD}/β_{loop} nor IRE1β^{LD}/α_{loop}

Once confirmed that AGR2 engages the loop peptide, we proceeded to express and test both chimeric proteins.

Similarly to Neidhardt and colleagues [5], we did not observe any significant binding between AGR2 and IRE1α^{LD} (Fig. 5, A, D). Furthermore, AGR2 did not engage IRE1α^{LD}/β_{loop} differently to IRE1α^{LD} (Fig. 5, B, D), thus giving further evidence for the importance of the loop in AGR2-IRE1β complex formation.

Surprisingly, AGR2 did not show any more affinity to IRE1α^{LD}/β_{loop} than to IRE1α^{LD} and IRE1β^{LD}/α_{loop}, suggesting that β loop alone might not be sufficient to form the complex (Fig. 5, C-E). This is further indicated by the IRE1α^{LD}/β_{loop} K_d value, which is larger than the K_d of IRE1β^{LD}-AGR2 binding (Table 2). These findings suggest that the LD of IRE1α prevents most AGR2 molecules from binding.

Conclusion and Future Directions

This investigation proves that the IRE1β_{loop} is indeed engaged by AGR2 (Fig. 4, Table 1); nonetheless, the mechanism of AGR2-IRE1β interaction seems to be more complex than the predicted lock-and-key binding with some LD, non-loop residues, playing an important role in the complex formation.

Currently, there is no structure of the complex to investigate the loop's role. This is due to the nature of the AGR2-IRE1β interaction which is not stable enough for crystallography studies. However, at the end of the studentship, we created a construct with AGR2 and IRE1β^{LD} joined with stabilising proteins (Fig. 6, A). The purified product remained in the complex form and had a sufficient yield for further structural studies (Fig. 6, B).

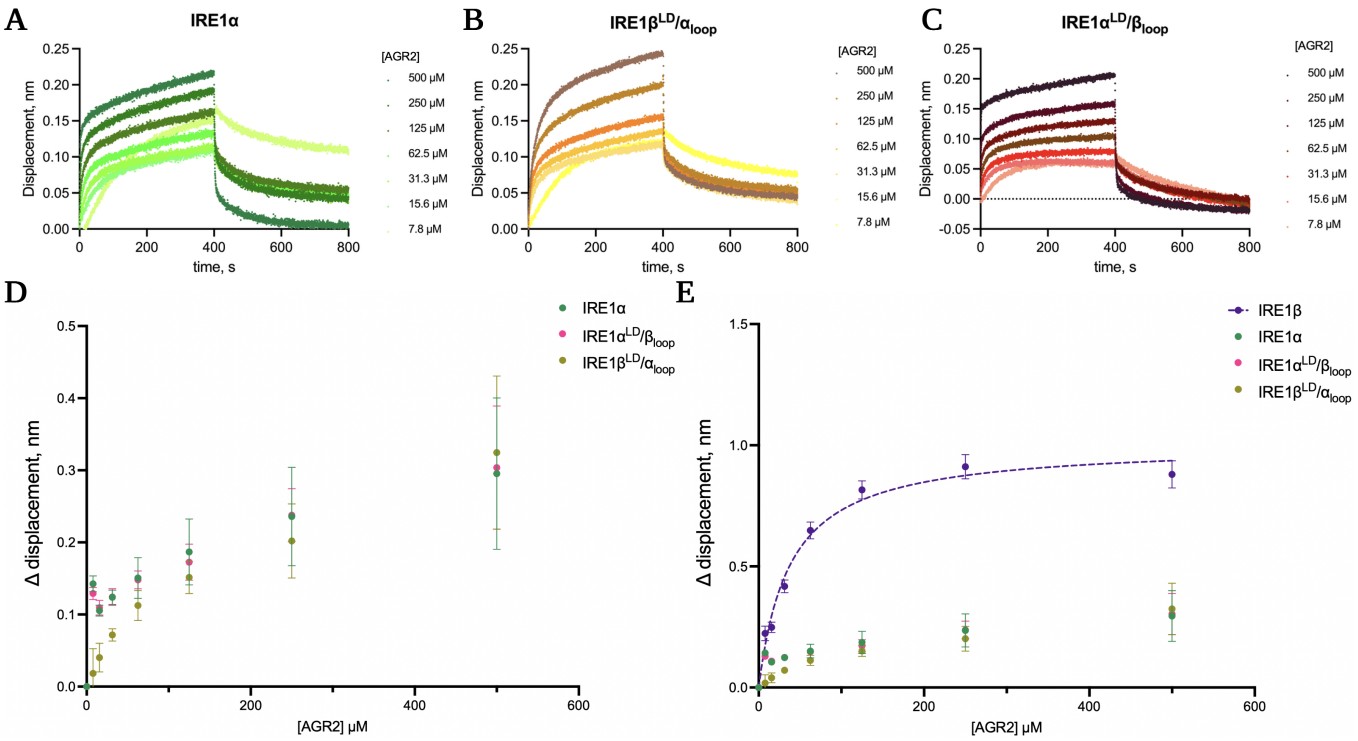

Figure 5: Investigation of AGR2's affinity for the βloop in context of the chimeric proteins
A: Association and dissociation phases of a selected IRE1α^{LD} replicate across the AGR2 titration. **B**: Association and dissociation phases of a selected IRE1β^{LD}/α_{loop} replicate across the AGR2 titration. **C**: Association and dissociation phases of a selected IRE1α^{LD}/β_{loop} replicate across the AGR2 titration. All three (A, B, C) show similar profiles of transient complexes as the loop peptide in Fig. 4, B(i). **D**: Steady-state binding of all three proteins (A, B, C) (three replicates each) shows no difference in the reaction kinetics and AGR2 affinity. **E**: Comparison of the steady-state binding of all the proteins in the study (except for the loop peptide due to its small size).

Materials and Methods

Protein expression

E. coli BL21 pLysS were transformed with corresponding plasmids and the standard IPTG induction protocol was followed [4]. All used plasmids were prepared beforehand by a lab member and confirmed with DNA sequencing. Furthermore, except AGR2, all proteins contained biotinylation tags.

Cell lysis and protein purification

After incubation, the cells were harvested, lysed, and the lysates collected for protein purification.

Depending on the protein, different steps were used. AGR2 purification involved nickel ion chromatography, an overnight SUMO cleavage, and reverse nickel ion chromatography; IRE1α_{LD}/β_{loop} required nickel ion chromatography and SEC, and IRE1β_{LD}/α_{loop} followed the same route as IRE1α_{LD}/β_{loop} with additional amylose purification (MBP-tag) preceding SEC.

The remaining proteins were kindly donated by Dr Lisa Neidhardt and Dr Yahui Yan.

Binding analysis

Binding analysis was conducted on BLI ForteBio Octet with Streptavidin Biosensors. All experiments included a 200s baseline step, followed by ligand loading of varied lengths, a 700s wash and association and dissociation steps across a seven-step AGR2 titration ($7.8 - 500\ \mu M$), 400s each. The ligand loading was adjusted for each protein, such that at the end of the step, the tip was saturated.

Data analysis

Association and dissociation steps were extracted for each concentration. At each concentration, the initial reading was subtracted from each subsequent value, thus each data set began from zero.

To obtain the Δ displacement values, the maximum change in the association phase was used.

Acknowledgements

I would like to thank the Biochemical Society for funding this 6-week studentship and for the opportunity I received to visit a different institution, which otherwise would be beyond my abilities. Furthermore, I am very grateful to Professor David Ron who agreed to accommodate me in his group and also to the rest of the group (Fig. 6, C), especially Dr Yahui Yan and Dr Lisa Neidhardt who together with Professor David Ron supervised this project. Lastly, I would like to thank Boris, my landlord, who managed to offer me accommodation on very short notice and my mum for supporting me during this project despite not understanding anything about it.

From the studentship, I learned two main skills that I believe will stay with me for life. These are the ability to take

good quality laboratory notes which are extremely useful when something does not go as planned, and the ability to manage work if the results show no expected outcomes (Fig. 5, C-E). Furthermore, I developed better communication and teamwork skills as the project required me to cooperate and communicate with three supervisors and work in a total team of five people who focus on the ER-mediated UPR.

The work which I did during the studentship, will complement a research paper by Dr Lisa Neidhardt and is going to be included in it. The AGR2-IRE1β complex construct gave a good yield and will be used for some initial structure-determining work. However, more investigation is required into the stabilising proteins which opens the doors for further projects by other summer or PhD students. Before beginning this project, I planned to go into a PhD, and after, I am even more certain that this path is right for me.

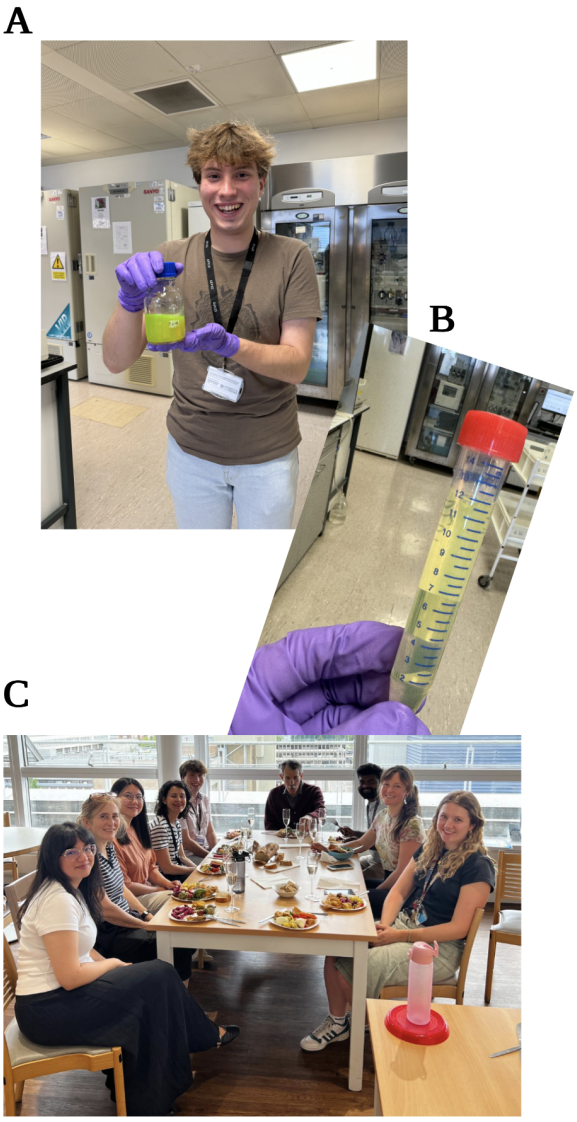

Figure 6: Pictures from the studentship
A: Jakub after collecting the cell lysate of the AGR2-IRE1β complex construct (high hopes for a good yield). **B**: The purified complex with approximately 30% yield. **C**: Jakub and all the group members at an "International Dinner" (a group's tradition).

References

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