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

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Background:

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

Aims:

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

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

Work undertaken:

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

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

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

Results and Discussion:

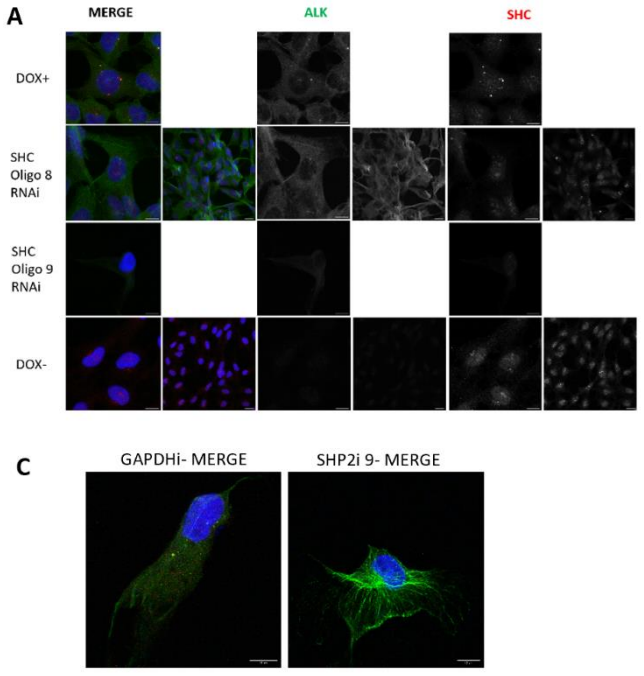

Figure 1: Immunofluorescence of BEAS2B V1 cells after transfection with SHC and SHP2 RNAi oligonucleotides.

A- Fluorescence confocal micrographs comparing phenotypes for each SHC RNAi condition. ALK (green), SHC (Red) and Nuclei (blue).

B- Bar graph representing the mean number of AlK foci per cell under each respective SHC RNAi condition.

C- Fluorescent confocal micrographs comparing phenotypes between a GAPDH RNAi control and transfection with SHP2 9 oligos.

D- Bar graph representing the mean number of AlK foci per cell under each respective SHC and SHP2 RNAi condition.

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

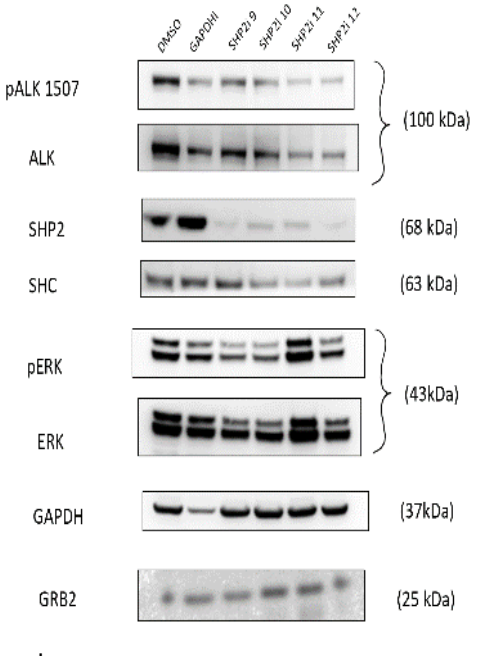

Figure 2: Western blot analysis of H3122 V1 cell lysates after transfection of SHP2 RNAi oligonucleotides. DMSO was used as a control. Lysates were analysed for the relative presence/phosphorylation of the proteins indicated above, using complementary antibodies. GAPDH was used as a loading control.

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

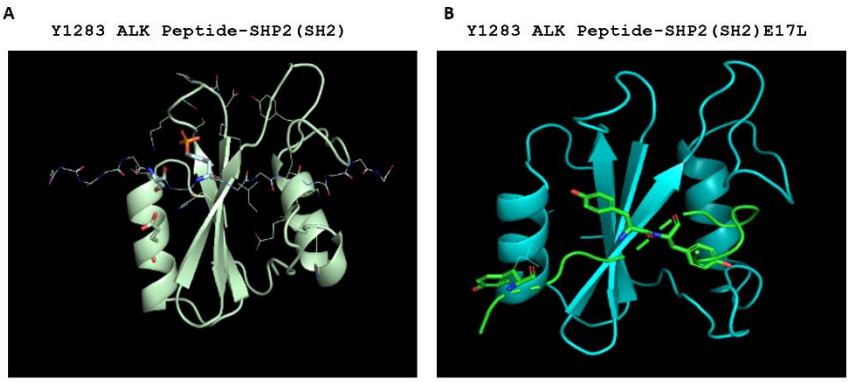

Figure 3. Pymod3 and AlphaFold models of SHP2 interactions with ALK SHP2 binding site (tyrosine 1283) A- Pymod3 prediction of SH2 domain of SHP2 bound to an ALK peptide (phosphorylated at Y1283). The prediction used the template: SHP2 interaction with GAB1 peptide. B- AlphaFold prediction (phosphotyrosine independent) of SHP2 (E17L point mutation) and the ALK Y1283 peptide.

The predicted binding site for SHP2 is Phosphotyrosine 1283, the third of three phosphotyrosine sites on the ALK peptide, DIyRASyyRKGGCAM. Looking at 3A, the phosphorylated tyrosine interacts with the peptide backbone of SHP2, but a Glut-Val interaction may hold the phosphotyrosine in place. Using AlphaFold to substitute Glut for Leu (blocking the interaction), the prediction favours the second tyrosine in the peptide instead, implying the interaction is key for the Y1283 SHP2 binding.

What next?

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

Impact

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

Skills and future career plans

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

References

1. Sampson, J., M. W. Richards, J. Choi, A. M. Fry, and R. Bayliss. 2021. Phase-separated foci of eml4-alk facilitate signalling and depend upon an active kinase conformation. *EMBO reports* 22.