Regulation of the Cell Cycle: Characterisation of the interaction between the transcription factor Runx1 and CDK6

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Introduction

Most types of cancer arise from dysregulation of the cell cycle which can cause uncontrollable growth of cells, forming tumours[1]. Cells complete one round of the cell cycle when they grow and divide into two. There are 4 main stages of the cycle: G1, S, G2 and M. A family of proteins called cyclin-dependent kinases (CDKs) control processes in the cell cycle, by interacting with another family of proteins called cyclins. Certain CDK-cyclin interactions are required to progress to the next stage of the cycle[2].

During the cell cycle, cyclin D pairs with CDK6 to allow the cell cycle to progress during G1 phase. CDK6 is also important in regulating the expression of genes that control cell fate. These different functions of CDK6 are executed by its binding (pairing) to different protein partners, such as the transcription factor Runx1[3].

Runx1 regulates gene expression and contains a domain called runt, which binds to DNA and CDK6. Runx1 does not contain a cyclin D binding motif, but can bind to CDK6, suggesting that the runt domain of Runx1 binds CDK6 at a different site to where cyclin D pairs with CDK6[4]. Figure 1 (A) is a structure of a CDK with various binding partners, and Figure 1 (B) is a crystal structure of Runx1 in complex with DNA and CBFβ. These structures show that different binding partners interact with CDKs at different sites.

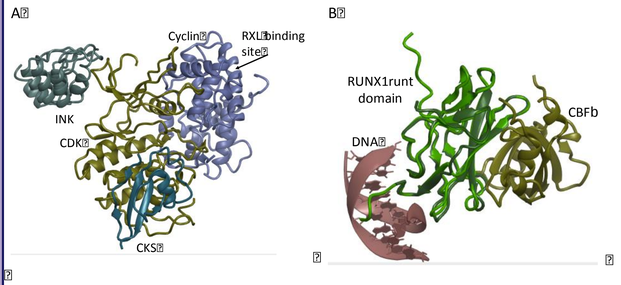


Figure 1: Structures of proteins to be identified in this project. (A) Locations of known sites of CDK protein interaction. CDK4/6 interact with INKs and cyclins. Other CDKs interact with CKS. **(B)** Structure of the Runx1 runt domain superimposed on the structure of a Runx1 runt-CBFβ-DNA complex.

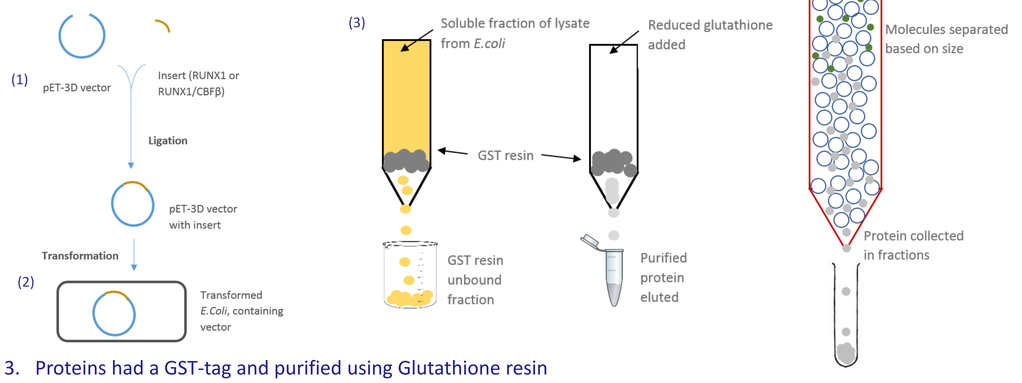
Hypothesis and Aims

The project aims to characterise the interaction of CDK6 with Runx1 and explore if proteins that regulate CDK6 utilise novel sites for protein interaction on the surface of CDK6-cyclin D:

- Produce recombinant Runx1 and Runx1/CBFβ complex
- Reproduce literature methods and explore alternative tags such as His-tag and GST-tag (solubilises protein) for protein production
- Use a range of biophysical techniques to characterise Runx1 binding to CDK6 and CDK6-cyclin D

Methods

1. Runx1 and Runx1/CBFβ constructs generated and cloned
2. Proteins expressed in *E.coli*



3. Proteins had a GST-tag and purified using Glutathione resin
4. Gel filtration using superdex 75 16/60 column was used as a final purification step. Bigger proteins elute faster than smaller proteins
5. Pull downs performed (a binding assay to identify binding partners, e.g. Runx1 and CDK6)
6. SDS-PAGE gel electrophoresis and Western Blot to confirm protein successfully expressed and purified

Results

SDS-PAGE results show Runx1 protein has been produced in *E.coli* from the pET-3d vector with a GST tag.

Figure 2: Runt domain of Runx1 purification using GST-tag.

1. RUNX1
2. RUNX1 cleaved with 3C protease

Runx1 and Runx1/CBFβ are very similar in size so it is difficult to differentiate between them on a gel. Western Blots allow confirmation of the presence of Runx1 in Runx1/CBFβ complex and on its own.

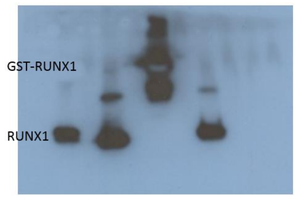


Figure 4: Western Blot using anti-Runx1 antibody.

Gel filtration separates proteins into fractions according to their size and shape.

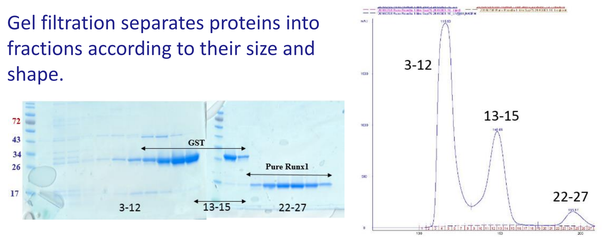


Figure 3: SDS-PAGE and gel filtration of Runx1 using superdex 75 26/60 at room temperature.

These pull downs identify interactions between GST-CDK6 and Runx1 and GST-CDK6 and Runx1/CBFβ. GST is used as a control.

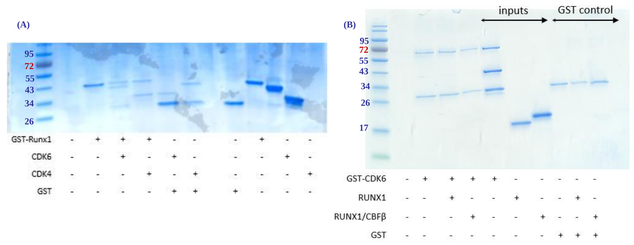


Figure 5: SDS-PAGE results of pull downs with different binding partners. **(A)** Pull down using GST-Runx1. **(B)** Pull down using GST-CDK6.

Discussion

Runx1 was produced with a GST tag. The gel in Figure 2 shows protein produced as a GST-Runx1 fusion and then Runx1 and GST are separated after GST tag cleavage using 3C protease. The protein was purified from a soluble fraction, an improvement to literature where Runx1 with a His-tag was insoluble and needed to be purified from inclusion bodies.

Figure 3 shows that Runx1 is stably expressed in *E.coli* using a GST-tag and produces a very good yield of 5 mg of pure protein per litre of *E.coli* culture. Gel filtration allowed separation into three peaks shown on the chromatogram. The peak containing fractions 3-12 was potentially nucleic acid contamination (Runx1 is a DNA binding protein) as it is not visible in the SDS-PAGE gel. The second peak (fractions 13-15) is cleaved GST tag and the third peak (fractions 22-27) contains pure Runx1 protein. It is possible that the contamination in the first peak could be resolved by extra washes of resin with 1 M NaCl. Another alternative method would be to use ion exchange chromatography as final step to ensure all DNA is removed.

The Western Blot shown in Figure 4 confirms the presence of Runx1 in the Runx1-CBFβ complex which is difficult to differentiate in SDS-PAGE gels. CBFβ presence could not be confirmed as we had no antibody for it. When expressing Runx1-CBFβ with a His-tag in *E.coli*, the expression was poor and the protein tended to aggregate and precipitate. A different tagging system could be explored for complex stabilisation and to obtain better yields, for instance a GST-tag or MBP-tag.

The pull-downs shown in Figure 5 show that there is no clear binding of Runx1 and Runx1-CBFβ to GST-CDK6. Proteins bound non-specifically to the resin which made pull down analysis difficult and the results inconclusive. Buffer optimisation will be explored to improve the pull down experiments.

In future, constructs with an Avi-tag could be produced for biophysical binding assays such as HTRF (Homogenous Time Resolved Fluorescence) and SPR (Surface Plasmon Resonance).

Conclusion and Future Work

- Runx1 Runt domain is stably expressed in *E.coli* using a GST tag
- Expression is reproducible with yield of 5 mg per litre of culture
- Results of binding between Runx1 and CDK6 or CDK6-D were inconclusive

- Explore different tag systems to produce Runx1-CBFβ with higher yield
- Test different fragments of Runx1 in binding assays as some key residues for interaction could have been omitted in the construct used
- Explore different binding assays such as SPR or HTRF
- Crystallise a Runx1-CDK6 complex to find molecular details of the interaction

References

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4 T Fujimoto KA, S E W Jacobsen, S-i Nishikawa, C Nerlov. Cdk6 blocks myeloid differentiation by interfering with Runx1 DNA binding and Runx1-C/EBPα interaction. The EMBO Journal. 2007 02/05/2007;26:2361-70.

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