NOB1 and PNO1 In The Intricate Composition Of Ribosome Biogenesis

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1. Introduction and Aims

Ribosomes are composed of a large (60S) and small (40S) subunit and contain both rRNA, transcribed in the nucleus, and ribosomal proteins. The small subunit includes a piece of rRNA called the 18S. Many trans-acting factors are involved in ribosome biogenesis, including NOB1 and PNO1. These are not included in the ribosome composition. Evidence suggests that NOB1, an endonuclease, is involved in 18S formation in yeast and that is forms a stable complex with PNO1.

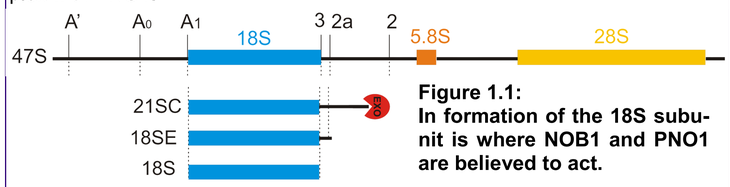


Figure 1.1: In formation of the 18S subunit is where NOB1 and PNO1 are believed to act.

Aims

- Express recombinant Nob1 and PNO1
- Purify the NOB1-PNO1 complex
- Optimise the conditions for complex purification.

Questions that can be addressed using a large stock of PNO1-NOB1 complex:

1. What is the reaction interface?

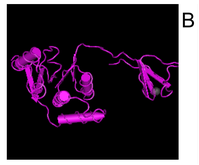
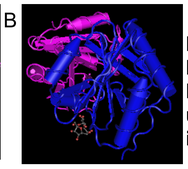
A  B 

Figure 1.2: A- Crystal structure of PNO1; B- Crystal structure of NOB1. Mass spectroscopy can be used to investigate the reaction interface of these two proteins.

2. Further investigation into proposed helix binding sites.

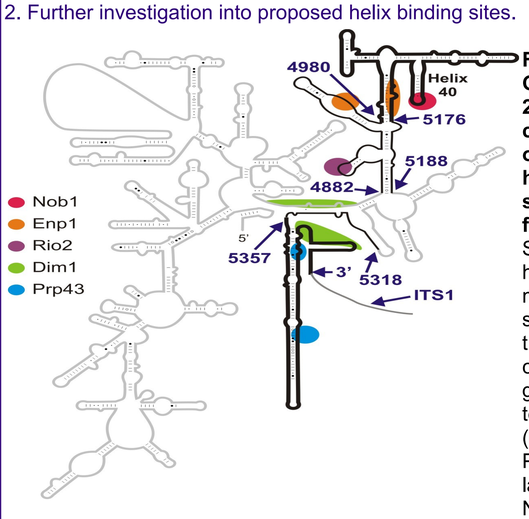


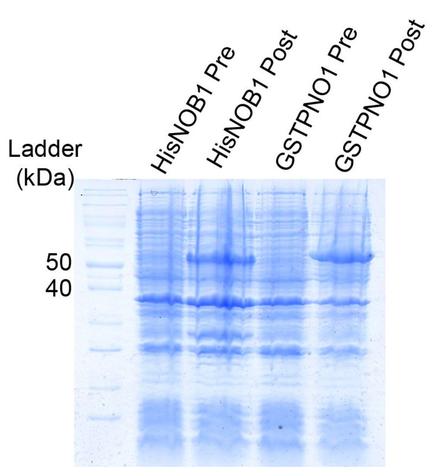
Figure 1.3: From Granneman *et al.* 2010, showing cross linking sites of various yeast homologues of ribosome biogenesis factors.

Similar investigations have shown that human NOB1 binds in a similar position and that PNO1 binds very close to NOB1, suggesting they may interact in vivo (Katherine Sloan, PhD, ongoing). A large stock of NOB1PNO1 complex will greatly benefit further studies.

3. Can previous cleavage assays be refined: does PNO1 presence improve cleavage by NOB1?

Cleavage studies are being carried out to investigate the extent to which PNO1 assists NOB1 in cleaving (Katherine Sloan, PhD). These can be refined using a PNO1-NOB1 complex stock.

2. Transformation and Purification



Individual transformations of BL21 E. coli were carried out to provide large amounts of each protein. The plasmid used to transform each gene contained Ampicillin resistance for selection. The NOB1 gene used contained a His tag to aid purification and the PNO1 gene a GST tag. Subsequent protein purification exploited these.

When cells reached an OD of ~0.3-0.4, i.e. were in mid-log phase, IPTG was added to a concentration of 1mM to induce protein expression. Samples were taken pre– and post– cell sonication and run using SDS PAGE. Subsequent Coomassie stain allows protein visualisation. Figure 2 shows a large band in each 'post' lane not present in the 'pre' lanes, suggesting successful protein overexpression.

After cell sonication and centrifugation to remove debris, GST beads were used to purify PNO1 and a nickel column on the AKTA to purify NOB1. Each protein solution was then desalted using the AKTA.

Figure 2.1: SDS PAGE gel of transformed E. coli, lysed, stained with Coomassie.

3. Pull Down Assays

A pull down assay is one form of affinity purification that can be used to study protein interactions. Tags added to a protein will attach on to beads which can be separated then eluted. A series of these, investigating conditions for NOB1-PNO1 purification, were carried out to investigate the optimal conditions for complex purification. Glutathione sepharose or nickel beads were both used. Unless otherwise stated, the complex was allowed to form in the cold room for 2 hours initially then beads were added. Western blots using anti-His or Anti-GST antibodies were used to detect any pulled down complex. The relevant complex yield was established through comparison with 20% inputs.

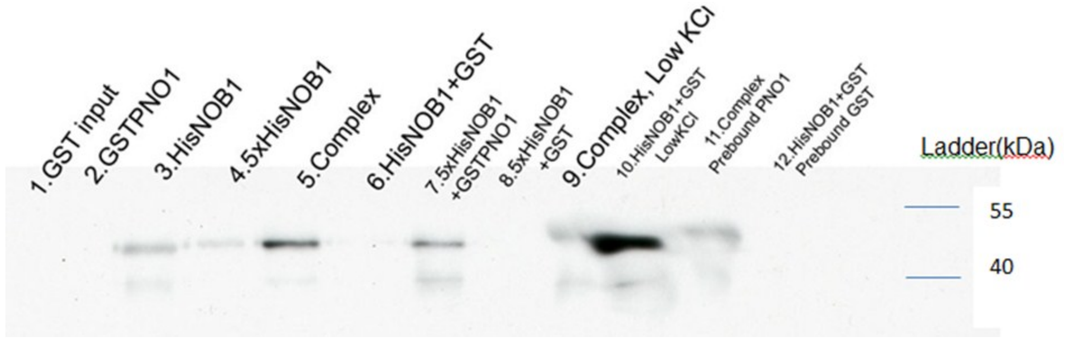


Figure 3.1: Western blot using donkey anti-His antibody showing presence of HisNOB1. 'Complex' refers to the GSTPNO1-HisNOB1 complex. Lanes 1, 2 and 3 are 20% inputs. Control lanes are 6, 8, 10 and 12.

Comparing lanes 5 and 7 then 5 and 11 in Figure 3.1 shows that neither saturating PNO1 nor pre-binding GSTPNO1 to the beads, then adding HisNob1 give a more efficient complex purification. However comparing 5 and 10 shows that lowering my initial KCl concentration dose (300mM to 100mM). Further experiments showed that the GST tag on PNO1 could bind nickel beads intended for HisNOB1 purification. This was eliminated with the addition of 50mM imidazole to the buffer. A double purification using both nickel beads and glutathione beads is necessary to ensure the greatest complex purity. Nickel bead purification leaves a high imidazole concentration (350mM)and glutathione sepharose beads leaves high glutathione concentration (50mM). The second purification must be carried out in either a high imidazole concentration or glutathione concentration. Figure 3.2 shows that complex binding to nickel beads is not affected by this high glutathione concentration, whereas a previous pull down showed that complex binding to glutathione sepharose beads is highly attenuated in the presence of a high imidazole concentration, therefore the glutathione sepharose purification should be carried out first. Comparing lanes 1A and 2A, it is apparent that the 50mM glutathione does not affect complex binding to the nickel beads, therefore the nickel bead purification should be carried out first. Additional bands are likely degradation products due to the fragile nature of the complex and vigorous nature of bead elution when running a gel, purely for complex purification optimisation.

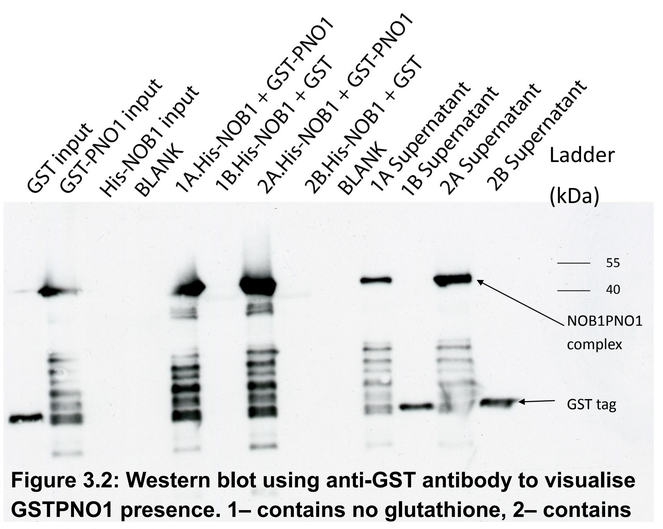


Figure 3.2: Western blot using anti-GST antibody to visualise GSTPNO1 presence. 1– contains no glutathione, 2– contains 50mM glutathione.

4. Conclusions and Significance

Clinical Significance

Half of the energy a cell produces is spent on ribosome biogenesis. In humans, NOB1 dysfunction can be involved in ovarian cancer. NOB1 is also involved in chronic myeloid leukaemia and possibly noise-injured cochlea (Veith *et al.,* 2011).

Conclusion

The optimal conditions for NOB1-PNO1 complex purification are:

- Use a low concentration of KCl e.g. 100mM
- Use equal protein concentrations
- Allow the complex to form, then add the beads
- 2 step purification: 1) via GST tag; 2) via His tag.

5. Accuracy and Precision

Transformation and Purification

C+ containing E.coli strains include human tRNAs making them an ideal strain to express human proteins.

A different column was used for each protein when desalting to avoid contamination.

Pull Downs

Prior to each pull down assay, samples were spun at 13000rpm to ensure no protein had precipitated. If this was apparent, a new sample would be used.

References: Granneman *et al.* (2010). Cracking pre-40S ribosomal subunit structure by systematic analysis of RNA-protein cross linking. *The EMBO Journal*, **29**(2026-2036). Veith *et al.* (2011). Structural and Functional Analysis of the Archaeal Endonuclease Nob1. *Nucleic Acids Research* (1-16).