Expression of Speedy A, a CDK activating protein required for meiosis

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Introduction:

Everyday millions of cells in your body must grow and divide. The cell cycle is divided into 4 phases and describes the process by which a cell duplicates its DNA (during S-phase) and then divides to form two daughter cells (M-phase). Cell growth and division must be tightly controlled; too little and you would die, too much and cancer can develop. Meiosis is a specialised type of cell division that reduces the DNA content of the cell by half and leads to the formation of gametes (sperm and eggs). To control mitosis and meiosis your body has a vast array of proteins. Key regulators of both mitosis and meiosis are a family of proteins called the cyclin-dependent protein kinases (CDKs) *(figure1).*

CDKs act at different points in the cell cycle. CDK activity is regulated by checkpoint pathways that ensure that CDKs are only activated when the cell is ready to proceed to the next cell cycle stage. CDKs are activated by binding to another set of proteins called cyclins. Each cyclin has specific CDK partners and is expressed at a particular stage of the cell cycle (Figure 1). Speedy (SPY1) is a cyclin that binds to CDK1 and CDK2. CDK-SPY1 complexes are expressed in cells undergoing mitosis and meiosis.

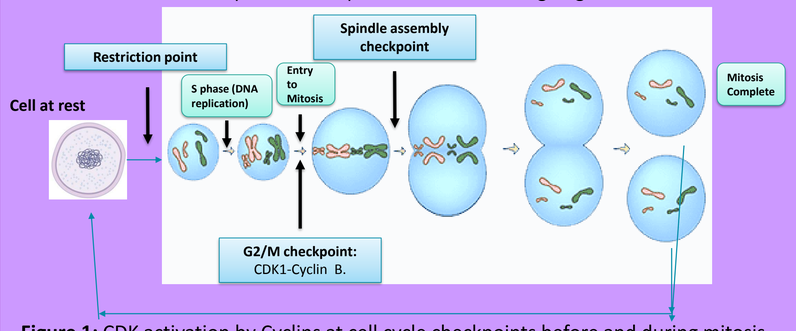

Figure 1: CDK activation by Cyclins at cell cycle checkpoints before and during mitosis.

Overexpression of SPY1 in cells allows a cell to overcome checkpoints usually activated in response to DNA damage[1,2]. When over-expressed SPY1 may have the capacity to induce uncontrolled cell division, in certain cells, under certain conditions[2].

Biochemical and structural studies can be carried out to gain greater insight into SPY1. For these experiments we need to be able to reliably make the protein in the lab. Proteins can be made recombinantly in *E. coli* (bacteria)cells by cloning the DNA encoding the protein into a Plasmid vector (circle of DNA) that can replicate in *E. coli* cells (figure 2). The recombinant protein can then be purified away from the *E. coli* proteins and used in assays. The purification is possible because the plasmid contains a piece of DNA encoding an MPB protein. When the E.coli express the SPY1 protein they also express the MPB protein, that is then then bound to the SPY1 protein. MBP binds to sugar, so by adding sugar to the solution you can isolate the MBP and thus the SPY1 attached to it. A protein named 3Cprotease can then be used to cleave the MPB from SPY1, leaving you with pure SPY1.

Aims:

1. To design 4 different DNA constructs to express different pieces of SPY1 protein.
2. To incorporate these DNA fragments into a vector to express SPY1 in *E. coli* cells.
3. To create DNA stocks of each SPY1 construct that can be used in future research.
4. To discover which *E.coli* strain, and under what conditions, would express these SPY1 constructs.
5. To prepare a stock of purified SPY1 protein using one of the recombinant *E.coli* strains.

Methods:

1. Creation of SPY1 plasmid vectors:

a. Transformation: *E.coli* bacteria (DH5alphas) transformed with pET21 plasmid*(Figure 2*). Plasmids are circular DNA that can be taken up by bacteria and can replicate.

b. Preparation of plasmid DNA: Lyse (burst) *E.Coli* and purify the DNA using a MiniPrep kit.

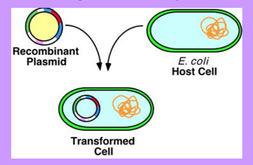

Figure 2: Transformation of E.coli with plasmid.

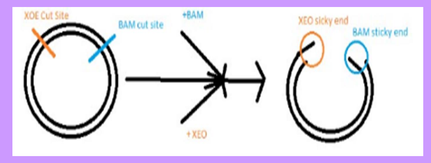

Figure 3: Enzyme digest of plasmid.

c. Restriction enzyme digestion: Digest vector DNA with enzymes (proteins that cut DNA at specific sites) called BamH1 and Xho1.This digest creates overhangs at the cut sites, called sticky ends. *(Figure 3).*

d. Gel Electrophoresis: Visualise the cut vector to check digest efficiency

e. Primer design: Create primers to complement the SPY1 DNA sequence. Designed 4 primers (F61/F68/R213/R199) to create 4 constructs *(Figure 4)*. Primers are short pieces of single stranded DNA. Each primer was designed to contain a sequence complementary to the sticky end of the pET21 vector, so each of our 4 constructs (pETSPY1-4) contained a sticky end complementary sequence.

f. PCR: Four constructs were made by amplifying the SPY1 sequence (in a pGEX vector) using the 4 primers in 4 separate amplification (PCR) reactions *(Figure 4)* to generate 4 PCR products.

g. Recombinant vector construction: Each PCR product was mixed with the cut pET21 vector stock and transformed into *E. coli* (DH5alpha). The cells combine the vector and PCR product to make a recombinant vector that can express the protein fragment.

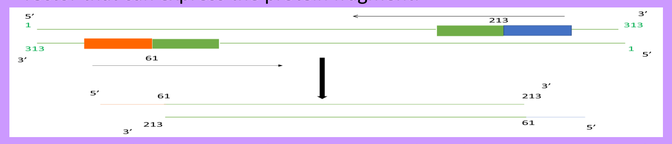

Figure 4: Example of creation of PCR primers and resultant DNA construct for Petspy1-2.

2. Creation of DNA stocks: Mini Prep of constructs transformed into alphaDH5.

3. Preparation of SPY1 proteins: small scale expression:

a. Transformation: of *E.coli* (Artic and Lemo strains) with each pET21 plasmid vector (pETSPY1-4).

b. Overnight culture: of transformed Artic and Lemo cells in two different media: AIM and LB to express the SPY1 proteins

c. Protein purification: Cell lysis using 'Bug Buster' and protein purification using 'Amylose Bead Pull downs'. MBP binds to Amysole (sugar) purifying the MBP tagged SPY1. MBP can be removed by 3C protease.

d. SDS PAGE: To analyse the purified proteins *(Figure 6).*

4. Creation of SPY1 proteins: large scale expression

a. Transformation: of Artic cells with pET21SPY3 vector.

b. Overnight culture: of transformed Artic cells in AIM.

c. Protein purification: Cell lysis using sonication and subsequent protein purification using affinity chromatography**.**

d. SDS PAGE: To analyse the purified proteins (*Figure 7).*

Results

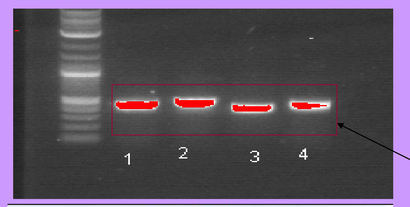

Figure 5: Digestion of pET21d by BamH1 and Xho1. Separation of the cut vector DNA by electrophoresis and visualization under UV light. There is one band at ~5,000bp consistent with successful digestion.

~5,000bp

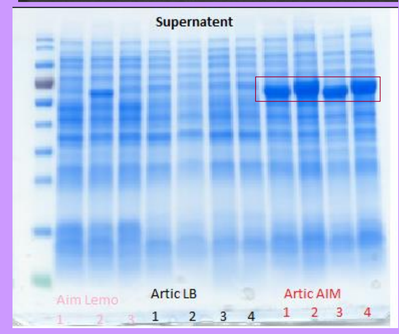

Figure 6: SDS-PAGE to assess protein expression. **Row 1:** Protein ladder. **Row 2**: Construct 2, Lemos in AIM media. **Row 3**: Construct 3, Lemos in AIM media. **Row 4**: Construct 4, Lemos in AIM media. **Row 5:** Construct 1, Artics, in LB media. **Row 6**: Construct 2, Artics, in LB media. **Row 7**: Construct 3, Artics, in LB media, **Row 8:** Construct 4, Artics, in LB media. **Row 9:** Construct 1, Artics, in AIM media. **Row 10:** Construct 2, Artics, in AIM media. **Row 11:** Construct 3, Artics, in AIM media. **Row 12:** Construct 4, Artics, in AIM media. Constructs 1-4 were best expressed in Artics in AIM, as seen by the large strong bands of MPB-tagged- SPY1 protein at ~70Kda on gel (boxed bands).

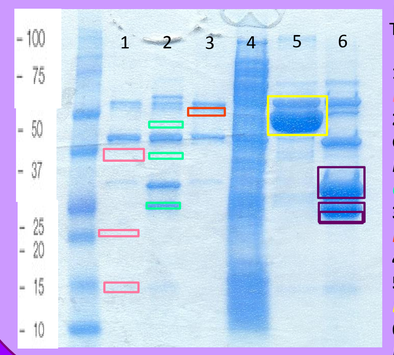

Figure 7: SDS-PAGE to assess SPY1 purification.
The protein was not apparent in samples, suggesting it was not expressed to detectable levels.

1= Spy1 with MBP tag cleaved by 3c protease.
Expect: 3 bands: 42.9- MBP tag, 22- 3cProtease, 16.9 SPY1.
2= Spy1 bound to CDK2 with spy1 MBP tag cleaved and CDK2 GST tag cleaved by 3c protease.
Expect: 3 bands: 50.8 -SPY1 bound to CDK2, 42.9- MBP tag, 26- GST tag.
3=MPB tagged Spy1.
Expect: 1 strong band at 59.8
4= Artic express cell supernatant.
5= GST tagged CDK2.
Expect= 1 strong band at 59.9
6= CDK2 with GST tag cleaved by 3c protease.
Expect= 3 bands: 33.9 -CDK2, 26- GST tag, 22- 3cProtease

Conclusions:

1. Speedy is a highly unstable protein making it challenging to express and purify in recombinant cells.
2. Artic are good at expressing SPY1: We found Artic to be excellent at expressing high levels of protein on a small scale when grown in AIM.
3. AIM is difficult to scale- up for large growths: We were unable to scale up SPY1 expression in Artic *E. coli* cells using AIM from 1ml to 1L growth. We hypothesize this may be because AIM is 'self inducing'. At such large volumes it is possible the bacteria never switched to protein synthesis within the time scale of the grow.

Future:

Alternative inoculation conditions, growth temperature and times will be explored to optimise large-scale expression.

References:

1. Elizabeth A. Barnes, Lisa. A. Porter, Jean-Luc Lenormand, Ryan. W. Dellinger, and Daniel J. Donoghue. *Cancer Research, 63, 3701–3707*. [Online]. Available from: https://pdfs.semanticscholar.org/7228/3efb3a6f4f71ee024c097e7565913fa6814a.pdf. [Accessed 15 September 2017].
2. Mohammad. A. Sorkhy, Rosa-Maria. Ferraiuolo, Espanta. Jalili, Agnes. Malysa, Andreea. R. Fratiloiu, Bonnie. F. Sloane, and Lisa. A. Portercorresponding. *BMC Cancer, 2407*. [Online]. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3294245. [Accessed 15 September 2017].
Figure 1. Mitosis and the cell cycle. [Adapted from] *"BBC GCSE Bitesize Mitosis"* .2014. BBC ©.
Figure 2. Bacterial transformation. [Taken from] *"FlickRiver"*. October 27, 2008. © All Rights Reserved.
Figure 3: Plasmid digest. [Created by] *Helen Timmins.* September 15, 2017.
Figure 4: Creation of PCR Primers. [Created by] *Helen Timmins.* September 15, 2017.
Figure 5: Gel-electrophoresis of plasmid digest product 1-4. [Photographed by] *Helen Timmins.* August 08, 2017.
Figure 6: SDS-Page-Gel- of Supernatant proteins from small scale expression. [Photographed by] *Helen Timmins.* August 23, 2017.
Figure 7: SDS-Page-Gel- of purified proteins from large scale expression. [Photographed by] *Helen Timmins.* August 29, 2017.