Developing a new destruction method of a

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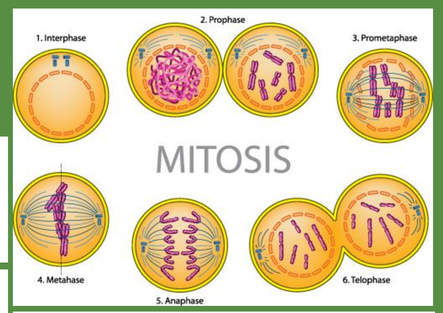
Introduction

Successful cell division requires the accurate replication and sorting of chromosomes to produce daughter cells, and the ability of replicated chromosomes to separate at the correct time and place. Errors in this process underlie birth defects and may also contribute to cancer. The aim of this investigation is to develop a method to artificially drive the destruction of the protein Shugoshin which is a protein involved in holding chromatids together until the correct time at anaphase. An artificial construct of Shugoshin, a degradation signal and a fluorescent tag will be assembled and used to transfect into human cells, to hopefully see expression and localisation in the expected places within the cell.

Aims

The aim of this investigation is to artificially drive the destruction process of the protective protein Shugoshin, to see expression, visualise the precise location and degradation of the protein due to the fluorescent tag associated.



A reminder of the stages of mitosis (3)

Method

- Competent *Escherichia coli* were used for cloning of N90-EGFP plasmid
- The plasmid was extracted from *E.coli* by a high speed plasmid midi kit
- N90-EGFP plasmid was linearized by BamH1 restriction enzyme (molecular scissors)
- Two isoforms of Shugoshin (A1 and A2) was amplified using KOD polymerase in a polymerase chain reaction (PCR)
- PCR products were screened to check the correct forms of Shugoshin were amplified by testing on agarose gel electrophoresis.
- A one step Sequence and ligation independent cloning (SLIC) method was used to join ends of N90-EGFP and one form of Shugoshin (SGO1A1 or SG01A2)
- Digestion with BamH1 to check ligation was successful
- The completed plasmid N90-SGO1A1-EGFP was transfected into U20S cells using lipid molecule transfection with Xtreme gene 9
- Localisation of exogenous shugoshin was seen from a Zeiss Axio imager microscope

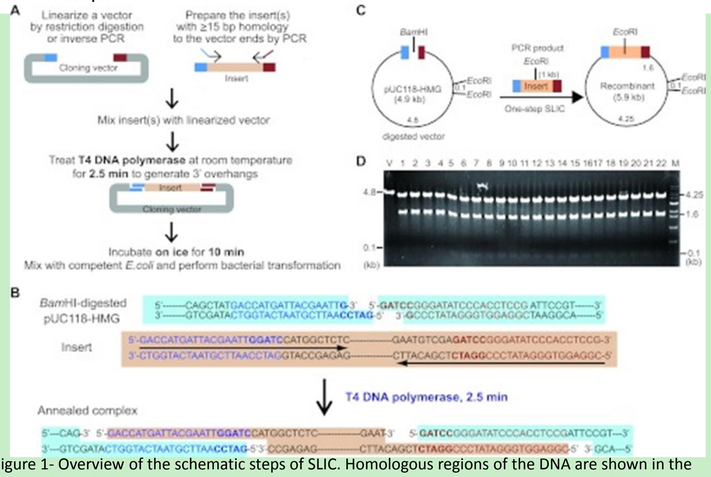


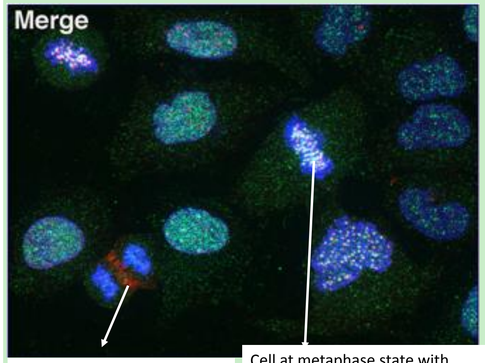
Figure 1- Overview of the schematic steps of SLIC. Homologous regions of the DNA are shown in the same colour. The BamH1 restriction sites created by BamH1 restriction enzyme are in bold. (1)

Discussion

- The N90-SGO1-EGFP plasmid was designed so that the anaphase promoting complex (APC) would recognise the fusion protein created and degrade Shugoshin specifically at anaphase. We were able to confirm that N90-SGO1-EGFP localises to centromeres as expected.
- However from live imaging and microscopy work expressed Sgo1 is found to stay at centromeres later than what was expected. A reason for this result is because the plasmid needed to contain more amino acids from cyclin B sequence (the destruction signal . Perhaps the first 90 amino acids was insufficient for the APC to recognise the degradation signal and also target Sgo1.
- Microscopy work showed a low number of mitotic cells, suggesting N90-SGO1-EGFP prevented cells from entering mitosis. U2OS cells were therefore treated with nocodozole prior to fixing to increase prometaphase cells.
- The results were encouraging but the system needs to be optimised by using a earlier degradation signal that does not disrupt mitosis.

Results

1. Shugoshin 1 localization



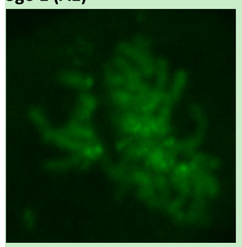
Cell at anaphase. Red Survivin shown in centre between the segregation of chromosomes

Cell at metaphase state with DNA shown in blue. Chromosomes are lined up at the centre ready for segregation

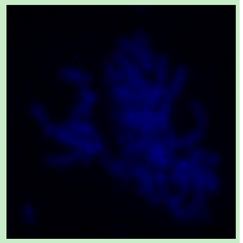
Figure 2- HeLa cells were fixed in 4% PFA and blocked with 1% BSA. Cells were stained for Survivin (red), Shugoshin 1 (green), DAPI (blue).

3. Localisation of N90 Shugoshin 1 A2

Sgo 1 (A1)



DNA



Merge

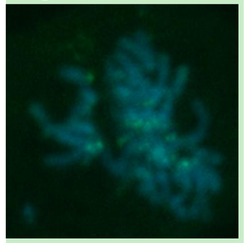


Figure 6 - U2OS were fixed in 4% PFA and blocked with 1% BSA. Cells were stained with DAPI DNA (blue) and artificial Shugoshin 1(A1) labelled with EGFP tag. Localisation of shugoshin (A1) can be seen at the centromeres on chromosome

Sgo1 A1 5 Prime
5′ 3′
TGCTGGTGTTGGATCCATGGCCAAGGAAAGATGC
BamHI

Sgo1 A1 3 Prime
5′ 3′
GGCGACCGGTGGATCCGATTGTATTTGTTTCATACT
BamHI

Sgo1 A2 5 Prime
As for A1, 5 prime sequence identical

Sgo1 A2 3 Prime
5′ 3′
GGCGACCGGTGGATCCGACCTCAAGCAGATGTGGGTTT
BamHI

Figure 3 - Oligonucleotide primers for PCR in cloning using SLIC (Sequence and ligation independent cloning)

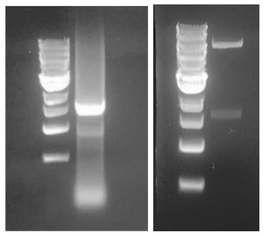


Figure 4- 1% agarose gel electrophoresis of Sgo1 PCR product (Left) and N90-SG01A2-EGFP digestion with BamH1 (right). Both used a 1KB DNA ladder which is shown on the left of each picture

2. Construction Of a new Degradation Method In Anaphase…

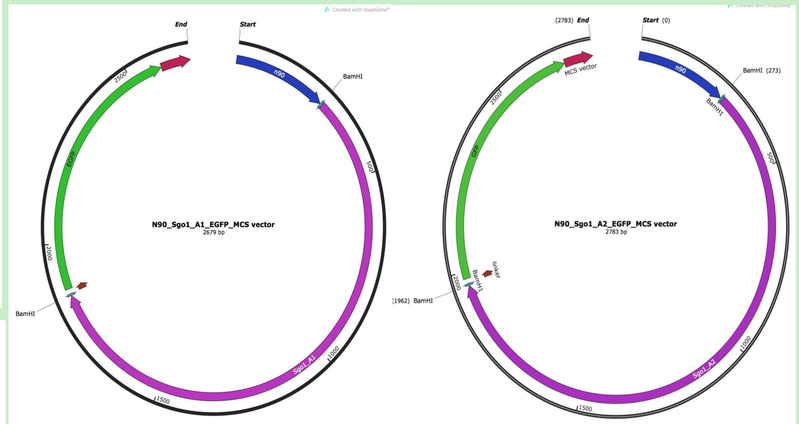


Figure 5 - Plasmid construct created of N90-SGO1A1-EGFP (left) and N90-SGO1A2-EGFP (right) showing BamH1 restriction sites, EGFP green fluorescent tag (green), destruction signal (blue) and isoforms of shugoshin (pink).

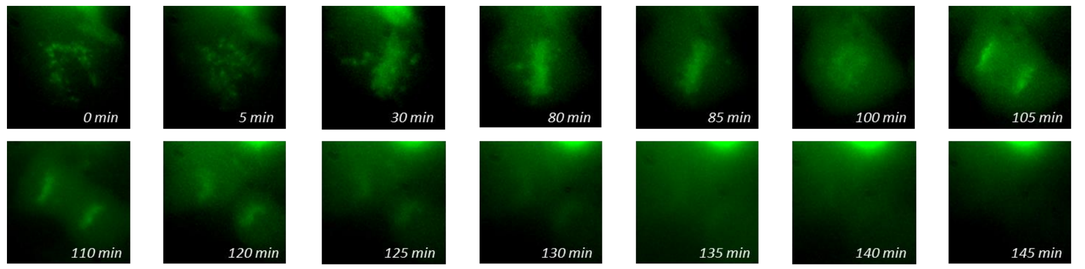


Figure 7 – Live imaging of U2OS cells over the course of 145 minutes during stages of mitosis. Localisation of Shugoshin (A1) is shown by the green fluorescence. Shugoshin can be seen to follow the movement of chromosomes centromere during prophase, metaphase and anaphase. Live imaging shows the degradation of shugoshin at anaphase between 130-135 minute into cell cycle.

Further work…

Further development of this research is to 'knock down' the gene for endogenous shugoshin and replace it with our modified artificial N90-SGO-EGFP plasmid. 'Knock down' will be achieved by small interference (siRNA) which interferes with the expression of specific genes resulting in no translation(2). In doing this, the effects of how the degradation of protective shugoshin can be visualised with live imaging and microscopy work.

References
1. doi: 10.1128/AEM.00844-12
2. https://en.wikipedia.org/wiki/Small_interfering_RNA
3. http://www.biology101.org/biologystudyguides/whatismitosis.php