MATERIALS AND METHODS.

2.1 Solutions and media

All chemical grade reagents were supplied by VWR International unless stated. A list of the suppliers is given in Appendix 1. Solutions were made up to volume with dH_2O unless stated. Solutions and media were sterilised by autoclaving at 115˚C and 200 kPa for 15 minutes. pH was altered by hydrochloric acid or sodium hydroxide as appropriate.

2.1.1 General solutions:

Solutions	Components
10X Phosphate Buffered Saline (PBS)	137mM NaCI, 3mM KCI, 10mM Na_2HPO_4. The pH was adjusted to 7.4 with Na_2HPO_4 and autoclaved to sterilise. Buffer was stored at room temperature.
RNase A stock solution	2mg/ml RNase A was dissolved in dH_2O and stored at 4˚C.
Protease inhibitor solution	1X Complete Protease Inhibitors Cocktail tablet was dissolved in 2ml dH_2O and stored at -20˚C for up to 2 months.
Puromycin stock solution	20mg/ml puromycin was dissolved in methanol and stored at -20˚C.
Freezing medium	90% (v/v) Foetal calf serum (FCS) and 10% (v/v) Dimethylsulphoxide (DMSO) were mixed and made fresh as required.

Calcium chloride solution	60mM $CaCI_2$, 15% (v/v) Glycerol, 10mM Piperazine-N,N'-bis(2-hydroxypropanesulfonic acid) (PIPES) pH 7. The solution was autoclaved to sterilise and stored at room temperature.

2.1.2 Agarose gel solutions:

Solution	Components
50X Tris acetate ethylenediamine tetra-acetic acid (EDTA) (TAE)	208mM Tris, 5.71% (v/v) Acetic acid, 10% (v/v) 0.5 M EDTA pH 8. The solution was stored at room temperature.
Agarose gel	1-2% (w/v) Agarose, 98-99% (v/v) 1X TAE. The agarose was dissolved by heating in a microwave oven. 0.5µg/ml ethidium bromide was added before being poured into a horizontal gel cast with a comb and left to set.
6X Agarose gel loading buffer	40% glycerol (v/v), 6% (v/v) 1X TAE. Bromophenol blue was added to colour the solution and kept at room temperature.

2.1.3 SDS-PAGE and Western blotting solutions:

Solutions	Components
5X Running buffer	25mM Tris, 125 mM Glycine, 0.5% (w/v) SDS. The solution was stored at room temperature.
1X Transfer buffer	5mM Tris, 25mM Glycine, 20% (v/v) methanol or ethanol, 0.05% (w/v) SDS. The solution was made fresh as required.
10X Tris Buffered Saline (TBS)	200mM Tris pH 7.6, 1.37 M NaCl. The solution was stored at room temperature.
TBST	1X TBS pH 7.6, 0.1% (v/v) Tween 20
Separation buffer	1.5M Tris-HCl pH 8.8, 0.4% SDS
Stacking buffer	0.5M Tris-HCl pH 6.8, 0.4% SDS
2X SDS-PAGE loading buffer	12.5ml stacking buffer, 10ml glycerol, 2.5ml Bromophenol blue (0.1%), 20ml 10% SDS, 5ml β-mercaptoethanol.
Blocking solution	5% (w/v) fat-free dried milk powder in 1X TBS/0.05% Tween-20. The solution was stored at 4˚C for up to one week.

2.1.4 Transfection solutions:

Solutions	Components
20X 2-Hydroxyethylpiperazine-2-ethanesulphonic acid (HEPES) Buffered Saline (HBS)	2.8M NaCl, 100mM KCl, 15mM Na_2HPO_4, 120mM Dextrose, 500mM HEPES. The solution was stored at room temperature.
2X HBS transfection buffer	2X HBS pH was adjusted to exactly 7.05 and made fresh as required.
5X Cell lysis buffer	500mM NaCl, 100mM HEPES pH 7.4, 5mM $MgCl_2$, 0.5mM EDTA, 5mM NaF, 35% (v/v) glycerol. 4% (v/v) of protease inhibitor solution and 1mM DTT were added prior to use.
RIPA buffer	150mM NaCl, 50mM Tris-HCl pH 8.0, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS. 1 tablet of protease inhibitor was added per 7-10ml of buffer.

2.1.5 Immunoprecipitation solutions:

Solution	Components
Immunoprecipitation (IP) buffer	50mM Tris-HCl pH 8, 150mM NaCl, 10% Glycerol, 0.5% Triton X-100, 1 Complete Protease Inhibitors Tablet per 50ml and was made fresh as required.
Buffer A (cytoplasmic protein extraction)	10mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 1mM 1mM DTT, complete protease inhibitors
Buffer B (Nuclear protein extraction)	20mM HEPES pH 7.9, 1.5mM $MgCl_2$, 10mM KCl, 450mM NaCl, 1mM NaF, 25% glycerol, 1mM DTT, complete protease inhibitors.

2.1.6 GST binding assay solutions

Solution	Components
EBC buffer	140mM NaCI, 100mM NaF, Na_2VO_4, 0.5% (v/v) NP-40 50mM Tris-HCI pH 8 and stored at -20°C.
NaCl-EDTA-Tris-NP-40 (NETN) Buffer	200mM NaCI, 1mM EDTA, 50mM Tris-HCI pH8 0.5% (v/v) NP-40 and made fresh as required

2.2 Molecular Biology

2.2.1 Synthetic oligonucleotides

All oligonucleotides primers were synthesised by Invitrogen Laboratory and used for real-time polymerase chain reaction (PCR). Lyophilised oligonucleotides were dissolved in dH_2O to obtain master stock of 100 µM. These were stored at -20˚C. Working stocks were made by diluting the master stock in dH_2O to a concentration of 10 µM. Details of oligonucleotides used in this study are given in Table 2.1.

Probe	**Sequence**	**T_M**
Lin-54	5'-TTAATACTACAACTCAGCCCTC-3'	60.83˚C
16E7	5'-GATATTGTAATGGGCTCTGCCTTTTTG-3'	61.0° C
Plk1	5'-AATATCCATTCACCGCAGCC-3'	63.03˚C
AurkA	5'-GCACCACTTGGAACAGTTTAT-3'	61.16˚C
CyclinB1	5'-CTGAGACAACTTGAGGAAGAG-3'	60.05˚C
P130	5'-GCACCACTTGGAACAGTTTAT-3'	62.1℃
Lin-9	5'-ACCAGTTGCCTGACGAGAGCTC-3'	60.0℃
ARP-PO	5'-CACCATTGAAATCCTGAGTGATGT-3'	63.33˚C

Table 2.1. Sequences of probes used for real-time PCR amplification

2.2.2 Total RNA extraction

Total RNA was extracted from the cell pellet using RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol. In brief, 15µl of β-mercaptoethanol was freshly added to 1.5ml buffer RLT as supplied in the kit. Six hundred (600) µl of this was mixed thoroughly with the pellet until the solution became viscous. The cells were homogenized by pipetting directly onto a QIAshredder mini spin column and spun for 2 minutes at maximum speed at room temperature. Following this, one volume of 70% ice-cold ethanol was added to the flow-through and the mixture was applied to an RNeasy mini spin column. The column was centrifuged for 15 seconds at 10000rpm at room temperature where RNA bound to the column and the flow-through was discarded.

Subsequently, 700 µl of Buffer RW1 and 500µl of Buffer RPE were sequentially added to wash the column, followed by centrifugation for 15 seconds at 10000 rpm between the two buffers. Both flow-through solutions were discarded. To ensure total elimination of ethanol, the column was centrifuged for 1 minute at maximum speed. Finally, the column was placed into a clean microcentrifuge tube and the RNA was eluted with 50 µl of RNase-free water supplied by the manufacturer.

2.2.3 Quantitation of RNA.

The concentration and purity of the total cellular RNA extracted from cells was estimated using NanoDrop ND-1000 spectrophotometer. Two microliters (2µl) of the total cellular RNA were dropped at the platform of the instrument and were automatically calculated from the 260nm/280nm absorbance readings with the 320 nm absorbance being the background reduction factor, assuming that OD of 1 was produced from 40µg/ml of RNA.

2.2.4 Generation of cDNA for real-time PCR

Total cellular RNA was reverse-transcribed to cDNA using SuperScript II reverse transcriptase (Invitrogen). Firstly, total cellular RNA were subjected to DNase-free treatment using Invitrogen DNase kit according to the manufacturer's instruction. A total 1µg DNase-free RNA was mixed with 50ng random hexamers, 10mM dNTP mix and adjusted to total volume of 12µl with steriled distilled water. The mixture was heated to 65˚C for 5 minutes and quickly chilled on ice. Then, the mixture was added with 5X First Strand buffer, 0.1M DTT and RNaseIN (40units/µl) and further incubated at 25˚C for 2 minutes. Subsequently, 200units of SuperScript™ II RT was added to the mixture and incubated at 25˚C for 10 minutes and further incubated at 42˚C for 50 minutes. The reaction was inactivated by heating at 70˚C for 15 minutes.

2.2.5 Real-time PCR.

The real-time PCR amplification was carried out using Absolute SYBR green ROX (ABgene) and an ABI 7900HT sequence detector. The reaction mixture was prepared by mixing 10µl of the master mix (containing SYBR® Green I Dye, AmpliTaq Gold® DNA Polymerase, dNTPs and optimized buffer components) with 1µM of each probe, 1µl of cDNA and distilled water to make up to 20µl final volume. The real-time amplification was performed using the ABI 7900HT sequence detector (Applied Biosystem), specifically using Relative Quantitative assay protocol. In order to ensure the integrity of the experiment, three to five independent experiments with each independent experiment consisting of triplicate samples were performed.

All PCR reactions were performed for 40 cycles consisting of the following steps:

1. Step: Denaturation at 95°C for 15 minutes
2. Step: Denaturation at 95°C for 15 seconds
3. Step: Annealing at 60°C for 20 seconds
4. Step: Extension at 72°C for 20 seconds
5. Step: Repeat of the steps from 2-4 for 40 times.

Relative quantification was calculated by normalizing against *ARP P0* gene amplification.

2.2.6 Cloning of short-hairpin RNA (shRNA).

2.2.6.1 Materials

2.2.6.1.1 Media for Bacterial Cell Growth

Media	Components
Luria-Bertani (LB) broth	10 g/l Tryptone, 5g/l Yeast Extract, 10g/l NaCl. The pH was adjusted to 7.0 with HCl and autoclaved to sterilise. The broth was kept at room temperature.
Luria-Bertani (LB) agar	1.5% agar was added to LB broth and autoclaved to sterilise. 50µg/ml ampicillin was added as appropriate. LB agar was poured into 10 cm sterile tissue culture dishes and left to set. Agar plates were inverted and stored at 4˚C.

The pH of the media was adjusted to 7.0 and sterilized by autoclaving at 121˚C in an autoclave machine. After cooling ~60˚C, before ampicillin was added at a concentration of 100µg/ml. The LB agar medium was poured onto sterile agar plates and allowed to solidify and stored at 4˚C until use.

2.2.6.1.2 Bacterial strains and plasmids

The bacteria host strain used in this study was DH5α competent cells (Invitrogen) with the genotype as follows: F- φ80*lac*ZΔM15 Δ (*lac*ZYA-*arg*F) U169 *rec*A1 *end*A1 *hsd*R17(rk-, mk+) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1 λ-. The plasmid vectors used were pMSCV puro and pLKO.1 vector and the restriction maps of these vectors are shown in Figures 2.1 and 2.2, respectively. However, the pLKO.1 is a replication-incompetent lentiviral vector which was used specifically for expression of shRNAs.

2.2.6.1.3 Antibiotic solution for Bacterial Culture

Ampicillin (100mg/ml) stock solutions was prepared using sterile distilled water, filter sterilized using 0.22μm filters and stored at -20˚C in 2 ml aliquots.

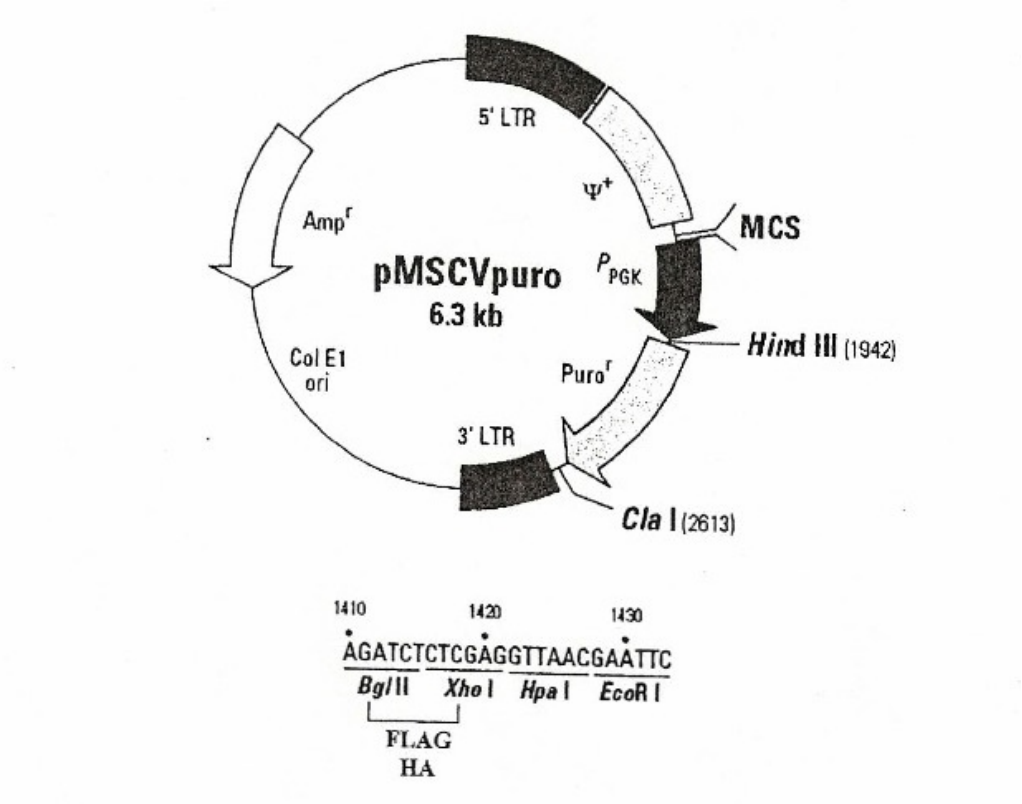
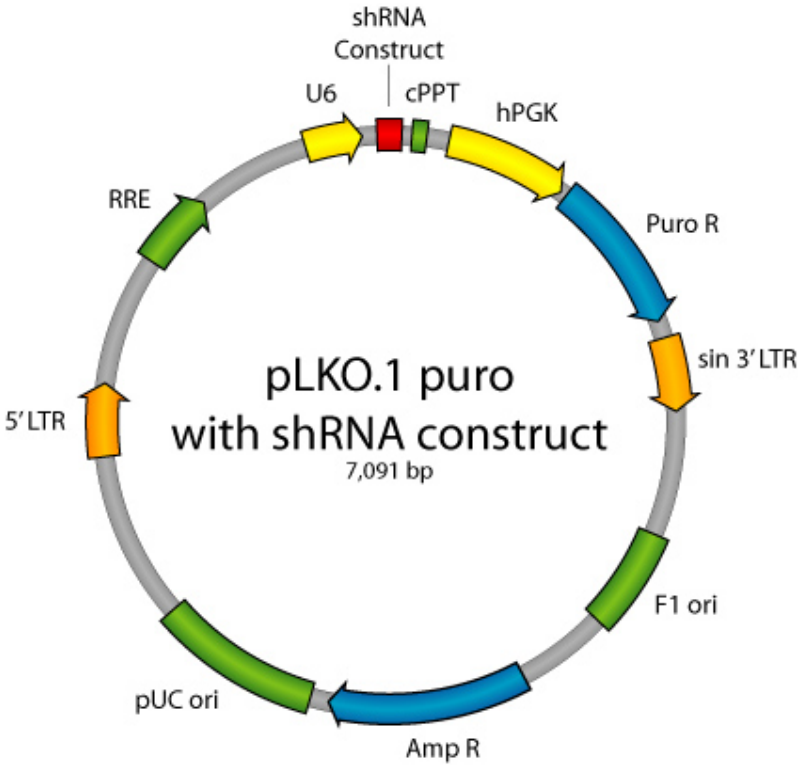


Figure 2.1. pMSCVpuro retroviral vector restriction map. The multiple cloning site is given. pMSCVpuro contains a puromycin selection gene and confers ampicillin resistance. Adapted from the Clontech pMSCVpuro vector information booklet (http://www.clontech.com).

a)



b)

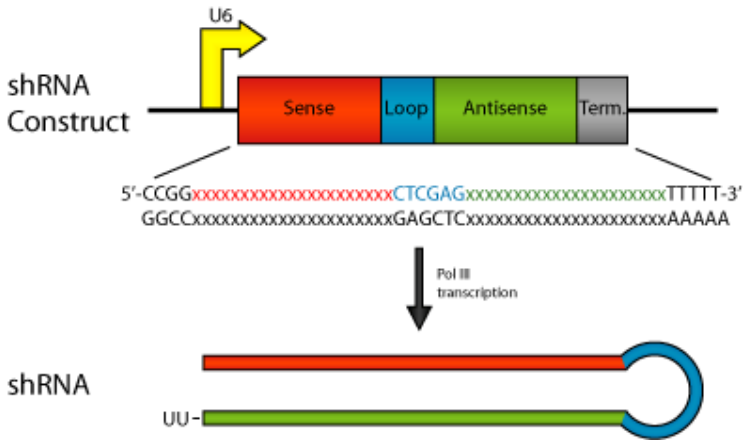


Figure 2.2. Vectors used for cloning a) Map of pLKO.1 containing shRNA insert**.** b) Detail of shRNA insert. The U6 promoter directs RNA polymerase III transcription of the shRNA. The shRNA contains 21'sense' bases that are identical to the target gene, a loop containing an XhoI restriction site, and 21 'antisense' bases that are complementary to the 'sense' bases. The shRNA is followed by polyT termination sequence for RNA Polymerase III.

2.2.6.2 Designing shRNA oligos for pLKO.1 vector

2.2.6.2.1 Determining the optimal 21-mer targets in selected genes

For an efficient gene silencing, a selection of 21-mer targets in the selected genes (16E7A, 16E7B, Lin-54, Lin-9 and p130) was performed. The siRNA Selection Program was accessed from the Whitehead Institute for Biomedical Research to determine a set of top-scoring targets for the genes. A summary of guidelines for designing siRNAs with effective gene silencing is as below:

a) Starting at 25 nucleotides downstream of the start codon (ATG) and search for 21 nucleotides sequences that match the pattern AA (N_{19}). If no suitable match is found, search for NAR (N_{17}) YNN, where N is any nucleotide, R is a purine (A,G) and Y is a pyrimidine (C,U).
b) G-C content should be 36-52%
c) Sense 3' end should have low stability, at least one A or T between position 15-19.
d) Avoid targeting introns.
e) Avoid stretches of 4 or more nucleotide repeats, especially repeated Ts because polyT is a termination signal for RNA polymerase III.

The NCBI's BLAST program was used to minimize the degradation of off-target mRNAs.

2.2.6.2.2 Ordering oligos compatible with pLKO.1 vector

To generate oligos for cloning into pLKO.1 vector, sense and antisense sequences from step above were inserted into oligos below.

Forward oligo:
5'CCGG-21bp sense-CTCGAG-21bp antisense-TTTTTG 3'

Reverse oligo:
5'AATTCAAAAA-21bp sense-CTCGAG-21bp antisense 3'

The generated oligos were followed specifically from both templates because these bases are important for cloning the oligos into pLKO.1 vector (Table 2.2).

Oligo Name	**Sequence**
16E7A	5'-CCGG**ATGCATGGAGATACACCTACA**CTCGAG**TGTAGGTGTATCTCCATGCA**TTTTTTG-3
16E7B	5'-CCGG**GGACAGAGCCCATTACAATAT**CTCGAG**ATATTGTAATGGGCTCTGTCC**TTTTTG-3'
Lin-54	5'-CCGG**CGGCTTCCATTCAATGGCATA**CTCGAG**TATGCCATTGAATGGAAGCCG**TTTTG-3'
p130	5'-CCGG**ATGGCCATGTTTTGGAAGCAA**CTCGAG**TTGCTTCCAAAACATGGCCA**TTTTTTTG-3'

Table 2.2. Oligonucleotides used for cloning into pLKO.1 vector. Underlined and bold indicate the designated gene sequences

2.2.6.3 Restriction digests

A typical restriction digest reaction consisted of 10 units of the appropriate restriction endonuclease(s) and 1x buffer, 2 μg DNA and 100 μg/ml BSA. The total reaction volume was made up to 10-50 μl with dH_20. The glycerol content of the reaction was minimised to no more than 10% total endonuclease solution to avoid star activity. Digests were incubated for 2 hours at 37 °C or the appropriate temperature for optimum enzyme activity. All restriction enzymes and buffers used were provided by New England Biolabs (NEB). DNA fragments were resolved by agarose gel electrophoresis and when required, purified by agarose gel extraction.

2.2.6.4 Agarose gel electrophoresis

DNA fragments were typically resolved on 1% agarose gels prepared in TAE buffer. Gels were run at 7.5 V/cm for the required time in 1x TAE containing 0.5 μg/ml ethidium bromide. Larger or smaller DNA fragments were run on lower or higher percentage gels as appropriate. To obtain optimum fragment resolution, gels were run at 5 V/cm. Samples were mixed with one sixth volume of 6x DNA loading buffer and run alongside 2 μl 1 kb and/or 100 bp DNA marker (NEB). DNA fragments were visualised under UV trans-illumination.

2.2.6.5 DNA purification from agarose gels

The DNA band of interest was visualised under UV trans-illumination and excised from agarose gels with a clean scalpel. The DNA fragment was purified using either the GENECLEAN®II kit supplied by Q-BIOgene or the QiaexII gel extraction kit supplied by Qiagen.

2.2.6.6 Ligations

A typical ligation consisted of 400 U T4 DNA ligase (NEB), 1x DNA ligase buffer (NEB), and a 1:3 ratio of plasmid:insert(s). As a rule, 200 ng of plasmid were used and the total reaction volume was made up to 10 μl with dH_20. Ligations were incubated at RT overnight.

2.2.6.7 Production of chemically competent bacterial cells

Two and a half millilitres of a freshly grown culture were inoculated into 250 ml LB and grown at 37°C to an OD_{590} of 0.375. The culture was aliquoted into five 50ml prechilled, sterile Falcon tubes and left on ice for 10 minutes. The cells were centrifuged

at 2600 rpm in a benchtop centrifuge for 7 minutes at 4°C an allowed to decelerate without the brake. The supernatant was discarded and the pellet resuspended in 10ml ice cold $CaCl_2$ solution. The cells were centrifuged at 2250 rpm in a benchtop centrifuge for 5 minutes at 4°C and the pellet resuspended in a further 10ml ice cold $CaCl_2$ solution. The resuspended cells were incubated on ice for 30 minutes and centrifuged at 2250rpm in a benchtop centrifuge for 5 minutes at 4 C. The pellet was resuspended in 2 ml ice cold $CaCl_2$ solution. After 20 minutes, the cells were aliquoted and frozen at -80 C.

2.2.6.8 Transformation of chemically competent bacterial cells

Fifty nanograms of plasmid DNA or all of a ligation reaction was incubated with 50 µl chemically competent bacterial cells for 30 minutes on ice. The cells were heated shocked at 37 °C for 5 minutes and placed on ice for 2 minutes. One millilitre of LB was added and the transformed cells incubated for an hour at 37 °C. A 100 µl aliquot and the remaining spun down cells were plated onto LB plates containing the appropriate antibiotic selection. Plates were incubated overnight at 37 °C.

2.2.6.9 Screening of colonies

After the transformation of ligations, the incorporation of the correct insert into the plasmid was verified by screening transformant colonies. Individual colonies were picked and grown in 3 ml LB with the appropriate antibiotic selection overnight. Plasmid DNA was extracted by miniprep and screened by endonuclease digestion. The presence of an insert was confirmed by agarose gel electrophoresis.

2.2.6.10 Small scale purification of plasmid DNA (miniprep)

Individual, freshly grown transformant colonies were picked and grown overnight in 3 ml LB plus the appropriate antibiotic selection. One and a half millilitres of overnight culture were microfuged for 5 minutes at 14,000 rpm and the supernatant removed by aspiration. The bacterial pellet was resuspended in 100 μl P1 resuspension buffer (Qiagen) by vortexing. One hundred microlitres of P2 lysis buffer (Qiagen) were added and the sample gently inverted to mix. One hundred microlitres of ice cold P3 neutralisation buffer (Qiagen) were added and the sample gently inverted to mix. The sample was incubated on ice for 15 minutes. The cellular debris was then microfuged at 14,000 rpm for 5 minutes and the supernatant removed to a fresh Eppendorf tube. To remove any contaminating ssRNA, 10 μg RNaseA were added and the sample incubated at 37 °C for 30 minutes. To remove any protein from the DNA solution, an equal volume of phenol:chloroform was added. The sample was vortexed briefly and microfuged for 5 minutes at 14,000 rpm. The upper aqueous phase was removed to a fresh Eppendorf tube, taking care not to disturb the lower organic phase or the interphase. The DNA was precipitated from aqueous solution by adding three volumes of ice cold ethanol. The sample was inverted to mix and placed at –20 °C for 60 minutes. The DNA was microfuged for 20 minutes at 14,000 rpm at 4 °C and the supernatant discarded. The pellet was washed twice with 200 μl of 70% ethanol and air dried. The DNA was resuspended in 30 μl dH_20 and stored at –20 °C.

2.2.6.11 Preparation of glycerol stock

The remaining overnight culture from Section 2.2.6.9 was used to prepare glycerol stock of the recombinant vector. Sterile glycerol (150 µl) was added to every 850 µl of the bacterial culture in several microcentrifuge tubes. The mixture was vortexed for 15 seconds and stored at -80˚C in 1 ml aliquots. Each aliquot was thawed only once and any remainder from the same tube was discarded if unused.

2.2.6.12 Large scale purification of plasmid DNA (maxiprep)

All large-scale plasmid DNA purification was carried out using the Mega Plasmid Purification kit (Qiagen) according to the manufacturer's instructions. DNA was resuspended in 1 ml dH_20, quantified using a UNICAM Heλos β spectophotometer and stored at –20 °C. For long term storage, glycerol stocks were prepared by combining 400 µl of the overnight culture with 600 µl of 20% sterile glycerol and stored at –80 °C.

2.2.6.13 Sequencing

All sequencing was carried out by the Advanced Biotechnology Centre (ABC), South Kensington.

2.2.7 Producing lentiviral particles.

For a stable transfections, it is recommended to generate lentiviral particles and infect the target cells. The infected cells will stably express the shRNA of interest.

Firstly, 7 x 10^5 HEK 293T cells were plated in 5 ml of media in a 6 cm^2 tissue culture plate and the cells were incubated at 37˚C, 5% CO_2 overnight. During this stage, the cells were passaged in DMEM + 10% FCS without antibiotics. The next day, the transfection was carried out in the late afternoon because the transfection mix should

only be incubated with the cells for 12-15 hours. In polypropylene microfuge tubes, a cocktail for each transfection was made as followings:

1µg pLKO.1 shRNA plasmid

750ng CMV pol packaging plasmid

250ng VSVG envelope plasmid

To 20µl serum-free OPTI-MEM

Then a master mix of FuGENE® 6 transfection reagent was created in serum-free OPTI-MEM. The amount of FuGENE® 6 and OPTI-MEM was calculated given that each reaction will require 6 µl FuGENE® 6 + 74µl OPTI-MEM. In a polypropylene tube, OPTI-MEM was added first, followed by pipetting FuGENE® 6 directly into the OPTI-MEM. The mixture was gently swirled and incubated for 5 minutes at room temperature. Subsequently, 80 µl of FuGENE® 6 master mix was added to each tube for a total volume of 100 µl. The master mix was carefully pipette directly into the liquid and not onto the walls of the tube. The tube mixture was gently swirled and further incubated for 20-30 minutes at room temperature. The HEK-293T cells was retrieved from incubator and the cells should be 50% confluency and in DMEM without antibiotics. Without touching the sides of the dish, the DNA:FuGENE® 6 mix was gently added to the cells and the plate was swirled to disperse the mixture evenly. The cells were left at 37˚C, 5% CO_2 for further 12-15 hours.

The next morning, the medium was changed to remove the transfection reagent by replacing with 5 ml fresh DMEM + 10% FCS + penicillin/streptomycin. The medium was pipetted onto the side of the plate so as not to disturb the transfected cells. The cells were further incubated in the same conditions for 24 hours.

After 24 hours of incubation, the medium was harvested from cells and transferred to a sterile polypropylene storage tube. The medium contained the lentiviral particles and

was stored at 4˚C. The former cells were added with 5ml of fresh media containing antibiotics and further incubated for 24 hours.

The following day, the medium was harvested from cells and pooled with medium harvested from the day before. The medium was spun at 1250 rpm for 5 minutes to pellet any HEK-293T cells that were inadvertently collected during harvesting. In lieu of centrifugation, the collected medium was filtered through cellulose acetate filter, 0.45µm to remove the cells. The virus was kept frozen at -20˚C or -80˚C for long term storage in small aliquots to prevent from freeze/thaw cycles since it can decrease the virus infectivity.

2.2.8 Site directed mutagenesis of p130

P130 point mutations were introduced by the QuickChangeR site-directed mutagenesis kit according to the protocol of Stratagene™. The p130 was mutagenized into three types of mutants, which are p130 mutant that is defective in binding the E7 LXCXE motif (p130mE7), mutant which cannot be phosphorylated by CDK (p130PM22) and a double mutant (p130PM22/mE7) were constructed. The p130mE7 mutant was designed based on the work of Dick and Dyson (2002), whereas the p130PM22 was designed by replacing 22 phosphorylation sites of serine and threonine with alanine (Farkas et al., 2002). Successful mutagenesis was confirmed by DNA sequence analysis.

Mutagenesis reaction:

Plasmid DNA (10 ng) 2.0 µl

Oligonucleotide primer #1 (10 µM)* 1.5 µl

Oligonucleotide primer #2 (10 µM)* 1.5 µl

Reaction buffer (10×) 5.0 µl

dNTP mix 1.0 µl

QuikSolution 3.0 µl

H2O, deionised ad 50.0 µl

Mutagenesis program:

95 °C		1.0 min
95 °C		50 sec
60 °C	18 cycles	50 sec
68 °C		5 min
68 °C		7.0 min

2.3 Cell culture

2.3.1 Types of cell lines

All cell lines are from human origin and described in detail as follows:

T98G

Tissue : brain

Morphology : fibroblast

Age stage : 61 years

Ethnicity : caucasian

Growth properties : adherent

HPV presence : no

Reference : Stein GH (1979)

C33A

Tissue : cervix

Morphology : epithelial

Age stage : 66 years

Ethnicity : caucasian

Growth properties : adherent

HPV presence : no

Reference : Yee C., et al (1985)

SiHa

Tissue : cervix

Morphology : epithelial

Age stage : 55 years

Ethnicity : asian

Growth properties : adherent

HPV presence : yes (HPV 16, 2 copies per cell)

Reference : Baker CC., et al (1987)

Caski

Tissue : cervix

Morphology : epithelial

Age stage : 40 years

Ethnicity : caucasian

Growth properties : adherent

HPV presence : yes (HPV 16, 600 copies per cell)

Reference : Baker CC., et al (1987)

2.3.2 Maintenance and sub-culturing of cell lines.

Human glioblastoma cell line (T98G), HPV-negative cervical cancer cell line (C33A), HPV16-positive cervical cancer cell lines (Caski and SiHa) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum (Invitrogen), 100 U/ml penicillin and 100 ug/ml streptomycin (Invitrogen) and 2mM L-glutamine (Invitrogen). All cell lines were routinely passaged by splitting 1:10 every three to four days.

All cells were passaged using 1 X 0.05% trypsin-EDTA (Invitrogen). As a rule, cells were grown in 225 cm^2 Corning® cell culture flasks (Fisher Scientific Limited). Briefly, the cell culture medium was aspirated and the cell monolayers were washed twice with 10mls of PBS. Then 2.5 ml of trypsin-EDTA was added to the flask and incubated for 2-3 minutes at 37˚C to dislodge the cells. When all the cells had dislodged, 1ml of 1X growth medium (GM) was dispensed into the flask to inactivate the action of trypsin-EDTA and the medium containing the cells were pipetted up and down several times to separate the cells. Following this, additional growth medium was added to the cell suspension and then dispensed (15ml) into each flasks. The cells were incubated at 37°C with 10% CO_2 and 95% humidity.

2.3.3 Preservation, storage and revival of cells.

Five flasks that were 95% confluent with cells were rinsed and trypsinized as previously described (Section 2.3.2). The cell suspension was transferred into a 15ml conical-bottom centrifuge tube and spun down by centrifugation at 1300 rpm for 4 minutes at room temperature. The supernatant was discarded and the cell pellet was resuspended in 10ml cryo-medium containing 90% FCS and 10% DMSO. The cell suspension was then dispensed into cryo-vials in 1 ml aliquouts and placed in an

isopropanol freezing box at -70˚C overnight. The next day, the cryo-vials were transferred onto cryo-canes and stored in the liquid nitrogen tank as frozen cells for longer storage.

Frozen cells stored in the liquid nitrogen were revived by thawing the cells to 37˚C in a water bath. The content of the cryo-vial was then dispensed into a centrifuge containing 2 ml 1XGM pre-warmed to 37˚C and mixed well. The cells were spun down at 1300 rpm for 3 minutes at room temperature. The supernatant containing cryo-medium with DMSO was discarded and the cell pellet was resuspended in 15ml 1XGM. The cell suspension was transferred to a 225 cm^3 flask and incubated accordingly.

2.3.4 Cell counting and seeding.

Cells were harvested as previously described in Section 2.3.2. The cell pellet was resuspended in a suitable 15 ml conical bottom tube containing 1 ml 1XGM without antibiotic supplementation. The cell suspension was pipetted up and down in the tube for 5-8 times using a small-bored 2 ml serological pipette to obtain a uniform suspension containing single cells.

Cell counting was performed with the use of a haemocytometer to estimate the number of cells in a suspension. Trypan blue was used as a vital dye to determine viable verses non-viable cells whereby the dye was excluded from viable cells. Viable cells would be seen as clear in a bluish background while non-viable cells appeared blue under the microscope. Subsequently, a 1:10 dilution of the cell suspension was prepared in trypan blue by combining 10 µl of cell suspension with 90µl of trypan blue dye placed in a clean microcentrifuge tube.

A clean cover slip was placed over the counting chambers. Thirty µl of the diluted cell suspension was loaded onto each counting chamber and allowed to fill the entire space underneath the cover slip by capillary action. The cells were observed

under a microscope at 100X magnification. The number of cells (total of viable) overlying the $1mm^2$ areas of all four corners of the counting chambers was determined (A, B, C and D). For accurate determination, the total number of cells overlying one $1mm^2$ corner should be between 15-50 cells, above which the cell suspension should be further diluted and below which less diluted sample should be used. The concentration of the cell suspension was then calculated using the formula as follows:

$$\text{Concentration of cells} = \frac{A + B + C + D}{\text{No. of quadrants}} \times \text{dilution factor} \times 10^4 = \text{No. of cells/ml}$$

When the concentration of the cell suspension has been determined, an appropriate volume of the cell suspension according to the desired number of cells was used for seeding.

2.4 *In vivo* protein expressions and analysis.

2.4.1 Transfection analysis

2.4.1.1 Transient transfection protocol 'FuGENE 6'.

The transient transfections of the T98G cells were performed according to the FuGENE 6 protocol. Approximately $2x10^6$ T98G cells were plated in 10 cm dishes the day before transfection and incubated at 37˚C with 5% CO_2. On the next day, 485 µl of OpTIMEM were mixed with 18ul of FuGENE 6 reagent and incubated for 5 minutes. Following reagent incubation, 6 µg of the plasmid DNA (pLKO.1 sh.scramble, pLKO.1 shLin54-1, pLKO.1 shLin54-2, pLKO.1shLin54-3, pLKO.1 shLin54-4 and pLKO.1 shLin54-5) was added to the transfection reagent diluents and further incubated for 15 minutes to allow complex formation. The transfection complexes were poured onto the cells and the cells were incubated overnight in the presence of the plasmid DNA. On the following day, cells were washed and replaced with fresh medium. The transfected cells were selected by supplementation of the growth medium with 2 µg/ml of puromycin (Merck Pharmaceuticals) 24 hours post-transfection and further incubated for 48 hours before being harvested.

2.4.1.2 Transient transfection by Calcium phosphate co-precipitation.

On the day before transfection, T98G cells were plated in 10 cm dishes to reach 70-80% confluency. On the following day, T98G cells were transiently transfected with 30 µg of the respective pMSCV puro plasmids (pMSCV puro, pMSCV puro 1E7HA, 11E7HA, 16E7HA, 18E7HA, 33E7HA and 48E7HA)

For each transfection the following mixture was prepared:

The required amount of DNA was mixed with dH_2O to a total volume of 440 µl. Five hundred microliters of HBS transfection buffer were gently added to the DNA mix and 62 µl of 2M $CaCl_2$ dropped onto the surface. The transfection mixture was tapped to mix and incubated at room temperature for 2-3minutes until the calcium phosphate precipitate formation. The final volume of 1ml was added to the cells and incubated at 37°C with 10% CO_2 overnight. On the following day, the culture medium was changed and 2 µg/ml of puromycin was supplemented and the cells incubated for a further 24 hours. After incubation, the medium was removed by aspiration and the cells washed with 10ml PBS. Finally, cells were harvested for protein and RNA extraction.

2.4.1.3 Stable transduction by lentiviral system.

The lentiviral system was utilised since it gives a higher percentage of the cells being transduced rather than conventional methods of transfections. This system was particularly necessary for Caski cell since it was difficult to transfect with the common transfection methods.

The lentiviral particle carrying the targeted genes was first generated following the manufacturer's instructions (Addgene). Two types of shRNA were used to generate lentiviral particles, 16E7A and 16E7B shRNAs, which were designed to target specifically the 16E7 mRNA in Caski and SiHa cells. Firstly, $7x10^5$ HEK 293a cells were plated in 6 cm dish with 5 ml of DMEM + 10% FCS without penicillin/streptomycin and incubated at 37˚C, 5% CO_2 overnight. On the next day, transfection was performed in the late afternoon because the transfection mix should only be incubated with the cells for 12-15 hours. For each transfection the following cocktail was prepared individually in polypropylene microfuge tubes:

1 µg pLKO.1 sh.16E7A/B

750 ng CMV pol

250 ng VSVG

to 20 µl serum-free OPTI-MEM

Next, a master mix of FuGENE 6 transfection reagent in serum-free OPTI-MEM was made, given that each reaction required 6 µl FuGENE 6 plus 74 µl OPTI-MEM and this was incubated for 5 minutes at room temperature. After incubation, 80 µl of FuGENE 6 master mix was added to each cocktail tube for a total volume of 100 µl and incubated for 20 -30 minutes at room temperature. The transfection complexes were poured onto the cells in DMEM that did not contain antibiotics and incubated at 37˚C, 5% CO_2 for 12-15 hours. The following morning, the medium was replaced with 5 ml fresh DMEM + 10% FCS + penicillin/streptomycin and further incubated at 37˚C, 5% CO_2 for 24 hours. After 24 hours of incubation, the medium already contained lentiviral particles and was harvested from the cells and stored at 4˚C. The cells were then replaced with fresh medium containing antibiotics and further incubated at 37˚C, 5% CO_2 for 24 hours. On the next day, the medium from the cells was harvested and pooled with the medium from the previous day. The medium was spun at 1250 rpm for 5 minutes to pellet any HEK 293a cells that were inadvertently collected during harvesting. Finally, the medium was filtered through a 0.45 µm filter and stored at -80˚C.

The day before the transduction was performed, $1x10^6$ SiHa and Caski cells were plated in 10cm dishes and incubated at 37˚C with 5% CO_2. On the next day, the medium was replaced with fresh medium containing 8 µg/ml polybrene. Polybrene was used to increase the efficiency of viral infection. The lentiviral stock was added onto plate with 1 ml for each 10cm dish and the cells were further incubated at 37˚C with 5% CO_2 overnight. Following the transduction, the medium was changed to fresh medium

supplemented with 2µg/ml of puromycin 24 hours post-transduction. Cells were harvested 48 hours after puromycin selection for RNA and protein extractions.

2.4.2 Nuclear extraction.

The cell monolayer was washed once with sterile PBS, then cells were scraped to detach and removed to a fresh 50 ml-falcon tube. The cells were spun down at 1300 rpm for 4 minutes in a Sorvell Legend RT centrifuge and washed in 1ml ice-cold PBS. The cells were resuspended in 200 µl buffer A and allowed to swell on ice for 15 minutes. The cells were lysed by drawing up slowly through 25G syringe needle and rapidly ejecting into Eppendorf tube. The nuclei were collected by brief microcentrifugation for 20 seconds at 14000 rpm in a cooled Eppendorf centrifuge (4˚C). The nuclei were then incubated on ice in one volume of buffer B for 30 minutes. Subsequently, the nuclei were microcentrifuged at 14000 rpm for 10 minutes. The supernatant was transferred to a fresh eppendorf tube and the protein concentrations were measured using the Bradford assay. The proteins were stored at -80˚C until used.

2.4.3 Protein estimation using Bradford assay.

The concentrations of total nuclear protein extracts were determined using the standard Bradford assay. The concentrated Bradford reagent was diluted 5X with distilled water to produce the diluted working solution of Bradford reagent. A standard curve was produced using suitable dilutions of 10mg/ml bovine serum albumin (BSA) solution ranging from 0.2 mg/ml to 3.0 mg/ml. Five microliters (5 µl) of each BSA dilution sample was placed in a well of 96 well clear micro-titre plate in triplicates, followed by the addition of 200 µl of diluted Bradford reagent. The samples were gently mixed by pipetting with special care to avoid air bubbles that would interfere with the

absorbance readings. The micro-titre plate was then agitated for 5 times in the microplate reader and incubated for 5 minutes at room temperature to equilibrate the mixture. Light absorbances were read at 590nm and a standard curve were generated from the readings.

For experimental protein samples, 5µl of each sample was used and placed in the wells in triplicates. This was followed by the addition of 200µl of diluted Bradford reagent and the absorbances were acquired. The actual protein concentrations of the experimental samples were calculated from the standard curve.

2.4.4 Proteins co-immunoprecipitation (co-IP).

Three hundred microgram (300 µg) of the cleared nuclear lysate was diluted with an equal volume of 20 mM HEPES-KOH, pH 7.9 and mixed with 2µg of antibody overnight at 4°C on a wheel. The next day, protein G Sepharose beads which was in 50% slurry IP buffer were added to the mixture and incubated for one hour at 4°C on a wheel. The immune complexes were collected by brief centrifugation for 2 minutes at 12000 rpm and washed four times with IP buffer and eluted in 50 µl 2xSDS-PAGE loading buffer. At this stage, the diluents were stored at -20˚C. Prior to SDS-PAGE gel electrophoresis, the immunoprecipitated proteins were heated at 100˚C for 5 minutes to denature the protein and 20 µl were loaded in each well of a mini-gel.

2.4.5 Antibodies

The dilution and/or concentration of antibodies used in Western blotting (WB) and immunoprecipitation (IP) are shown in Table 2.3.

Antibody	Dilution/concentration	
	WB	IP
Rabbit α-Lin-9 polyclonal (Santa Cruz)		5µg/ml
Rabbit α-Lin-9 polyclonal (Abcam)	1:1000	
Mouse α-B-myb monoclonal (LX015.1) (Watson's Lab)	1:2	
Rabbit α-B-myb polyclonal (Santa Cruz)		5µg/ml
Rabbit α-p107 polyclonal (Santa Cruz)	1:1000	
Rabbit α-p130 polyclonal (Santa Cruz)	1:1000	
Rabbit α-actin polyclonal (Sigma-Aldrich)	1:1000	
Rabbit α-Lin-54 polyclonal (a kind gift from Stephan Gaubatz)		5µg/ml
Mouse α-HA monoclonal (Roche)	1:1000	
Mouse α-p53 polyclonal (Watson's Lab)	1:5000	

Table 2.3: The dilution and/or concentration of antibodies used in western blotting (WB) and immunoprecipition (IP).

2.4.6 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), immunoblotting and Enhanced Chemiluminescent (ECL) detection of proteins

Protein samples to be analysed were mixed with the appropriate volume of 4x SDS loading buffer, boiled at 100 °C for 5 minutes and microfuged at 13,000 rpm for 1 minute before being loaded onto an SDS-PAGE minigel. Samples were run against the Precision Plus protein molecular weight marker (Bio-Rad). SDS-PAGE minigels were composed of a resolving gel and a stacking gel (Table 2.4) and assembled using BioRad minigel apparatus. The percentage resolving gel used depended upon the molecular weight of the protein to be analysed.

Minigels were run at 100 V for 90 minutes. After the proteins had been resolved, a section of Hybond Polyvinylidene difluoride (PVDF) membrance (GE Healthcare) was soaked in methanol for 10 seconds, washed in dH20 for 5 minutes and soaked in transfer buffer for 10 minutes. The gel was removed from the minigel apparatus and the stacking gel discarded. The separation gel was soaked in transfer buffer for 10 minutes. Protein from the gel was transferred onto a Hybond PVDF membrane (GE Healthcare) by forming a sandwich of triple layers of Whatman 3 mm filter paper and a sponge either side of the membrane and the gel. The sandwich was placed in a transfer tank, immersed in 1x transfer buffer and run at 100 V for 1 hour. Following transfer, the membrane was blocked in blocking solution for an hour at room temperature with agitation and probed with the primary antibody. The primary antibody was diluted as specified in 10 ml of blocking solution and incubated either overnight or over the weekend at 4 °C with agitation. BSA (Sigma-Aldrich) was substituted for Marvel

	% Separation gel			
	7%	10%	12%	15%
Acrylamide:Bis-acrylamide (30:08)	4.7 ml	6.7 ml	8 ml	10 ml
Separation buffer	5 ml	5 ml	5 ml	5 ml
dH_2O	10.2 ml	8.2 ml	6.9 ml	4.9 ml
10% APS	100µl	100µl	100µl	100µl
TEMED	20µl	20µl	20µl	20µl

Stacking gel	
Acrylamide:Bis-acrylamide (30:08)	1.3 ml
Stacking buffer	2.5 ml
dH_2O	6.1 ml
10% APS	100µl
TEMED	20µl

Table 2.4: The composition of the separation gels and the stacking gel used in SDS-PAGE. Volumes shown are enough for 2 minigels.

for phospho-specific primary antibodies. The membrane was washed for three 15 minute intervals in 1x TBS/0.05% Tween-20 (Sigma-Aldrich) and probed with the appropriate secondary HRP conjugated antibody. The secondary antibody was diluted as specified in 10 ml of blocking solution and incubated for an hour at RT with agitation. The membrane was washed for a further four 15 minute intervals in 1x TBS/0.05% Tween-20 and the proteins visualised using the SuperSignal® West Pico Chemiluminescent Substrate provided by Pierce Biotechnology according to the manufacturer's instructions. The membrane was exposed to autoradiography film and developed using the RGII FUJI X-ray film processor.

2.5 *In vitro* GST-E7 binding assay

Hundred fifty microgram nuclear extracts of p130 transfected cells were added to 200 µl EBC buffer, 20 µl of BSA (10mg/ml, NEB), 20 µl Glutathione-Sepharose G beads (GE Healthcare) and 5 µl GST bound beads on ice. The samples were incubated at 4 °C on an orbital rotor for an hour, the beads microfuged at 13,000 rpm for 1 minute at RT and the supernatant removed to a fresh, cold Eppendorf tube. An equal amount of GST bound beads or GST-fusion bound beads were added to the supernatant and incubated at 4 °C on an orbital rotor for two hours. The beads were washed four times with cold NETN buffer with a 1 minute spin of 13,000 rpm in a microfuge in between washes. Ten microlitres of 4x SDS loading buffer were added, the sample boiled at 100°C for 5 minutes, spun down and loaded onto an SDS-PAGE gel alongside a 10% input control.

2.6 Flow cytometry analysis

2.6.1 Cell cycle analysis

Cells in 10 cm dishes were harvested by trypsinization and then centrifuged at 1500rpm for 5 minutes. Subsequently, cells were washed three times with PBS and fixed with 90% ethanol and 10% PBS for a minimum of 12 hours.

Cells were then spun down at 7000 rpm in a microfuge for 2 minutes and the fixation solution was discarded. The cell pellet was resuspended in a 982 µl of PBS, 10 µl of RNAse A stock solution (10 µg/µl) and 8µl of propidium iodide (5mg/ml) and incubated for 30 minutes at room temperature. FACS was performed using a FAC Canto II analyser and analysed with FlowJo (Tree Star Inc., Ashland, Or, USA) software. At least 10000 cells were counted.

2.6.2 Bromodeoxyuridine (BrdU) staining.

Cells were treated with 10 µM BrdU for an appropriate time (30 minutes – 48 hours) and washed twice with 1% BSA/PBS at 4˚ C. The cells were resuspended thoroughly in 250 µl ice cold 80% ethanol and incubated for 30 minutes on ice. At this stage, the samples were stored at -20°C or used immediately. The ethanol was discarded gently and the cells were washed with PBS. Subsequently, 750 µl of 2M HCl/0.5% Triton X-100 was added to the cells and it was mix by gentle pipetting and incubated at room temperature for 15 minutes. The cells were agitated by vortexing briefly and incubated for another 15 minutes. The cells were spun at 3000 rpm for 3 minutes and HCl/Triton X-100 was removed and 750 µl of 100mM $Na_2B_4O_7$ (Borax) pH 8.5 was added. The cells were left for approximately 1 minute to neutralize the acid. Then, the

cells were spun at 3000 rpm for 3 minutes and Borax was discarded. The cells were resuspended thoroughly in 20µl anti BrdU-FITC conjugated. The propidium iodide (PI) only and the unstained were resuspended in PBS. The samples were kept at 4˚ C until FACs was performed.

2.6.3 Mitotic index

The cells were harvested and fixed in 90% ethanol and 10% PBS for 24 hours at – 20 C. The cells pellet down by spun at 7000 rpm for 2 minutes and washed twice with PBS. Then, the cells were resuspended in 1ml of 0.25% Triton X-100 and were placed on ice for 15 minutes, followed by centrifuged the cells at 7000rpm for 2 minutes. The cells were then resuspended in 80 µl PBS/1% BSA containing 1µg rabbit polyclonal anti-histone H3 phospho-serine 28 and incubated for 30 minutes at room temperature in the dark. The cells were washed in PBS/1% BSA and resuspended in 0.5ml PBS containing 25 µg PI and 10µg RNase A and incubated for 30 minutes at room temperatures in the dark. The samples were analysed on a FACS Canto II analyser.

2.7 Statistical tests

2.7.1 Students t-test

Statistical significance between two sets of data was determined using the two-tailed Students t-test for independent samples. P-values of <0.05 were considered to be significant.