2 METHODS

The methods presented here are in one of two formats. Format one is step by step instructions for each of standard protocols used. Format two is a description of how these protocols were applied to complete each experiment. Unless otherwise stated all reagents were from Sigma. .

2.1 Common Solutions and Media

2.1.1 LB Broth

10g	Tryptone
5g	Yeast extract
10g	Sodium chloride
Upto 1l	Double-distilled water
	… pH to 7.0

2.1.2 2XLB Broth (low salt)

20g	Peptone
10g	Yeast Extract
5g	Sodium chloride
Upto 1l	Double-distilled water

2.1.3 Phosphate Buffered Saline (PBS)

36.65 g	Sodium chloride
11.80 g	Disodium hydrogen phosphate (Na_2HPO_4)
6.60 g	Sodium dihydrogen phosphate (NaH_2PO_4)
up to 5 l	Double-distilled water

2.1.4 Creosol Red Loading Buffer

28g	Sucrose
100ml	Filter sterilised T0.1E
8mg	Creosol Red

2.1.5 T0.1E

10mM	Tris ($NH_2C(CH_2OH)_3$)
0.1mM	EDTA
	…. pH 8

2.1.6 siRNA Suspension Buffer

11.78g	Potassium Acetate
2.56g	HEPES-KOH
800µl	1M Magnesium Acetate

398.2µl HPLC grade Water

… pH to 7.4 with KOH

2.1.7 HeLa Growth Media

50ml Modified Eagle's Medium (Sigma #M2279)

5ml Fetal Bovine Serum (Gibco #10270-106)

0.5ml 200mM L-Glutamine

0.5mls 100 Uml^{-1} Penicillin

5g Streptomycin

0.5ml 100x Non-essential amino acids (Gibo, # 11140-035)

HeLa Seed Media

50ml Modified Eagle's Medium (Sigma #M2279)

5ml Fetal Bovine Serum (Gibco #10270-106)

0.5ml 200mM L-Glutamine

0.5ml 100x Non-essential amino acids (Gibo, # 11140-035)

2.1.8 Treatment/Assay media

10ml Modified Eagle's Medium (Sigma #M2279)

100µl 200mM L-Glutamine

100µl 20 Uml^{-1} Penicillin

1g Streptomycin

100µl 100 x Non-essential amino acids (Gibo, # 11140-035)

TRAIL/FAS/H_2O_2 was added as required to make treatment media. 5mls alamarBlue was added to 45mls treatment media to make alamarBlue assay media. .

2.2 Common cell culture methods

2.2.1 Routine Passage of cells

Cells were routinely grown in a Galaxy R incubator (Scientiifc Laboratory Supplies) at 37°C, 5% CO_2 in 75 cm^2 culture flasks with 0.2µm vent caps (Corning #430641). Cells were passaged every 3 or 4 days. All solutions are warmed to 37°C. Growth media was made fresh each time cells were passaged.

1. Media was aspirated using a disposable glass pipette attached to a vacuum trap in 2% virkon.
2. Cells were washed with approximately 20mls of PBS, PBS was aspirated off.

3. Cells were washed with 3mls of 1XTypsin-EDTA solution and wash apirated off.
4. Cells were washed with a further 3mls of 1XTypsin-EDTA solution and the wash decanted into 1% virkon.
5. Flask was incubated for 5 minutes at 37°C.
6. 10mls of HeLa growth media was added to the flask and cells suspended by pippetting up and down.
7. Cells were counted using an Improved Neubauer Haemocytometer (Assistant #403002400).
8. Cells were diluted to $1*10^5$ $cellsml^{-1}$ in 20mls of growth media per new flask to be seeded.
9. 20mls of diluted cells were added to each new flask required. .

2.2.2 Defrosting of cells

All solutions were warmed at 37°C before use. Cells were stored in Liquid Nitrogen in Fetal Bovine Serum plus 10% DMSO.

1. 10mls of Growth Media was added to a T25 0.2µm vented flask (Corning #430639) and the flask marked passage 0.
2. Cells were defrosted in 37°C water bath.
3. A plastic pasteur pipette was used to add 1ml growth media to the cells.
4. Cells were transferred to flask containing media.
5. Cells were incubated overnight in 37°C, 5% CO_2 incubator.
6. Cells were passaged as described above (section 2.2.1).

2.2.3 RNA transfection of cells

Media was warmed to 37°C before use and transfection reagent was warmed to room temperature.

1. 2.5pmol per well of the siRNA(s) to be transfected was arrayed in a 96 well, round bottomed plate allowing one extra well per siRNA excess. .
2. 0.12µl of Lipofectamine 2000 (Invitrogen #11668-019) was diluted in 12.5µl of Opti-MEM I (Invitrogen #31985-047) per well to be transfected plus 10% excess and incubated for 15 minutes.
3. siRNA were diluted in 12.5µl Opti-MEM I per well to be transfected .
4. 12.5µl of Lipofectamine 2000/Opti-MEM I mixture per well to be transfected was added to the diluted siRNAs and incubated for a further 15 minutes. .
5. 25µl of siRNA/Lipofectamine 2000/Opti-MEM I mixture was added to the well of a

96 well plate containing cells seeded 24 hours earlier.

This protocol is for transfecting cells with 2.5pmol of siRNA in a 96 well plate. Unless otherwise stated this is how cells were transfected with siRNA. In other cases differing amounts of siRNA were used as specified in the text. Where cells were transfected in 24 well plates, 12.5µl of siRNA was diluted in 50µl Opti-MEM I and 0.6µl of Lipofectamine was mixed with 50µl Opti-MEM I.

2.2.4 Transfection of plasmid DNA

Media was warmed to 37°C before use. siPort XP-1 was warmed to room temperature before use.

1. 80ng of plasmid DNA per well to be transfected was aliquoted into the well of a round bottomed 96 well plate allowing one well extra excess for each different plasmid.
2. 0.24µl of siPort XP-1 per well to be transfected, plus 10% was diluted in 20µl Opti-MEM I and incubated for 10 minutes.
3. 20µl of siPort XP-1/Opti-MEM I per well to be transfected was added to plasmid DNA and incubated for a further 10 minutes.
4. 20µl of DNA/Opti-MEM I/siPort XP-1 mixture was added to each well of cells to be transfected.

The above protocol is for the transfection of 80ng of plasmid DNA using 0.24µl siPort XP-1 into cell grown in 96 well plates. Unless otherwise stated in the text this is how DNA transfection were performed. In some cases different quantities of DNA and siPort XP-1 were used as indicated in the text. Where cells were transfected in 24 well plates, 400ng of DNA was used per well and 1.2µl of siPort XP-1 was diluted in 100µl of Opti-MEM I.

2.2.4.1 Transfection Protocol using GeneJuice

Media was warmed to 37°C, and the GeneJuice reagent to room temperature before beginning. Protocol presented is for transfection in 24 well plates/8-well slides.

1. 200ng per well of DNA to be transfected was aliqouted into the well of a round bottomed 96 well plate, allowing one well extra excess.
2. 0.75µl GeneJuice (Novagen #70967-3) per well was mixed with 20µl Opti-MEM I per well, allowing one well excess, and incubated for 10 minutes at room temperature.
3. GeneJuice/Opti-MEM I mixture was added to plasmid DNA and incubated for 10

minutes.

4. 20µl GeneJuice/Opti-MEM I/DNA mixture was added to each well to be transfected. .

2.2.4.2 Transfection Protocol using Lipofectamine 2000

Media was warmed to 37°C and Lipofectamine 2000 reagent to room temperature before beginning. Protocol presented is for transfection in 24 well plates/8-well slides.

1. 1µg per well of plasmid DNA was aliquoted into a well of a round bottomed 96 well plate, allowing one well excess.
2. 1.5µl per well of Lipofectamine 2000 was added to 50µl per well of Opti-MEM I, allowing one well excess and incubated for 5 minutes.
3. 50µl per well of OptiMEM I was added to plasmid DNA, allowing one well excess.
4. 50µl per well of Lipofectamine 2000/OptiMEM I mixture was added to DNA/OptiMEM I mixture, allowing one well excess, and incubated for 20 minutes.
5. 100µl of Lipofectamine 2000/plasmid DNA/OptiMEM I mixture was added drop-wise to each well of cells and mixed by rocking.

2.2.4.3 Transfection Protocol using Effectene

Effectene used directly from the 4°C, but was not kept on ice during procedure. Buffers mentioned come from the Effectene Transfection Kit (Qiagen #301425). Protocol presented is for transfection in 24 well plates/8-well slides. .

1. 0.5µl per well of plasmid DNA was aliquoted into a well of a round bottomed 96-well plate and diluted to 146µl per well in Buffer EC. 4µl per well of Enhancer reagent was added.
2. Mixture was incubated at room temperature for 5 minutes.
3. 5µl per well Effectene reagent was added to 95µl per well of Buffer EC and this mixture added to the DNA/Enhancer Mixture and mixed by pipetting up and down 5 times.
4. Mixture was incubated for 10 minutes at room temperature.
5. 250µl of Effectene/DNA/Enhancer was added to each well of cells.

2.2.5 Isolation of total cellular RNA

Unless otherwise specified reagents are from the SV Total RNA isolation kit (Promega #Z3100). After lysis, lysates were stored as -70°C until purification. Samples were generally

processed in batches of 12.

1. 300µl of RNA Lysis Buffer was added to each well of cells .
2. Cells were left to lyse for 5 minutes on ice.
3. Plates were sealed with Parafilm (Fisher #SEL-400-050J) and stored at -70°C.
4. Plates were defrosted at room temperature.
5. Lysates were transferred to a 1.5ml tube.
6. Lysates were incubated at 70°C for 3 minutes in a water bath.
7. Lysates were centrifuged at 14,000g for 10 minutes.
8. Cleared lysates were transferred to a fresh 1.5ml tube.
9. 200µl 95% Ethanol was added to lysate and mixed by pipetting 3-4 times.
10. Half of lysate/ethanol mixture was transferred to SV RNA columns.
11. Columns were centrifuged at 14,000g for 1 minute.
12. Steps 10 and 11 were repeated for second half of lysate and elute discarded.
13. 600µl of SV RNA wash solution was added to each column.
14. Columns were centrifuged at 14,000g for 1 minute and elute discarded.
15. DNAse mix was made up as the following master mix:

	1X	12x
0.009M $MnCl_2$	5µl	60µl
SV Yellow Core Bufer	40µl	480µl
SV DNase	5µl	60µl

and 50µl added to each column.

16. Columns were incubated at room temperature for 15 minutes.
17. 200µl of SV DNase stop solution was added to each column.
18. Columns were centrifuged at 14,000g for 1 minute.
19. 600µl SV RNA wash solution was added to each column.
20. Columns were centrifuged at 14,000g for 1 minute and elute discarded.
21. 250µl SV RNA wash solution was added to each column.
22. Columns were centrifuged at 14,000g for 2 minutes and collection tube discarded.
23. Cap was removed from the columns and the columns were placed in elution tubes.
24. 100µl nuclease water was added to each column and the columns were centrifuged for 1 minute at 14,000g.
25. 2µl of 5M NaCl and 250µl ice-cold 100% Ethanol was added to each elute.
26. Elute was incubated for 1 hour at -20°C.
27. RNA was collected by centrifugation at 14,000g for 30 minutes at 4°C.
28. Supernatant was carefully removed by pipetting.

29. Pellet was air-dried for 5 minutes and dissolved in 13.2µl Nuclease free water .

2.2.6 Preparation of samples for determination of mRNA knock-down by qRT-PCR

2.2.6.1 siRNA mediated knock-down

1. Passage 3 cells were passaged as described in 2.2.1 and resuspended in HeLa seed media.
2. Cells were diluted to $3x10^4$ $cellsml^{-1}$ in HeLa seed media and 0.5mls seed into enough wells of a 24 well cell culture cluster (Corning #3524) to allow one well per siRNA, plus one extra. Cell were allowed to adhere to the plate for 15 minutes and then incubated at 37°C, 5% CO_2 for 24 hours.
3. Cells were transfected with 12.5pmol of each siRNA plus 12.5pmol of siNeg control.
4. Cells were incubated at 37°C, 5% CO_2 for 24 hours.
5. Media was removed by aspiration and replaced with HeLa growth media and cells were grown for a further 24 hours.
6. Media was aspirated and total cellular RNA isolated as described above (2.2.5).

2.2.6.2 shRNA mediated knock-down

1. Passage 3 cells were passaged as described in 2.2.1 and resuspended in HeLa seed media.
2. Cells were diluted to $1.2x10^5$ in HeLa seed media and 0.5ml seeded into enough wells of a 24 well cell culture cluster to allow one well per shRNA encoding plasmid, plus one extra. Cells were allowed to adhere to the plate for 15 minutes and then incubated at 37°C, 5% CO_2 for 24 hours.
3. Cells were transfected with 200ng of each shRNA encoding plasmid plus pSM2.shControl and 200ng pIRES-P plasmid.
4. Cells were incubated at 37°C, 5% CO_2 for 24 hours.
5. Media was removed by aspiration and replaced with 0.5ml HeLa growth media plus 2µg/ml puromycin. Cells were incubated at 37°C, 5% CO_2 for a further 48 hours.
6. Media was removed by aspiration and total cellular RNA isolated as described above (2.2.5).

2.2.7 Screening Methods

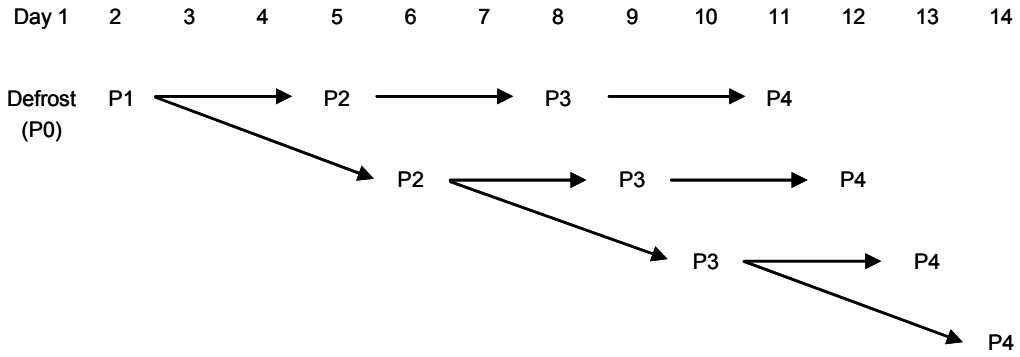

Figure 2.1 Scheme for growing cells for use in screening
Scheme ensures that cells, passaged four times since removal from liquid nitrogen storage, are available on four consecutive days. Cells are passaged every 3 or 4 days. Flasks passaged after four days are split into two flasks, with one flask passaged after 3 days and the other after 4 days. P - Passage

2.2.7.1 Seeding Cells for Library Transfection

1. Cells were trypsinized as described (2.2.1) and resuspended in 10ml Seed Media.
2. Cells were counted and sufficient cells for 10mls per plate diluted to $3x10^4$ cell/ml

$$cells(ml) = \frac{3 \times 10^4 \, cells/ml \times 10ml \times plates}{cell\_density}$$

3. A 1250µl electronic repeating multi-channel pipette (Matrix Impact 1250, Thermo Scientific) was used to add 100µl of cell suspension to the first 11 columns of each plate, and 6 of the 8 wells of the 12th column of a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947).
4. Plates were left on bench for 20-30 minutes.
5. Plates were placed in a polystyrene box containing sterile water and the lid sealed, with one corner unsealed (semi-sealed).

2.2.7.2 Transfection of Library siRNAs

Aliquots of original library plates were diluted to 1.25µM in siRNA suspension buffer and stored at -20°C. Sufficient control siRNAs for 100 wells were diluted to 1.25µM in siRNA and arrayed in a 96 well plate in layout used in screen (see Figure 4.1).

The following protocol is based on 12 plates. If more plates than this were processed in a day, the protocol was repeated.

1. Diluted library plates plus the plate contain controls were removed from freezer and defrosted on bench. .

2. Plates were centrifuged at 3,000 rpm for 5 minutes in a bench top centrifuge (Sorvall RT7).
3. 2µl of each siRNA (1.25µM) was transferred to a labelled V bottomed plate (VWR # 732-2702) excluding wells to contain controls. .
4. 2µl of control siRNAs added to 2 empty columns on each plate.
5. 13.75µl per plate Lipofectamine 2000 was diluted in 1.43ml per plate OptiMEM I , for first 6 plates and incubated for 15mins.
6. 13µl OptiMEM I was added to each well of the siRNA contain plates using 125µl electronic repeating pipette (Matrix Impact2 125, Thermo Scientific) .
7. Step 5 was repeated for second 6 plates.
8. Lipofectamine 2000/OptiMEM I mixture from step 5 was added to first six siRNA containing plates and the mixture was incubated for 15 minutes.
9. Plates containing cells were checked for contamination. .
10. Lipofectamine 2000/OptiMEM from step 7 was added to second six siRNA containing plates and mixture incubated for 15 minutes.
11. Lipofectamine 2000/OptiMEM I/siRNA mixture from step 8 was added to wells of cell containing plates and mixed by gentle pipetting.
12. Step 11 repeated for mixture from step 10.
13. Cells were returned to 37°C, 5% CO_2 incubator for 24 hours.
14. Media was removed using 8-channel aspirator and replaced with 100µl HeLa growth media using electronic repeating pipette.

2.2.7.3 Transfection of library DNA

Library was provided by the Chromosome 22 ORF team at the Wellcome Trust Sanger Insititute as six plates of DNA prepared using Qiagen QIAspin miniprep kits at a concentration of 50µg/ml. The following protocol is for three plates. If more plates were required, the protocol was repeated.

1. Construct containing plates were defrosted on bench.
2. Construct containing plates were spun down at 3000rpm for 5 minutes in a benchtop centrifuge (Sorvall RT7).
3. 1.6µl (80ng) of DNA was transferred from each well of the library plate to a fresh 'V-bottom' 96 well plate (VWR # 732-2702).
4. 26.4µl per plate siPort XP-1 (Ambion # AM4507) was mixed with 2.2ml per plate OptiMEM I and incubated for 10 minutes at room temperatue.

5. 20µl siPort XP-1/OptiMEM I mixture was added to each well of plasmid DNA. .
6. Complex/DNA was collected in the base of the well and mixed by gently tapping the plate and incubated for 10 minutes at room temperatue.
7. Complex/DNA was transferred to plates containing pre-plated cells and mixed by gentle pipetting.
8. Cells were returned to 37°C, 5% CO_2 incubator for 24 hours.
9. Media was removed using 8-channal aspirator and replaced with 100µl HeLa growth media using electronic repeating pipette. .

2.2.7.4 alamarBlue Assay

The protocol here is based on 12 plates. If more plates were to be assayed, the protocol was repeated as necessary. Sufficient assay media was made to assay each plate twice immediately prior to pre-treatment reading.

1. Media was aspirated from wells using 8-channel aspirator and replaced with 100µl alamarBlue assay media.
2. Plates were incubated at 37°C, 5% CO_2 for 3 hours.
3. Condensation was removed for inside of plate lid using a tissue immediately before reading.
4. Level of fluorescence was detected by plate reader with the following settings:.

   Excitation wavelength: 544nm.

   Emission wavelength: 590nm.

   Reads: 6.

   Temperature: 37°C.

2.3 Common molecular biology methods

2.3.1 Transformation of plasmid DNA into *E.coli*

1. 0.5µl of each DNA to be transformed was aliquoted into the well of a 96 well PCR plate, plus one well containing 0.5µl 1ng/µl pUC19 DNA as a positive control.
2. Plate was spun down briefly.
3. Plate was incubated at -80°C to allow DNA to freeze.
4. Competent cells were thawed for 5 minutes on a metal cold block (Stratagene).
5. Competent cells were mixed by gently flicking the tube.
6. Plate was placed on a metal cold block inside a benchtop cooler (StrataCooler) to

maintain plate at 4°C.

7. 10µl of cells was added to each well of the plate containing DNA.
8. Plate was incubated at 4°C for 20 minutes.
9. Cells were heat shocked at 42°C for 45 seconds in a thermocycler (MJ, PTC-225).
10. Cells were incubated for 2 minutes at 4°C.
11. 90µl of ice cold LB broth was added to each well.
12. Cells were incubated for 90 minutes at 37°C.
13. Cells were spread onto LB agar plates containing appropriate antibiotic to select for transformants. The pUC19 sample was spread on LB agar plates containing 100µg/ml ampicillin.
14. Plates were grown over night at 37°C, colonies counted and stored at 4°C until needed. .

2.3.2 Digestion with Restriction Enzymes

Protocol presented here is for a 100µl reaction. Reactions of different sizes were scaled directly from this protocol except for quantity of enzyme(s) which was always 1µl unless otherwise stated.

1. Reaction pre-mix sufficient for each reaction required, plus one extra was made up

Reagent	*1X*
10x Buffer	10µl
Creosol Red Loading Buffer	34µl
Bovine Serum Albumen	1µl
Enzyme 1	1µl
(optional) Enzyme 2	1µl
Double distilled water	.. upto (100-x)µl.

   Where x is the volume of DNA to be added.
2. DNA is added to pre-mix and incubated for 1-16 hours at 37°C.
3. Reactions were terminated by incubating reaction at 80°C for 15 minutes.

2.3.3 Agarose Gel electrophoresis

Strength and size of gel is specified in text.

1. Gel was prepared in 50mls (mini-gel) or 250mls (maxi-gel) 1XTBE containing 250ng/ml ethidium bromide and appropriate quantity of agarose (Invitrogen #15510027.
2. Samples were run in 30% Creosol Red loading buffer at either 80v for 20minutes (mini-gel) or 200v for 1 hour (maxi-gel).
3. Products were visualised using UV illumination and recorded using a digital gel

documentation system (UVP #GelDoc-IT).

2.3.4 KOD Polymerase Chain Reaction (KOD PCR)

Unless otherwise stated in text, PCR reactions carried out were done so with KOD Hot Start Polymerase (Novagen #71086-3) using the following protocol.

1. A PCR reaction premix sufficient for the number of reactions to be carried out plus one was made up

Reagent	1Xl
10x KOD Buffer	2.5µl
2mM dNTPs	2.5µl
25mM $MgSO_4$	1µl
Creosol Red loading buffer	8.3µl
Double distilled water	3.7µl
KOD polyermase (1 $U\mu l^{-1}$)	1µl
Forward Primer (15µM)*	0.5µl
Reverse Primer (15µM)*	0.5µl

* Primers were only included in master mix if all reactions required the same primers.

2. Mix was briefly vortexed and spun down.
3. 20µl of premix was added to wells of a 96 well PCR plate (ABgene #AB800).
4. 5µl template diluted in double distilled water was added to each well.
5. PCR was carried out using the following program on a 96 well thermocycler (MJ #PTC-225).
    - 95°C for 2 minutes.
    - 95°C for 20 seconds, 60°C for 30 seconds, 70°C for 1 minutes - 30 cycles.
    - 68°C for 5 minutes.
    - 10°C forever.

.

2.3.5 Quantitative Polymerase Chain Reaction (qPCR)

All reactions were carried out in triplicate. Pre-mixes were made up for the number of reactions required plus 10%. Reactions were performed using reagents from the SYBR green qPCR MasterMix Plus kit (Eurogentec # RT-QP2X-03-075+).

1. PCR pre-mix was diluted with double distilled water:

Reagent	*1X/µl*
2x qPCR MasterMix	12.5µl
Double distilled water	6.5µl
Primer Mix (7.5µM)*	2µl

* Primer mix was only added it only one primer was used.

2. 19µl (without primer) or 21µl (with primer) of pre-mix was added to 96 well optical reaction plate (ABI # N8010560) using a multichannel pipette.
3. If primers not already added 2µl primer was added to each well.
4. 4µl template was added to each well.
5. Plate sealed with an optical plate seal (ABI #4311971).
6. Plate spun at 3000 rpm for 3 minutes in a bench top centrifuge (Eppendorf 5403) and inspected to ensure no wells contained bubbles.
7. PCR reactions carried out on either an ABI7000 or ABI7900 sequence detection systems using the following program:.
    - 50°C for 2 minutes.
    - 95°C for 10 minutes.
    - 95°C for 15 seconds, 60°C for 1 minute, 40 cycles*.
    - Melt Curve: 95°C for 15 seconds, 60°C for 15 seconds, 95°C for 15 seconds, ramping at 2% of maximal speed*.

    * data was collected during these stages.

    .

.

2.3.6 Reverse Transcription

All reagents are defrosted in metal cold blocks cooled to 4°C. Work is carried out in an RNase free environment with RNase free solutions and pipettes. Unless otherwise stated reagents come from Superscript II Reverse Transcription Kit (Invitrogen # 18064-071).

1. 11µl of each RNA was aliquoted into a 96 well PCR plate (ABgene #AB800).
2. 1µl 12.5µg/µl oligo dT (Invitrogen #18418012) was added to each well.
3. Plate was headed to 70°C for 10 minutes and then rapidly cooled to 42°C in a 96 well thermocycler (MJ, PTC-225).
4. To each well the following was added

Reagent	*1X/µl*
5x First Strand Buffer	4µl
0.1 M DTT	2µl
10 mM dNTP mix (Invitrogen, #18427013)	1µl

5. Plate was heated to 42°C for 2 minutes.
6. 1µl Superscript II reverse transcriptase was added to each well.

7. Plate was incubated at 42°C for 50 minutes.
8. Enzyme was inactivated by heating at 70°C for 15 minutes.

2.3.7 Measuring efficiency of qRT-PCR oligonucleotide primers

1. cDNA was prepared from HeLa cells by extracting total cellular RNA from $5x10^6$ HeLa cells and Reverse Transcription of 5µg as described in 2.2.5 and 2.3.6.
2. KOD PCR was performed on 25ng HeLa cDNA as a template using the primer being tested. Products were analysed by agarose gel electrophoresis to ensure one and only one product of the correct size was present.
3. Dilutions of HeLa cDNA were prepared to allow 3µl of 25ng/µl, 5ng/µl, 1ng/µl and 0.2ng/µl cDNA per primer to be tested plus 3µl excess, by serial dilution.
4. Diluted HeLa cDNA was used as a template to perform SYBR green qPCR. Three reactions were run with each dilution of the template for each primer to be tested, plus one reaction for each primer using double distilled water as a template.
5. Melt-curves were examined to ensure the absence of primer-dimers.
6. Primer efficiencies were calculated using the qBase software.

2.3.8 Measurement of RNAi mediated mRNA knock-down by qRT-PCR

1. RNA samples were prepared as described above (2.2.6).
2. RNA samples were reverse transcribed into cDNA (2.3.6).
3. Success of reverse transcription was confirmed by KOD PCR with 1µl of each sample as template using primers designed to amplify a section of the ARSA gene and products analysed by agarose gel electrophoresis on a 2% gel.
4. Samples were diluted 1:10 with double distilled water, except negative controls which were diluted 1:20.
5. 4µl of each sample was used in qPCR for the following genes: the gene of interest, GAPDH and ACTB in triplicate as described in 2.3.5.
6. 4µl of negative control sample was used in qPCR for all genes of interest plus GAPDH and ACTB.
7. Where possible all reactions involving a gene were kept on the same plate.
8. Ct values were calculated using an automatic baseline and a threshold of 0.2 in the ABI SDS software.
9. Data was analysed using the qBase software (Hellemans et al. 2007): Replicates more

than 0.5 Ct from other replicates were excluded from analysis. GAPDH and ACTB were used as normalization standards and all results are presented relative to the negative sample. .

2.4 Computational methods

2.4.1 Design of oligonucleotide primers for qRT-PCR

Where possible oligonucleotide primer sequences were taken from RTPrimerDB (Pattyn et al. 2006) or existing publication as specified in Appendix B. .

1. Sequence and exon/introns boundaries for transcript was extracted from the Ensembl human database (www.ensembl.org, release 45 at time of writing).
2. Primers were designed using the Primer3 software (Rozen, Skaletsky 2000) using the default setting except:

Setting	*Value*
Max Tm difference	1
Max self complementarity	4.00
Product Size Ranges	50-100 150-250

3. Primers were designed so that either the amplicon included an introns of at least 5kb or one of the oligonucleotides contained sequence from two exons.
4. Specificity of primers was checked using the In-silico PCR tool at http://genome.brc.mcw.edu/cgi-bin/hgPcr.

2.4.2 Analysis of screening data

The files output from the plate reader are in the incorrect format to be processed by the R/Bioconductor package cellHTS. A Perl script was used to subtract the background fluorescence from each value and reformat the plate reader output file so that they could be read by cellHTS.

Screening data was analysed in the R/Bioconductor package cellHTS using scripts available in description file that is attached to the HTML reports for each screen, available in the appropriate appendix (on the included CD or on-line).

Briefly, each script:.

1. Loads plate data into a single cellHTS object x.
2. Loads plate layouts, screen description and log files.
3. Removes values of blank wells and siKIF11 wells.
4. (for Druggable genome and chromosome 22 screens only) Normalizes each plate in each channel to the plate median and removes wells in channel 1 which are below a

threshold.

5. Calculates survival percentages, and normalizes these survivals to the plate median and log transforms data (Druggable genome screen and Chromosome 22 screen).
6. Calculates scores for each "probe" by calculating z-scores and selecting the minimum of the two replicates.
7. Loads gene annotation information.
8. Outputs a HTML report.
9. Calculates the results summarised by gene.

Mean/Sd plots were generated using the R/Bioconductor package vsn's function meanSd.

2.4.3 Analysis of seed sequences

siRNA sequences for siRNAs in the library are provided as the sequences of the target, which is equivalent to the sense strand of the siRNA. Seed sequences are therefore bases 15-20 or 14-20 of this sequence.

2.4.3.1 Assessment of significance of 'hit' seeds

The hexamer and heptamer seed sequences of the siRNAs in the Qiagen Druggable genome V2 library were extracted and read into R. The sample and replicate functions were used repeatedly select 20 seed sequences at random from the list of siRNA seed sequences. The table function was used to count the appearances of each seed in each sample. P values were calculated by counting the number of samples in which x seeds occurred more than y times and dividing by the total number of samples. For calculations of the hexamer seeds 5,000 samples were used. For calculations of the heptamer seeds only 2,000 samples were used due to memory restraints. .

2.4.3.2 GSEA of ranked seeds

A ranked list of siRNA sequences was extracted from the screen results along with the score the siRNA obtained. The hexamer/heptamer for each siRNA was determined. siRNAs were placed into sets depending on the seed and formatted for the GSEA-P software using a Perl script.

See section 0 for details of GSEA. GSEA preranked analysis was performed using 1,000 repetitions, using the ranked list of siRNAs as the ranked list and the seed sequence sets for the gene set database. All other parameters were set to default. Seed sequences were said to

be significant if FWER < 0.05.

2.4.3.3 Assessing average frequency of seed matches in 3'UTRs

The set of human 3'UTRs from ensembl was retrieved using the BioMart data mining software. The average frequency of each possible 6nt, 7nt and 8nt sequence in each 3' UTR and also the frequency of matches in total 3' UTR sequence was calculated using the perl script, written by Dr. A. Enright using the following formulas:.

$$\overline{Frequency}_s = \frac{\sum_n^N \frac{count(s, utr_n)}{length(utr_n)}}{N}$$

$$Bulk\_Frequency_s = \frac{\sum_n^N count(s, utr_n)}{\sum_n^N length(utr_n)}$$

where s is some sequence, N is the number of UTRs in the set, and utr_n is the nth UTR in that set. count(s,utr_n) is the number of occurrences of s in utr_n and length(utr_n) is the length of utr_n in kilobases. count(s.utr_n) was implemented using a regular expression search, implemented with the code snippet:.

```
$matches = () =~ ($utrseq =~ /$seedseq/g) .
```

This script takes as its input a set of UTRs and a length donating the length of sequence to be searched for. It then generates all possible sequences of that length, and calculates their average frequency in the set of UTRs and also their bulk frequency as described above. It outputs a table of sequences follow by each of the different measures of frequency. This output was analysed and plotted in R.

2.4.3.4 Searching for possible transcripts with matches to hit and enriched seeds

A Perl script was implemented which takes a set of 3' UTR sequences and a list of seeds and outputs the number of seeds found in each of the 3' UTRs and the frequency with which matches are found:.

$$MatchFrequency_{S,utr} = \frac{\sum_{s \in S} count(s, utr)}{length(utr)}$$

where S is the set of seeds being examined, utr is some utr being examined and count and length are as described above.

A second Perl script takes a list of gene names, a list of seed sequences and a list of 3'UTRs and outputs a list of genes from gene list that contain a match for each of the seeds in the seed list. .

2.5 Chapter 3 methods

2.5.1 TRAIL cytotoxicity assays

Many of the parameters in this protocol were changed during the optimisation process. Presented here is a template protocol, the values of various parameter used is specified in the text when non-standard.

2.5.1.1Treated vs. Untreated assay

1. Passage 3 cells were passaged (see 2.2.1) and resuspended in HeLa Seed Media.
2. Cells were diluted to $3x10^4$ $cellsml^{-1}$ in HeLa Seed Media.
3. 100µl of cells were seeded into a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947).
4. Two wells were left empty for blanking measurements.
5. Plates were placed in a semi-sealed polystyrene box stood on petri-dishes of sterile water.
6. Cells were allowed to adhere to plate for 15 minutes at room temperature.
7. Cells were incubated at 37°C, 5% CO_2 for 24 hours.
8. (optional) Cells were transfected with amount of siRNA or plasmid DNA indicated (see 2.2.3 and 2.2.4 respectively).
9. Cells were incubated for 48 hours at 37°C for 24 hours.
10. Media was removed by pipetting with a multi-channel pipette and discarded into 1% virkon.
11. 100µl TRAIL treatment media containing appropriate concentration of TRAIL and serum was added half of the wells.
12. 100µl TRAIL treatment media containing no TRAIL and appropriate concentration of serum was added to the remaining wells.
13. Plates were incubated for 24 hours.
14. Media was removed by pipetting with a multi-channel pipette and discarded into 1% virkon.
15. 100µl alamarBlue assay media was added to each well.
16. Plates were incubated at 37°C, 5% CO_2 for 3 hours.

17. Plates were read in a Gemini SpectraMAX plate reader (Molecular Devices #XPS) using the following settings:.

    Temperature: 37°C.

    Excitation Wavelength: 544nm.

    Emission Wavelength: 590nm.

    Accuracy: 10 readings.

18. Average value of fluorescence in the blank wells was subtracted from the fluorescent reading in all other wells.

19. Survival for each condition was calculated: $survival = \frac{\bar{fl}_{treated}}{\bar{fl}_{untreated}} x100\%$ where $\bar{fl}_{treated}$ and $\bar{fl}_{untreated}$ are the average fluorescence for treated and untreated wells for a particular condition or transfected with a particular construct. .

2.5.1.2 Before vs. After assay

1. Passage 3 cells were passaged (see 2.2.1) and resuspended in HeLa Seed Media.
2. Cells were diluted to $3x10^4$ $cellsml^{-1}$ in HeLa Seed Media.
3. 100µl of cells were seeded into a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947).
4. Two wells were left empty for blanking measurements.
5. Plates were placed in a semi-sealed polystyrene box stood on petri-dishes of sterile water.
6. Cells were allowed to adhere to plate for 15 minutes at room temperature.
7. Cells were incubated at 37°C, 5% CO_2 for 24 hours.
8. (optional) Cells were transfected with amount of siRNA or plasmid DNA indicated (see 2.2.3 and 2.2.4 respectively).
9. Cells were incubated for 24 hours at 37°C for 24 hours.
10. If cells were transfected, media was removed by pipetting with a multi-channel pipette and discarded into 1% virkon and replaced with 100µl HeLa growth media.
11. Cells were incubated for 24 hours at 37°C.
12. Media was removed by pipetting with a multi-channel pipette and discarded into 1% virkon and replaced with 100µl alamarBlue assay media.
13. Plate was incubated for 3 hours.
14. Plates were read in a Gemini SpectraMAX plate reader (Molecular Devices #XPS)

using the following settings:.

Temperature: 37°C.

Excitation Wavelength: 544nm.

Emission Wavelength: 590nm.

Accuracy: 10 readings.

15. Media was removed by pipetting with a multi-channel pipette and replaced with 100µl TRAIL treatment media with appropriate concentration of TRAIL ligand.
16. Plates were incubated for 20 hours at 37°C, 5% CO_2.
17. Steps 10-12 repeated.
18. Average value of fluorescence in blank wells was subtracted from the fluorescence values in all other wells.
19. Survival of cells in each well was calculated: $survival = \frac{fl_{after}}{fl_{before}} x100\%$ where fl_{after} and fl_{before} are the flourescence readings for that well after and before treatment respectively. .

.

2.5.2 Assessment of plasmid transfection efficiency

1. Passage 3 cells were passaged as described in 2.2.1 and resuspended in HeLa seed media.
2. Cells were diluted to $5x10^4$ $cellsml^{-1}$ and 0.5ml seeded into each well of an 8-well tissue culture slide (BD Falcon #354118), allowed to adhere for 15 minutes and incubated at 37°C, 5% CO_2 for 24 hours.
3. Cells were transfected with the pEGFP-N1 plasmid using the indicated transfection reagent as described in 2.2.4.
4. Cells were grown for 48 hours.
5. Cells were fixed by washing once with PBS and then incubating for 15 minutes in 3.7% Paraformaldehyde:

370mg	Paraformaldehyde
400mg	Sucrose
10mls	PBS
A few drops	1M Sodium Chloride

6. Cells were washed three times in PBS and the well divider removed.
7. Slide was dried and coverslip mounted using Vectashield + DAPI (Vector Laboratories #H-1200).
8. Slides were visualised on a fluorescent light microscope (Zeis Axioplan 2 microscope fitted with a Hamamastsu ORCA-ER camara) and images of three random fields of view captured from each well using both FITC and DAPI filters using the SmartCapture X software (Digital Scientific). .
9. Number of green fluorescent cells (number of transfected cells) and number blue fluorescent DAPI stained nuclei (total number of cells) was counted in each field of view and used to calculate transfection efficiency.

For experiments involving selection, cells were transfected with 100ng pEGFP-N1 and 100ng pIRES-P vectors using siPort XP-1. Cells were grown for 24 hours and the media changed for HeLa growth media containing 2μg/ml puromycin. Cells were grown for a further 48 hours before fixation. .

2.5.3 Cloning of hairpins targeting Caspase 8

Sequences of hairpin oligonucleotides were designed using the shRNA retriever tool http://katahdin.cshl.org:9331/RNAi/html/rnai.html. Sequences of these oligonucleotide templates were:

shCasp8.1 TGCTGTTGACAGTGAGCG**AGGATACTGTCTGATCATCAAC**TAGTGAAG
CCACAGATGTA**GTTGATGATCAGACAGTATCCC**TGCCTACTGCCTCGGA

shCasp8.2 TGCTGTTGACAGTGAGCG**CTCTCGGACTCTCCAAGAGAAC**TAGTGAAG
CCACAGATGTA**GTTCTCTTGGAGAGTCCGAGAT**TGCCTACTGCCTCGGA

shCasp8.3 TGCTGTTGACAGTGAGCG**CTGCCTTGATGTTATTCCAGAG**TAGTGAAGC
CACAGATGTA**CTCTGGAATAACATCAAGGCAT**TGCCTACTGCCTCGGA

Sequence of mature siRNA sense strand shown in blue, antisense in bold.

1. 97bp hairpin oligonucleotide templates were amplified in a KOD PCR reaction using the primers 5'mirR30EcoRIA and 3'miR30XhoI:

5'mirR30EcoRIA CAGAAGGCTCGAGAAGGTATATTGCTGTTCACAGTGAGCG
3'miR30XhoI CTAAAGTAGCCCCTTGAATTCCGAGGCAGTAGGCA

Using the following reaction pre-mix:

Reagent	*1X*
10x KOD Buffer	10µl
DMSO	5µl
Betane	5µl
2mM dNTPs	10ul
5' primer	1µl
3' primer	1µl
Template (100ng/µl)	1µl
25mM $MgSO_4$	4µl
Double distilled water	70µl
KOD polymerase (1 $U\mu l^{-1}$)	2µl

Each reaction was set up in a 96 well PCR plate (ABgene #AB800).

2. PCR reactions were carried out on a 96-well thermocycler (MJ, PTC-225) using the following program:
    - 94°C for 1 minutes, 54°C for 30 seconds, 75°C for 1 minutes – 1 cycle.
    - 94°C for 30 seconds, 54°C for 30 seconds, 75°C for 1 minutes – 23 cycles.
    - 94°C for 30 seconds, 54°C for 30 seconds, 75°C for 11 minutes – 1 cycle.
    - 4°C forever.
3. PCR products were purified by extracting once in 100µl of phenol: chloroform:isoamyl alcohol (25:24:1) and once in 100µl of chloroform:isoamyl alcohol (24:1).
4. Products were precipitated by addition of 250µl of 96% ethanol and 10µl 3M Sodium Acetate and incubation at -20°C for 30 minutes. Pellets were collected by centrifugation at 16,000 x g for 20 minutes and washed in 70% ethanol and centrifuged at 16,000 x g for 20 minutes and resuspended in 20µl 0.1M TrisCl.
5. An A-tailing reaction pre-mix was made up sufficient for A-tailing reactions:

Reagent	1X
NEB Buffer	1µl
1mM dATP	2µl
Taq Polymerase	1µl
Double Distilled Water	4µl

6. 3µl (approximately 1.5µg) of each PCR product was added to 7µl A-tail pre-mix and incubated at 70°C for 30 minutes.
7. Ligation pre-mix sufficient to ligate each product in the pGEM-T easy vector was made up using reagents from the Roche Rapid Ligase Kit (Roche #11 635 379 001):

Reagent	*1Xl*
5x Dilution Buffer	2µl
Double distilled water	5.5µl
pGEM-Teasy vector (Promega #A1360)	0.5µl
2x Ligation Buffer	10µl
T4 DNA Ligase	1µl

8. 1µl of each A-tailed product was added to 19µl to ligation pre-mix and incubated for 10 minutes at room temperature.
9. 0.5µl of each ligation product was transformed into JM109 competent cells (Promega #L2001) and transformants plated on X-gal/AMP/LB agar plates:

   1 litre LB agar at 52°C
   160µl 500mg/ml X-gal (Fisher # BPE1615-100) in demethylformide
   2ml 25mg/ml Ampicillin
   5ml 0.1mM Isopropyl-β-d-Thiogalactopyranoside (IPTG)

10. 12 white colonies from each transformation were picked into 1.8ml of LB broth + 50µg/ml ampicillin and incubated overnight at 37°C in a 2ml 96 well plate (costar #3961).
11. 240µl 50% glycerol was added to each well. Plate stored at -70°C.
12. 100µl of each clone sent for sequencing by the in-house sequencing service with the primers:

    Forward: GTAAAACGACGGCCAGT

    Reverse: GGAAACAGCTATGACCATG.

13. Sequences were check for expected insert using the cross_match algorithm.
14. One clone for each hairpin was cultured overnight in 10mls of LB broth plus 50µg/ml ampicillin. Each culture was split in two and plasmid DNA prepared using the Qiaquick Spin Miniprep kit (Qiagen, #27104) as per manufacturer's instructions. Each plasmid was eluted in 50µl EB buffer supplied with kit.
15. 10µg Plasmid DNA was digested for 1 hour using XhoI (New England Biolabs # R0146S) and EcoRI (New England Biolabs #R0101L) restriction enzymes in 50µl 1xEcoRI Buffer (New England Biolabs #B0101S).
16. Restriction products were size separated using agarose gel electrophoresis on a 2% agarose gel. The fragment of the correct size (121bp) was excised and purified using the Qiagen QIAquick gel extraction kit (Qiagen, #28704) as per manufacturer's instructions.
17. Vector was prepared by digesting 3.5µg of pSM2 vector for 2 hours with EcoRI and XhoI restriction enzymes in 200µl of EcoRI buffer.
18. Digested vector was precipitated in 2 volumes of 96% ethanol and 0.1 volumes of 3M Sodium Acetate at 4°C for 30 minutes. Pellet was collected by centrifugation at 16,000 x g for 30 minutes. Pellet was washed in 70% ethanol and resuspended in 60µl 0.1M Tris-EDTA.
19. Digested vector was size selected by agarose gel electrophoresis on 1% gel, and the

band of the correct size (approx 7kb) was excised and purified using the Qiagen QIAquick gel extraction kit as per manufacturer's instructions.

20. Hairpin inserts were ligated into prepared pSM2 vector using the following mix:

Reagent	*1X*
5x Dilution Buffer	2µl
pSM2 vector	2µl
Insert	2µl
Double distilled water	3µl
2x Ligation Buffer	10µl
T4 DNA Ligase	1µl

21. Ligation reactions were incubated for 10 minutes at room temperatue.
22. 0.5µl of ligation product was transformed into PIR1 chemically competent cells (Invitrogen #C1010). Transformants were plated on LB agar plates containing 50µg/ml chloramphenicol and 25µg/ml kanamycin and incubated over night at 37°C.
23. 8 colonies from each transformation were picked into 1.8ml 2XLB (low salt) plus 50µg/ml chloramphenicol and 25µg/ml kanamycin in a 2ml 96 well plate and cultured overnight at 37°C.
24. 240µl of 50% glycerol was added to each well and culture stored at -70°C.
25. 100µl of each culture was sequenced by the in-house sequencing service using the primer: GTAACTTGAAAGTATTTCG.
26. Sequences were compared to expected insert sequences using the cross_match algorithm. .

2.5.4 Comparison of effect of siRNA and shRNA mediated knock-down of Caspase – 8

For siRNAs, 12 wells of HeLa cells were transfected with 2.5pmol siCasp8 and 12 with 2.5pmol siNeg and their sensitivity to TRAIL measured as described in 2.5.1.2. Assays for the effect of shRNAs were carried out on the same plate by transfecting 12 wells with 80ng pSM2.shCasp8.2 and 12 with 80ng pSM2.shControl and measuring their sensitivity to TRAIL as described in 2.5.1.2. Assays were carried out using 0µg/ml, 0.25µg/ml, 0.5µg/ml and 1µg/ml in triplicate. This procedure was repeated using 3 separate aliquots of cells defrosted and grown independently. .

The test for the effect of selection was carried out by transfecting 12 wells with 40ng pSM2.shCasp8.2 and 40ng pIRES-P and 12 wells with 40ng pSM2.shControl and 40ng pIRES-P and measuring their sensitivity to TRAIL as described in 2.5.1.2 except that 24 hours after transfection media was removed and replaced with 100µl HeLa growth media

plus 2µg/ml puromycin and grown for a further 48 hours before assaying for theeffect of TRAIL.

2.5.5 Blind pseudo-screens

All DNA used was prepared using the Qiagen HiSpeed Maxi prep kit (Qiagen #12663) as per manufacturers instructions. Sequences of siRNAs can be found in Appendix A. For siRNAs, siRNAs were arrayed in a 96 well plate by experimenter 1 such that 5-15 wells contained 2.5pmol of siCasp8 and the rest 2.5pmol of siNeg. Plate was passed to experimenter 2 who transfected siRNAs into cells and measured their sensitivity to TRAIL as described in 2.5.1.2, using 1µg/ml TRAIL. Data was processed by dividing survival value in all wells by median survival of all wells on plate and standardised by calculating a score, z, thus:.

$$z = \frac{x - \mu}{\sigma}$$

.

shRNA experiment was carried out identically except cells were transfected with pSM2.shCasp8.2 or shControl. .

Experiment using selection was carried out as described for shRNAs except cells were seeded at 12,000 cells per well, media was removed 24 hours after selection and replaced with 100µl HeLa growth media plus 2µg/ml puromycin and cells were incubated for a further 48 hours at 37°C, 5% CO_2 before the effect of TRAIL was measured.

2.5.6 Comparison of siRNA mediated and shRNA mediated knock-down of 18 genes associated with the TRAIL pathway

Reqseq nucleotide IDs specified by Aza-blanc *et al* (Aza-Blanc et al. 2003) were used to retrieve mRNA sequences. Sequences were used to search a database of the hairpin sequences contained in the Expression Arrest shRNA library (v1.3) using the BLAST algorithm, via an in house tool for automating BLAST searching (written by Dr. D Beare). Plasmid DNA from each clone was prepared using the Millipore Montage 96 miniprep kit (Millipore #LSKP 096 04) as per manufacturer's instructions. 40ng of pSM2 plasmid DNA for each clone and 40ng pIRES-P was transfected into cells at a density of 12,000 cells per well in triplicate and the sensitivity of cells to TRAIL assayed as described in 2.5.1.2 except media was removed 24 hours after transfection and replaced with HeLa growth media containing 2µg/ml puromycin. Cells were incubated at 37°C, 5% CO_2 for a further 48 hours

before sensitivity to TRAIL was assessed. .

2-4 siRNAs targeting each transcript were ordered from a variety of suppliers. Details of both sequence and source of siRNAs can be found in Appendix A. siRNAs were tested in batches on separate plates with each plate containing siCasp8 and siNeg positive and negative controls in triplicate as described in 2.5.1.2. The siRNAs for each transcript which had the largest effect on TRAIL-induced cytotoxicity were retested on a single plate in triplicate with siCasp8 and siNeg controls as described in 2.5.1.2. .

Statistics were performed by log transforming survival values and performing a one tailed, heteroscedastic t-test between values for each siRNA/shRNA and the siNeg negative control. P-values were adjusted by multiplying by the total number of siRNAs/shRNAs tested.

2.5.7 qRT-PCR of positive control knock-downs

shRNA encoding plasmids and siRNAs targeting TRAIL pathway genes were selected and prepared as described in 2.5.6. Oligonucleotide primers were designed and tested and efficiencies calculated as described in 2.3.7. Primers with more than one product, primer dimers or efficiency below 80% or above 110% were re-designed and re-tested up to three times. Knock-down by each siRNA/shRNA encoding plasmid was measured as described in 2.3.8.

2.6 Chapter 4 and 5 methods

2.6.1 siRNA library screens

For siRNA screening, HeLa cells were grown according to the scheme laid out in Figure 2.1, allowing for cells passaged four times to be available on consecutive days. On the first day cells were seeded for the required number of plates, usually 24 (2.2.7.1). On the second day Library siRNAs were transfected into the plates seeded the day before (2.2.7.2). On the third day media was removed from the first set of plates and replaced with fresh HeLa growth media. On day four the first set of plates were assayed for the viability of cells (2.2.7.4). Plates where siKIF11 did not significantly reduce the viability of cells were discarded. Media was removed and replaced with TRAIL treatment media. On the fifth day viability was reassessed. Sets of plates were overlapped such that day one for the second set of plates was day two for the first set of plates and day one for the third set of plates was day two for the second etc.

For the kinase and phosphatase screen plates 78a through 89b of the Qiagen Druggable Genome siRNA library v2 were transfected into cells in batches of six (replicate 1), 12 (replicate 2) or 24 (replicate 3).

For the Druggable genome screen plates 1a through 77b of the Qiagen Druggable genome library were transfected into HeLa cells in batches of 24. Data was processed (2.4.2) and dynamic ranges for plates calculated. Plates with a dynamic range of less than 2 were repeated.

2.6.2 Reconfirmation of siRNAs from Kinase and Phosphatase screen

siRNAs for confirmation experiments were ordered as pre-arrayed FlexiPlate siRNAs (Qiagen). siRNAs were resuspended in RNase Free Water (Qiagen, included with siRNAs) to a final concentration of 1.25µM. siRNAs targeting the first eight genes, plus siCasp8, siNeg and siKIF11 were transfected in quadruplicate on one plate and siRNAs targeting the remaining genes (plus siCasp8, siNeg and siKIF11) were transfected in a second 96 well plate and assayed for sensitivity of 0.5µg TRAIL as described in 2.5.1.2.

Statistics were calculated by log transforming survival values for each well and performing a one-tailed, heteroscedastic student's t-test for each siRNA compared with the values for siNeg on the same plate.

2.6.3 qRT-PCR of screen hit siRNAs

Primers were designed to amplify from selected hit transcripts as described in 2.4.1. Primers were tested for specificity and efficiency as described in 2.3.7. Primers that amplified a single band and had an efficiency of between 80% and 110% were used for quantification. Failed primers were not redesigned. Knock-down mediated by hit siRNAs was calculated as described in 2.3.8, except that since measurement of GAPDH and ACTB was split across several qPCR plates, three wells containing reactions with HeLa RNA as a template and either ACTB or GAPDH were included as inter-run controls to allow normalisation of plate differences. .

2.6.4 Luminescent Caspase assays

Protocol described below describes that used for measurement of effect of confirmed hits from kinase and phosphotase screen and effect of over-expression screen hits on Caspases 8,9,3 and 7 and protocol used for measurement of confirmed hits from Druggable gene screen on TRAIL dependent Caspase 8 and Caspase 9 activation. For measurement of effect

of knock-down of hits from druggable genome screen, only three wells per siRNA were transfected and all were treated with TRAIL in step 10.

1. Passage 3 cells were passaged as described in 2.2.1 and resuspended in HeLa Seed media.
2. Cells were diluted to 3 x 10^4 cellsml^{-1} in HeLa Seed media.
3. 100µl of cells was added to wells of a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947).
4. Plates left on bench to allow cells to adhere to plate for 20 minutes.
5. Plates were placed in a semi-sealed polystyrene box stood on petri-dishes of sterile water.
6. Cells were incubated at 37°C, 5% CO_2 for 24 hours.
7. Cells were transfected with 2.5pmol of siRNA as described in 2.2.3 or 80ng plasmid DNA as described in 2.2.4. Each construct was transfected in to six wells.
8. Cells were incubated at 37°C, 5% CO_2 for 24 hours.
9. Media was removed and replaced with 100µl HeLa growth media and incubated for at 37°C, 5% for a further 24 hours.
10. Media was removed. TRAIL treatment media containing 0.5µg/ml TRAIL was added to three of the six wells for each siRNA. TRAIL treatment media containing no TRAIL was added to the remaining three.
11. Cells were incubated for 6 hours at 37°C, 5% CO_2.
12. Caspase-Glo 8 Substrate (Promega # G8200), Caspase-Glo 9 Substrate (Promega #G8210) or Caspase-Glo 3/7 Substrate (Promega, #G8090) was resuspended in Caspase-Glo 8, Caspase-Glo 9 or Caspase-Glo 3/7 buffer (all Promega, catalogue numbers as before).
13. (Caspase-Glo 8 and Caspase 9-Glo only) 7.5µl MG-132 Protease Inhibitor added to substrate.
14. 100µl assay reagent added to each well of plate.
15. Plate sealed and mixed on plate-mixer at lowest speed setting for 2 minutes.
16. Plates incubated for 1 hour.
17. Tape seal removed and luminescence read on Berthold LB96V luminometer with a 10 second integration time.

2.6.5 Measurement of sensitivity of cells to inducers of apoptosis

1. Passage 3 cells were passaged (see 2.2.1) and resuspended in HeLa Seed Media.

2. Cells were diluted to $3x10^4$ $cellsml^{-1}$ in HeLa Seed Media.
3. 100µl of cells were seeded into a 96 well OptiLux white walled tissue culture plate (BD Bioscience #353947). Two wells were left empty for blanking measurements.
4. Plates were placed in a semi-sealed polystyrene box stood on petri-dishes of sterile water.
5. Cells were allowed to adhere to plate for 15 minutes at room temperature.
6. Cells were incubated at 37°C, 5% CO_2 for 24 hours.
7. Cells were transfected with 2.5pmol of siRNA or 80ng plasmid DNA (see 2.2.3 and 2.2.4 respectively). Each siRNA or DNA was transfected into an entire row of cells.
8. Cells were incubated for 24 hours at 37°C for 24 hours.
9. Media was removed by aspiration with a multi-channel aspirator and replaced with 100µl HeLa growth media.
10. Cells were incubated for 24 hours at 37°C.
11. Media was aspirated from cells and replaced with 100µl of alamarBlue assay media.
12. Plates were read in a Gemini SpectraMAX plate reader (Molecular Devices #XPS) using the following settings:.

    Temperature: 37°C.

    Excitation Wavelength: 544nm.

    Emission Wavelength: 590nm.

    Accuracy: 10 readings.

2.6.5.1 TRAIL, FAS and H_2O_2

13. Treatment media was prepared.
    a. TRAIL media was prepared by diluting 5µg TRAIL in 5ml treatment media per plate to give 1µg/ml TRAIL and serially diluting to give required concentrations.
    b. FAS media was prepared by diluting 500ng FAS-ligand Plus™ (Calbiochem, # PF092) in 5ml treatment media per plate to give 100ng/ml FAS and serially diluting to give required concentrations.
    c. H_2O_2 media was prepared by adding 1.13µl 0.3% H_2O_2 solution to 5ml treatment media to give 200nM H_2O_2 and serially diluted to give required concentrations.
14. Media was removed from plate and treatment media added.
15. Plates were incubated at 37°C, 5% CO_2 for 24 hours.

16. Steps 11 and 12 were repeated.
17. Average value of fluorescence in blank wells was substracted from the fluorescence values in all other wells.
18. Survival of cells in each well was calculated: $survival = \frac{fl_{after}}{fl_{before}} x100\%$ where fl_{after} and fl_{before} are the flourescence readings for that well after and before treatment respectively. .

2.6.5.2 UV

13. Media was aspirated and replaced with 30µl warm PBS.
14. Plate was placed in a Stratalinker UV cross-linker (Stratagene #400075) and lid removed. Columns 10-12 were covered with card.
15. Cells were exposed to 50 Jm^{-2} UV.
16. Card was moved to cover columns 7-12 and cells were exposed to 50 Jm^{-2} UV.
17. Card was moved to cover columns 4-12 and cells were exposed to 100 Jm^{-2} UV.
18. PBS was removed by aspiration and replaced with treatment media.
19. Plates were incubated for 24 hours at 37°C, 5% CO_2.
20. Steps 10 and 11 were repeated.
19. Average value of fluorescence in blank wells was subtracted from the fluorescence values in all other wells.
21. Survival of cells in each well was calculated: $survival = \frac{fl_{after}}{fl_{before}} x100\%$ where fl_{after} and fl_{before} are the flourescence readings for that well after and before treatment respectively.

2.6.6 Re-screen of candidate hits from the druggable genome screen.

Aliquotes of the 40 siRNAs targeting the genes targeted by the top 20 scoring siRNAs were taken from the original library plates. These siRNAs were transfected in triplicate into HeLa cells on several 96 well plates and tested for their sensitivity to 0.5µg/ml TRAIL as described in 2.5.1.2. Each plate also contained three wells transfected with siCasp8, siNeg and siKIF11 respectively.

Statistics were calculated by log-transforming data and then performing a one-tailed, heteroscedastic student's t-test and adjusting the resulting p values by applying Hommel's correction for multiple testing using the p.adjust() function in R. .

2.7 Chapter 6 methods

2.7.1 Chromosome 22 ORF screen

The library was provided in 6 plates containing plasmid DNA prepared using Qiagen QIAspin minipreps and diluted to 50µg/ml. Plates were processed in batches of three plates. HeLa cells, passaged 4 times after removal from liquid nitrogen storage, were seeded into assay plates as described in 2.2.7.1. Cells were transfected with 80ng plasmid DNA as described in 2.2.7.3. 48 hours after transfected cells were assayed for viability using the alamarBlue assay (2.2.7.4). Media was removed and replaced with treatment media containing 0.5µg/ml TRAIL. After 24 hours the cells were assayed for viability using the alamarBlue assay (2.2.7.4). Each plate was transfected in two separate replicates.

2.7.2 Creation of the pcDNA3.GW.NoTag vector

The starting point for creating this vector was pcDNA3.GW.V5N adapted from the pcDNA3 (Invitrogen #V790-20) vector by Dr. J. Collins. Since no unique restriction sites exist that will remove tag without also removing the Gateway Cassette, the Gateway cassette was removed first by EcoRV digestion and religation. The V5N tag could then be removed using HindIII and EcoRV double digestion and the Gateway cassette re-inserted. .

1. 2.5µg of pcDNA3.GW.V5N vector digested with 2µl EcoRV in 100µl Buffer 2 for 2 hours at 37°C .
2. Vector religated for 5 minutes at room temperature using the following ligation mix:

Reagent	*1X*
Digested vector DNA	1µl
5x Roche Dilution buffer*	2µl
Double distilled water	7µl
2x Roche Ligation buffer*	10µl
Roche T4 ligase*	1µl

* From Roche rapid ligation kit (Roche #11 635 379 001).

3. Ligation product was transformed into ccdB sensitive MACH1 chemically competent cells (Invitrogen #C8620-03) and the transformants plated on gentomycin/LB agar plates.
4. Plates were incubated at 37°C for 16 hours.
5. 4 colonies were picked and cultured in 1ml LB/Gentomycin for 8 hours.

6. 6µl of each starter culture was added to 3mls of LB/Gentomycin and cultured at 37°C for 16 hours.
7. Plasmid DNA was prepared from cultures using Qiagen QIAprep spin mini kit (Qiagen #27104) according to manufacturer's instructions.
8. 5µg of plasmid was digested with 5µl HindIII and 5µl EcoRV in 100µl 1x NEB Buffer 2 for 1 hour at 37°C.
9. Reaction product was cleaned up using Qiagen QIAquick PCR clean-up kit (Qiagen #28104) according to manufacturer's instructions.
10. HindIII sticky ends were blunted using T4 polymerase using the following reaction mix assembled on ice:

Reagent	*1X*
Vector DNA	30µl
NEB Buffer 2	10µl
BSA	0.5µl
2mM dNTPs	5µl
Double distilled water	52.5µl
T4 polymerase (NEB #M0203S)	2µl

11. Reaction was incubated for 15 minutes at 12°C and stopped by adding 10µl 100mM EDTA and heating to 75°C for 20 minutes.
12. Reaction was product purified using QIAquick PCR cleanup kit according to manufacturer's instructions.
13. Product was dephosphorylated by adding 2.5µl Antarctic Phosphatase Buffer and 1.5µl Antarctic phosphatase and incubating for 30 minutes at 37°C.
14. Gateway cassette was ligated into the vector using the following ligation mix:

Reagent	*1X*
Vector DNA	1µl
Gateway C.1 cassette*	2µl
5x Dilution Buffer^	2µl
Double Distilled Water	6µl
2X Ligation Buffer^	10µl
T4 ligase^	1µl

* from Gateway vector conversion kit (Invitrogen #11828029).

^ from Roche Rapid Ligation kit.

15. Reaction was incubated at room temperature for 5 minutes.
16. 0.5µl Ligation product was transformed into ccdB resistant DB3.1 chemically competent cells (Invitrogen #11782-018) and transformants plated on chloramphenicol/LB agar plates.
17. Plates were Incubated at 37°C for 16 hours.
18. Ten were colonies picked and cultured in 3mls 25µg/ml chloramphenicol /LB for 16

hours at 37°C.

19. Plasmid DNA was prepared using QIAspin mini prep kit as per manufacturer's instructions.
20. Orientation of Gateway cassette was checked by digesting 1µl plasmid DNA with 1µl EcoRV and 1µl EcoRI in 10µl 1x NEB EcoRI buffer.
21. Products were run on a 1% maxi-gel. If gateway cassette is in correct orientation digestion results in a 1259bp band, if in the wrong orientation results in a 412bp band. 4 clones had cassette in correct orientation.
22. Absence of V5N tag was confirmed by digesting 1µl of plasmid DNA with 1µl EcoRV and 1µl HindIII in 10µl 1x NEB buffer 2. .
23. Products run on a 1% maxi-gel. Presence of V5N tag would have shown as a 1700bp band. No clones contained V5N tag.

2.7.3 Gateway LR recombination reactions

Gateway LR recombination reactions move an insert from a pENTR entry clone into a pDEST destination clone. pENTR vectors and pDEST vectors contain different antibiotic resistance markers allowing for the selection of one or the other in transformation. Un-recombined pDEST clones carry the gateway cassette which contains the ccdB gene which is toxic to most bacterial cell lines, allowing for selection against un-recombined vectors.

1. pENTR DNA was diluted to 7.4µl with double distilled water.
2. pENTR DNA was linearised with 1µl PvuI enzyme in 10µl 1x NEB buffer 3 for 1 hour.
3. LR reactions set up for each of the reactions plus negative and positive (pENTR-gus*) controls from master-mix:

Reagent	*1x*	*7x*
pcDNA3.GW.NoTag	0.36µl	2.55µl
100mM Tris EDTA pH 7.4	6.64µl	46.48µl
LR Clonase II*	1µl	7µl
pENTR clone	2µl	-

   * from the LR Clonase II kit (Invitrogen #11791020).
4. Reactions were incubated at 25°C for 24 hours and terminated by adding 1µl proteinase K and incubated for 20 minutes at 37°C.
5. 0.5µl of each reaction was transformed into MACH1 cells and transformants plated on gentomycin/LB plates.
6. Plates was incubated for 16 hours at 37°C.
7. Colonies were picked and cultured in 150mls gentomycin/LB for 16 hours at 37°C.

8. Plasmid DNA prepared using Qiagen HiSpeed Maxi kit (Qiagen #12663) according to manufacturer's instructions.
9. Inserts were end sequenced by the in house sequencing service using the primers:
   Forward: AATGTCGTAACAACTCCGCC
   Reverse: GCGATGCAATTTCCTCATTT.
10. Sequences were checked against expected ORF end sequences using the cross_match algorithm.

2.7.4 qPCR of ORF expression

Primers were designed to amplify sequences from the ORFs using the primers 3 software. The efficiency of the primers was determined as described in 2.3.7, except cDNA template was supplemented with 1µl of each pENTR clone before serial dilution. .

Cells were seeded and transfected as described in 2.2.6.2 except that cells were transfected with 400ng of pcDNA3.ORF.T7N, pcDNA3.ORF.T7C or pcDNA3.ORF.NoTag for each ORF except for MTMR3 where pcDNA3.ORF.T7N was unavailable, or pcDNA3.T7 as a negative control. RNA was prepared from each sample as described in 2.2.5. RNA isolated from each sample was divided in two. One half was used in a reverse transcription reaction (2.3.6,RT+ sample) while the other half was used in a mock reverse transcription reaction that contained no reverse transcriptase enzyme (RT- sample). The expression of the ORFs in the RT+ sample was determined as described in 2.3.8. In addition 4µl of each RT- sample was used as a template for qPCR using primers for the gene of interest in triplicate (2.3.5). Expression levels were calculated using qBase (Hellemans et al. 2007). Repeats whose Ct value differed more than 0.5 from the other two repeats were excluded. Expression levels were standardised using ACTB and GAPDH levels in RT+ sample, and results were normalized to levels in pcDNA3.T7 sample.