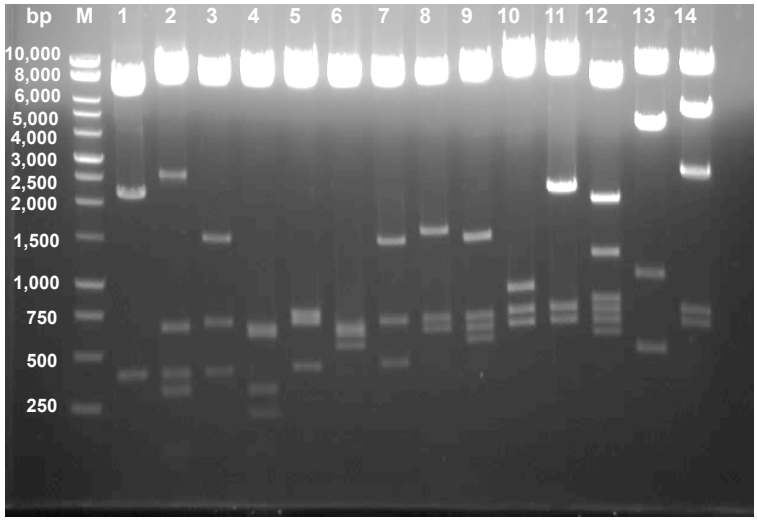
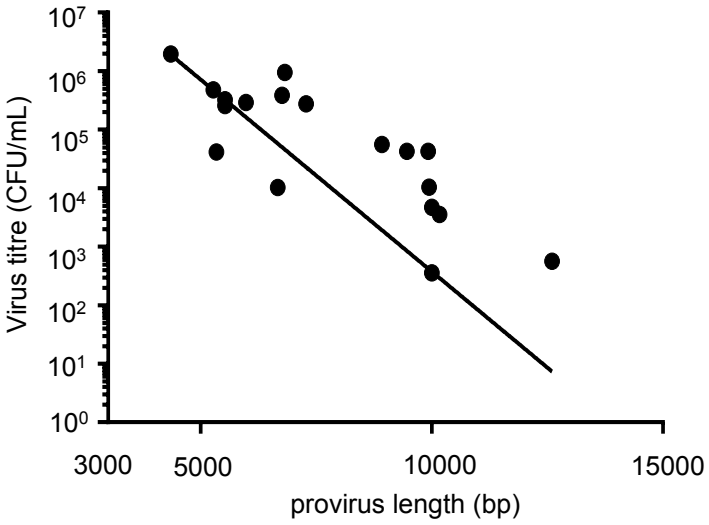


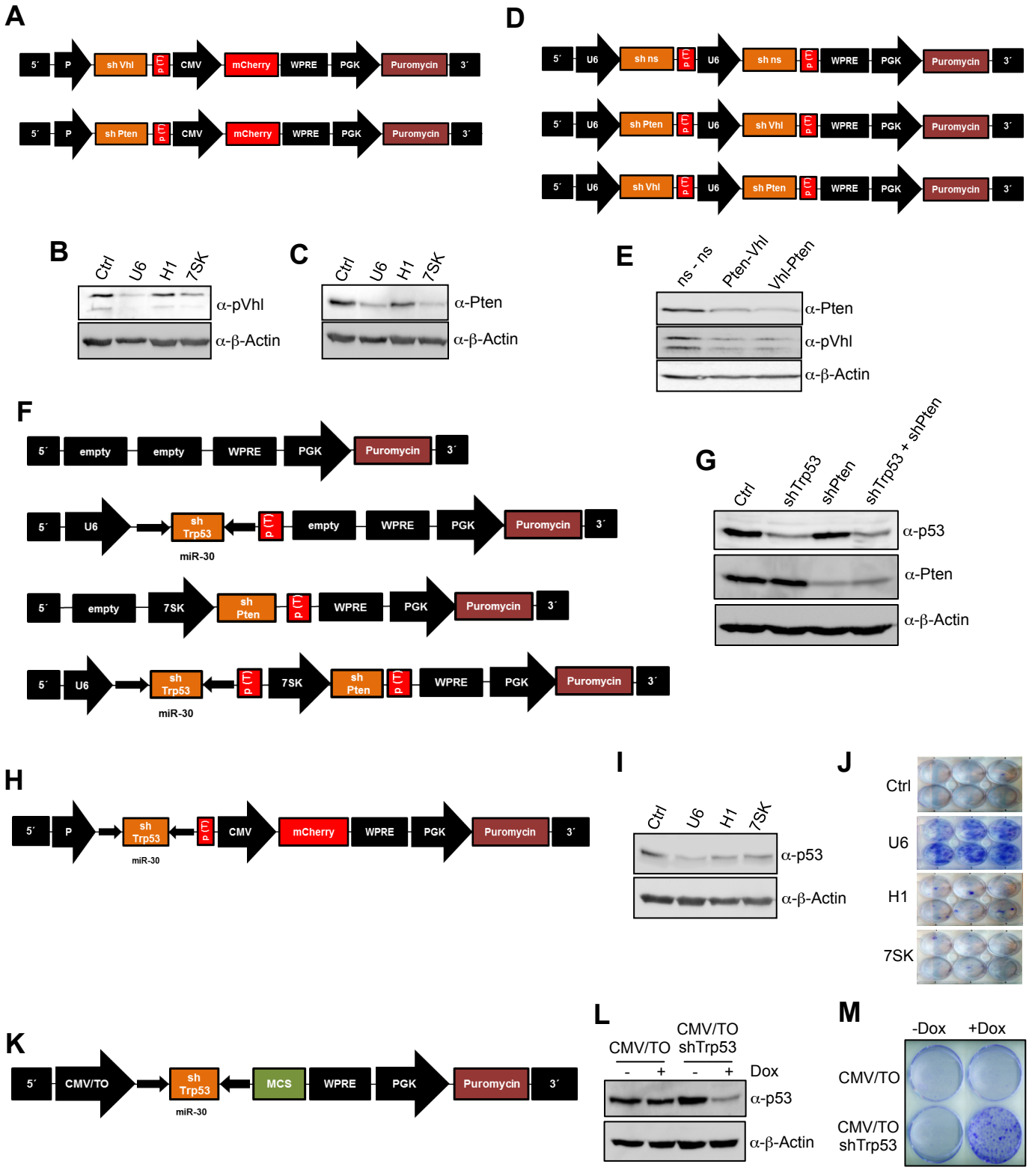
1. mCherry/ns shRNA/Puro Dest) - 6670, 2165, 389
2. shCdkn2a/empty/Luc Dest) - 7763, 2536, 684, 389, 276
3. shCdkn2a/H-Ras^{G12V}/Luc Dest – 7763, 1436, 686, 416
4. miR30 Trp53/ns shRNA/Luc Dest - 7763, 655, 594, 354, 199
5. shPTEN/CMV empty/Luc Dest – 7763, 759, 701, 416
6. miR30 Trp53/shPTEN/Luc Dest – 7763, 655, 532, 514
7. shPTEN/H-Ras^{G12V}/Luc Dest – 7763, 1436, 686, 416
8. miR30 Trp53/H-Ras^{G12V}/Luc Dest – 7763, 1565, 686, 514
9. miR30 Trp53/shPTEN//H-Ras^{G12V}/Luc Dest – 7763, 1436, 686, 514, 435
10. miR30 Trp53/empty/Luc Dest – 7763, 888, 701, 514
11. miR30 Trp53/c-Myc/Luc Dest - 7763, 2183, 701, 514
12. miR30 Rb/shPTEN/CreERT2/H-Ras^{G12V}/Puro Dest - 6670, 2005, 1004, 762, 679, 532, 514
13. sgTrp53/sgNF-1/hCas9/Luc Dest - 7763, 4227, 1310, 984, 450,
14. miR30 Trp53/hMED12/Luc Dest – 7763, 4784, 2621, 701, 514

Supplemental Figure 1: Absence of recombination of complex MuLE vectors in bacteria. EcoRI digestions of midi-prep DNA of 14 different complex MuLE vectors, lanes 1-14. Listed below the gel is a description of each vector and the predicted size of each EcoRI restriction fragment in base pairs. M: BenchTop 1kb DNA ladder.



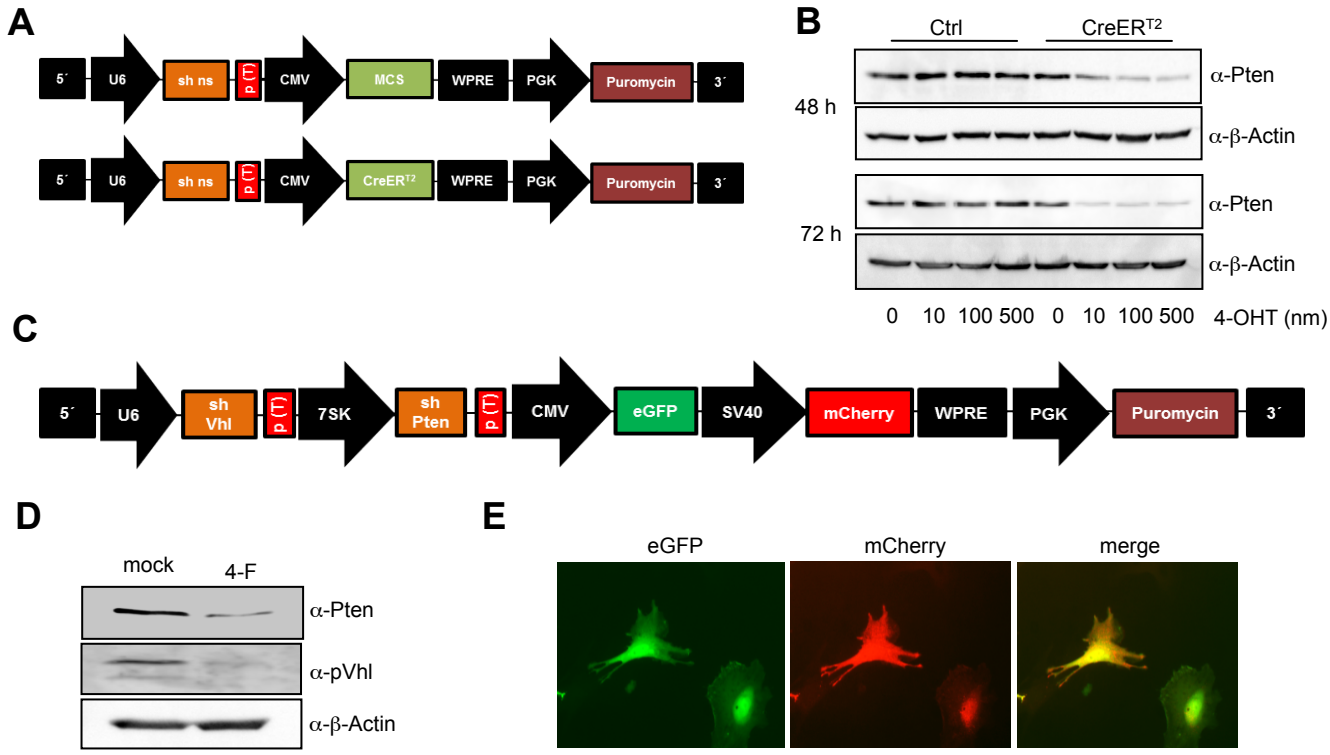
Supplemental Figure 2: Relationship between provirus length and virus titre. A series of 18 different MuLE vectors of varying sizes were packaged using an ecotropic envelope and titred using serial dilution and drug resistance of infected NIH3T3 cells as a readout of infection.

Supplemental Figure 3



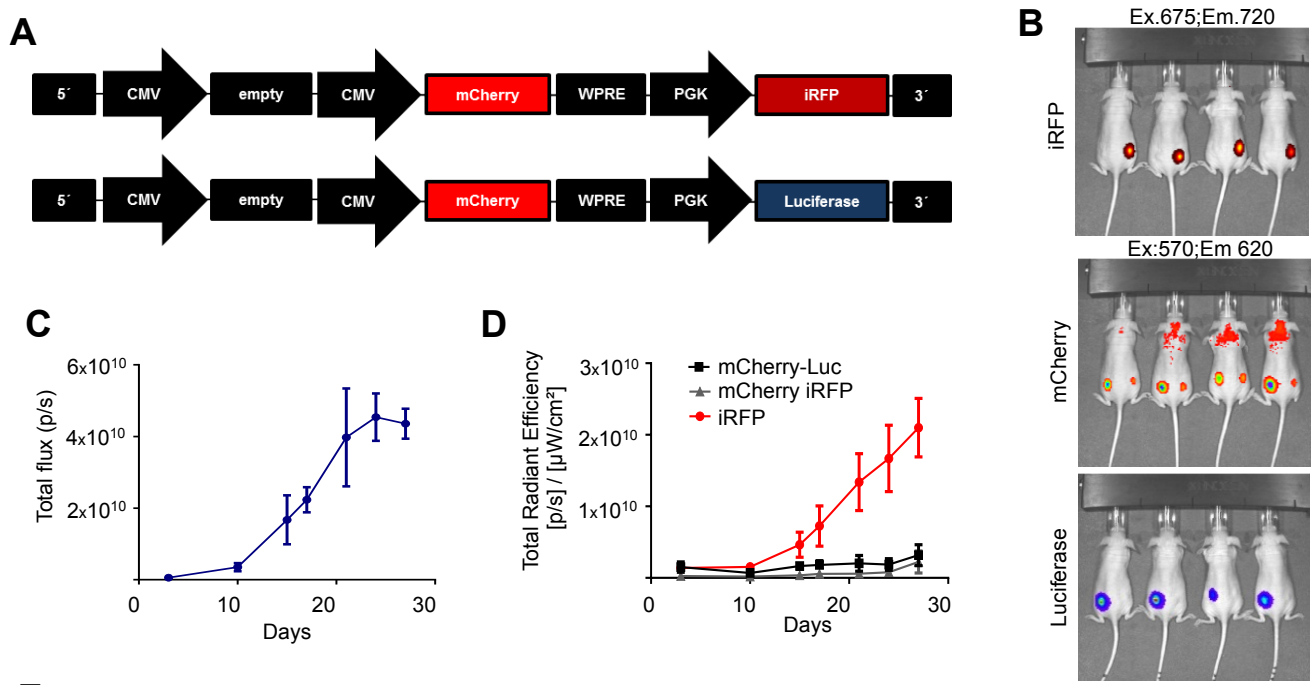
Supplemental Figure 3: Validation of shRNA-mediated knockdown from MuLE vectors. (A) To compare the shRNA knockdown efficiency of the three poll III promoters we generated tricistronic MuLE vectors expressing shRNAs against the von Hippel-Lindau (*Vhl*) or Phosphatase and tensin homolog (*Pten*) tumour suppressor genes from different promoters in combination with expression of mCherry and a Puromycin resistance gene. P = Promoter (U6, 7SK or H1). (B and C) Western blot analysis of primary MEF lysates transduced with lentiviruses generated from vectors shown in panel A. β-Actin was used as a loading control in all experiments. U6 and 7SK promoters resulted in efficient knockdown of both proteins while the knockdown induced by the H1 promoter was only weak in this cell type. (D) To test the possibility of expressing both shRNAs from the same construct and whether the order of the shRNA expression constructs in the lentiviral expression vector influences the knockdown efficiency we generated tricistronic vectors using two U6-driven shRNA cassettes expressing shRNAs against *Vhl* and *Pten*. (E) Western blot analysis of primary WT MEFs transduced with vectors shown in panel (D) demonstrating that the location in the expression vector has no influence on the knockdown efficiency. (F) Schematic of MuLE expression vectors expressing shRNA against Pten or Trp53 alone or together. (G) Western blot showing that the extent of knockdown achieved by expressing two shRNAs from a single MuLE vector is equivalent to knockdown when only a single shRNA is expressed, indicating that the presence of additional elements does not affect the efficiency of expression of the other elements. (H) The functionality of the shRNA-miR-30 expression vectors was tested by cloning a previously established miR-30-based shRNA against *Trp53* (Dickins *et al.* 2005. Nat. Genetics, 37:1289-95) into MuLE Entry vectors with the different pol III promoters. P = Promoter (U6, 7SK or H1) (I) Western blot analysis of MEFs transduced with lentivirus generated from vectors shown in panel F. (J) Crystal violet staining of the same cells seeded at low density and quantification of crystal violet staining, demonstrating functionality of the *Trp53* knockdown in causing cellular immortalisation. (K) Scheme of a vector generated to test the Doxycyclin (DOX)-inducible expression of an shRNA-miR-30 that targets *Trp53.* (L) MEFs were transduced at high MOI with pLenti-CMV-TetR-Blast (Addgene #17492) and western-blot analysis of Blasticidin resistant TetR-expressing primary MEFs demonstrated decrease in p53 protein levels upon addition of DOX to the culture medium. (M) Crystal violet staining of the same cells seeded at low density in the presence of DOX or vehicle control.

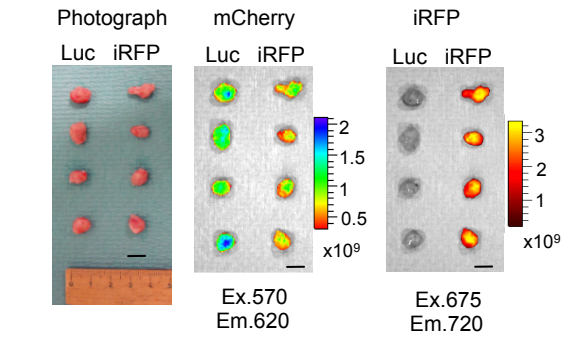
Supplemental Figure 4



Supplemental Figure 4: Validation of CreER^{T2} function and expression of five independent elements from MuLE vectors. (A) Schematic representation of the vectors generated to test the function of an inducible Cre-recombinase (CreER^{T2}) in the MuLE-System. (B) MEFs isolated from *Pten$^{fl/fl}$* mice were transduced with the tricistronic constructs shown in panel L and treated with different concentrations of 4-Hydroxytamoxifen (4-OHT) for 48 h or 72 h then subjected to western blot analysis. The efficient reduction of Pten protein abundance demonstrates the functionality of the CreER^{T2} vector construct. (C) Scheme of a pentacistronic MuLE expression vector generated by 4-Fragment MultiSite L/R- recombination to simultaneously overexpress the fluorescent proteins eGFP and mCherry and shRNAs against *Vhl* and *Pten*. (D) Western blot analysis of puromycin-selected primary MEFs transduced with lentivirus generated from this vector demonstrating efficient knockdown of *Pten* and *Vhl*. (E) Representative fluorescence image of transduced cells showing simultaneous expression of eGFP and mCherry. These studies demonstrate that all genetic elements are functionally expressed from this complex vector.

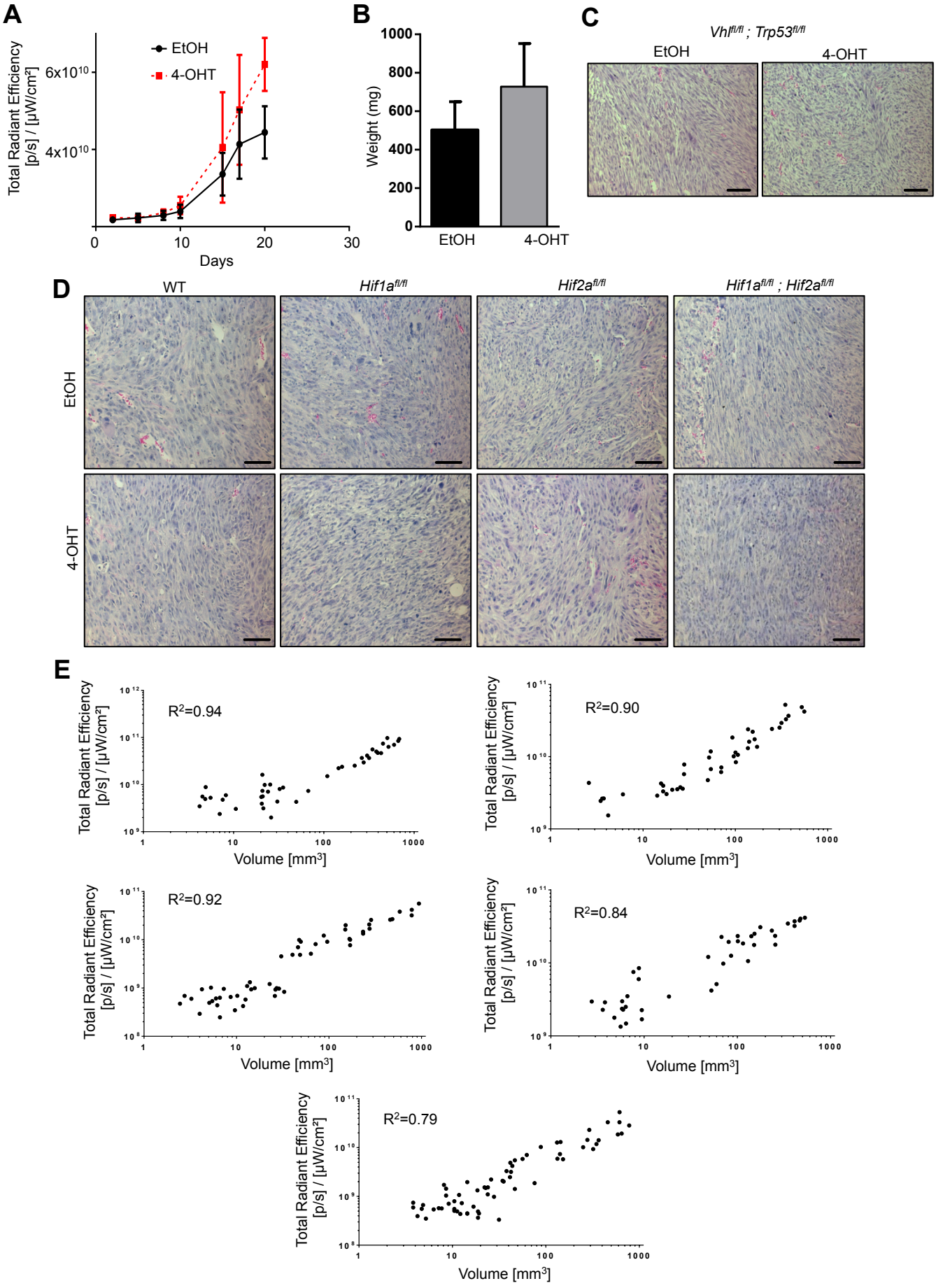
Supplemental Figure 5





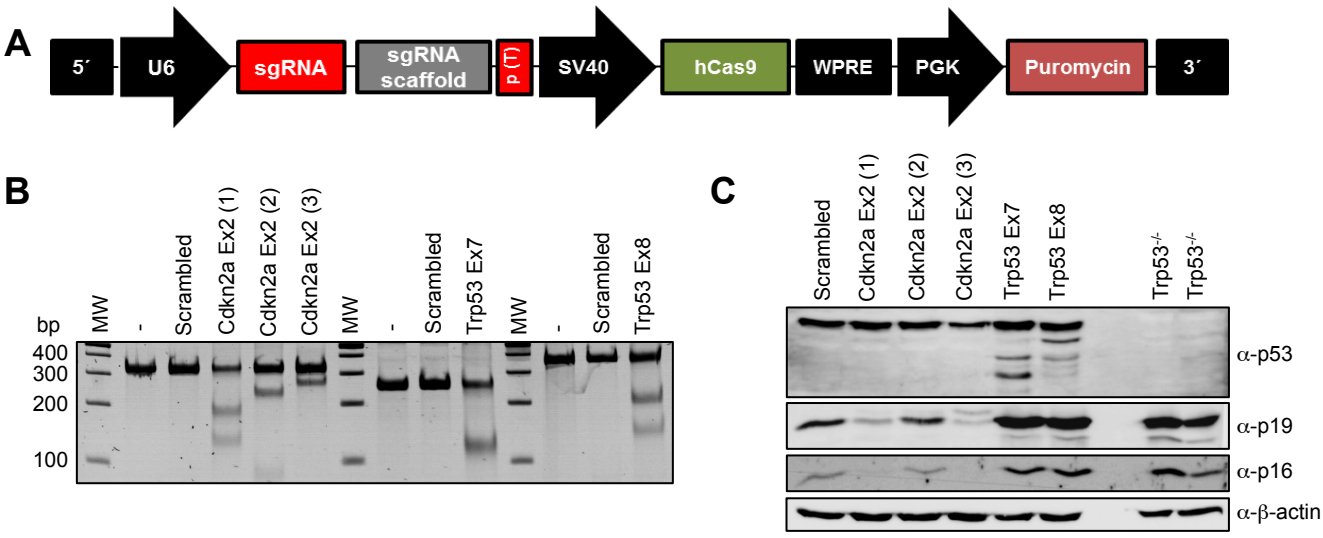
Supplemental Figure 5: *In vivo* function of MuLE lentiviruses in monitoring tumour burden. (A) Scheme of amphotropic lentiviral vectors that were used to transduce human A-375 melanoma cells to overexpress mCherry and iRFP or mCherry and Luciferase. (B) Representative images of athymic nude mice (nude-*Foxn1*^{nu}) that were subcutaneously injected with the same cells and imaged using an IVIS Spectrum imaging device (Caliper). Images were taken 3 weeks after injection of 1x10^6 cells in the left and right flank of nude mice. (C and D) Quantitative monitoring of tumour burden of the same mice demonstrated that luciferase and iRFP, but not mCherry, show a good relationship between signal and tumour volume. Nevertheless all tumours still coexpressed mCherry and luciferase or mCherry and iRFP during the timecourse of tumour formation and also after excision (E) demonstrating permanent coexpression of these proteins from MuLE expression vectors *in vivo*.

Supplemental Figure 6



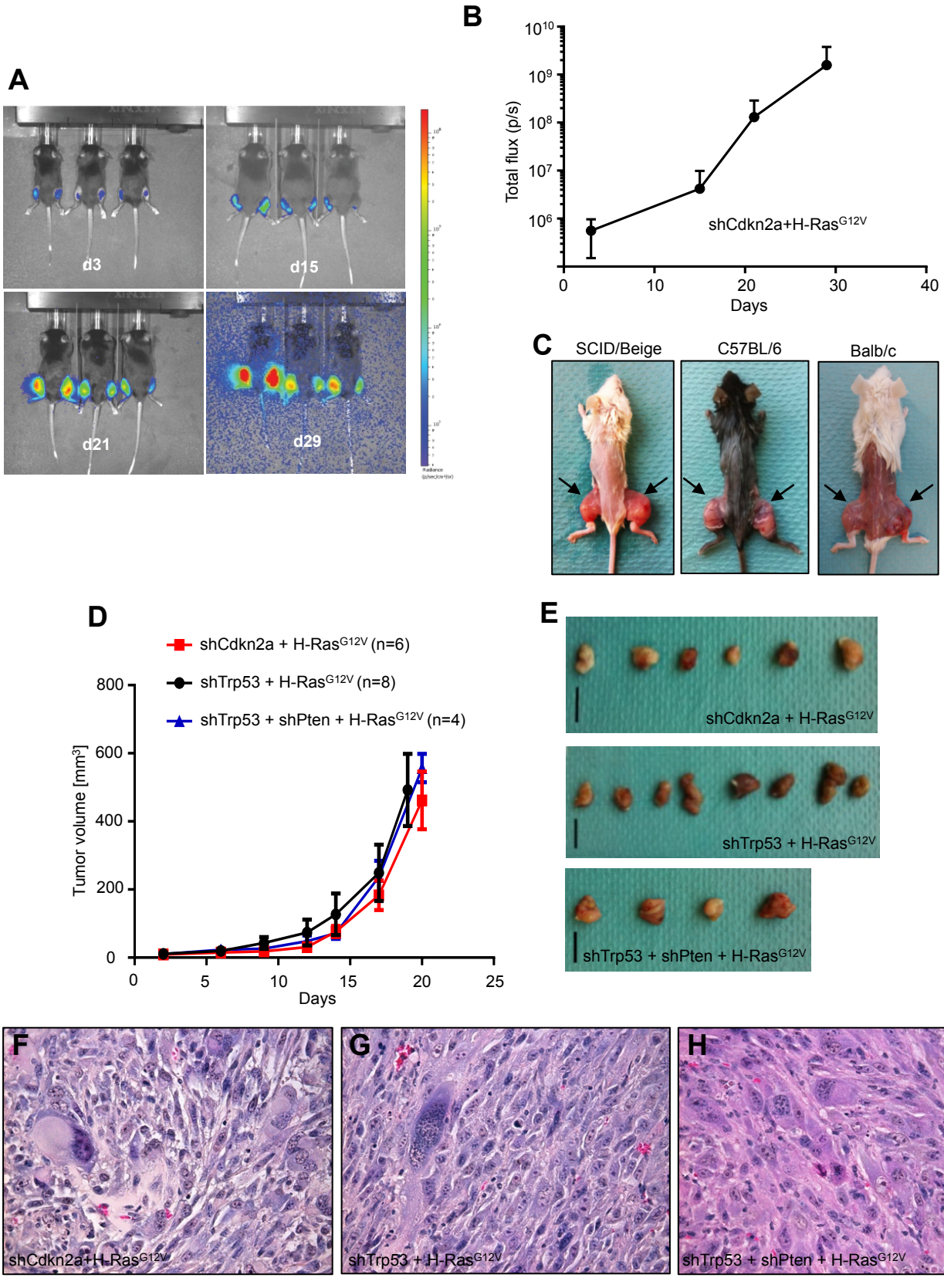
Supplemental Figure 6: Generation and monitoring of genetically complex tumours. (A) Longitudinal tumour growth measured by *in vivo* iRFP fluorescence imaging of SCID/Beige mice that have been injected with *Vhl*$^{fl/fl}$*;Trp53*$^{fl/fl}$ MEFs transduced with a pentacistronic vector to simultaneously express shRNAs against *Rb1* and *Pten* tumour suppressor genes and express Cre-ER^{T2}, oncogenic H-Ras^{G12V} and the Puromycin resistance gene and treated with 4-OHT or vehicle for 3 days. (B) Weight of resulting tumours after excision. Representative histological images of tumurs derived from various genotypes of MEFs that were infected with MuLE virus expressing shRNA against *Rb1*, *Pten* and overexpressing H-Ras^{G12V} and Cre-ER^{T2}, followed by ethanol (EtOH) or tamoxifen (4-OHT) treatment, as control and to activate Cre activity, respectively. (C) *Vhl*$^{fl/fl}$ *; Trp53*$^{fl/fl}$ MEFs, (D) wild type, *Hif1a*$^{fl/fl}$, *Hif2a*$^{fl/fl}$ and *Hif1a*$^{fl/fl}$ *; Hif2a*$^{fl/fl}$ MEFs. All tumours displayed a similar histological appearance and are classified as sarcomatoid malignant lesions with storiform growth pattern containing numerous tumour giant cells with bizarre nuclei and eosinophilic cytoplasm. Scale bars = 100 μm. (E) Correlation of iRFP fluorescence signal intensity and tumour volume with Pearson correlation coefficient (R^2) values for the five sets of xenograft experiments performed in Figure 6C and 7B-E

Supplemental Figure 7



Supplemental Figure 7: CRISPR/Cas9-mediated gene mutation using MuLE vectors. (A) Scheme of lentiviral vectors coexpressing a single sgRNA, hCas9 and puromycin resistance. (B) MEFs were infected with viruses expressing scrambled sgRNA or sgRNAs against *Cdkn2a* Exon 2, *Trp53* Exon 7 or *Trp53* Exon 8 and genomic DNA was isolated 10 days after puromycin selection. PCRs of the loci targeted by the indicated sgRNAs were reannealed and subjected to Surveyor nuclease digestions. The presence of cleaved products in cells infected with sgRNA-containing vectors but not in the vector expressing scrambled control sgRNA indicate mutation of the targeted loci. (C) Western blotting analysis of cells 10 days after puromycin selection showing loss of p19 and p16 expression and presence of truncated forms of p53 in the relevant cell populations.

Supplemental Figure 8



Supplemental Figure 8: Generation of sarcoma mouse models. (A and B) Longitudinal measurement of tumour growth in C57BL6 mice. Mice were injected with concentrated ecotropic lentivirus (10^7 TU/mL) into the gastrocnemius muscle analogously to the SCID/Beige mice shown in Figure 10 at 20 days of age. Tumour growth could be observed with similar kinetics to that seen in SCID/Beige mice (Figure 8). (C) Representative images of tumours generated by injection of SCID/Beige, C57/BL6 and Balb/c mice. (D) *In vivo* growth curves of tumour cells isolated from the primary sarcomas of the different genotypes in Figure 10 and subcutaneously injected in SCID/beige. (E) Images of excised xenograft tumours. (F-H) Representative histological images of xenograft tumours derived from shCdkn2a + H-Ras^{G12V} (F), shTrp53 + H-Ras^{G12V} (G) or shTrp53 + shPten + H-Ras^{G12V} (H) cell lines.

Supplemental Table 1: MuLE Entry Vectors for cloning inserts

Annotation	Promoter	5´att	3´att	Addgene ID
pMuLE ENTR MCS L1-R5	-	L1	R5	62084
pMuLE ENTR MCS L5-L2	-	L5	L2	62085
pMuLE ENTR MCS R4-R3	-	R4	R3	62086
pMuLE ENTR MCS L1-L4	-	L1	L4	62087
pMuLE ENTR MCS L3-L2	-	L3	L2	62088
pMuLE ENTR MCS L5-L4	-	L5	L4	62089
pMuLE ENTR CMV L1-R5	CMV	L1	R5	62090
pMuLE ENTR CMV L5-L2	CMV	L5	L2	62091
pMuLE ENTR CMV L3-L2	CMV	L3	L2	62092
pMuLE ENTR CMV R4-R3	CMV	R4	R3	62093
pMuLE ENTR CMV loxP L5-L2	CMV	L5	L2	62094
pMuLE ENTR SV40 L5-L2	SV40	L5	L2	62095
pMuLE ENTR SV40 L3-L2	SV40	L3	L2	62096
pMuLE ENTR SFFV L1-R5	SFFV	L1	R5	62097
pMuLE ENTR SFFV L5-L2	SFFV	L5	L2	62098
pMuLE ENTR CMV/TO L1-R5	CMV/TO	L1	R5	62099
pMuLE ENTR CMV/TO R4-R3	CMV/TO	R4	R3	62100
pMuLE ENTR H1 L1-R5	H1	L1	R5	62101
pMuLE ENTR H1 L5-L4	H1	L5	L4	62102
pMuLE ENTR H1 L5-L2	H1	L5	L2	62103
pMuLE ENTR H1 L1-L4	H1	L1	L4	62104
pMuLE ENTR H1 R4-R3	H1	R4	R3	62105
pMuLE ENTR 7SK L1-R5	7SK	L1	R5	62106
pMuLE ENTR 7SK L5-L4	7SK	L5	L4	62107
pMuLE ENTR 7SK L5-L2	7SK	L5	L2	62108
pMuLE ENTR 7SK R4-R3	7SK	R4	R3	62109
pMuLE ENTR 7SK L1-L4	7SK	L1	L4	62110
pMuLE ENTR U6 L1-R5	U6	L1	R5	62111
pMuLE ENTR U6 L1-L4	U6	L1	L4	62112
pMuLE ENTR U6-miR-30 L1-R5	U6	L1	R5	62113
pMuLE ENTR U6-miR-30 L1-L4	U6	L1	L4	62114
pMuLE ENTR U6-miR-30 L5-L2	U6	L5	L2	62115
pMuLE ENTR U6-miR-30 L5-L4	U6	L5	L4	62116
pMuLE ENTR U6-miR-30 R4-R3	U6	R4	R3	62117
pMuLE ENTR 7SK-miR-30 L1-R5	7SK	L1	R5	62118
pMuLE ENTR 7SK-miR-30 L5-L4	7SK	L5	L4	62119
pMuLE ENTR 7SK-miR-30 L5-L2	7SK	L5	L2	62120
pMuLE ENTR 7SK-miR-30 R4-R3	7SK	R4	R3	62121
pMuLE ENTR 7SK-miR-30 L1-L4	7SK	L1	L4	62180
pMuLE ENTR H1 miR-30	H1	L1	R5	62181
pMuLE ENTR CMV/TO-miR-30 L1-R5	CMV/TO	L1	R5	62122
pMuLE ENTR CMV/TO-miR-30 L5-L2	CMV/TO	L5	L2	62123
pMuLE ENTR CMV/TO-miR-30 L1-L4	CMV/TO	L1	L4	62124
pMuLE ENTR CMV/TO-miR-30 R4-R3	CMV/TO	R4	R3	62125
pMuLE ENTR CMV/TO-miR-30 L5-L4	CMV/TO	L5	L4	62126
pMuLE ENTR U6 stuffer sgRNA scaffold L1-R5	U6	L1	R5	62127
pMuLE ENTR U6 stuffer sgRNA scaffold L1-L4	U6	L1	L4	62128
pMuLE ENTR U6 stuffer sgRNA scaffold L5-L4	U6	L5	L4	62129
pMuLE ENTR U6 stuffer sgRNA scaffold L5-L2	U6	L5	L2	62130
pMuLE ENTR U6 stuffer sgRNA scaffold R4-R3	U6	R4	R3	62131

Supplemental Table 2: Ready-to-use MuLE Entry Vectors

Annotation	Promoter	5´att	3´att	Addgene ID
pMuLE ENTR CMV-hCas9 R4-R3	CMV	R4	R3	62132
pMuLE ENTR SV40-hCas9 L3-L2	SV40	L3	L2	62133
pMuLE ENTR SV40-hCas9 L5-L2	SV40	L5	L2	62134
pMuLE ENTR U6 n.s shRNA L1-R5	U6	L1	R5	62135
pMuLE ENTR U6 n.s shRNA L1-L4	U6	L5	L4	62136
pMuLE ENTR U6 n.s shRNA R4-R3	U6	L5	L2	62137
pMuLE ENTR U6 n.s shRNA L5-L4	U6	L1	L4	62138
pMuLE ENTR U6 n.s shRNA L5-L2	U6	R4	R3	62139
pMuLE ENTR CMV eGFP L5-L2	CMV	L5	L2	62140
pMuLE ENTR CMV eGFP R4-R3	CMV	R4	R3	62141
pMuLE ENTR CMV eGFP L1-R5	CMV	L1	R5	62142
pMuLE ENTR CMV eGFP L3-L2	CMV	L3	L2	62143
pMuLE ENTR SV40 eGFP L5-L2	SV40	L5	L2	62144
pMuLE ENTR SV40 eGFP L3-L2	SV40	L3	L2	62145
pMuLE ENTR eGFP L5-L2	-	L5	L2	62183
pMuLE ENTR CMV mCherry L5-L2	CMV	L5	L2	62146
pMuLE ENTR CMV mCherry R4-R3	CMV	R4	R3	62147
pMuLE ENTR CMV mCherry L1-R5	CMV	L1	R5	62148
pMuLE ENTR SV40 mCherry L5-L2	SV40	L5	L2	62149
pMuLE ENTR SV40 mCherry L3-L2	SV40	L3	L2	62150
pMuLE ENTR mCherry L5-L2	-	L5	L2	62184
pMuLE ENTR CMV tdTomato R4-R3	CMV	R4	R3	62151
pMuLE ENTR CMV tdTomato L5-L2	CMV	L5	L2	62152
pMuLE ENTR SV40 tdTomato L3-L2	SV40	L3	L2	62153
pMuLE ENTR SV40 tdTomato L5-L2	SV40	L5	L2	62154
pMuLE ENTR SFFV tdTomato L5-L2	SFFV	L5	L2	62155
pMuLE ENTR tdTomato L5-L2	-	L5	L2	62185
pMuLE ENTR CMV iRFP L1-R5	CMV	L1	R5	62156
pMuLE ENTR CMV iRFP L5-L2	CMV	L5	L2	62157
pMuLE ENTR CMV iRFP R4-R3	CMV	R4	R3	62158
pMuLE ENTR CMV iRFP L3-L2	CMV	L3	L2	62159
pMuLE ENTR SV40 iRFP L3-L2	SV40	L3	L2	62160
pMuLE ENTR CMV PuroR L5-L2	CMV	L5	L2	62161
pMuLE ENTR CMV PuroR R4-R3	CMV	R4	R3	62162
pMuLE ENTR CMV PuroR L3-L2	CMV	L3	L2	62163
pMuLE ENTR SV40 PuroR L3-L2	SV40	L3	L2	62164
pMuLE ENTR SV40 PuroR L5-L2	SV40	L5	L2	62165
pMuLE ENTR CMV LacZ L5-L2	CMV	L5	L2	62166
pMuLE ENTR SV40 LacZ L5-L2	Sv40	L5	L2	62167
pMuLE ENTR CMV Luc2 (FF-Luciferase) L5-L2	CMV	L5	L2	62168
pMuLE ENTR CMV Luc2 (FF-Luciferase) R4-R3	CMV	R4	R3	62169
pMuLE ENTR SV40 Luc2 (FF-Luciferase) L5-L2	SV40	L5	L2	62170
pMuLE ENTR SV40 Luc2 (FF-Luciferase) L3-L2	SV40	L3	L2	62171
pMuLE ENTR CMV Renilla Luciferase L5-L2	CMV	L5	L2	62186
pMuLE ENTR CMV CreERT2 L5-L2	CMV	L5	L2	62172
pMuLE ENTR CMV CreERT2 R4-R3	CMV	R4	R3	62173
pMuLE ENTR SV40 CreERT2 L3-L2	SV40	L3	L2	62174

Supplemental Table 3: Restriction enzyme recognition sites in Multiple Cloning Sites of MuLE vectors

Annotation	Restriction Enzyme recognition sites in Multiple Cloning Site
pMuLE ENTR MCS	HindIII-SacI-BamHI-SpeI-EcoRI-XhoI-SphI-XbaI
pMuLE ENTR CMV	HindIII-BamHI-EcoRI-XhoI-SphI-XbaI
pMuLE ENTR SV40	HindIII-SacI-BamHI-SpeI-EcoRI-XhoI-XbaI
pMuLE ENTR SFFV	BamHI-SpeI-EcoRI-XhoI-SphI-XbaI
pMuLE ENTR CMV/TO	BamHI-SpeI-EcoRI-XhoI-SphI-XbaI

Supplemental Methods

LR Recombination

The MultiSite Gateway LR recombinations were performed using the Gateway LR Clonase® II Plus Enzyme mix (Life Technologies #12538-120). 2-Fragment recombinations were performed in a PCR tube in a final reaction volume of 5 µl with 0.5 µl of each Entry vector (5 fmoles), 0.5 µl of the Destination vector (10 fmoles), 2.5 µl of 1x TE buffer pH 8.0 and 1 µL of the LR Clonase enzyme mix. 3-and 4-fragment recombinations were performed in a final reaction volume of 10 µl in a 1.5 ml microcentrifuge tube using 10 fmoles of each Entry vector and 20 fmoles of the Destination vector and 2 µl of the LR Clonase enzyme mix. Reactions were incubated at 25°C for 16-20 h followed by a 10 min proteinase K digestion at 37 °C and transformation into chemically competent One Shot® Mach1 E.coli cells (Life Technologies #C8620-03).

BP Recombination

BP recombinations were performed using Gateway BP Clonase II enzyme Mix (Life Technologies #11789-020). 15-150 ng (50 fmoles) of the PCR product with the designed attB sites was mixed with 150 ng of the Donor Vector with the respective attP sites in a total volume of 8 µl in TE buffer pH 8.0. 2 µl of the BP Clonase II enzyme mix was added to the reaction mix and incubated for 1 h at 25 °C. After 10 min Proteinase K digestion at 37 ° C, 1 µl of the reaction mix was used to transform chemically competent One Shot® Mach 1 E.coli cells (Life Technologies #C8620-03).

The following Donor vectors were used for the generation of MultiSite Gateway compatible Entry vectors:

pDONR^{TM} 221 P1-P5R (Life technologies #12537-100)

pDONR^{TM} 221 P2-P5 (Life technologies #12537-100)

pDONR^{TM} 221 P1-P4 (Life technologies #12537-100)

pDONR^{TM} 221 P4R-P3R (Life technologies #12537-100)

pDONR^{TM} 221 P3-P2 (Life technologies #12537-100)

pDONR^{TM} 221 P5-P4 (Life technologies #12537-100)

Cloning of Gateway Destination Vectors

pLenti X1 Luciferase DEST

The synthetic firefly luciferase gene (luc2), which has been codon optimized for high expression in mammalian cells, was PCR amplified from pQUAS-Luc2 (Addgene, #24337) using primers that incorporate a 5´- EcoRI and a 3´- NsiI restriction enzyme cutting site into the PCR product which was then ligated into a 6kb vector fragment resulting from EcoRI/NsiI digestion of pLenti X1 Puro DEST (Addgene, #17297). In a second cloning step the attR1-CMR/ccDB-attR2-WPRE-pGK- fragment was PCR amplified from pLenti X1 Puro DEST using primers that incorporate a 5´-MfeI and a 3´-SbfI restriction enzyme cutting site and cloned into the EcoRI/SbfI digested vector that was generated in the first step. Bacterial transformation was done using chemically competent DB3.1 E.coli cells.

pLenti X1 Neomycin DEST

The Neomycin resistance gene was PCR amplified from pCMV-Neo-Bam (Addgene, #16440) using primers that incorporate a 5´- SbfI and a 3´- MluI restriction enzyme cutting site into the product which was then cloned into the SbfI/MluI digested pLenti X1 Luciferase DEST. Bacterial transformation was done using chemically competent DB3.1 E.coli cells.

pLenti X1 eGFP DEST

The eGFP cDNA was PCR amplified from the 7TGC vector, (Addgene #24304) using primers that incorporate a 5´- SbfI and a 3´- MluI restriction enzyme cutting site into the product which was then cloned into the SbfI/MluI digested pLenti X1 Luciferase DEST. Bacterial transformation was done using chemically competent DB3.1 E.coli cells.

pLenti IFP1.4 DEST

The IFP1.4 cDNA was PCR amplified from the pcDNA3-IFP1.4 vector (kind gift of Prof. R Tsien, Department of Pharmacology, Department of Chemistry and Biochemistry, and Howard Hughes Medical Institute, University of California at San Diego, La Jolla, CA 92093-0647) using primers that incorporate a 5´- NsiI and a 3´- MluI restriction enzyme cutting site into the product which was then cloned into the SbfI/MluI digested pLenti X1 Luciferase DEST. Bacterial transformation was done using chemically competent DB3.1 E.coli cells.

pLenti iRFP DEST

The iRFP cDNA was PCR amplified from the pShuttle-CMV-iRFP vector (Addgene, #31856) using primers that incorporate a 5´- NsiI and a 3´- MluI restriction enzyme cutting site into the product which was then cloned into the SbfI/MluI digested pLenti X1 Luciferase DEST. Bacterial transformation was done using chemically competent DB3.1 E.coli cells.

Cloning of the MultiSite Gateway compatible MuLE entry vector toolbox

pMuLE ENTR MCS

The multiple cloning site of pcDNA3 (Invitrogen) was amplified by PCR using primer pairs that incorporate different combinations of attB sites into the product. The PCR products were then recombined in a BP recombination with the respective pDONR^{TM} 221 vectors to generate the respective MultiSite Gateway compatible Entry vectors.

pMuLE ENTR CMV

The CMV promoter and the multiple cloning site (MCS) was amplified by PCR from pcDNA3 (Invitrogen) using primers that incorporate the respective attB sites. BP recombination with the respective pDONR^{TM} 221 vectors generated the desired Entry vectors.

pMuLE ENTR CMV loxP

The multiple cloning site of pcDNA3 (Invitrogen) was amplified by PCR using primers that incorporate loxP sites and 5´-HindIII and 3´- XbaI restriction enzyme cutting sites into the product. This was then cloned into HindIII/XbaI of pMuLE ENTR CMV (Nr. 8 and 10)

pMuLE ENTR SV40

The SV40 promoter was PCR amplified from the 7TGC vector, (Addgene #24304) using a forward primer that incorporates the respective attB5 site and a reverse primer that incorporates the respective attB2 site plus 3´-XbaI-HindIII restriction enzyme cutting sites. The resulting PCR product was recombined in a BP recombintion with pDONR^{TM} 221 P2-P5 to generate the subcloning vector in which the MCS of the pcDNA3 Vector was cloned using HindIII/XbaI. This vector was then used as a template for the SV40-MCS PCR amplification that was performed to generate pMuLE ENTR SV40(L3-L2) by BP recombination.

pMuLE ENTR SFFV

The SFFV promoter was PCR amplified from LeGO-T2 (Addgene, #27342) using primers that incorporate a 5´-HindIII restriction enzyme cutting site and a 3´-BamHI restriction enzyme cutting site. The PCR product was the cloned into HindIII/BamHI of pMuLE ENTR MCS vectors.

pMuLE ENTR CMV/TO

The doxycycline inducible CMV/TO promoter was PCR amplified from pENTR/pSM2(CMV/TO) (Addgene, #17388) using primers that incorporate a 5´-HindIII and a 3´-KpnI restriction enzyme cutting site into the PCR product which was then cloned into HindIII/KpnI of the respective pMuLE ENTR MCS vectors.

pMuLE ENTR H1

The H1 promoter plus part of the stuffer was PCR amplified from pENTR/pSUPER+ (575-1), (Addgene #17338) using forward primers that incorporate the necessary attB site as well as a 5´-ClaI restriction enzyme cutting site and reverse primers that incorporate the respective attB site and a 3´-EcoRI restriction enzyme cutting site. The PCR product was then used for BP recombination with the respective pDONR^{TM} 221 vector to generate the final Vector. The plasmids are propagated in methylation deficient GM2163 bacteria to avoid methylation of the ClaI restriction enzyme cutting site.

pMuLE ENTR 7SK

The 7SK promoter was PCR amplified from psiRNA-h7SK G1 Hygro (Invivogen, # ksirna3-h21) using forward primers that incorporate a 5´-ClaI restriction enzyme cutting site and reverse primers that incorporate 3´- SacI and BglII restriction enzyme cutting sites in the PCR product. The PCR product was then digested with ClaI/SacI and cloned into ClaI/SacI of pMuLE ENTR H1 vectors.

pMuLE ENTR U6

The U6 promoter was PCR amplified from pLKO.1 puro (Addgene, #8453) using forward primers that incorporate a 5´-ClaI restriction enzyme cutting site and reverse primers that incorporate a 3´- BglII restriction enzyme cutting sites in the PCR product. The PCR product was then digested with ClaI/BglII and cloned into ClaI/BglII of the respective pMuLE ENTR H1 vectors.

pMuLE ENTR U6 miR-30

The U6 promoter- 5´-miR-30 context shRNA cloning site 3´-miR-30 context were PCR amplified from pENTR/pSM2(U6) (Addgene, #17387) using primers that incorporate the necessary attB sites. The PCR product was then used for BP recombination with the respective pDONR^{TM} 221 vectors to generate the final Vector.

pMuLE ENTR 7SK miR-30

The miR-30 context including the shRNA cloning site was PCR amplified from pENTR/pSM2(U6) (Addgene, #17387) using primers that incorporate a 5´- BglII restriction enzyme cutting site and a 3´-MfeI restriction enzyme cutting site in the PCR product. Using these restriction sites, the PCR product was then cloned into BglII/EcoRI of the respective pMuLE ENTR 7SK vectors.

pMuLE ENTR H1 miR-30

A ~300bp fragment was excised from the pMuLE ENTR H1 vector (L1-R5) using BamHI and BglII following religation. Then the H1 promoter was PCR amplified using primers that incorporate a 5´-ClaI and 3´-MfeI and XbaI restriction enzyme cutting

sites. The PCR product was cloned into the ClaI/EcoRI sites of the religated subcloning vector. Then the miR-30 context plus shRNA cloning site was cutted out of the pENTR/pSM2(U6) vector (Addgene, #17387) using SpeI/XbaI and cloned into SpeI/XbaI of the H1 containing subcloning vector to generate pMuLE ENTR H1 miR-30.

pMuLE ENTR CMV/TO-miR-30

The CMV/TO-miR-30 fragment was excised from pENTR/pSM2(CMV/TO) (Addgene, #17388) using DraI/XbaI and ligated into Ecl136II/XbaI of pMuLE ENTR MCS (L1-R5). From this vector the CMV/TO-miR-30 fragments were excised using HindIII/XbaI and ligated into the HindIII/XbaI of the respective pMuLE ENTR MCS vectors.

pMuLE ENTR U6 stuffer sgRNA scaffold

The U6 promoter and the sgRNA cloning cassette including the sgRNA scaffold was excised from pLKO.1-puro U6 sgRNA BfuAI stuffer (Addgene,#50920) using ClaI/EcoRI and ligated into ClaI/EcoRI digested pMuLE ENTR H1 vectors. To facilitate cloning of sgRNAs a 703 bp DNA stuffer was inserted between the two BfuAI sgRNA cloning sites.

Ready to use MuLE MultiSite Gateway ENTRY vectors

pMuLE ENTR CMV/SV40 hCas9

The human codon optimized Cas9 cDNA was excised from pCAG-hCas9 (Addgene, #51142) using KpnI/XbaI and ligated into KpnI/XbaI of pMuLE ENTR CMV and pMuLE ENTR SV40.

pMuLE ENTR U6/7SK/H1 ns shRNA

Control vectors expressing a non silencing scramble shRNA driven by different PolII promoters were generated by cloning of the scramble shRNA from the pLKO.1 scramble shRNA vector (Addgene, #1864) into the respective pMuLE entry vectors.

pMuLE ENTR CMV/SV40 eGFP

The eGFP cDNA was PCR amplified from the 7TGC vector, (Addgene #24304) using primers that incorporate a 5´-BamHI and a 3´- XhoI restriction enzyme cutting site into the PCR product which was then cloned into BamHI/XhoI of the respective pMuLE ENTR vectors.

pMuLE ENTR CMV/SV40 mCherry

The mCherry cDNA was PCR amplified from the 7TGC vector, (Addgene #24304) using primers that incorporate a 5´-BamHI and a 3´- XhoI restriction enzyme cutting site into the PCR product which was then cloned into BamHI/XhoI of the respective pMuLE ENTR vectors.

pMuLE ENTR CMV/SV40/SFFV tdTomato (Nr. 78-83)

The tdTomato cDNA was cut out from pCSCMV:tdTomato (Addgene, #30530) using BamHI and XbaI restriction enzymes and ligated into BamHI/XbaI of the respective pMuLE entry vectors.

pMuLE ENTR CMV/SV40 iRFP

The iRFP cDNA was excised from pShuttle-CMV-iRFP (Addgene, #31856) using BglII and XbaI restriction enzymes and ligated into BamHI/XbaI of the respective pMuLE entry vectors.

pMuLE ENTR CMV/SV40 PuroR

The Puromycin cDNA was PCR amplified from the pLenti X1 Puro DEST vector, (Addgene, #17297) using primers that incorporate a 5´-HindIII and a 3´-SalI restriction enzyme cutting site into the PCR product which was then cloned into HindIII/XhoI of the respective pMuLE ENTR vectors.

pMuLE ENTR CMV/SV40 LacZ

The LacZ cDNA was PCR amplified from the pcDNA3.1/nV5-GW/lacZ vector (Invitrogen, #12290-010) using primers that incorporate a 5´-HindIII and a 3´- XbaI restriction enzyme cutting site into the PCR product which was then cloned into HindIII/XbaI of the respective pMuLE ENTR vectors.

pMuLE ENTR CMV/SV40 Firefly Luciferase

The firefly Luciferase cDNA was PCR amplified from pQUAS-Luc2 (Addgene, #24337) using primers that incorporate a 5´- EcoRI and a 3´- XbaI restriction enzyme cutting site into the PCR product which was then cloned into EcoRI/XbaI of the respective pMuLE ENTR vectors.

pMuLE ENTR CMV Renilla Luciferase

The renilla Luciferase cDNA was PCR amplified from the pRL-SV40 Vector (Promega #E2231) using primers that incorporate a 5´- HindII and a 3´-XhoI restriction enzyme cutting site into the PCR product which was then cloned into HindIII/XhoI of pMuLE ENTR CMV (L5-L2).

pMuLE ENTR CMV/SV40 Cre-ER^{T2}

The CreER^{T2} cDNA was excised from pBabe-puro-CreER^{T2} (kind gift of S.W. Lowe, Howard Hughes Medical Institute, Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York 10065, USA) using EcoRI and cloned into the EcoRI site of the respective pMuLE ENTR vectors.

Cloning of additional MultiSite Gateway compatible MuLE entry vectors used for experiments

pMuLE ENTR CMV/SV40 H-Ras^{G12V}

The H-Ras^{G12V} gene was excised from pBabe puro HRasV12 (Addgene, #9051) using BamHI/SalI and cloned into BamHI/XhoI of the respective pMuLE ENTR vectors.

pMuLE ENTR CMV c-Myc

The c-Myc gene was excised from pBabe-c-Myc-puro (kind gift of W. Krek, Institute of Molecular Health Sciences, ETH Zurich, CH-8093 Zurich, Switzerland; Competence Center for Systems Physiology and Metabolic Diseases, ETH Zurich, CH-8093 Zurich, Switzerland.) using BamHI/EcoRI and cloned into BamHI/EcoRI of the respective pMuLE ENTR vector.

Cloning of miR-30 based shRNAs into pMuLE ENTR vectors

The miR-30-based shRNAs targeting murine *Trp53* (clone 1224) and murine *Rb1* (clone 577) were excised from LMP-p53.1224 and LMP-Rb1.577 (kind gift of S.W. Lowe, Howard Hughes Medical Institute, Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York 10065, USA) using XhoI/EcoRI and cloned into XhoI/EcoRI of the respective pMuLE ENTR miR-30 vectors.

Cloning of pLKO.1-derived shRNAs into pMuLE ENTR vectors

The U6 promoter and the shRNAs targeting murine *Pten*, *Vhl* or *Cdkn2a* were PCR amplified from the respective pLKO.1 vectors using primers that incorporate ClaI/EcoRI restriction enzyme cutting sites in the PCR product and then cloned into ClaI/EcoRI digested pMuLE ENTR H1 vectors (propagated in methylation deficient GM2163 bacteria) to result in an entry vector in which the shRNA is driven by the U6 promoter. Cloning of shRNAs can be also performed by ligation of double stranded oligonucleotides with the respective overhangs into BglII/SacI digested pMuLE ENTR 7SK/U6/H1 vectors to generate Entry vectors in which the shRNA is driven by the 7SK, H1 or U6 promoter.

Cloning of sgRNAs into pMuLE ENTR U6 stuffer sgRNA scaffold

sgRNAs used in this study were designed according to the CRISPR Design Tool (crispr.mit.edu) and depicted in Table 1 below, note that the PAM sequence depicted in green was not included in the cloned sgRNA. A 5´ACCG was added to the 5´end of the forward direction oligo and 5´AAAC to the 5´end of the reverse complement oligo. Oligonucleotides were annealed to generate double stranded DNA fragments and ligated into the BfuAI digested Entry vectors. The sequence of the resulting Entry vectors was verified by Sanger sequencing.

Table 1: sgRNA sequences used in this study

sgRNA target	Target sequence 5'-sgRNA-PAM-3'
Scrambled control	GTCATGTCACTTATCAAGTC
Trp53 Exon 7	GTGTAATAGCTCCTGCATGG**GGG**
Trp53 Exon 8	GTTCGTGTTTGTGCCTGCCC**TGG**
Cdkn2a Exon 2 (1)	GGGTCGCCTGCCGCTCGACT**TGG**
Cdkn2a Exon 2 (2)	CCCGCGCTGCGTCGTGCACC**GGG**
Cdkn2a Exon 2 (3)	CGGTGCAGATTCGAACTGCG**AGG**
Vhl Exon 1 (1)	ACAAAGGCAGCACGACGCGC**GGG**
Vhl Exon 1 (2)	GCCCGGTGGTAAGATCGGGT**AGG**
Vhl Exon 1 (3)	ACCGAGCGCAGCACCGGCCG**CGG**
Pten Exon 1 (1)	GCTAACGATCTCTTTGATGA**TGG**
Pten Exon 1 (2)	AGATCGTTAGCAGAAACAAA**AGG**
Pten Exon 2 (3)	AAAGACTTGAAGGTGTATAC**AGG**

Surveyor assays

Genomic DNA was isolated using the Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). PCRs for Surveyor assays were run using 20ng genomic DNA, 0.5μL 10mM dNTP mix, 2.5μL 10x High Fidelity Buffer with 15mM MgCl2 (Roche), 0.4μL High Fidelity Enzyme Mix (Roche), 0.75μL mix of forward and reverse primers (10mM) in a 25μL reaction. 35 cycles of 94^{o}C 15 sec, 55^{o}C 30 sec, 72^{o}C 45 sec were performed. 15μL of PCR product was used for formation of heteroduplexes and 7μL of this was subjected to Surveyor nuclease digestion.

Table 2: Sequences of primers used for Surveyor assays:

Primer	Olignucleotide (5'-3')	Product size (bp)
Trp53 Exon 7 forward	ATTCCCGGCTGCTGCAGGTC	258
Trp53 Exon 7 reverse	GGCGGGACTCGTGGAACAGAA	
Trp53 Exon 8 forward	GGACGTCTCTTATCTGTGGCT	350
Trp53 Exon 8 reverse	GACTTTGGGGTGAAGCTCAAC	
Cdkn2a Exon 2 forward	GTGATGATGATGGGCAACGT	334
Cdkn2a Exon 2 reverse	TGCTTGAGCTGAAGCTATGC	
Vhl Exon 1 forward	CACGTCCAGCTTGCGAAT	366
Vhl Exon 1 reverse	TAGATGCAGTGGGTAGGG	
Pten Exon 2 forward	GCTCAAGGAGCAGACAAGTA	403
Pten Exon 2 reverse	GCCAGTTCTCATCCAGTGA	

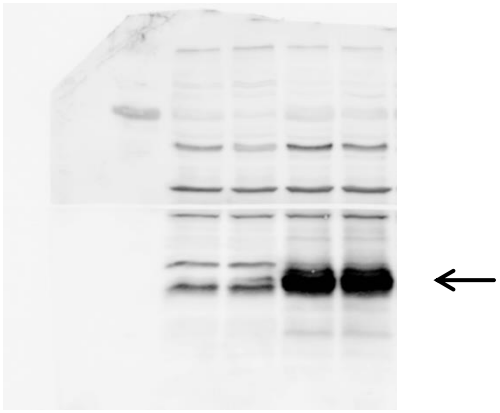
Next Generation sequencing

Genomic DNA was isolated using the Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). Regions for sequencing were amplified using the same primers that were used for Surveyor assays (Table 2 above). PCR amplified target regions from one sample were mixed in equimolar ratio and purified using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany). Subsequently, Ion Torrent specific adapters including barcodes were ligated to the amplicon mix after a DNA end repair step (all barcodes and reagents from ThermoFisher Scientific, Waltham, Massachusetts, USA) and purified again with the Agencourt beads. Quality control included a Bioanalyser run using the High Sensitivity DNA assay from Agilent Technologies (Agilent Technologies, Waldbrann, Germany) showing the size distribution of the input amplicon mix before adapter ligation and the adapter ligated amplicon mix. After this quality control step, the amplicon mixes from different samples were quantified using the Ion Library Taqman Quantitation Kit (ThermoFisher Scientific), diluted to 8 pM, and pooled. This pooled amplicon mix was then used as input for the Ion PGM Template 400 preparation according to the protocol (Ion PGM Template OT2 400 Kit, ThermoFisher Scientific). Subsequently, the amplicons were loaded on a 318v2 chip (ThermoFisher Scientific) and run on the PGM platform using the Ion PGM Sequencing 400 Kit (ThermoFisher Scientific). NGS data was analysed using the Torrent Suite Version 4.2.1 (ThermoFisher Scientific). The amplicons were aligned to mouse mm10 genome and the variants called with the variant caller version 4.2.1.0 (ThermoFisher Scientific).

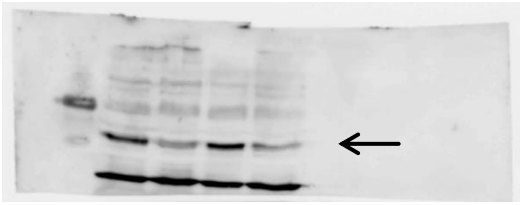
SUPPLEMENTAL UNCUT GELS

Figure 4 B

α- H-Ras (Santa Cruz #sc-520)



α-p53 (Novocastra, NCL-p53-CM5p)



α-ß-Actin (Sigma-Aldrich, A2228)

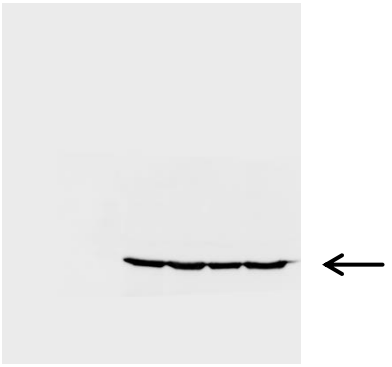
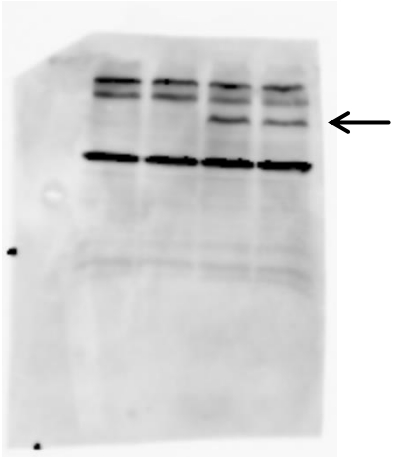
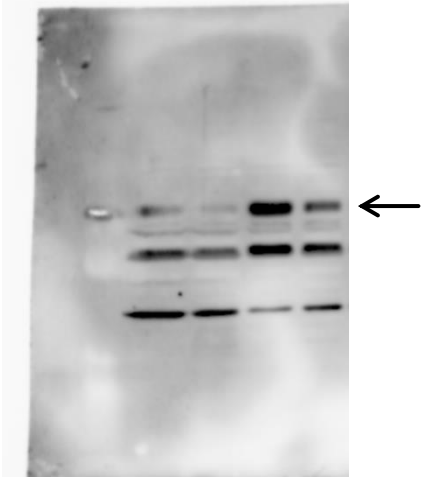


Figure 4H

α-c-Myc (Sigma, , #M5546)



α-p53 (Novocastra, NCL-p53-CM5p)



α-ß-Actin (Sigma-Aldrich, A2228)

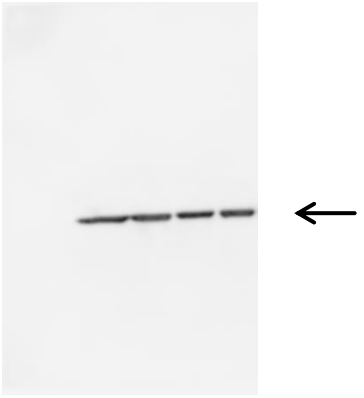
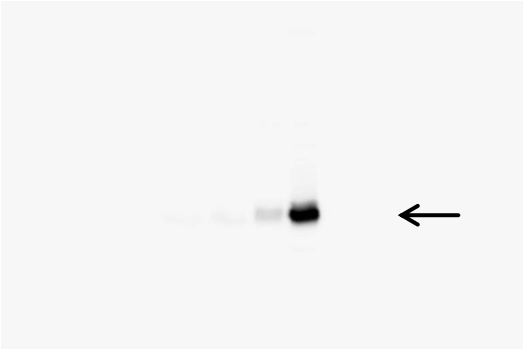
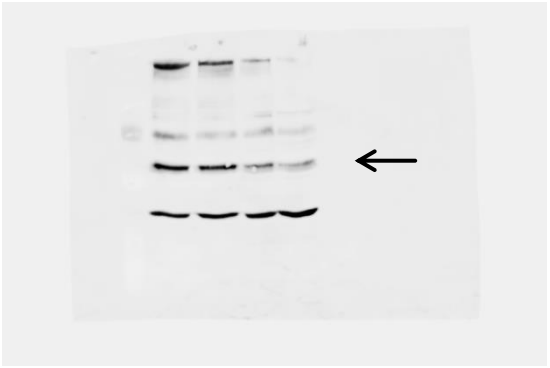


Figure 5 B

α- H-Ras (Santa Cruz #sc-520)



α-p53 (Novocastra, NCL-p53-CM5p)



α-ß-Actin

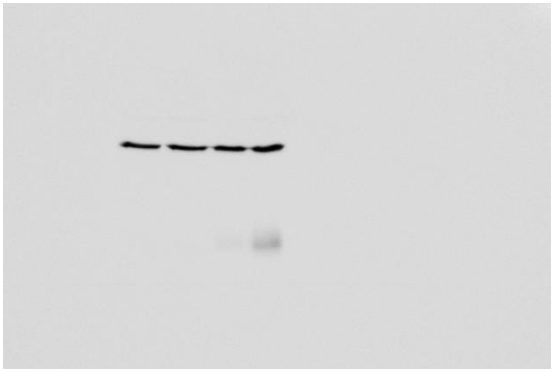
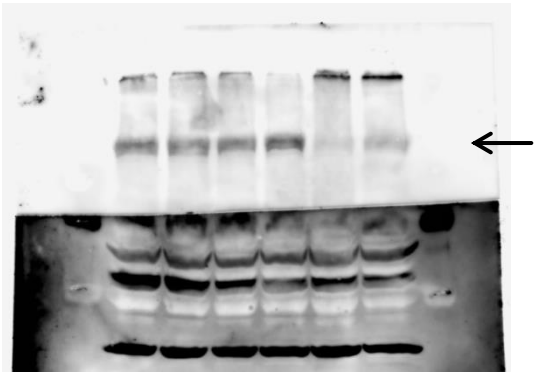
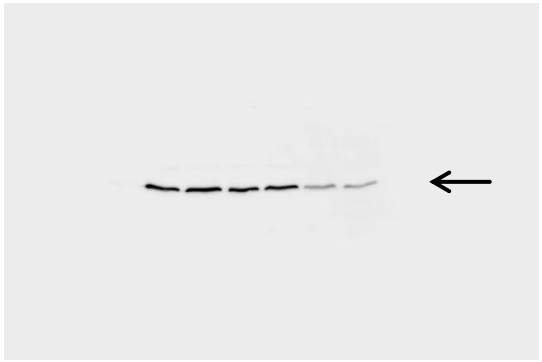


Figure 6 B

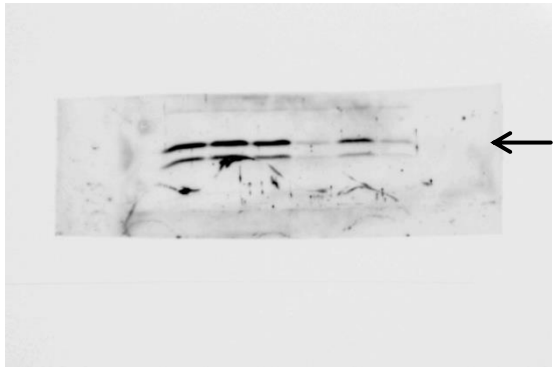
α-Rb1(Cell Signaling, #9313)



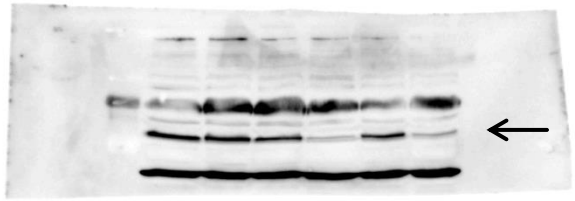
α-Pten (Santa Cruz, #sc-7974)



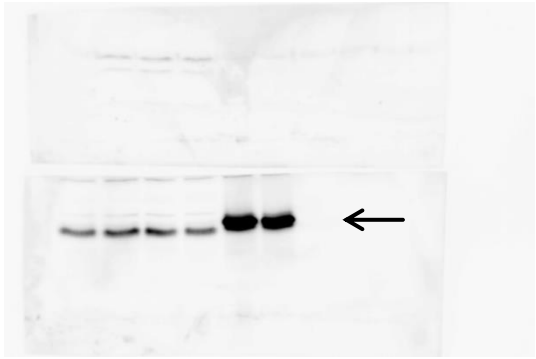
α-pVhl (Santa Cruz, #sc-5575)



α-p53 (Novocastra, NCL-p53-CM5p)



α- H-Ras (Santa Cruz #sc-520)



α-ß-Actin (Sigma-Aldrich, A2228)

α-Hif1a (Novus, #NB100-479)

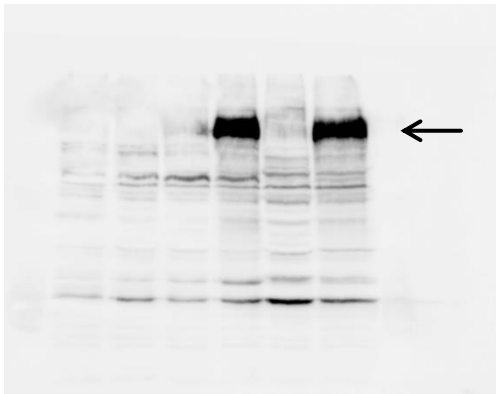
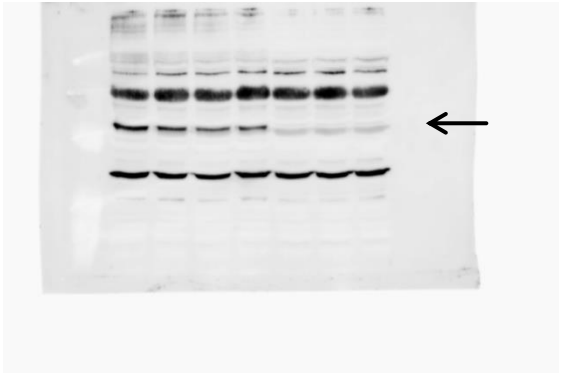


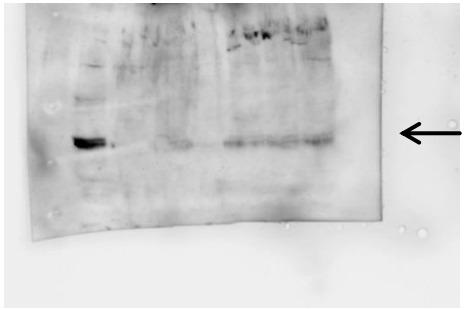
Figure 6 D

α-Pten (Santa Cruz, #sc-7974)

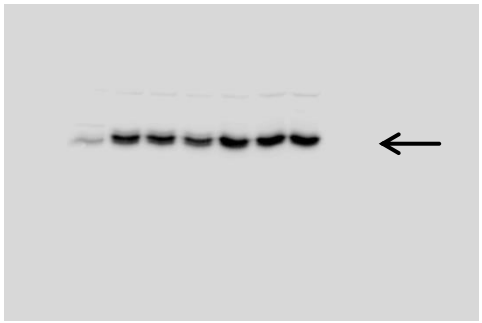
α-p53 (Novocastra, NCL-p53-CM5p)



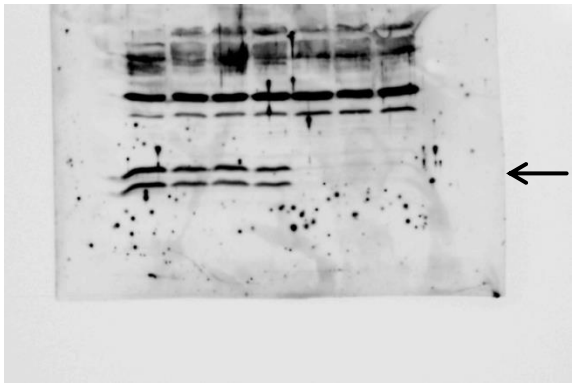
α-Rb1(Cell Signaling, #9313)



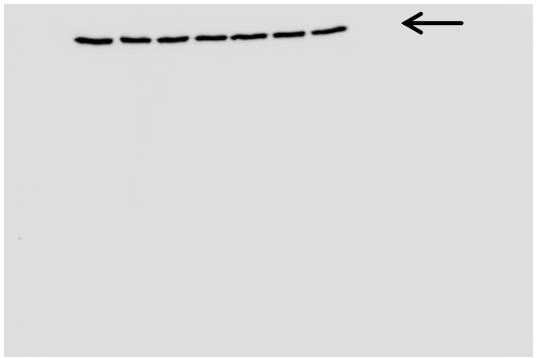
α- H-Ras (Santa Cruz #sc-520)



α-pVhl (Santa Cruz, #sc-5575)



α-ß-Actin (Sigma-Aldrich, A2228)



α-Hif1a (Novus, #NB100-479)

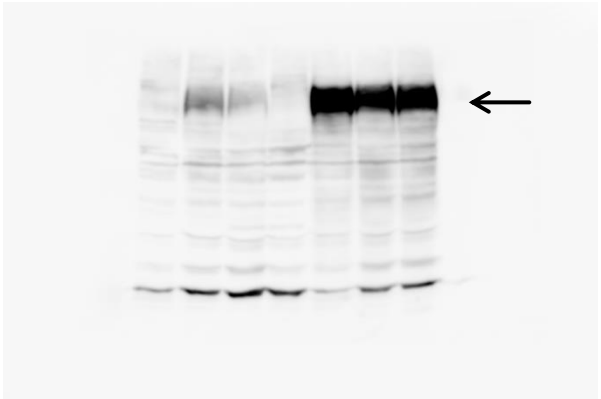
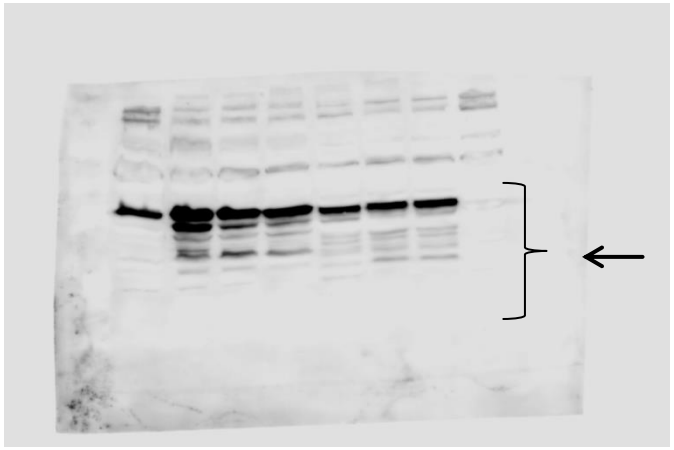
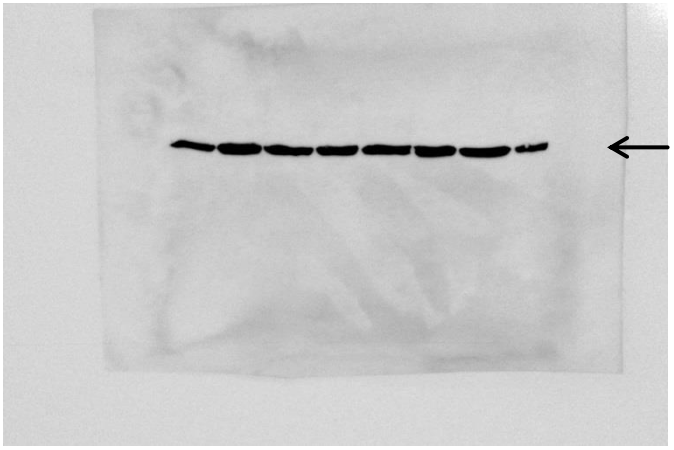


Figure 8 D

α-p53 (Novocastra, NCL-p53-CM5p)



α-ß-Actin (Sigma-Aldrich, A2228)



α- H-Ras (Santa Cruz #sc-520)

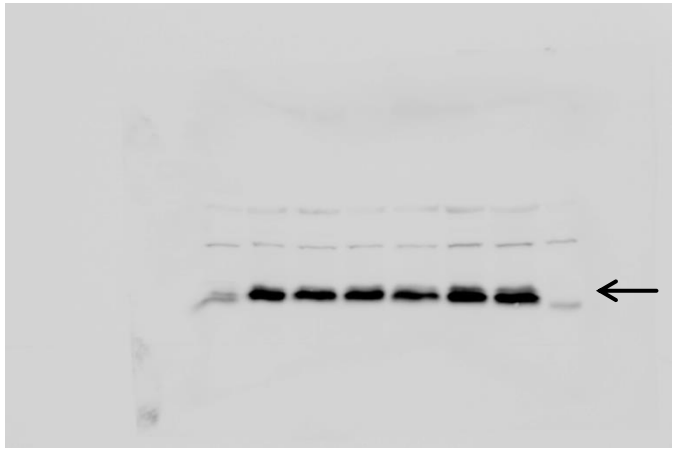
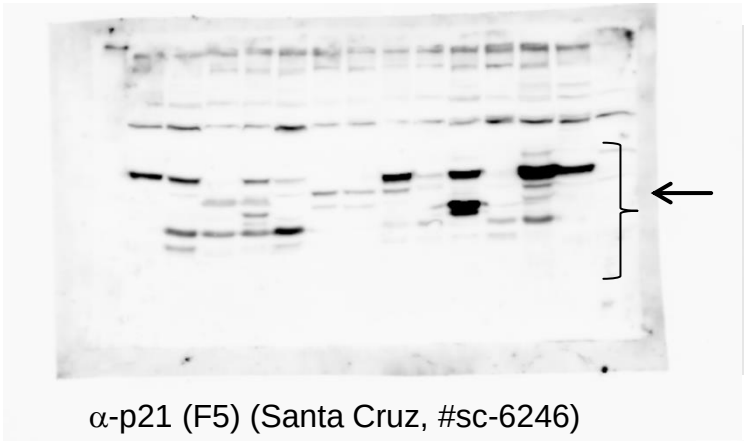


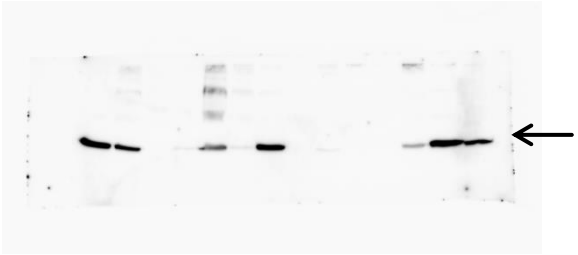
Figure 8 G part 1

α-p53 (Novocastra, NCL-p53-CM5p)

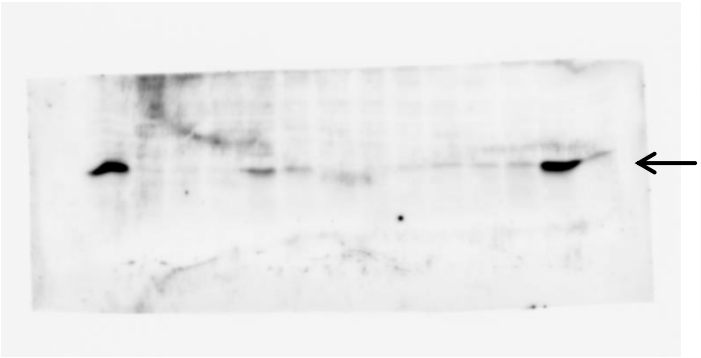


α-ß-Actin (Sigma-Aldrich, A2228)

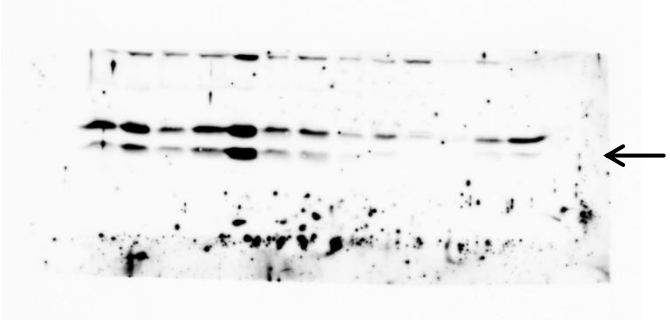
α-Pten (Santa Cruz, #sc-7974)



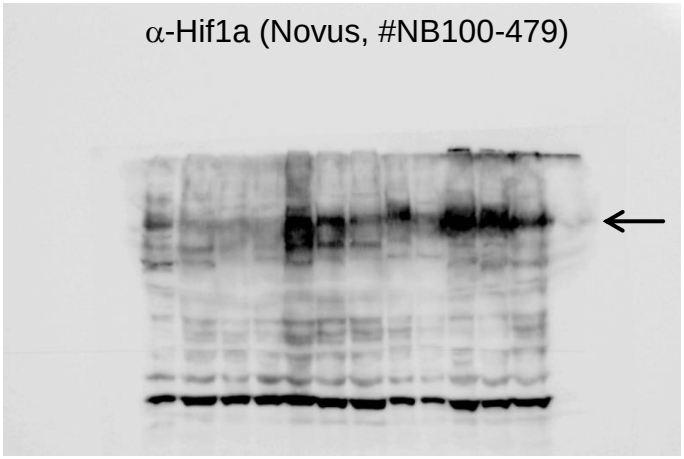
α-p21 (F5) (Santa Cruz, #sc-6246)



α-pVhl (Santa Cruz, #sc-5575)



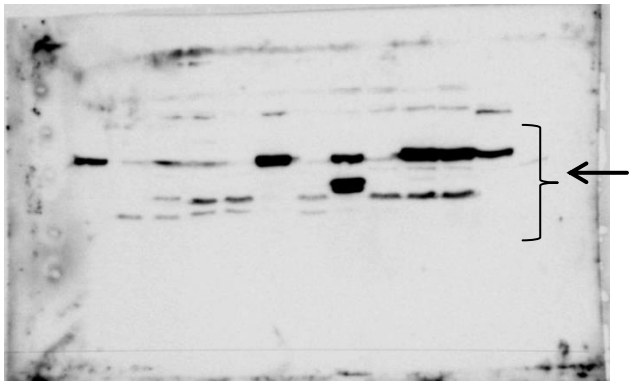
α-Hif1a (Novus, #NB100-479)



α-ph-Akt (Ser473)(193H12) (Cell Signaling, #4058))

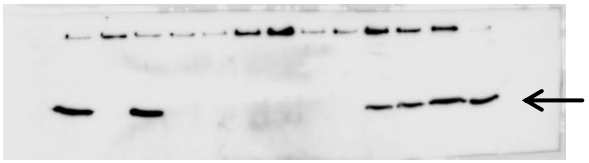
Figure 8 G part 2

α-p53 (Novocastra, NCL-p53-CM5p)

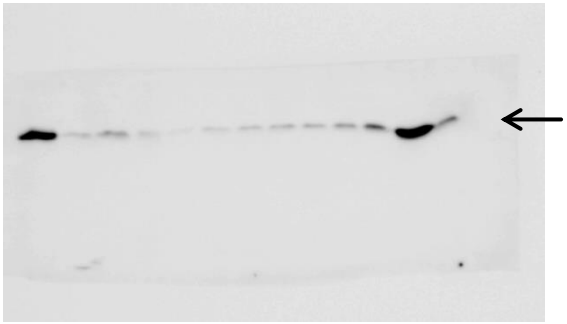


α-ß-Actin (Sigma-Aldrich, A2228)

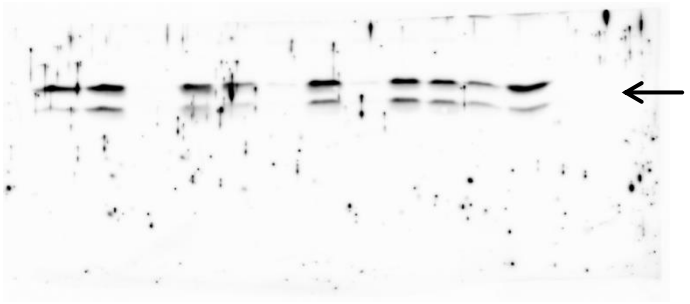
α-Pten (Santa Cruz, #sc-7974)



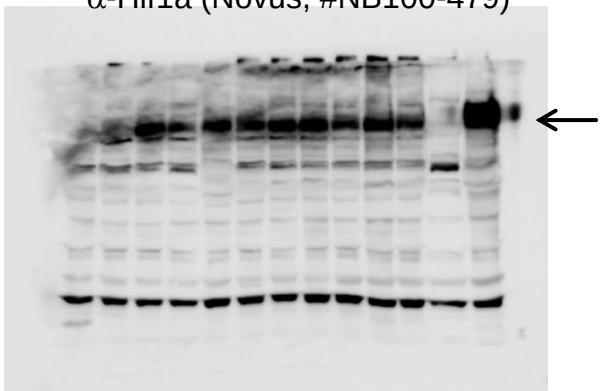
α-p21 (F5) (Santa Cruz, #sc-6246)



α-pVhl (Santa Cruz, #sc-5575)



α-Hif1a (Novus, #NB100-479)



α-ph-Akt (Ser473)(193H12) (Cell Signaling, #4058))

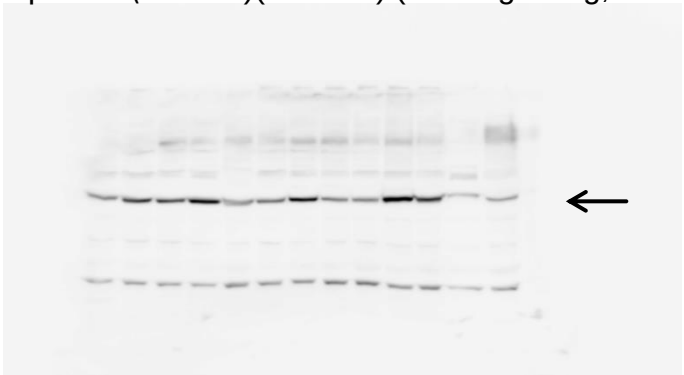
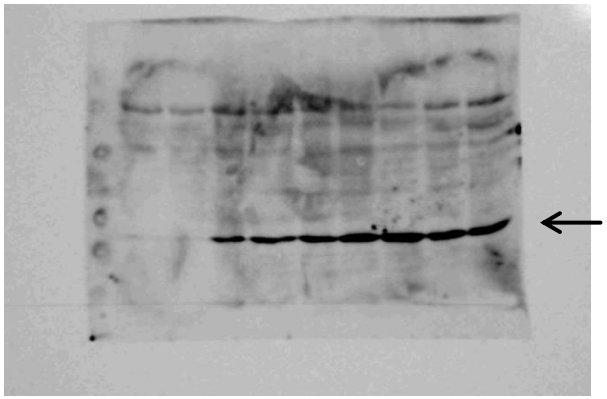
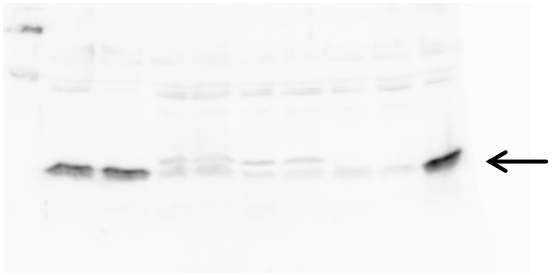


Figure 9C

α-GFP (Life Technologies, #G10362)

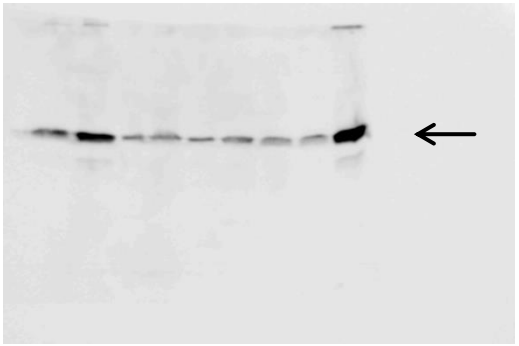


α-p16 (Santa Cruz, #sc-1207)

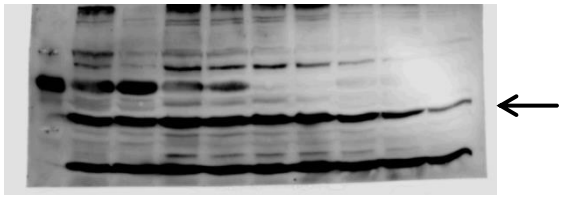


α-ß-Actin (Sigma-Aldrich, A2228)

α-p19 (Santa Cruz, #sc-32748)



α-p53 (Novocastra, NCL-p53-CM5p)



α- H-Ras (Santa Cruz #sc-520)

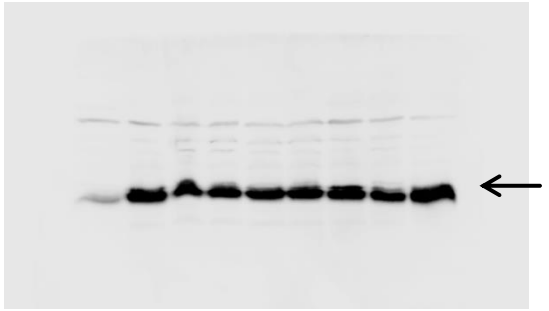
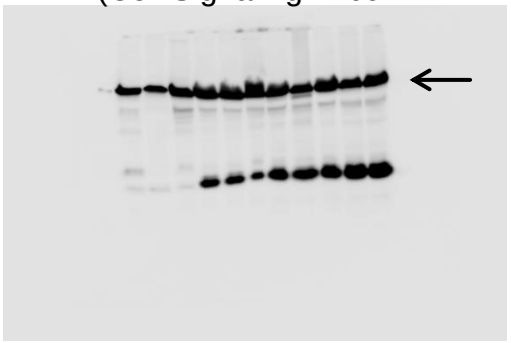
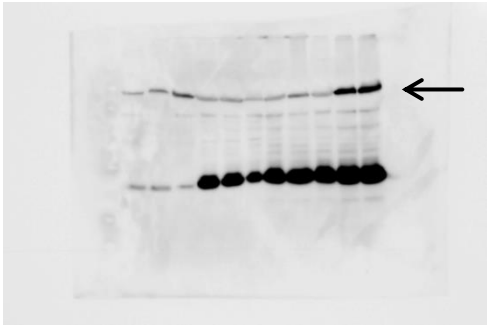


Figure 10 O

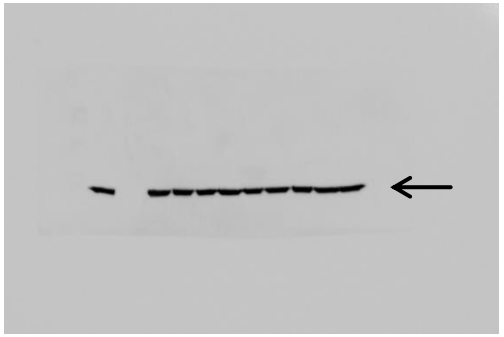
α-Akt (pan) (C67E7)
(Cell Signaling #4691



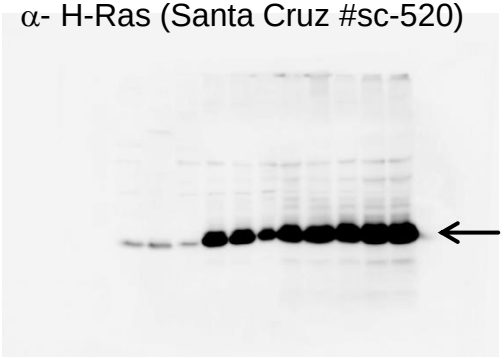
α-ph-Akt (Ser473)(193H12)
(Cell Signaling, #4058))



α-ß-Actin (Sigma-Aldrich, A2228)

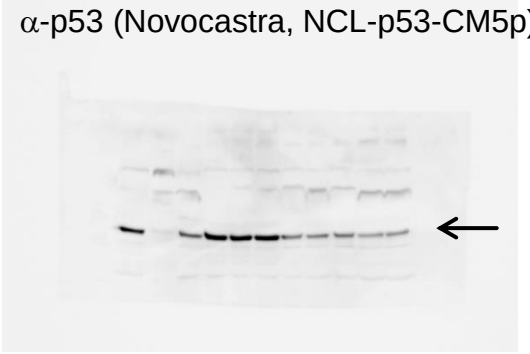


α- H-Ras (Santa Cruz #sc-520)

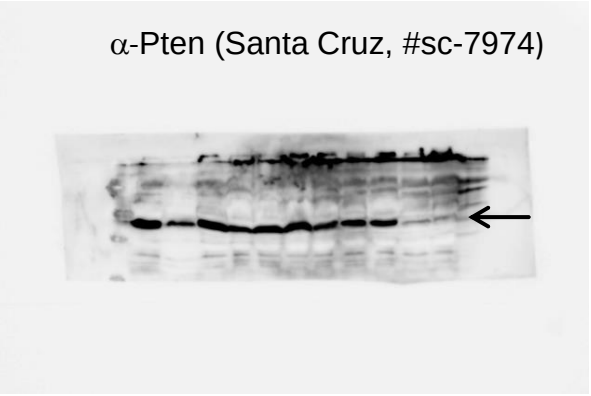


α-p19 (Santa Cruz, #sc-32748)

α-p53 (Novocastra, NCL-p53-CM5p)



α-Pten (Santa Cruz, #sc-7974)



α-p16 (Santa Cruz, #sc-1207)

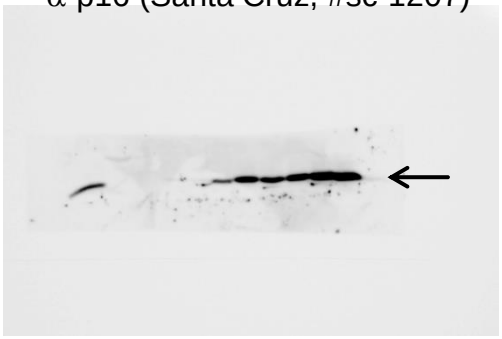
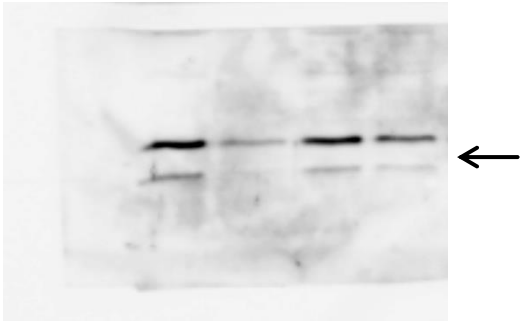


Figure S3 B

α-pVhl (Santa Cruz, #sc-5575)



α-ß-Actin (Sigma-Aldrich, A2228)

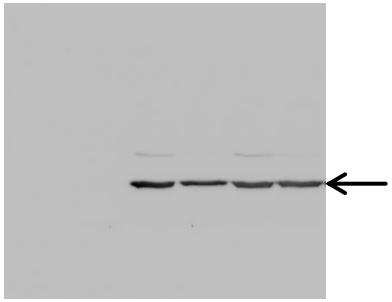
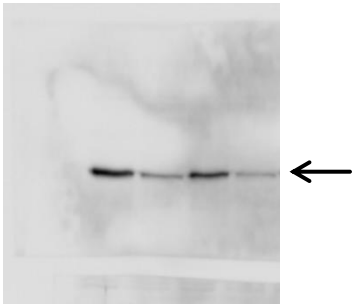


Figure S3 C

α-Pten (Santa Cruz, #sc-7974)



α-ß-Actin (Sigma-Aldrich, A2228)

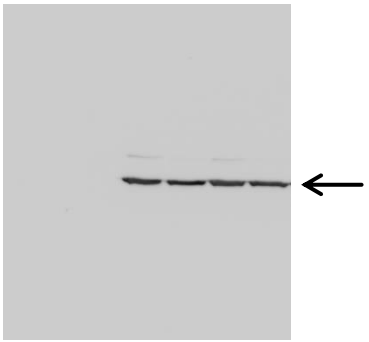
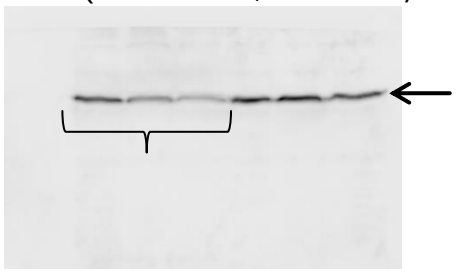
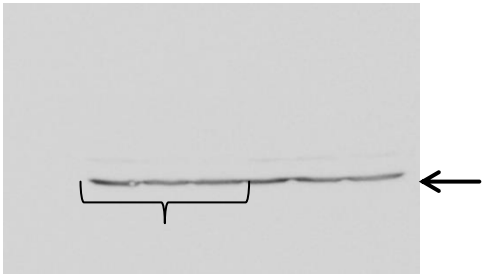


Figure S3 E

α-Pten (Santa Cruz, #sc-7974)



α-ß-Actin (Sigma-Aldrich, A2228)



α-pVhl (Santa Cruz, #sc-5575)

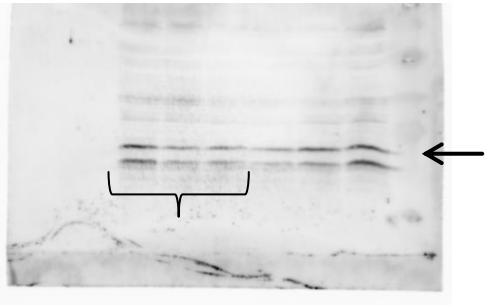
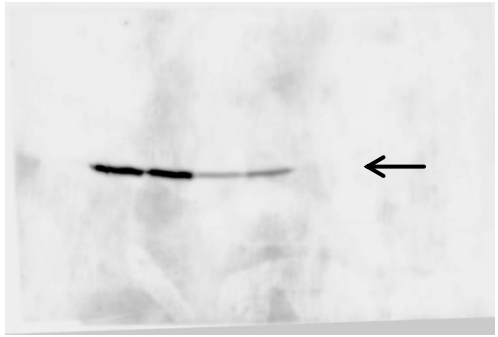
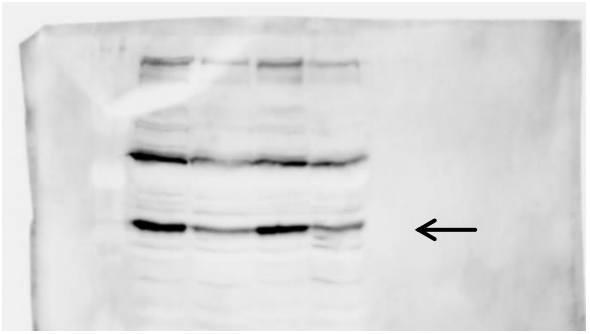


Figure S3 G

α-Pten (Santa Cruz, #sc-7974)



α-p53 (Novocastra, NCL-p53-CM5p)



α-ß-Actin (Sigma-Aldrich, A2228)

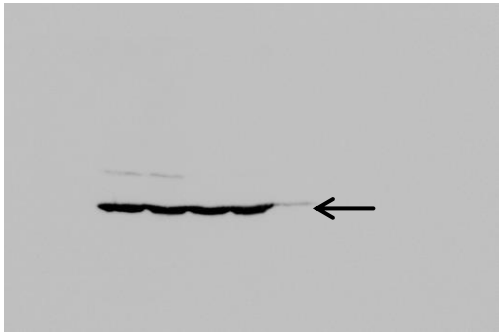
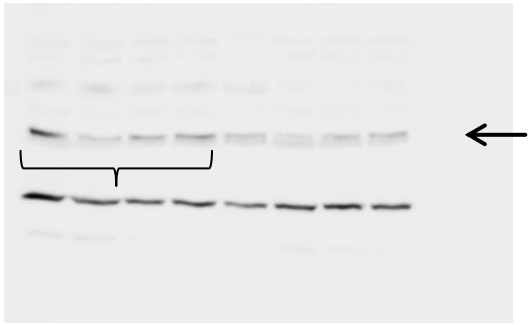


Figure S3 I

α-p53 (Novocastra, NCL-p53-CM5p)



α-ß-Actin (Sigma-Aldrich, A2228)

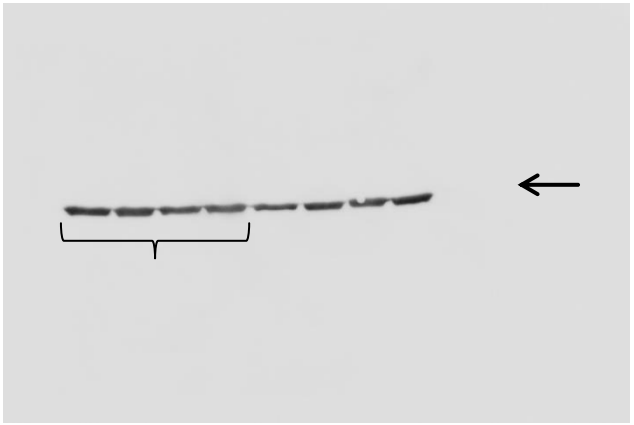
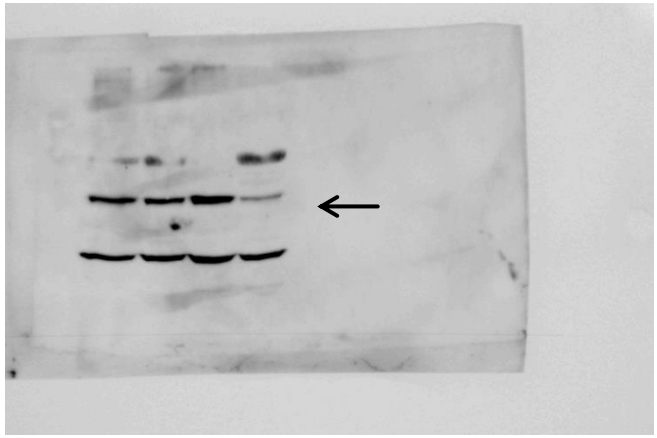


Figure S3 L

α-p53 (Novocastra, NCL-p53-CM5p)



α-ß-Actin (Sigma-Aldrich, A2228)

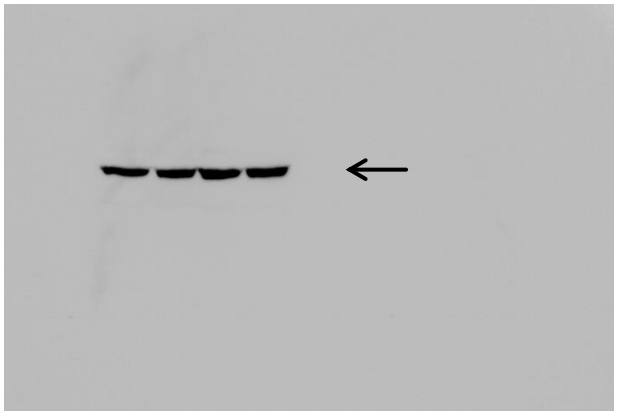


Figure S4 B

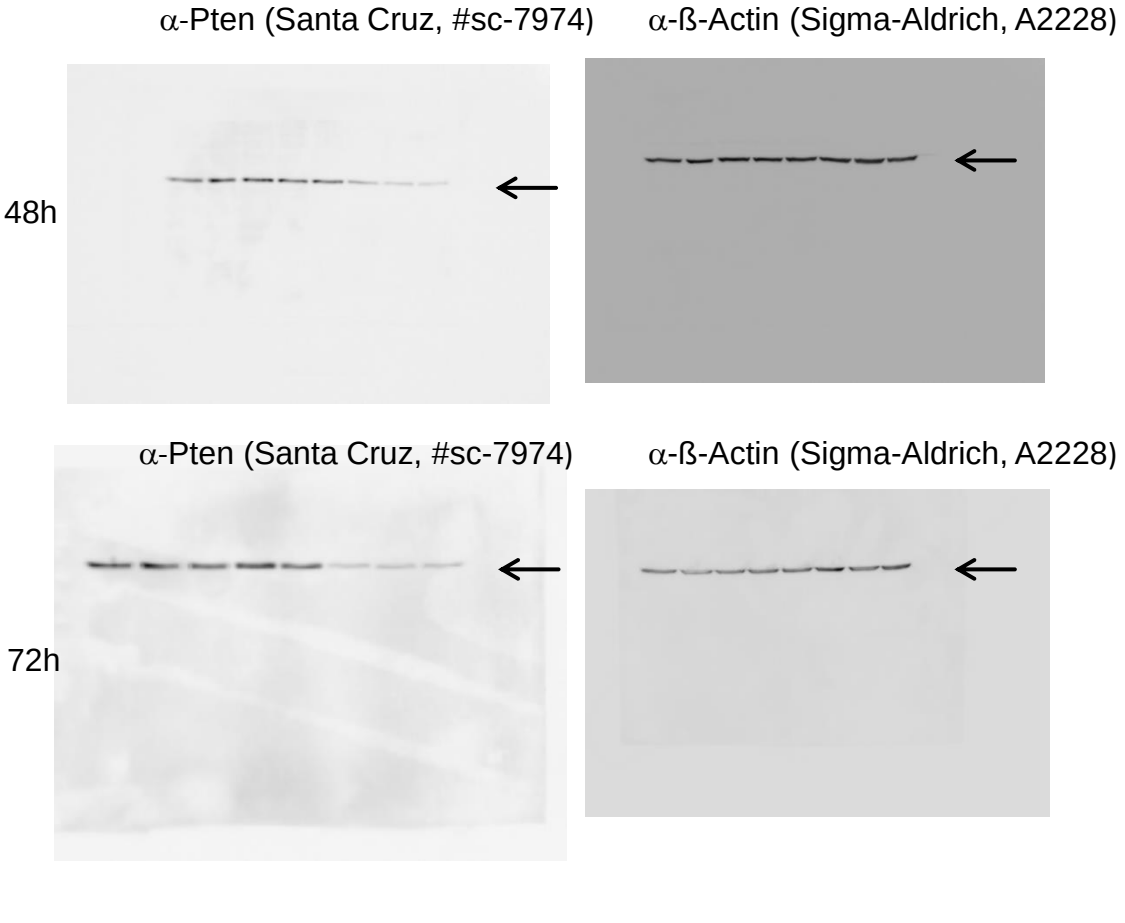


Figure S4D

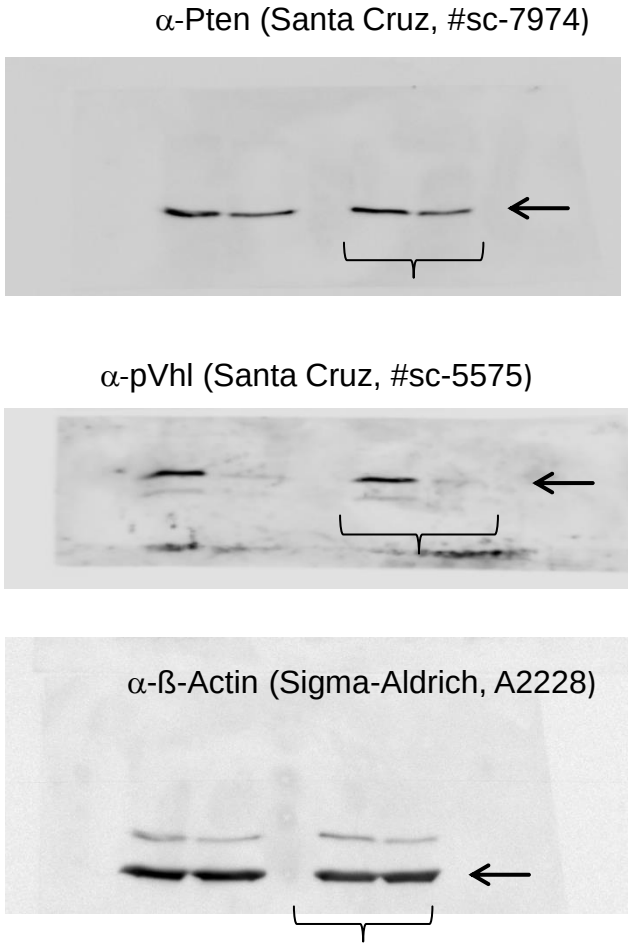
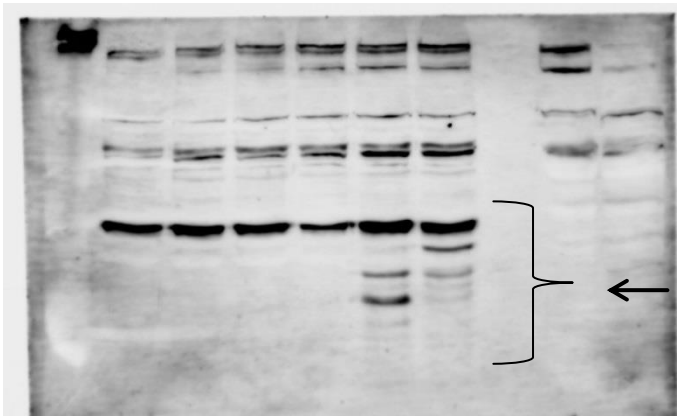
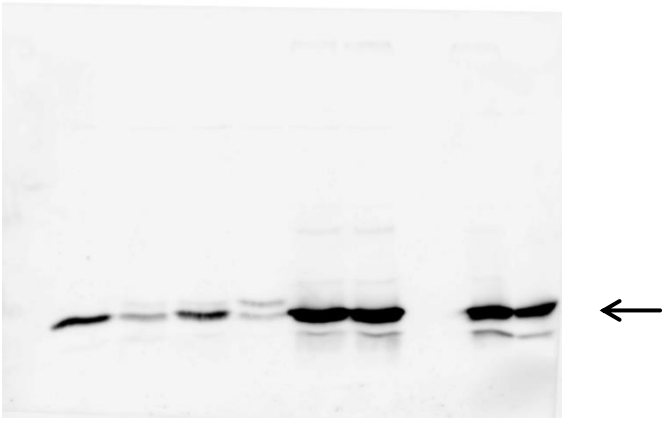


Figure S7 C

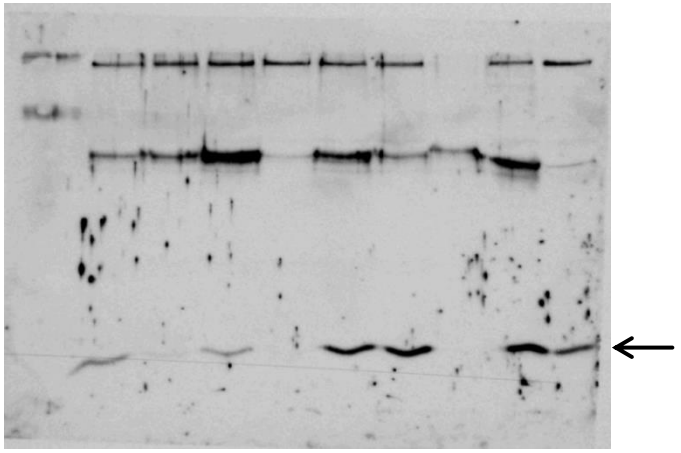
α-p53 (Novocastra, NCL-p53-CM5p)



α-p19 (Santa Cruz, #sc-32748)



α-p16 (Santa Cruz, #sc-1207)



α-ß-Actin (Sigma-Aldrich, A2228)