Supplemental Experimental Procedures

Cell Culture, transfection and viral infections

U2OS, MDAMB231, MCF7, HCC1937, HeLa S, and HCT116 cells were kept in Dulbecco modified Eagle medium (DMEM, GIBCO) or RPMI medium (Lonza) supplemented with 10% fetal calf serum. HeLa S cells in spinner cultures were kept in phosphate buffered Dulbecco modified Eagle medium with 10% bovine serum for large scale nuclear extract preparation. Murine embryonic stem cells were described previously (Rathmell et al., 2004) and were grown in DMEM supplemented with 15% fetal calf serum, 0.1 mM non-essential amino acids, 0.1 mM 2-mercaptoethanol, 2mM glutamine, penicillin-strepromycin, nucleosides, and 10 ng/ml LIF. Transfections were conducted according to manufacturers' instructions using lipofectamine 2000 (Invitrogen). HCT116 cells were infected with shRNA lentiviruses directed against VHL (Open Biosystems) and selected with puromycin.

Expression vector cloning and immunoblots

Full length or partial cDNAs for human VHL and KLF4 were subcloned into a CMV promoter driven vectors with or without FLAG-HA tag for mammalian expression. Point mutants were created by site-directed mutagenesis (Stratagene). Full length VHL was subcloned into the pGEX-4T3 vector (GE Healthcare) for bacterial purification. Antibodies used were the following: VHL (Cell Signaling), KLF4 (Santa Cruz and R&D Systems), actin, HA, tubulin from Santa Cruz. For whole cell extracts cells were washed in PBS and harvested on the plates in standard RIPA buffer with protease inhibitor. After freeze thawing thrice, cell debris was cleared by centrifugation at 14,000 x g for 10 minutes. Protein concentration of the supernatant was measured by BCA Assay (Novagen). After SDS polyacrylamide gel electrophoresis (PAGE), proteins were transfered to nitrocellulose membranes (Optitran, Schleicher and Schüll).

Proliferation assays and BrdU staining

Cell proliferation was measured by the MTS assay. Briefly, HCT116 cells or knockdown derivatives were seeded into 96-well plates (1000-5000 cells per well). Following the

next day, the absorbance at 490 nm was measured in 24 h intervals after addition of 100 µl fresh medium and 20 µl CellTiter 96 Aqueous One Solution (Promega) and incubated at 37°C for 2 h. BrdU staining was done with a kit from Roche Applied Science according to manufacturer's instruction. The incubation time for BrdU incorporation was 45 minutes. For FACS analysis a kit from BD Pharmingen was used according to manufacturer's instruction.

Soft agar colony formation assays

HCT116 were infected with lentiviruses expressing wild type or mutant KLF4. The soft agar is sandwiched with 0.6%-0.3%-0.6% agar and cells are seeded in the 0.3% agar. 10,000 cells were seeded on soft agar in 6-well plates and incubated at 37°C for 10 days. Plates were stained with Crystal violet. 5 random microscope views at two different magnifications were used to count colony density in each plate. Data represent the average of three experiments.

Immunohistochemical staining

Tissue sections were dewaxed with xylene and rehydrated through gradient ethanol into water. For antigen retrieval, sections were heated in citrate buffer (pH6.0) for 10 min at 95°C in a microwave oven. After cooling to room temperature, the sections were then digested with 0.05% trypsin for 10 min at 37°C. Endogenous peroxidase activity was quenched with 0.3% H_2O_2 in methanol for 30 min at room temperature. After PBS washes, nonspecific antibody binding was blocked by pre-incubating slides with 10% normal goat non-immune serum at 37° for 30 min. After blotting off the blocking serum, sections were incubated with primary antibody against KLF4 (1:100 dilution) as well as primary antibody against VHL (1:100 dilution) at 4°C overnight. After PBS washes again, sections were incubated with biotinylated secondary antibody at 1:200 dilution for 30 min at room temperature. After incubating with Vectastain ABC reagent (Vector Laboratories, Inc., Burlingame, CA) for 30 min at room temperature, the sections were developed with DAB (Sigma-Aldrich). Sections were washed in running tap water and lightly counterstained with hematoxylin, followed by dehydration and coverslip mounting. Negative controls were obtained by omitting the primary antibody.

To evaluate the expression KLF4 and VHL, the percentage of positive tumor cells was determined semi-quantitatively by assessing the entire tumor section. Each sample was assigned to one of the following categories: 0 (0-4%), 1 (5-24%), 2 (25-49%), 3 (50-74%), or 4 (75-100%). The intensity of immunostaining was determined as 0 (negative), 1+, (weak), 2+, (moderate) or 3+, (strong). A final immunoreactive score between 0 and 12 was calculated by multiplying the percentage of positive cells with the staining intensity score. All slides were blind evaluated for immunostaining without any knowledge of the clinical outcome of other clinical or pathological data. Statistical analysis was performed using the SPSS statistical software (SPSS Inc., Chicago, IL). The results were presented as means±SD. $P < 0.05$ was considered statistically significant.

Supplemental Figure Legends

Figure S1. **pVHL immunoblot and Coomassie stain of baits for interaction study**
(**A**) Immunoblot of the large and small isoforms of pVHL. Anti-FLAG (M2 agarose) immunoprecipitates from nuclear extracts of HeLa cells (control) or HeLa cells stably transfected with a retrovirus expressing FLAG-tagged KLF4 were peptide eluded and analyzed by immunoblot for pVHL. Both pVHL isoforms are detected by the antibody in the nuclear extract input, but only the large isoform (ISO 1) co-precipitates with KLF4. (**B**) Endogenous pVHL binds to endogenous KLF4. Nuclear extract of HCT116 was incubated with an antibody against KLF4 or a control immunoglobulin (Ig). Precipitates were tested for pVHL by immunoblot. (**C**) Coomassie stain of GST and GST-pVHL proteins used for interaction studies. The asterisk denotes the band for the full-length protein. (**D**) KLF4 levels in wild type HeLa cells and HeLa cells stably expressing tagged KLF4. Untagged (u) and tagged (t) KLF4 levels in whole cell lysates of wild type HeLa cells and HeLa cells stably transfected with a retrovirus expressing FLAG-tagged KLF4 were probed for KLF4 expression by immunoblot with an antibody against KLF4. (**E**) Bacterially purified KLF4 interacts with bacterially purified pVHL. His-tagged KLF4 was expressed in E. coli and partially purified over a NiNTA column. GST-pVHL and GST purified from E. coli were immobilized on GTT-sepharose and incubated for 4 hours with His-tagged KLF4 in BC buffer containing 150mM KCl. After four washes

with the binding buffer beads were directly boiled in SDS loading buffer before analysing by immunoblotting for KLF4. GST-pVHL without added KLF4 served as negative control.

Figure S2. **Efficiency of VHL knockdown in HCT116**

(**A**) Three different shRNA lentiviruses against human VHL were tested for the efficiency to knockdown VHL by real time PCR. #3 was chosen as vector for knockdown studies.

Figure S3. **KLF4 levels and turnover in cancer cells**

(**A**) KLF4 levels vary in cancer cell lines from different tissues. Equal protein amounts of whole cell lysates from cancer cells from colon (HCT116), lung (U2OS), and breast (HCC1937, MCF7, MDAMB231) were tested for KLF4 by immunoblot with two different antibodies (1, goat derived; 2, rabbit derived). (**B**) Quantitation of KLF4 levels in HCT116 and MDAMB231 after cycloheximide (CHX) addition.

Figure S4. **The amino-terminus of KLF4**

(**A**) Alignment of the LAP sequence in KLF4 with known degrons in HIF1α and HIF2α. (**B**) Proline 81 independent degradation of KLF4 by pVHL. An expression vector for FLAG-HA-tagged wild type (wt) or proline 81 to alanine mutated (P81A) KLF4 was co-transfected with empty vector or FLAG-HA-tagged VHL vector. Equal protein amounts of whole cell lysates were probed for KLF4 and pVHL levels by immunoblot with an antibody against the hemagglutinin (HA) tag. (**C**) Sequence of the KLF4 amino-terminus. The potential ubiquitylation sites, lysines 23 and 43, are highlighted.

Figure S5. **KLF4 knockdown efficiency by siRNA transfection**

(**A**) Two different siRNA sets against human KLF4, either alone or in combination, were tested for the efficiency to knockdown KLF4 by immunoblot. **(B**) p21 levels in HCT116 cells after KLF4 silencing. Total RNA from HCT116 cells was purified after transfection with siRNA against KLF4 or luciferase and cDNA was prepared. p21 mRNA levels were

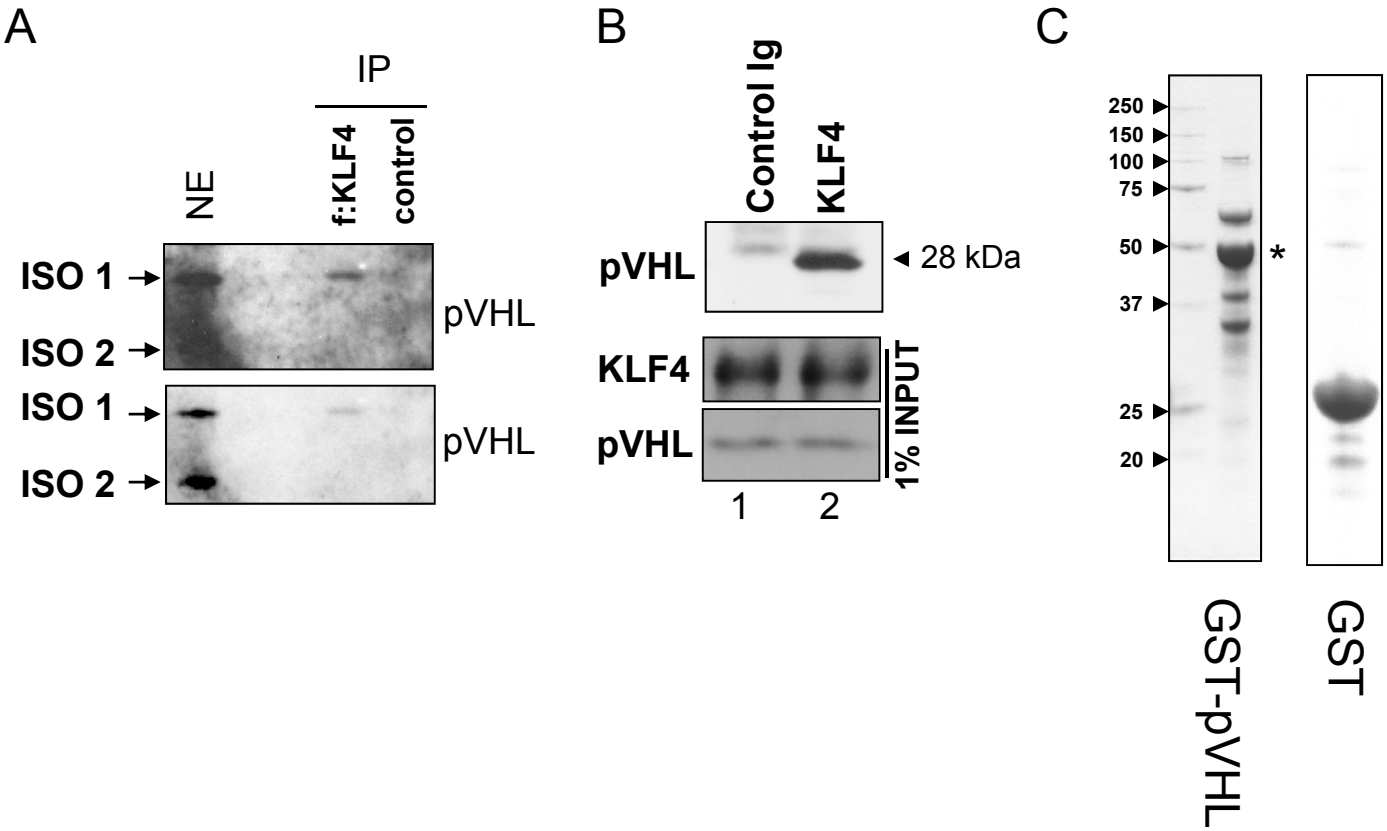
quantified by real time PCR and normalized to β-actin levels. Error bars represent standard deviations.

Figure S6. **Aberrant KLF4 levels in RCC4 cells**

(**A**) Immunoblot of renal cell carcinoma cells. Renal cell carcinoma cells RCC4 and RCC4 cells stably complemented with pVHL were tested for KLF4 expression by imunoblot. HeLa cells served as positive control. No detectable KLF4 band could be observed at the expected size. A faster migrating band could be a cross-reacting protein or a KLF4 truncation/splice mutant.

Figure S7. **Raw data for soft agar assay**

(**A**) Raw data for soft agar assay. Soft agar plates with HCT116 or HCT116 infected with lentiviruses expressing the indicated KLF4 mutant (wt, wild type) were inspected by randomly choosing 5 views under 2 different magnifications and colonies counted. Data from 3 experiments were pooled. (**B**) Immunoblot of infected HCT116. Cells used for the soft agar assay were tested for KLF4 expression.


A
IP
NE
f:KLF4
control
ISO 1
ISO 2
pVHL
ISO 1
ISO 2
pVHL
B
Control Ig
KLF4
pVHL
28 kDa
KLF4
pVHL
1% INPUT
1
2
C
250
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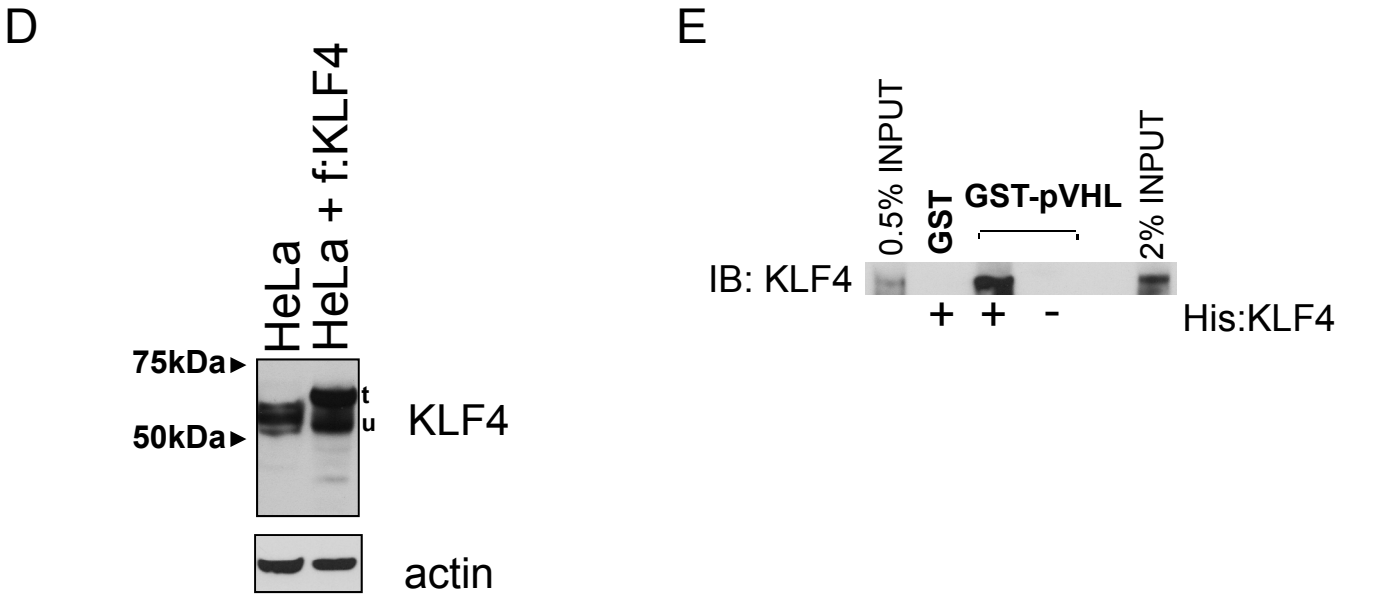
25
20
*
GST-pVHL
GST


D
HeLa
HeLa + f:KLF4
75kDa
50kDa
t
u
KLF4
actin
E
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GST
GST-pVHL
2% INPUT
IB: KLF4
+
+
-
His:KLF4

Fig.S1

A

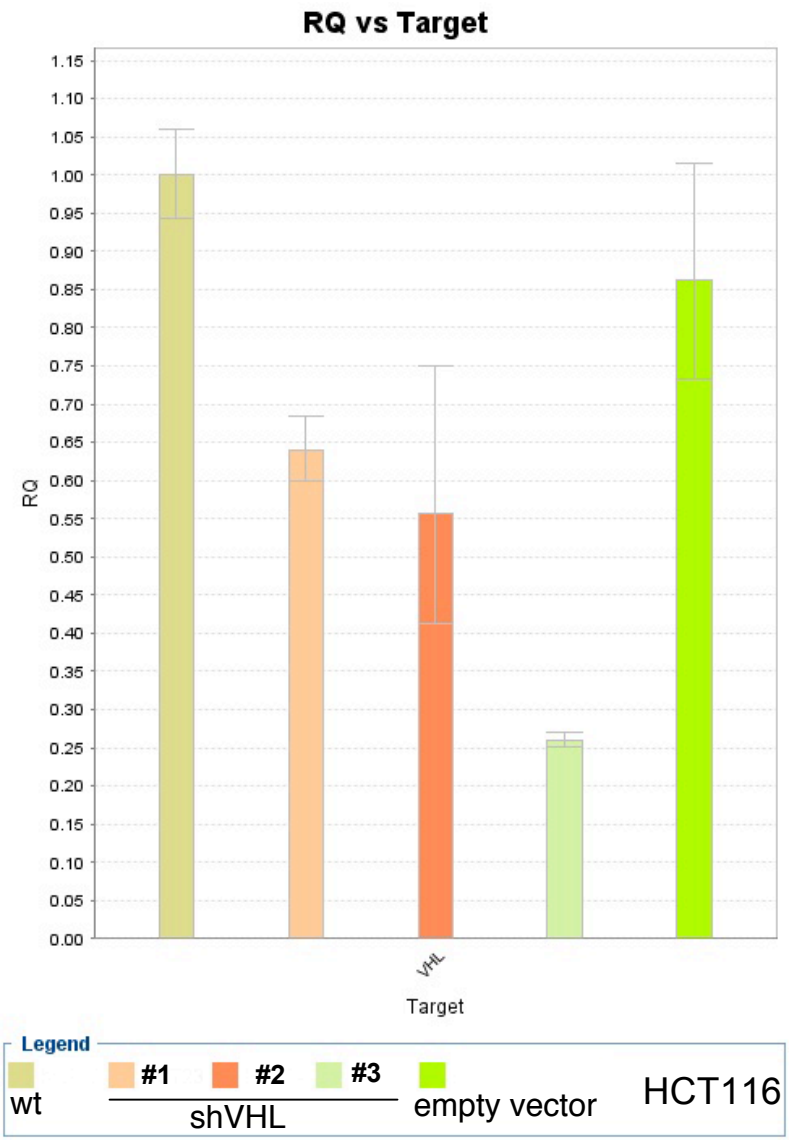
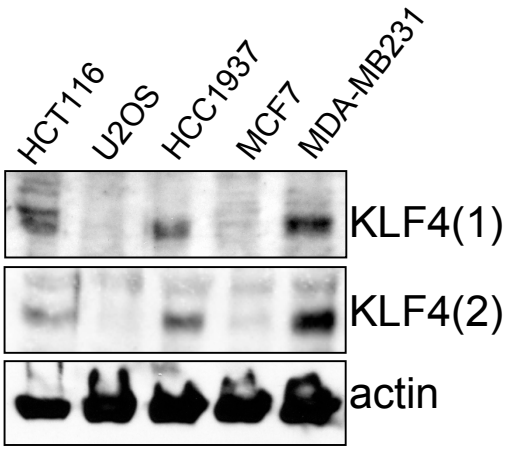

RQ vs Target
RQ
1.15
1.10
1.05
1.00
0.95
0.90
0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
VHL
Target
Legend
wt
#1
#2
#3
shVHL
empty vector
HCT116

Fig.S2

A


HCT116
U2OS
HCC1937
MCF7
MDA-MB231
KLF4(1)
KLF4(2)
actin

B

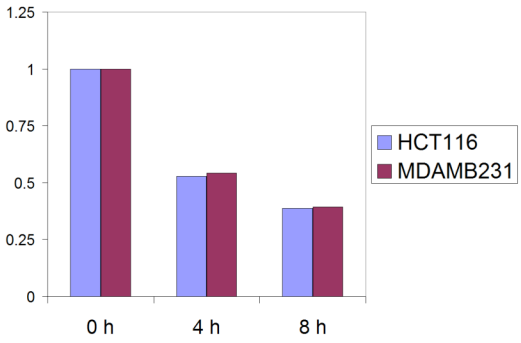
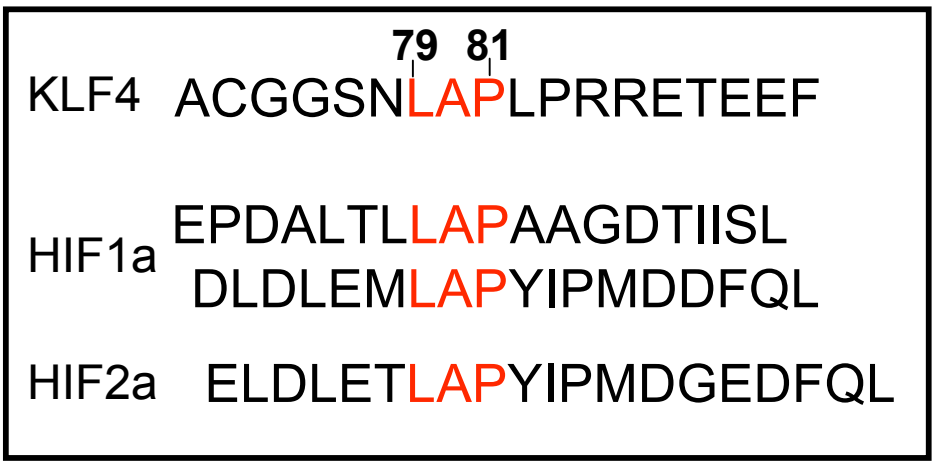
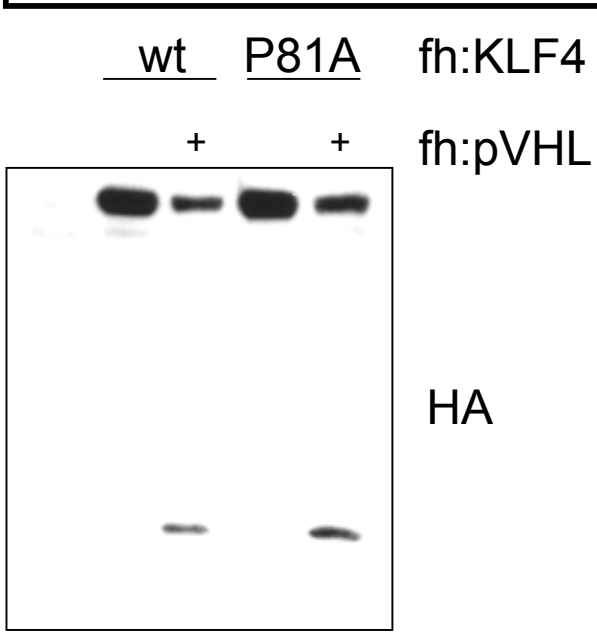

1.25
1
0.75
0.5
0.25
0
0 h
4 h
8 h
HCT116
MDAMB231

Fig.S3

A


79
81
KLF4 ACGGSNLAPLPRRETEEF
HIF1a EPDALTLLAPAAGDTIISL
DLDLEMLAPYIPMDDFQL
HIF2a ELDLETLAPYIPMDGEDFQL

B


wt
P81A
fh:KLF4
+
+
fh:pVHL
HA

C

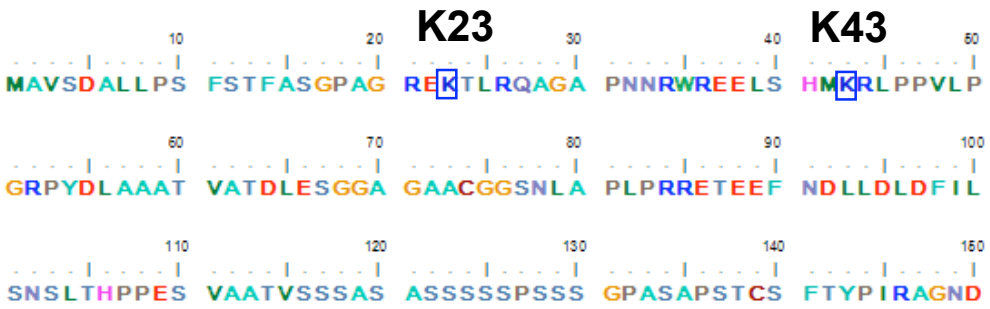
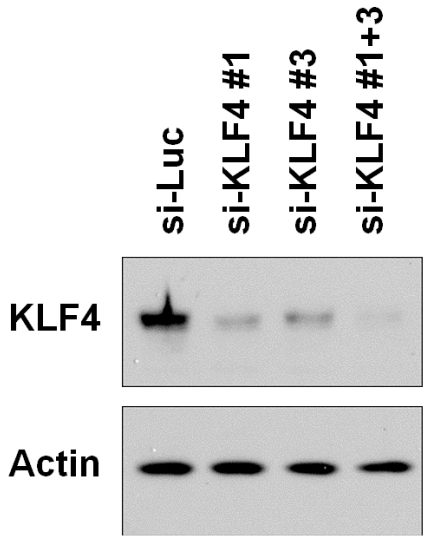

K23
K43
10 20 30 40 50
MAVSDALLPS FSTFASGPAG REKTLRQAGA PNNRWREELS HMKRLPPVLP
60 70 80 90 100
GRPYDLAAAT VATDLESGGA GAACGGSNLA PLPRRETEEF NDLLDLDFIL
110 120 130 140 150
SNSLTHPPES VAATVSSSAS ASSSSSPSSS GPASAPSTCS FTYPIRAGND

Fig.S4

A


si-Luc
si-KLF4 #1
si-KLF4 #3
si-KLF4 #1+3
KLF4
Actin

B

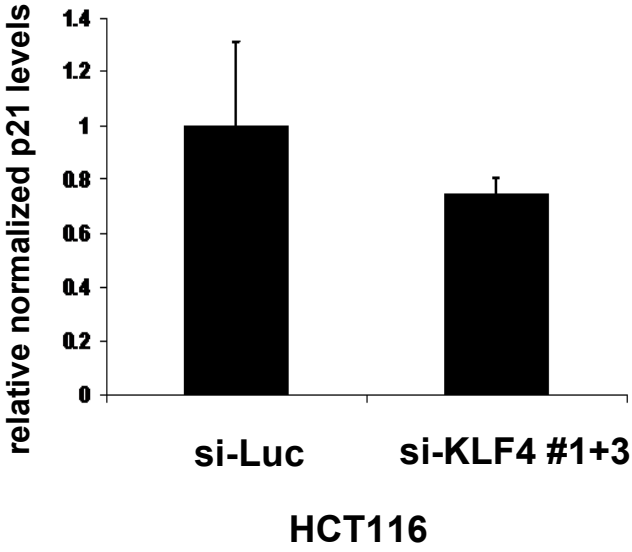

relative normalized p21 levels
1.4
1.2
1
0.8
0.6
0.4
0.2
0
si-Luc
si-KLF4 #1+3
HCT116

Fig.S5

A

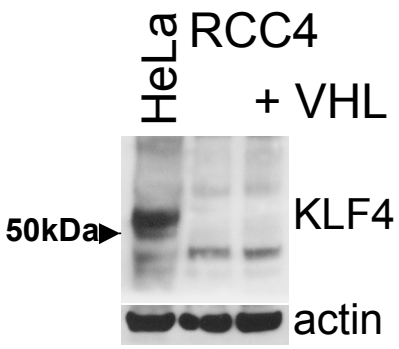


Fig.S6

A

Raw Data

HCT116		+ wt KLF4		+ KLF4 K43R		+ KLF4 K23,43R	
718	145	367	83	32	6	44	10
809	148	286	59	43	9	17	3
827	127	220	85	29	4	29	6
807	141	294	94	35	9	44	10
776	169	247	77	30	7	48	11
914	166	295	74	34	8	32	9
685	150	380	60	33	8	15	3
729	184	301	48	31	6	28	6
760	194	297	71	39	8	34	7
825	182	441	67	42	8	32	9
857	194	312	70	54	11	18	5
693	185	302	68	38	7	39	9
680	109	341	66	37	7	41	9
774	128	379	95	57	12	40	8
658	163	306	84	36	6	55	11

5 random views were chosen under two different magnifications per experiment (15 views per magnification in total). Numbers in black represent colonies observed under low magnification, blue numbers under high (30x) magnification.

B

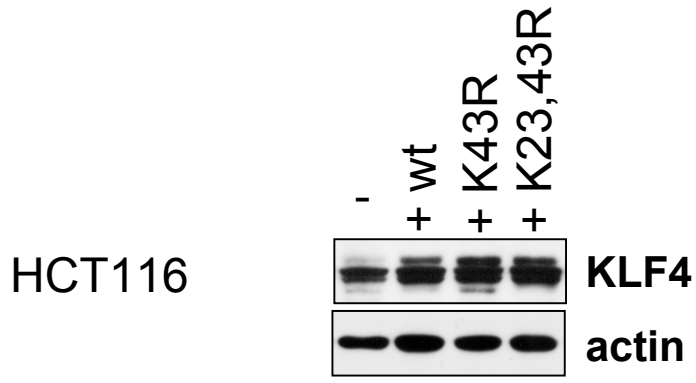


Fig.S7