CHAPTER 2

Materials and methods

2.1 Materials

2.1.1 Plasmids

Lentiviral vectors (pLKO.1) encoding various shRNA sequences against human *IP6K1* and mouse *Ip6k2* were obtained from Sigma-Aldrich to generate transient and stable knockdowns. shRNA clone IDs and their representation in the thesis are given below.

Table 2.1: Plasmids expressing shRNA against human *IP6K1* used for generating stable cells in HeLa and HCT116.

S.No	TRC number of shRNA	Clone ID	Representation in the text
1	SHC016	Non-targeting shRNA control	NT
2	TRC0000013508	NM_153273.1-1513s1c1	sh*IP6K1-1*
3	TRC0000013511	NM_153273.1-1172s1c1	sh*IP6K1-2*
4	TRC0000199665	NM_153273.3-1287s1c1	sh*IP6K1-3*
5	TRC0000196808	NM_153273.3-571s1c1	sh*IP6K1-4*
6	TRC0000199561	NM_153273.3-421s1c1	sh*IP6K1-5*

Table 2.2: Plasmids expressing shRNA against mouse *Ip6k2* used for generating stable cells in MEFs are listed below.

S.No	TRC number of shRNA	Clone ID	Representation in the text
1	TRCN0000202175	NM_029634.1-317s1c1	sh*Ip6k2*
2	TRCN0000191589	NM_029634.1-866s1c1	sh*Ip6k2*
3	TRCN0000202257	NM_029634.1-1151s1c1	sh*Ip6k2*
4	TRCN0000202065	NM_029634.1-311s1c1	sh*Ip6k2*

Other plasmids used for lentivirus generation: VSV-G, VSV-GP (gifts from Dr. Renu Wadhwa, AIST, Japan) and psPAX2 (a gift from Dr. Didier Trono, Addgene plasmid # 12260).

2.1.2 Cell lines:

The cell lines used in the study are mouse embryonic fibroblasts (MEFs) derived from wild type (WT) and *Ip6k1* knockout mouse embryos. The MEFs were immortalized with SV40 large T antigen (Bhandari *et al.*, 2008) and single cell derived lines were generated in the lab. *Ip6k1* knockout MEFs display 70% lower levels of IP_7 compared with wild type MEFs (Bhandari *et al.*, 2008). *Ip6k1*$^{-/-}$ MEFs expressing kinase active or inactive forms of IP6K1 were generated in the lab (Rescue MEFs). MEFs were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies) supplemented with 10% fetal bovine serum (FBS, Life Technologies), 1 mM L-Glutamine (Life Technologies), 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies). Rescue MEFs were cultured in complete DMEM supplemented with G418 (200 µg/mL) as selection marker. HCT116 (colon cancer cells, a gift from Dr. Sagar Sengupta, NII, New Delhi) or HeLa (cervical cancer cells) expressing non-targeting control and shRNA against human *IP6K1* were cultured in complete DMEM containing puromycin (2µg/mL). The amphotropic Phoenix cells (a gift from Dr. Shweta Tyagi, CDFD, Hyderabad) and HEK293T packaging cells were used for generating lentiviral particles containing shRNA against human *IP6K1* or mouse *Ip6k2* and were maintained in complete DMEM.

2.1.3 Mice

All animal experiments were conducted as per guidelines provided by the Committee for the Purpose of Control and Supervision of Experiments on Animals, Ministry of Environment, Forest, and Climate Change, Government of India, and these experiments were approved by the Institutional Animal Ethics Committee (Protocol numbers PCD/CDFD/02-version 2 and PCD/CDFD/08). Mice used for this study were housed in the Centre for DNA Fingerprinting and Diagnostics animal facility located within the premises of Vimta Labs, Hyderbad. *Ip6k1*$^{+/-}$ heterozygous mice were bred to generate age and sex matched *Ip6k1*$^{+/+}$ and *Ip6k1*$^{-/-}$ littermates for experiments. *Foxn1*^{nu} mice were generated by breeding homozygous males with heterozygous females. These mice were used for *in vivo* tumourigenic assays.

2.1.4 Chemicals:

Cell culture reagents: fetal bovine serum (FBS, 26140-079), L-glutamine (25030-081), penicillin-streptomycin (15140-122) and trypsin (25200-056) were from Life Technologies. DNA damaging agents used for this study were obtained from following sources: hydroxyurea (HU; H8627, Sigma-Aldrich), neocarzinostatin (NCS, N9162, Sigma-Aldrich), mitomycin C (M0503, Sigma-Aldrich). Reagents used for cell spreading assays: methyl cellulose (Sigma-Aldrich), fibronectin (F2006, Sigma-Aldrich) and fluorophore conjugated phalloidin (Molecular Probes Inc). Antibiotic selection markers: puromycin (Sigma-Aldrich), G418 (Sigma-Aldrich), transwell inserts (24 well, 8 µm pore size, Costar, Corning), Invasion chambers (BioCoat Matrigel invasion chamber, 24 well, 8 µm pore size, Corning). Other chemicals: Propylene glycol (151957, MP Biomedicals), 4-Nitroquinoline-1-Oxide (4NQO; N8141**,** Sigma-Aldrich), MTT [(3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide], TNP [N^2-(*m*-(trifluoromethy)lbenzyl) N^6-(*p*-nitrobenzyl)purine], DMSO (dimethyl sulfoxide), ethanol, paraformaldehude, vectashield DAPI (Vector labs), Tris, PMSF, NP-40, PBS, Tween-20, BSA, $MgCl_2$, colcemid, KCl, methanol, glacial acetic acid, giemsa, SDS, sodium bicarbonate (S5761, Sigma-Aldrich), polyfect transfection reagent, crystal violet, propidium iodide, Triton X-100 and PEI were obtained from Sigma-Aldrich. Low melting agarose (Difco), ECL detection system (GE Healthcare).

2.1.4 Kits:

Click-iT cell proliferation kit (C35002, Invitrogen),

Apo-BrdU TUNEL assay kit (A35127, Invitrogen).

2.1.5 Antibodies

Table 2.3: Antibodies used in this study

Antibody	Catalog no	Company	Dilution	Application
		Primary antibodies		
Rabbit anti-BLM	A310-167A	Bethyl laboratories	1:400	Immunoflourescence (IF)
Rabbit anti-Rad51	PC130	Calbiochem	1:250	IF
Rabbit anti- γH2AX	ab2893	Abcam	1:400	IF
Rabbit anti-H3S10	ab5176	Abcam	1:250	IF
Mouse anti-γH2AX	ab26350	Abcam	1:200	IF
Rabbit anti-Mus81	Ab97391	Abcam	1:200	IF
Rabbit anti-IP6K1	HPA040825	Sigma-Aldrich	1:1000	Immunoblot
Goat anti-IP6K1	sc-10419	Santa Cruz Biotechnology	1:1000	Immunoblot
Goat anti-IP6K2 (C-17)	sc-10425	Santa Cruz Biotechnology	1:1000	Immunoblot
Mouse anti-Myc tag ascites	IMG-80155	Imgenex		Immunoblot
Mouse anti-α tubulin	T9026	Sigma-Aldrich	1:5000	Immunoblot
Mouse anti-GAPDH	G8795	Sigma-Aldrich	1:10,000	Immunoblot
Rabbit anti-pFAK (Tyr397)	3283	Cell Signaling Technology	1:1000	Immunoblot
Rabbit anti-FAK	3285	Cell Signaling Technology	1:1000	Immunoblot
Rabbit anti-pPaxillin (Tyr118)	2541	Cell Signaling Technology	1:1000	Immunoblot
Mouse anti-Paxillin	610051	BD Biosciences	1:1000	Immunoblot
Mouse anti-E Cadherin	14472S	Cell Signaling Technology	1:1000	Immunoblot
		Secondary antibodies		
Alexa-488 anti-rabbit	A11034	Life Technologies	1:400	IF
Alexa-594 anti-rabbit	A11012	Life Technologies	1:400	IF
Alexa-488 anti-mouse	A11029	Life Technologies	1:400	IF
Donkey anti-goat HRP conjugated antibody	sc-2020	Santa Cruz biotechnology	1:5000	Immunoblot
Goat anti-rabbit HRP conjugated antibody	4010-05	Southern Biotech	1:5000	Immunoblot
Goat anti-mouse HRP conjugated antibody	1031-05	Southern Biotech	1:10,000	Immunoblot

2.1.6 Buffers

Hypotonic lysis buffer
10 mM Tris Cl (pH 7.4)
2.5 mM $MgCl_2$,
1 mM PMSF
0.5% NP-40

Hypotonic buffer
Prewarmed 0.075 M KCl

Fixatives used in this study
100% ethanol kept overnight at -20°C for immunofluorescence by prelysis protocol

70% ethanol kept overnight at -20°C used for PI based cell cycle analysis

Methanol: glacialactetic acid (3:1) for cytogenetic analysis

4% Paraformaldehyde- 4 g of paraformaldehyde dissolved in 100 mL water

PI staining solution

PBS containing,
0.1% Triton X-100
0.2 mg RNase
20 μg propidium iodide

Reagents required for Tris-Glycine SDS-PAGE

SDS-PAGE 30% Acrylamide solution
29 g of acrylamide and 1 g bi-acrylamide (29:1 ratio) dissolved in 100 mL water

10% SDS
10 g SDS dissolved and 100 mL water

Laemmli buffer
40% Glycerol
240 mM Tris/HCl pH 6.8
8% SDS
0.04% bromophenol blue
5% beta-mercaptoethanol

Stacking gel solution (5%) 5 mL

3.4 mL H_20
0.63 mL 30% acrylamide solution (acrylamide:bis-acrylamide; 29:1)
0.83 mL 1 M Tris (pH 6.8)
0.05 mL 10% SDS
0.05 mL 10% ammonium persulfate (APS)
0.005 mL N,N,N',N'-Tetramethylethylenediamine (TEMED)

Resolving gel solution (12%) 10 mL
3.3 mL H_20
4 mL 30% acrylamide solution (acrylamide:bis-acrylamide; 29:1)
2.5 mL 1.5 M Tris (pH 8.8)
0.1 mL 10% SDS
0.1 mL 10% ammonium persulfate
0.004 mL N,N,N',N'-Tetramethylethylenediamine (TEMED)

SDS-Running buffer (Tris/Glycine/SDS)
25 mM Tris-Cl
192 mM glycine
0.1% SDS

Transfer Buffer
25mM Tris-Cl
190 mM glycine
20% Methanol

MTT dye

MTT dye was dissolved in PBS at 5 mg/mL concentration. Filtered through 0.45 μm syringe filters, and stored in dark at 4ºC.

2.2 Methods

2.2.1 Cell proliferation assay

Cells were seeded in 24 well plates at 10% confluence in triplicates and allowed to grow for different lengths of time. Spent medium was replaced with fresh medium every 48 h. Cells were incubated for different lengths of time ranging from 24 h to 120 h, to allow them to grow and cell survival was monitored by the MTT assay. Metabolically active

cells convert MTT to insoluble purple formazan dye crystals which can be solubilized using detergent or DMSO and measured by colorimetry. At each time point media containing MTT dye (250 µg/mL) was added to each well and incubated at 37°C. After 2 h cells were lysed with 200 µL DMSO for 15 min on a rocker at room temperature to solubilize the formazan and absorbance was monitored at 570 nm using the EnSpire multimode plate reader (PerkinElmer). Doubling time was determined by plotting log [A_{570}] *vs* time and conducting a linear regression analysis using GraphPad Prism 5.

2.2.2 DNA damage and recovery assay

Cells were plated at a density of 30,000 cells per well in triplicates in a 24-well plate. After 24 h, cells at a confluency of 30-40%, were treated with different genotoxic agents such as hydroxyurea (0.5 mM), neocarzinostatin (0.25 µg/mL) and mitomycin-C (1 µg/mL) for 12 h. Spent media containing drug was removed and cells were washed gently twice with PBS. Cells were then incubated for different lengths of time ranging from 24 h to 120 h, in fresh complete DMEM to allow them to recover from genotoxic stress. At each time point cell survival was analyzed by MTT assay as described in 2.2.1. Cell survival was expressed as a fold increase in cell population relative to cells treated with drug for 12 h.

2.2.3 Cell cycle analysis by PI staining

Cells were grown in 35 mm dishes at 20% initial confluence. At 40-50% confluence, cells were treated with different genotoxic agents as described in Section 2.2.2. Post treatment, cells were washed twice with PBS and replaced with fresh media to allow recovery for different lengths of time. At each time point, cells were harvested and fixed with 70% ethanol at -20°C overnight. Fixation was carried out by adding 70% ethanol drop by drop, while the cells were being vortexed at a low speed. The fixed samples were brought to room temperature, pelleted down at 2000 rpm for 3 min and washed twice with PBS. Cells were stained with PI solution containing 0.1% Triton X-100, 0.2 mg RNase and 20 µg propidium iodide and incubated at 37°C for 30 min in the dark. Samples were analyzed by flow cytometry (FACS ARIA, BD). Data was analyzed using FACS DIVA (BD) and FlowJo (FLOWJO, LLC) softwares to identify different stages of the cell cycle.

2.2.4 PI live dead analysis

Cell viability can be monitored by changes in the morphology of the cell or by membrane permeability to certain dyes such as PI. When exposed to PI, viable cells do not take up PI due to the presence of an intact membrane whereas dead cells take-up PI due to loss of membrane integrity. Briefly, cells were treated with HU for 12 h, and allowed to recover for different lengths of time as described in Section 2.2.2. At each time point, cells were harvested by trypsinisation and washed with PBS. Approximately 10^6 cells were resuspended in 1 ml PBS and stained with 2 µg /mL (final concentration) PI for 2 min at room temperature. The extent of PI staining was used to determine the viable and dead population by flow cytometry analysis (Accuri C6, Becton Dickenson). Nonviable cells were approximately 100 fold brighter than the unstained (viable cells) cells.

2.2.5 Cell cycle analysis by EdU labeling

Cell cycle analysis by PI staining is based on the DNA content of cells and cannot distinguish G0 from G1, and G2 from M phase. One more limitation is that it provides overlapping cell populations in different phases. Alternatively, multi parameter based cell cycle analysis can be performed using EdU (5-ethynyl-2'-deoxyuridine, a BrdU alternative) labeling which exclusively distinguishes cells in S phase from other phases of cell cycle.

EdU labeling and cell cycle analysis was conducted using the Click-iT cell proliferation assay kit (C35002, Invitrogen), as per the manufacturer's instructions. Briefly, cells were grown in 35 mm dishes at 30% initial confluence. At 50-60% confluence, cells were treated with 0.2 mM HU for 12 h. After treatment, media containing drug was removed, gently washed twice with PBS and replaced with fresh media. Cells were allowed to recover for different time periods such as 3, 6, 9 and 12 h to observe arrest and release into S-phase. At each time point cells were labeled with 25 µM EdU for 30 min before harvesting by trypsinisation. Harvested cells were washed with 1% BSA in PBS and fixed with 100 µL of ClickiT-fixative containing 3% paraformaldehyde for 15 min at room temperature. After fixation, cells were again washed with 1% BSA in PBS, resuspended in

the same solution and stored overnight at 4ºC. Fixed cells were permeabilized with 100 µL saponin based permeabilisation buffer and wash buffer for 2 min in the dark. Cells were pelleted down and washed twice with saponin based permeabilisation buffer, centrifuged to pellet the cells and the supernatant was aspirated leaving 30-40 µL buffer to dislodge the pellet. EdU was detected by adding 350 µL of Click-iT reaction cocktail and incubated for 30 min at room temperature in the dark. Cells were washed once with permeabilisation buffer and DNA content was measured by adding 5 µL Ribonuclease A and 2 µL of cell cycle dye 633 red incubated for 15 min at room temperature in the dark. Cells were analyzed by flow cytometry (FACS ARIA, Becton Dickenson). Data analysis to determine the stages of the cell cycle was performed using FACS Diva (Becton Dickenson), and results were plotted using GraphPad Prism 5.

2.2.6 Immunofluorescence

Localization of the DNA damage response proteins γH2AX, Rad51 and BLM and mitotic marker H3S10 (Histone H3 phosphorylated on Ser10) upon genotoxic stress and recovery was analyzed by immunofluorescence, following hypotonic lysis. Cells were seeded on cover slips in 12 well plates at 10-15% confluence and incubated overnight. Cells were treated with 0.5 mM hydroxyurea and 0.25 µg/mL neocarzinostatin for 12 h. Coverslips were washed twice with PBS and cells were incubated in hypotonic lysis buffer containing 10 mM TrisHCl (pH 7.4), 2.5 mM $MgCl_2$, 1 mM PMSF and 0.5% NP-40, on ice for exactly 8 min on a shaker at 50-60 rpm. Cells were washed twice on ice, with ice cold PBS for 2 min, with continuous shaking. Fixation was carried out with ice-cold 100% ethanol (which had been kept overnight in -20°C) for 4 min, shaking on ice. 500 µL of ethanol was added to each cover slip during fixation. Cells were washed thrice with 1 mL PBS containing 0.2% Tween-20 (PBST), for 8 min each, at room temperature with shaking. Nonspecific interactions were blocked by incubating the cells for 30 min with 2% BSA diluted in 1X PBS. Cover slips were again washed twice with 1 mL PBST, incubated with appropriate primary antibody (Table-2.3) diluted in PBS + 0.2% BSA for 2 h at room temperature. 200 µL of antibody was placed directly on the cover slips. Post incubation, the cover slips were washed thrice with 1 mL PBST for 3 min each and the cells were

incubated with appropriate Alexa-488 conjugated secondary antibodies diluted in PBST with 2% BSA at room temperature for 1 h (for dilutions refer to Table-2.3). The cover slips were washed with PBST, mounted on glass slides using Vectashield mounting medium with DAPI. Images were acquired on a LSM 510 META confocal microscope (Zeiss, LSM acquisition software, 63x 1.4 N.A. objective) at a 0.7 scan zoom to collect maximum number of cells per field. The number of foci in each nucleus was manually counted by changing contrast identically across all the samples. A minimum of 10 random fields were imaged per sample. Data are represented as average number of foci per nuclei.

2.2.7 Inhibition of IP6Ks in MEFs

The IP6K inhibitor TNP was dissolved in DMSO at a concentration of 22.6 mM and stored at -20°C. MEFs were seeded on coverslips in 12 well plates at 15% confluence. Once, cells adhered and attained their morphology, they were pre-treated with 5 μM TNP and DMSO 3 h prior to HU treatment. After 3 h, cells were treated with 0.5 mM HU for 12 h in presence or absence of TNP. Similarly, post drug removal, cells were incubated with or without TNP to monitor recovery for 6 h. At each time point cells were processed for presence of nuclear BLM as described in Section 2.2.6. BLM was used as readout for initiation and completion of repair. Images were acquired as described in Section 2.2.6.

2.2.8 Detecting DNA DSBs by TUNEL assay

Cells were grown in 35mm dishes at 30% initial confluence. At 60% confluence, cells were treated with 0.2 mM hydroxyurea for 12 h. After treatment, media containing drug was removed and fresh media was added for recovery of the cells from genotoxic stress. DNA DSBs were monitored during the treatment period and recovery period of 3, 6, 9 and 12 h. At each time point cells were harvested and fixed using 70% ice cold ethanol by gentle vortexing at very low speed and kept overnight at -20°C. A TUNEL assay to detect DSBs was conducted using Apo-Direct Tunel assay kit (A35127, Invitrogen). After overnight fixation, cells were washed with 800 μL of wash buffer, and 50 μL of DNA labeling solution [10 μL reaction buffer, 0.75 μL of TdT enzyme, 8 μL of BrdUTP and 31.25 μL of dH_2O (Sigma)] was added to each sample, incubated at 37°C for 4 h. Samples

were mixed or shaken every 15 min during the incubation as to keep them in suspension. At the end of the incubation, cells were washed again with 800 µL of rinse buffer by centrifugation at 500 x g for 6 min and the supernatant was aspirated. BrdU incorporation was detected by adding 100 µL of antibody staining solution containing 5 µL Alexa488 dye labeled anti-Brdu antibody with 95 µL of rinse buffer (1:20 dilution), and the cell suspension was incubated for 30 min at room temperature in the dark. At the end 350 µL of PI staining buffer was added to each sample and incubated for an additional 30 min at room temperature in the dark. Cells were analyzed for the presence of DSBs by flow cytometry on a FACS ARIA instrument (BD). Viable cells were analyzed for the presence of DNA DSBs by excluding the hypodiploid (<G0/G1) population, using FACS DIVA software (BD). Median fluorescence values of the treated cells were plotted as a fold difference over untreated controls, using GraphPad Prism 5.

2.2.9 Cytogenetic analysis

MEFs plated in 6 cm dishes were treated with MMC (300 nM) for 12 h. Post damage cells were treated with colcemid (0.1 µg/mL) for 4 h to arrest cells in metaphase. After 4 h cells were washed twice with PBS to remove traces of colcemid and harvested by trypsinisation, pelleted down at 100 x g for 5 min in 15 mL conical tubes. The supernatant was removed by leaving 0.5 mL and resuspended thoroughly by pippeting. Cells were subjected to hypotonic swelling in pre-warmed 0.075 M KCl for 15 min at 37°C [Note: key to this step is to add hypotonic solution drop by drop slowly otherwise cell clumps will form which are impossible to disperse. Add a few drops first then pipette the cells up and down to mix them thoroughly and add few more drops. Invert tubes 2-3 times during the incubation]. After 15 min cells were fixed by adding few drops of chilled fresh fixative (methanol: glacial acetic acid; 3:1) kept in the deep freezer. Cells were pelleted down at 100 x g for 8 min, the supernatant was removed, 0.5 mL of fixative was left behind and the pellet was resuspended very gently. Slowly fresh fixative was added in the same manner as the KCl solution and cells were pelleted down at 100 x g for 8 min; this step was repeated twice. The supernatant was removed by leaving 0.5 mL fixative and the cells were resuspended and mixed well before preparing metaphase spreads. Cell suspensions were

dropped on clean prechilled slides held at a 45° angle from a height of 18 inches, dried at 55°C on a hot plate and stained with 3% geimsa in phosphate buffer (pH 6.8) for 10 min. Slides were washed three times and dried, individual metaphase spreads were analyzed using bright field microscopy (Zeiss Axio Scope), and chromosomal aberrations were scored manually from each spread.

2.2.10 Western blot analysis

Dishes containing adherent cultures were placed on ice and washed once with cold PBS. Cells were scraped in 1x sample buffer on ice. Samples were sonicated in a probe sonicator (Misonix ultra sonic liquid processor, S-4000) 3 times for 15 sec each to complete cell lysis and shear DNA to reduce viscosity. Equal volumes of lysates were loaded and resolved using 12% SDS-PAGE. Samples were stored at -80ºC if necessary. Protein transfer onto PVDF membrane (GE Healthcare) was carried out at 200mA constant current for 2 h by placing the transfer tank in ice. Membranes were blocked with appropriate blocking buffer according to manufacturer's instructions provided for each antibody. Preferentially TBST with 5% non-fat dry milk was used for blocking and for antibody dilutions. Briefly, non-specific interactions were blocked with blocking buffer (TBST+5% non-fat dry milk) for 1 h at room temperature. Membranes were probed with appropriate primary (overnight at 4°C) and HRP-conjugated secondary antibodies (2 h at room temperature). Proteins were detected using ECL detection system. Chemiluminiscence was detected using the LAS4000 (GE Healthcare) or FlourChem E (Protein Simple) documentation system. Densitometry analysis of bands was done using ImageJ documentation software (Fiji) or the multiplex band analysis tool in AlphaView software (Protein Simple).

2.2.11 IP6K2 knockdown by shRNA-Plasmids encoding either a non-targeting shRNA (Sigma- Aldrich SHC016) or shRNA directed against mouse *Ip6k2* (Sigma-Aldrich, TRCN0000202175 or TRCN0000202065), were co-transfected with VSV-G and VSV-GP encoding plasmids into the Phoenix amphotropic packaging cell line, using Polyfect reagent (Qiagen), and incubated at 37°C and 5% CO_2 for virion formation. After 48 h, the

supernatant was collected and filtered through a 0.45 μm syringe filter unit. Lentiviral particles carrying either non-targeting shRNA or both IP6K2 directed shRNA constructs were used to infect MEFs for 48 h. At the 36th hour, cells were treated with HU for 12 h, followed by recovery for 6 h, and immunofluorescence was carried out as described in 2.2.6. Immunoblotting with an IP6K2 antibody (Table 2.3) was performed to analyse knockdown levels as described in Section 2.2.10.

2.2.12 Generation of stable cells expressing shIP6K1 in HeLa and HCT116

2.2.12a Generation of lentiviral particle containing shRNA against human IP6K1- HEK293T packaging cells were seeded at 30-40% confluency in 60 mm dishes. After 24 h, cells were co-transfected with three plasmids required for viral production i.e. VSV-G, psPAX2 (Addgene plasmid # 12260) and pLKO.1-puro-non-targeting and shIP6K1 clones using polyethylenimine reagent (PEI) and incubated at 37°C and 5% CO2 for virion formation. After 48 h, viral particles were harvested by collecting supernatant and filtered through a 0.45 μm syringe filter unit. Viral stock was aliquoted and stored at -80°C for further use. Viral titer was approximated on the number of cells plated for the production of lentivirus. Calculations were done as per Cell Bio Labs instruction. 2 x 10^6 cells will yield 10^7 infectious units/mL. All necessary precautions were taken while generating lentiviral particles such as wearing mask, double gloves, and sterile filter tips. All the consumables used were bleached (1% sodium hypochlorite solution) at least 1 h before being discarded.

2.2.12b Generation of stable cell lines expressing shIP6K1- HeLa and HCT116 cell lines were used for stable knockdown of IP6K1 expression. Viral particles harboring either non-targeting control or *IP6K1* directed shRNA were used to infect HeLa or HCT116 cell lines at 0.5 MOI, following treatment with polybrene (8 μg/mL) for 2 h. After 48 h, transduced cells were selected with 2 μg/mL puromycin. Medium was changed twice a week and observed for colony formation. After reaching the optimum growth, selected cells were maintained in DMEM supplemented with 10% FBS and 1 μg/mL puromycin as

the selection marker. Knockdown was confirmed by immunoblot analysis with an IP6K1 specific antibody (Table 2.3) as described in Section 2.2.10.

2.2.13 Cell adhesion assay

Cell adhesion assays were performed as described previously (Hocking *et al.*, 1998) with slight modifications. Fibronectin coating was done overnight at 4°C. 5×10^4 cells were seeded per well onto fibronectin (2 μg/mL) coated 24 well plates in triplicates. Cells were allowed to adhere for different time periods. At each time point, unadhered cells were washed away with PBS; adhered cells were trypsinized and counted with a hemocytometer. Percentage of adhesion was calculated by normalizing total number of adhered cells at each time point to number of cells adhered after 5 h.

2.2.14 Cell spreading

Cell spreading assays were done as described previously (Balasubramanian *et al.*, 2010). Briefly, cells were cultured in complete medium (10% FBS) or subjected to serum starvation (0.2% FBS for 12-14 h), trypsinized, counted using a hemocytometer, and held in suspension in the same medium containing 1% methylcellulose at 37°C and 5% CO_2 in a 50 mL tube in a slanted position. After 90 min, cells were pelleted down at 1350 rpm for 8 min at 4°C and the supernatant was discarded leaving 5 mL at the bottom. Cells were dislodged or displaced gently without touching the walls of the tubes, which might lead to activation of adhesion dependent signalling. Now, cells were washed once with excess of cold media (4°C) and one more time with warm media (37°C). A day before the experiments coverslips were coated with fibronectin (2 μg/mL) overnight at 4°C and washed once with PBS before plating the cells. Cell suspension equivalent to 10^5 cells was plated onto each fibronectin (2 μg/mL) coated coverslip and allowed to spread for 15 min or 24 h in serum depleted (0.2% FBS) or complete medium. At each time point, unadhered cells were washed off with PBS and the coverslips were fixed with 3.5-4% paraformaldehyde for 20 min at room temperature. After fixation coverslips are washed thrice with PBS and cells were permeabilized with 0.1% Triton X-100 for 5 min, followed by blocking with 5% BSA for 30 min at room temperature. Fixed cells were stained with

fluorophore-conjugated phalloidin (Alexa Fluor 488 or rhodamine) for 45 min, followed by DAPI staining for 2 min. Cover slips were mounted onto glass slides using Fluoromount G (Southern Biotech) or Vectashield (Vector Labs), and imaged using an LSM 710 laser confocal-anisotropy microscope (Zeiss, Zen acquisition software, 40x 1.3 N.A. objective) for 15 min spreading, or LSM 510 laser confocal microscope (Zeiss, LSM acquisition software, 63x 1.4 N.A. objective) for 24 h spreading. To measure the cell spread area for serum depleted cells (0.2% serum), images captured at identical zoom settings were analyzed using the Image J software (NIH) as follows: threshold values were set to define the cell edge and a mask was then created for each cell to get the total cell area (with arbitrary units) within the mask. For 15 min spreading (10% serum) and 24 h spreading, the exact spread area was calculated based on pixel dimensions during image acquisition.

2.2.15 Immunoblot analysis to detect pFAK

Cells were seeded in six well plates. After attaining optimal growth, cell lysates were prepared by scraping cells in 1x Laemmli buffer and samples were processed by standard western blot techniques. To detect FAK activation samples were processed as described in Section 2.2.13. Briefly, 2 $x10^6$ cells (6 $x10^5$ cells per time point) were held in suspension in complete medium containing 1% methylcellulose for 90 min (Susp), and replated on fibronectin (2 μg/mL) coated surfaces for 20 min (+FN) or for 4 h (SA - stably adherent). Cells at each time point were lysed in 1x Laemmli buffer and subjected to immunoblotting. Membranes were probed with specific antibodies (Table 2.3) and detected using the ECL detection system (GE Healthcare) as described in Section 2.2.10.

2.2.16 Transwell migration assay

Transwell migration assays were conducted as described previously (Rao *et al.*, 2015). Transwell inserts (24 well, 8 μm pore size, Costar, Corning) were used to conduct migration assays. Inserts were equilibrated by adding 1% FBS containing media to the upper chamber, as well as lower chamber of the insert (companion plate) and placed in a 37ºC incubator with 5% CO_2 till use. Cells were harvested, counted using hemocytometer

and pelleted down at 1200 rpm for 5 min. The supernatant containing 10% FBS and trypsin were discarded and the cell pellet was resuspended in media containing 1% FBS. 5×10^4 cells suspended in 200 µL DMEM (1% FBS) was added to the upper chamber and carefully transferred to the well of the companion plate containing 700 µL of complete DMEM (10% FBS) to serve as chemotactic agent. Inserts were lifted with sterile forceps and placed in the companion plate by avoiding any bubbles at the bottom surface of the insert. Cells were incubated at 37ºC with 5% CO_2 for 18 h (MEFs) and 24 h (HeLa and HCT116 cells) to allow migration. At the indicated time, cells on the upper surface of the filter were removed carefully by scrubbing with wet cotton swabs and inserts were dunked twice in excess PBS. Cells that migrated to the lower surface of the filter were rinsed twice with PBS, fixed with 4% paraformaldehyde for 15 min at room temperature. Post fixation inserts were rinsed in PBS twice and air dried at room temperature. Filters were cut carefully through the edges using a scalpel blade and placed inverted on a clean slide so that the migrated cells faced the upward direction. Filters were mounted with vectashield DAPI and covered with a clean coverslip. Migrated cells stained with DAPI were counted by imaging multiple fields using an epifluorescence inverted microscope (Olympus lX51, Image-Pro AMS 6.0 acquisition software, 20x 0.45 N.A. objective). The number of cells migrated to the lower surface was quantified by counting the total number of DAPI positive nuclei in at least 10 random fields per sample.

2.2.17 Invasion assay

Invasion assays were conducted as described previously (Rao *et al.,* 2015). Invasion chambers with pre-coated matrigel (BioCoat Matrigel invasion chamber, 24 well, 8 µm pore size, Corning) were used to conduct the invasion assays. Packages containing invasion chambers were kept outside and allowed to come to room temperature. Inserts were equilibrated by adding with 500 µL warm (37ºC) 1% FBS containing DMEM to the upper chamber of the insert and bottom well of the companion plate, and rehydrated for 2 h in a humidified tissue culture incubator at 37ºC with 5% CO_2. The rehydration media was removed carefully without disturbing the pre-coated matrigel matrix on the membrane. Meanwhile, HCT116 cells expressing either non-targeting or IP6K1 targeting

shRNA were processed for invasion studies. A suspension of 1×10^5 cells in 200 µL of 1% FBS containing DMEM was prepared and plated as described in Section 2.2.16. Cells were incubated at 37ºC with 5% CO_2 for 24 h to allow invasion. Downstream processing of the samples i.e. removing non-invading cells, staining and imaging were followed as described in Section 2.2.16. The number of cells invaded to the lower surface was quantified by counting the total number of DAPI positive nuclei in at least 10 random fields. The total number of cells invaded was normalized to non-targeting control cells and expressed as percentage invasion.

2.2.18 Scratch wound healing assay

Scratch wound healing assay was performed as described previously (Rao *et al.*, 2015). Cells were seeded in 6 well plates in triplicates to attain confluence. On the next day, a scratch was made on the confluent monolayer culture using a 200 µL pipette tip. Cells were washed gently twice with PBS to remove floating cells, replaced with complete DMEM and incubated at 37ºC with 5% CO_2. Images were acquired using a Zeiss phase contrast inverted microscope (ProgRes CapturePro v2.8 acquisition software, 5x 0.12 N.A. objective). Images were acquired immediately after scratch (0 h) and after a period of 4 h (MEFs) or 24 h (HeLa and HCT116) to monitor wound closure. The area of wound closure was analyzed using ImageJ software, and the data was plotted as total area covered in square µm (µm^2).

2.2.19 Anchorage independent growth

Anchorage-independent cell growth was conducted in 6 well plates at least in triplicates. 1% of low temperature melting agarose (Difco) was prepared in sterile MQ water and mixed with 2X DMEM containing 20% FBS kept warm at 37ºC. Both the solutions were mixed in equal proportion to get 0.5% agarose in 1X DMEM and 10% FBS. This solution was kept warm throughout the experiment at 37ºC in a water bath or incubator. To prepare base agar (0.5% agarose), 2 mL of this solution (0.5% agarose+1x DMEM+10% FBS) was dispensed in 6 well plate to spread evenly without any bubbles and allowed to solidify at room temperature at least for 30 min. Cells were harvested and counted using a

hemocytometer. 2 mL of cell suspension containing 2×10^4 cells/mL were aliquoted in 15 mL conical tubes. To prepare top agarose (0.3%), 3 mL of prewarmed 0.5% agarose mixture was added to the 2 mL cell suspension, mixed gently by pipetting up and down to avoid air bubbles. Quickly 2 mL of this mixture (cells+0.5% agarose mixture) was transferred to the plate containing 2 mL top agarose. All the samples plates were kept at room temperature (TC laminar flow hood) for at least 60 min to solidify the top agarose. After agarose solidification, plates were incubated at 37°C and 5% CO_2 for 28 days. Every 2 days, spent media was replaced with 0.5 mL fresh media to prevent it from drying. After 28 days, colonies were stained with 0.005% crystal violet (in 2% ethanol) for 1 h at 37°C. Excess stain was washed with 2% ethanol until clear visible colonies were seen. Plates were imaged using AlphaImager (Alpha Innotech), and the number of colonies was determined by ImageJ software. Multiple images of stained colonies were acquired on a phase contrast inverted microscope (Zeiss) at 5x objective with 1.2 N.A using ProgRes CapturePro v2.8 acquisition software. Area of the individual colonies was determined using ImageJ software.

2.2.20 *In vivo* xenograft model

HCT116 cells stably expressing NT or shRNA against *IP6K1* were grown to subconfluence. Logarithmic phase monolayer cultures were harvested by trypsinization, cell number was determined using a hemocytometer, and resuspended in complete DMEM. Before injection, cells were washed and resuspended in PBS at 2×10^7 cells/mL. Approximately 2×10^6 cells were injected subcutaneously into either flank of 6 week old female homozygous *Foxn1*^{nu} athymic nude mice (n= 8 mice) and tumor size was monitored every 3 days for a period of 4 weeks. Mice were euthanized 4 weeks after injection and tumors were surgically excised and weighed.

2.2.21 Carcinogenesis studies

Carcinogenesis studies were conducted as described previously for *Ip6k2*$^{-/-}$ mice (Morrison *et al.*, 2009). Briefly, the carcinogen, 4NQO stock solution (5 mg/mL) was prepared fresh in propylene glycol every week. Four-week old mice [11 *Ip6k1*$^{+/+}$ (5 male, 6 female) and 9

Ip6k1$^{-/-}$ (4 male, 5 female)] were exposed to 4NQO (100 µg/mL) in their drinking water. Mice were allowed free access to drinking water containing the carcinogen, and the water was changed every week. Water bottles containing carcinogen was covered with foil to avoid exposure to light. All the apparatus used for this study and remaining water were decontaminated using 1% sodium hypochlorite for 30 min. Every week water consumption and weight of the each animal was monitored. Mice displaying any characteristics of weight loss or dehydration before 24 weeks were euthanized and examined for lesions. After 24 weeks, mice were euthanized by CO_2 inhalation and a complete necropsy was performed. Tissues from the aerodigestive tract (tongue, esophagus, stomach, and duodenum) were fixed in formalin and paraffin-embedded sections were stained with hematoxylin and eosin (H&E) to examine the lesions by light microscopy. Images were acquired using a bright field light microscope (Nikon ECLIPSE N*i*, NIS Elements acquisition software, 20x 0.5 N.A. objective). During the pathological examination, the lesions observed in the various tissues were categorized into hyperplasia, dysplasia, and invasive carcinoma. Hyperplasia was defined as an increase in the layers or thickening of the epithelium with hyperkeratinization. Dysplasia was defined as loss of epithelial cell polarity, nuclear pleomorphism and abnormal mitoses confined to the epithelium. Invasive carcinoma was defined as invasion of dysplastic cells or lesions into subepithelial tissues such as submucosa and muscle.

2.2.22 Data analysis

Statistical analysis was performed using GraphPsssad Prism 5. Data are presented as mean ± SEM. The difference between two groups was analyzed using either a two-tailed Student's *t*-test or a nonparametric two-tailed Mann-Whitney test, as appropriate. The differences between multiple groups were analyzed by one-way ANOVA, using Tukey's multiple comparison test for parametric data and Kruskal-Wallis test for non-parametric data. $P<0.05$ was considered as statistically significant. The cell numbers used to obtain quantitative data (*n*) and the number of independent experiments performed is indicated in the respective figure legends.