Supplementary Materials Index

Figures & Legends:

Tables & Legends:

Detailed Materials & Methods

Supplementary References

Supplementary Materials & Methods

Mice & procedures

The Villin-CreER^{T2} (51); Floxed Apc (52); LSL-KRas^{G12D} (53), floxed Nuak1 (11) & Rosa26-CAAGS-rtTA3 (54) allelic mice were described previously. Mice bearing the doxycycline-inducible shRNA alleles targeting Nuak1 were generated by Mirimus Inc., as described previously (55): Briefly, shRNA candidates were selected using the sensor assay (56). Two shRNAs were chosen that scored >5 and produced knockdown >90%. The selected shRNA sequences were subsequently cloned in the miR-E backbone (57) within the 3'UTR of a turboGFP cDNA downstream of a TRE in the Col1a1 TtGM vector and targeted to the Col1a1 locus using standard protocols (54). Targeted ES cells were injected using blastocyst injection technique. Resulting shRNA mice were of mixed C57BL/6 × 129/SV background.

For tumor enumeration, small intestines and colons were flushed with PBS, mounted 'en face' and fixed overnight in formalin. Tumor area was measured as width by length, omitting depth as negligible/immeasurable in many instances. For histological examination, fixed tissue was then rolled ('swiss roll method') and embedded in paraffin for sectioning, followed by standard staining in hematoxylin and eosin (H&E). Additionally, prior to fixation and immediately upon harvest, a small number of representative tumors and adjacent normal tissue were dissected and flash-frozen for RNA analysis. For immunohistochemistry, FFPE tissue sections were deparaffinized in 3 changes of xylene and rehydrated in graded ethanol solutions. Antigen retrieval was performed by microwaving in 10mM Sodium Citrate, pH6.0. Endogenous peroxidases were quenched in 3% H_2O_2 and non-specific binding was blocked with 1 or 3% BSA solution. The following antibodies were used: Ki67 (Thermo Fisher, RM-9106-S0); Cleaved Caspase 3 (Cell Signaling, 9661); Lysozyme (Dako, A099); BrdU (Beckton Dickenson, BD347580); 8-Oxo-dG (Abcam, AB48508). Goblet cells were stained with Periodic acid Schiff (PAS). TUNEL staining was performed using ApopTag peroxidase kits (Millipore). For quantification of IHC, 3-5 20X fields were scored on representative sections from at least 3 mice of each genotype. In situ hybridization (RNA-Scope) was performed according to the manufacturers directions (Advanced Cell Diagnostics; ACD): 4μm FFPE tissue sections were baked at 60^{o}C for 1hr, then de-paraffinized and rehydrated. Endogenous peroxidases were blocked in H_2O_2, followed by antigen retrieval (100^{o}C for 8 min.) and protease digestion (H_2O_2 & Protease Plus kit, ACD). For in-situ hybridization, sections were hybridized with ACD-designed probes for Nuak1 (434281), positive ctrl PP1β (313911) or negative ctrl DapB (322330) for 2hrs at 40^{o}C. Probe detection was performed using RNA-Scope kit reagents (ACD 322310) and counterstained with Hematoxylin.

Cell culture & analysis

U2OS, HCT-116; SW620; LS174T and SW480 cells were obtained from the ATCC and maintained in DMEM (25mM Glucose) with Penn/Strep and 10% FBS. All cell lines were validated using an approved in-house validation service (CRUK-BICR) and tested periodically for mycoplasma. Primary mouse embryo fibroblasts (MEFs) were generated as previously described (58) by interbreeding heterozygous floxed *Nuak1* mice, generating homozygous Nuak1$^{FL/FL}$ and Nuak1$^{+/+}$ MEFs, and maintained as above. To delete floxed Nuak1, MEFs were infected overnight with 300pfu/cell Adeno-CRE (Uni. Iowa). Alternatively, MEFs were infected with retrovirus expressing CreER^{T2}, selected on Puromycin for 48hrs, and CreER^{T2} was subsequently activated by treatment with 100nM 4-OHT. Cells in log phase growth were treated with the indicated concentrations of Nuak1 inhibitors (HTH-01-015 and WZ4003). Where indicated, cells were pre-incubated with 500μM of Trolox for 8hrs prior to treatment with Nuak1 inhibitor. Equivalent volumes of DMSO were used as vehicle controls. Reactive Oxygen species were measured after 8hrs Nuak1 inhibition using CellROX Deep Red or MitosOX reagents (Thermo) according to the manufacturers directions, followed by FACS analysis. For cell death measurements, cells were trypsinized, quenched with 1% BSA followed by replacement of original supernatant, and centrifuged at 300 x G for 5mins; 200ul of Annexin binding buffer (10mM HEPES (PH 7.40, 140mM NaCl, 2.5mM $CaCl_2$) and 2μl of Annexin V-APC (Biolegend 640920) were

added to the pellet and incubated for 15mins. Propidium iodide (PI, 10μg/ml) was added immediately prior to FACS analysis. Alternatively, cells were imaged by Incucyte (Essen Bio) time-lapse video-microscopy in the presence of Sytox green reagent (Thermo Fisher). For immunoblotting, whole cell lysates were prepared in RIPA buffer (150mM NaCl, 50mM Tris, pH 7.5, 1% NP-40, 0.5% sodium deoxycholic acid, 1% SDS, plus complete protease and phosphatase inhibitor cocktail) followed by sonication (40% Amp for 5s). Cytoplasmic and Nuclear fractions were prepared in low salt buffer (20mM KCL, 10mM HEPES, pH 7.5, 1mM $MgCl_2$, 1mM $CaCl_2$, 0.1% Triton X-100) followed by centrifugation at 3300 rpm for 3min. NUAK1 (CST 4458, 1:750); NUAK2 (Hs: MRC, S225B, Mm: CST 4100); NRF2 (Novus NB100-80011, 1:1000); pMYPT1 (MRC S5087, 1:400); total MYPT (BD 612164, 1:1000); β-Actin (Sigma A5441, 1:5000); Lamin A/C (Santa Cruz, 6215), Keap1 (Santa Cruz, 15246); phosphor AMPK substrate (CST 5759) were used as primary antibodies. Secondary horseradish peroxidase conjugated antibodies (α-mouse IgG NA931V; α-rabbit NA934V, both GE Healthcare; α-goat IgG, Vector Labs PI-9500) were detected by chemiluminescence (Pierce ECL western blotting substrate 32106). For immuno-precipitations (IPs), $2.5x10^6$ cells were seeded per 15cm dish. Next day, 5ug of NRF2-Flag or empty vector was transfected using Lipofectamine 3000 reagent (1:1.8 ratio for Lipofectamine, 1:2 for p3000 reagent). 48hrs post transfection, cells were trypsinized and $5x10^6$ cells were seeded per 15cm dish. Cells were treated with 10μM HTH or DMSO for 8hrs, then washed with ice cold PBS and scraped in 400μl of NP-40 lysis buffer (150mM NaCl, 50mM Tris Ph7.5, 1mM EDTA, 1mM EGTA, 1% NP-40, plus complete protease and phosphatase inhibitor cocktail). Lysates were incubated on ice for 10min and centrifuged at 12k rpm for 10min and the supernatant was used for IP. 1.5mg of total protein was used per condition. Lysates were incubated with Flag-M2 resin (20μl /μg of protein) overnight. Beads were pelleted at 3000rpm/5 minutes and washed 3X with the lysis buffer (3000 rpm/5mins/4°C). IP'ed proteins were eluted in 60μl of 1x Laemlli buffer. The following siRNAs were used at 20nM, except where noted: NUAK1 D-004931-01-002 (40nM, Dharmacon) SI00108402 (10nM, Qiagen); NUAK2 SI02660224 (Qiagen); NRF2 SI03246950, SI04223009 (Qiagen); KEAP1 S104155424, S103246439 (Qiagen); MYC SI03101847 (Qiagen); PTEN SI00301504, SI03048178 (Qiagen). For Dimedone detection of Cysteine oxidation, USOS cells transiently overexpressing FLAG-tagged NUAK1 were treated with H_2O_2 for 5 minutes, then lysed in RIPA buffer containing 1mM Dimedone (Sigma, D153303) followed by α-FLAG IP. Dimedone incorporation was detected using α-Dimedone antibody (Millipore, 07-2139). Iodo-acetamide labeling was performed similarly by addition of 55mM labeled ($^{13}C_2D_2H_2INO$; Sigma-Aldrich/ Merck KGaA, 721328) or unlabeled (C_2H_4INO; Sigma-Aldrich/ Merck KGaA, I6125) iodoacetamide in RIPA buffer, followed by anti-FLAG IP. IPs were washed twice in lysis buffer, followed by H_2O, prior to combining H_2O_2 treated and untreated samples for MS analysis (see below). Label incorporation was normalized to the level of IP'ed NUAK1, measured by immunoblotting of 10% of each IP.

Gene expression analysis

U2OS cells were depleted of Nuak1 by shRNA-4977 as previously described (9) and selected on puromycin for 48hrs. Control cells were similarly selected after infection with non-targeting shRNA expressing retrovirus. SW480 and HCT116 cells were depleted of NRF2 or NUAK1 by siRNA. 24hrs after re-seeding in the absence of selection, total RNA was isolated using the RNEasy Mini Kit (Qiagen) according to manufacturer's instructions and DNA was depleted with the RNase-Free DNase Set (Qiagen). RNA-integrity was checked using the RNA ScreenTape assay (Agilent Technologies) and cDNA was synthesized with the TruSeq Stranded mRNA Library Prep Kit (Illumina). Following library quantification (D1000 ScreenTape, Agilent Technologies), libraries were standardized to 10nM, denatured, diluted to 10pM and analyzed by paired-end sequencing using an Illumina NextSeq500 platform. RNA-Sequencing reads were aligned to the GRCh38 version of the human genome or the GRCm38 version of the murine genome and differential expression determined using DESeq2 (59). Pathway modulation analysis was performed using Metacore

GeneGO (Thompson Reuters). For analysis of individual genes, RNA was isolated using TRIZOL (Invitrogen) as per manufacturer's directions. cDNA was synthesized using Quantitect reverse transcription kit (Qiagen 205313) followed by real time PCR using SYBR Green method (VWR QUNT95072). The following primer sets were used to detect indicated mRNA transcripts:

Human

GCLC F: 5'atgccatgggatttggaat; R: 5'gatcataaaggtatctggcctca
GCLM F: 5'gttggaacagctgtatcagtgg; R: 5'gttggaacagctgtatcagtgg
GSHR F: 5'atgatcagcaccaactgcac; R: 5'cttccaagcccgacaaagt
MGST F: 5'accacaccattgcatatttgac; R: 5'gccatggaaagagtaactcca
TXN F: 5'ttacagccgctcgtcaga; R: 5'ggcttcctgaaaagcagtctt
β-ACTIN F, 5'ccaaccgcgagaagatga; R: 5'ccagaggcgtacagggatag
NUAK1 F: 5'acatgatctcaatctctcgtctg; R: 5'acctacggcaaagtcaagc

Mouse

Gclc L: 5'agatgatagaacacgggaggag; R: 5'tgatcctaaagcgattgttcttc
Gclm L: 5'tgactcacaatgacccgaaa; R: 5'tcaatgtcagggatgctttct
Gshr L: 5'ctatgacaacatccctactgtggt; R: 5'cccatacttatgaacagcttcgt
Mgst L: 5'gcccttctccctggattc; R: 5'ggccatcaacacctcattgt
Txn L: 5'tgaagctgatcgagagcaag; R: 5'agaagtccaccacgacaagc
B2m F: 5'agccgaacatactgaactgctacg; R: 5'cggccatactgtcatgcttaactc
Nuak1 F: 5'gagcccacacaaccctca; R: 5'tctgcgatcgggattcac

A human CRC tissue micro-array comprising 650 tissue cores was stained for *NUAK1* mRNA expression by RNA-Scope (as described above) and scored blindly using Halo image analysis software (Indicalab). Omitting 47 results that failed quality control (undetectable PP1β expression), results were subdivided into equal quartiles from low to high NUAK1 expression and P values were determined by Chi-Square test.

Proteomic analysis

SILAC labeling: U2OS cells were cultured in light ($^{12}C_{61}{}^{4}N_4$ L-arginine and $^{12}C_{61}{}^{4}N_2$ L-lysine, Sigma) and heavy ($^{13}C_{61}{}^{5}N_4$ L-arginine and $^{13}C_{61}{}^{5}N_2$ L-lysine, Cambridge Isotope Laboratories) SILAC medium (SILAC DMEM, Life Technologies) supplemented with 10 KDa dialysed serum (Sigma) until full labeling (>98%) of the proteome was reached. For "forward analysis" heavy labeled cells were treated for 1hr with 10 μM HTH-01-015, and light labeled cells were vehicle treated. Cells were then harvested and lysates mixed in equal amounts. For the reverse replicate experiment, light labeled cells were treated for 1hr with 10 μM HTH-01-015, and heavy labeled cells were vehicle treated. **Sample Preparation**: 1.5 mg of each cell lysate from light and heavy SILAC labeled cells were mixed 1:1, light Ctl : heavy HTH in the forward experiment and light HTH and heavy Ctl in the reverse experiment. Proteins were precipitated overnight at -20 C in acetone, re-dissolved in 8M Urea, 0.1 M TrisHCl pH 8.5 buffer with phosphatase inhibitors (Halt phosphatase cocktail, Thermo Scientific) and digested with Lys-C (Alpha Laboratories) and Trypsin (Promega) using Filter-Aided Sample Preparation (FASP) (60). To remove salts, peptides were loaded onto C18 Sep-Pak column (Waters) and eluted with increasing concentration of acetonitrile (ACN, 10%, 15%, 20%, 25%, 30%, 40%, 60%) in 01% trifluoroacetic acid. ACN was removed using a speed vacuum centrifuge and peptides were resuspended in MOPS 50 mM, sodium phosphate 10 mM, sodium chloride 50 mM pH 7.2. Fresh ACN was added to 30% final concentration and TFA to pH down to 2.5. The enrichment for phosphorylated peptides was performed incubating the peptide solution with TiO_2 beads, 5:1, peptide:TiO_2, as previously described (60). Four subsequent incubations with TiO_2 were performed. Peptides were eluted from the TiO_2 beads using a solution 15% ammonium hydroxide 40% ACN and loaded onto a C18 StageTip (62). Peptides were eluted from StageTip with 80% ACN, speed vac and resuspended in 1% ACN, 0.05% TFA for MS analysis. Peptides

recovered from the four incubations were run separately at the MS. **MS analysis**: Digested peptides were injected on an EASY-nLC system coupled on line to a LTQ-Orbitrap Elite via a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were separated using a 20 cm fused silica emitter (New Objective) packed in house with reversed-phase Reprosil Pur Basic 1.9 µm (Dr. Maisch GmbH) and eluted with a flow of 200 nl/min from 5% to 25% of buffer containing 80% ACN in 0.5% acetic acid, in a 190 min linear gradient. The top ten most intense peaks in the full MS were isolated for fragmentation with high collision energy dissociation. MS data were acquired using the XCalibur software (Thermo Fisher Scientific) and .RAW files processed with the MaxQuant computational platform (63) version 1.5.0.36 and searched with the Andromeda search engine (64) against the human UniProt database (65) (release-2012 01, 88,847 entries). MaxQuant was run with the following settings: To search the parent mass and fragment ions we required a mass deviation of 4.5 ppm and 20 ppm. The minimum peptide length was 7 amino acids and maximum of two missed cleavages and strict specificity for trypsin cleavage were required. Carbamidomethylation (Cys) was set as fixed modification, and oxidation (Met), N-acetylation and phosphoSTY as variable modifications. For peptide and phosphorylation site identification, a false discovery rate (FDR) of 1% was required. The re-quantification and match between runs features were enabled and the relative quantification of the peptides against their SILAC-labeled counterparts was performed by MaxQuant. For phosphorylation sites to be quantified, we required at least two ratio counts. The MaxQuant output file Phospho (STY)Sites was analyzed with the Perseus software (66), the reverse and contaminant hits were excluded and only class I phosphorylation sites (localization probability = probability that the phosphorylation site has been accurately localized > 0.75 and score difference > 5) used for the analysis. Raw MS data is available via the PRIDE repository under accession number PXD008229. **Cysteine oxidation:** tryptic peptides were separated by nanoscale C18 reverse-phase liquid chromatography on an EASY-nLC II 1200 (Thermo Scientific) coupled online to a Q-Exactive HF mass spectrometer (Thermo Scientific) and data acquired using the XCalibur software (Thermo Fisher Scientific). The .RAW files were processed with the MaxQuant computational platform version 1.6.0.16 as described above, but with Cysteine carbamidomethyl Heavy and Light as variable modifications. The Oxidation (M)Sites.txt was analyzed with the Perseus software.

Kinase Assays

GST-tagged full length human NUAK1 (product number 02-126) was purchased from Carna Biosciences, Japan. Kinase assays were performed using an IMAP fluorescence polarization assay format (Molecular Devices Inc.). a titration of kinase from 470nM down to 45nM was incubated for 60 min at room temperature with 100 nM 5Fam-LKKQLSTLYL, NRF1-derived peptide or 5Fam-VSRSGLYRSPSMPENLNRPR, CDC25C- derived peptide (synthesized by Alta Biosciences, University of Birmingham UK) in the presence of 50 µM ATP and 50 mM MgCl2 in 20 mM HEPES buffer (pH 7.5) containing 0.01% Brij-35). Reactions were stopped by adding 2 assay volumes of 0.25% (v/v) IMAP progressive binding reagent in 1x progressive binding buffer A for the NRF1 peptide or 0.16% (v/v) IMAP progressive binding reagent in 85:15% IMAP progressive binding buffer A: Buffer B for the CDC25C peptide. After 60 min incubation to allow binding reagent to bind phosphorylated peptide, fluorescence polarization was measured on a Tecan Infinite M1000 Pro plate reader at excitation (470 nm) and emission (530 nm) wavelengths. Net fluorescence polarization values were calculated by subtractions of the control samples with no ATP.

References (continued from main text)

51. el Marjou F, Janssen KP, Chang BH, Li M, Hindie V, Chan L, et al. Tissue-specific and inducible Cre-mediated recombination in the gut epithelium. Genesis. 2004;39(3):186-93.
52. Shibata H, Toyama K, Shioya H, Ito M, Hirota M, Hasegawa S, et al. Rapid colorectal adenoma formation initiated by conditional targeting of the Apc gene. Science. 1997;278(5335):120-3.
53. Jackson EL, Willis N, Mercer K, Bronson RT, Crowley D, Montoya R, et al. Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. Genes Dev. 2001;15(24):3243-8.
54. Premsrirut PK, Dow LE, Kim SY, Camiolo M, Malone CD, Miething C, et al. A rapid and scalable system for studying gene function in mice using conditional RNA interference. Cell. 2011;145(1):145-58.
55. Dow LE, Premsrirut PK, Zuber J, Fellmann C, McJunkin K, Miething C, et al. A pipeline for the generation of shRNA transgenic mice. Nat Protoc. 2012;7(2):374-93.
56. Fellmann C, Zuber J, McJunkin K, Chang K, Malone CD, Dickins RA, et al. Functional identification of optimized RNAi triggers using a massively parallel sensor assay. Mol Cell. 2011;41(6):733-46.
57. Fellmann C, Hoffmann T, Sridhar V, Hopfgartner B, Muhar M, Roth M, et al. An optimized microRNA backbone for effective single-copy RNAi. Cell Rep. 2013;5(6):1704-13.
58. Muthalagu N, Junttila MR, Wiese KE, Wolf E, Morton J, Bauer B, et al. BIM is the primary mediator of MYC-induced apoptosis in multiple solid tissues. Cell Rep. 2014;8(5):1347-53.
59. Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):550.
60. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009;6(5):359-62.
61. van den Biggelaar M, Hernandez-Fernaud JR, van den Eshof BL, Neilson LJ, Meijer AB, Mertens K, et al. Quantitative phosphoproteomics unveils temporal dynamics of thrombin signaling in human endothelial cells. Blood. 2014;123(12):e22-36.
62. Rappsilber J, Mann M, Ishihama Y. Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. Nat Protoc. 2007;2(8):1896-906.
63. Cox J, Mann M. MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol. 2008;26(12):1367-72.
64. Cox J, Neuhauser N, Michalski A, Scheltema RA, Olsen JV, Mann M. Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res. 2011;10(4):1794-805.
65. UniProt C. The Universal Protein Resource (UniProt) in 2010. Nucleic Acids Res. 2010;38(Database issue):D142-8.
66. Tyanova S, Temu T, Sinitcyn P, Carlson A, Hein MY, Geiger T, et al. The Perseus computational platform for comprehensive analysis of (prote)omics data. Nat Methods. 2016;13(9):731-40.