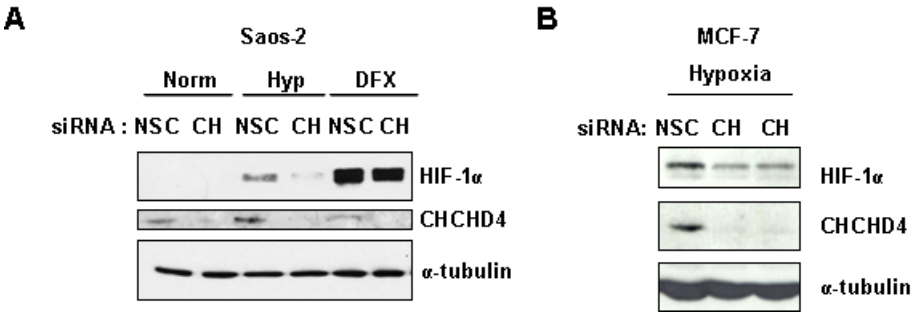
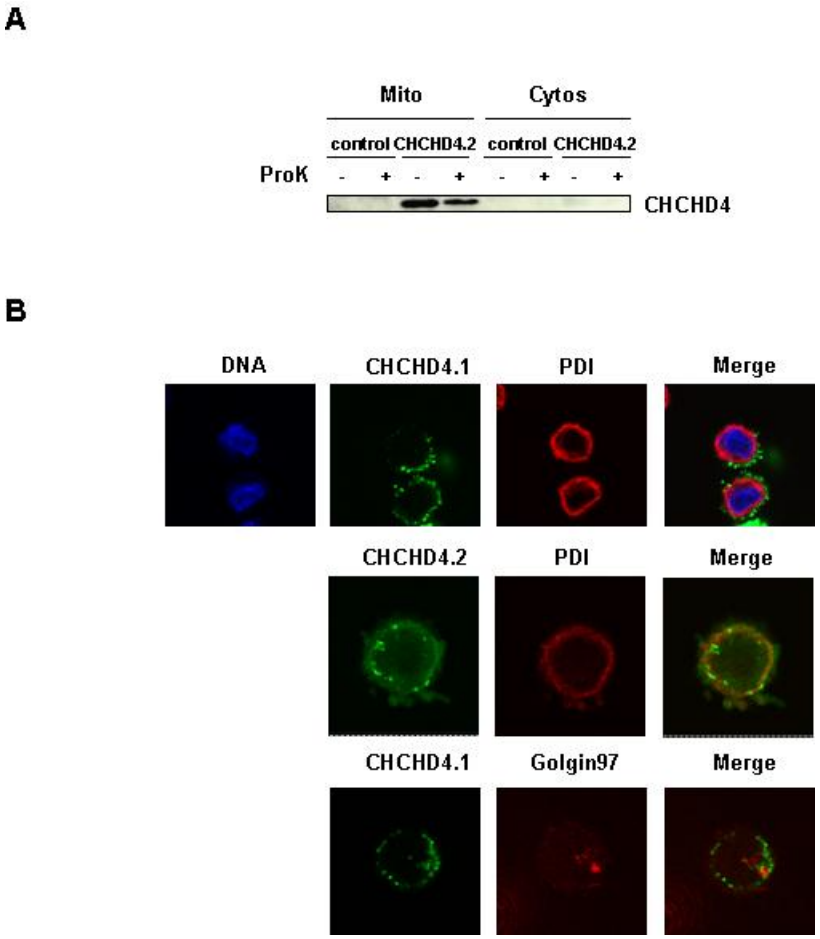
SUPPLEMENTAL INFORMATION: Yang et al.

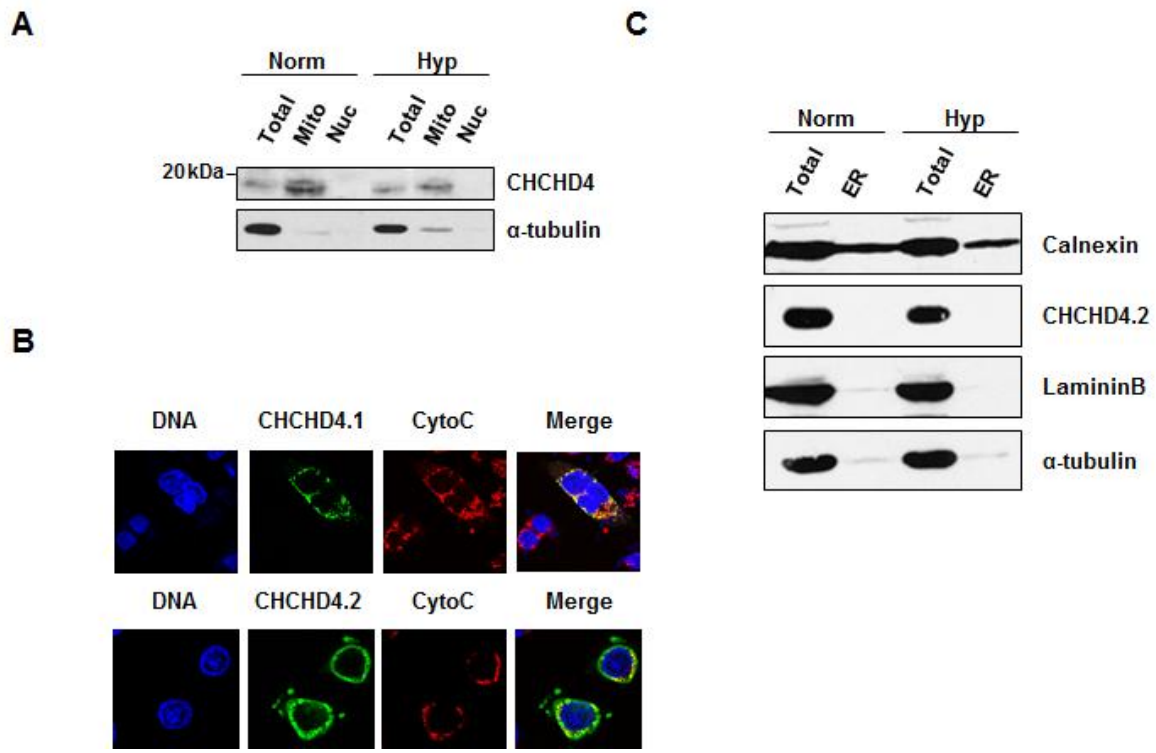
SUPPLEMENTAL DATA:



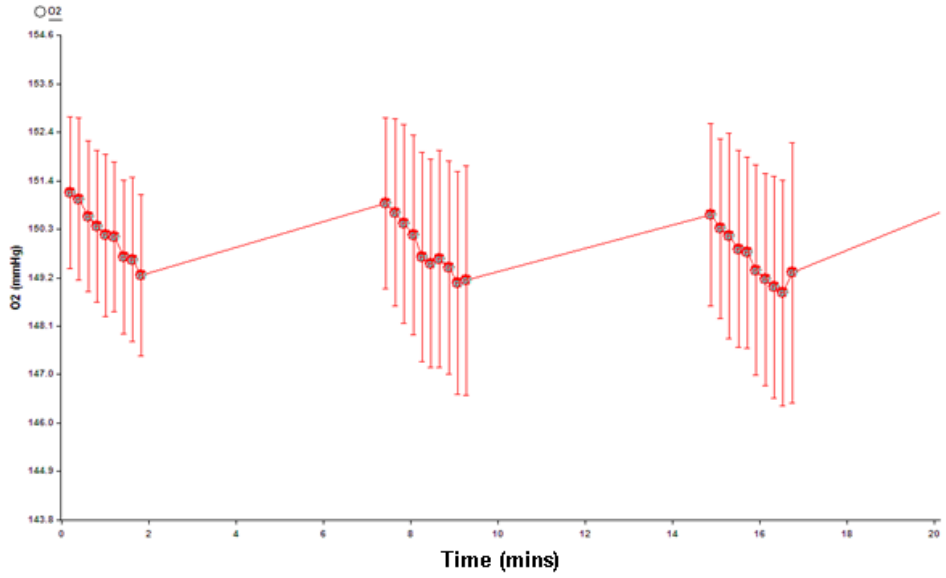
Supplemental Figure 1: CHCHD4 knockdown blocks HIF-1α induction in hypoxia. CHCHD4 siRNA or a non-silencing control (NSC) siRNA were transfected into (**A**) Saos-2 and (**B**) MCF-7 cells. Cells were incubated in hypoxia (1% O_2) or treated with DFX (500 μM) for 16 hours as indicated. Whole cell lysates were analyzed by western blot for HIF-1α and CHCHD4 proteins. Tubulin was used as a load control.



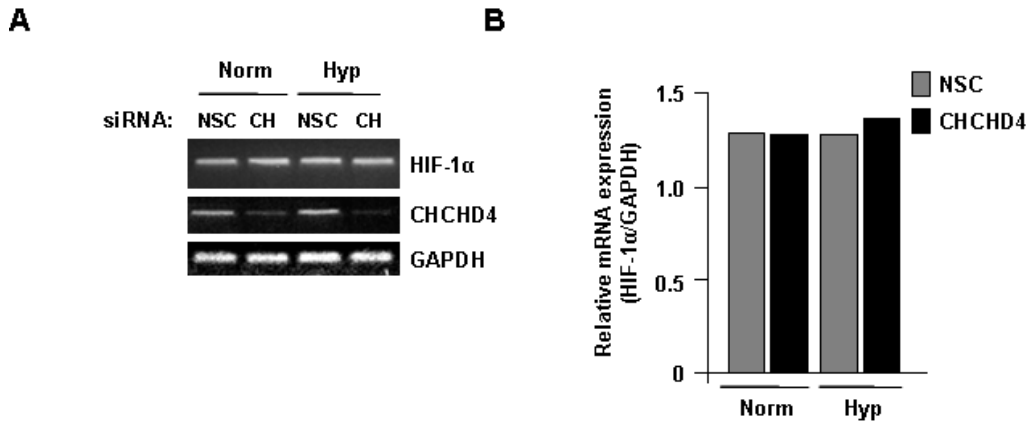
Supplemental Figure 2: CHCHD4 proteins localise to the mitochondria and are not detected in ER or Golgi. (**A**) HCT116 cells were transfected with myc-tagged CHCHD4.2, lysed and treated with (+) or without (-) proteinase K (ProK, 200 µg/mL) on ice for 30 minutes. Western blots were assessed for CHCHD4.2 (CHCHD4, myc) protein levels. (**B**) HCT116 cells transfected with myc-tagged CHCHD4 were stained with anti-myc antibody and imaged by confocal microscopy. The ER was visualised using an antibody to PDI. The Golgi was visualised using an antibody to Golgin97.



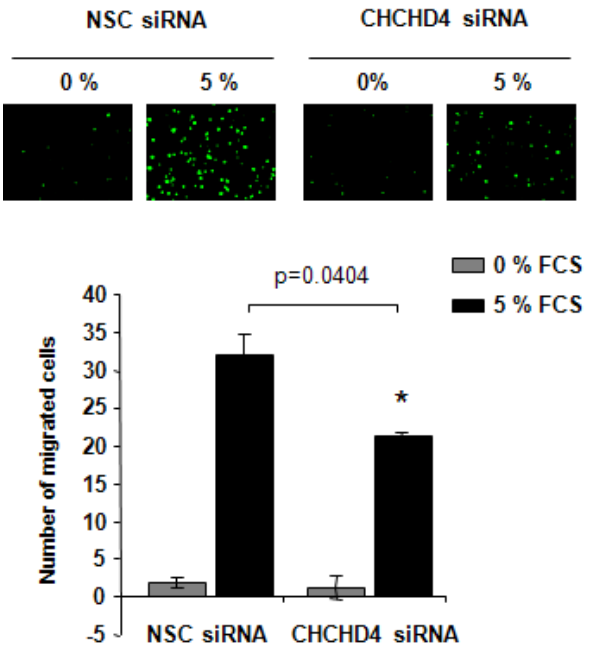
Supplemental Figure 3: Subcellular localization of CHCHD4 proteins in normoxia and hypoxia. (**A**) HCT116 cells were incubated in normoxia (Norm), hypoxia (1% O_2, Hyp) for 16 hours. Subcellular fractionation was performed and the fractions loaded at equal volume (Nuc= nuclear; Cytos = cytosolic; Mito= mitochondrial) were analyzed by western blot for HIF-1α and CHCHD4 proteins. Cytochrome c (CytoC) was used as a fractionation control. (**B**) HCT116 cells were transfected with myc-tagged CHCHD4.1/MIA40 or CHCHD4.2 vectors and exposed to hypoxia (1% O_2) for 16 hours. Cells were fixed and stained with an anti-myc antibody (CHCHD4, green) and imaged by confocal microscopy. The mitochondria were visualized using an antibody to cytochromeC (red). The nuclei were stained with TO-PRO-3 (blue). The overlay shows co-localized proteins in yellow. (**C**) Subcellular fractionation of HCT116 cells transfected with myc-tagged CHCHD4.2 after incubation in normoxia (Norm) or hypoxia (1% O_2, Hyp) for 16 hours. Western blots were assessed for the ER marker Calnexin, CHCHD4.2 (myc), lamin B and α-tubulin in total and ER subcellular fractions.



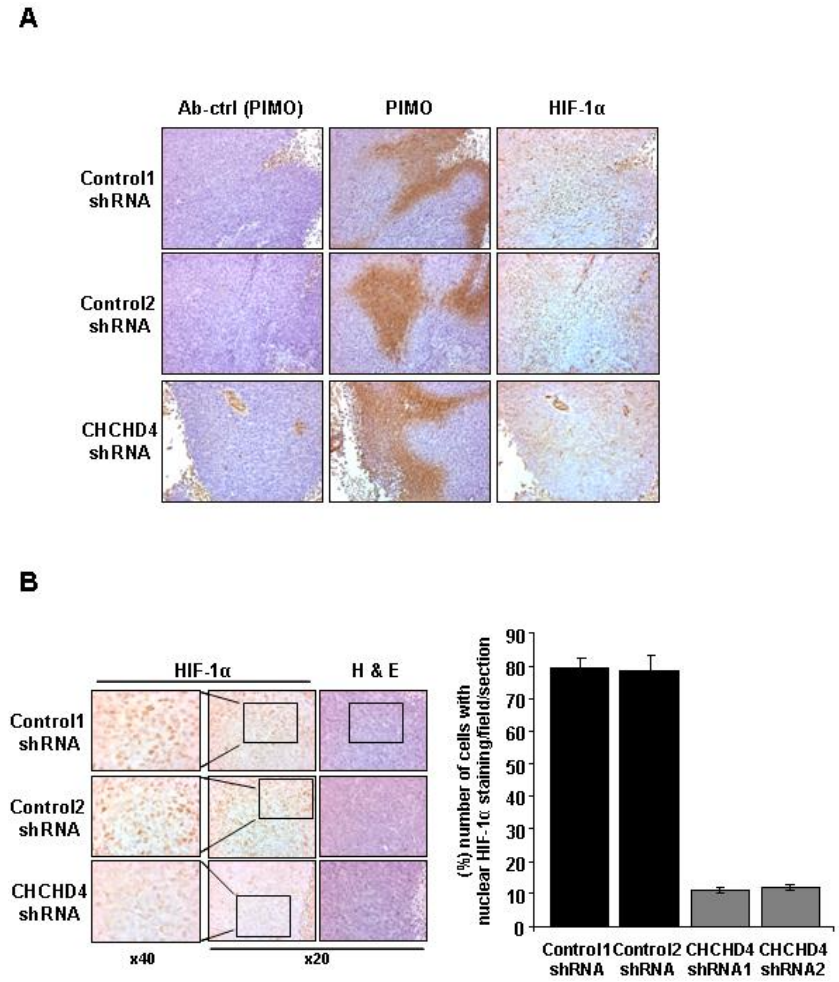
Supplemental Figure 4: Control cellular oxygen concentration measurements for HCT116 cells. Graph shows basal cellular oxygen concentration (mmHg) measurements for control HCT116 cells indicating consecutive mix, wait and measure cycles over the time period indicated using the Seahorse Bioscience XF24 analyser. Values are expressed as average ± SD (n=5).



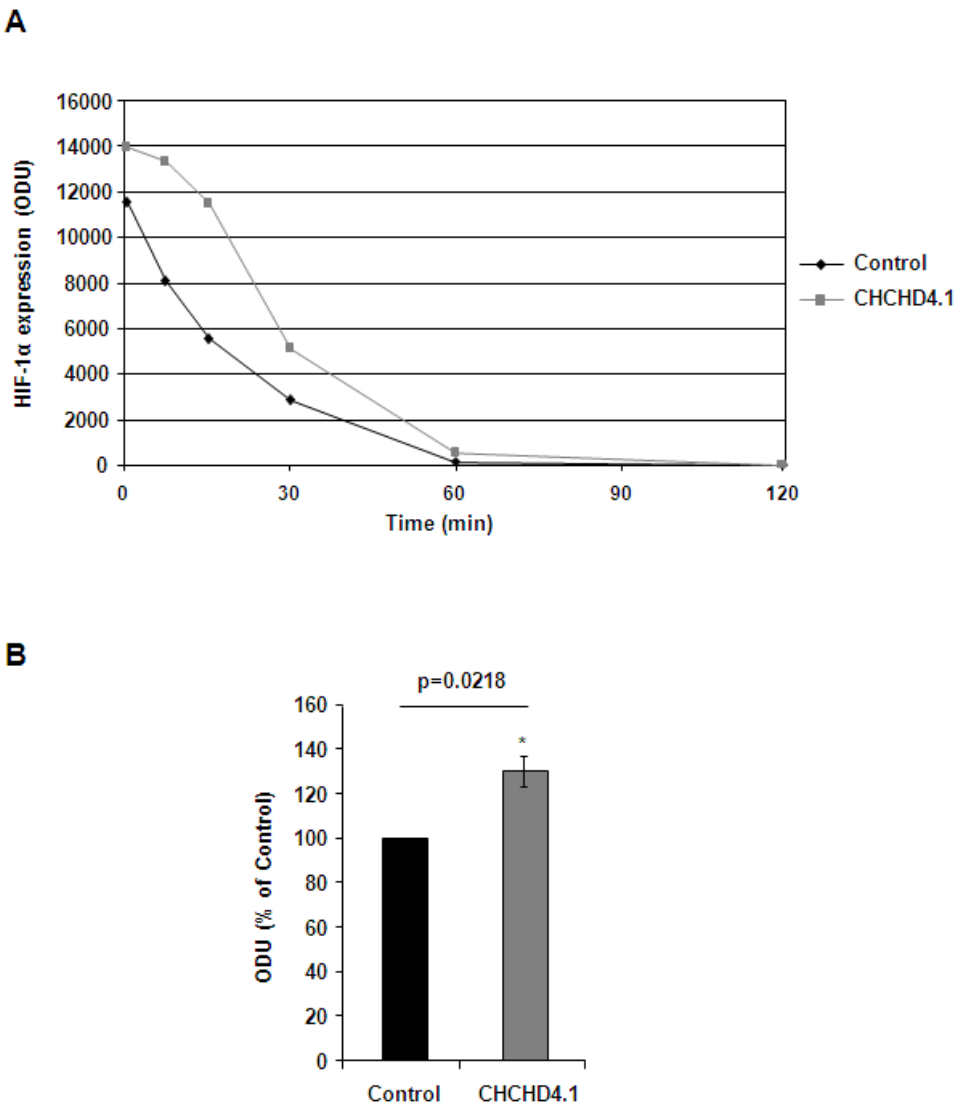
Supplemental Figure 5: CHCHD4 knockdown does not affect *HIF-1α* mRNA expression. HCT116 cells expressing a CHCHD4 (CH) siRNA or a non-silencing control (NSC) siRNA were incubated in normoxia (Norm) hypoxia (1% O_2) (Hyp) for 16 hours. Expression of *CHCHD4* and *HIF-1α* genes in (**A**) were analysed by real time quantitative PCR relative to GAPDH (**B**).



Supplemental Figure 6: CHCHD4/MIA40 proteins are critical for HIF-α function. Tumour cell migration is inhibited by loss of CHCHD4/MIA40. At 36 hour post-transfection with CHCHD4 or non-silencing control (NSC) siRNAs, HCT116 cells were trypsinised and labeled with the fluorescence tracker CMFDA. Cells were serum-starved for 4 hours and assessed for their ability to migrate towards FCS (5%) as a chemoattractant using an HTS FluoroBlok insert (BD Biosciences). Graphs show the average number of migrated cells (values are expressed as average ± SEM). Cells were counted from 10 independent fields of view with significance (*) p=0.0404, as indicated.



Supplemental Figure 7: CHCHD4/MIA40 proteins are required for HIF-1α induction in response to hypoxia in vivo. HCT116 cells stably expressing a CHCHD4 shRNA vector or two independent control shRNA vectors were subcutaneously injected into mice (n=8 mice/group) and grown as xenografts. Animals were treated with pimonidazole prior to sacrifice. Immunohistochemical analysis of HCT116 xenograft tumour sections. (**A**) Tumour sections show pimonidazole staining (PIMO) to identify areas of hypoxia, a respective antibody control (Ab-ctrl), and HIF-1α protein levels. (**B**) Tumour sections show nuclear HIF-1α protein levels in tumour cells (at x20 and x40 magnification) and tumour cell viability (H&E). Representative tumour sections are shown. Graph (right) shows mean percentage (%) number of cells scored for nuclear HIF-1α staining/field/section. Values are taken from at least three independent fields/section (two independent sections) for each condition and expressed as mean ± SEM.

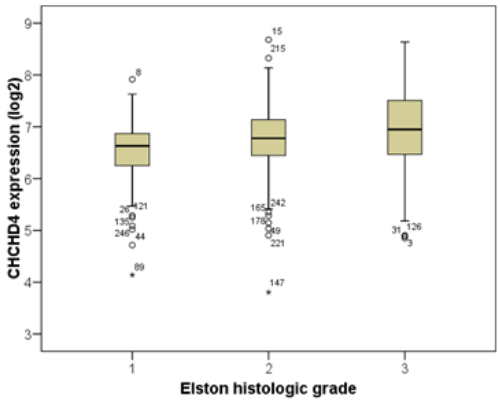


Supplemental Figure 8: Overexpression of CHCHD4/MIA40 enhances HIF-1α protein stability and increases cellular lactate levels. (**A**) Control HCT116 cells or HCT116 cells stably expressing myc-tagged CHCHD4.1 were exposed to hypoxia (1% O_2) for 16 hours then treated with cycloheximide (CHX, 50 µg/ml) for the times indicated. Graph shows HIF-1α protein levels relative to actin measured by densitometric analysis (optical density units, ODU) from western blot data shown in Figure 5A. (**B**) HCT116 cells were treated as described in A and cellular lactate levels were measured using a colorimetric assay kit (Abcam). Graph shows optical density units (ODU) represented a mean percentage (%) of control as indicated. Values are expressed as mean ± SEM and significance (*) p=0.0218, as indicated.

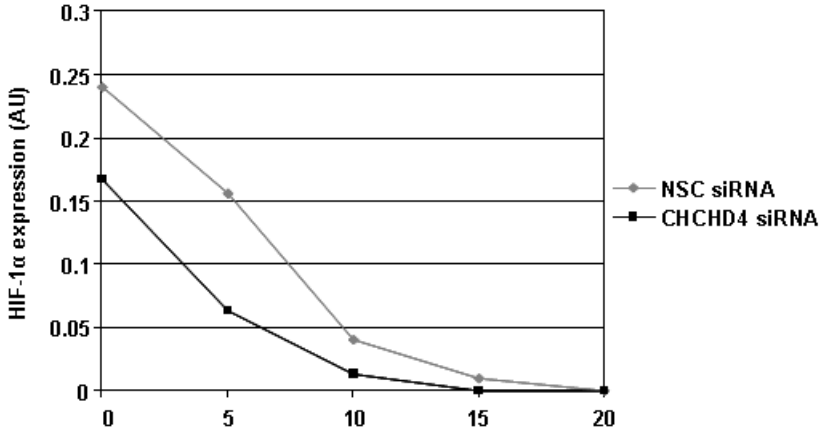
A

Continous variables: Spearman correlation				
		Age at operation	Tumour size	Elston Grade
CHCHD4	rho	-0.02	0.23	0.21
	p	0.7585	0.0002	0.0007
	N	251	251	249

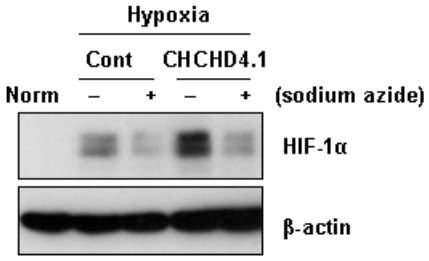
B



Supplemental Figure 9: Increased expression of CHCHD4 is associated with increasing tumour grade. CHCHD4 expression was correlated with tumour size and grade in up to 251 breast cancer samples. (**A**) We compared CHCHD4 expression with tumour size as a continuous variable and there was a highly significant correlation [Spearman rho=0.23, p=0.0002,]. Similarly, with Elston grade there was a strong correlation with poor grade, [Spearman rho 0.21, p=0.0007]. There was no relationship to oestrogen receptor status [Mann-Whitney Test p=0.432]. There was a relationship to node positivity [Mann-Whitney Test p=0.023]. (**B**) Whisker box plot showing CHCHD4 expression (log2) and Elston histological grade.



Supplemental Figure 10: CHCHD4/MIA40 regulates HIF-1α protein stability. HCT116 transiently expressing either a non-silencing control (NSC) siRNA or CHCHD4 siRNA were exposed to hypoxia (1% O_2) for 16 hours then treated with cycloheximide (CHX, 50 µg/ml) for the times indicated. Graph shows HIF-1α protein levels relative to actin measured by densitometric analysis (arbitrary units, AU) from western blot data shown in Figure 6A.



Supplemental Figure 11: CHCHD4/MIA40-mediated enhancement of HIF-1α protein levels is blocked with the complex IV inhibitor, sodium azide. Control (Cont) HCT116 cells or HCT116 cells stably expressing myc-tagged CHCHD4.1 were exposed to hypoxia (1% O_2) for 16 hours in the absence or presence of the sodium azide (5 mM). Whole cell lysates were analyzed by western blot for HIF-1α protein levels. Actin was used as a load control.

SUPPLEMENTARY METHODS:

Identification of CHCHD4 transcript variant 2

Functional genomics approaches have been developed to attempt to make use of the vast wealth of data produced by genome sequencing to describe gene functions and interactions. One such approach called Rosetta stone is a computation method of de novo protein function prediction, based on the hypothesis that some proteins involved in a given physiological process may exist as two separate genes in one organism and as a single gene in another (20-22). If two genes have fused (or are associated with one another), it is predicted that they have co-regulatory functions, making it evolutionarily advantageous. Based on this rationale, we used the human HIF-1α protein sequence (NP_001521.1) to search the Genbank database with the Position-specific Iterated BLAST (PSI-BLAST) program and initially identified the Apis mellifera nucleotide sequence, XM_392382.1, GI:48096026; http://www.ncbi.nlm.nih.gov/nuccore/48096026, protein sequence XP_392382.1, GI:48096027. Using this sequence, further BLAST analyses identified the human sequence *CHCHD4* transcript variant 2 (GenBank accession number, NM_144636.2, GI:148612891), indicating the possibility CHCHD4 and HIF-1α proteins may be functionally linked.

Cloning of CHCHD4 cDNA and construction of vectors

Total RNA from HCT116 cells was prepared using the RNAeasy Kit (QIAGEN) according to the manufacture's instructions. 5 µg of total RNA was used for cDNA synthesis using the SuperScript First-Strand synthesis system (Invitrogen). 2 µL of first strand reaction was used for each PCR reaction with the gene specific sense primers for *CHCHD4.1* (5-GATGGTTCCTTCTGGAATGTCTAT-3) and *CHCHD4.2* (5-GCCATGTCCTATTGCCGG-3) and gene specific antisense primers for *CHCHD4.1* (5-TAACTTGATCCCTCCTCTTCTTTG-3) and *CHCHD4.2* (5-CTGGTCTACACAGTCTGA CCC-3). The PCR products were cloned into the TA cloning vector (Invitrogen) and sequenced with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems/Hitachi) by MWG-Biotech.

For the construction of expression vectors, the open reading frame of *CHCHD4.1* or *CHCHD4.2* was reamplified from the TA clones using Pfu Turbo polymerase (Stratagene). *CHCHD4.1* and *CHCHD4.2* were cloned into the EcoRI and BamHI

sites of the mammalian expression vector pcDNA3.1-myc/his (Invitrogen) to generate the CHCHD4.1 and CHCHD4.2 myc-tagged constructs.

Real-Time quantitative PCR assays

Real-time quantitative PCR using primers for *HIF-1α* (forward: 5-GCAAGCCCTGAAAGCG-3, reverse: 5-GGCTGTCCGACTTTGA-3) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5-ATGGGGAAGGTGAAGGTCG-3 and reverse: 5-TAAAAGCAGCCCTGGTGACC-3) was performed as described previously (42). PCR primers for *CHCHD4.1* (forward 5-GCCATGTCCTATTGCCGG-3, reverse 5-CTGGTCTACACAGTCTGACCC-3) and *CHCHD4.2* (forward: 5-GATGGTTCCTTCTGGAATGTCTAT-3, reverse 5-TAACTTGATCCCTCCTCTTCTTTG-3) were purchased from Invitrogen (Paisley, UK). Expression of target gene mRNA relative to reference gene mRNA (*GAPDH*) was calculated using the Relative Expression Software Tool (REST™) and statistical significance was determined using the Pair Wise Fixed Reallocation Randomization Test™ available from http://www.gene-quantification.com.

VEGF ELISA

Following treatment of cells, total VEGF in whole cell lysates or in the conditioned culture media as indicated were measured directly by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's protocol (QuantiGlo, R&D Systems) and described by us recently (44). Calibration curves were performed for all experiments.

Migration and invasion assays

After 36 hours post-transfection with CHCHD4 siRNAs as described above, $p53^{+/+}$ HCT116 cells were serum-starved for 4 hours. The fluorescence tracker CMFDA (Molecular Probes) was added to a final concentration of 5 μM and left incubating for one hour. Cells were then trypsinized using Tryple Express (Invitrogen), spinned down and diluted in phenol-free DMEM medium (Gibco) without serum to $1x10^5$/mL. 800 μL of serum-free medium containing 5% FCS as a chemoattractant was added to the bottom of the well, while 300 μL of labelled cells was added in to the HTS FluoroBlok Insert (BD Biosciences) with 8.0 μm pore size. Cells were incubated in

normoxia or hypoxia (1% O_2) for 16-20 hours and the migrated cells were examined and triplicate counts were taken using a fluorescent microscope. Three independent experiments were carried out. For tumour cell invasion, the BioCoat™ tumour system (BD Biosciences) was used according to the manufacturer's recommendations.

Localization and subcellular fractionation analyses of CHCHD4

For immunofluorescence analysis of CHCHD4, HCT116 cells were transfected with CHCHD4-myc expression constructs either using calcium phosphate precipitation or SuperFect (QIAGEN). Cells on the coverslips were washed three times with PBS and fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature. Cells were permeabilized for 10 minutes at room temperature in a staining buffer containing Triton X-100 (0.5%), and then incubated with primary antibody (1:100) in the buffer (1% BSA, 2% FCS in PBS) for 1 hour. After washing three times with PBS, cells were incubated with secondary antibody (1:500) for 1 hour. The cells were washed three times with PBS, and coverslips were dipped twice in water then mounted onto slides using mounting media (VectaShield; Vector Laboratories). Imaging of the cells was carried out using a Zeiss LSM510 META laser scanning confocal microscope.

For subcellular fractionation experiments, the cytoplasmic and nuclear fractions were prepared using the NE-PER kit (Sigma) according to the manufacturer's protocol. The mitochondria were extracted using the Mitochondria Isolation kit purchased from Perbio. Subcellular fractions were separated by SDS-PAGE and assessed by western blot analysis.

Immunohistochemical analyses

For HIF-1α, and Hematoxylin and Eosin (H&E) staining, 3 μm serial sections from paraffin embedded tissue blocks were placed on Snowcoat Xtra slides (Surgipath) and dried at 37^{o}C overnight. Tissues were dewaxed in xylene and rehydrated using graded ethanol washes. For Hematoxylin and Eosin staining, slides are thoroughly washed in distilled water and then placed into Hematoxylin and Eosin solution. After this slides are quickly dipped into acid (1%)/alcohol (70%) solution. Slides are then dehydrated in graded ethanol washes and mounted with DePex (VWR International). For HIF-1α staining, an appropriate positive control was used and mouse or rabbit serum was used as a negative control. Antigen retrieval was carried out using a pressure cooker with

1x target retrieval solution (Dako). Antigen/antibody complexes were detected using either the CSA I kit (Dako) and a mouse biotinylated secondary (Dako) following the manufacturer's instructions. After developing the slides with DAB chromagen, sections were counterstained with haematoxylin (Sigma) for 30 seconds, dehydrated in graded ethanol washes and mounted with DePex moutant (VWR International). All washes were performed in TBS (trisbase and sodium chloride, pH 7.6) and 0.1% Tween (Sigma).

For CD34 staining, sections were deparaffinized, rehydrated and subjected to microwave antigen retrieval in a 10mM citrate buffer solution for 20 minutes. Following blocking of endogenous peroxidase with 3% H_2O_2/methanol for 15 minutes at room temperature, sections were first incubated in 3% normal rabbit serum (Dako) for 30 minutes, followed by a 1 hour incubation at room temperature with a 1:5 dilution of a rat anti-mouse anti-CD34 antibody (Hycult Biotech). After washing with phosphate buffered saline (PBS), sections were incubated for 30 minutes with a 1:200 dilution of a biotin conjugated rabbit anti-rat antibody (Dako), rinsed in PBS, and then incubated with the avidin–biotin complex (Vectastain ABC Kit, Vector Laboratories Ltd) following the manufacturer's instructions. Sections were developed with 3,3-diaminobenzidine (Sigma-Aldrich) and counterstained with hematoxylin. Sections were mounted in dibutyl phthalate xylene and visualized under a light microscope.

For immunostaining of pimonidazole, mice were injected with 60mg/kg pimonidazole HCl (NPI-Hypoxyprobe) intraperitoneally 30 minutes before sacrifice. Tumours were isolated, formalin fixed for 24hrs after which tissue was paraffin embedded. Immunohistochemistry was performed on 3μm thick serial sections. Sections were deparaffinized, rehydrated in graded ethanol concentrations and subjected to microwave-based antigen retrieval in a 10 mM citrate buffer solution for 15 minutes. Following blocking of endogenous peroxidase with H_2O_2 (ARK^{TM} Dako) for 5 minutes at room temperature, sections were first incubated in normal horse serum (Vectastain ABC Kit, Vector Laboratories Ltd) for 30 minutes, followed by an overnight incubation at 4ºC with a 1:50 dilution of a mouse monoclonal anti-pimonidazole antibody (NPI-Hypoxyprobe). After washing with phosphate buffered saline (PBS), sections were then incubated with a biotin conjugated horse anti-mouse antibody. (Vectastain ABC Kit, Vector Laboratories Ltd), Sections were washed in

PBS, and then incubated with the avidin–biotin complex (Vectastain ABC Kit, Vector Laboratories Ltd) following manufacturer's instructions. Sections were developed with 3,3-diaminobenzidine (Sigma-Aldrich) and counterstained with hematoxylin and dehydrated in graded ethanol concentrations. Sections were mounted in dibutyl phthalate xylene and visualized under a light microscope.