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Supplemental Data

Article

HIF-α Effects on c-Myc Distinguish

Two Subtypes of Sporadic *VHL*-Deficient

Clear Cell Renal Carcinoma

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Supplemental Experimental Procedures

Antibodies

Antibodies for IHC were as follows: HIF-1α (Novus monoclonal NB100-131, 1:600 for frozen material, Labvision HIF-1A67 1:10,000 for FFPE material), HIF-2α (Novus mouse monoclonal NB100-132, 1:200 for frozen material, Novus rabbit polyclonal 1:5,000 for FFPE material), Ki-67 (Novocastra mouse monoclonal MIB-1 1:50), Skp2 (Zymed mouse monoclonal 2C8D9, 1:200), phospho-S6 (Cell Signaling rabbit polyclonal 1:100), phospho-ERK (Cell Signaling rabbit polyclonal 1:200), γH2AX (Cell Signaling rabbit polyclonal 1:100, Upstate mouse monoclonal 1:5000), phospho-Thr68-Chk2 (Calbiochem rabbit polyclonal 1:200), BARD1 (Novus rabbit polyclonal 1:500) and BRCA1 (Cell Signaling rabbit polyclonal 1:50).

Antibodies for immunoblotting were as follows: HIF-1α (Novus mouse monoclonal NB100-131), HIF-2α (Novus rabbit polyclonal), phospho- and total S6, γH2AX and total H2AX, total Chk2, and Skp2 (Zymed mouse monoclonal 2C8D9), Cdc7 (Novus mouse monoclonal SPM171), BRCA1 (Cell Signaling rabbit polyclonal), BARD1 (Zymed rabbit polyclonal), phospho-Thr68-Chk2 (Calbiochem rabbit polyclonal), FLAG tag (Santa Cruz rabbit polyclonal) phospho-Ser345-Chk1 (Cell Signaling rabbit monoclonal) and total Chk1 (Cell Signaling mouse monoclonal).

Immunohistochemical Protocols

For both fresh frozen and paraffin embedded sections, Avidin/Biotin blocking, secondary antibodies, ABC reagents, and DAB, were used following the manufacturers specifications (Vector Labs). Hematoxylin provided counterstaining. For immunofluorescence, Alexa Fluor labeled secondary antibodies (Invitrogen) and DAPI mounting media (Vector Labs) were used following the manufacturers' specifications. FFPE specimens were unmasked for 20 minutes in a vegetable steamer with Vector Lab's Antigen Unmasking Solution, except for Ki-67 and γH2AX where samples were boiled. Avidin/Biotin blocking, secondary antibodies, ABC reagents, and DAB, were used as above, except for HIF-1α and HIF-2α staining, where the Dako Catalyzed Signal Amplification kit was used.

Analysis of Microarray Results

For the initial date set, Affymetrix Microarray Analysis Suite (MAS5) statistical algorithm was used to extract numerical data. Data were normalized with the GC-RMA algorithm (Array Assist Lite version 3.4). Probe sets that were scored as not detectable in more than 50% of samples, or showing a variance less than 0.15, as well as control probe sets, were removed before further analysis. Class distinction analyses were performed using the comparative marker selection algorithm from the Gene Pattern analysis suite (Broad Institute) (Reich et al., 2006). Clustering analysis was performed with Cluster 3.0, and gene ontology defined with Ingenuity Pathway Analysis (Ficenec et al., 2003).

The raw aCGH data were processed using Illumina's BeadStudio software and then exported to the Partek Genomics Suite (version 6.4) for further analysis using Copy Number Workflow. The segmentation was measured using Partek's Genomic Segmentation algorithm with the default parameters. Segments were called aberrant either if their copy number was greater than 2.3 or if their copy number was less than 1.7. A p-value of 10^{-6} was used to make these calls. The amplified and deleted segments together are referred to as "aberrant segments." The percent of the genome that was aberrant in each sample was determined by adding up the number of probes covered by all the aberrant segments in that sample and dividing by the total number of probes on the chip. A one-tailed t-test was performed to compare the different sub-groups of clear cell renal tumors based on the percent to the genome that was aberrant.

Finally, for the validation expression arrays, Agilent Feature Extraction software with Lowess Normalization was employed, and probes with a raw value lower than 175 were excluded as absent. These data were analyzed with SAM, as they could be interpreted by Gene Pattern. For comparison to the primary data set, gene lists were reduced to those found to be significant in the primary analysis and comparison was performed. All genes with a q value less than 0.15 were considered significant.

Cell Cycle and Proliferation Analysis

For cell cycle analysis, cells were plated to achieve <50% confluency when assayed, with each point measured in triplicate. They were pulsed with 10 μM BRDU for 20 minutes, and then stained for with Alexa-Fluor488 conjugated anti-BRDU (Invitrogen) following standard protocols. Proliferation was measured by serial cell counts on duplicate plates. All experiments were performed in media containing 10% serum.

Protein and RNA Extraction

RNA was extracted with Trizol Reagent (Invitrogen) following the manufacturer's specifications. Tumor derived material was put through a second Trizol extraction and further purified using RNeasy spin columns (Qiagen). Protein was extracted with RIPA, complete proteinase inhibitors (Roche), β-Glycerophosphate, Sodium Fluoride and Sodium Vanadate. RNA electrophoresis was performed to confirm sample quality, and standard SDS-PAGE and protein transfer techniques were used. Antibodies are listed in Supplemental Methods.

siRNA Analysis

siRNAs against c-Myc (Hs_Myc_2, Hs_Myc_5), or a control siRNA were obtained from Qiagen. Transfection was performed using Hiperfect (Qiagen) as directed. 40 hr after transfection, cells were harvested for protein and RNA as above.

qRT-PCR

The Superscript II RT kit (Invitrogen) was used with random hexamer primers (Roche) to produce cDNA from 2 mg total RNA. 18S and β–actin were used as endogenous controls for tumor samples. All primers for analysis were generated with Primer Express (Applied Biosystems); sequences available on request. c-Myc mRNA expression was confirmed with a TaqMan primer set (Hs00153408_m1, Applied Biosystems). Analysis was performed using an Applied Biosystems 7900HT Sequence Detection System, with SYBR green labeling. Microarray results were confirmed directly in those tumors analyzed, with pooled tumor margin samples from 14 cases as normal control.

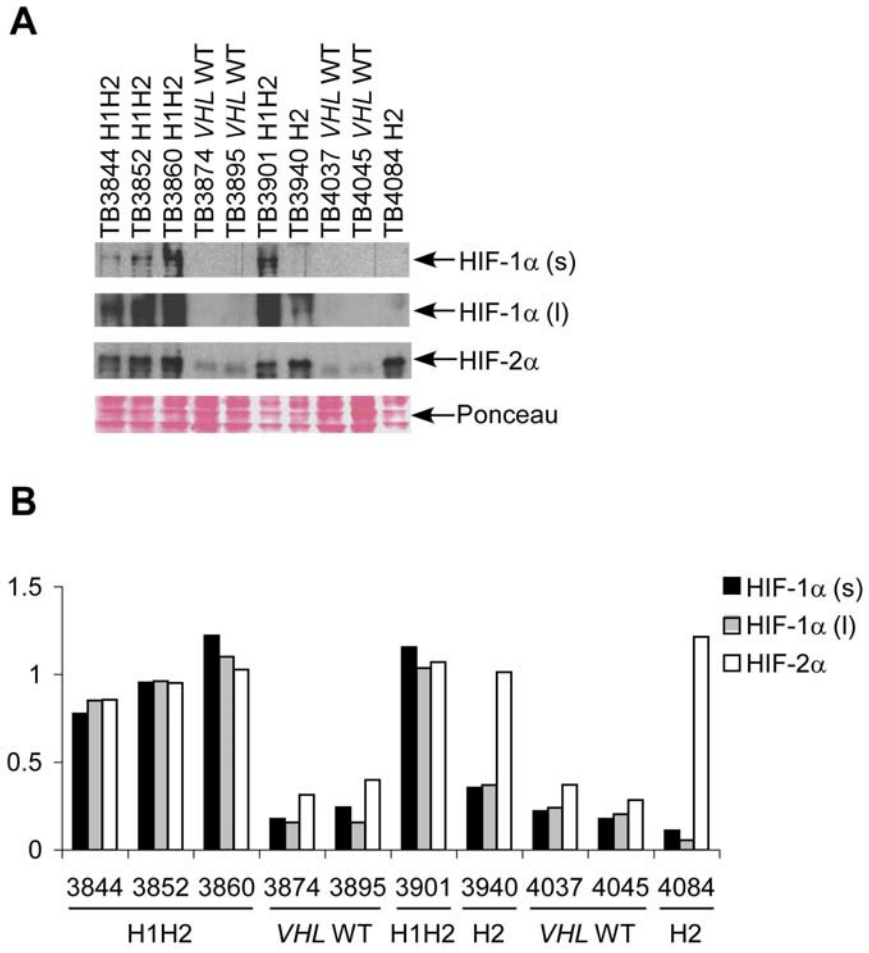


Figure S1. Immunoblot Analysis of HIF-α Expression

(A) Western blot analysis of HIF-α expression in a subset of tumor specimens selected for microarray. Short (s) and long (l) exposures of HIF-1α are shown. A faint background band migrating faster than HIF-2α was observed in the HIF-2α immunoblots of *VHL* WT proteins. Ponceau S staining of total protein is used as a loading control.

(B) HIF-α western blots were scanned, and band intensity was quantified with Image J. Quantification was performed on short (s) and long (l) exposures of HIF-1α. Values were normalized to Ponceau S staining intensity, as well as equivalent amounts of FLAG-tagged, in vitro translated HIF-1α and HIF-2α proteins (data not shown). Together, these data indicate that HIF-1α and HIF-2α are present at low but approximately equivalent levels in *VHL* WT tumors, and at higher but equivalent levels in H1H2 tumors. In contrast, H2 tumors have elevated levels of only HIF-2α protein.

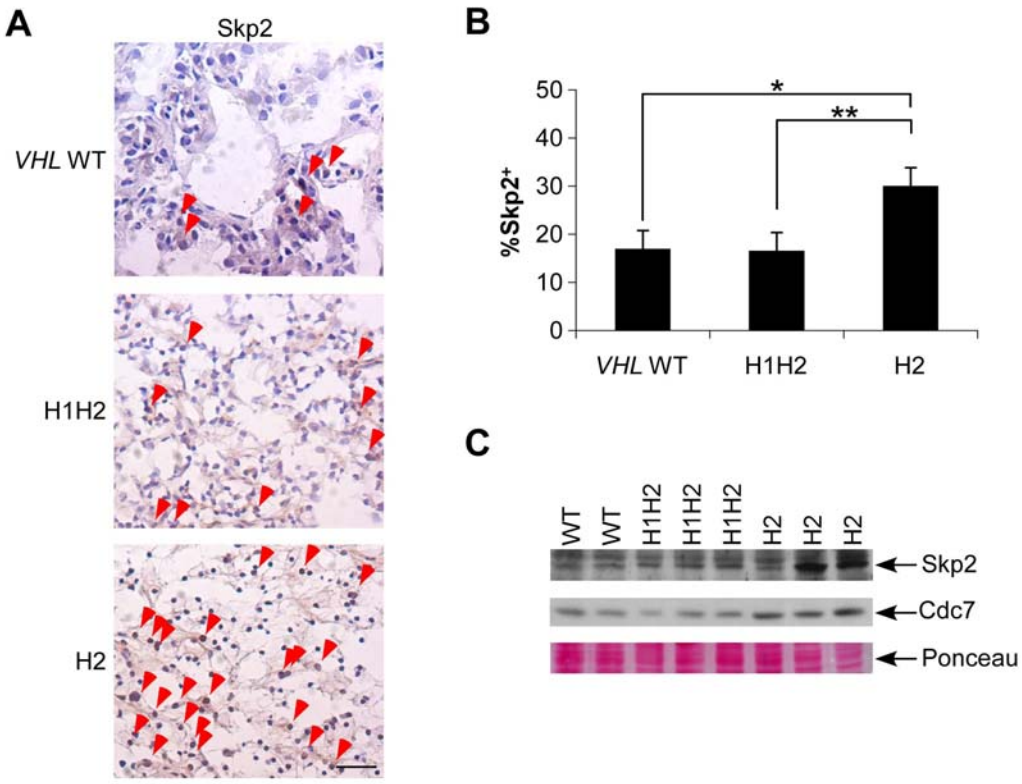


Figure S2. Measurement of Skp2 Protein Expression by IHC and Western Blot

(A) Representative Skp2 staining in fresh frozen tumors. Scale bar is 0.5 μM.

(B) Summary of Skp2 staining from 4 *VHL* WT, 12 H1H2 and 12 H2 tumors. Data are shown as mean percentage $Skp2^{+}$ from each tumor, ±1 SEM. *$p < 0.05$, **$p < 0.01$.

(C) Western blot of Skp2 and Cdc7 in VHL WT, H1H2 and H2 tumors. Ponceau S staining shown as a loading control.

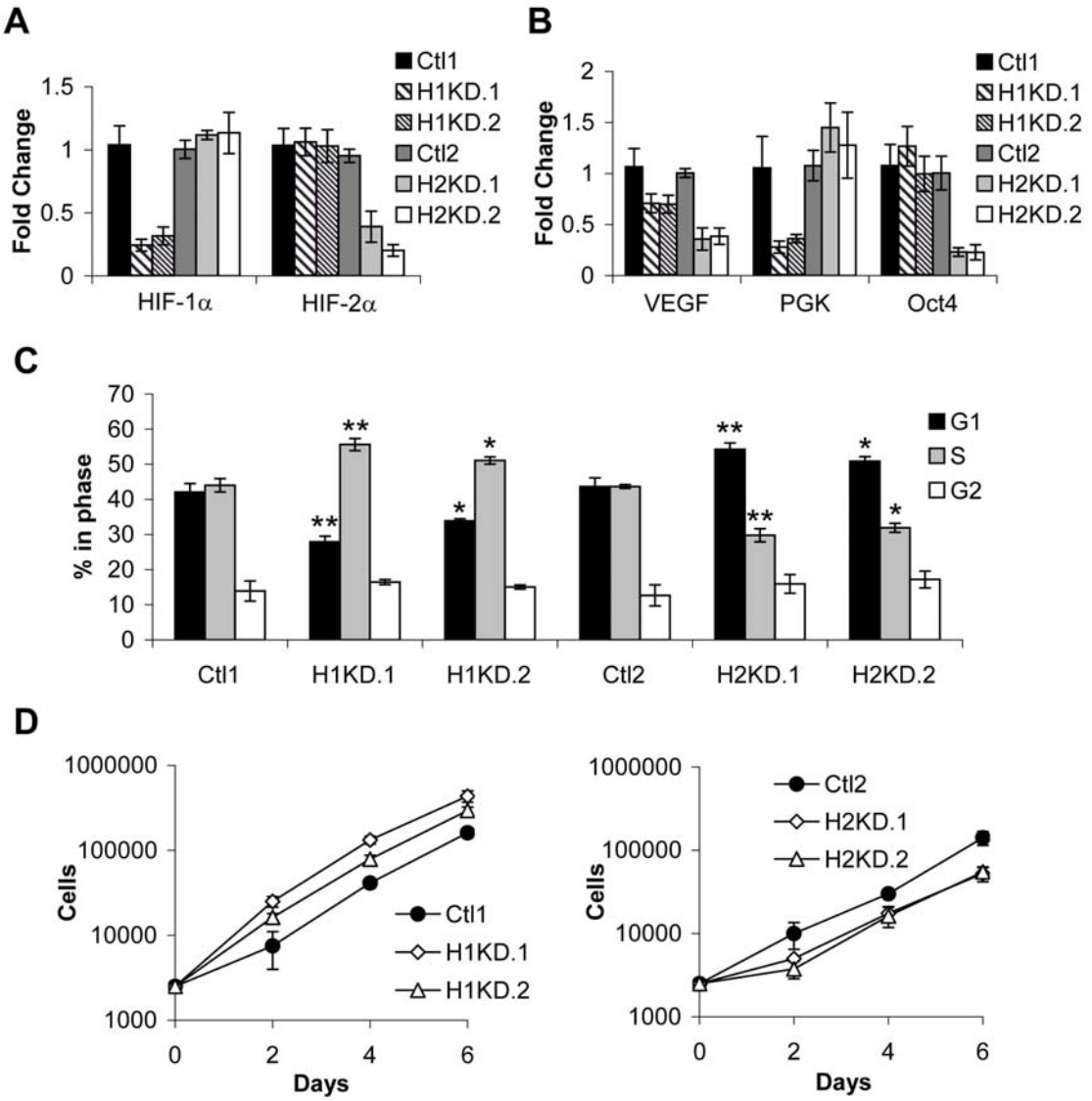


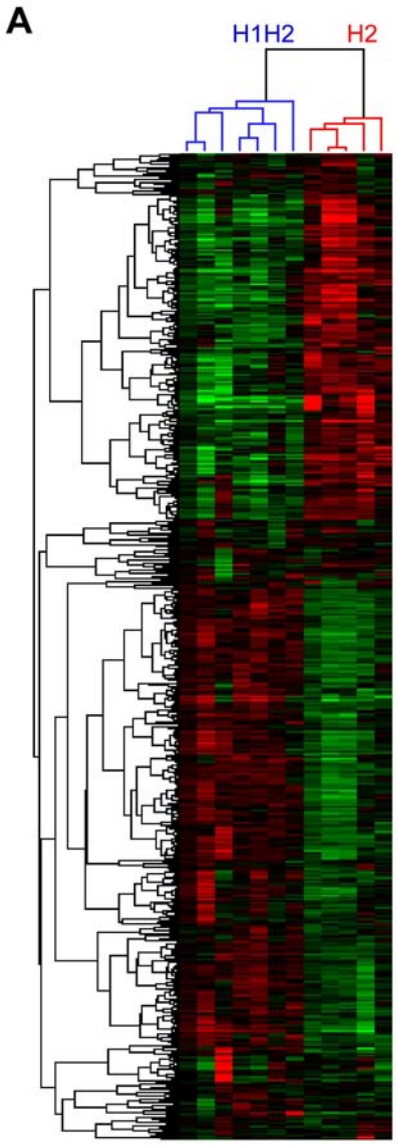
Figure S3. Characterization of RCC4 Cells with HIF-1α and HIF-2α Knockdown

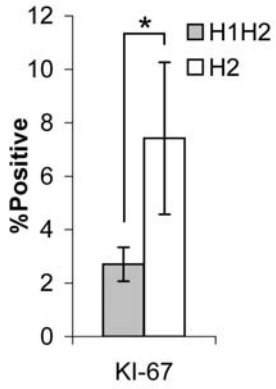
(A) qRT-PCR of HIF-1α and HIF-2α expression in RCC4 clones. "H1KD.1" and "H1KD.2" represent HIF-1α knockdown clones, whereas "H2KD.1" and "H2KD.2" are HIF-2α knockdown clones. Average expression from 4 experiments is shown, ±1 SEM.

(B) qRT-PCR of VEGF (HIF-1α/HIF-2α target), PGK (HIF-1α target) and Oct4 (HIF-2α target) expression in RCC4 clones. Average values from 4 experiments, ±1 SEM.

(C) Cell cycle distribution by BRDU incorporation. Data averaged from 4 experiments, ±1 SEM. Statistical comparisons were performed between Ctl1 and H1.1, Ctl1 and H1.2, Ctl2 and H2.1, Ctl2 and H2.2 for % of cells in G1 and S-phase, *$p < 0.05$, **$p < 0.01$.

(D) Proliferation in RCC4 clones measured by serial counting. Data from one representative experiment are shown logarithmically, ±1 SD. Statistically significant differences ($p < 0.05$) were noted between control and knockdown clones at each time point.





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Name	Q val.	Fold Change H2 vs. H1H2
Asp-like	0.000	8.65
DCLRE1C	0.011	2.09
E2F1	0.013	3.07
Cyclin D2	0.021	3.20
BuB1	0.025	2.24
p73-like	0.025	2.14
Cyclin B1	0.034	1.77
CDC7	0.037	1.93
BARD1	0.043	1.85
MCM3	0.043	1.89
CENPE	0.067	1.72
DHFR	0.082	1.20
BRCA1	0.088	1.43
Skp2 (p45)	0.110	1.64
CDC5-like	0.122	1.30
PMS2-L4	0.126	1.57
V-myb	0.144	1.29

Figure S4. Validation of Primary Microarray Results in a New Data Set

(A) Two-way clustering of 700 most significantly altered genes, $q < .05$. H1H2 tumors ($n = 7$) in blue, H2 ($n = 5$) in red.

(B) Quantification of Ki-67 staining in H1H2 and H2 tumors, data are shown ±1 SEM.
*$p < 0.05$.

(C) Partial list of overlapping cell cycle/DNA repair genes upregulated in H2 tumors in each data set. q values for significance and fold change (H2 vs. H1H2) are from the data set presented here.

Supplemental Tables

(See Excel workbook.)

Table S1. Detailed Description of VHL Status and Clinical Information for CHTN Material

Patient information was summarized from CHTN provided pathology reports, and VHL status summarized from sequencing, methylation analysis and qPCR for copy number.

Table S2. Overview of Expression Profiling Results

Microarray results were analyzed for differentially expressed targets with Gene Pattern 2.0. Differentially expressed genes were organized into groups using Ingenuity Pathways analysis. Only genes with FDR < 0.15 are shown.