Supplemental Table 1. Homer Motif Analysis of Regions Gaining 5-hmC in Hypoxia, Related to Figure 2

Transcription Factor	Observed (%)	Expected (%)	Log(p-value)
ZFX	80	33	-53
Eomes	83	49	-29
ZNF711	91	61	-29
NeuroD1	58	25	-28
N-Myc	50	18	-27
PIF5ox	57	25	-26
Foxo1	66	35	-23
Max	45	17	-23
c-Myc	35	11	-22
Stat3	40	15	-21
AR-halfsite	97	75	-21
Pho2	33	10	-20
c-Myc	40	16	-19
TRa	45	20	-19
E2F4	28	8	-19
Znf263	89	65	-18
STAT4	45	20	-18

Supplemental Experimental Procedures

Cell Culture

Neuroblastoma cell lines SK-N-BE(2), NBL-WN, La1-55n, SK-N-AS, and SHEP were maintained in RPMI 1640 media supplemented with 10% fetal bovine serum. For hypoxia exposures, both control and hypoxic cells were seeded 18-24 hours prior to the exposure at 35-45% confluency and cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum and 1 mM sodium pyruvate. For hypoxic conditions, cells were incubated under 1% O_2 and 10% CO_2 in modular incubator chambers (Billups-Rothenberg). For normoxic conditions cells were incubated at 10% CO_2 and ambient oxygen levels (20%). At the completion of hypoxia experiments, media was immediately aspirated and cells snap frozen in liquid nitrogen to avoid reoxygenation of cells cultured at 1% O_2.

HIF-1α Immunoblot

Cells were lysed in 100 mM Tris (pH=6.8), 4% SDS, 20% glycerol, and 0.56 M β-MeOH. After boiling for 10 minutes, proteins were separated on 8% polyacrylamide-SDS gels and transferred onto Amersham-Hybond ECL membranes (GE Healthcare Life Sciences). Blots were blocked with 5% milk in TBS-T for 1 hour and then sequentially probed with 1:1,000 polyclonal rabbit anti-HIF-1α antibody (Novus, 100-479), and HRP conjugated anti-rabbit secondary antibody diluted 1:10,000 (Jackson Immunological Research, 111-036-047). Bands were detected using Western Lightning ECL reagent (PerkinElmer).

Quantification of 5-methylcytosine and 5-hydroxymethylcytosine by High Performance Liquid Chromatography Coupled With Tandem Mass Spectrometry (HPLC-MS/MS)

HPLC-MS/MS analysis of 5-methylcytosine and 5-hydroxymethylcytosine was performed as described previously (Madzo et al., 2014; Vasanthakumar et al., 2013). Two micrograms of genomic DNA were denatured by heating at 100°C. Five units of Nuclease P1 (Sigma, Cat # N8630) were added, and the mixture incubated at 37°C for 2 hours. A 1/10 volume of 1M Ammonium bicarbonate and 0.002 units of venom phosphodiesterase 1 (Sigma, P3243) were added to the mixture and the incubation continued for 2 hours at 37°C. Next, 0.5 units of alkaline phosphatase (Invitrogen, 18009-027) were added and the mixture incubated for 1 hour at 37°C. The reactions were diluted 10-fold to dilute out the salts and enzymes, and injected into a Zorbax-26 4.6 mm x 50 mm column (3.5 μm particle size) (Agilent, 927700-902). Samples were run on an Agilent 1200 Series liquid chromatography machine in tandem with the Agilent 6410 Triple Quad Mass Spectrometer. LC separation was performed at a flow rate of 220 μL/min. Quantification was performed using the Agilent MassHunter Workstation software using the multiple reaction monitoring (MRM) mode. All runs included standard curves for 5-mC and 5-hmC, which were used for subsequent normalizations. Each standard curve was composed of five concentrations: 0.0025, 0.025, 0.25, 2.5, and 10% 5-mC (or 5-hmC), and the peak intensities obtained were used to calculate the observed %5-mC and %5-hmC by the formula %5-mC (or %5-hmC)= 5-mC (or 5-hmC) signal *100 /Sum of signals from dC, 5-mC and 5-hmC. Linear regression was performed using the known input percentages and the calculated percentages as above, to obtain slopes and intercepts. Using the above formula, %5-mC and %5-hmC was calculated from the samples, and the slopes and intercepts derived from linear regression were used to calculate the actual %5-mC and %5-hmC values.

Quantification of 5-hydroxymethylcytosine by Dot Blot

Genomic DNA was sonicated to 200-500 base pairs. 5-hmC was conjugated to a previously described uridine diphosphoglucose derivative (UDP-6-N3-Glu) using β-glucosyltransferase (β-GT) (Song et al., 2010). The glycosylation reaction was carried out in a solution of 50 mM HEPES (pH 7.7), 25 mM $MgCl_2$, 250 uM UDP-6-N3-Glu, and 2.25 μM β -GT with a final volume of 20 μL. DNA was purified from this reaction with Micro Bio-Spin Columns (BioRad). 5-hmC was subsequently labeled with biotin by addition of 4.5 nm Click-IT Biotin DIBO Alkyne (Life Technologies) followed by incubation at 37°C for 2 hours. The biotin labeled DNA was purified using Qiagen MinElute reaction cleanup kit. The purified product was quantified by NanoDrop (Thermo) and spotted on HyBond N+ membrane (GE Healthcare Life Sciences) in 2-fold serial dilutions. DNA was crosslinked to the membrane using a UV Stratalinker 2400 (Stratagene). Membranes were blocked in 5% BSA in PBS overnight, washed in TBS-T, and probed with Avidin-HRP diluted 1:50,000 in 5% BSA in PBS. Membranes were washed in TBS-T, and dots visualized with Western Lightning ECL reagent (PerkinElmer).

Quantitative Real Time PCR

Total RNA was isolated with Trizol Reagent (Life Technologies) according to the manufacturer's protocol. cDNA was synthesized using random hexamers according to the SuperScript III Reverse Transcriptase protocol (Life Technologies). Quantitative PCR was performed on Applied Biosystems Fast 7500 machines using Power SYBR Green reagents (Life Technologies). Relative quantification was performed by the standard curve method.

5-hmC Selective Chemical Labeling (hMe-Seal)

5-hmC rich sequences were affinity purified from genomic DNA according to a previously described protocol (Song et al., 2010). Briefly, 20 μg genomic DNA was sonicated to 250 base pairs (Covaris), conjugated to UDP-6-N3-Glu with β-GT, and purified with Micro Bio-Spin Columns (BioRad). 5-hmC was subsequently biotinylated by addition of DMCO-S-S-PEG3-Biotin Conjugate (Click Chemistry Tools) to the eluate. Biotinylated DNA was isolated using streptavidin-coated magnetic beads (Invitrogen). After washing beads, labeled DNA was released with 50 mM DTT and purified sequentially with Micro Bio-Spin Columns (BioRad) and MinElute reaction cleanup kit columns (Qiagen). The isolated DNA was quantitated by PicoGreen (Invitrogen).

hMe-Seal Read Mapping and Peak Calling

100 and 50 bp reads were mapped to the hg19 genome using the BWA-mem and BWA-backtrack algorithms, respectively. Peak calling was performed using MACS 1.4 with default parameters and options -wS.

RNA-Seq Read Mapping and Peak Calling

Reads were mapped to the hg19 genome using Bowtie2 and TopHat2. Gene expression analysis was performed using Cufflinks to calculate fragments per kilobase of transcript per million mapped reads (FPKM). The Cufflinks Cuffdiff algorithm was used to compare expression between normoxia and hypoxia samples.

Enrichment of 5-hmC at Genomic Annotations

Genomic coordinates of 5' UTRs, 3' UTRs, exons, introns, and CpG islands were obtained from the UCSC Table Browser (Karolchik et al., 2004). Promoter regions were defined by downloading TSS sites from the UCSC Table Browser and using the bedtools suite to generate a bed file of coordinates 2kb downstream to 500 bp upstream of TSSs. Genomic coordinates of CpG shores were deduced from the locations of CpG islands by using the bedtools suite to define regions 2kb adjacent to each CpG island. Positions of HIF-1 and HIF-2 binding sites were defined by remapping previously published ChIP-seq data obtained in MCF7 cells to hg19 (Schödel et al., 2011). Positions of DNase hypersensitivity sites were obtained from ENCODE experiments using SK-N-SH cells (ENCODE Project Consortium et al., 2012).

To calculate absolute gains over each annotation, we created a bed file containing genomic coordinates of positions with a 5-hmC peak in either or both control and hypoxia samples. We named these regions peak islands. Each peak island was then annotated with the number of sequencing tags (outputted by MACS 1.4) mapping to that peak island from the control and hypoxia samples. For each peak island gaining sequencing tags, we calculated the number of sequencing tags gained over that entire peak island and divided this by the length of the peak island to give the number of tags gained per base at each peak island. We then intersected the peak island bed file against genomic annotations. For each intersection of a peak island and genomic annotation, we multiplied the length of the intersection in bases with the number of tags gained per base over that peak island to obtain the number of sequencing tags gained at each intersection. We summed these values for all intersections genome wide.

To calculate enrichment, we compared the observed absolute number of sequencing tags gained at each annotation to the expected value determined by scrambling regions gaining 5-hmC across the genome using the bedtools shuffle command and repeating the calculation

described above. Shuffling was performed 1,000 times for each enrichment analysis and the average value obtained from these random permutations was used as the expected value in enrichment calculations.

For all calculations, each biological replicate was treated independently and results averaged.

Generation of Short Hairpin RNA Expressing Cells

Production of lentiviral particles and subsequent infection of neuroblastoma cells followed the pLKO.1 protocol found at http://www.addgene.org/tools/protocols/plko/. Briefly, 293T cells maintained in DMEM supplemented with 10% FBS (GIBCO) in 10 cm dishes were co-transfected with 2.77 μg pLKO.1, 2 μg psPAX2, and 0.7 μg pMD2.G plasmids using Fugene HD transfection reagent (Promega). Media was replaced 15 hours post-transfection and 293T cells incubated for 24 additional hours. Media was then harvested, filtered through 0.45 μm filters, supplemented with polybrene to a final concentration of 4 μg/mL, and used to infect target cells over 8 hours. Two days post-infection, cells expressing the desired shRNAs were selected using puromycin (4 μg/mL, Santa Cruz).

***TET1* siRNA Transfection**

Three days before hypoxic treatment, cells were transfected with either *TET1* or non-targeting control siRNA (Thermo ON-TARGETplus SMARTpools L-014635 and D-001810-10) using Mirus TransIT-siQUEST reagent (MIR 2114) according to the manufacturer's protocol. Two days prior to hypoxic treatment, cells were passaged. 16 hours prior to hypoxic exposure cells were re-transfected with siRNA.

HIF-1 Electrophoretic Mobility Shift Assay (EMSA)

HIF-1 EMSA assays followed a previously published protocol (Camenisch et al., 2002) with modifications described below. Nucleotide probes were designed based off of the previously characterized *CA9* HRE (Wykoff et al., 2000): 5'-CCAATGCA**C**GTACAGCCC-3' and 5'-GGGCTGTA**C**GTGCATTGG-3'with the bolded cytosine being unmodified, methylated, or hydroxymethylated. Probes were annealed in 1 mM Tris HCl (pH=7.4), 1mM EDTA, and 50 mM NaCl by heating to 90 °C in a heat block followed by cooling to room temperature over an hour. Probes were labeled with γ–[^{32}P]-ATP using T4 polynucleotide kinase (NEB) at 37°C for 1 hour, and purified through mini Quick Spin Oligo Columns (Roche).

HIF-1α and ARNT were produced by in vitro transcription/translation (IVTT) using the TNT T7 Quick Coupled Transcription/Translation (Promega) kit according to the manufacturer's protocol. HIF-1α IVTT reactions used Addgene plasmid #18949 as template. *ARNT* was amplified from SK-N-BE(2) cDNA and TOPO TA cloned into the PCR4-Topo vector (Life Technologies). Kozak and polyadenylation sequences were inserted upstream and downstream of the *ARNT* insert respectively to facilitate IVTT. Equal volumes of the HIF-1α and ARNT IVTT reactions were incubated at 4°C for 1 hour to allow for heterodimerization before addition of binding buffer and labeled DNA probe. Binding reactions were performed in 10 mM Tris (pH=7.5), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 200 ng salmon sperm DNA, 0.1 mg/mL bovine serum albumin, and 5% glycerol in a total volume of 40 μL. Binding occurred over 30 minutes at 4°C. Supershifts were produced by addition of 2 μg monoclonal HIF-1α antibody (BD Biosciences 610958). Binding reactions were run on 4.5% non-denaturing polyacrylamide gels for 600V-h. Bands were visualized by autoradiography.

Tet Assisted Bisulfite Sequencing (TAB-Seq)

TAB-Seq was performed using the Wisegene 5-hmC TAB-Seq kit. 10 μg genomic DNA was sonicated to 500 bp (Covaris), and conjugated to UDP-glucose using β-glycosyltransferase for 2 hours at 37°C. Following conjugation, DNA was purified using PCR Clean-up Kits (Sigma). 600 ng of conjugated DNA was then oxidized with mTet1 for 2 hours at 37°C. Following oxidation, mTet1 was digested with proteinase K at 55 °C. DNA was sequentially purified from these reactions with Micro Bio-Spin Columns (BioRad), and PCR Clean-up Kits (Sigma). DNA was then bisulfite treated using the MethylCode Bisulfite Conversion Kit (Life Technologies, MECOV-50). DNA that was conjugated with β-GT, but not mTet1 oxidized, was bisulfite treated to measure the amount of unmodified cytosines at each CpG. Bisulfite treated DNA was PCR amplified. PCR products were TOPO TA cloned into the PCR4-TOPO vector (Life Technologies K4575-02) and sequenced.

Supplemental References

Camenisch, G., Wenger, R.H., and Gassmann, M. (2002). Methods in Molecular Biology. 1–15.

Karolchik, D., Hinrichs, A.S., Furey, T.S., Roskin, K.M., Sugnet, C.W., Haussler, D., and Kent, W.J. (2004). The UCSC Table Browser data retrieval tool. Nucleic Acids Res *32*, D493–D496.