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Supporting Online Material for

The Obesity-Associated *FTO* Gene Encodes a 2-Oxoglutarate–Dependent Nucleic Acid Demethylase

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Supporting information

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Methods

Fto and ABH3 *expression constructs*

The cDNA sequence encoding full-length murine Fto was PCR-amplified from IMAGE clone ID: 4237261 (Geneservice UK Ltd.) and cloned into vector pET-28a(+) (Novagen) to generate a His-tagged fusion protein. Full-length cDNA for ABH3 was obtained from IMAGE clone ID: 4519083 and cloned into pGEX-6P-1 (GE Healthcare).

Mutant production

The Fto H304A and R313A mutants were created from the wild-type construct using the QuickChange® Site-Directed Mutagenesis Kit (Stratagene). The integrity of the mutations was confirmed by DNA sequencing and in-gel trypsin (Roche Diagnostics Ltd.) digestion followed by MALDI-TOF mass spectrometry (data not shown). The primers for each of the mutations were (bold codons indicate the site of mutation):

mftoH304Afwd: 5'-CAATGCCACCCACCAG**GCG**TGTGTTTTGGCTGGC-3'

mftoH304Arev: 5'-GCCAGCCAAAACACA**CGC**CTGGTGGGTGGCATTG-3'

mftoR313Afwd: 5'-GCTGGCTCACAGCCT**GCG**TTTAGTTCCACTCACCG-3'

mftoR313Arev: 5'-GCGTGAGTGGAACTAAA**CGC**AGGCTGTGAGCCAGC-3'

Constructs for fluorescent Fto fusion proteins

A YFP-Fto chimera was generated by fusing the YFP gene to the N-terminus of Fto (GenBank BC022222) by overlap PCR. The amplified PCR product was first cloned into the pGEM®-T Easy vector (Promega) and fully sequenced. It was then excised by digestion with NotI/XhoI and cloned into the pcDNA3 vector (Invitrogen), creating the pcDNA3/YFP-Fto plasmid. A similar approach was used to generate the pcDNA3/YFP plasmid.

Protein expression and purification

Expression plasmids were transformed into *E. coli* BL21-Gold(DE3) (Strategene). Cells were grown at 37ºC till OD_{600} 1.0, then at 15ºC and protein production was induced by addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG). His-tagged

recombinant Fto was purified using His-Bind® Ni^{2+} affinity chromatography (Novagen) and gel filtration on Superdex S200 (GE Healthcare, Fto) gel filtration columns, respectively. ABH3 was purified to homogeneity using Glutathione Sepharose™ (GE Healthcare) affinity chromatography followed by gel filtration using a Superdex S75 column.

2OG decarboxylation assays

Decarboxylation assays of [1-^{14}C]-labelled 2OG were performed as described (*1*). Methylated oligonucleotide substrates of the sequence 5'-TTXTTTTTTTTTTT-3', where X is 3-methylthymine, 3-methylcytosine, 1-methyladenine and 1-methylguanine, respectively, were synthesized by ATDBio Ltd. (University of Southampton, UK). Potential Fto substrates other than nucleic acids were obtained as reported (*2-5*). The inhibitors used in this study have been shown to inhibit other 2OG oxygenases [reviewed in (*6, 7*)].

4 μM Fto, 100 μM 1-meA oligonucleotide, 288 μM 2OG, 12 μM [1-^{14}C]-2OG (2.1016 GBq/mmol specific activity, PerkinElmer), 4 mM sodium ascorbate, 50 μM $(NH_4)_2Fe(SO_4)_2$ and 1 mM DTT were mixed in a total volume of 100 μl with 50 mM TRIS, pH 7.5. A 500 μl Eppendorf tube containing 200 μl hyamine hydroxide (Fisher Scientific) was added to each tube and tubes were closed with a rubber septum. After incubation in an environmental shaker at 37ºC for 30 minutes, 200 μl methanol was added to the contents and the tubes were incubated on ice for 30 minutes to quench the reaction. The Eppendorf tubes containing the hyamine hydroxide were transferred to scintillation vials, mixed with OptiPhase Hisafe 2 Liquid Scintillation Cocktail (Fisher

Scientific) and total ^{14}C counts quantified using a LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter). Uncoupled 2OG turnover assays were performed similarly in the absence of the oligonucleotide substrate.

LC-MS assays

Preparation and incubation of samples was performed as described above for 2OG decarboxylation assays, but without [1-^{14}C]-2OG. For ABH3, the incubation conditions were 5.5 μM enzyme, 11 μM oligonucleotide substrate, 160 μM 2OG, 4 mM sodium ascorbate, 80 μM $(NH_4)_2Fe(SO_4)_2$ and 1 mM DTT in a total volume of 100 μl with 50 mM TRIS, pH 7.5. Samples were incubated at 37ºC for one hour. Oligonucleotides containing 3-meT were used for all cofactor and mutant protein assays. Samples were run on a 5 μm Luna C18 column connected to a Waters 600 HPLC system in 10 mM ammonium acetate, 5% methanol in H_2O at 1 ml/min. Elution employed a linear gradient into 10 mM ammonium acetate, 5% H_2O in methanol. Mass analysis was performed on a ZMD electrospray ionization mass spectrometer (Micromass) connected to the HPLC system in negative ion mode.

Demethylase assay using radiolabelled DNA substrates

Proteins were assayed for their ability to demethylate 1-methyladenine or 3-methylthymine in [^{14}C]-methylated polydeoxyribonucleotides. The substrates were generated by treatment of poly(dA) or poly(dT) with [^{14}C]-methyl iodide (54 mCi/mmole, GE Healthcare). Demethylation was assayed by measuring the release of [^{14}C]-formaldehyde as described previously (*8*). Briefly, reaction mixtures contained 50

mM TRIS-HCl pH 7.5, 1 mM 2-oxoglutarate, 2 mM ascorbate, 75 μM $(NH_4)_2Fe(SO_4)_2$, 50 μg/ml BSA and the [^{14}C]-methylated polydeoxyribonucleotides (approximately 1000 c.p.m.) in a total volume of 100 μl, and were incubated at 37ºC for 15 minutes. The reactions were stopped by the addition of EDTA to a final concentration of 10 mM and ethanol-soluble radioactive material was monitored by scintillation counting.

Cell culture and confocal microscopy

COS-7 cells (passage number 40-50) were grown in Dulbecco's Modified Eagle Medium (D-MEM, 4500 mg/L D-Glucose; Gibco BRL, Invitrogen) supplemented with 10% (v/v) foetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere. $5x10^4$ cells were plated onto glass cover slips one day before transient transfection with YFP-Fto or YFP constructs, using the TransIT-LT1 transfection reagent (Mirus, Cambridge BioScience). Two days after transfection, live cells were stained for 20 minutes with 500 nM MitoTracker Deep Red 633 dye (Molecular Probes, Invitrogen, Paisley, UK), then fixed for 15 minutes with 3.7% formaldehyde and permeabilized for five minutes in ice-cold acetone before mounting on glass slides using Vectashield hardset mounting medium with 4',6-diamidino-2-phenylindole (DAPI, VECTOR Laboratories).

COS-7 cells expressing YFP-Fto or YFP were visualized by confocal imaging using an LSM 510 META confocal microscope and a Plan-Apochromat 63x/1.4 oil objective (Carl Zeiss). YFP was excited using the 514 nm line of the Argon laser and emitted light was collected between 522 and 565 nm in the Meta channel of the confocal system.

MitoTracker Deep Red was excited at 633 nm with light from a HeliumNeon laser, and emission was detected in the Meta channel at wavelengths >640 nm. DAPI was excited in the two-photon mode with the 740 nm line of a Chameleon laser and emitted light detected between 437 and 480 nm in the Meta channel.

Animal studies

All animal studies were carried out in male SV 129 mice purchased from Charles River (Kent UK). Prior to all procedures animals were allowed to acclimatize for at least one week. Animals were kept at 22°C on a 12 hour light/dark cycle (lights on 0700-1900). The animals were fed on standard laboratory Chow (SDS diet) and had free access to water throughout. All experimental procedures were in accordance with regulations and guidelines of the United Kingdom Home Office.

RNA extraction from whole tissue

Six 8 weeks old mice were sacrificed by cervical dislocation at the onset of the light cycle. The tissues of interest were rapidly dissected and snap frozen in liquid nitrogen. RNA was extracted using Stat-60 (Tel-Test Inc.) according to manufacturer's protocol and column purified using RNeasy^{TM} (QIAGEN). RNA concentration was measured using a ND-1000 Spectrophotometer (Nanodrop Technologies).

In situ hybridisation

Six series of one in six 14-μm brain sections were cut through the hypothalamus of five weeks old mice on a cryostat and placed onto poly-L-lysine-coated slides (Fisher

Scientific), dried overnight and then stored at -80°C. Hybridization histochemical localization was carried out using ^{35}S-labeled cRNA probes. Antisense and sense (control) cRNA probes for FTO were generated from the full length cDNA template that was cloned into pcDNA3 transcription vector (Invitrogen) in a 5' to 3' orientation with respect to the T7 promoter. Labeled antisense and sense probes for Fto mRNA were synthesized following linearization with *Hind*III or *Xho*I using SP6 or T7 RNA polymerase, respectively. Probes were labeled with ^{35}S-UTP (GE Healthcare) following in vitro transcription according to the manufacturer's protocols (Promega). Unincorporated nucleotides were removed using a Sephadex G50 spin column (GE Healthcare). Before hybridization with riboprobe, the sections were fixed in 4% formaldehyde for 20 minutes, followed by a 10 minutes microwave pretreatment with 10 mM sodium citrate buffer, pH 6.0. Slides were dehydrated through graded concentrations of ethanol (50-100%) and air-dried. Hybridization was carried out in 150 μl of hybridization solution (20 mM Tris pH 7.4, 2 mM EDTA pH 8.0, 300 mM NaCl, 50% formamide, 10% dextran sulfate, 1x Denhardt's solution, 100 μg/ml salmon sperm DNA, 0.1% yeast total RNA, 250 μg/ml yeast tRNA, 0.0625% SDS, 0.0625% sodium thiosulfate, 100 mM DTT) containing 2x 10^6 cpm/ml denatured ^{35}S-labeled riboprobe. After overnight hybridization at 57°C, the sections were rinsed in 2 x SSC. After treatment with 20 μg/ml RNase-A and a series of washes in increasing stringencies of SSC, the slides were dehydrated through graded concentrations of ethanol (50-100%). The sections were air-dried and exposed to X-ray film for 7 days.

In vivo murine studies

Three months old mice were divided into three weight-matched groups (N = 6-8 in each group). Group one (fed) had ad libitum access to chow and group two (fasted) had all food removed at the onset of light cycle, remained fasted for 48 hours. Both groups received twice daily intraperitoneal (i.p.) injection of saline. Group three (fasted plus leptin) had all food removed at the onset of light cycle, remained fasted for 48 hours but received twice daily i.p. injection of recombinant murine leptin (Amgen) at a dose of 1 μg/g total body weight. All animals were sacrificed by cervical dislocation 12 hours after the last injection. The brains were removed and immediately frozen on powdered dry ice and stored at -80°C until further processing.

Laser capture microdissection and total RNA amplification

Coronal sections of 14 μm thickness were prepared on a cryostat and mounted on RNase free membrane-coated glass slides (P.A.L.M. Membrane Slides, P.A.L.M. Microlaser Technologies). Slides were kept in a slide box embedded in dry ice until sectioning was completed. Within 24 hours after sectioning, the frozen sections were thawed and fixed for 30 seconds in 95% ethanol, then rehydrated (75% and 50% ethanol, 30 seconds each). After fixation, the slides were stained with 1% cresyl violet for 1 min. The sections were then dehydrated in a graded ethanol series (50%, 75%, 95% and 2 x 100%, 30 seconds each) followed by Histoclear for 5 minutes. All the solutions were prepared with RNase-free water (Ambion). Laser microdissection was performed using a P.A.L.M. Microlaser System (P.A.L.M. Microlaser Technologies). The hypothalamic PVN were microdissected covering the region from -0.7 to -1.22 mm caudal to bregma (37 sections),

ARC covering from -1.46 to -1.97 mm (36 sections) and VMN covering -1.34 mm to -1.94 mm (43 sections) as defined by Franklin and Paxinos (*9*). All evenly spaced sections within each nucleus were pooled to elimilate any potential rostrocaudal gene expression bias. Following each microdissection, the captured cells were kept in RNA*later* (Ambion) prior to RNA isolation. Total RNA was isolated according to the manufacturer's protocol using the RNAqueous®-Micro Kit (Ambion). Quality and quantity of the total RNA samples were determined by electrophoresis using the Agilent BioAnalyzer. Before RNA amplification the more sensitive Agilent BioAnalyzer PicoChip was used (Agilent; according to manufacturer's instructions). To determine the RNA concentration a dilution series of a control with known concentration was run alongside the nuclei samples.

To minimize amplification bias, 500 pg of isolated total RNA from each nucleus was subjected to two rounds of T7-based linear amplification. Briefly, RNA was primed with a T7 promoter-oligo (dT) primer and reverse transcribed to generate first stand cDNA, which was used as the template to synthesize second strand cDNA by DNA polymerase. The T7 RNA polymerase promoter within the double-stranded cDNA (ds-cDNA) was then used by T7 polymerase to transcribe antisense amplified RNA (aRNA; MEGAscript T7 kit, Ambion). The aRNA was then randomly primed to make single-stranded cDNA with a 3'-poly-A tail to serve as the template for second-strand cDNA synthesis primed with a T7 promoter-oligo dT primer to make ds-cDNA containing a T7 promoter site. A second in vitro transcription step using T7 polymerase produced the second round of aRNA resulting in 1,000,000 fold amplification.

Quantitative PCR analysis of Fto mRNA

Fto mRNA quantitative PCR analysis was performed using TaqMan® Gene Expression assays on unpooled purified RNA samples. Either 500 ng of total RNA from each tissue or 100 ng of amplified RNA from laser capture microdissected samples were used in a random-primed first strand cDNA synthesis reaction, using reverse transcriptase from Promega. The resulting first strand cDNA reaction was diluted 2.5-fold and 2 μl used in each 12 μl TaqMan® reaction. Quantitative PCR reactions were performed in triplicate on an ABI 7900HT (Applied Biosystems) and using ABI PCR master mix, according to manufacturer's protocols. Expression results were normalized to β-actin. Quantitative PCR statistical analysis was performed using Microsoft Excel. P-value was calculated using a two-tailed distribution unpaired Student's T-Test. Data is expressed as mean +/- SEM.

Figure legends (Supporting information)

Figure S1. Multiple sequence alignment of FTO homologues

Multiple sequence alignment of putative FTO homologues: H.s. *Homo sapiens* (gi|122937263), P.t. *Pan troglodytes* (gi|114662524), Ma.m. *Macaca mulatta* (gi|109128525), C.f. *Canis familiaris* (gi|73950384), B.t. *Bos taurus* (gi|119910109), Mu.m. *Mus musculus* (gi|18490097), R.n. *Rattus norvegicus* (gi|89337260), M.d. *Monodelphis domestica* (gi|126296336), X.t. *Xenopus tropicalis* (gi|62859671), D.r.

Danio rerio (gi|125821796), O.t. *Ostreococcus tauri* (gi|116060758), O.l. *Ostreococcus lucimarinus* (gi|145352974).

Figure S2. Uncoupled 2OG turnover experiments

(a) Assays used the 2OG turnover method; no methylated DNA substrate was added. Experimental setup and procedure were as described above for 2OG decarboxylation assays with 1-meA substrate. Values shown are averages of two independent experiments, 100% corresponds to 130,000 counts of [^{14}C]-CO_2; (b) Reduction of uncoupled 2OG turnover in the presence of inhibitors. DMSO = dimethylsulfoxide. Although *N*-oxalylglycine can chelate Fe(II), this does not fully account for its Fto inhibitory effect because *N*-oxalyl-D-phenylalanine (*10*), an inhibitor of a 2OG dependent asparagine-hydroxylase, has similar Fe(II) chelating abilities to *N*-oxalylglycine but did not inhibit Fto activity. Values shown are averages of two independent experiments, 100% corresponds to 130,000 counts of [^{14}C]-CO_2; (c) Structures of inhibitors used. Compound 1 was previously identified as a potent 2OG oxygenase inhibitor (*11*) and synthesized in this laboratory using standard synthetic methodology (*12*).

Figure S3. Homology model of hFTO based on hABH3

Homology model of human FTO built with MODELLER v8.2 in automodel mode (*13*) based on sequence and secondary structure prediction (*14*) in alignment with that of the ABH3 crystal structure (*15*). Dotted lines indicate imaginary un-modelled loops where sequence alignment was difficult or large insertions take place. The color scheme of secondary structures matches that in Figure 1 of the main text. Residues His231, Asp233,

His307, Arg316, Leu205, Arg 324 in human FTO are equivalent to residues His228, Asp230, His304, Arg 313, Leu202, Arg321 in mouse Fto, respectively.

Figure S4. No nutritional regulation of *Fto* expression in the VMN and PVN

Bar graphs show no change in expression of *Fto* in the ventromedial nucleus and in the paraventricular nucleus of the hypothalamus in the fed, fasted and leptin-treated-while-fasted state. Response is expressed in terms of fold induction of the fasted and leptin-treated expression over the fed expression. Data is represented as the mean (+/-SE) of at least 5 independent mice per group.

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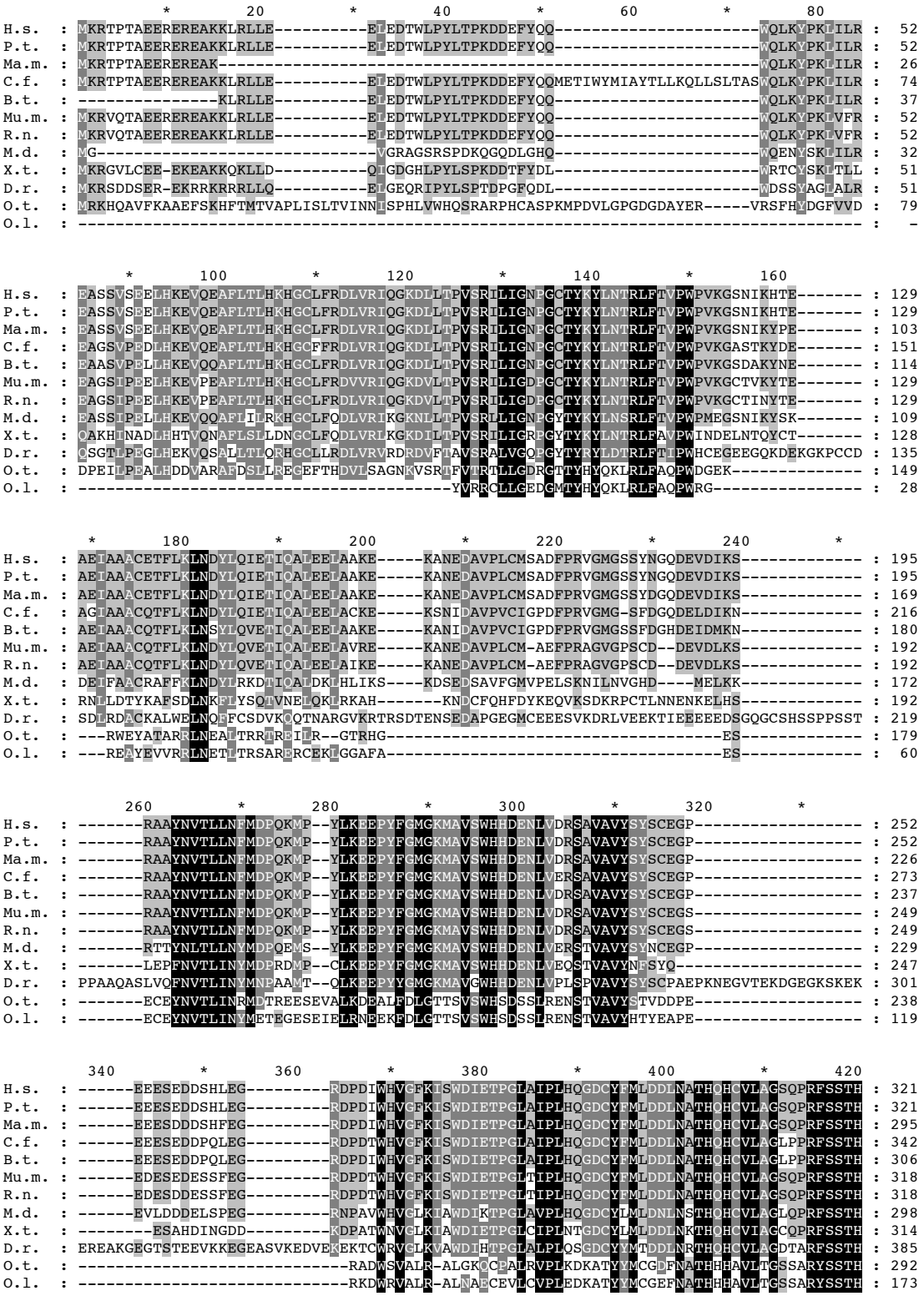
Figure S1



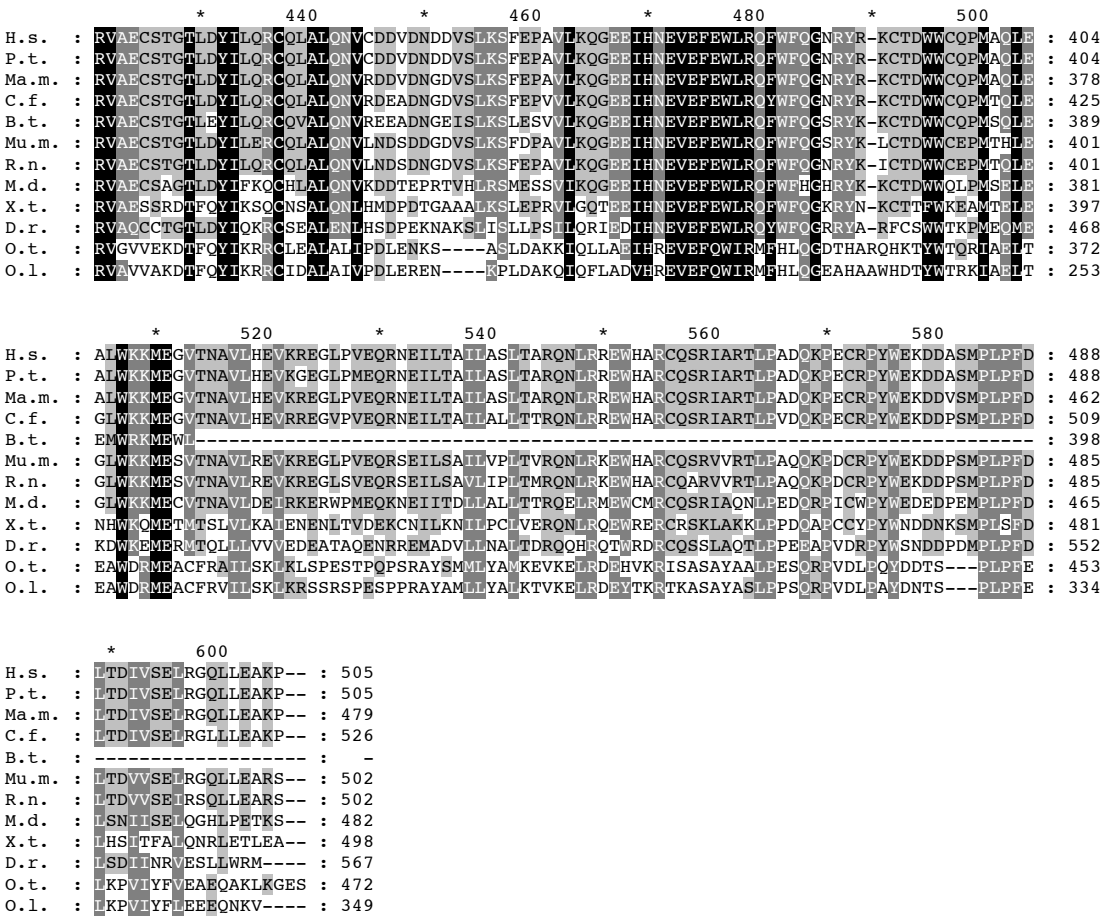


Figure S2

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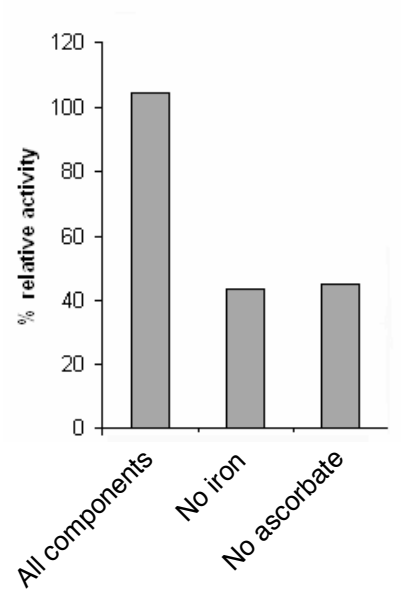

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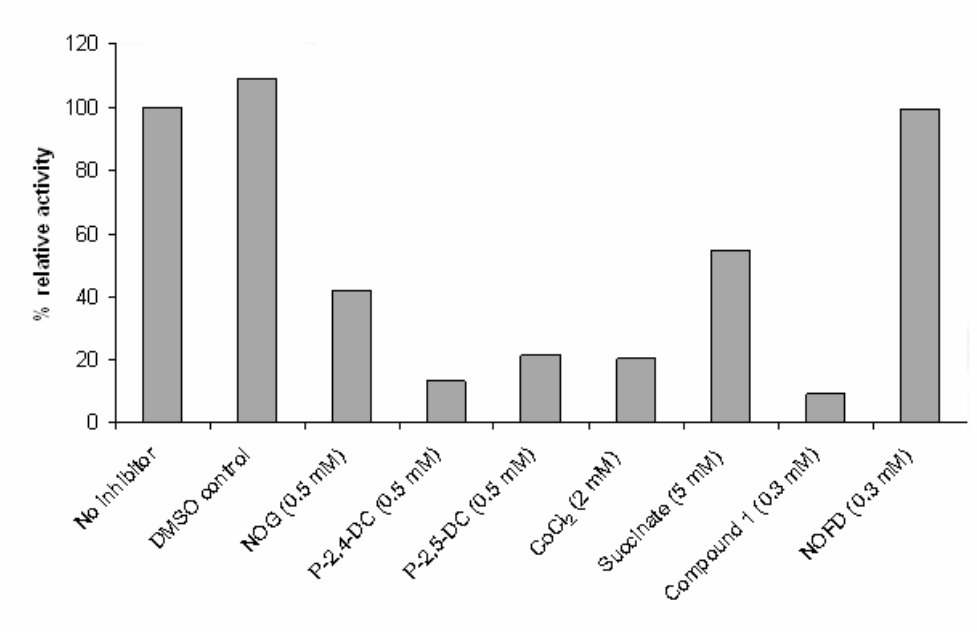

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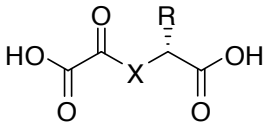

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X = NH, R = CH_2Ph,
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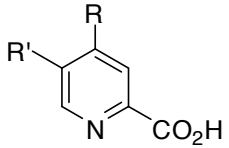

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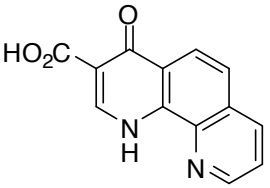
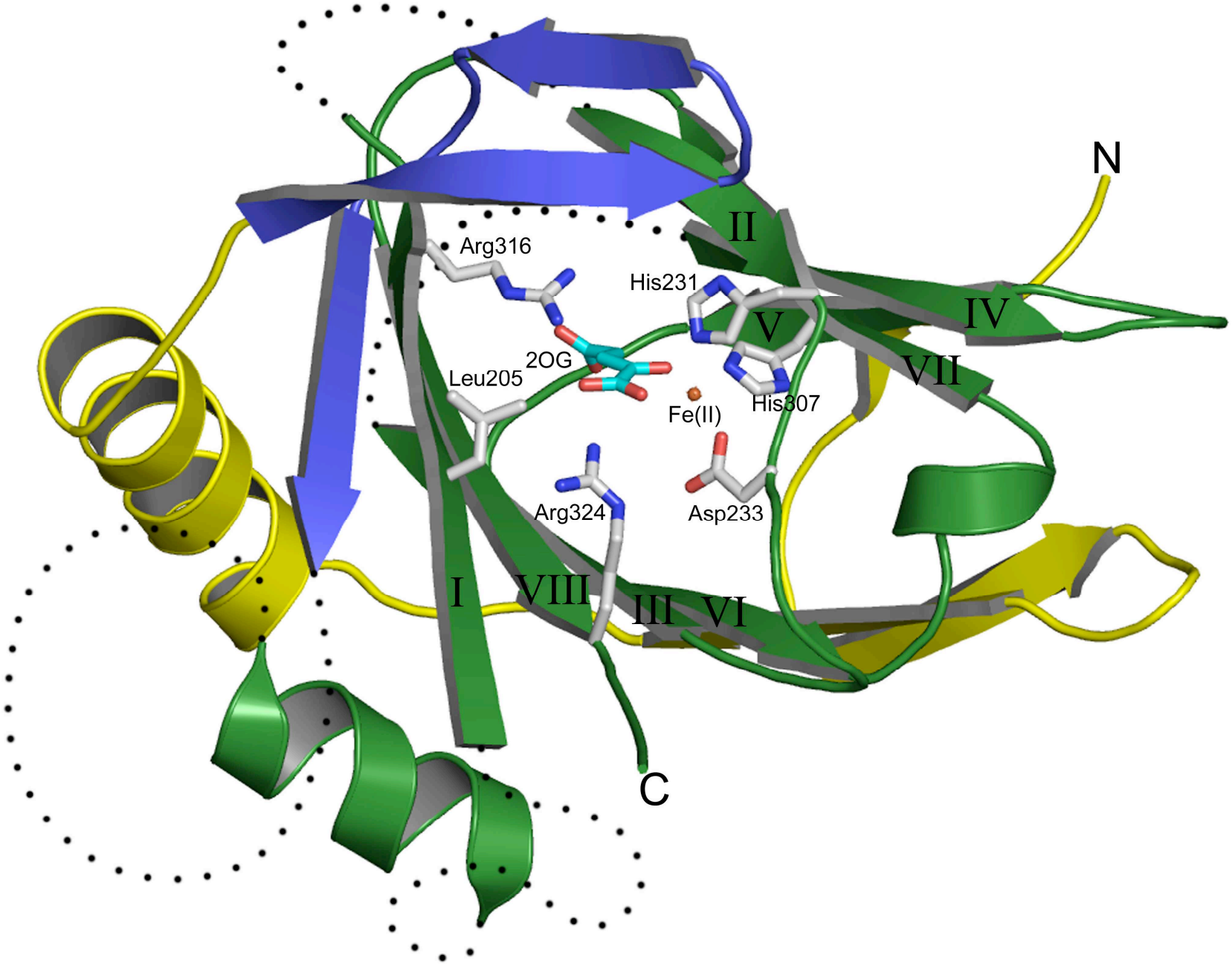
Pyridine-2,5-dicarboxylate (P-2,5-DC)



Compound 1

Figure S3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VI
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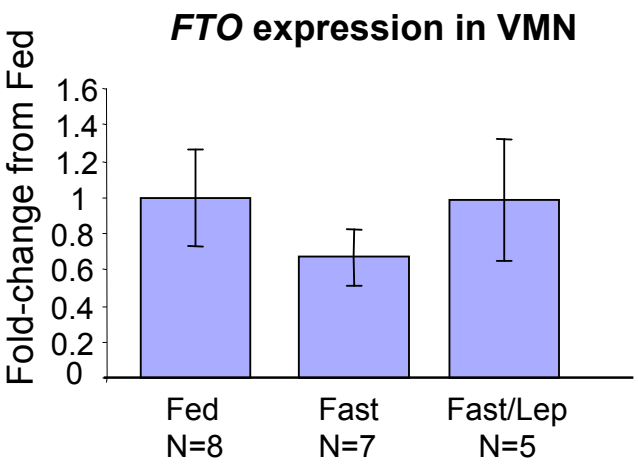
Figure S4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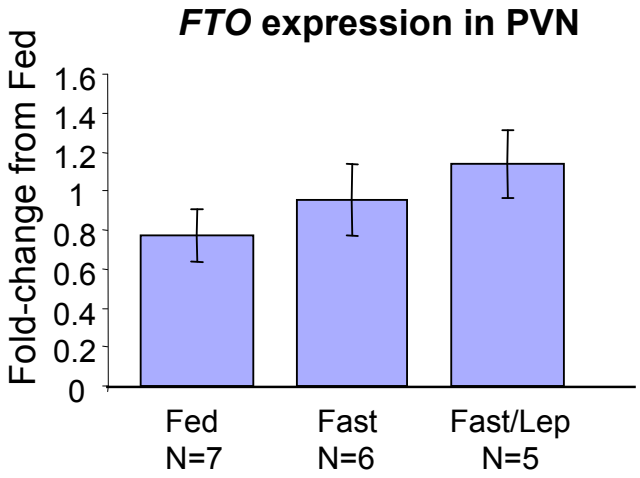
Fast
N=7
Fast/Lep
N=5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0.4
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N=6
Fast/Lep
N=5