Electronic Supplementary Information (ESI)

Self-hydroxylation of the splicing factor lysyl hydroxylase, JMJD6

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Experimental Details

Cloning, expression and purification of recombinant JMJD6 and U2AF65

Full-length human JMJD6 was cloned, expressed and purified as described.[1] To generate recombinant U2AF65 the DNA encoding the open reading frame of human U2AF65 was sub-cloned into the pGEX-2T vector to generate a GST-tagged fusion protein. The plasmid was transformed into *E. coli* BL21(DE3) cells and the cells were grown in 2TY media supplemented with ampillicin (100 $\mu g mL^{-1}$) at 37 °C with shaking (200 rpm) until mid-logarithmic phase (OD_{600} 0.6-0.8). To induce expression isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM and cells were harvested *via* centrifugation (5K for 20 min) 3 hours post-induction and stored at -80 °C until lysis.

U2AF65 was purified by resuspending the cell pellet in lysis buffer (50 mM Tris, 100 mM NaCl and 1mM DTT, pH 7.5), sonicating on ice, followed by centrifugation (22 K for 20 min). The filtered crude lysate (0.45 µM) was then purified by glutathione *S*-tranferase, GST affinity chromatography (GE Healthcare). The protein was eluted from the column using 15 mM reduced L-glutathione (Sigma) and then incubated over night with thrombin (Promega) to cleave off the GST tag. The native U2AF65 protein was separated from the GST tag by use of a second GST affinity column. The protein was purified to >90 % purity, as determined by SDS-PAGE analysis.

[1-^{14}C]-2OG turnover enzyme assays

2OG consumption by JMJD6 was determined by a [1-^{14}C]-2OG turnover assay[2]. Enzyme assays measured the release of [^{14}C]-CO_2 from 1-[^{14}C] 2OG. JMJD6 (20 µM) was incubated in a reaction volume of 100 µl at 37 ºC for 15 minutes with 100 µM Luc7like2$_{(267-278)}$, 400 µM Fe(II), 500 µM 2OG, 1mM ascorbate, 1mM dithiothreitol, 0.3 mg ml^{-1} catalase, 50 mM Tris-HCl pH 7.5. Control reactions lacking the peptide substrate and enzyme were performed

for comparison. Reactions were quenched by adding 200 µl of methanol followed by 30 minute incubation on ice. For each data set, three reactions were carried out.

^{1}H-NMR analysis showing succinate formation

Reaction components (20 µm JMJD6, 100 µm $(NH_4)_2Fe(SO_4)_2$, 500 µm 2OG, and 100 µm ascorbate) were prepared in deuterated Tris buffer (pD 7.5, 50 mm in D_2O, D = ^{2}H). The reaction was carried out at 310 K in a 2 mm NMR tube, and initiated by the addition of JMJD6. ^{1}H-NMR spectra were recorded using a Bruker AVIII 700 machine (with inverse cryoprobe optimized for ^{1}H observation and running topspin 2 software; Bruker, Germany) and reported in p.p.m. relative to D_2O (δ_H 4.72). The deuterium signal was also used as an internal lock signal and the solvent signal was suppressed by presaturating its resonance. Spectra were obtained at 75 s intervals and integrated using absolute intensity scaling to monitor changes in the intensity of signals of interest. The reaction was also carried out without adding ascorbate to the assay mixture.

The specific activity calculated by monitoring succinate formation in the NMR assay is 0.202 µmol.min^{-1}.mg^{-1} as compared to a value of 1.2 µmol.min^{-1}.mg^{-1} for the $^{14}CO_2$ turnover assays (Fig. 1A); however, note that the assay conditions are different.

Amino acid analyses

For amino acid analyses, the reactions were initiated by mixing JMJD6 (20 µM), 2OG (500 µM), Fe(II) (400 µM) and ascorbic acid (100 µM) in MOPS buffer (50 mM, pH 7.5) followed by incubation at 37°C for 1 hour. The assay mixture was treated first with trypsin and then with carboxypeptidase Y in an enzyme based hydrolysis procedure to give rise to mixture of peptides which were derivatised by 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) in acetonitrile according to the AccQTag Solution Protocol (Waters) and analysed by LC-MS as reported.[3]

Peptide synthesis and MALDI analysis

Peptides were synthesized by solid phase peptide synthesis using a CS Bio peptide synthesizer and analysed by MALDI assays as reported.[1, 3]

Determination of hydroxylation of recombinant JMJD6

To investigate whether recombinant JMJD6 ((20 µM) is hydroxylated in the presence of 2OG (500 µM) and Fe(II) (400 µM) an incubation of these components was performed as described above. The incubation mixture was then subjected to LysC protease (Roche) digestion using a standard protocol[4] and analysed by LC-MS/MS.[1]

Electronic Supplementary Material (ESI) for Medicinal Chemistry Communications

Isolation and mass spectrometric analysis of endogenous JMJD6

To investigate whether endogenous JMJD6 from HeLa cells is hydroxylated JMJD6 was extracted from HeLa (human) cells by immunoprecipitation with polyclonal anti-JMJD6 antibody (Abcam 10526). Extracts from 4x 10^8 cells were prepared in lysis buffer (20 mM Tris/HCl pH 8.0, 500 mM NaCl, 0.5% NP40 supplemented with protease (Pefabloc (Boehringer), pepstatin A, Aprotinin, Leupeptin) and phosphatase inhibitors (100 nM okadaic acid and phosphatase inhibitor cocktails 1 + 2 (Sigma)). After centrifugation supernatants were incubated with 30µl of the anti-JMJD6 antibody for 1 hour under agitation at 4°C. 60 µl of Protein-G Sepharose 4 Fast Flow (GE Healthcare, NJ, USA) was added for another hour. After three wash steps with 20 mM Tris/HCl pH 8.0, 500 mM NaCl, the beads were re-suspended in SDS sample buffer and subjected to SDS-PAGE (12 % acrylamide). As described previously, JMJD6 was digested with LysC protease and analysed by LC-MS/MS.[1, 5]

Supplementary Figures

Figure S1. MS/MS shows that Lys167$_{JMJD6}$ is not (at least substantially) hydroxylated in as purified recombinant JMJD6 that has not been incubated with 2OG and Fe(II). Recombinant JMJD6 (20 µM) was separated by SDS-PAGE gel, excised, then digested with LysC protease and analysed by LC-MS/MS.[5] No peptide corresponding to the mass of hydroxylated peptides that include Lys167$_{JMJD6}$ was identified by MS.

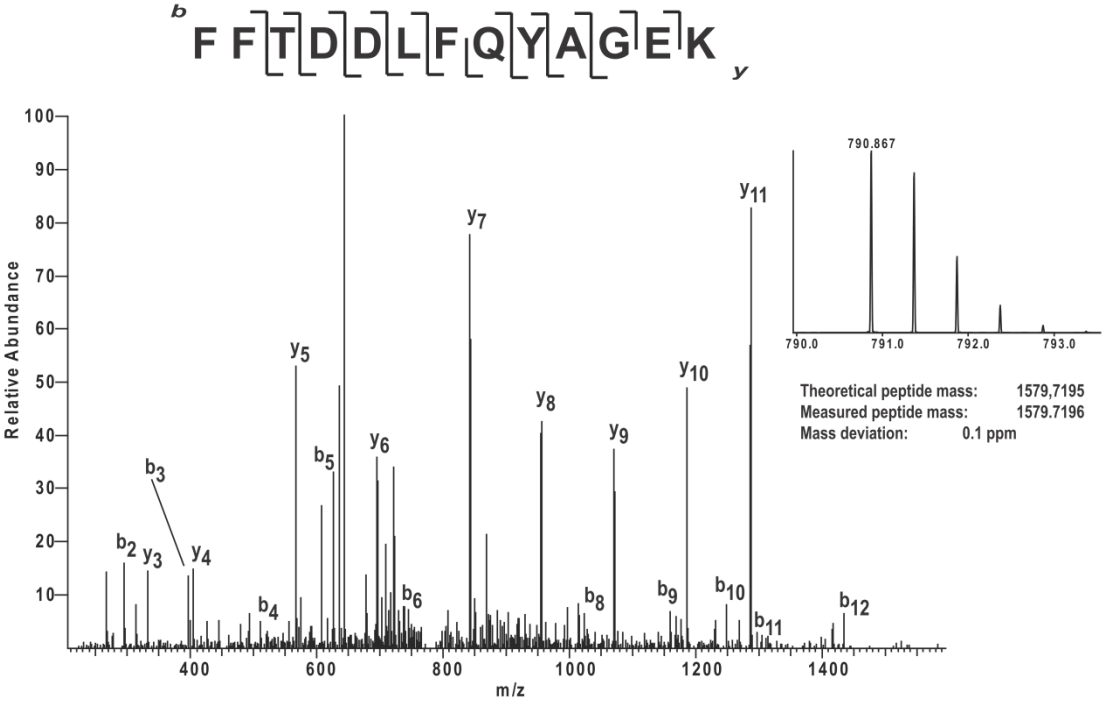


Figure S2. Lys167$_{JMJD6}$ of JMJD6 is hydroxylated in the presence of recombinant U2AF65 protein. LC-MS/MS analyses support hydroxylation of Lys167$_{JMJD6}$ of JMJD6 (20 µM) in the presence of 2OG (500 µM), Fe(II) (400 µM) and U2AF65 (40 µM). After two hours incubation at 37 °C the JMJD6 was separated by SDS-PAGE. The protein band corresponding to the molecular weight of JMJD6 was excised, then digested with LysC protease and analysed by LC-MS/MS.[5]

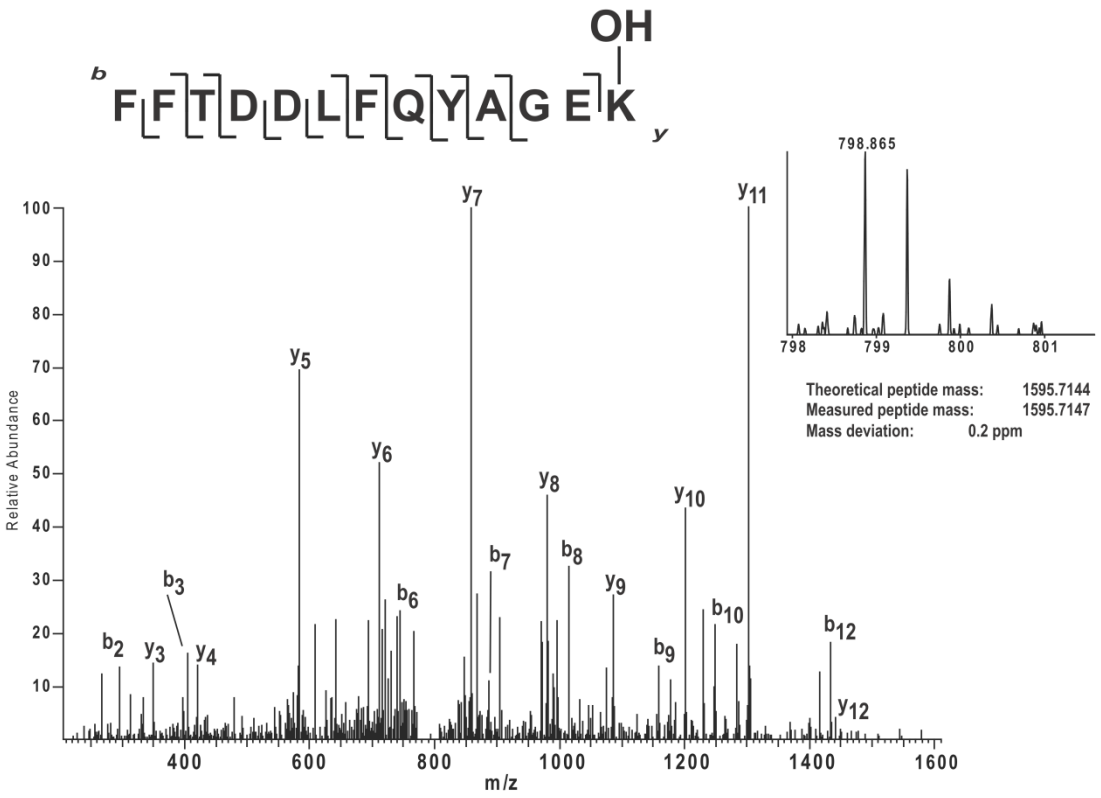


Figure S3. MS/MS analysis of endogenous JMJD6 showing unhydroxylated peptide containing Lys167$_{JMJD6}$.

JMJD6 was immunoprecipitated from HeLa cells, digested with LysC protease and analysed by LC-MS/MS.

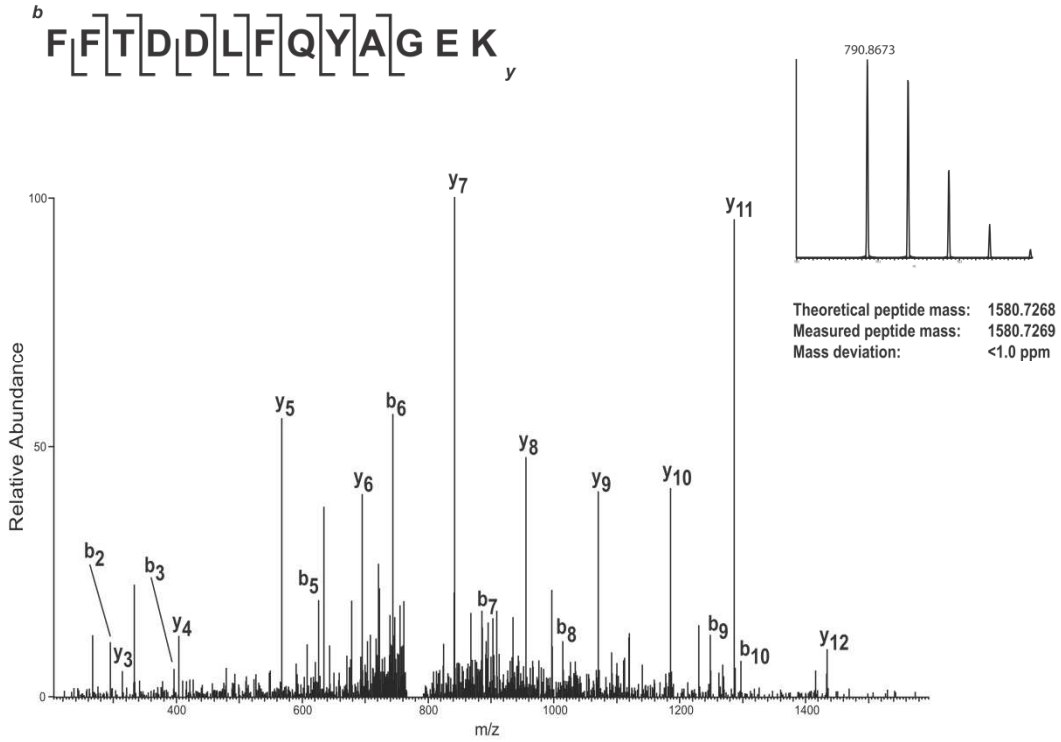


Figure S4. MALDI-TOF analysis of peptides synthesised for identifying possible lysine hydroxylation sites. Peptides were incubated with and without JMJD6, Fe(II) and 2OG. **(A1)** $JMJD6_{1-14}$ with JMJD6 **(A2)** $JMJD6_{1-14}$ without JMJD6 **(B1)** $JMJD6_{105-120}$ with JMJD6 **(B2)** $JMJD6_{105-120}$ without JMJD6 **(C1)** $JMJD6_{160-176}$ with JMJD6 **(C2)** $JMJD6_{160-176}$ without JMJD6 **(D1)** $JMJD6_{301-314}$ with JMJD6 **(D2)** $JMJD6_{301-314}$ without JMJD6.

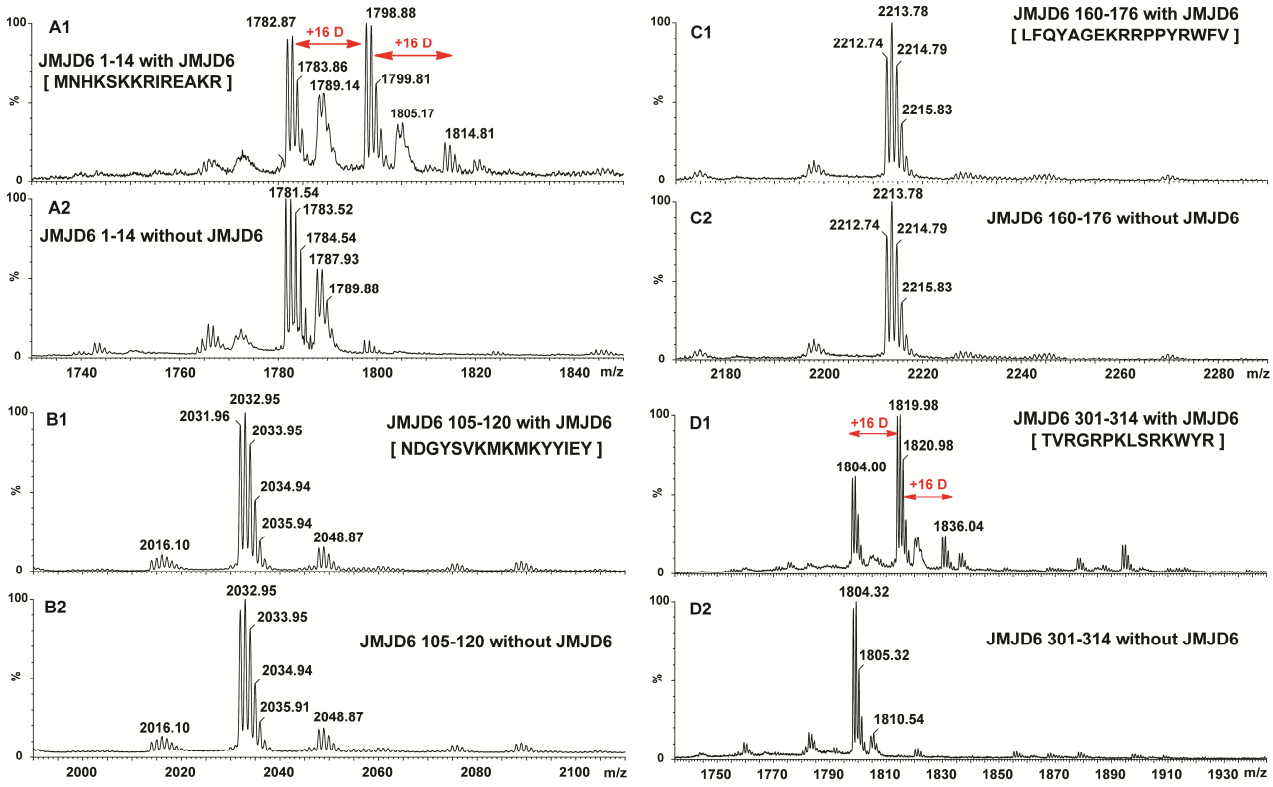


Figure S5. MALDI-TOF analysis of peptides synthesised for identifying possible lysine hydroxylation sites. All the peptides were incubated with and without JMJD6, Fe(II) and 2OG. **(A1) and (A2)** $JMJD6_{14\text{-}30}$ on incubation with and JMJD6 without JMJD6 **(B1) and (B2)** $JMJD6_{78\text{-}95}$ on incubation with and without JMJD6 **(C1) and (C2)** $JMJD6_{86\text{-}104}$ on incubation with and without JMJD6 **(D1) and (D2)** $JMJD6_{135\text{-}150}$ on incubation with and without JMJD6 **(E1) and (E2)** $JMJD6_{363\text{-}379}$ on incubation with and without JMJD6 **(F1) and (F2)** $JMJD6_{363\text{-}381}$ on incubation with and without JMJD6.

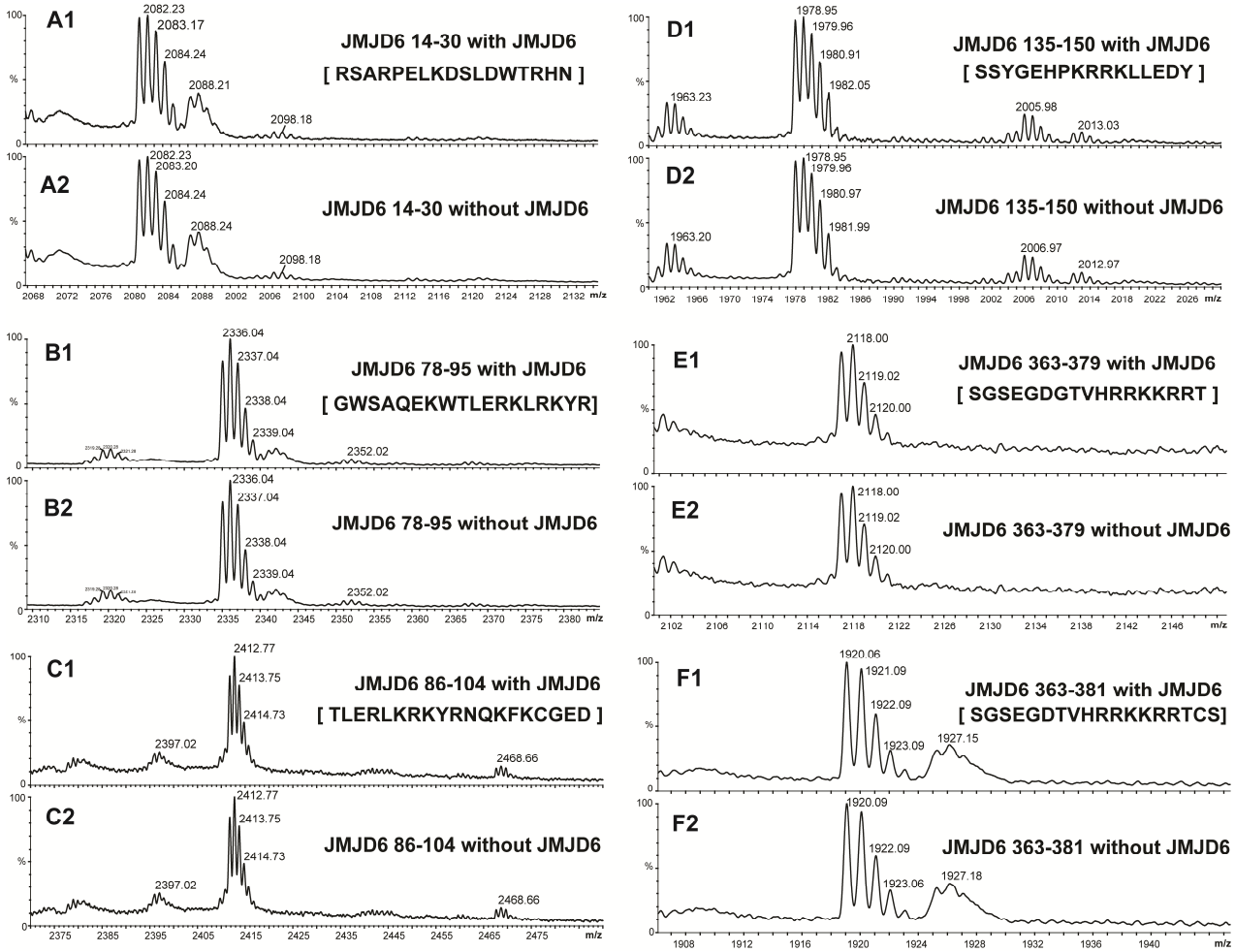
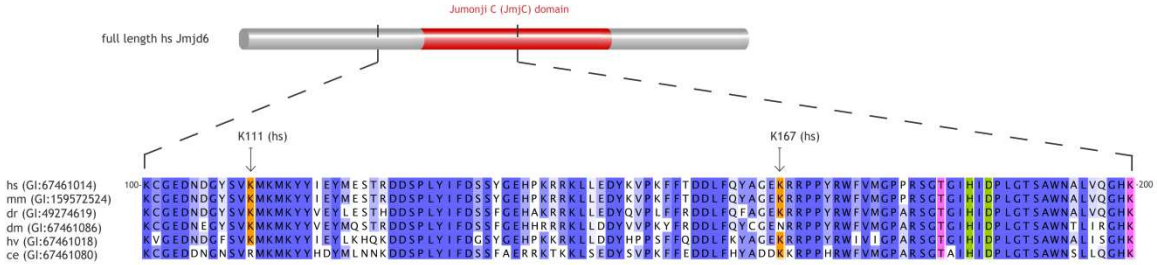


Figure S6. Multiple sequence alignment of JMJD6 proteins (amino acids 100-200) from different species. $Lys111_{JMJD6}$ and $Lys167_{JMJD6}$ are highlighted in orange, residues proposed to bind Fe(II) and 2OG are shown in green and pink, respectively. The domain structure of the full length human JMJD6 is shown at the top. Sequences from *Homo sapiens* (hs), *Mus musculus* (mm), *Danio rerio* (dr), *Drosophila melanogaster* (dm),) *Hydra vulgaris* (hv) and *Caenorhabditis elegans* (ce).



References

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