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Jmjd6 catalyses lysyl-hydroxylation of U2AF65, a protein associated with
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Jmjd6 catalyses Lysyl-hydroxylation of U2AF65 – a protein associated with RNA splicing.

Jmjd6 catalyses Lysyl-hydroxylation of U2AF65 - a protein associated with RNA splicing.

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Abstract

The metazoan hypoxic response is regulated by the oxygen-dependent post-translational hydroxylation of the α -subunits of the hypoxia inducible factor (HIF) system. This identification has raised the question of whether other protein-hydroxylases are involved in the regulation of gene expression. Here we demonstrate that the splicing factor protein U2AF65 undergoes post-translational lysyl-5-hydroxylation as catalysed by the Fe(II) and 2-oxoglutarate dependent dioxygenase Jumonji domain-containing protein 6 (Jmjd6). Jmjd6 is a nuclear protein that has an important role in vertebrate development and is a close human homologue of the hypoxia inducible factor asparaginyl-hydroxylase. Jmjd6 is shown to regulate alternative RNA splicing of endogenous and reporter genes. The ability of Jmjd6 to influence the splicing pattern of all endogenous genes investigated supports a specific role for Jmjd6 in regulating RNA splicing.

Main text

Metazoan cells respond to limiting oxygen by activation of the hypoxia inducible factor (HIF) system(1). The HIF α subunits are regulated by Fe(II) and 2-oxoglutarate

(2OG) dependent oxygenases; prolyl-hydroxylation promotes HIF α degradation via the ubiquitin-proteasome system, and asparaginyl-hydroxylation reduces the HIF transcriptional activity by blocking its interaction with p300(2). Sequence analyses suggest that Jmjd6 (3, 6-8) is a homologue of the HIF asparaginyl hydroxylase (Factor inhibiting HIF, FIH)(4, 5) (9).

To investigate the function of Jmjd6 we assayed for proteins that interact with Jmjd6 *in vivo* using tandem-affinity purification coupled with MS analyses (10, 11). A significant proportion (22/39) of the potential Jmjd6 interacting proteins are connected to RNA metabolism, processing and splicing (Table S1) with approximately a quarter being nuclear proteins possessing Arg-Ser (RS) domains (12/39). We confirmed interaction of Jmjd6 with U2 snRNP auxiliary factor 65 kDa subunit (U2AF65)(12) and cisplatin resistance-associated over-expressed protein (CROP)(13), using GFP (green fluorescent protein) pull-down assays(14), (Fig. 1A and S1A) in human HEK293T cells. Endogenous U2AF65 and Jmjd6 co-immunoprecipitate in HeLa cells with anti-U2AF65 antibody (Fig. S1B). The interaction is disrupted upon treatment with RNase A (Fig. S2).

Jmjd6 has recently been reported to function as a histone arginine demethylase #(9). However, we considered the possibility that arginine rich sequences present in RS domains (Fig. 1B) of many of the potential Jmjd6 binding proteins (see Table S1) may be a substrate of Jmjd6. RS domain sequences derived from U2AF65, CROP and LUC7-like2 (LUC7L2) were synthesised with dimethylated arginine residues, incubated with recombinant Jmjd6 in the presence of oxygen, ascorbate, Fe(II) and 2OG and analysed by MALDI MS. Peptides U2AF65₂₈₋₄₃(R35Me_{2sym}), U2AF65₂₈₋

⁴³(R33Me_{2sym}) and LUC7L2₂₆₇₋₂₈₂(R274Me_{2sym}) did not show dimethyl arginine-residue demethylation (-14 shift in mass). Recombinant Jmjd6 did not demethylate arginine histone H4 and H3 fragment peptides. Instead a clear +16 Da. or +32 Da. mass shift relative to unreacted peptide was observed for RS-rich and histone peptides (Fig. S3, Fig. S4). This observation is consistent with the incorporation of one or two oxygen atom(s) into the peptide and has been previously observed by Chang et al.(9). No hydroxylation was observed in the absence of oxygen. The CROP₃₂₁₋₃₃₆ peptide did not contain any lysine residues and was not hydroxylated. The +16 Da. mass shift observed upon incubation of LUC7L2₂₆₇₋₂₇₈ was shown to be 2OG and Fe(II) dependent, and its formation was completely inhibited by an established 2OG and Fe(II) dependent oxygenase inhibitor, N-oxalylglycine (NOG)(15), as well as the citric acid cycle intermediates succinate and fumarate (1 mM) (Fig. 1C). Substitution of one or two of the proposed Fe(II) binding residues(4) with non-chelating residues (H187A and H187A-D189A) inactivated Jmjd6, implying an intact Fe(II) binding centre is required for catalysis. Incubation under an ¹⁸O₂ atmosphere reveals the origin of the Jmjd6 introduced hydroxyl group to be from dioxygen gas (Fig. S5).

MS/MS analyses of LUC7L2₂₆₇₋₂₇₈ indicated oxidation of Lys-3, corresponding to Lys-269 in LUC7L2 (Fig. S6). ¹H NMR analyses confirmed the oxidation to be at C-5 on the hydrocarbon side-chain of Lys-269 (Fig. S7).

The shortest U2AF65 and LUC7L2 peptide (Fig. IB) substrate with which we observed hydroxylation possessed 12-residues (LUC7L2₂₆₇₋₂₇₈). Shorter peptides were not observed (<5 %) to be hydroxylated under standard conditions. When LUC7L2₂₆₃₋₂₇₈ was tested both +16 and a +32 mass shifts were observed. When either of the two

target lysines in LUC7L₂₆₃₋₂₇₈ was individually substituted for an arginine-residue only a single hydroxylation is observed. The simultaneous substitution of both of these lysines to arginines prevented hydroxylation by Jmjd6 (Fig. S8) as did substitution with a tri-, di- and mono- methylated lysine-residue (Fig. S9). The peptides U2AF65₁₂₋₂₆ (NENKQERDKENRHRK) and U2AF65₂₇₄₋₂₈₇ (NDDQVKEILTSFGPL) were not substrates of Jmjd6. Thus, Jmjd6 does not require a clearly defined consensus sequence, but appears to prefer very basic sequences. LC-MS/MS analyses of full-length recombinant U2AF65 after incubation with recombinant Jmjd6, 2OG and iron (ascorbate is not required for Jmjd6 activity) indicates that U2AF65 is a Jmjd6 substrate (Fig. S10, S11). LC-MS/MS analysis of endogenous U2AF65 from HeLa cells revealed hydroxylation at Lys-15 and Lys-276 at ratios of ~1:100 and 1:250 hydroxylated:unhydroxylated residues (Fig. 2A and 2B), respectively. Over-expression of Jmjd6 in HeLa cells resulted in increased hydroxylation of U2AF65 (5-fold at Lys-15) (Fig. S12).

The available data suggests that factors in addition to the local sequence determine the substrate selectivity of Jmjd6. For example, Lys-15 and Lys-276 in intact proteins appear to be substrates of Jmjd6, whereas the peptides U2AF65₁₂₋₂₆ and U2AF65₂₇₄₋₂₈₇ are not. We note that all the synthetic peptides found to be hydroxylated in vitro are very basic in sequence and carry a predicted overall charge of at least +4. Precedent for structural factors other than local sequence selectivity comes from work on FIH(19).

U2AF65 is required for mRNA splicing #(17, 18) etc. Modulation of splice site recognition by U2AF65 also influences alternative splicing(20). Jmjd6 knock-down

(but not knock-down of a non-specific DNA-binding protein, MAZ4) altered the alternative splicing pattern of the endogenous tumour antigen gene MGEA6, which is regulated by the splicing regulatory protein SF2/ASF(21), with an increase in the amount of exon 19 skipping (-19) of the MGEA6 gene (Fig. 3A, S13, 3B). A similar splicing pattern was also observed when HeLa cells were treated with the hypoxia mimicking reagent, desferrioxamine (DFO)(22), a general inhibitor of 2OG oxygenases (Fig. 3C).

The α -tropomyosin (α -TM) minigene construct (23) (Fig. 3D) recapitulates splicing regulation of the α -TM gene and contains exons 1, 3 and 4 surrounded by their intronic regulatory sequences #23). Splicing of the α -TM minigene construct generates two mRNA isoforms: 1-3-4 and 1-4. We over-expressed the α -TM construct both in mock-treated cells and cells treated with siRNA targeting Jmjd6 and MAZ4. The Jmjd6 knock-down cells, but not mock-treated, or cells treated with siRNA targeting the MAZ4 protein, produced a clear increase in the amount of 1-4 spliced isoform (Fig. 3D). Together these results imply a role for Jmjd6 in the regulation of alternative splicing.

Lysyl hydroxylation is the dominant oxidative catalytic activity of Jmjd6 (Fig. S14). Jmjd6 catalysed arginine demethylation cannot be ruled-out but would have to occur at a level below our current levels of detection. Although synthetic histone sequences were found to be Jmjd6 substrates; our sub-cellular localisation and interaction analyses imply that histones, if interacting with Jmjd6 at all, are not a major focus of Jmjd6 activity. Furthermore, in extensive MS-based analysis on endogenous histones we have accrued no evidence for hydroxylation of any lysine residue on histone H2A,

H2B, H3 and H4. Instead, we have shown that U2AF65, a protein important for pre-mRNA splicing, is an endogenous substrate for Jmjd6.

Our results on lysyl-hydroxylation of nuclear proteins support proposals (24) that post-translational hydroxylation in both the cytoplasm and the nucleoplasm is ubiquitous and changes in oxygen tension elicit systems wide-effects on proteins and protein biosynthesis and may provide a regulatory mechanism for alternative splicing.

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Conflict of Interest

C.J.S was a cofounder of ReOx, a company that aimed to exploit the hypoxic response for therapeutic benefit.

Figure legends

Fig. 1. (A) GFP-pulldown demonstrates interaction of Jmjd6 with U2AF65 and CROP. Cell lysates from HEK293 cells expressing GFP-Jmjd6 (left hand panels) or GFP (right hand panels). Input (In), flow-through (F) and bead (B) fractions were analysed by Western blot probed with anti-GFP antibody, anti-U2AF65 antibody and anti-CROP antibody. **(B) RS domains of LUC7L2, U2AF65 and CROP.** Lysyl residues are in red. Peptides (10-16 residues) were derived from these RS domains and residues observed to be hydroxylated by Jmjd6 are underlined. **(C) MALDI TOF analysis of LUC7L2₂₆₇₋₂₇₈ in the presence of Jmjd6.** Assays were comprised of

LUC7L2₂₆₇₋₂₇₈ (100 μ M), ascorbate (100 μ M), Fe(II) (100 μ M), 2OG (500 μ M), Tris (50 mM, pH 7.5) and Jmjd6 ((b) and (i)). (a) and (f) are negative controls without Jmjd6. (c)-(h) include all assay components except 2OG (c), Fe(II) (d), and with the addition of NOG (12 mM) (e), succinate (1 mM) (g) and fumarate (1 mM) (h).

Fig. 2. MS/MS analysis of endogenous U2AF65 reveals hydroxylation of Lys-15 (A) and Lys-276 (B). U2AF65 was immunoprecipitated from HeLa cells, digested with LysC protease, then analysed by LC/MS and MS/MS. MS data for the unmodified (top spectrum) and hydroxylated peptides (bottom spectrum). The MH^{2+} peptides that were fragmented to give the resulting MS spectra are shown on the right hand side of each spectrum.

Fig. 3. Jmjd6 regulates alternative splicing. (A) RNAi-mediated depletion of human Jmjd6 in HeLa cells. Western blot analyses of total protein extract from mock and Jmjd6 siRNA-treated HeLa cells with anti-Jmjd6 and anti-actin antibody. **(B)** Left panel: Exon structure of human MGEA6 gene showing exons 18, 19 and 20 as boxes and introns as lines. Splicing patterns are represented as diagonal lines. Arrows indicate the location of primers used in RT-PCR analyses. The forward primer used for qPCR quantification of -ex19 spliced isoform is shown as a dashed line. Middle and right panel: RT-PCR analyses detecting endogenous MGEA6 nuclear RNA from mock, Jmjd6 siRNA-treated or MAZ4 siRNA-treated HeLa cells. Histogram on right shows average amount of -ex19 spliced isoform ($\pm$ SD) as analysed by qPCR from 3 independent experiments. **(C)** RT-PCR analysis detecting MGEA6 RNA from HeLa cells treated with 100 μ M of DFO for 24 hours. Splicing products +ex19 and -ex19 are indicated. Histogram on right shows the average amount of -ex19 spliced isoform

($\pm$ SD) as analysed by qPCR from 3 independent experiments. **(D) Top panel:** α -TM minigene construct, with exons 1, 3 and 4 shown as boxes and introns as lines. Splicing patterns are shown as diagonal dashed lines. Arrows indicate location of primers used in RT-PCR analysis. **Bottom panel:** The α -TM minigene construct transfected in mock-treated HeLa cells and cells treated with Jmjd6 siRNA or siRNA targeting MAZ4 was analysed from nuclear RNA by RT-PCR. Histogram on right shows average percentage of α -TM exon 3 skipping ($\pm$ SD) from 3 independent experiments.

Supporting Online Material

Methods

Expression constructs

For full-length Jmjd6 cDNA was cloned from IRAKp961F08140Q, provided by RZPD (Deutsches Ressourcenzentrum fuer Genomforschung GmbH, Berlin, Germany), and inserted into the pEGFP-N1 and pEGFP-C1 (Clontech, Palo Alto, CA, USA) vectors, and the pcDNA3 vector (Invitrogen, CA, USA). For the tandem affinity purification (TAP) analyses full length Jmjd6 was cloned into the pECFP-N1-TAPc vector(25), provided by Heiko Hermeking (Max-Planck-Institute of Biochemistry, Martinsried, Germany).

Tandem affinity purification (TAP) and mass spectrometry (MudPIT)

Human embryonic kidney (HEK) 293T cells were transiently transfected with pECFP-N1-TAPc:Jmjd6. Extracts of 2×10^8 cells were prepared on ice for 15 minutes in lysis buffer (10 mM 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris), pH 8.0,

150 mM NaCl, 1% NP-40, 5 mM dithiothreitol (DTT) supplemented with protease inhibitors (Pefabloc (Boehringer), pepstatin A, aprotinin, leupeptin) and phosphatase inhibitors (100 nM okadaic acid and protease inhibitor cocktails 1 + 2 (Sigma)). TAP-tagged Jmjd6 and associated proteins were purified as described(25), dissolved in digestion buffer, digested with trypsin, and analysed by LC/LC/MS/MS according to published protocols(10, 11).

GFP-pulldown experiments

Human embryonic kidney (HEK) 293T cells were transiently transfected as described below. Extracts from 1×10^7 cells were prepared in 100 μ l lysis buffer (20 mM Tris/HCl pH 8.0, 150 mM NaCl, 0.5% NP40 supplemented with protease (Pefabloc (Boehringer), pepstatin A, Aprotinin, Leupeptin) and phosphatase inhibitors (100 nM okadaic acid and protease inhibitor cocktails 1 + 2 (Sigma)). After centrifugation supernatants were diluted to 200 μ l with lysis buffer without NP40, incubated with 50 μ l of GFP-nanotrap(14) for 1 hour at 4 °C with constant mixing. After centrifugation the supernatant was removed, the beads washed twice with 1 ml of dilution buffer containing 300 mM NaCl and the proteins were eluted in SDS-sample buffer and subjected to SDS-PAGE/Western blotting. As primary antibodies we used monoclonal anti-U2AF65 (SIGMA (U4758), Saint Louis, USA), monoclonal anti-GFP (Roche Applied Science, Mannheim, Germany) and anti-CROP (a gift from Kazumitsu Ueda, Kyoto University, Kyoto, Japan). For RNase treatment RNaseA (Quiagen) was added to the lysis buffer (final concentrations of 50 μ gml⁻¹, 250 μ gml⁻¹ or 500 μ gml⁻¹) and incubated for 30 minutes on ice.

Co-immunoprecipitation experiments

A monoclonal anti-U2AF65 antibody was used for immunoprecipitation experiments. Control experiments were performed without adding the anti-U2AF65 antibody. Extracts of 2×10^7 HeLa cells (untransfected or 24 hours after transfection with pcDNA3:jmjd6) were prepared as described for GFP-pulldown and incubated with or without (control) anti-U2AF65 antibody for 1 hour under agitation at 4°C. Protein-G Sepharose 4 Fast Flow (GE Healthcare, NJ, USA) (100 μ l) was added for another hour. After 3 wash steps with 20 mM Tris/HCl, pH 8.0, 150 mM NaCl, the beads were re-suspended in SDS sample buffer and subjected to SDS-PAGE/Western blotting.

Cell culture, transfection and immunostaining experiments

HeLa cells and human embryonic kidney (HEK) 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum, penicillin (100 Uml⁻¹) and streptomycin (100 μ gml⁻¹) at 37 °C, 5 % CO₂. For microscopy cells were grown to 50-70 % confluence on 18x18 glass coverslips and transfected with the indicated expression constructs using jetPEI (Polyplus transfection, Illkirch, France) or Nanofectin (PAA, Pasching, Austria) according to the manufacturer's instructions. 24 hours after transfection cells were fixed with 4 % paraformaldehyde in PBS for 15 minutes at room temperature, permeabilised with methanol for 2 minutes and 1 % Triton X-100 in PBS for 10 minutes, stained with antibodies, counterstained with To-Pro3 (Invitrogen, CA, USA) and mounted in Vectashield (Vector Laboratories, CA, USA) As primary antibodies we used polyclonal anti-Jmjd6 (ab10526).

Consistent with previous reports(4) immunofluorescence with anti-Jmjd6 antibody revealed nuclear localisation (Fig. S15).

Cloning, expression and purification of human Jmjd6 (residues 1-343)

The gene encoding human Jmjd6 (residues 1-343) was cloned into the pET-28a(+) vector (Novagen) and was expressed in *E. coli* BL21-rosetta cells in 2TY growth medium supplemented with kanamycin (25 μgml^{-1}) and chloramphenicol (30 μgml^{-1}). Cells were grown at 37 °C until an OD₆₀₀ of 0.6; the temperature was then reduced to 25 °C and isopropyl β -D-1-thiogalactopyranoside (IPTG) (0.5 mM) was added. Cells were then harvested 16-18 hours post-induction and lysed by sonication in tris buffer (50 mM, pH 7.0), NaCl (300 mM), tris(2-carboxyethyl) phosphine hydrochloride (TCEP) (0.2 mM) and glycerol (10 %) (lysis buffer). Purification of the N-terminally His₆-tagged (N-terminus: MGSSHHHHHHSSGLVPRGSH) protein was carried out using nickel-affinity chromatography. Jmjd6 was eluted from the column using a gradient from 0 to 100% imidazole (500 mM). Fractions were analysed by SDS-PAGE and those containing protein of the correct molecular weight and the best purity were pooled and concentrated using a 10,000 MWCO filter (Millipore). The protein solution was buffer-exchanged into lysis buffer and concentrated to 10-20 mgml^{-1} measured by a NanoDrop[®] ND-1000 spectrophotometer (LabTech International, UK). Further purification of Jmjd6 (>95 % as determined by SDS-PAGE analysis) was performed using size-exclusion chromatography (SEC), using a Superdex-75[™] (300 ml, GE Healthcare, UK) preparative grade column.

The H187A and H187A-D189A Jmjd6 variants were created from the wild-type construct (pET-28a(+)-Jmjd6 (residues 1-343)) using the QuikChange[®] II Site-

directed mutagenesis kit (Stratagene). The integrity of the mutations was confirmed by DNA sequencing (Geneservice, Oxford).

Finding a substrate for Jmjd6

Standard assays consisted of the substrate mixture: substrate (100 μ M), 2OG (500 μ M), ascorbate (100 μ M) in Tris (50 mM, pH 7.5); and the enzyme mixture: FeNH₄SO₄ (100 μ M) and Jmjd6 (10 μ M) in Tris (50 mM, pH 7.5). The reaction was initiated by mixing the substrate and enzyme mixtures and incubating at 37°C for 30-60 min. The reaction was quenched by adding CF₃CO₂H to a final concentration of 0.1 % and incubating on ice. Hydroxylation of the peptide was assayed by a MALDI TOF micro MX mass spectrometer (Waters Micromass); where the assay mixture (1 μ l), along with α -cyano-4-hydroxycinnamic acid (CHCA) MALDI matrix in 60% acetonitrile/0.1 % CF₃CO₂H (1 μ l), was spotted directly onto the target plate. To determine whether modification of peptide was Jmjd6-dependent, assays were performed omitting either 2OG or Fe(II). To determine whether Jmjd6 was inhibited by N-oxalylglycine (NOG), NOG (12 mM) was added to the substrate component of the assay system.

MS/MS analysis of hydroxylated LUC7L2₂₆₇₋₂₇₈

MS/MS analyses were carried out using a Ultraflex III MALDI TOF-TOF (Bruker Daltonics) mass spectrometer and experiments were acquired using the potential LIFT technique (Bruker Daltonics, Germany), based on post source and post-decay acceleration of fragment ions. MS/MS spectra were annotated using FlexAnalysis software and transferred to BioTools for sequence evaluation (Bruker Daltonics, Germany). The Sequence Editor tool was used to define the peptide sequence variants

to be matched to the actual MS/MS spectra in BioTools. Assay mixtures (with and without Jmjd6) (1 μ l) were spotted directly onto the MALDI target plate, followed by CHCA matrix (1 μ l), and then allowed to dry. Mass spectra were acquired in the positive reflector mode with a 17 kV acceleration voltage. The mass corresponding to LUC7L2₂₆₇₋₂₇₈ and LUC7L2₂₆₇₋₂₇₈ +16, equivalent to one hydroxylation, were identified and subsequent MS/MS spectra were acquired by post source decay experiments in positive reflector mode.

NMR analysis of hydroxylated LUC7L2₂₆₇₋₂₇₈

LUC7L2₂₆₇₋₂₇₈ was hydroxylated (>80 %) by incubation with Jmjd6 in the presence of ascorbate, 2OG and Fe(II) overnight at 22 °C. The reaction was quenched by the addition of methanol (20%). Precipitate was removed by centrifugation and the supernatant purified by HPLC using a Synergi™ Hydro-RP (100 x 21.2 mm) (Phenomenex, UK). Peptide was eluted using a gradient of acetonitrile in 0.1% CF₃CO₂H and then lyophilised. To prepare the peptide for NMR analysis the sample was lyophilised a second time from ²H₂O and re-suspended in 6 μ l of this solvent. 1D ¹H and 2D COSY NMR experiments were performed on a Bruker AVII 500 spectrometer using a 1 mm microprobe.

¹⁸O₂ dioxygen experiment with LUC7L2₂₆₇₋₂₇₈

The hydroxylation of LUC7L2₂₆₇₋₂₇₈ by Jmjd6, under standard assay conditions, was performed under an atmosphere of ¹⁸O₂. A rubber septum sealed reaction vessel, containing the substrate mixture, was evacuated and flushed with argon no less than three times before being filled with ¹⁸O₂ gas (>98 % CK gases). The reaction was initiated by injecting the enzyme mixture (Jmjd6 and Fe(II)) through the septum into

the vessel. The reaction was carried out for one hour before it was quenched by freezing and then addition of $\text{CF}_3\text{CO}_2\text{H}$ (0.1 %). Products were analysed by MALDI-TOF mass spectrometry as above.

Peptide synthesis

All peptides tested as potential substrates of Jmjd6 were synthesised in-house using a Intavis MultiPep automated multiple synthesiser and Tentagel amide resin (Intavis, Germany). Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids were coupled with *N*, *N'*-diisopropylcarbodiimide (DIC)/1-hydroxybenzotriazole (HOBt). Cleavage of the completed peptides from the resin used $\text{CF}_3\text{CO}_2\text{H}$ /triethylpropylsilane (95/2.5). Predicted masses were confirmed by MALDI-TOF mass spectrometry.

Substrate selectivity assays

Standard assay conditions were used and are described above. To determine the pH profile of Jmjd6 standard peptide assays were performed over a pH range of 6 to 9. Buffers used were piperazine-*N,N'*-bis(ethanesulfonic acid) (PIPES) (pH 6.0 and 6.5), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0 to 8.0) and *N,N*-bis(2-hydroxyethyl) glycine (BICINE) (pH 8.5 and 9.0). Inhibition assays were performed under identical conditions as described for the standard assays except either succinate (1mM) or fumarate (1 mM) were included in the substrate mixture.

In-gel digestions with LysC protease

Immunoprecipitated U2AF65 and recombinant U2AF65 were separated 1D SDS-PAGE (12 % acrylamide), the gel was then stained with Coomassie Blue using Colloidal Blue Staining Kit (Invitrogen), and protein bands corresponding to the

proteins of interest were excised and digested with LysC protease (Roche) according to standard protocol(26). The extracted peptides were desalted and concentrated using RP-C18 StageTip columns(27) and the eluted peptides used for mass spectrometric analysis.

Mass spectrometric analysis of recombinant and endogenous U2AF65

MS experiments on recombinant and endogenous U2AF65 were performed using a nanoflow high-performance liquid chromatography (HPLC) system (Agilent Technologies 1200, Waldbronn, Germany) connected to a hybrid LTQ-Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Odense, Denmark) as described(28). The peptide mixtures were loaded onto a 15 cm analytical column (75 μ m inner diameter) packed in-house with 3 μ m reverse-phased, fully endcapped C18 beads (Reprosil-AQ Pur, Dr. Maisch). Mobile phases for HPLC consisted of (A) 99.5 % Milli-Q water and 0.5 % acetic acid (v/v) and (B) 19.5 % Milli-Q water, 80 % acetonitrile and 0.5 % acetic acid (v/v). 5 μ L of prepared peptide mixture was loaded onto the analytical column for 20 minutes in 2 % buffer B at a flow rate of 500 nLmin⁻¹ followed by reverse-phased separation through a 90 minute gradient ranging from 5 % to 40 % acetonitrile in 0.5 % acetic acid. The eluted peptides from the HPLC column were directly electrosprayed into the mass spectrometer for detection. The MS instrument was operated in data-dependent mode by automatically switching between full survey scan MS and consecutive MS/MS acquisition. Survey full scan MS spectra (from mass range m/z 300 – 2000) were acquired in the orbitrap section of the instrument with a resolution of R=60,000 at m/z 400 (after accumulation to a “target value” of 1,000,000 in the linear ion trap). The ten most intense peptide ions in each survey

scan with an ion intensity above 500 counts and a charge state ≥ 2 were sequentially isolated to a target value of 5,000 and fragmented in the linear ion trap by collisionally induced dissociation (CID/CAD). All peaks selected for fragmentation were automatically put on an exclusion list for 90 seconds, which ensured that the same ion would not be selected for fragmentation more than once. For optimal duty cycle the fragment ion spectra were recorded in the LTQ mass spectrometer “in parallel” with the orbitrap full scan detection. For all survey scan measurements with the orbitrap detector a lock-mass ion from ambient air (m/z 391.284286, 429.08875, and 445.120025) was used for internal calibration as described(29) ensuring an overall sub-ppm mass accuracy for all detected peptides. For all mass spectrometric experiments data was saved in RAW file format (Thermo Scientific, Bremen, Germany) using the Xcalibur 2.0 with Tune 2.4. All data was analyzed using in-house written software MaxQuant.

Identification of U2AF65 peptides

The data analysis was performed with the MaxQuant software as described in(30) supported by Mascot as the database search engine for peptide identifications. MS/MS peak lists were filtered to contain at most six peaks per 100 Dalton intervals and searched by Mascot (www.matrixscience.com) against a concatenated forward and reversed version of the IPI human database (version 3.37, www.ebi.ac.uk). Protein sequences of common contaminants, e.g. keratins, were added to the database. The initial mass tolerance in MS mode was set to 5 ppm and MS/MS mass tolerance was 0.5 Da. Cysteine carbamidomethylation was searched as a fixed modification, whereas N-acetyl protein, N-pyroglutamine, oxidized methionine, and lysine hydroxylation were searched as variable modifications. Relative peptide quantitation

was based on extracted ion chromatograms (XICs) with a mass window of +/- 5 mDa. Furthermore, peptide assignments were statistically evaluated in a Bayesian model based on sequence length and Mascot score. We only accepted peptides with a false discovery rate (FDR) of less than 1%, estimated based on the number of accepted 'reverse' hits. Due to the technical limitations of the MS analyses on the LysC digests from intact proteins, we are unable to obtain complete coverage of the target proteins (including in the RS domain region) via our current MS system. Therefore the number of hydroxylated residues may be greater than those we have identified.

Effect of RNAi-mediated depletion of Jmjd6 on alternative splicing

The α -TM minigene construct contains 6 tandem URE copies to increase skipping of exon 3 in HeLa cells(31) and has been described elsewhere(31). Although the α -TM minigene lacks the mutually exclusive exon 2, this does not interfere with the splicing regulation of exon 3. In most cells the generated splicing product is 1-3-4, while in smooth muscle (SM) cells it is 1-4. Polypyrimidine tract (PPT), downstream and upstream regulatory elements are involved in the repression of exon 3 in SM cells. Transient transfections in HeLa cells and RT-PCR RNA analysis using radioactively labelled primers has been performed as described in (32). MGEA6 gene analysis was carried out as described previously(21). RNAi procedure was carried out as described in (33). RT-PCR primers for detection of actin mRNA were 5'-CGTGATGGTGGGCATGGGTCAG-3' and 5'-CTTAATGTCACGCACGATTTC-3' and for Jmjd6 mRNA- 5' GGCACAACTACTACGAGAGC-3' and 5'-TTTGGCACCTTG TAGTCTTCC-3'. Western blotting was performed by standard immunoblotting procedure with ECF western blotting kit (GE Healthcare) on 25-50 μ g of total HeLa cell protein extract. Antibodies for Western blot analysis were rabbit

polyclonal anti-Jmjd6 antibody (Abcam), rabbit polyclonal anti-actin antibody (Sigma) and anti-rabbit fluorescein-conjugated antibody (Sigma). DFO (Sigma) was added at final concentration of 100 μ M for 24 hours.

Supporting Tables 1

Table S1. Potential interacting proteins of Jmjd6, as identified by tandem-affinity purification and mass spectrometry.

Supporting Figures 1-15

Fig. S1. (A) GFP-pulldown demonstrates interaction of Jmjd6 with U2AF65.

Lysates from HEK293 cells over-expressing untagged Jmjd6 and GFP-U2AF65 (left hand panels) or GFP (right hand panels) were subjected to GFP-pulldown experiments. Input (In), flow through (F) and beads (B) fractions were probed with anti-GFP antibody and anti-Jmjd6 antibody. **(B) Co-immunoprecipitation of U2AF65 and Jmjd6.** U2A65 immunoprecipitation experiment in HeLa cell lysates expressing endogenous Jmjd6 (left panel) or over-expressing Jmjd6 from pcDNA3/Jmjd6 plasmid (right panel). Samples were analysed on Western blot probed with anti-U2AF65 antibody (top panel) and anti-Jmjd6 antibody (lower panel). -/+ refers to the absence and presence of anti-U2AF65 antibody; * is endogenous Jmjd6; ** is over-expressed Jmjd6; IgG is the heavy chain of immunoglobulin.

Fig. S2. The interaction of Jmjd6 with U2AF65 is dependent on RNA. Lysates from HEK293 cells over-expressing Jmjd6-GFP were treated with increasing amounts

of RNase A and subjected to GFP-pulldown. Untreated lysates were used for comparison (upper panel). Input (In), flow through (F) and bead (B) fractions were probed with anti-GFP antibody and anti-U2AF65 antibody. In a control experiment the interaction of DNMT1-GFP with PCNA (14) was not disrupted after RNase A treatment.

Fig. S3. MALDI TOF analyses of U2AF65₂₈₋₄₃(R35Me_{2sym}), U2AF65₂₈₋₄₃(R33Me_{2sym}) and LUC7L2₂₆₇₋₂₈₂(R274Me_{2sym}) in the presence of Fe(II), 2OG, ascorbate and Jmjd6. Peptides were prepared with an N-terminal Fmoc group (+222 Da.). The -17 Da. mass shift from unmodified peptide observed in all spectra is most likely due to aspartimide formation, a common side reaction in Fmoc-based solid phase peptide synthesis(35).

Fig. S4. MALDI TOF analysis of histone peptides with Jmjd6. Incubation of Jmjd6 (10 µM) with H3R2 peptides (AR*TKQTARKSTGGKAPRK) and H4R3 peptides (SGR*GKGGKGLGKGGAK) (100 µM) (where * is both asymmetrically dimethylated and symmetrically dimethylated arginine) in the presence of ascorbate (100 µM), 2OG (500 µM) and Fe(II) (100 µM). A +16 shift in mass is observed (hydroxylation) over a -14 shift (demethylation).

Fig. S5. MALDI TOF mass spectrum of LUC7L2₂₆₇₋₂₇₈ after incubation with Jmjd6 under ¹⁸O₂. Incubation of LUC7L2₂₆₇₋₂₇₈ (100 µM) with Jmjd6 (10 µM), under aerobic (a) and ¹⁸O₂ conditions (c). The spectrum in (b) is a negative control of LUC7L2₂₆₇₋₂₇₈ (100 µM) under ¹⁸O₂, without Jmjd6.

Fig. S6. MS/MS spectra from post source decay (PSD) fragmentation of unmodified (a) and hydroxylated (b) LUC7L2₂₆₇₋₂₇₈. The 'b' (red) and 'y' (black) series of fragmented ions are labelled with the mass and the corresponding residue. *N*-Terminal sequence analysis by the Edman method, using DL and DL allo-hydroxylysine as a reference compound, further supports hydroxylation at Lys-269 of the LUC7L2₂₆₇₋₂₇₈ peptide.

Fig. S7. 2D COSY experiment comparing hydroxylated LUC7L2₂₆₇₋₂₇₈ (A) with unhydroxylated LUC7L2₂₆₇₋₂₇₈ (B) and DL allo-hydroxylysine (C). The new resonances in the hydroxylated LUC7L2₂₆₇₋₂₇₈ are shown in a red box and are consistent with resonances observed in the hydroxylysine reference experiment.

Fig. S8. MALDI TOF analysis of LUC7L2₂₆₄₋₂₇₈ and variations of LUC7L2₂₆₄₋₂₇₈ with Jmjd6. Incubation of Jmjd6 (10 μ M) with peptides (100 μ M) LUC7L2₂₆₃₋₂₇₈ (a), LUC7L2₂₆₃₋₂₇₈ (K266R) (b), LUC7L2₂₆₄₋₂₇₈ (K269R) (c) and LUC7L2₂₆₄₋₂₇₈ (K266R-K269R) (d) in the presence of ascorbate (100 μ M), 2OG (500 μ M) and Fe(II) (100 μ M). Residues within the peptides that have been mutated from the wild-type LUC7L2 sequence are underlined.

Fig. S9. MALDI TOF analysis showing the effect of lysine modifications on Jmjd6-catalysed hydroxylation. Incubation of Jmjd6 (20 μ M) with either (a) H3K9Me₃ (15 mer), (b) H3K9Me₂ (15 mer), (c) H3K9Me₁ (15 mer), (d) H3K9 (15 mer) (100 μ M) in the presence of ascorbate (100 μ M), 2OG (500 μ M), Fe(II) (100 μ M); where the sequence of H3K9 is ARTKQTARKSTGGKA.

Fig. S10. MS/MS spectra showing hydroxylation of recombinant U2AF65 at Lys-276. The Fig. shows the spectra for the hydroxylated Lys-276 peptide. The 911.47 Da. (MH^{2+}) peptide that was fragmented to give the resulting MS spectra is located on the right hand side of the figure. U2AF65 (40 μ M) was incubated with Jmjd6 (20 μ M monomer concentration) in the presence of 2OG (500 μ M) and Fe(II) (400 μ M) for 2 hours at 37 °C. The assay mixture was then separated by 1D SDS-PAGE and the band corresponding to the molecular weight of U2AF65 was excised and digested with LysC protease. The sample was analysed using an LC-MS/MS system.

Fig. S11. Mapping hydroxylated Lys-276 on recombinant U2AF65 by MS/MS (MS^E) analysis. Recombinant U2AF65 was incubated with recombinant Jmjd6, digested with LysC and subjected to tandem mass spectrometry analysis using nano-UPLC- MS^E . Reconstituted MS/MS (MS^E) spectrum of the observed parent ion mass $[M+H]^+$ 1706.84 Da that was assigned to the peptide 272-276 (DDQVK(OH)ELLTSFGPLK) containing hydroxylated Lys-276. Fragments are indicated as b, b* (loss of NH^3), y and im (immonium) ions.

Samples were subjected to separation by ultra performance liquid chromatography tandem mass spectrometry analysis (nano-UPLC- MS^E) using a 75 μ m I.D.x 25 cm C18 nanoAcquityTM UPLCTM column 1.7 μ m particle size (Waters, Milford, MA, USA) and a 90 minute gradient: 2 % to 45 % solvent B (solvent A: 99.9 % H_2O , 0.1 % formic acid; solvent B: 99.9 % MeCN, 0.1 % HCO_2H) with a Waters nanoAcquityTM UPLCTM system (final flow rate 250nlmin⁻¹, 7000 psi) coupled to a Waters QTOFpremierTM tandem mass spectrometer (Waters, Milford, MA, USA).

Data was acquired in high-definition MS^E mode (low collision energy: 4 eV, high collision energy ramping from 15 eV to 40 eV, switching every 1.5 seconds) and processed with ProteinLynx Global Server (PLGS version 2.2.5, Waters, Milford, MA, USA) to reconstruct MS/MS spectra by combining all masses with identical retention times. Note that some fragment ions may not always be assigned to the right parent ion in the case when several ions co-elute. The mass accuracy of the raw data was corrected using glu-fibrinopeptide (GFP, 200fmol μ l⁻¹, 700nlmin⁻¹ flow rate, 785.8426 Da. [M+2H]²⁺) that was infused into the mass spectrometer as a lock mass during sample analysis. Low and high collision energy MS data was calibrated at intervals of 30 seconds. The raw data sets were processed including deisotoping, deconvolution, and peaklists generated on the basis of assigning precursor ions and fragments based on similar retention times. MS/MS spectra (reconstituted peaklists) were searched against the SwissProt database (release 54.0, 07/2007, number of entries 276256) using Mascot version 2.2 (Matrixscience, London, UK) and the following parameters: peptide tolerance 0.2 Da., ¹³C=2, fragment tolerance 0.1 Da., missed cleavages: 3, variable modifications: carbamidomethylation C, oxidation M/R/K, instrument type: ESI-QUAD-TOF.

Because of the highly basic amino acids in the RS-domain sequences we have been unable to detect sequence with some potential target hydroxylation sites, thus it is likely that there are hydroxylation sites additional to those we have detected in recombinant and endogenous U2AF65.

Fig. S12. XIC of non-modified and hydroxylated Lys-15 extracted from a Mock-transfected HeLa cell line and a cell line over-expressing Jmjd6. Comparison of both cell lines shows that hydroxylation of Lys-15 is 5-fold higher in cells over-expressing Jmjd6. The top chromatogram shows the unmodified Lys-15 peptide at identical intensities between the two different cell lines. The bottom Fig. shows the hydroxylated Lys-15 peptide intensities. The ion chromatogram was extracted from the data in a narrow mass range corresponding to the masses of the observed peptides +/- 5 mDa. The intensities of these extractions were normalised and the results from the normal cell line (black) and from the cell line over-expressing Jmjd6 (red) are overlaid.

Fig. S13. RNAi-mediated depletion of human Jmjd6 in HeLa cells. RT-PCR analysis of β -actin and Jmjd6 mRNAs in Mock and Jmjd6 siRNA-treated cells. Numbers below the gel represent the number of PCR cycles.

Fig. S14. Jmjd6 catalyses lysyl hydroxylation in an oxygen, Fe(II) and 2OG dependent manner. All the peptide assays described in this paper were performed using C-terminally truncated Jmjd6 (residues 1-343). Full-length (residues 1-403) and protein with C- and N- terminal truncations (residues 25-338) were also found to hydroxylate peptides to a similar extent, and no demethylation of arginine residues was observed.

Fig. S15. Localisation of Jmjd6 to the nucleus. Endogenous Jmjd6 was observed to localise in the nucleoplasm in a diffuse fashion and overlapped with nuclear speckles/interchromatin granules (dynamic structures enriched in RNA splicing

factors located in the interchromatin regions of the nucleoplasm(34)) in areas excluding the nucleolus. Immunofluorescence of HeLa cells co-stained with anti-Jmjd6 antibody and anti-SC-35 antibody reveals partial co-localisation of Jmjd6 with SC-35 (non-snRNP spliceosomal component) (confocal sections, counterstaining with DNA dye To-Pro3, scale bar 5 μ m).

figure 1

figure 2

A

figure 2

B

figure 3

table S1

ATP-dependent RNA helicase DDX41	Q9UJV9	pre-mRNA processing
CROP protein	O95232	pre-mRNA processing
Poly-U binding splicing factor PUF60	Q9UJY7	pre-mRNA processing
Putative RNA-binding protein Luc7-like 1	Q9NQ29	pre-mRNA processing
Putative RNA-binding protein Luc7-like 2	Q9Y383	pre-mRNA processing
Splicing factor arginine/serine rich 11 (p54)	Q05519	pre-mRNA processing
Arginine/serine-rich coiled coil protein 1	Q96IZ7	pre-mRNA processing
Probable ATP-dependent RNA helicase DDX46	Q7L014	pre-mRNA processing
RNA binding motif protein 25	P49756	pre-mRNA processing
Acinus (Apoptotic chromatin condensation inducer in the nucleus)	Q9UKV3	pre-mRNA processing
Probable ATP-dependent RNA helicase DDX17 (p72)	Q92841	pre-mRNA processing
RNA binding protein	Q9UQ39	pre-mRNA processing
Pre-mRNA-processing factor 40 homolog A	O75400	pre-mRNA processing
Splicing factor U2AF 35kD subunit	Q01081	pre-mRNA processing
Splicing factor U2AF 65kD subunit	P26368	pre-mRNA processing
RNA-binding protein 39 (Splicing factor HCC1)	Q14498	pre-mRNA processing
RNA polymerase-associated protein RTF1 homolog	Q92541	pre-mRNA processing
Cleavage and polyadenylation specificity factor 6	Q16630	pre-mRNA processing
Nucleolar RNA helicase II (DDX21)	Q9NR30	pre-mRNA processing / Nucleolus
Nucleolar phosphoprotein p130	Q14978	pre-mRNA processing / Nucleolus
Treacle protein (Treacher Collins syndrome protein)	Q13428	pre-mRNA processing
H/ACA ribonucleoprotein complex subunit 4 (Dyskerin)	O60832	pre-mRNA processing
Nuclease sensitive element binding protein 1	P67809	
DNA-binding protein A	P16989	
Parafibromin (Cell division cycle protein 73 homolog)	Q6P1J9	
Bromodomain-containing protein 4	O60885	
Eukaryotic translation initiation factor 3 subunit 4	O75821	
Eukaryotic translation initiation factor 3 subunit 8	Q99613	
Elongation factor 1- α 2	Q05639	
NF- κ B-activating protein	Q8N5F7	
Phosphatidylinositol-4 phosphate 5-kinase type II α	P48426	
Casein kinase 2A1	P68400	
Phosphatidylinositol-4-phosphate 5-kinase type-II γ	Q8TBX8	
Phosphatidylinositol-4 phosphate 5-kinase type II β	P78356	
Hypothetical protein FLJ32377	Q96MH4	
RNF187 protein	Q6PJR0	
Small acidic protein	O00193	
Multiple myeloma tumor-associated protein 2	Q9BU76	
Hepatoma derived growth factor 2	Q7Z4V5	

figure S1

figure S2

figure S3

figure S4

figure S5

NPKRSRSREHRR
LUC7L2₂₆₇₋₂₇₈

figure S6

figure S7

figure S8

(a) SHSKNPKRSRSREHRR
LUC7L2₂₆₃₋₂₇₈

(b) SHSRNPKRSRSREHRR
LUC7L2₂₆₃₋₂₇₈ (K266R)

(c) SHSKNPRRSRSREHRR
LUC7L2₂₆₃₋₂₇₈ (K269R)

(d) SHSRNPRRSRSREHRR
LUC7L2₂₆₃₋₂₇₈ (K226R-K269R)

figure S9

ARTKQTARKSTGGKA (H3K9)

figure S10

figure S11

figure S12

figure S13

figure S14

figure S15