Supplementary Information

Investigations on the Oxygen Dependence of a 2-Oxoglutarate Histone Demethylase

Elena M. Sánchez-Fernández, Hanna Tarhonskaya, Khalid Al-Qahtani, Richard J. Hopkinson, James S. O. McCullagh, Christopher J. Schofield and Emily Flashman*

Chemistry Research Laboratory, University of Oxford, 12 Mansfield Road, Oxford, OX1 3TA, United Kingdom.

Corresponding Author: E-mail: emily.flashman@chem.ox.ac.uk
Telephone: +44 (0)1865 285110
Fax: +44 (0)1865 285002

Table of Contents

SUPPLEMENTARY METHODS

Demethylation assays: MALDI-TOF MS

Relative proportions of a particular peptide species (i.e. trimethylated, dimethylated or monomethylated peptide) were calculated by taking the ratio of one methylation state's peak intensity in the mass spectrum to the sum of all three methylation states' peak intensities (Equation 1). Equivalent concentrations of H3K9 me3/me2/me1/me0 peptides are known to demonstrate equal peak intensities upon MALDI-MS analysis (data not shown).

$$\%me3 = 100 \times \frac{I_{me3}}{I_{me3} + I_{me2} + I_{me1}}$$

Equation 1
Formula for calculating relative percentage of the trimethyl methylation state from MALDI-TOF MS data, where I_{mex} is the intensity (in arbitrary units) of a particular peak in the MALDI-TOF mass spectrum after background substraction had been applied to the raw spectrum.

SUPPLEMENTARY FIGURES

Figure S1

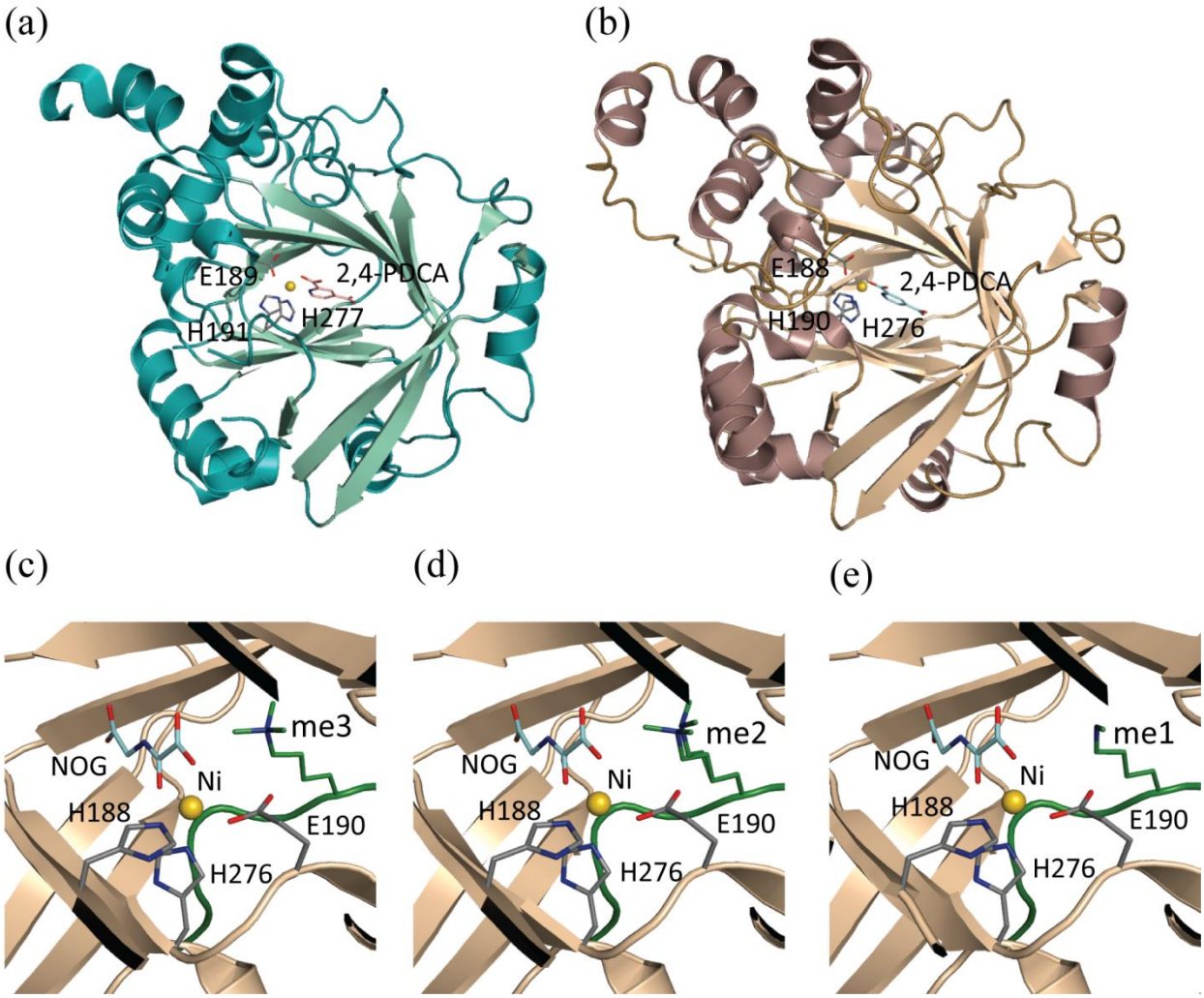


Figure S1 Comparison of structural features of KDM4E and KDM4A
(**a**) KDM4E has a core double stranded beta helix motif and a catalytic triad (H189, E191 and H277) coordinating a Ni atom (gold); Ni was used in place of Fe(II) for crystallisation purposes. The 2OG analogue pyridine 2,4-dicarboxylate (2,4-PDCA) is also coordinated to the Ni. The KDM4E crystal structure (PDB ID: 2W2I) is reported in reference [1]. (**b**) A view of the crystal structure of KDM4A (PDB ID: 2VD7) [2], shows its structural homology with KDM4E. The double stranded beta helix motif and HXD/E…H catalytic triad are common features of all 2OG oxygenases [3]. (**c-e**) Views from the crystal structure of KDM4A (wheat) in the presence of H3K9me3 (**c**), H3K9me2 (**d**) and H3K9me1 (**e**) peptide substrates (green) [2], PDB IDs: 2OQ6, 2OX0 and 2OT7, respectively; the view shown in (**c**) implies that for the H3K9me3 substrate, one of the methyl groups is directed towards the active site metal in a position that is favourable for demethylation, whilst for the H3K9me1 substrate (**e**), the single methyl group is directed away from the metal (note, in solution this situation may change - at least with some peptide

sequences low levels of H3K9me1 demethylation are observed) [4]. Interestingly, two conformations for the H3K9me2 substrate are observed (**d**), suggesting a degree of flexibility in substrate binding. Although structural data for KDM4E in the presence of substrates are not available, the homology between KDM4E and KDM4A suggests that the H3K9 substrates may bind similarly.

Figure S2

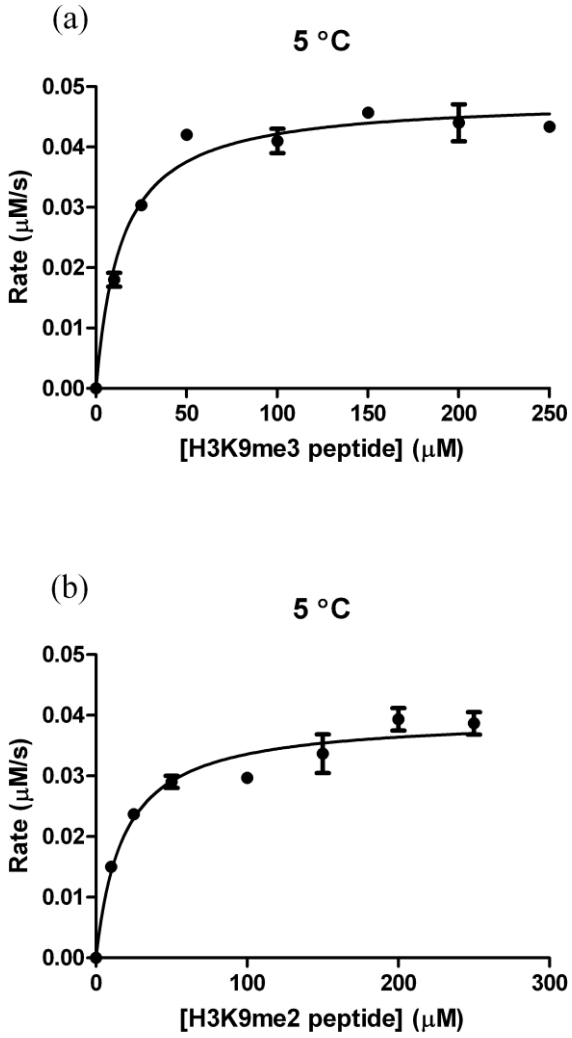


Figure S2 Steady-state kinetic parameters for KDM4E

K_M and k_{cat} values for KDM4E with both H3K9me3 and H3K9me2 8mer peptides were determined at 5 °C by MALDI-TOF MS spectrometry. (**a**) For H3K9me3: k_{cat} = 0.048 ± 0.0012 s^{-1}; K_M = 14.0 ± 1.9 μM. (**b**) For H3K9me2: k_{cat} = 0.040 ± 0.0014 s^{-1}; K_M = 18.2 ± 3 μM. Michaelis-Menten curves were fitted using GraphPad Prism 5.0™. Data points represent the mean of three replicate experiments, and error bars are given as standard error of the mean.

Figure S3

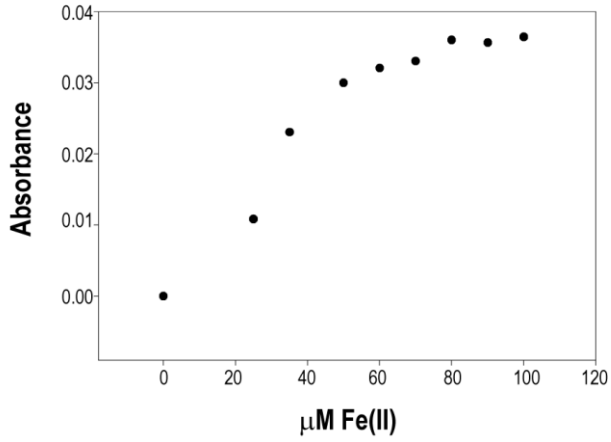


Figure S3 Titration of Fe(II) into the KDM4E:2OG complex under anoxic conditions
Fe(II) was titrated (0, 25, 35, 50, 60, 70, 80, 90, 100 μM) into a solution containing KDM4E (0.4 mM) and 2OG (4 mM) in deoxygenated 50 mM Hepes/500 mM NaCl, pH 7.5, at room temperature in an anaerobic glove box and analyzed by UV-visible spectroscopy. The points in the figure depict the absorbance at 541nm as a function of Fe(II) concentration.

Figure S4

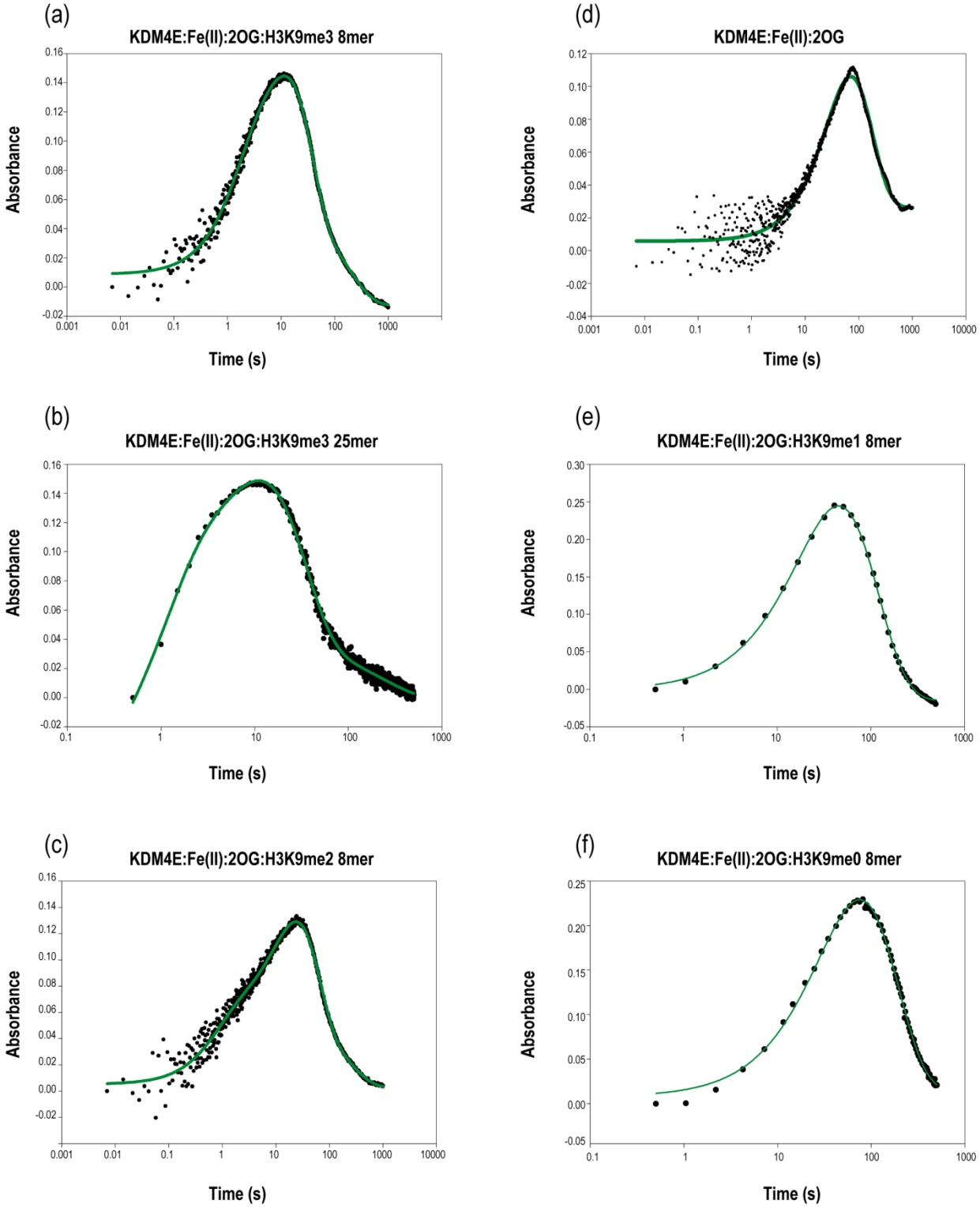

(a)
KDM4E:Fe(II):2OG:H3K9me3 8mer
Absorbance
Time (s)
(b)
KDM4E:Fe(II):2OG:H3K9me3 25mer
Absorbance
Time (s)
(c)
KDM4E:Fe(II):2OG:H3K9me2 8mer
Absorbance
Time (s)
(d)
KDM4E:Fe(II):2OG
Absorbance
Time (s)
(e)
KDM4E:Fe(II):2OG:H3K9me1 8mer
Absorbance
Time (s)
(f)
KDM4E:Fe(II):2OG:H3K9me0 8mer
Absorbance
Time (s)

Figure S4 Exponential fits of data collected at 320nm monitoring the reaction of KMD4E:Fe(II):2OG ± H3K9 me3/me2/me1/me0 complexes with O_2 at 5 °C

Stopped flow UV-vis spectroscopy was used to observe the formation of species absorbing at 320nm over time in the absence and presence of the H3K9 peptide substrates with different methylation states (me3/me2/me1/me0) after mixing with oxygen-saturated buffer at 5 °C. Data collected at 320nm (black circles, shown on a logarithmic scale) were fitted (green lines) using SigmaPlot^{TM} to a sum of four exponentials (f = a*exp(-b*x) + c*exp(-d*x) + e*exp(-g*x) + h*exp(-i*x) + j; for (**a**), (**b**) and (**c**)) or to a sum of two exponentials when demethylation is not involved in the process (f = a*exp(-b*x) + c*exp(-d*x) + e; for (**d**), (**e**) and (**f**)). For formation and decay rate constants of the 320nm species see Table 2 in the main text.

Figure S5

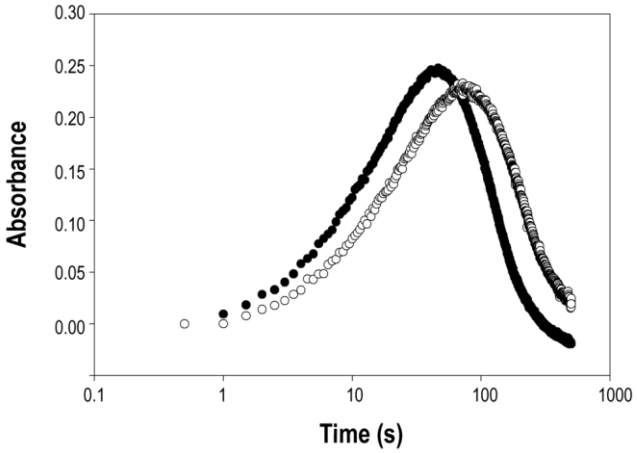


Figure S5 Development of absorption at 320nm in the presence of H3K9me1 and H3K9me0 8mer peptides

Stopped flow UV-vis spectroscopy was used to observe the formation of species absorbing at 320nm over time in the presence of H3K9me1 (black filled circles) and H3K9me0 (white circles) peptides after mixing with oxygen-saturated buffer at 5 °C. Maximum accumulation (t_{max}) was observed at 46 s and 72 s respectively (shown on a logarithmic scale).

Figure S6

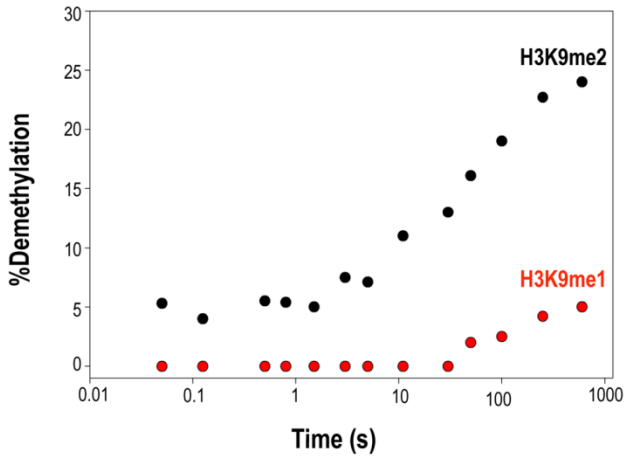
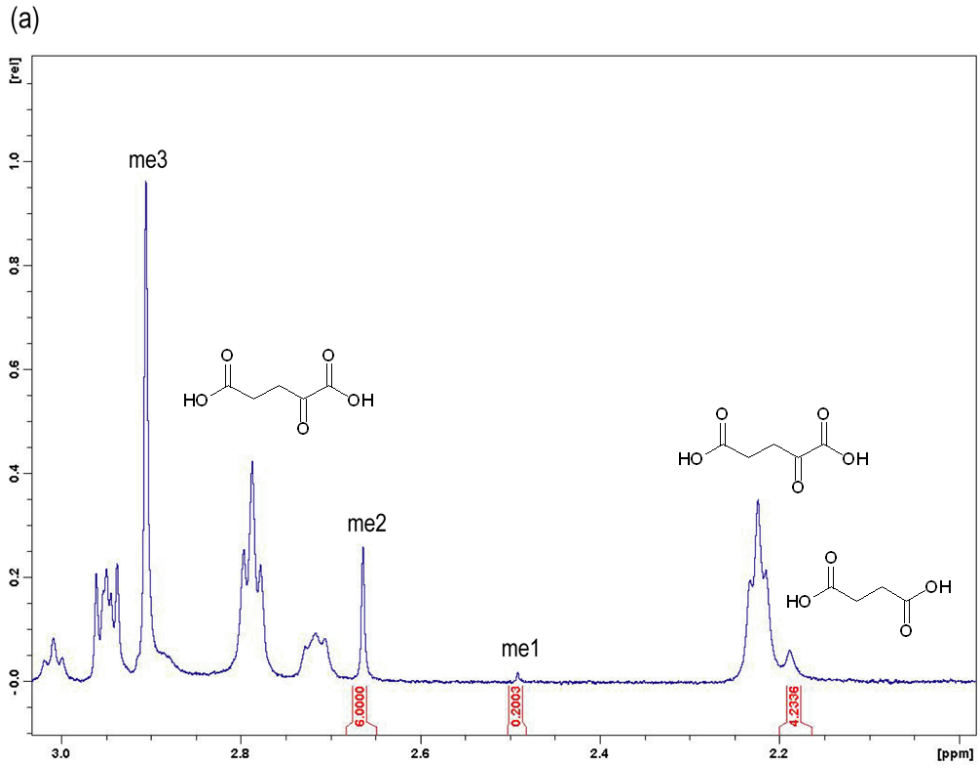


Figure S6 Rapid chemical quench experiments in the presence of H3K9me3 8mer peptide MALDI-TOF MS was used to quantify demethylation of H3K9me3 substrate to both H3K9me2 (black circles) and H3K9me1 (red circles) peptide products with time at 5 °C after rapid chemical quench experiments were conducted. In order to determine rate constants the data obtained from the sum of demethylation levels (%H3K9me2 + %H3K9me1) were fitted with the equation f = y0 + a*(1-exp(-b*x)), using SigmaPlot^{TM}. Overall demethylation of H3K9me3 to H3K9me2 and H3K9me1 occurs at 0.014 ± 0.001 s^{-1}. H3K9me1 peptide is detected from ≈50 s.

Figure S7



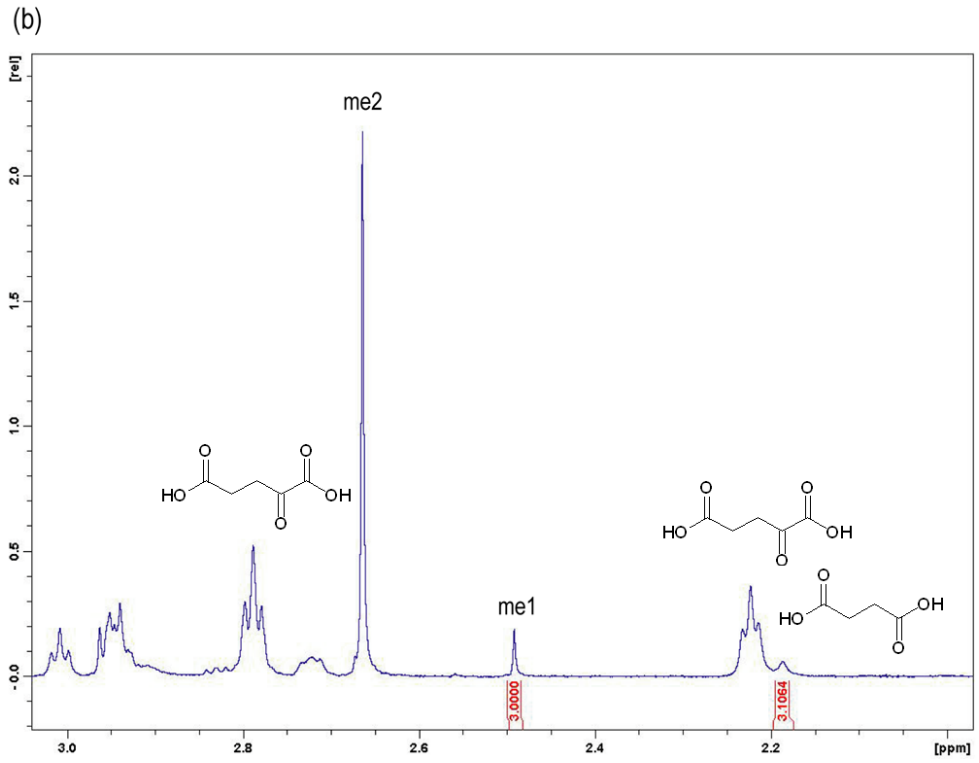


Figure S7 CPMG-edited ^{1}H NMR spectrum of KDM4E-catalysed demethylation of (a) H3K9me3 and (b) H3K9me2

The CPMG-edited ^{1}H NMR (700 MHz) spectrum shows the first acquisition 307 s (**a**) or 300 s (**b**) after mixing the assay components. Peak integration of the ^{1}H NMR spectra reveals that near-equivalent amounts of me2 (**a**) or me1 (**b**) and succinate were produced, indicating minimal uncoupled turnover by KDM4E under conditions similar to those used in the pre-steady state experiments.

NMR analyses employed a Bruker AVIII 700 spectrometer equipped with an inverse TCI cryoprobe optimised for ^{1}H observation and installed with TOPSPIN 2 software. Chemical shifts are reported in ppm relative to D_2O (δ_H = 4.72 ppm). The spectrometer conditions were optimised by using a control sample with all the components of the reaction except KDM4E. Solutions of 0.5 mM KDM4E, 5 mM 2OG, 0.45 mM NH_4FeSO_4 and 2.5 mM of the corresponding H3K9me3/H3K9me2 peptides were prepared in deuterated ammonium formate and mixed at 5 °C. The enzyme was added to the assay mixture (75 µL) directly prior to transfer to a 2 mm diameter NMR tube. The NMR spectroscopy tube was centrifuged for a few seconds in a hand centrifuge. The sample was introduced to the magnet and acquisition of the first spectrum began after 300 s (total time lapse between adding the enzyme and the start of data acquisition). The samples were monitored using a PROJECT-CPMG (Carr–Purcell Meiboom–Gill) sequence [7], composed of 6 cycles with τ = 4 ms. The total CPMG duration was 48 ms. The deuterium signal was used as an internal lock signal and the HDO signal was reduced by presaturation during a 2 s recovery delay. The time course data were collected over a period of 30 min, at 50 s intervals by using an automated routine. The sample temperature was maintained at 5 °C throughout the run.

Figure S8

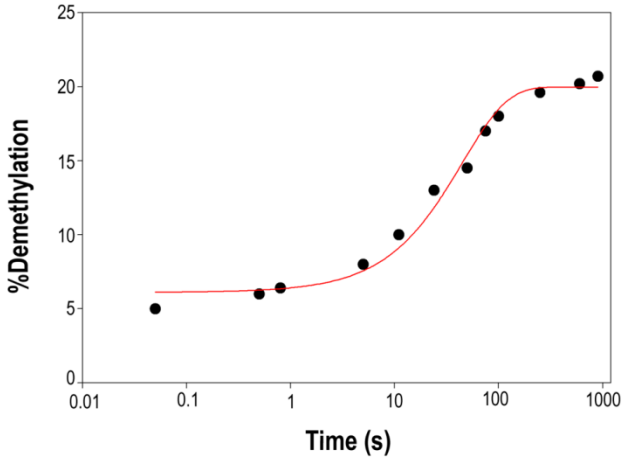


Figure S8 Rapid chemical quench experiments in the presence of H3K9me2 8mer peptide

MALDI-TOF MS was used to quantify demethylation of H3K9me2 with time at 5 ℃ after rapid chemical quench experiments (black circles). Data were fitted by the equation f = y0 + a*(1-exp(-b*x)), (red line). Demethylation apparent first order rate constant of H3K9me2 substrate to H3K9me1 product occurs at 0.022 ± 0.003 s^{-1}.

Supplementary Information References

1 Hillringhaus, L., Yue, W. W., Rose, N. R., Ng, S. S., Gileadi, C., Loenarz, C., Bello, S. H., Bray, J. E., Schofield, C. J., Oppermann, U. (2011) Structural and Evolutionary Basis for the Dual Substrate Selectivity of Human KDM4 Histone Demethylase Family. J. Biol. Chem. **286** (48), 41616-41625

2 Ng, S. S., Kavanagh, K. L., McDonough, M. A., Butler, D., Pilka, E. S., Lienard, B. M., Bray, J. E., Savitsky, P., Gileadi, O., von Delft, F., Rose, N. R., Offer, J., Scheinost, J. C., Borowski, T., Sundstrom, M., Schofield, C. J., Oppermann, U. (2007) Crystal structures of histone demethylase JMJD2A reveal basis for substrate specificity. Nature **448** (7149), 87-91

3 Clifton, I. J., McDonough, M. A., Ehrismann, D., Kershaw, N. J., Granatino, N., Schofield, C. J. (2006) Structural studies on 2-oxoglutarate oxygenases and related double-stranded beta helix fold proteins. J. Inorg. Biochem. **100** (4), 644-669

4 Rose, N. R. (2009) Structural, Mechanistic and Inhibition Studies on the Histone Lysine Demethylases. In. *Chemistry Department*, University of Oxford

5 Schofield, C. J., Zhang, Z. Z. (1999) Structural and mechanistic studies on 2-oxoglutarate-dependent oxygenases and related enzymes. Curr. Opin. Struct. Biol. **9** (6), 722-731

6 Hausinger, R. P. (2004) Fe(II)/α-Ketoglutarate-Dependent Hydroxylases and Related Enzymes. *Crit. Rev. Biochem. Mol. 39* (1), 21-68.

7 Aguilar, J. A., Nilsson, M., Bodenhausen, G., Morris, G. A. (2012) Spin echo NMR spectra without J modulation. Chem. Commun. **48**, 811-813