Published in final edited form as:

Nat Chem Biol. 2012 December ; 8(12): 960–962. doi:10.1038/nchembio.1093.

Oxygenase Catalysed Ribosome Hydroxylation Occurs in Prokaryotes and Humans

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The finding that oxygenase-catalysed protein-hydroxylation regulates animal transcription raises questions as to whether the translation machinery and prokaryotic proteins are analogously modified. *E.coli* ycfD is a growth regulating 2-oxoglutarate oxygenase catalysing arginyl hydroxylation of ribosomal protein Rpl16. Human ycfD homologues, MINA53 and NO66, are also linked to growth and catalyse histidyl hydroxylation of Rpl27a and Rpl8, respectively. The work reveals new therapeutic possibilities *via* oxygenase inhibition and by targeting modified over unmodified ribosomes.

Following from the identification of roles for Pro-/Asp-hydroxylation of the hypoxia inducible transcription factor (HIF) in animals¹, related oxygenases have been shown to

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Author Contributions

W.G. led *in vitro* assays and identified substrates by peptide screening. A.W. performed *in vivo* ycfD experiments and proteomics. C.H. discovered ycfD Arg hydroxylase activity. T.F. and A.Z. performed *in vivo* NO66 and MINA53 experiments, respectively. R.S. analysed ribosome structures and performed and analysed all whole protein mass spectrometry with advice from Y. G. and C. V. R.; N.G. and M.E.C. performed MINA53 proteomics. C.L. undertook evolutionary analysis. N.L. performed amino acid analyses. T.D.W.C. and R.B.H. undertook NMR. L.G., M.M.M., D.C.T., J.S.M., Y.G., M.M., and B.M.K. performed or supervised mass analyses. M.J. synthesized hydroxylated standards. P.J.R. and G.M.P. contributed to MINA53/NO66 and ycfD project design, respectively. M.L.C. and C.J.S. designed and supervised the study, analyzed data and wrote the manuscript with assistance of other authors.

Competing Financial Interests

The authors declare no competing financial interests.

catalyse demethylation of *N*- ϵ -methyl Lys histone residues ('JmjC' demethylases)² and lysyl 5-hydroxylation of splicing-related proteins (JMJD6)³. These modifications are catalysed by 2-oxoglutarate (2OG) oxygenases^{2,4,5}. We report the discovery of a new set of 2OG oxygenases - the ribosomal oxygenases (ROXs).

To investigate whether oxygenase-catalysed prokaryotic protein-hydroxylation occurs we employed structure-informed bioinformatic analyses. In *E. coli* we identified 4 candidate oxygenases, in addition to the assigned AlkB, and taurine dioxygenase (TauD)⁴: csiD (GI: 90111476), ycfD (GI: 90111217), ybiU (GI: 16128789), ybiX (GI: 16130149), as identified in another study⁶. We focused on ycfD because it is related to human oxygenases, including hydroxylases (FIH, JMJD6)^{1,3} and 2 enzymes assigned as histone demethylases (Myc-induced nuclear antigen (MINA53) and NO66)^{7,8}.

Following the demonstration that ycfD catalyses 2OG turnover (Supplementary Fig. S1a), we screened known human 2OG hydroxylase (hypoxia inducible factor prolyl-hydroxylase domain (PHD), factor inhibiting HIF (FIH), JMJD6) peptide substrates and *N*- ϵ -methyl-lysine demethylase histone (H3 K4/K9/K27/36me_n) as potential substrates using mass spectrometry (MS). None displayed evidence of the +16/-14 Da shifts characteristic of modification. Screening of other peptides led to the finding that one (RLLPAVSEATIRRL) gives a +16Da shift (Supplementary Fig. S1b). Further MS-analyses implied hydroxylation at the Arg at the -3 relative to the C-terminus (Supplementary Fig. S1c), as supported by 'alanine scanning' (Supplementary Fig. S2a). Of the tested peptides, 6-residues were required for efficient hydroxylation (Supplementary Fig. S2b). However, hydroxylation was ablated when the C-terminus of this peptide was extended to more closely resemble a protein (Supplementary Fig. S2b). NMR studies with an 8-mer peptide (AVSEATIR) revealed C-3 hydroxylation (Supplementary Fig. S2c,d). Hydroxylation occurs with incorporation of >90% ¹⁸O from ¹⁸O₂, consistent with studies on human 2OG hydroxylases⁴ (Supplementary Fig. S2e).

We then focused on identifying endogenous ycfD substrates using co-immunoprecipitation-MS analyses; we found ribosomal proteins interacting with ycfD, including Rpl16 from the large subunit (Supplementary Fig. S3a,b). Analysis of endogenous Rpl16 after immunoprecipitation with tagged ycfD revealed Arg81 to be >95% hydroxylated (Fig. 1a, b, and Supplementary Fig. S4a-c). Although ~10% of Rpl16 residues are Arg (14/136), Arg81 is the only residue observed to be hydroxylated (Supplementary Fig. S5). No detectable (<5%) Arg81 hydroxylation was observed in a ycfD gene-deleted strain (JW1114)⁹ (Fig. 1b and Supplementary Fig. S4a, b). When ycfD was ectopically expressed in JW1114, hydroxylation of Arg81 was restored. Expression of a catalytically inactive ycfD variant did not produce hydroxylation (Fig. 1b and Supplementary Fig. S4a,b). The tryptic digest MS analyses (Fig. 1b and Supplementary Fig. S4b) were supported by Rpl16 whole protein studies (Supplementary Fig. S4a). The results imply that the only differential modification between wildtype and JW1114 strains, among 52/55 ribosomal proteins (3 not observed) was Rpl16 hydroxylation (Supplementary Fig. S5). *E. coli* grown in anoxia display near complete suppression of Rpl16 hydroxylation (<5%) (Supplementary Fig. S4d).

NMR analysis of a hydroxylated peptide (Fig. 1c and Supplementary Fig. S6a) demonstrates that, as with the initially identified substrate, hydroxylation occurs at the C-3 position (Supplementary Fig. S6b). Amino acid analyses demonstrate the stereochemistry of the hydroxylated Arg residue to be *2S,3R* (Supplementary Fig. S6c), contrasting with that of VioC-catalysed Arg hydroxylation where the (*2S,3S*)-stereoisomer is produced¹⁰.

Since growth is linked to translation¹¹, we compared wildtype and JW1114 growth. Whereas under standard conditions both strains grow similarly, under low nutrient conditions reduced growth of JW1114 was observed (Supplementary Fig. 7a): This correlates with a 3–4 fold lower bulk protein translation rate in JW1114 (Supplementary Fig. S7b). The observation that *ycfD* deletion restricts growth is interesting because reduced expression of its human homologues, NO66 and MINA53 (Supplementary Figs. S8 and S9), also suppresses cell proliferation^{12–15}. Consistent with roles in cell proliferation, MINA53/NO66 interact with the Myc oncogene pathway^{12,14}, are highly expressed in several cancers, and correlate with poor prognosis^{12,13,15–19}.

We considered whether the finding that *ycfD* is a ROX could be used to shed light on NO66/MINA53 substrates. We first demonstrated 2OG oxygenase activity for NO66 and MINA53 (Supplementary Fig. S10a,b), then carried out substrate searches. Proteomic studies using tagged NO66 identified specific ribosomal interactors in HEK293T cells (Supplementary Fig. S10c), consistent with nucleolar localisation of NO66/MINA53 (Supplementary Fig. S11)^{20,21}. We made 19–20 mer peptides corresponding to overlapping sequences of these proteins (~200 peptides) and incubated them with NO66. This screen identified an Rpl8 fragment giving +16 and -2Da (desaturation) mass shifts (Fig. 2a). No such shifts were observed for other tested peptides. MSMS assays suggest that His216 of Rpl8 is oxidised *in vitro*, with +16 and -2Da shifts being observed (Fig. 2b and Supplementary Fig. S12a). The site of Rpl8 hydroxylation was validated by H216A substitution (Supplementary Fig. S12b). NMR revealed hydroxylation at C-3 of His216, without detectable desaturation (Fig. 2c and Supplementary Fig. S12c). Thus, the -2Da shifts likely occurs during MS. Amino acid analyses assigned the product stereochemistry as *2S,3S* (assuming maintenance of *2S*-stereochemistry) (Supplementary Fig. S12d). MSMS of endogenous Rpl8 revealed His216 hydroxylation in tumour cell lines (Supplementary Fig. S13a). LC-MS quantitation indicated abundant hydroxylation in HeLa cervical carcinoma cells (Fig. 3a), A549 lung adenocarcinoma cells (Supplementary Fig. S13b) and in normal mouse liver (>95%) (Supplementary Fig. S13c,d). ShRNA-mediated knockdown indicated that Rpl8 His216 hydroxylation is NO66-dependent (Fig. 3a and Supplementary Fig. S13b). Furthermore, similar to *ycfD*/Rpl16, cellular Rpl8 His216 hydroxylation is impaired by hypoxia (Supplementary Fig. S13e).

Peptide screening of MINA53-interactors (Supplementary Fig. S10d) defined an Rpl27a fragment giving +16 and -2Da shifts (Supplementary Fig. S14a). Rpl27a His39 was identified as the site of the MINA53 catalysed modification (Fig. 2b and Supplementary Fig. S14) with, as for NO66, *2S,3S* 3-hydroxy-His production (Supplementary Fig. S12c). The site of Rpl27a hydroxylation was validated by H39A substitution (Supplementary Fig. S14e). NMR of the product demonstrated hydroxylation at C-3 of His39 Rpl27a (Supplementary Fig. S14f). MS analyses of endogenous cellular Rpl27a confirmed the

presence of hydroxylated His39 in human embryonic kidney HEK293T (Supplementary Fig. S15a). Whole protein MS quantitation indicated that Rpl27a was >90% hydroxylated in A549 lung adenocarcinoma cells (Fig. 3b), HEK293T cells (Supplementary Fig. S15b), several mouse tissues (Supplementary Fig. S15c-e), normal human placenta and tumour samples from a human Hodgkin's lymphoma (Supplementary Fig. S15f). ShRNA-mediated knockdown indicated that Rpl27a hydroxylation is MINA53-dependent (Fig. 3b and Supplementary Fig. S15b).

Identified ROX substrates have basic side chains as do JmjC demethylase substrates, JMJD6 and, in some cases, FIH, suggesting related substrate selectivities, consistent with an evolutionary relationship (Supplementary Fig. S8 and S9). However, we did not detect cross-reactivity between the human ROXs (Supplementary Fig. S16a,b); Peptide studies infer that HXHR and NHXH are preferred motifs for MINA53 and NO66 hydroxylation, respectively (Supplementary Fig. S16c,d). NO66 interacts with endogenous Rpl8 *in vivo*, but not Rpl27a (Fig. 3c). Furthermore, MINA53 co-precipitates Rpl27a, but not Rpl8 (Fig. 3d); Consistent with this, endogenous human Rpl8 and Rpl27a hydroxylation are unaffected by MINA53 or NO66 ShRNA, respectively (Supplementary Fig. S16e,f). Because some oxygenases hydroxylate multiple proteins^{4,22}, we cannot exclude the possibility that the ROXs have other substrates. Indeed, NO66 and MINA53 are reported histone demethylases^{7,8}; However, our current evidence suggests that the ROXs are selective within our set of identified substrates; we have not observed MINA53/NO66-catalysed demethylation of histone peptides under conditions where JmjC demethylases are active.

Overall, our results reveal that ROXs are likely conserved in animals and present in other life forms including pathogenic prokaryotes (Supplementary Fig. S9). Because of their links to proliferation^{12–15}, ROXs are targets for cancer and inflammatory disease; the identification of their substrates will enable inhibitor discovery. The molecular mechanism by which ROX hydroxylation is linked to cell growth can now be investigated; Ribosome hydroxylation sites are close to important regions, e.g. Rpl16 (Arg81) and Rpl8 (His216) are adjacent to the peptidyl transferase centre (Supplementary Fig. S18). Given the roles of 2OG oxygenases in chromatin modification², splicing³, transcriptional regulation⁵, and, of interest in relation to ROXs, in t-RNAs hydroxylation^{6,23,6,24} the possibility of translational regulation by oxygenases is attractive. The findings that the activities of some oxygenases involved in transcription are limited by elevated levels of tricarboxylic acid intermediates/2-hydroxyglutarate in tumors²⁵ raises the possibility of analogous interfaces between altered metabolism and translation *via* ROX inhibition. Importantly, ROX activity is limited by hypoxia; hence molecules selectively targeting unhydroxylated ribosomes are of interest for targeting hypoxic cells in tumours. The knowledge that resistance to antibiotics that target ribosomes is achieved by point mutations suggests such an approach is viable.

Supplementary Material

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Acknowledgements

We thank the Biotechnology and Biological Research Council (BB/G014124/1 to P.J.R. and C.J.S), Wellcome Trust (091857/Z/10/Z to P.J.R. and C.J.S), Cancer Research UK for studentships (to R.S. and A.Z.), the Slovenian Academy of Sciences and Arts (R.S.), and the OAK Foundation (M.L.C) for funding. We thank Christian Ducho for the kind gift of standards of hydroxyarginine stereoisomers and Ulrike Ackermann and Claudia Tessmer for technical assistance in antibody preparation.

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Figure 1.

E. coli ycfD is an Arg hydroxylase of the 50S ribosomal protein Rpl16 required for growth. (a) Reaction scheme for Arg hydroxylation (red). (b) Endogenous Rpl16 Arg81 hydroxylation requires ycfD. Rpl16 was purified from either wildtype (BW25113) or ycfD-null (JW1114) *E. coli* reconstituted with wild-type GFP-ycfD or inactive GFP-ycfD (H125A/D127A, Supplementary Fig. S8) and Arg81 hydroxylation measured by LC-MS. Data represent mean values $\pm$ s.d. (c) ycfD hydroxylates a 20mer Arg81 peptide *in vitro*. ycfD was incubated with Rpl16 peptide plus cosubstrate prior to LC-MS. In the absence of ycfD the triply-charged ($[M+H]^{3+}$) Rpl16 peptide showed the predicted mass (m/z 717.8). Following ycfD incubation the triply-charged Rpl16 peptide mass is 723.2 (+16Da $[M+H]^{3+}$), consistent with hydroxylation.

Figure 2.

NO66 catalyses β -carbon His hydroxylation of 60S ribosomal protein Rpl8. **(a)** NO66 hydroxylates a peptide derived from Rpl8 (CNPVEHPFGGGNHQHIGKPST). Recombinant NO66 was incubated with the above Rpl8 peptide prior to chymotrypsinolysis and LC-MS. Without NO66 the doubly-charged ($[M+H]^{2+}$) chymotryptic Rpl8 peptide has the predicted mass (m/z 644.8). Following incubation the doubly-charged chymotryptic peptide displayed a mass of m/z 652.8 ($+16\text{Da } [M+H]^{2+}$) consistent with hydroxylation, plus a mass of m/z 643.8 ($-2\text{Da } [M+H]^{2+}$), consistent with hydroxylation followed by dehydration. **(b)** Reaction

scheme showing His hydroxylation (red). (c) Partial 2D ^1H - ^{13}C HSQC spectra of Rpl8 peptide (CNPVEHPFGGGNHQHIGKPST; left panel) and hydroxylated (CNPVEHPFGGGNH(OH)QHIGKPST; right panel) Rpl8 peptide labelled with amino acid assignments for the alpha resonances. The NMR assignment is consistent with beta-hydroxylation at His. Blue labels highlight His residues. Red labels highlight alpha and beta residues of the hydroxylated His residue. The purple labelled residue (N) shows significant proton deshielding on hydroxylation as this precedes the modified His. See Supplementary Fig. 12c for the $\text{H}\alpha$ - $\text{H}\beta$ coupling correlation of the alpha and beta residues of the hydroxylated His residue in a region of the 2D ^1H - ^1H COSY experiment.

Figure 3.

Histidyl hydroxylation of endogenous human Rpl8 and Rpl27a is enzyme-dependent and specific. **(a)** Endogenous Rpl8 hydroxylation is NO66-dependent. HeLa cells expressing inducible control or NO66 ShRNA were treated with doxycycline prior to immunoblot (left). Endogenous Rpl8 was purified from control (upper right) or NO66 knockdown (lower right) cells as described in methods prior to trypsinolysis and LC-MS quantitation of His216 hydroxylation. **(b)** Endogenous Rpl27a hydroxylation is MINA53-dependent. A549 cells expressing control or MINA53 ShRNA were immunoblotted as shown (left). Endogenous Rpl27a was purified from control (upper right) of MINA53 knockdown (lower right) cells prior to whole protein MS quantitation of hydroxylation. **(c)** NO66 binds Rpl8, but not

Rpl27a. Complexes were immunoprecipitated from HA-tagged NO66 or empty vector control (EV) HEK293T cells prior to immunoblot. The interaction was specific as Rpl8 was not detected in control pulldowns (anti-V5). **(d)** MINA53 binds Rpl27a, but not Rpl8. Complexes were immunoprecipitated from doxycycline-treated FLAG-tagged MINA53 or empty vector control (EV) U2OS inducible cells prior to immunoblot. The interaction was specific as Rpl27a was not detected in control pulldowns (anti-V5). Uncropped immunoblots from panels **c** and **d** are presented in Supplementary Fig. S17).