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REVIEW ARTICLE

The oxygenase Jmjd6 – a case study in conflicting assignments

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The Jumonji domain-containing protein 6 (Jmjd6) is a member of the superfamily of non-haem iron(II) and 2-oxoglutarate (2OG)-dependent oxygenases; it plays an important developmental role in higher animals. Jmjd6 was initially assigned a role as the phosphatidylserine receptor responsible for engulfment of apoptotic cells but this now seems unlikely. Jmjd6 has been shown to be a nuclear localized protein with a JmjC domain comprising a distorted double-stranded β-helical structure characteristic of the 2OG-dependent oxygenases. Jmjd6 was subsequently assigned a role in catalysing *N*-methyl-arginine residue demethylation on the N-terminus of the human histones H3 and H4; however, this function is also subject to conflicting reports. Jmjd6 does catalyse 2OG-dependent C-5 hydroxylation of lysine residues in mRNA splicing-regulatory proteins and histones; there is also accumulating evidence that Jmjd6 plays a role in splicing (potentially in an iron- and oxygen-dependent manner) as well as in other processes regulating gene expression, including transcriptional pause release. Moreover, a link with tumour progression has been suggested. In the present review we look at biochemical, structural and cellular work on Jmjd6, highlighting areas of controversy and consensus.

Key words: alternative splicing, arginine-demethylase, epigenetic regulation, Fe(II) and 2-oxoglutarate dependent oxygenases, hydroxylysine, JmjC, Jmjd6, jumonji, lysine-hydroxylase, SR-proteins.

INTRODUCTION

Jmjd6 is a ferrous iron [Fe(II)] and 2-oxoglutarate (2OG)-dependent enzyme, and thus a member of the largest identified family of non-haem oxygenases, examples of which are ubiquitously distributed in both eukaryotes and prokaryotes [1,2]. Mammalian 2OG oxygenases catalyse hydroxylations to give stable alcohol products and *N*-methyl demethylation reactions probably occurring via initial hydroxylation, referred to hereafter as hydroxylase and demethylase reactions (Figure 1) [2].

Jmjd6 has a JmjC (Jumonji C) domain (residues Pro^{141} to Gln^{286} of human Jmjd6), which is conserved in proteins from eukaryotes to bacteria [3]. With this domain it is a member of a widely distributed metalloenzyme family characterized by the presence of a cupin fold [4]. In addition to its JmjC domain, human Jmjd6 contains other motifs conserved in predicted Jmjd6 proteins in animals from mammals to cnidarians [5]. These include three apparent nuclear localization signals (NLSs) (Pro^{141} to Lys^{145}, Lys^{167} to Pro^{171} and Arg^{373} to Arg^{378} in human Jmjd6), an AT hook (Lys^{300} to Ser^{309}) [4], a putative sumoylation site (Leu^{316} to Glu^{319}) [6] and a polyserine (polyS) domain.

The common core protein structural fold of all 2OG oxygenases comprises a distorted double-stranded β-helix (DSBH or cupin) fold surrounded by characteristic secondary structure elements [7]. This DSBH fold forms a barrel-type structure with two β-sheets, which support highly, 'but not completely', conserved binding motifs for Fe(II) and, to a lesser extent, 2OG [8,9]. The metal is most commonly bound by the side chain of three residues, which form an HXD/E(X)$_n$H motif. In reported 2OG oxygenase crystal structures, the 2OG C-5 carboxylate is bound by a basic (Lys/Arg), and at least one alcohol residue (Figure 2) [7]. The substrates of 2OG oxygenases are known to include both macromolecules, i.e. proteins and nucleic acids, and smaller molecules, including amino acid derivatives and lipids [1]. The 2OG oxygenases were first identified as prolyl and lysyl hydroxylases involved in collagen biosynthesis [10]. Subsequently, they have been found to play many other important roles in animals including in epigenetic regulation, hypoxia sensing, fatty acid metabolism and DNA repair [11].

The catalytic mechanism for 2OG oxygenases comprises an ordered sequential process in which 2OG, substrate and finally oxygen bind to the active site, the latter containing a single Fe(II) [1]. Oxidative decarboxylation of 2OG generates an Fe(IV) oxo species. This reactive oxidizing species then reacts with the carbon–hydrogen bonds of substrates to give an alcohol as the other oxidized product [12,13]. In case of *N*-demethylation, the initial reaction on the methyl group occurs to yield a hemiaminal, which can fragment to yield the demethylated product and formaldehyde (see Figure 1) [13–15]. Some, but not all, 2OG oxygenases display catalytic flexibility in terms of the types of oxidative reaction that they catalyse, e.g. hydroxylation versus desaturation (as has been observed for plant and microbial enzymes) [1]. Some animal 2OG oxygenases, probably including Jmjd6, can accept multiple protein substrates. Perhaps the best-studied case of this is the factor inhibiting hypoxia-inducible factor (FIH), which accepts hypoxia-inducible transcription factor

Abbreviations: 2OG, 2-oxoglutarate; Brd4, bromodomain-containing protein 4; CDK9, cyclin-dependent kinase 9; DSBH, distorted double-stranded β-helix or cupin; ER, oestrogen receptor; FIH, factor inhibiting hypoxia-inducible factor; HA, haemagglutinin; HEK, human embryonic kidney; HIF, hypoxia-inducible transcription factor; KDM, lysine demethylase; LH, lysyl hydroxylase; NLS, nuclear localization signal; P-TEFb, positive transcription elongation factor b; PLM, posterior lateral mechanosensory; Pol II, RNA polymerase II; PSR, phosphatidylserine receptor; snRNA, small nuclear RNA; TM, transmembrane; VEGF, vascular endothelial growth factor.

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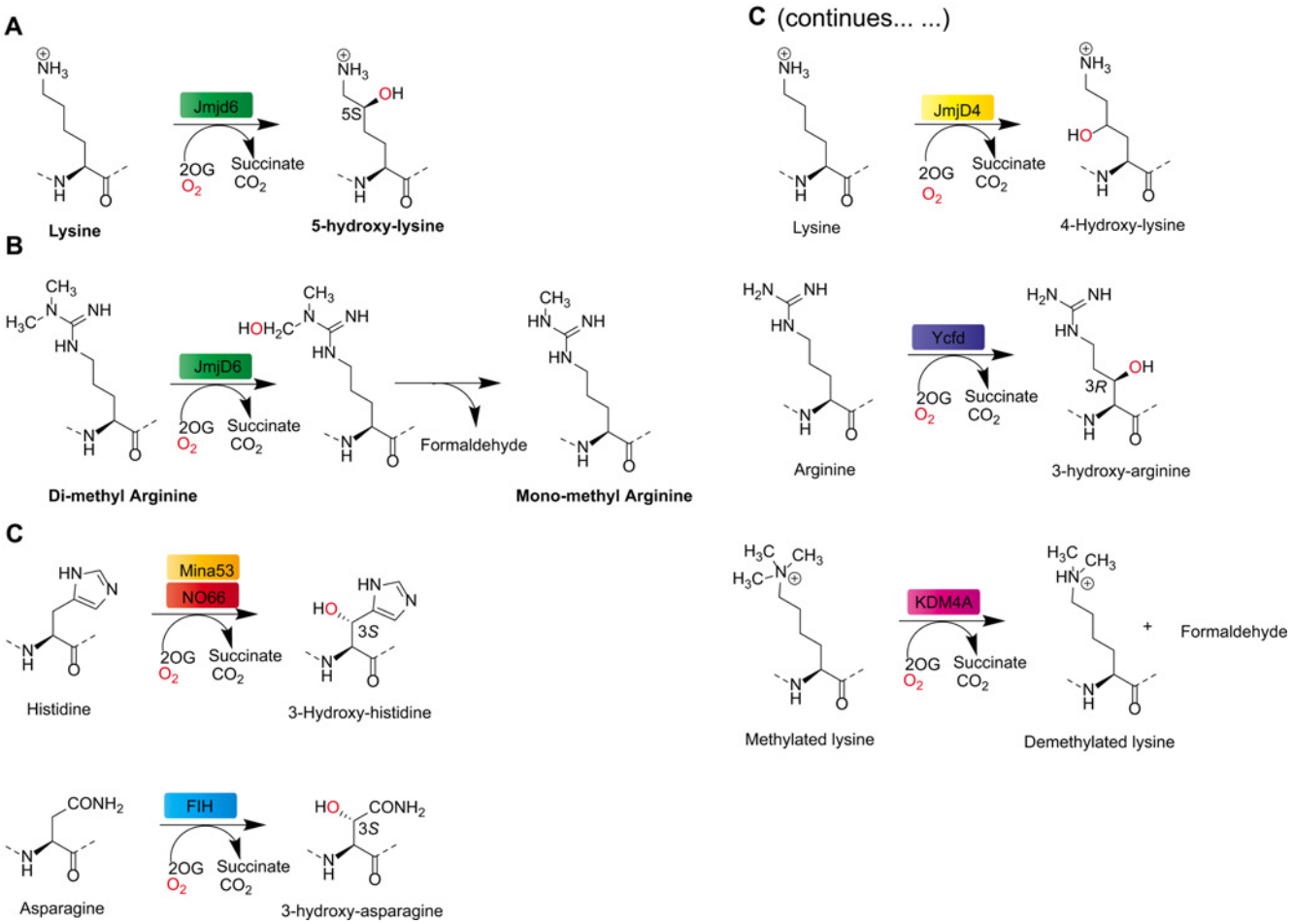

Figure 1 Reactions catalysed by Jmjd6 and related 2OG-dependent oxygenases

(**A**) Lysyl 5-hydroxylation reaction catalysed by Jmjd6, (**B**) proposed demethylation reaction of dimethyl arginine catalysed by Jmjd6 (exemplified for demethylation of asymmetric *N*-dimethylated arginine) and (**C**) respective hydroxylation and demethylation reactions catalysed by other 2OG-dependent oxygenases.

α subunit protein (HIF-1α, HIF-2α) as a substrate, resulting in C-3 hydroxylation of the asparagine residue [16]. FIH also accepts multiple ankyrin repeat domain proteins as substrates, and probably interacts with others that are not substrates [17,18]. In addition FIH can hydroxylate residues other than asparagines, including histidine and aspartate residues, at least in the ankyrin repeat-domain protein context [18,19]. To what extent such relaxed substrate selectivity is true for Jmjd6 is controversial. In the present review we look at the evidence for the different suggested biochemical and biological roles of Jmjd6.

THE DAWN OF WORK ON JMJD6: MISASSIGNMENT AS THE PHOSPHATIDYLSERINE RECEPTOR

In 2000, Jmjd6 was misassigned as a phosphatidylserine receptor (PSR or PTDSR) in the plasma membrane of phagocytes (Figure 3) [20], most probably as a result of a lack of antibody selectivity. In 2004, Cikala et al. [5] identified the DSBH fold as being present in Jmjd6 (PSR) together with potential NLSs. Jmjd6 was suggested to be a 2OG-dependent dioxygenase localizing to the nucleus, based on comparison of the predicted Jmjd6 structure with the 2OG-dependent HIF-α asparaginyl hydroxylase FIH [21]. Due to this prediction, the PSR was later renamed Jmjd6.

Continuing biological interest in the function of Jmjd6 came from observations with transgenic mice carrying Jmjd6 null mutations [22–24]. Three independent Jmjd6 mouse lines have been described, in each of which the Jmjd6 knockout mice were observed to die around birth. They showed severe developmental defects including malformations of the brain, lack of one or both eyes, insufficient lumen formation in the lungs, impairment of erythroid and thymocyte differentiation, and defects in development of the kidneys and intestine. Death of the knockout mice was attributed to heart defects, especially impaired heart muscle differentiation [22–24]. Similar phenotypes were subsequently shown to occur in zebrafish after treatment with 'morpholinos' targeted against Jmjd6 [25]. In contrast, Jmjd6 loss-of-function experiments in *Caenorhabditis elegans* and *Drosophila melanogaster* did not display lethal defects [26,27]. In *C. elegans Jmjd6* gene ablation was reported to lead to a delay in the engulfment of apoptotic cells and, more recently, to a defect in regenerative axon fusion [27,28]. *Drosophila* mutants ubiquitously over-expressing Jmjd6 (dPSR) exhibited a 'rotated male genitalia' phenotype. This was similar to mutants with loss of *hid* and *dronc* genes, both encoding regulators of developmental apoptosis in *Drosophilamelanogaster*. Furthermore, the Hid- and Grim-induced 'small rough eye' phenotype, reflecting increased cell death in the *Drosophila* eye, was reversed by Jmjd6. On the

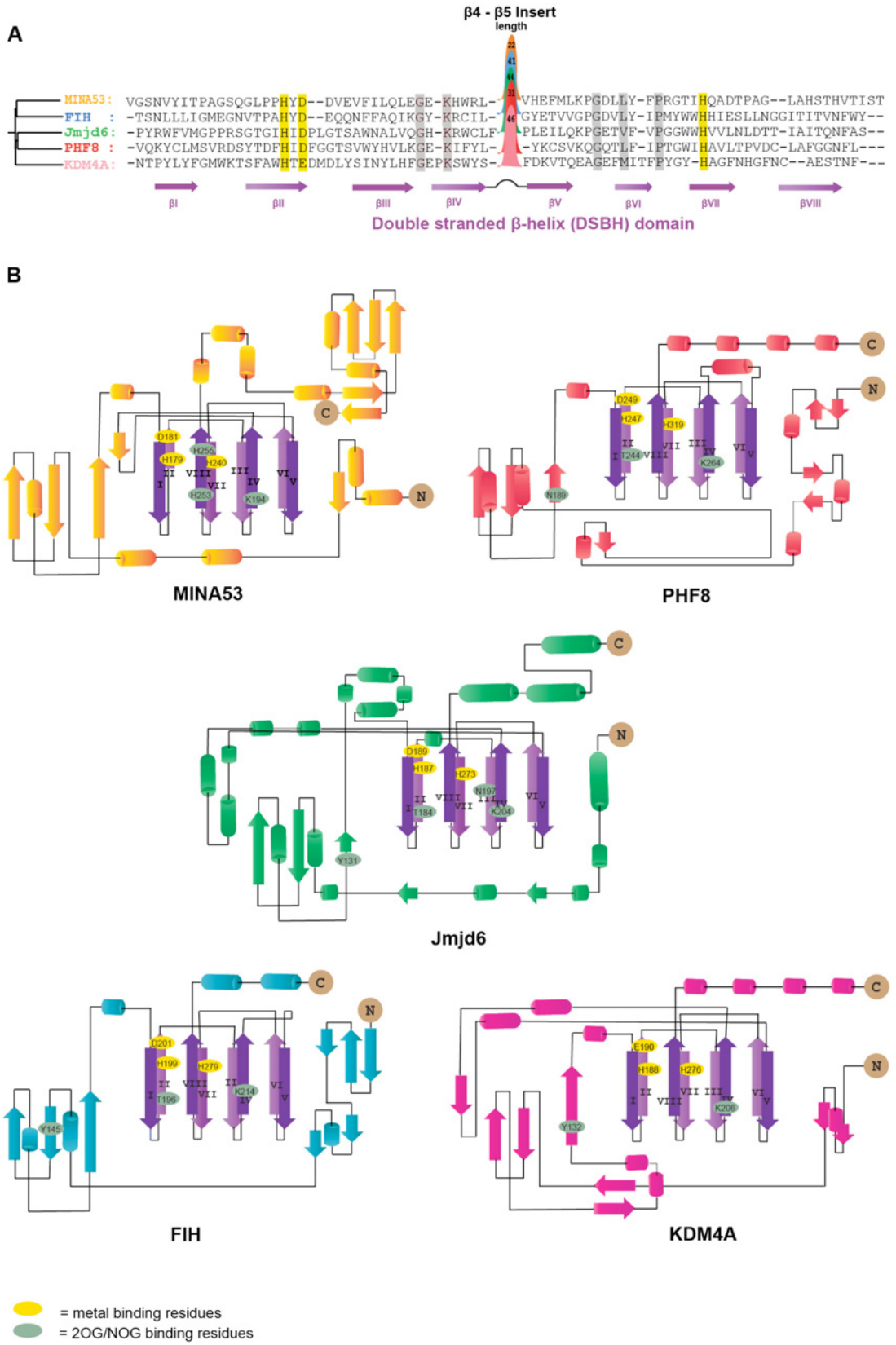

Figure 2 Comparison of the Jmjd6 fold with related JmjC enzymes

(**A**) Clustal W [73] alignment of the core DSBH domain of human Jmjd6 with a few other human JmjC proteins: two hydroxylases, Mina53 (MYC-induced nuclear protein 1) and FIH, and two demethylases KDM4A (JmjC domain-containing protein 2A) and PHF8 (PHD finger protein 8). (**B**) Comparison of the topologies of the same proteins as observed in the reported crystal structures. Helices are shown as cylinders and sheets as arrows. NOG, *N*-oxalyl-glycine, an analogue of 2OG.

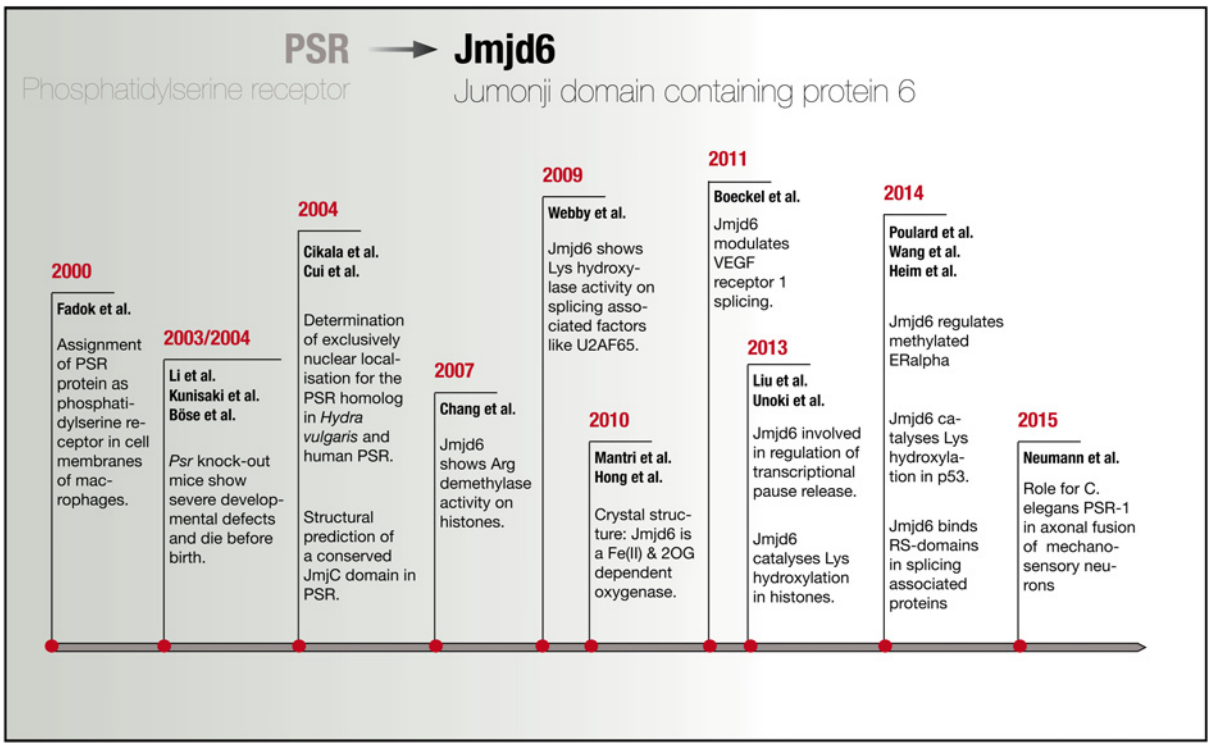

Figure 3 Timeline of functional assignments for Jmjd6

other hand, loss of Jmjd6 enhanced the phenotype. Jmjd6 was thus suggested to suppress apoptosis in several fly tissues, probably acting through c-Jun N-terminal kinase (JNK) activity [26].

Most of this work has been previously reviewed [29]. However, a clear explanation for the phenotypes observed in vertebrates is still not available. We now focus on advances in Jmjd6 research in the 'post-PSR' era.

JMJD6–PROTEIN INTERACTION STUDIES

Initial attempts to investigate functions of Jmjd6 in cells involved immunoprecipitation/affinity purification approaches to identify Jmjd6-interacting proteins [30]. The first approach used identification based on a tandem-affinity approach coupled with MS; this study led to the identification of approximately 40 potential Jmjd6-interacting proteins in human embryonic kidney (HEK) 293T cells [30]. Notably, 22 of these proteins were known to relate to mRNA processing and splicing, including the U2 small nuclear ribonucleoprotein (snRNP) auxiliary factor 35 and 65-kDa subunits (U2AF35 and U2AF65). Additional proteins that potentially interact with Jmjd6 include three DEAD-box (protein family sharing the tetrapeptide, Asp–Glu–Ala–Asp, abbreviated as DEAD from the one-letter notation) helicases and three DNA-binding proteins, including bromodomain-containing protein 4 (Brd4) [30]. Similar sets of Jmjd6-binding proteins have been identified in co-immunoprecipitation experiments of haemagglutinin (HA)-tagged Jmjd6 from HeLa cell lysates, in more recent green fluorescent protein (GFP) pull-down experiments, and by immunoprecipitation of endogenous Jmjd6 [31,32]. Several of these Jmjd6 target proteins have been analysed for Jmjd6-binding regions; it was found that Jmjd6 bound to the Arg/Ser-rich (RS) domains of U2AF65, SRSF11, Luc7-like protein 3 (Luc7L3) and acinus protein [31]. The SR proteins, which are characterized by the presence of RS domains, are RNA-binding proteins involved in splicing regulation [33]. SRSF11 is one of the twelve 'core' SR proteins, whereas U2AF65, Luc7-like protein 3 and acinus are SR-like proteins [34]. Notably, the Jmjd6–SR protein interactions seemed to be RS domain selective, because the RS domain of one 'core' SR protein, SRSF1, did not bind to Jmjd6 [31].

Further studies have demonstrated Jmjd6 interacts with proteins unrelated to SR proteins. As discussed later, interaction of Jmjd6 with histone tails, including H3 (residues 1–20) and H4 was shown by *in vitro* pull-down assays [35]. Wang et al. [36] reported that Jmjd6 can interact with the tumour-suppressor protein p53 in human colon carcinoma HCT116 cells. The C-terminal region of p53 (residues 290–393) was necessary and sufficient for its interaction with Jmjd6. This region contains a stretch of basic amino acids and two SR dipeptides (**SR**AHSSHLKSKKGQST**SR**). Furthermore, recent work has confirmed the interaction of Jmjd6 with Brd4 in HEK 293T cells and mapped the binding region to residues 471–730 of Brd4 [37]. Finally, work by Poulard et al. [38] indicates that Jmjd6 interacts with the hinge domain region of the oestrogen receptor ERα. Although not all of these interactions have been fully validated as being of biological relevance, they suggest that Jmjd6 may have a broad substrate specificity.

JMJD6: ENZYMATIC ACTIVITY

In 2007, Chang et al. [39] were the first to experimentally demonstrate 2OG oxygenase activity for Jmjd6. They proposed a role for Jmjd6 in epigenetic regulation by a Jmjd6-catalysed demethylation of *N*-dimethylated (both symmetrical and asymmetrical) histone H3 ($H3Arg^2Me_2$) and *N*-dimethylated histone H4 ($H4Arg^3Me_2$). The evidence for *N*-dimethylated arginine residue demethylation was provided by using a reportedly methylation-specific antibody (for the monomethylated state of

Arg^3 of histone H4) after incubation of bulk histones with or without recombinant Jmjd6 and also after over-expression of V5-tagged Jmjd6 in HeLa cells. The demethylation reaction catalysed by Jmjd6 was reported to be dependent on and stimulated by the presence of Fe(II), 2OG and ascorbate. An iron-binding Jmjd6 mutant did not show any demethylation activity. MS analysis of arginine-methylated peptide sequences of histones H4 and H3 after incubation with recombinant Jmjd6 provided further evidence of 2OG oxygenase activity. The MS analyses were conducted after immunoprecipitation using the α-H4Arg^3Me_1 and α-H3Arg^2Me_1 antibodies; hence, contamination with proteins potentially cross-reacting with the antibody was not entirely excluded. Notably, the MS spectra of the histone H4 peptides, on Jmjd6 incubation, also show evidence of oxidation (+16 Da) on two lysine residues (H4Lys^5 and H4Lys^8), indicating the possibility of a lysine hydroxylation on peptides catalysed by Jmjd6 [39].

More recently, evidence for Jmjd6-mediated arginine *N*-demethylation of histone H4Arg^3 has come from work studying the Brd4–Jmjd6 interaction [37]. In support of the earlier study, histone H4Arg^3 demethylation (monomethylated and symmetrical/asymmetrical dimethylated form) was corroborated in the work of Liu et al. [37]; however, histone H3 arginine demethylation could not be detected. Poulard et al. [38] have reported Jmjd6-catalysed demethylation of an asymmetrically *N*-dimethylated arginine residue (Arg^{260}) in the transcription factor ERα. However, their evidence was accrued through use of antibodies reported to distinguish between methylated and unmethylated ERα peptides, and the result was not corroborated by MS or other methods [38].

The initial report on the histone arginine demethylation activity of Jmjd6 has, in the meantime, been challenged by other results [30,35,36,40] (see Figure 3). Three groups have independently reported that they do not observe *N*-methyl arginine demethylation activity for Jmjd6 when using histone H3 and H4 fragment peptides as analysed by matrix-assisted laser desorption/ionization (MALDI) MS experiments [30,35,41]. However, consistent with some of the MS evidence of Chang et al. [39], they all observed lysine hydroxylation of histone peptides instead. It is also notable that in endothelial cells, the arginine methylation status at Arg^3 in histone 4 was not observed to change on knockdown of Jmjd6 [40]. Further work in cells has indicated no obvious role for Jmjd6 in histone lysine demethylation, by comparing with lysine methylation (H3Lys^4, H3Lys^9, H3Lys^{27}, H3Lys^{36} and H4Lys^{20}) states in wild-type and Jmjd6-depleted mouse embryonic fibroblasts [42].

In 2009 LC-MS/MS analyses revealed lysine residues in splicing associated proteins to be hydroxylated by Jmjd6 in an Fe(II)- and 2OG-dependent manner [30]. The essential human splicing regulatory factor U2AF65 was shown to be hydroxylated by Jmjd6 on Lys^{15} and Lys^{276} in studies with recombinant Jmjd6 and U2AF65. These hydroxylated lysines were also observed in endogenous U2AF65 protein from HeLa cells as shown by LC-MS/MS analyses. Over-expression of Jmjd6 in HeLa cells was observed to result in an increase in the amount of lysine hydroxylation on endogenous U2AF65 [30].

NMR studies on peptides revealed that Jmjd6-catalysed hydroxylation of lysine occurs at the C-5 position [30]. Lysyl hydroxylation is a well-characterized, post-translational modification in collagens and proteins with collagenous domains. It is catalysed by Fe(II)- and 2OG-dependent oxygenases in the endoplasmic reticulum, including the lysyl hydroxylases (LHs or PLODs) 1, 2 and 3 in humans [43]. Jmjd6, in contrast, is predominantly localized to the nucleus [5,30,44]. Significantly the stereochemistry of 5-hydroxylysine differs significantly between PLOD- and Jmjd6-catalysed hydroxylation reactions. The procollagen PLODs give products with 2*S*,5*R* stereochemistry [45,46], whereas Jmjd6-catalysed lysine hydroxylation on Luc7-like 2 peptides resulted in products with 2*S*,5*S* stereochemistry [47].

The lysine hydroxylation activity of Jmjd6 was further supported by the discovery that Jmjd6 catalyses autohydroxylation of internal lysine residues, as shown by studies with isolated proteins and cellular work [48]. Recombinant Jmjd6 protein was found to undergo self-hydroxylation via either inter- or intra-molecular reactions on (at least) Lys^{111} and Lys^{167}. These modifications also occurred in the presence of U2AF65 substrate and were identified by MS analyses on endogenous Jmjd6 purified from human HeLa cells [48]. Importantly, Jmjd6 catalyses self-hydroxylation in the same mode as substrate hydroxylation, i.e. hydroxylation of lysine residues at an unactivated carbon in the lysyl side chain. In contrast, in 2OG oxygenases that have been previously reported to undergo self-(auto)catalysed oxidation [49], this was linked to reactions of reactive oxidizing species (sometime related to intermediates in catalysis) that can cause damaging modifications to the active site.

In 2013, Unoki et al. [35] confirmed the previously reported hydroxylation of lysines in histone peptides by Jmjd6 *in vitro*. Moreover, they reported studies comparing histone modifications in whole embryos at embryonic day 14.5 of wild-type and Jmjd6 knockout mice [35]. Amino acid composition analysis detected 0.097 % and 0.08 % C-5-hydroxylated lysines (of undetermined stereochemistry) among the total lysyl residues in histones H2A/H2B and 0.094 % and 0.046 % in histones H3/H4 of wild-type mice [35]. These decreased to 0.004 % and 0.011 % for H2A/H2B and to 0 for H3/H4 in Jmjd6 knockout mice. Comparing different tissues of a 6-month-old wild-type mouse, the highest levels of hydroxylated lysine in histones were found in testes, correlating with the relatively high expression levels of Jmjd6 in testes [35].

Lysine hydroxylation at Lys^{382} of the p53 protein has also been described by Wang et al. [36] in work on colon cancer. These authors reported that recombinantly expressed GST-tagged Jmjd6 protein hydroxylated recombinant p53 protein and a C-terminal p53 peptide including Lys^{382}, as shown by MS-based assays. However, the level of activity was low, and further interpretation of the results is complicated by the presence of a methionine residue in the substrate because such residues are known to undergo oxidation to give sulfoxides by non-enzymatic oxidation under the incubation conditions of some 2OG oxygenases [50]. Although Lys^{382} in endogenous p53 appeared to be hydroxylated in HCT116 cells, the relative proportion of hydroxylated versus non-hydroxylated peptide was low [36].

Overall, at this stage, the catalytic studies clearly identify Jmjd6 as a functional 2OG oxygenase. The evidence for C-5 lysine-hydroxylase activity is also very strong, most importantly arising from work with recombinant Jmjd6 employing MS, including detailed fragmentation studies, NMR and amino acid analysis, which reveal the site and selectivity of hydroxylation.

In contrast, a *direct* role for Jmjd6 in *N*-methyl-arginine demethylation presently appears less secure, although for such reactions, given the promiscuous nature of some, but by no means all, 2OG oxygenases, it cannot be ruled out. The evidence that arises from antibody-based studies in cells is inconclusive because the selectivity of the antibodies has not been fully defined. Furthermore there is a possibility that the cellular observations arise from indirect effects, e.g. caused by antibody precipitation steps before MS analysis [39]. In the more recent MS-based work on Jmjd6, the assays measured single demethylation reactions, and did not include MS fragmentation or NMR studies

[37,38]. Thus, at least, in our view although the lysine-residue hydroxylation activity of Jmjd6 should be regarded as having been validated, its direct *N*-methyl-arginine demethylation activity should be regarded as being provisional.

The C-5 hydroxylation of Jmjd6 raises the question of whether such reactions can affect further post-translational modifications on the same residues. Given the proximity of the C-5 hydroxyl group, as produced by Jmjd6, to the *N*-amino group, it is reasonable to propose that it may affect N^{ε} modifications, e.g. acetylation, methylation, ubiquitylation and sumoylation. Unoki et al. [35] reported that *N*-acetylated or *N*-monomethylated lysine residues are not hydroxylated by Jmjd6 and lysyl 5-hydroxylation in H3 and H4 histone peptides completely inhibited subsequent SMYD3 (SET and MYND domain-containing protein 3)-catalysed lysyl *N*-methylation.

Although, so far there is no evidence for this, it is possible that, as within the context of collagen biosynthesis, C-5 lysyl hydroxylation can evaluate further modifications including glycosylation and, as more recently described, sulfilimine formation, via reaction at the N^{ε}-amino group of the hydroxyl group in the lysyl residue with a sulfoxide of the methionine residue [51,52].

CRYSTALLOGRAPHIC STUDIES ON Jmjd6

Two crystallographic studies on Jmjd6 have been reported, both revealing that the DSBH that is characteristic of the 2OG oxygenases is also present in Jmjd6 [53,54] (Figure 4). These studies also revealed the triad of iron-binding residues and residues involved in 2OG binding in Jmjd6. Mantri et al. [54] used a C-terminally truncated Jmjd6 lacking amino acids 344–403, including the polyS domain for X-ray crystallography. This work resulted in the observation that Jmjd6 homodimerizes via a 'pseudo-4-helix bundle'. Topology comparison of the Jmjd6 structure with available 2OG oxygenase structures [including histone lysine demethylases (KDMs) and others] supports a hydroxylase, rather than a demethylase, activity for Jmjd6 [54]. A second crystallographic analysis by Hong et al. [53] was achieved by co-crystallization of full-length Jmjd6 complexed with an Fab antibody fragment from a Jmjd6-specific antibody. In this case Jmjd6 crystallizes as a monomer, with well-defined electron density for amino acids 1–334 and a disordered C-terminus including the polyS domain [53]. The Fab fragment binds at the C-terminal region of Jmjd6 and might therefore interfere with the Jmjd6 'pseudo-4-helix bundle'-mediated dimerization as observed by Mantri et al. [54].

An initially suggested transmembrane (TM) domain for human Jmjd6 (amino acids 266–288) [20] and the *C. elegans* Jmjd6 homologue PSR-1 (amino acids 270–293) [55] is not yet supported by any structural analysis (Figure 5). In both observed crystallographic studies, the human sequence of amino acids 266–288 forms β-strands, as part of the core DSBH fold, and includes the second histidine residue (H273) of the iron-binding motif [53,54].

The proposal that Jmjd6 is an alysine-hydroxylase is supported by comparison of the Jmjd6 structure with the substrate structures reported for JmjC hydroxylases and demethylases [7]. JmjC hydroxylase structures in complex with substrates have been reported for FIH in complex with HIF-1α and ankyrin repeat-domain substrates [17–19], and for Mina53 (MYC-induced nuclear antigen, also known as MINA) and NO66 (nucleolar protein 66) with their ribosomal protein substrates [56]. Comparison of these structures with those reported for various JmjC KDMs complexed with their *N*-methylated lysine histone fragment substrates reveals differences in the substrate-binding modes for the JmjC hydroxylases and JmjC KDMs, i.e. the *N*-methyl groups do not penetrate the active site cavity as the side chains of hydroxylated residues do [7]. However, there appear to be some unusual features in the Jmjd6 active site [53,54], hence on the basis of structural studies demethylase activity for Jmjd6 cannot be completely ruled out.

Hong et al. [53] identified a positively charged area in a groove containing a helix-turn/helix-like motif in the Jmjd6 crystal structure; they thus speculated that RNA and/or DNA may bind to Jmjd6. Electrophoretic mobility shift assays (EMSAs) with recombinant Jmjd6 protein (full-length) demonstrate that Jmjd6 can interact with ssRNA. In contrast, ssDNA, dsRNA and dsDNA were not observed to bind to Jmjd6 [53].

The binding of RNA to Jmjd6 is supported by several recently reported studies. Jmjd6 has been shown to precipitate pre-mRNA of a splice reporter [31] and mRNA of the vascular endothelial growth factor (VEGF) receptor 1 [40] in RNA co-precipitation assays. In addition RNase treatment disrupted most of the interactions of Jmjd6 with target proteins [30,31]. Recent work by Liu et al. [37] has provided data suggesting a 7SK small nuclear (sn)RNA-decapping activity for Jmjd6. However, further structural approaches are necessary to analyse the precise nature of the interactions between Jmjd6 and proteins (in particular, Brd4) and RNA.

OLIGOMERIZATION OF Jmjd6

Several independent analyses revealed that Jmjd6 adopts an oligomeric structure in solution [41,57,58]. Western blot analyses of full-length recombinant Jmjd6 result in the detection of both monomeric and oligomeric forms, the latter corresponding to apparent trimeric, pentameric and larger oligomeric forms [41,42,57,58]. The Jmjd6 oligomers are partially resistant to SDS-PAGE treatment [42,58]. Co-immunoprecipitation and fluorescence two-hybrid assays have confirmed that Jmjd6 oligomerizes in cells [42,58]. Moreover, transmission electron microscopy (TEM) studies of recombinant Jmjd6 have revealed ring-like structures formed by Jmjd6 oligomerization. These structures turned into fibrils when the polyS sequence in the C-terminal region of Jmjd6 was deleted [58]. Importantly, these structural changes influence the subnuclear localization of Jmjd6. In the absence of the polyS domain, Jmjd6 was prominently found in the nucleolus and nuclear speckles, whereas, with the polyS domain, Jmjd6 was found in the nucleoplasm [58]. This is probably biologically relevant because an alternatively spliced Jmjd6 mRNA that lacks a polyS domain has been isolated from human cells [6,31].

BIOLOGICAL FUNCTIONS OF Jmjd6

Following from the protein–protein interaction studies and the enzymatic assays described above, several biological functions for Jmjd6 have been suggested. First, the activity of Jmjd6 towards SR proteins, especially U2AF65, indicates a function of Jmjd6 as a pre-mRNA splice modulator [30,31,40] (Figure 6). Second, the potential activity of Jmjd6 on histone substrates indicates a possible function in transcriptional/epigenetic regulation [35,39] (Figure 6). Third, the interaction of Jmjd6 with Brd4 has been connected with a function of Jmjd6 in transcriptional pause release [37] (Figure 6). Moreover, Jmjd6 derived from an alternatively spliced transcript has been demonstrated to be enriched in the nucleoli and to interact with nucleolar proteins

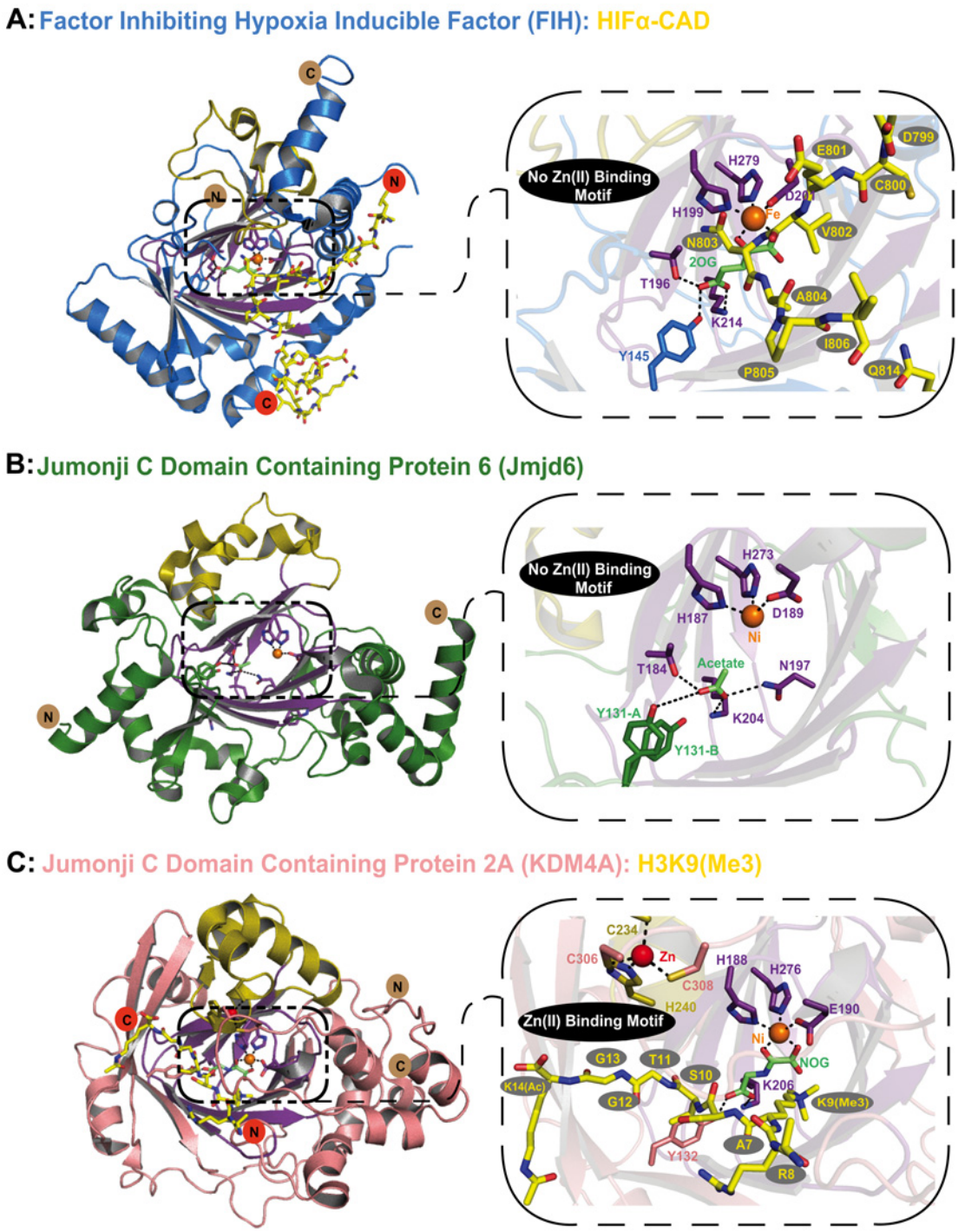

Figure 4 Comparison of the Jmjd6 crystal structure with those of a JmjC hydroxylase and a demethylase

Views from crystal structures of (**A**) the JmjC hydroxylase FIH (PDB: 1H2L), (**B**) Jmjd6 (PDB: 3K2O) and (**C**) the JmjC demethylase KDM4A (PDB: 2OQ6). The DSBH is in purple, the β4–β5 insert is in olive, and rest of the protein is in blue (FIH), green (Jmjd6) and light pink (KDM4A). In the case of Jmjd6 and KDM4A, nickel substitutes for iron. NOG, *N*-oxalyl-glycine, an analogue of 2OG. Note the two refined conformations for Tyr^{131} in the Jmjd6 structure.

[58]. Studies on the cellular functions of Jmjd6 are summarized below.

There is evidence that Jmjd6 can regulate mRNA splicing activity at least using model systems [30,40]. Knockdown of Jmjd6 in HeLa cells increased exon skipping of a α-tropomyosin 'minigene' [30]. In addition, the endogenous tumour antigen gene *MGEA6* (meningioma-expressed antigen 6) shows an altered splicing pattern in response to Jmjd6 knockdown in HeLa cells. A similar effect has been observed after treatment of HeLa cells with the broad-spectrum 2OG oxygenase inhibitor and iron chelator desferrioxamine [30].

There is also evidence for a regulatory role for Jmjd6 in alternative pre-mRNA splicing in human umbilical vein endothelial cells and mice [40]. Jmjd6 knockdown with siRNA

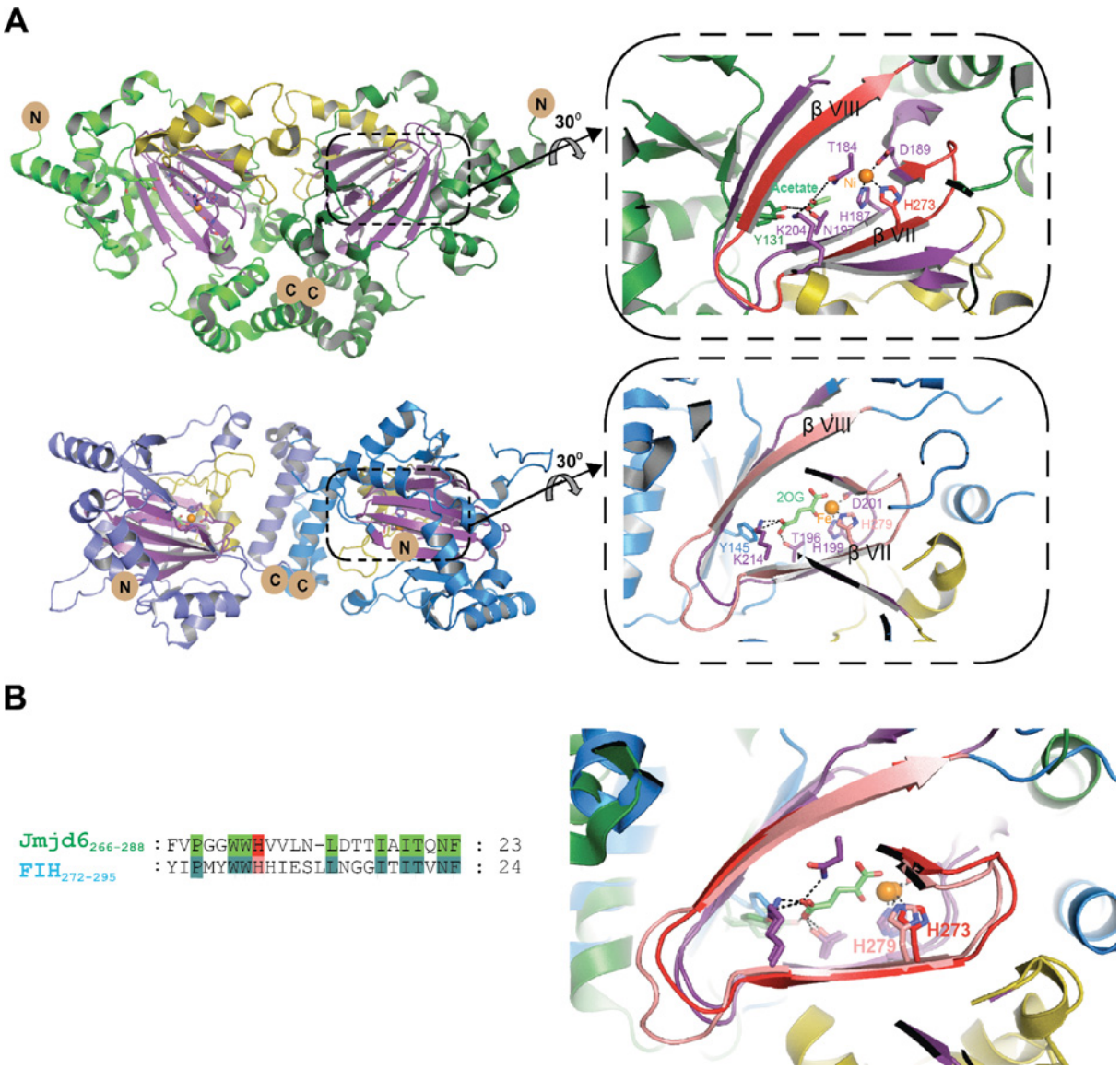

Figure 5 Dimerization of Jmjd6

(**A**) Views from crystal structures of Jmjd6 (PDB: 3K2O) and FIH (PDB: 1H2L) showing how their C-termini enable dimerization. Note that a proposed TM region of Jmjd6 (amino acids 266–288) contains one of the iron-binding triads (His^{273}) and is shown in the inset as red. This region is found conserved in Jmjd6 from *C. elegans* and all animals as well as in other 2OG oxygenases, including FIH (salmon in the FIH region). (**B**) Alignment of the proposed TM region, i.e. $Jmjd6_{266–288}$ and $FIH_{272–295}$ using Clustal W [73] with a superimposed ribbon presentation of the proposed TM region of Jmjd6 with the same region in FIH. Colour coding was maintained as used in Figure 4 except for the βVII and βVIII sheets.

resulted in increased production of an alternatively spliced transcript of the *vegfr1* gene (*Fms-related tyrosine kinase 1* or Flt1), which includes an extension of exon 13 and a premature stop codon, resulting in the production of a C-terminally deleted VEGFR-1 protein that lacks the TM domain and thus is secreted into the extracellular space (soluble VEGFR-1 or sFlt1) [59–61]. The soluble VEGFR-1 then sequesters VEGF leading to inhibition of angiogenic sprouting of endothelial cells. *In vivo* it was shown that lung epithelial cells from heterozygotic $Jmjd6^{+/-}$ mice showed impaired *ex vivo* capillary network formation. Moreover, the effect of Jmjd6 knockdown could be mimicked with hypoxia and the iron chelator desferrioxamine. To further characterize the effect of Jmjd6 on *vegfr* splicing Boeckel et al. [40] immunoprecipitated U2AF65 and found co-precipitation of *vegfr* mRNA and Jmjd6, suggesting that the effect of Jmjd6 on *vegfr* gene expression was linked to U2AF65. Moreover, later work revealed that the cytokine TNFSF15, a member of the tumour necrosis factor family, facilitates alternative splicing to produce soluble VEGFR through down-regulation of Jmjd6 protein [62].

Evidence for Jmjd6–U2AF65-linked regulation of alternative pre-mRNA splicing has also been described by Barman-Aksözen et al. [63]. They investigated the inherited disease erythropoietic protoporphyria, which is caused by a partial deficiency of ferrochelatase, the enzyme catalysing the insertion of Fe(II) into protoporphyrin IX, the precursor for haem [63]. In this case haem cannot be formed and toxic intermediates, including protoporphyrin IX, accumulate, causing the symptoms of the disease. In patients, a single nucleotide polymorphism at −48 from intron 3 (IVS3-48C) leads to aberrant splicing of 30–40 % of total transcripts, resulting in a premature termination codon and nonsense-mediated decay [63]. In combination with

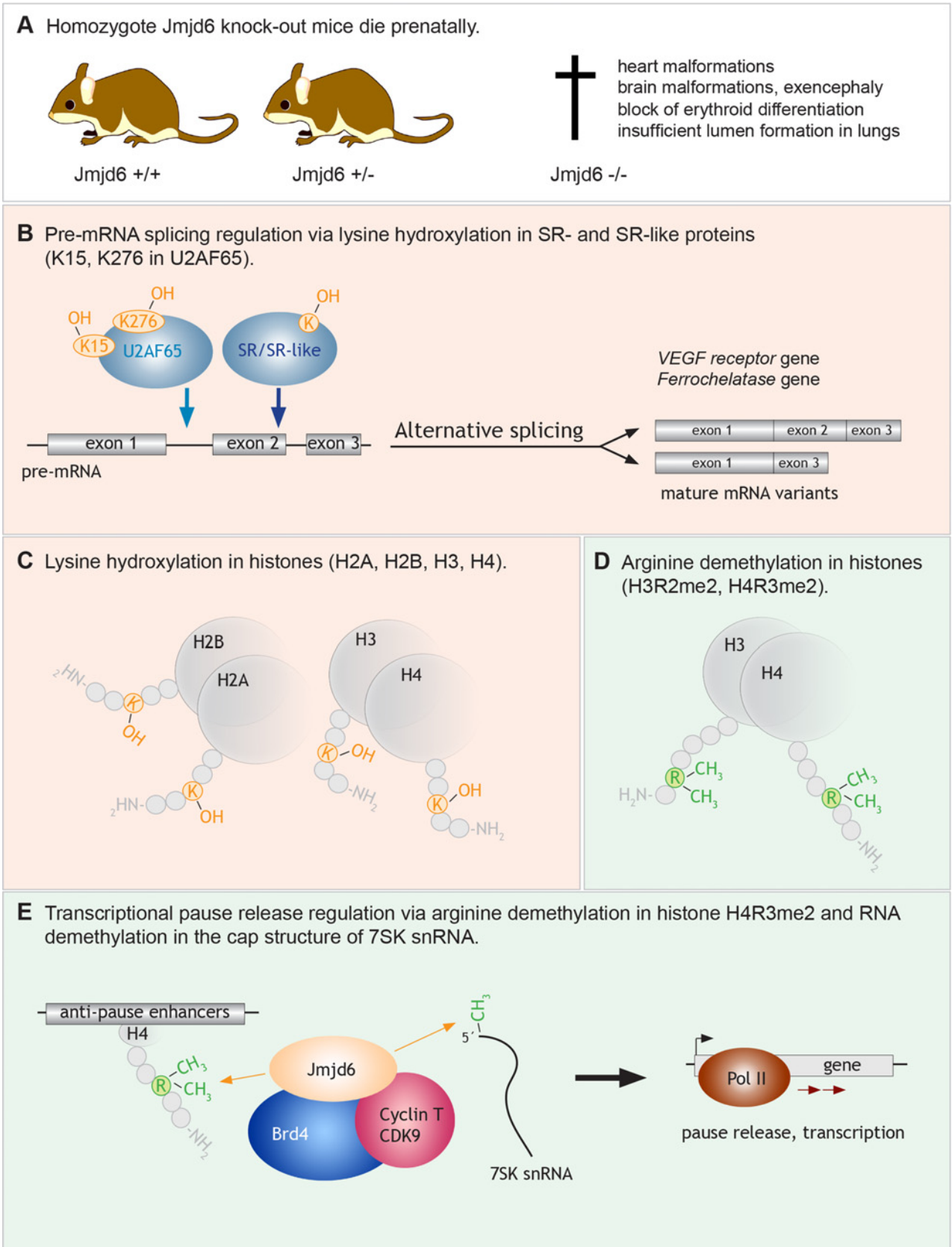

Figure 6 Biological functions of Jmjd6

(**A**) The homozygote Jmjd6 knockout in mice resulted in severe developmental defects and the mice died before birth. A biochemical explanation for this phenotype is still missing. However, proposed roles for Jmjd6 in several biological functions have emerged in recent years, including (**B**) pre-mRNA splicing, (**C**, **D**) epigenetic regulation and (**E**) transcriptional pause release.

a deleterious mutation in the second allele of the ferrochelatase gene this causes disease. On the other hand, a IVS3-48T allele, in the same combination, does not cause the disease symptoms. The authors showed that, in cell lines under iron deficiency, aberrant splicing of this latter allele increased considerably [63]. The same splicing pattern was observed when Jmjd6 or U2AF65 levels were depleted by siRNA. It was suggested that iron availability was important for Jmjd6 functioning in the modification of U2AF65 (2OG oxygenases including Jmjd6 need iron for activity), which is in turn responsible for 'correct' splicing of the ferrochelatase gene [63].

The evidence suggesting that Jmjd6 activity is regulated by hypoxia and iron is of physiological interest. The HIF hydroxylases have been identified as major regulators of the hypoxia response, probably in all animals [64]. Under some conditions, their activities can also be limited by iron and 2OG availability, although the physiological importance of these observations is presently unclear [13]. Furthermore, in some tumours, elevated levels of some tricarboxylic acid cycle intermediates (succinate, fumarate and the associated metabolite 2-hydroxyglutarate) can inhibit 2OG oxygenases (including chromatin-modifying enzymes such as the JmjC *N*-methyl lysine demethylases) [13,65], in a physiologically relevant manner. Thus, although Jmjd6 is unlikely to be a 'master' regulator of the hypoxic response, there is a possibility that it contributes to its regulation by fine-tuning expression of specific sets of hypoxically regulated genes, possibly in an oxygen- and iron-dependent manner in some circumstances

Matters on the biological role(s) of Jmjd6 have become even more complex with recently published results [37]. Liu et al. [37] investigated the significance of the previously described interaction between Jmjd6 and Brd4. Brd4 is a bromodomain-containing DNA-binding protein of the BET (bromo- and extra-terminal) domain family of proteins [66]. It has been identified in multiprotein complexes including the mediator complex [67], and is of major therapeutic interest as a target for some cancers [68]. Brd4 is involved in transcriptional pause release by interacting with the positive transcription elongation factor b (P-TEFb) complex, specifically in its active form [37]. Brd4 extracts the P-TEFb heterodimer [cyclin-dependent kinase 9 (CDK9) and a cyclin component of cyclin-T1, cyclin-T2 or cyclin-K] from its inhibition complex with 7SK snRNA and Hexim1/2. This then allows P-TEFb to phosphorylate, among other targets, serine 2 of RNA polymerase II (Pol II), permitting promoter-proximal pause release and transcriptional elongation [69,70]. Liu et al. [37] showed that Brd4 and Jmjd6 interact with each other on distal enhancers, so-called anti-pause enhancers. They report that Jmjd6 demethylates both the H4Arg^{3}Me$_2$(s) and the methyl cap of 7SK snRNA, thus helping to dismiss the 7SK snRNA/Hexim inhibitory complex on a subset of Brd4 transcriptional targets. After removal of repressive histone marks, Jmjd6 and Brd4 remained co-bound to anti-pause enhancers and active P-TEFb. The role of Brd4 in this context was investigated by comparing the Pol II-pausing index in cells with and without Brd4 knockdown. The presence of Jmjd6 also exhibited an effect on P-TEFb activation and promoter-proximal Pol II pause release, albeit much more modestly. In chromatin immunoprecipitation (ChIP)-sequence profiling of Jmjd6 and Brd4, genomic-binding sites revealed overlap on gene-distal regions. Moreover, it was demonstrated that Jmjd6 interacts with CDK9 in immunoprecipitation experiments with HA-tagged and Flag-tagged proteins [37]. It is possible that the role of Jmjd6 in regulating transcriptional pause release has an effect on splicing because splicing depends on the kinetics of transcription.

Recently the Jmjd6 homologue PSR-1 in *C. elegans* has been reported to be involved in the regulation of axonal fusion of posterior lateral mechanosensory (PLM) neurons [28]. Worms lacking a functional *psr-1* gene displayed a strong defect in axonal fusion. Wild-type PSR-1 expression rescued the defect, whereas a PSR-1 variant with a defective Fe(II) binding site was unable to [28]. These results indicate the importance of PSR-1 enzymatic activity for axonal fusion of PLM neurons; although the detailed molecular basis has to be uncovered. The results presented in this work [28] certainly do not exclude a nuclear function of Jmjd6 in worms. In support of this, ectopically expressed mCherry-tagged PSR-1 localized to PLM neurons; some was also found co-localizing with mitochondrial markers.

Jmjd6 AND CANCERS

Emerging evidence implicates Jmjd6 in different types of cancer [36,71,72]. Zhang et al. [72] reported increased levels of Jmjd6 mRNA and protein expression in lung adenocarcinomas; they correlated high Jmjd6 levels with a poor prognosis by analysing data from 154 patients. A similar correlation was demonstrated for breast cancer. Jmjd6 knockdown in breast cancer cell lines decreased invasion and suppressed proliferation, whereas Jmjd6 over-expression correlated with the opposite effect [71]. This effect on cell growth may relate to the interaction between Jmjd6 and p53 [36]. Up-regulation of Jmjd6 was also correlated with aggressive clinical behaviour of colon adenocarcinomas. This observation was proposed to relate to Jmjd6-catalysed hydroxylation of p53. In HCT116 cells, Jmjd6 depletion enhanced p53 activity, induced G1 arrest of the cell cycle and promoted apoptosis. These studies have led to the suggestion that Jmjd6 may be useful as a biomarker for tumour aggressiveness [36,71].

CONCLUSIONS

In summary, we have aimed to provide an overview of work on Jmjd6. Structural and biochemical evidence clearly assigns Jmjd6 a function as an Fe(II)- and 2OG-dependent C-5 lysine-hydroxylase. Subcellular localization studies in cells of mammals, *Caenorhabditis elegans* and *Hydra vulgaris*. consistently reveal nuclear localization of this protein. All recent evidence strongly argues against the original idea that Jmjd6 is a transmembrane protein functioning as a phosphatidylserine receptor on the plasma membrane of phagocytes. Nevertheless, with regard to biological functions, the emerging picture is still complex and probably reflects multiple roles for Jmjd6 which are very probably context dependent. Investigating the biological relevance of the biochemical activity of Jmjd6 is challenging due to limitations of available methods for investigating post-translational modifications of proteins, especially when they occur at low frequencies. Developing new methods for protein and nucleic acid analysis is of general interest. Ultimately, we believe that secure functional assignments will be possible only by combining biochemical studies (i.e. studies with isolated components) with physiological work (i.e. with intact organisms in a natural habitat). The history and present state of Jmjd6 research illustrate the challenges in such endeavours.

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