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Cellular and Molecular Life Sciences

REVIEW

Structure and function insights into the NuRD chromatin remodeling complex

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Abstract Transcription regulation through chromatin compaction and decompaction is regulated through various chromatin-remodeling complexes such as nucleosome remodeling and histone deacetylation (NuRD) complex. NuRD is a 1 MDa multi-subunit protein complex which comprises many different subunits, among which histone deacetylases HDAC1/2, ATP-dependent remodeling enzymes CHD3/4, histone chaperones RbAp46/48, CpG-binding proteins MBD2/3, the GATAD2a (p66α) and/or GATAD2b (p66β) and specific DNA-binding proteins MTA1/2/3. Here, we review the currently known crystal and NMR structures of these subunits, the functional data and their relevance for biomedical research considering the implication of NuRD subunits in cancer and various other diseases. The complexity of this macromolecular assembly, and its poorly understood mode of interaction with the nucleosome, the repeating unit of chromatin, illustrate that this complex is a major challenge for structure–function relationship studies which will be tackled best by an integrated biology approach.

Keywords NuRD · Remodeling · Deacetylase · Chromatin · Nucleosome · Structural biology

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Introduction

In 1942, Waddington [1] coined the term "epigenetic", the branch of biology that studies "the causal interactions between genes and their products, which brings the phenotype into being". Indeed, genes and more generally, chromatin, are targeted by covalent modifications such as acetylation, methylation or phosphorylation, among others, which can be recognized by protein effectors, allowing the recruitment of enzymes and other partners involved in chromatin remodeling, a process that is thought to be related with the accessibility of the DNA of target genes to transcription factors or RNA polymerase in particular. In 1998, several groups described a complex exhibiting an ATP-dependent remodeling activity, similar to that of ySWI/SNF from *Saccharomyces cerevisiae*, and coupled to a histone deacetylation function. This complex, called NURD, NRD, Mi-2 complex, and finally, NuRD, standing for "Nucleosome Remodeling and histone Deacetylation", is, to date, one of the only two known complexes coupling two independent chromatin-regulating activities [2–6], along with Tip60/p400 [7, 8]. One possible reason for that could be that the ATP-remodeling activity is necessary for the Histone Deacetylase (HDAC) subunits to access their target [9]. This idea is supported by the observation that in absence of ATP, deacetylation is only possible on histone octamers, and not on nucleosomes. The binding site of HDACs could be somehow protected by the DNA, and thus inaccessible. Experiments carried out to determine whether ATP could stimulate deacetylase activity did not show any significant effect on free histone octamers. By contrast, when nucleosomes were tested, ATP was shown to stimulate deacetylase activity by twofold: without ATP, 30–35 % of acetylated H4 histones were deacetylated, while in the presence of ATP, 60–70 % were [3].

The NuRD complex is highly conserved among superior eukaryotes, and is expressed in a large variety of tissues. It forms a large macromolecular assembly that consists of different protein subunits (Fig. 1); however, different homologs and isoforms have been described for each of these subunits, among which some are found to be mutually exclusive. This is particularly the case for MBD and MTA proteins, thus leading to a horde of coexisting NuRD complexes [10, 11], depending on the cellular, tissue, physiological or pathological context. Moreover, the stoichiometry of the different subunits remains an open question. Recently, the development of a new label-free quantitative mass spectrometry method, applied to the analysis of NuRD, suggested that it is composed of one CHD3 or CHD4 protein (Chromodomain, Helicase, DNA binding domain), one HDAC1 or HDAC2, three MTA1/2/3 (Metastasis Associated), one MBD3 (Methylated CpG-Binding), six RbAp46/48 (Retinoblastoma Associated protein), two GATAD2b (p66α) or GATAD2a (p66β) and two DOC-1 (Deleted in Oral Cancer) (Fig. 1) [12]. Those data are nevertheless in contradiction with the structural analysis of the HDAC1/MTA1 complex showing a dimerization of MTA1, suggesting the presence of two MTA1/2/3 and two HDAC1 or HDAC2 in NuRD [13]. The specificities of each isoform, together with the sharing of competences such as the activities of deacetylation and remodeling, ensure that NuRD is a major actor in various biological processes, like embryonic development, cellular differentiation, haemato- and lymphopoeisis, tumor growth inhibition, or the general repression of transcription.

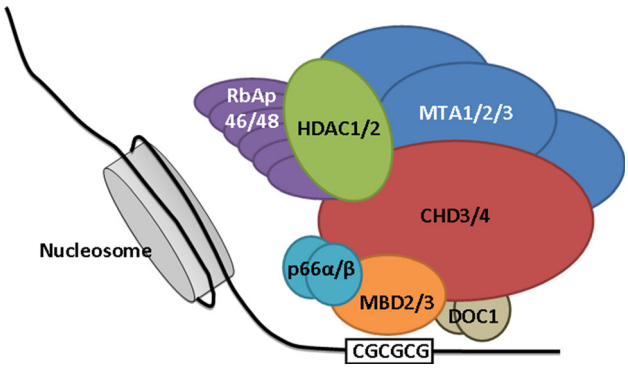

Fig. 1 Schematic description of the NuRD complex. The three-dimensional structure of the overall complex has not been determined to date and precise subunit interactions are unknown. All seven proteins of the complex are represented and annotated, and the stoichiometry obtained from mass-spectrometry analysis was taken into account, suggesting that the NuRD complex contains one CHD3 or CHD4 protein, one HDAC1 or HDAC2, three MTA1/2/3, one MBD2 or MBD3, six RbAp46/48, two p66α/β and two DOC-1 [12] subunits. Out of these proteins, the three paralogs of MTA (MTA1, 2 and 3) are found to be mutually exclusive, as well as the two paralogs of MBD (MBD2 and MBD3). Whether the other proteins are also part of distinct NuRD complexes is currently unknown

Furthermore, it directly interacts with various partners, like the lysine specific demethylase 1 (LSD1/KDM1A) [14], Ikaros, Aiolos, Helios [15–17], B cell lymphoma 6 (BCL6) [18, 19], the oestrogen receptor α (ERα/NR3A1) [20–22] or Oct4/Sox2/Klf4/c-Myc (OSKM) [23, 24]. This highlights the very broad and general role of NuRD, especially given that it is the most abundant form of deacetylase in mammals. The aim of this review is to give an up-to-date and comprehensive overview of the NuRD complex and the structure–function relationships of its different subunits.

CHD3/4: the ATP-dependent chromatin-remodeling

ATP-dependent chromatin-remodeling enzymes are helicases, which utilize the energy brought by the hydrolysis of ATP to destabilize interactions between DNA and histone proteins that constitute the core of the nucleosome. The chromatin structure is thus altered, by displacement of nucleosomes along the DNA, assumingly to make specific sequences available, or by eviction or replacement of histones with histone variants. These enzymes are part of the SF2 superfamily and Snf2 family [25]. In this group, the CHD subfamily is composed of two tandem chromodomains in the N-terminal part, and an ATPase domain. In yeast, only one CHD protein has been identified, yCHD1, while four in *Drosophila melanogaster* (dCHD1–4) and nine in mammals (hCHD1–9) exist. yCHD1 is closely related to d/hCHD1 and d/hCHD2 with approximately 35 % of overall sequence identity. The other CHDs on the contrary share only sequence similarity with yCHD1 within their defined domains, their extremities being highly variable.

In the context of NuRD, CHD3 and CHD4, also called Mi-2α and Mi-2β, are the two homologs found to ensure ATP-dependent chromatin remodeling. The latter is the most abundant in the NuRD complex, although it seems that both proteins can coexist within the same complex [3, 4]. At least three molecular species can thus be found: Mi-2α/NuRD, Mi-2α/Mi-2β/NuRD and Mi-2β/NuRD. This raises the question whether this protein is present in the NuRD complex in two or else copies, which is still unclear. The Mi-2 protein was initially identified as an autoantigen. About 25 % of patients affected with dermatopolymyositis [26, 27] appeared to be positive to anti-Mi-2 antibodies [26, 27]. While no correlation between the presence of these antibodies and tumor development have been proven formally, one anti-Mi-2-positive patient out of four develops an ovarian, colorectal, lung, pancreatic, stomachic or lymphatic cancer [25, 28].

CHD3 and CHD4 are large ATPases, with a molecular mass of about 220 kDa. Their domain organization (Fig. 2a) comprises two conserved plant homeodomains (PHD) fingers, two tandem chromodomains (Chromatin Organization Modifier), and a SWI2/SNF-like helicase domain [29]. They are highly conserved among the all eukaryotes, although absent in *Saccharomyces cerevisiae*. The ATPase activity of the Mi-2 subunit, from three different species (*Drosophila melanogaster*, *Xenopus laevis*, *Homo sapiens*), was shown to be stimulated by chromatin but not by free DNA or histones [2, 30, 31]. This implies that these enzymes are implicated in the recognition of the nucleosome rather than of its individual components. NMR solution structures of individual chromodomain (Fig. 2d) and the two PHD domains have been determined (Fig. 2b, c), revealing a bivalent mode of binding to histone H3 tail [32, 33]. Indeed, the two PHD domains of CHD4 are able to bind two distinct H3 tails, within a single nucleosome or on adjacent nucleosomes [34]. The post-translational modifications of these tails govern the binding affinity of CHD4: H3K9 trimethylation promotes the binding of the enzyme (Fig. 2), while H3K4 methylation abolishes it [35]. Recently, a low resolution SAXS envelope suggested domain arrangements of CHD4 [36, 37] and helped getting some understanding of the concerted action of the ATPase, PHD and chromodomains of this large protein. Functional studies suggest that these domains are mutually required to enhance their binding affinity and/or activity: the PHD domains prevent the chromodomains from non-specific binding to the DNA, while the chromodomains enhance the affinity of the PHD domains towards H3 tails. Finally, the ATPase domain itself enhances the affinity of the PHD domains, and vice versa. The shape of the complex derived from SAXS suggests a close interaction of all the domains, leading to a structural stabilization, independent of the presence of ATP (Fig. 2).

Additionally, two isoforms of CHD3, CHD3.1 and CHD3.3, exhibit a C-terminal SUMO-interaction motif

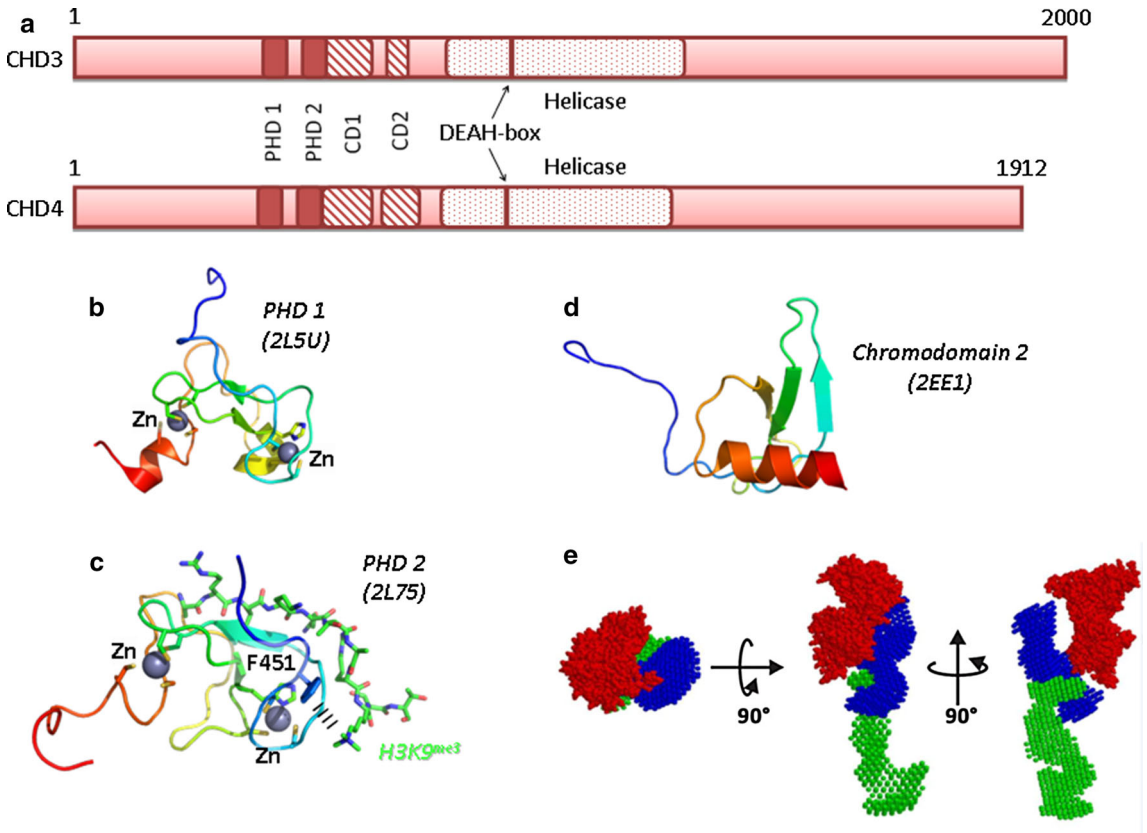

Fig. 2 **a** Schematic description of the CHD3 and CHD4 domains. *CD* chromo domain, *DEAH-box* Asp-Glu-Ala-His-box. **b–d** The published NMR structures of the two PHD domains and the second chromodomain of CHD4 are represented with their pdb accession number. Zinc ions are represented by *gray spheres*. In particular, one can notice the π-cation stacking interaction (represented with *black dashes*) allowing discrimination between the methylated and non-methylated state of H3K9 by residue F451 of the second PHD domain of CHD4 (2c). **e** The SAXS envelope of CHD4 suggests an elongated topology comprising ATPase (in *red*), PHD (in *blue*) and chromo domains (in *green*), as proposed by Morra et al. [36]. The structure can be divided into a head (ATPase domain) and a stalk (chromodomains), with the tandem PHD domain linking both. The tight association of these three domains explains their functional interdependence

(SIM) allowing them to interact with the sumoylated form of the KRAB-associated protein-1 (KAP-1), a major component of heterochromatin. KAP-1 phosphorylation by the ataxia telangiectasia mutated protein (ATM), as observed in the case of DNA double strand breaks, inhibits this interaction with CHD3 and leads to chromatin decompaction [38–40].

The Mi-2 proteins have also shown their crucial role in the development of some model organisms. In *Caenorhabditis elegans*, both CHD3 and CHD4 are implicated in the Ras signaling pathway, regulating cell fate in the hermaphrodite vulva and male tail [41]. In *Arabidopsis thaliana*, the CHD3 homolog PICKLE is implicated in the auxin signaling pathway, required for lateral root initiation and development [42]. In human, both CHD3 and CHD4 interact with transcription factors Ikaros, Aïolos and Helios, and target NuRD to specific promoters involved in lymphocytic development and proliferation [15–17]. Among those genes, one could mention CD179b, for progenitor B cells to precursor B cells differentiation; dntt, required for the V-DJ recombination; or CD4 and CD8a, for thymocytes maturation. These data suggest that Mi-2 could also have an important role in mammal development, but the lack of genetic models remains today a crucial bottleneck to further study these enzymes.

HDAC1/2: deacetylating histone lysines

During a ligand screen aiming at blocking the tumorigenic effect of the v-sis gene on 3T3 fibroblasts, a new molecule, trapoxin, was discovered [43]. Trapoxin-treated cells were shown to be hyperacetylated and their deacetylation function was inhibited, but the binding target remained unknown [44]. Finally, in the mid-1990s, this question was solved using trapoxin as a bait to purify its target by affinity chromatography. Mass spectrometry studies revealed that it was a homolog of the yeast deacetylase Rpd3p [45]. Since then, eighteen histone deacetylases have been identified and divided into three classes, HDACs I, II and III. The first one comprises the nuclear HDACs 1–3 and HDAC 8, based on a strong homology with yRpd3p. The second class gathers the cytosolic and nuclear HDACs 4–7 and HDACs 9–11. Finally, the third class, called sirtuins, comprises SIRT 1 to 7, homologs of the ySir2 histone deacetylase, and can be either cytosolic, nuclear, nucleolar or mitochondrial.

In the NuRD complex, the subunits ensuring histone deacetylation are HDAC1 and HDAC2 (Fig. 3a). These 55 kDa proteins are highly conserved and ubiquitous in all eukaryotes. They share 83 % of sequence identity, and their double knock-out in T-cells or embryonic stem cells leads to a decrease by half of the total deacetylase activity of these cells [46]. They are thus the two predominant enzymes in terms of histone deacetylation activity in mammalian cells.

Sequence alignments of class I HDACs showed major differences in the C-terminal domain, which is entirely missing in HDAC8. This domain is required in HDAC1 and 2 (the specific HDAC subunits of NuRD) to bind to partners in the context of protein complexes. Furthermore, it is post-translationally modified to regulate HDACs catalytic activity, in particular, by the kinase CKII which phosphorylates HDAC1 S421 and S423 to enhance the transcriptional repression activity [47–49]. Nevertheless, the first crystal structure of a HDAC, that of HDAC8 in complex with different inhibitors, paved the way for structural understanding of the class I HDACs [50, 51]. These proteins are composed of a single α/β domain (Fig. 3b), consisting of an eight-stranded-parallel-β-sheet at the centre of thirteen α-helices. These secondary structures are connected through long loops, thus creating the catalytic core domain of these enzymes. The active site consists of a long tunnel with a minimum depth of 8 Å, also referred to as lipophilic tube leading to the catalytic machinery. This tunnel is occupied by the four carbons of the side chain of the acetylated lysine, stabilized by hydrophobic contacts with residues G151, F152, H180, F208, M274 and F306 (HDAC8 numbering). All these residues are conserved among the class I HDACs, with the exception of M274 being a leucine in all other class I HDACs. Finally, the end of the tunnel accommodates a zinc ion, chelated by five coordination bonds in a trigonal bipyramidal fashion, and stabilized by the carboxylic oxygen of residues D178 and D267, and by the Nδ1 atom of the H180 side-chain. The carbonyl oxygen of the acetyl moiety carried by the acetylated lysine, as well as a water molecule, occupy the two other coordination sites. More recently, the structures of HDAC2 in complex with inhibitors [52, 53], and the one of HDAC1 in complex with the ELM (Egl-27 and MTA1 homology) and SANT (Switching-defective protein 3, Adaptor 2, Nuclear receptor co-repressor, Transcription factor IIIB) domains of MTA1 [13] (described later in this paper) shows the same global structure of the core HDAC protein.

It has been observed that inhibitors of the hydroxymate class, in the manner of suberoylanilide hydroxamic acid (SAHA) or trichostatin A, bind to the catalytic site in roughly the same way as acetylated lysines, with fast-binding kinetics and nanomolar K_d range over a large majority of class I and II HDACs. This is explained by the direct access of the ligand through the lipophilic tube, chelating the zinc ion with its hydroxamic group (Fig. 3c). In contrast, inhibitors of the benzamide class, like entinostat and mocetinostat, are also located in the lipophilic tube, but their thiophene group is accommodated in a

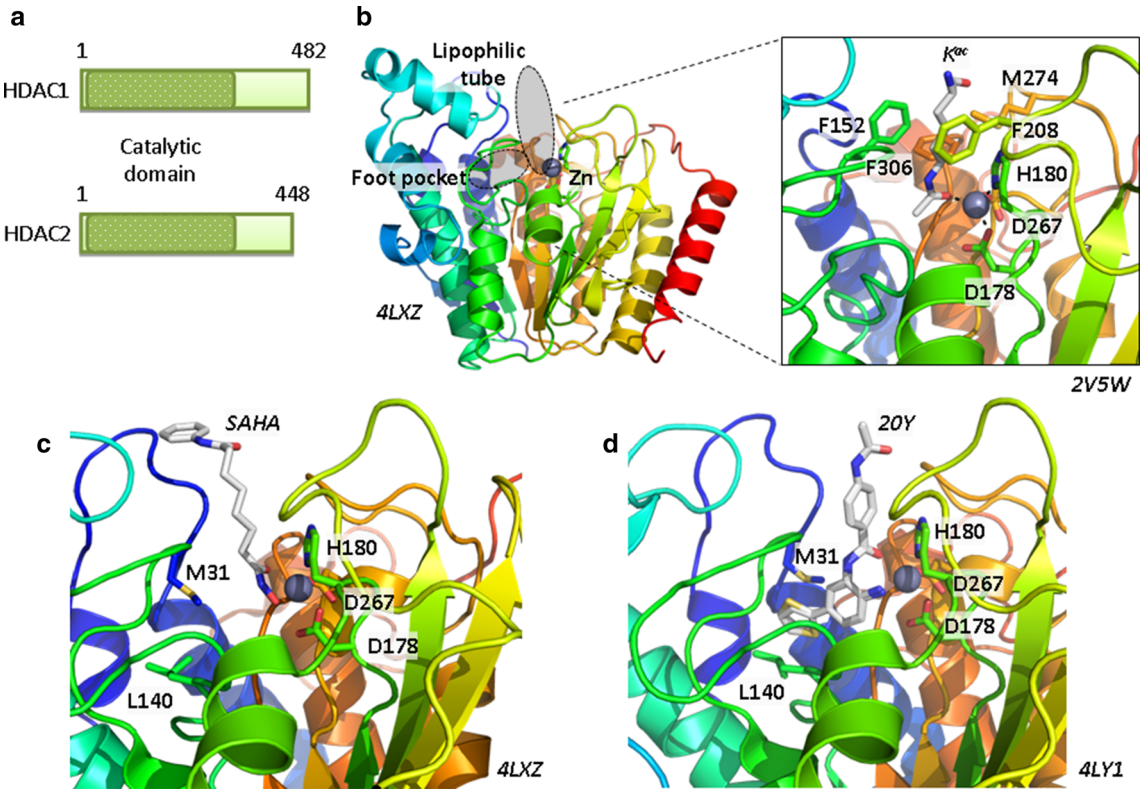

Fig. 3 **a** Schematic description of the HDAC1 and HDAC2 domains. **b** The global X-ray structure of HDAC2 highlights the lipophilic tube as well as the foot pocket forming the active site of this enzyme. On the *right*, a closer view of the active site of HDAC8 (not found in NuRD but structurally highly similar, and the only one for which the structure in complex with acetylated lysine, K^{ac}, is available) shows crucial residues for the zinc ion coordination and for natural substrate interaction. Zinc ions involved in coordinated bonds with the ligand are represented by *gray spheres*. **c**, **d** The two structures of HDAC2 in complex with antagonist inhibitors of the hydroxymate class (SAHA) and benzamide class (20Y: 4-acetylamino-*N*-2-amino-5-thiophen-2-ylphenylbenzamide) show the M31 and L140 residues, forming the gate of the foot pocket which opens up to accommodate the thiophene group of benzamide inhibitors. This results in slower kinetics but also higher specificity towards this class of inhibitors, buried deeper in the active site than hydroxymates

deeper pocket, named "foot pocket" (Fig. 3d). This pocket is formed by flipping and shifting of the two M31 and L140 residues (HDAC2 numbering). Those two residues are conserved between HDAC1–HDAC3 but not HDAC8 and class II HDACs, giving rise to a higher specificity of this class of inhibitors. Finally, the central secondary amide moiety of these inhibitors chelates the zinc ion, locking the molecule in place. This explains the slower kinetics of benzamides, compared to hydroxymates, together with the higher specificity for class I HDACs, and in particular, HDAC1 and HDAC2.

Although deacetylation is largely associated with gene repression, knock-out experiments showed that several genes become repressed in the absence of HDAC 1 or HDAC2 [54–57]. This suggests that these two enzymes could also have a role in gene activation. Further studies carried out by treating embryonic stem (ES) cells with trichostatin A showed both a decreased expression of pluripotency-related genes and an increase of lineage-specific genes, indicating a negative as well as positive regulation activity. By chromatin immunoprecipitation (ChIP), it has been shown that these enzymes can localize at some transcriptionally active loci in human [58], mouse [59] and yeast [60], corresponding to DNase I hypersensitive sites. In particular, HDAC1 has been detected in promoter regions, on pluripotency genes in ES cells (like fgf4, mbd3, nanog, oct4, sox2, tbx3 or zfp42) and trophoblast-lineage genes in trophoblast stem cells (like bmpr1a, cdkn1c, cdx2, elf5, hand1, msx2 or tcfap2c) [59], while HDAC2 is present in both promoters and gene bodies.

A commonly observed phenomenon when knocking-out HDAC1 and HDAC2 is the decrease of cell proliferation [56, 61–63]. The loss of these enzymes induces an overexpression of the kinases p21/WAF1/CIP1 [61, 64] and p57/Kip2 [56] inhibitors, preventing G1/S phase transition. HDACs inhibitors have been tested in numerous cases of cancers, with the aim of limiting tumor growth [65], but most of these inhibitors, in the manner of SAHA (approved and commercialized under Vorinostat or Zolinza) are large-

spectrum inhibitors of class I and II HDACs and therefore lead to significant side-effects. Studies carried out on mice showed that the use of specific HDAC1 and/or HDAC2 inhibitors, like benzamides described above are equally efficient with respect to antiproliferative effects, but with potentially reduced side-effects [62, 66]. Given their biochemical and genetic identity, it is not surprising that HDAC1 and HDAC2 are functionally redundant enzymes: deletion of both enzymes is required to produce a substantial phenotype [46, 55–57, 61, 67–70]. The molecular rationale for the redundant roles remains unclear.

MTA1/2/3: reading histone tails and promoters

MTA proteins were the last ones to be characterized within the NuRD complex. The first representative in this family, temporarily called p70, then MTA1, was isolated after the observation of its differential expression pattern observed by cDNA library screening using the 13762NF rat mammary adenocarcinoma metastatic system [71]. But despite the overexpression of this protein, one had to wait for the discovery of NuRD and the presence of MTA proteins in this complex to start understanding the role of this family [3, 5].

Phylogenetic studies suggested that the mta gene underwent duplications to lead to the three loci found in vertebrates (mta1 on chromosome 14q, mta2 on chromosome 11q and mta3 on chromosome 2q), mta2 being the nearest relative to the ancestral non-vertebrate gene [72]. Those three genes encode the three proteins MTA1, MTA2 and MTA3, and also three alternative-splicing products: MTA1S, MTA1-ZG29p and MTA3L [73]. The three canonical MTA proteins have a molecular weight of 80, 70 and 65 kDa, respectively, and share 68 % of sequence homology between MTA1 and MTA2 and 73 % between MTA1 and MTA3. This strong homology is especially due to the N-terminal domains, the C-terminal parts being more variable. With the exception of MTA1-ZG29p, all the MTA proteins possess various highly conserved domains (Fig. 4a): a bromo adjacent homology domain (BAH; 70 % of identity between $MTA1^{BAH}$ and $MTA2^{BAH}$ and 76 % of identity between $MTA1^{BAH}$ and $MTA3^{BAH}$), an Egl-27 and MTA1 homology domain (ELM; 76 % of identity between $MTA1^{ELM}$ and $MTA2^{ELM}$ and 78 % of identity between $MTA1^{ELM}$ and $MTA3^{ELM}$), a SANT domain (87 % of identity between $MTA1^{SANT}$ and $MTA2^{SANT}$ and 94 % of identity between $MTA1^{SANT}$ and $MTA3^{SANT}$) and a GATA domain (89 % of identity between $MTA1^{GATA}$ and $MTA2^{GATA}$ and 89 % of identity between $MTA1^{GATA}$ and $MTA3^{GATA}$). The role of these domains has not been fully studied yet in the context of MTA proteins within NuRD. Nevertheless, some functional insights come from related proteins. For example, the SANT domain in Ada2 and the second SANT domain in SMRT seem to interact primarily with unmodified histone tails [74, 75], while the first SANT domain of SMRT and the one of MTA1 recruit HDAC through direct interaction [13, 76, 77]; the BAH domain of Rsc2 is implicated in histone H3 binding [78], while $ORC1^{BAH}$ recognizes H4K20me2 [79] and GATA domains interact with specific DNA sequences [80].

Expression regulation for the mta genes is still little-known to date; however, preliminary results are available. For example, heregulin, a growth factor which binds to the human epidermal growth factor receptors 3 and 4 (HER3 and HER4) transmembrane receptors, is able to induce MTA1 expression in breast cancer cells [21]. It has also been shown that the c-Myc proto-oncogene could bind directly to the mta1 gene to activate its expression [81]. Moreover, MTA1 is overexpressed in hypoxia, and is responsible for hypoxia inducible factor 1 (HIF-1) stabilization by deacetylation, becoming then resistant to degradation by the 26S proteasome [82]. Additionally, MTA proteins are intimately linked to the oestrogen receptor ER [22, 83], in breast cancer and mammary gland development [84]. The short MTA1S isoform, which is produced by alternative splicing inside a cryptic site of exon 14 [85], directly interacts with ER and is responsible for its sequestration in the cytoplasm [85]. MTA1 also blocks ER-driven gene activation, by antagonizing the effect of oestradiol [21], while MTA2 can make breast cancer cells insensitive to oestrogens and tamoxifen, by deacetylation of ER itself [22]. Finally, the promoter of mta3 is directly activated by ER-α, thanks to the presence of a half response-element ERE, and MTA3 seems to be involved in repression of some genes involved in invasive growth, like Snail [11] or Wnt4 [86]. Consequently, MTA1 and MTA3 seem to have an opposite role. Expression patterns of those two proteins support this idea: MTA3 is largely expressed in healthy epithelial cells, and its expression decreases along with tumor growth, until complete shutdown at the carcinoma stage; on the contrary, MTA1 is gradually expressed, concomitantly with tumorigenesis. Finally, isoform MTA1-ZG29p is a product of the mta1 gene, including only the seven last exons. For this reason, it does not exhibit the three domains described previously, and its location seems to be restricted to zymogenic granules in the pancreas [87].

Recently, a first 3-Å-resolution crystal structure of HDAC1 in complex with MTA1 has been published [13, 77] (Fig. 4b, c). It shows the ELM and SANT domains of MTA1 (residues 162–335, i.e., one quarter of the protein), wrapping around HDAC1, with an interaction interface of 5185 $Å^2$ surface area. Three regions can be distinguished: the first one corresponds to the N-terminal part of the ELM domain, the second one to three-quarters of the C-terminal

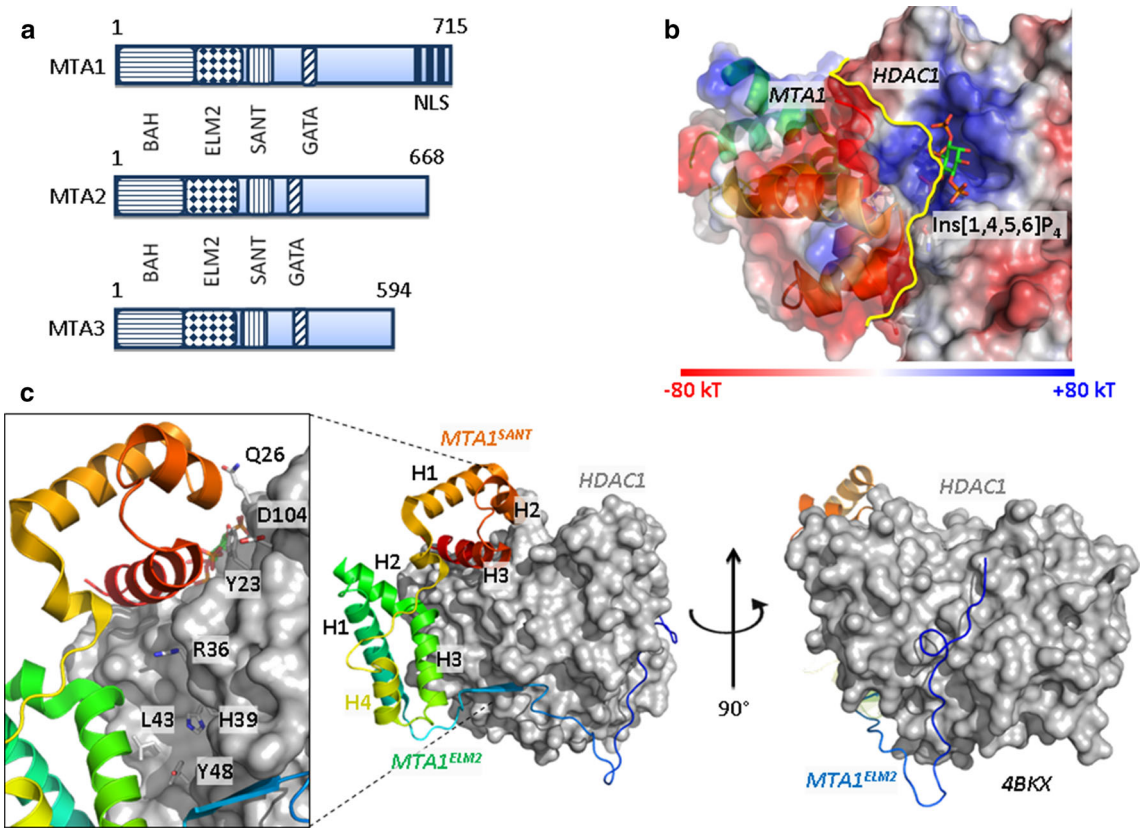

Fig. 4 **a** Schematic description of the MTA1, MTA2 and MTA3 domains. *NLS* nuclear localization sequence. **b** The structure shows how an inositol phosphate molecule (Ins[1, 4, 5, 6]P4) can accommodate in the basic pocket (in *blue*) formed at the interface between HDAC1 and MTA1. In *yellow*, the limitation of the HDAC1–MTA1 interface. Structure superimposed and docked on 4A69 (HDAC3-SMRT). Negative, neutral and positive surface electrostatic potentials are displayed in *red*, *white*, and *blue*, respectively. **c** The global X-ray structure highlights HDAC1, represented in *gray* (see also Fig. 3), and shows how MTA1 peptide (represented in *colour*) is wrapped around the deacetylase (in *orange*, the SANT domain; in *green*/*blue*, the ELM2 domain). Crucial residues involved in the HDAC1–MTA1 interaction are annotated

region of the ELM domain, and the third one to the SANT domain (Fig. 4c). The first region comprises a specific and conserved motif (EIRVGxxYQAxI), and forms an extended loop conformation. This long thirty-amino-acid-chain runs on the surface of HDAC1, inside a long apolar groove. The second region is folded with four α helices (H1–H4). The isolated ELM domain shows no folded secondary structure in circular dichroism, implying a radical structural reorganization upon binding to HDAC1 [13]. Helices H1 and H3 mediate the interaction interface with HDAC1 (1278 $Å^2$). Simultaneously, this region is responsible for dimerization of two MTA1 proteins, mediated by interactions between helices H1 and H4, and to a lesser extent, H2, of the two MTA1 molecules. Up to twenty-eight apolar residues (14 for each monomer) are involved in this dimerization, with an important interaction interface of 2332 $Å^2$. This is a rather clear confirmation that this dimerization interface is physiologically relevant, and that in terms of stoichiometry, the NuRD complex probably contains two MTA proteins, as well as two HDAC proteins.

The third region is composed of three α-helices (H1–H3, Fig. 4c). Surprisingly, the interface of MTA1 with HDAC1 contains many positively charged residues which would normally lead to mutual repulsion; however, the positively charged pocket that forms accommodates an inositol tetraphosphate molecule (Ins[1, 4, 5, 6]P_4) that neutralizes the charges and stabilizes the MTA1-HDAC1 protein complex, through residues K31, R270 and R306, among others (Fig. 4b). This observation had been made previously on a HDAC3-$SMRT^{SANT}$ complex, copurified from mammalian cells with endogenous Ins[1, 4, 5, 6]P_4 while direct complex formation from the individually purified proteins failed [76]. Further studies showed that mutations of the $MTA1^{SANT}$ residues involved in coordination of Ins[1, 4, 5, 6]P_4 lead to a reduced interaction between the SANT domain and HDAC1. However, MTA1 can still be tethered to HDAC1 in absence of Ins[1, 4, 5, 6]P_4, through interaction of the ELM domain as described earlier. Studies on the HDAC3-SMRT showed a direct link between ageing of the complex, loss of Ins[1, 4, 5, 6]P_4 moiety and

decreased HDAC activity. However, addition of exogenous Ins[1, 4, 5, 6]P_4 recovered the HDAC activity with level higher than endogenous complexes. Similarly, the same observation has been made on the HDAC1–MTA1 complex, with an activation K_d around 5 μM. These elements tend to confirm Ins[1, 4, 5, 6]P_4 as having a regulatory role of class I HDACs in vivo.

MBD2/3: DNA-binding and the connexion to methylation

The study of Methylated CpG-Binding domain Proteins (MBPs) started in 1989, after the fortuitous discovery of two proteins binding to methylated DNA. At this time, Bird and collaborators were seeking proteins able to bind to non-methylated DNA and likely to protect CpG islands from methyltransferases. Electromobility shift assays from mouse liver nuclear extracts identified two distinct proteins, either MeCP1 or MeCP2 (Methylated CpG-binding Protein 1 and 2) [88, 89]. MeCP2 was the first to be purified from mouse brain extracts and mutations of its gene in the locus ceruleus of the brain turned out to be responsible for neurological disorders, such as the Rett syndrome, lethal in men and causing psychiatric disorders in women [90], or autism [91]. This 53 kDa protein exhibits what has then been described as a 90 residues N-terminal MBD domain, as well as a C-terminal transcription repression domain (TRD) [92, 93] (Fig. 5a). Sequence similarity searches in databases identified four other proteins, MBD1, MBD2, MBD3 and MBD4, all very conserved in vertebrates [94]. Among those MBPs, MBD2 and MBD3 share the highest sequence identity (77 %). Furthermore, a single homolog of these two proteins, MBD2/3, is found in invertebrates. It is encoded by a single gene, in contrast to vertebrates where this gene probably underwent a duplication event. Indeed, mbd2 and mbd3 genes have a very similar genomic structure, varying only by the size of their introns. This supports the idea that MBD2 and MBD3 are probably the ancestral representatives of this family [95, 96]. Later, the protein MeCP1 initially discovered along with MeCP2 turned out to be a MBD2/HDAC1 complex [97]. From these various MBD proteins, only MBD2 and MBD3 are part of the NuRD complex.

With a mass of approximately 43 and 33 kDa respectively, MBD2 and MBD3 are the smallest subunits of the complex, which are exclusive yet interchangeable within NuRD [10]. While MBD2 binds to methylated DNA [94], MBD3 has lost this ability in mammals. Indeed, the appearance of this class was accompanied by a point mutation in the mbd3 gene, leading to the incorporation of a new amino acid in position 34 (a phenylalanine instead of a tyrosine). This abolishes the selectivity of this protein for methylated DNA [98–100]. While the very first studies 15 years ago credited MBD2 with only a transient role in the complex, being in particular a NuRD recruiter to methylated DNA before its eviction and replacement by MBD3 [101, 102], other studies since have shed light on a MBD2/NuRD complex, biochemically and functionally distinct from the MBD3/NuRD complex [10, 103]. In that sense, MBD2 knock-out experiments showed only little effects at the phenotype level, whereas MBD3 knock-out leads to embryonic lethality [104].

Recently, it has been proposed that MBD3 and, to a lesser extent, MBD2, were able to specifically bind to hydroxymethylated CpG islands. Notably, MBD3 seems to colocalize with TET1 (10–11 translocation methylcytosine dioxygenase 1), the protein responsible for hydroxylation of methylcytosines [105]. Additional experiments however failed to show an interaction between MBD3 and hydroxymethylated DNA [106]. Instead, MBD2 and MBD3 appear to be preferentially localized at CpG-rich transcription start sites (TSS). At TSS's, MBD2 predominantly binds methylated CpG islands, leading to a repression of gene expression; whereas MBD3 binds to non-methylated DNA, and is associated with active transcription [107, 108]. Recently, NMR spectroscopic analysis suggested that MBD3 could counterbalance the action of MBD2. The latter does not only bind methylated DNA but also to a lesser extend unmodified DNA. Being specifically targeted towards unmethylated CpG's, MBD3 could compete efficiently with MBD2 to avoid extensive repression of active genes by MBD2 [109].

Several X-ray and NMR structures of MBDs in complex with DNA have been solved, revealing a common interaction pattern for all the MBPs [109–115]. In particular, two solution structures of MBD2 and one solution structure of MBD3 have been solved, showing a quasi-structural identity between the two [109, 114, 116]. The MBD is characterized by an α/β sandwich, composed of an N-terminal four-stranded antiparallel β-sheet (β1: residues 6–8 in MBD3; β2: residues 15–20; β3: residues 32–37; β4: residues 41–43), and a C-terminal α-helix (residues 47–53). This α-helix is kept antiparallel against the β4 strand by hydrophobic contacts. Furthermore, the MBD exhibits three loops L1, L2 and C-terminal hairpin. L2 connects the α-helix and the C-terminal hairpin and is well-defined in solution. In contrast, the long L1 loop between β2 and β3, composed of a dozen of residues, is more flexible. This appears to be a necessary prerequisite for binding to DNA (Fig. 5b). Indeed, seven residues of this loop make contacts with one of the DNA strand, at the level of the major groove. The other DNA strand interacts mainly with residues in the α-helix and L2-loop.

Recognition of the methylation site occurs independently for each methylcytosine of the CpG site, consistent

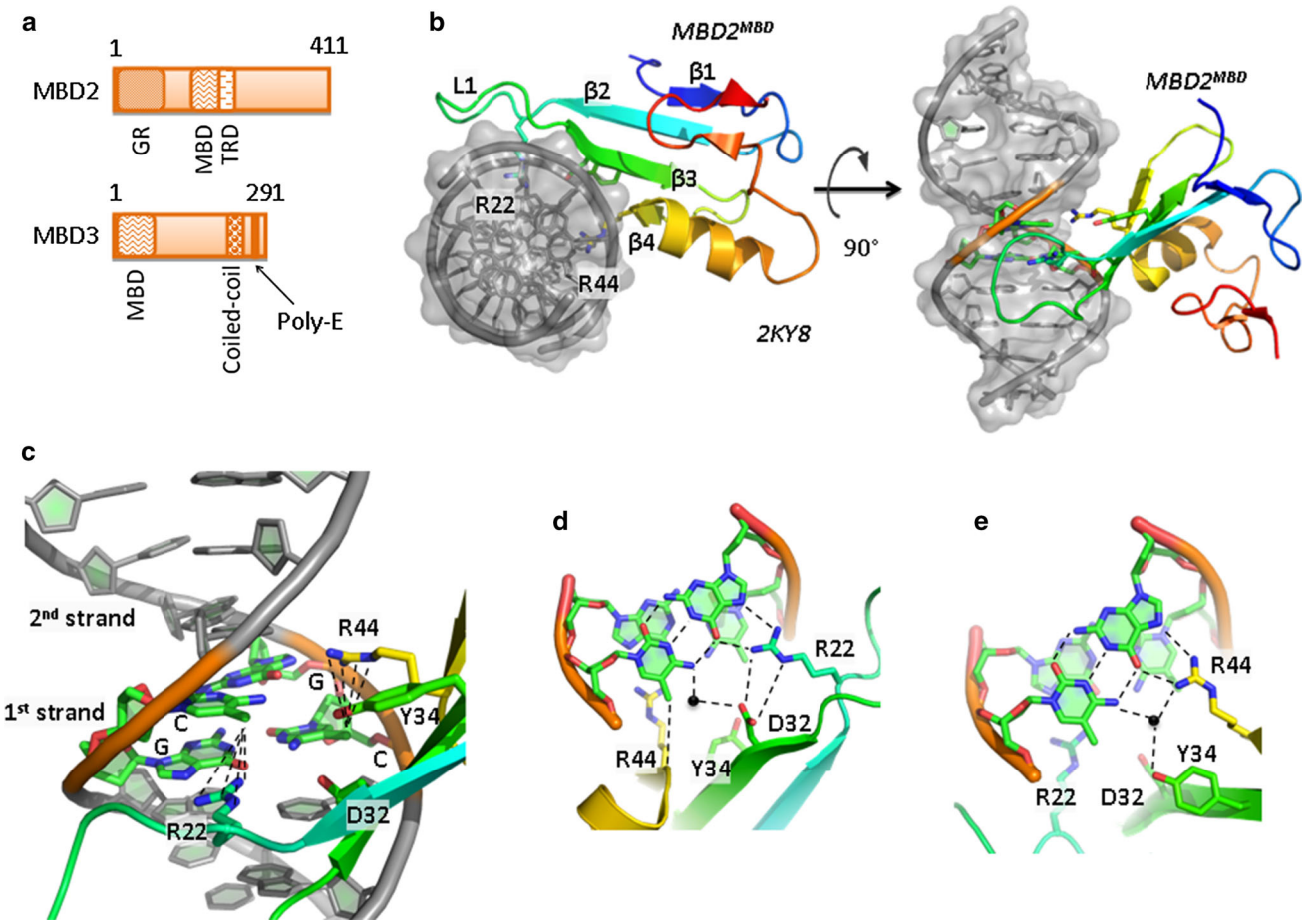

Fig. 5 **a** Schematic description of the MBD2 and MBD3 domains. *GR* glycine-arginine-rich region, *MBD* methyl-CpG binding domain, *TRD* transcription repression domain, *Poly-E* poly-glutamate. **b** The NMR structure of $MBD2^{MBD}$ shows the MBD domain of MBD2 interacting with a symmetrically methylated CpG island within an 11-bp DNA. Residues R22 and R44 are shown pointing at guanines inside the major groove of the DNA. **c** *Close-up* of the interaction interface highlights the crucial residues, and shows Van der Waals forces (represented with *black dashes*) between the methylated cytosines and the guanidinium group of arginines. **d**, **e** Complementary CG-base pairs and their specific hydrogen bonds with MBD2 are shown. Water molecules engaged in water-mediated hydrogen bonds are represented by *black dots*. The displayed arginine residues interact with guanines within the CpG island and are stabilized by residues D32 and Y34. The latter are also involved in cytosine recognition within the CpG

with the observation that the interaction domain shows an asymmetric arrangement in its fold. Arginines 22 and 44, which are conserved among all MBPs, interact with symmetrically arranged guanines inside a CpG island (Fig. 5d, e). Both arginines lie in a plane with their interacting guanines, stabilized and locked by direct hydrogen bonding of residue D32 and water-mediated hydrogen bonding of residue Y34. This flat orientation allows the two arginine residues to pack against the methylated cytosine bases neighboring their interacting guanines, and permitting weak van der Waals contacts (Fig. 5c). The integrity of these residues is crucial to ensure the binding to methylated DNA, as proven by mutagenesis experiments. In particular, Y34 turned out to be a key-residue in the recognition of the methylation state, although the molecular mechanism remains unclear as no interactions with the methyl group of the cytosine are observed in the crystal structure. Its mutation into a phenylalanine, as found in mammals, leads to a loss of affinity of methylated CpG islands. On the contrary, *Xenopus laevis* MBD3 does not exhibit this evolutionary mutation, and is thus still able to bind to methylated DNA. Also, the crystal structures of $MBD4^{MBD}$ in complex with different modified DNA show that Y96 (Y34 in xMBD3, F34 in m/hMBD3) is flipped out of the DNA interface, which leads to a loss of specificity of MBD4 towards methylated DNA, at the cost of an increased binding of 5mCG/TG and 5mCG/hmCG islands [113].

MBPs are ubiquitous proteins, nevertheless exhibiting strong disparities depending on the cellular type and development stage. For example, in embryonic stem cells, MBD3 is the only predominantly expressed MBP. At the

blastula stage of organismal development, MBD2 and MBD4 become detectable, and finally MeCP2 after the blastocyst implantation [117, 118]. In adults, expressions patterns depend on the cellular type: MBD3 (along with MeCP2 and MBD1) is highly expressed in the brain, notably in the olfactory bulb, cerebellum, hippocampus and prefrontal cortex [119, 120], while MBD2 has an almost opposite expression pattern, with mRNA quantities up to twenty times higher in some tissues, such as breast cells or cultured HeLa cells [121].

Recently, the central role of MBD3 in somatic cell reprogramming and cellular differentiation was suggested, interacting in particular with OSKM proteins, transcriptions factors responsible for maintaining totipotent state until blastocyst stage [23, 24, 122, 123]. However, opposite data obtained out of two different reprogramming systems suggest a context-dependent role of MBD3 in reprogramming, albeit further studies will be needed to confirm this theory. Another functional aspect, although in a completely different context, is the role of MeCP2 in the Rett syndrome. Seeing as mutations in the mecp2 gene are responsible for this neurodevelopmental disorder, lethal in men and causing neurological and psychiatric conditions in women, it has been thus suggested that mutations in other MBPs could also be linked to neurological disorders. In this respect, the DNA of 226 Caucasian and Afro-American autistic patients and their relatives was thus analysed and alterations were found in mbd1–4 genes in 198 of them [124]. Interestingly, one of those alterations was found in exon 1 of the mbd3 gene. It corresponds to a point mutation (G>T at 1,543,563 in locus 19p13.3), leading to the incorporation of a new amino acid inside the MBD domain (R23M). This mutation, inducing the loss of a positive charge, has been observed in two Afro-American half-brothers, displaying late and unfunctional language acquisition. This mutation seems to be inherited from their disease carrier maternal grandmother, suggesting a sex-related effect. This residue is semi-conserved in MBD2 were it correspond to K167. Though published structures have not shown any relevant role of this arginine in DNA binding, it is located right after the crucial R22 residue binding the CpG island. A new structure of the mutated gene will thus be needed to answer the question raised by the phenotype observed in R23M patients.

RbAp46/48: ensuring a stable platform and binding histones

RbAp46 and RbAp48 (also called Rbbp7 and Rbbp4, respectively) were first identified because of their interaction with the tumor suppressor factor retinoblastoma (Rb) [125–127]. Later, studies showed their affinity for histones, and their presence in various deacetylation and remodeling complexes [72, 128–130]. Although those two proteins share 90 % of sequence identity [126], they exhibit different biochemical activities. Thus, RbAp46 associates with other proteins, notably histone acetyltransferase 1 (HAT1), involved in de novo histone H4 acetylation, on its lysine 5 and 12 residues [9, 131]. This acetylation pattern is conserved among all eukaryotes, from yeast to human [132]; whereas RbAp48 is an essential chaperone for histone H3-H4 tetramer deposition on newly replicated DNA [133], and is especially found in the assembly complex CAF-1 (chromatin assembly factor 1), with p150/CHAF1A and p60/CHAF1B. Nevertheless, RbAp46 and RbAp48 can be jointly found inside complexes, for example in association with HDAC1 and/or HDAC2, within the Sin3A or NuRD complexes, where they promote gene repression, including the one regulated by Rb [101, 125, 134]; they are also found within the polycomb repressive complex (PRC2 and PRC3), with the histone-lysine N-methyltransferase EZH2, to methylate H3K27 or H1K26 [135]; or in the *Drosophila melanogaster* nucleosome remodeling factor (NURF) complex, along with ISWI (SNF2L in human), where RbAp proteins homologs are called NURF55 [136].

RbAp46/48 are 48 kDa proteins that share a WD40 repeat sequence (Fig. 6a). The great stability of these proteins allowed to date determining several crystal structures: RbAp46 in complex with a histone H4 peptide, at 2.4 and 2.6 Å resolution [137] (Fig. 6b); RbAp48 alone, at 2.3 Å resolution [138]; RbAp48 in complex with a FOG-1 (Friend of GATA) peptide at 1.9 Å resolution [139] (Fig. 6d); and RbAp48 in complex with an MTA1 peptide at 2.5 and 2.15 Å resolution [140] (Fig. 6c). Predictably, the RbAp proteins showed a structure similar to other WD40 proteins: a donut-shaped seven-bladed β-propeller, with a long N-terminal α helix (residues 9–28), lying on the seventh blade of the barrel, and a short C-terminal α-helix (residues 405–409), which is placed above and seems to extend the N-terminal helix. Finally, one particularity of these WD40 proteins is the presence of a seventeen residues loop, negatively charged, inside the sixth blade of the barrel, called PP loop (because of two successive prolines P362 and P363) (Fig. 6b).

The crystal structure of RbAp46 in complex with a small histone H4 peptide shows an interaction interface of approximately 700 $Å^2$. This H4 peptide corresponds to residues 25–42 of the human isoform, i.e., the first α-helix of the histone fold and a part of the N-terminal tail. Though the structures previously described in other WD40 proteins displayed an interaction interface on the front of the barrel, or even sometimes, inside it, histone H4 preferentially binds in a unique pocket located on the side of the barrel, and formed by the PP loop and the long N-terminal helix. Thus, hydrophobic residues I34, L37

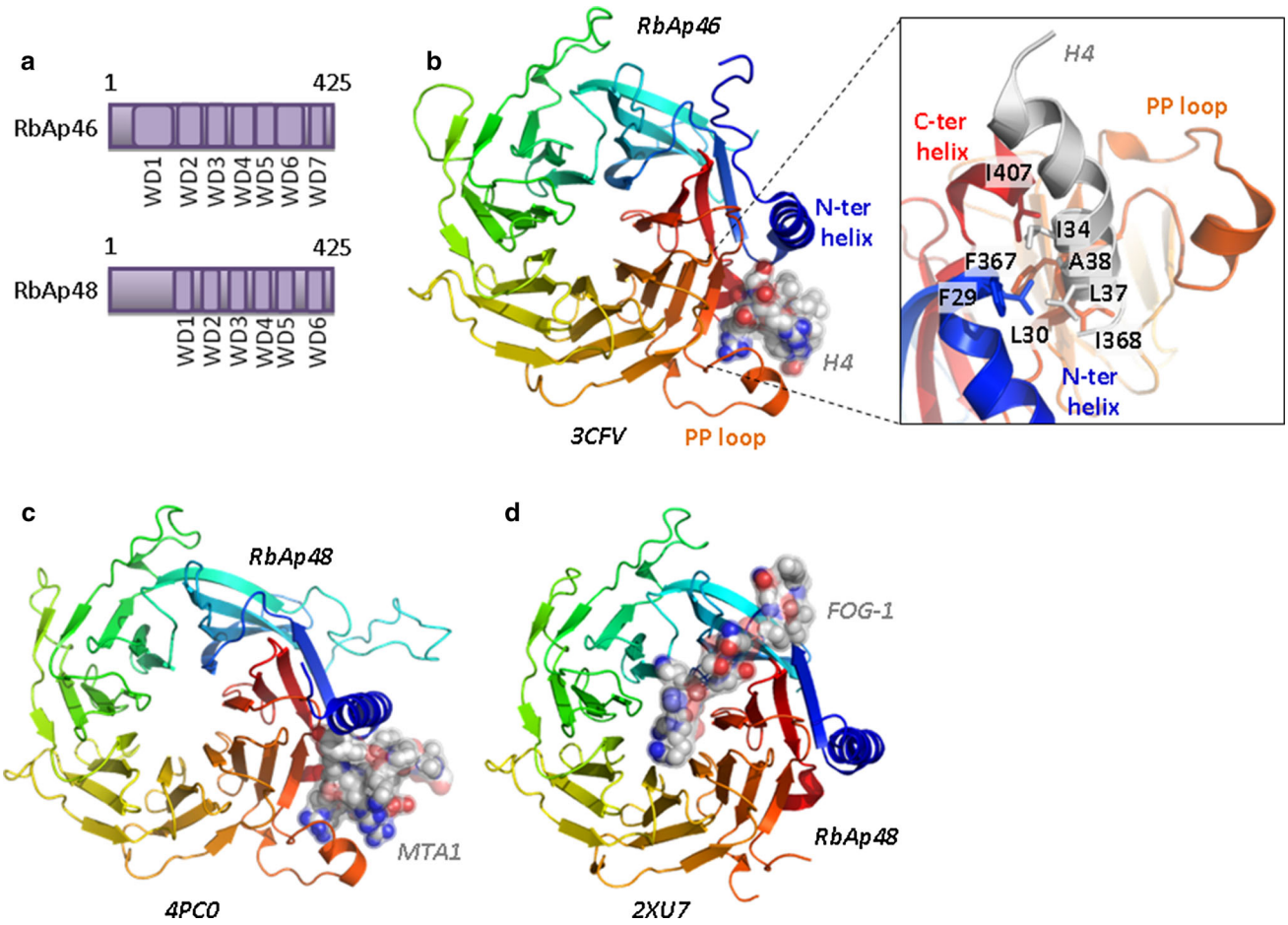

Fig. 6 **a** Schematic description of the RbAp46 and RbAp48 domains. *WD* tryptophan-aspartate domain. **b** RbAp46/H4 complex shows a binding interface located on the side of the barrel, in a pocket formed by the PP loop (in *orange*) and the long N-terminal helix (in *dark blue*). Crucial hydrophobic residues involved in the RbAp46–H4 interaction are annotated in the *close-up* view. **c** The structure of the RbAp48/MTA1 complex shows a noticeably similar interaction interface, on the side of the barrel, suggesting RbAp proteins cannot interact with histones and MTA proteins at the same time. **d** The RbAp48/FOG1 complex shows a different binding interface than the previous two, on the *top* of the barrel and extending towards the central channel

and A38 in helix α1 of histone H4 interact with a hydrophobic patch composed of residues F29, L30, F367, I368 and I407 of RbAp46 (Fig. 6b). A complex network of salt bridges and hydrogen bonds is also described between Q27, K31, R35, R36, R39 and R40 of histone H4; and E356, D357, D360, G361, P362, L365, N406, I407 and D410 of RbAp46 [137]. All these residues are conserved in RbAp48 and the yeast homolog p55, suggesting that the binding mechanism of these three proteins with histone H4 is similar. Finally, it has been shown that, to promote a proper interaction with RbAp46, the α1 helix of histone H4 must partially open, abolishing interactions with the α2 helix as well as those with histone H3, in particular through residues I34, L37 and A38. This observation suggested that RbAp proteins cannot interact with the nucleosome [137]; however, pulsed electron–electron double-resonance (PELDOR) experiments have shown recently that RbAp48 can interact with an H3-H4 dimer, suggesting an accrued flexibility of the nucleosome [141].

The structure of RbAp48 in complex with the fifteen N-terminal amino acids of the GATA-1 cofactor FOG-1, involved in erythroid and megakaryocytic cell differentiation, shows a binding interface located on the face of the barrel, which extends into the central channel [139] (Fig. 6d). The binding site is different from the one observed in the RbAp46/H4 complex and shows a high affinity probably because eight out of the thirteen FOG-1 residues are involved in hydrogen or ionic bonds with RbAp48. In particular, this interface is composed of numerous acidic residues of RbAp48 (E231, E319, E179, E126, E395, E41), allowing the stabilization of a basic triade of FOG-1 (R3, R4 and K5). This interaction pattern can be extrapolated to RbAp46, as it shares those same conserved residues. Finally, the RbAp48 structure, in complex with a short peptide of the C-terminal end of

MTA1, shows a very similar binding site to the one observed in the complex with H4 [77, 140] (Fig. 6c). This suggests that RbAp46/48 cannot simultaneously interact with MTA1 and histones.

Misregulations of RbAp46 and RbAp48 seem to be linked to tumorigenesis in several localizations, among which mammary and cervical tissues [142–144]. They indeed were shown to directly interact with the nuclear receptor ERα, and to affect ERα-regulated-gene expression [145]. For example, siRNA silencing experiments in MCF-7 cells were carried out, and Sox9 transcription factor and G2-cyclin gene activity were recorded. These genes are normally repressed by ERα in presence of estradiol, but it was shown that RbAp46 leads to their activation in presence of estradiol; in contrast, RbAp48 appears to maintain their repression in the absence of a ligand. Furthermore, a prolonged estradiol exposure of those cancer cells leads to a two to threefold increase of RbAp46 levels. Together, these data suggest that RbAp46 could be a mediator favouring a continuous ERα activity, while RbAp48 could ensure the basal repression of these genes in the absence of a ligand. Previous studies corroborate this idea, showing that repression of RbAp48 is involved in cervical cancer formation [146], and that an increase of RbAp46 levels prevents breast cancer development [142, 144, 147]. RbAp48 therefore appears to be a key therapeutic target for cervical cancer treatment [148]. Indeed, it has been shown that RbAp48 expression is favoured by radiotherapy irradiations, and that SiHa, HeLa and Caski cells were radiosensitive, the more the level of RbAp48 is high.

In another context, a recent study carried out on eight persons, aged 33–88, showed a differential expression pattern of RbAp48 in their brain, reduced along with the age, specifically in the dentate gyrus, a subregion of the hippocampus known for its lifelong neurogenesis, and foreseen to be the seat for episodic memory [149]. Additional studies carried out on mice confirmed the role of RbAp48 in the memorization process. A young knock-out mouse has indeed less potential in memorizing new objects and environments; on the contrary, lentivirus-induced re-expression of RbAp48 in old mice helped increase their cognitive capacities. Those phenomena seem to be closely related to the activity of the RbAp48-binding partner CREB-binding protein (CBP)/p300, nuclear receptor-bound transcription coactivators increasing gene expression through their intrinsic histone H4 and H2B acetyltransferase activities.

GATAD2A/B: potentializing repression

In 2002, a two-hybrid screening on MBD2 highlighted the interaction of two proteins, namely p66α and p66β, and later, GATAD2A and GATAD2B, respectively (GATA Zinc Finger Domain Containing 2A/B) [150]. These two proteins, initially thought to be two isoforms of the same gene, appear to derive from an ancestral gene duplication, undergone with the emergence of mammals. Indeed, a unique orthologue, named p66, is found in *Drosophila melanogaster*, *Caenorhabditis elegans* and *Xenopus laevis* [102, 150]. The human gene p66α could be localized on chromosome 19p13.11, while the p66β gene is localized on chromosome 1q23.1.

These proteins have shown to interact and colocalize with MBD2 and MBD3 [151]. Later, functional assays showed that GATAD2A/B are recruited through two domains: on one hand, to MBD2, via their CR1 domain; on the other hand, to DNA and deacetylated histones, via their GATA zinc finger-like CR2 domain [150]. Moreover, the over-expression of both p66 proteins induces an increase of repressive action by MBD2; whereas p66 knock-outs allow a partial recovery of MBD2-repressed genes [152]. GATAD2A/B can be targeted by post-translational modifications, like sumoylation. Thus, residues K30 and K487 of p66α, and K33 of p66β, when sumoylated, enhance the interaction of these proteins with other partners within the NuRD complex, like HDAC1 or RbAp46 [153].

DOC-1: the overlooked tumor-suppressor

Recently, copurification experiments carried out on recombinant MBD2 and MBD3-expressing stable cell lines revealed the presence of a new 12 kDa subunit called CDK2AP1 (Cdk2-associated protein 1) or DOC-1 (Deleted in oral cancer-1) inside both NuRD/MBD2 and NuRD/MBD3 complexes [10]. As its name suggests, this protein, a putative tumor suppressor interacting with CDK2, is inhibited in oral and colorectal cancers [154, 155]. Later, mass spectrometry experiments confirmed the presence of this protein in NuRD [12, 156].

The role of DOC-1 is still unclear; nevertheless, it was shown that overexpression in 293T cells would lead to a partial arrest of the cell cycle phase G1/S, together with a significant growth retardation [157]. This can be offset against the consequences of MBD2 overexpression promoting cell proliferation. This suggests thus that an opposite role for those two proteins exists inside the NuRD complex.

Structure–function relationship within NuRD and future prospects

From a functional point of view, it remains unclear today why evolution chose to assign two enzymatic activities within a single complex. Indeed, even though HDACs have

proved their capacities to activate a subset of genes, these subunits still persist in being considered as general repressors, which raises the question of the apparent contradiction with the ATP-dependent remodeling activity of CHD3 and CHD4, known to allow breathing of the chromatin and thus, potentially activate gene expression. A long date proposal suggests that ATP-dependent remodeling of the chromatin is a prerequisite to allow other subunits of the NuRD complex, in particular HDACs, to access their substrate. However, this has never been clearly proven, and further experiments will be needed to confirm the mechanism underlying the function of NuRD.

From a structural point of view, it is intriguing how so many different proteins can interact with a complex. Structural studies of the whole NuRD complex will be needed to address the accessibility of factors to this macromolecular complex, and determine the molecular basis of inter-protein interactions, such as with factors involved in cancer progression. Furthermore, relatively little is known about the intramolecular interactions within the entire NuRD complex, as illustrated by the remaining open question of the stoichiometry. Some works nevertheless constitute the blueprint for a better comprehension of the NuRD architecture, in the manner of the HDAC1/MTA1 complex or RbAp46/H4 complex structures. This indeed suggests the presence of two MTA and two HDACs subunits within the complex, as well as potentially two RbAp46/48 per nucleosome. Whether the latter function in synergy with CHD3/4 to destabilize histone octamers, as suggested by the binding of RbAp46 to H3-H4 dimer only, also remains a question to be answered.

Taken together, great efforts have been made these past few years to lift the veil regarding biochemical, genetic and structural data to fully understand the precise action of a given NuRD complex in vitro but also in its environment, as justified by the quasi-ubiquitous role that it plays. In this regard, numerous studies have focused on isolated subunits, and the results obtained tend to be extrapolated to the whole complex, leading to a multitude of scopes of activities. Future work will thus have to place back these results into the context of the entire NuRD complex and address the molecular mechanism by which the subunits cooperate to regulate gene expression. A key for a better understanding of NuRD function will be to analyze its overall architecture using integrated structural biology approaches and cryo-electron microscopy in particular, localize the individual subunits within the complex, and address their interactions in the chromatin context.

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Conflict of interest The authors declare no competing financial interests.

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