CDK2AP1/DOC-1 is a bona fide subunit of the Mi-2/NuRD complex†‡

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The Mi-2/NuRD (NUcleosome Remodeling and histone Deacetylase) chromatin remodeling complex is a large heterogeneous multiprotein complex associated with transcriptional repression. Here we apply a SILAC based quantitative proteomics approach to show that all known Mi-2/NuRD complex subunits co-purify with Cyclin Dependent Kinase 2 Associated Protein1 (CDK2AP1), also known as Deleted in Oral Cancer 1 (DOC-1). DOC-1 displays *in vitro* binding affinity for methylated DNA as part of the meCpG binding MBD2/NuRD complex. In luciferase reporter assays, DOC-1 is a potent repressor of transcription. Finally, immunofluorescence experiments reveal co-localization between MBD2 and DOC-1 in mouse NIH-3T3 nuclei. Collectively, these results indicate that DOC-1 is a bona fide subunit of the Mi-2/NuRD chromatin remodeling complex.

Introduction

In eukaryotic cells, DNA is packed in a structural polymer called chromatin. Nucleosomes form the fundamental building blocks of chromatin and in general these nucleosomes are inhibitory to processes that require access to the DNA template, such as transcription and DNA repair. During the last two decades many protein complexes have been identified and characterized that use ATP hydrolysis to alter the position of nucleosomes on DNA. In doing so, these protein complexes can regulate the accessibility of transcription factors or repair proteins to DNA.[1,2] One of these ATP dependent chromatin remodeling complexes is the Mi-2/NuRD complex (NUcleosome Remodeling and histone Deacetylase complex). This complex was biochemically purified by a number of labs more than a decade ago.[3–5] The two highly homologous proteins CHD3 and CHD4 (or Mi-2α and Mi-2β) represent the catalytic ATP hydrolyzing subunits in the complex. In addition, the complex contains two histone deacetylases, HDAC1 and HDAC2, RbAp48 and RbAp46, MTA1-3, p66 α and β and MBD2 or MBD3. MBD2 and MBD3 were first described as common subunits within the NuRD complex[6] but our subsequent study revealed that MBD2 and MBD3 each assemble into a Mi-2/NuRD like complex in a mutually exclusive manner.[7] MBD2, unlike MBD3, binds to methyl CpG residues and it has been proposed that this protein forms the link between the MBD2/NuRD complex and transcriptionally silent CpG methylated promoters. In addition to the reported (core) subunits, a number of transcription factors have been shown to interact with the Mi-2/NuRD complex.[8–14] These transcription factors could serve to recruit the Mi-2/NuRD complex to specific loci in the genome.

Previously, we identified DOC-1 (Deleted in Oral Cancer-1) peptides in MBD2/NuRD and MBD3/NuRD complex purifications,[7] indicating that this protein may be an interactor or a novel subunit of the Mi-2/NuRD complex. DOC-1 was first described as a protein that is commonly mutated or deleted in various malignancies.[15,16] In addition, DOC-1 has been characterized as a Cyclin Dependent Kinase 2 Associated Protein (CDK2AP1).[17] In this study it was shown that over-expression of DOC-1 in 293T cells results in a G1 arrest and significant growth retardation compared to wild-type cells consistent with loss of the protein in tumors. Recently, interactions between MBD3 and DOC-1 were shown by co-immunoprecipitation and western blot analyses.[18] However, convincing evidence that DOC-1 is a general Mi-2/NuRD interactor or a core subunit of the complex is still lacking.[19] Using a variety of biochemical and functional experiments, we here show that DOC-1 is indeed a bona fide subunit of the MBD2/NuRD and MBD3/NuRD complexes.

Results

DOC-1 exclusively associates with Mi-2/NuRD complex subunits in the nucleus

To investigate a putative interaction between DOC-1 and the Mi-2/NuRD complex, we tagged and purified DOC-1 from human cells. We made use of the recently developed BAC-transgeneOmics approach[20] to obtain a HeLa cell line expressing DOC-1-GFP from its own promoter at endogenous levels (Fig. 1A).

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This cell line and wild-type HeLa cells were SILAC labeled 'heavy' and 'light', respectively, subjected to single step affinity purification on GFP-nanotrap beads[21] after which bound proteins were digested with LysC and measured in a single LC-MS run on an LTQ-Orbitrap mass spectrometer. Computational analysis of the data was done using the MaxQuant software.[22] In this approach, GFP-tagged proteins and proteins interacting with the bait are more abundant in the heavy compared to the light form and can therefore easily be distinguished from background binders that have a one to

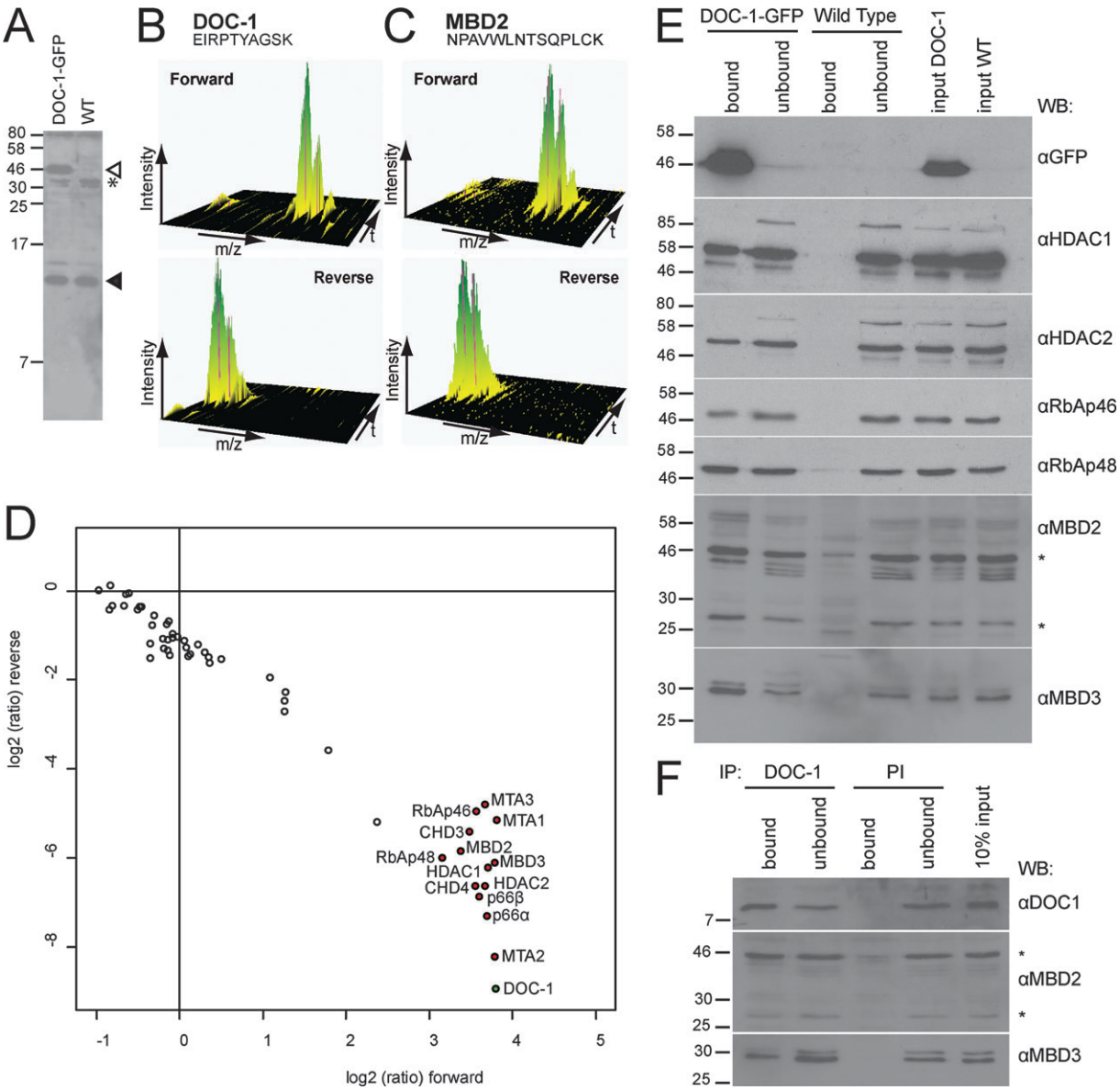

Fig. 1 DOC-1 is a subunit of the Mi-2/NuRD complex. (**A**) Nuclear extracts from DOC-1-GFP and wild-type HeLa cells were analyzed by western blotting using a DOC-1 antibody. Endogenous DOC-1 and DOC-1-GFP are indicated by ◀ and ◁, respectively. Note that the signal intensities for endogenous DOC-1 and DOC-1-GFP are about equal, indicating that DOC-1-GFP is expressed at roughly endogenous levels. The asterisk indicates antibody cross-reactivity. (**B and C**). Three dimensional representations (m/z = x-axis, chromatographic retention time = y-axis and MS intensity = z-axis) of MS signals from DOC-1 (B) and MBD2 (C) peptides that were obtained in the forward (upper spectra) and reverse (lower spectra) DOC-1-GFP pull-downs. The indicated MBD2 peptide shows a high ratio in the forward pull-down and a low ratio in the reverse pull-down, indicating that MBD2 specifically interacts with DOC-1-GFP. (**D**) Ratio *versus* ratio plot of all the proteins that were identified and quantified with at least two peptides in the DOC-1-GFP pull-downs. In this plot, background proteins appear around the centre of the axes with ratios close to 1 in both the forward and the reverse pull-down. In contrast, DOC-1-GFP and associated proteins show a high ratio in the forward pull-down and a low ratio in the reverse pull-down and therefore cluster together in the bottom right quadrant of the graph. Note that all the identified DOC-1-GFP interacting proteins are known subunits of the Mi-2/NuRD complex. (**E**) Nuclear extracts from DOC-1-GFP and wild-type HeLa cells were subjected to GFP pull-downs using GFP nanotrap beads and tested for the presence of the indicated proteins by western blotting. The eluates from the beads as well as 12.5% of the non-bound fraction and 10% of input extract was loaded on gel. Asterisks indicate MBD2a and b. (**F**) Endogenous DOC-1 was immunoprecipitated from HeLa nuclear extracts using a DOC-1 antibody. Immunoprecipitates were tested for the presence of DOC-1, MBD2 and MBD3. PI = immunoprecipitation using pre-immune serum.

one ratio. As a control, a 'label swap' experiment is performed in which the GFP-tagged cell line is labeled light and the wild-type cells are labeled heavy. In this case, the bait and associated proteins have a low heavy/light ratio. Plotting ratios of the 'forward' experiment against ratios of the 'reverse' experiment results in four quadrants in which the GFP-tagged protein and its interactors cluster together in a single quadrant. As expected, DOC-1-GFP derived peptides had a high ratio in the forward and a low ratio in the reverse pull-down, indicating that the bait protein was specifically enriched in both pull-downs consistent with the SILAC labeling scheme (Fig. 1B). MBD2 derived peptides showed a similar pattern, indicating that MBD2 is a DOC-1-GFP interacting protein in this experiment (Fig. 1C). A ratio *vs.* ratio plot of all the proteins that were identified and quantified in the pull-downs revealed that DOC-1-GFP interacts specifically with essentially all Mi-2/NuRD complex subunits that have been described in the literature to date, including both MBD2 and MBD3 (Fig. 1D and Supplementary Table 1‡). To further validate these findings we used nuclear extracts derived from DOC-1-GFP cells for pull-downs with GFP-nanotrap beads, which were then tested for the presence of Mi-2/NuRD complex subunits using western blotting (Fig. 1E). Consistent with our mass spectrometry data, all the Mi-2/NuRD complex subunits we tested were specifically enriched on DOC-1-GFP containing beads, whereas no enrichment could be observed on beads that were incubated with wild-type HeLa nuclear extract. Finally, to study the interaction between endogenous DOC-1 and MBD2/MBD3 we used an antibody against DOC-1 to precipitate the protein from HeLa nuclear extract (Fig. 1F, upper panel). MBD2 and MBD3 were specifically co-immunoprecipitated with endogenous DOC-1 (Fig. 1F, middle and lower panel). Taken together, these experiments show that DOC-1 interacts with the MBD2/NuRD and MBD3/NuRD complexes. Furthermore, no additional protein-protein interactions could be detected for DOC-1 in HeLa nuclear extracts by mass spectrometry, indicating that, at least in mammalian nuclei, the protein is primarily and exclusively associated with the Mi-2/NuRD complex.

DOC-1 and MBD2 specifically interact with methylated CpGs *in vitro*

DOC-1 is a small (115 aa) protein that does not carry an obvious methyl CpG binding motif. However, our biochemical data now clearly indicate that DOC-1 is part of the MBD2/NuRD complex. We therefore hypothesized that DOC-1 would indirectly bind to methylated DNA *via* an interaction with the MBD2/NuRD complex. To address this question we applied a DNA pull-down approach in combination with

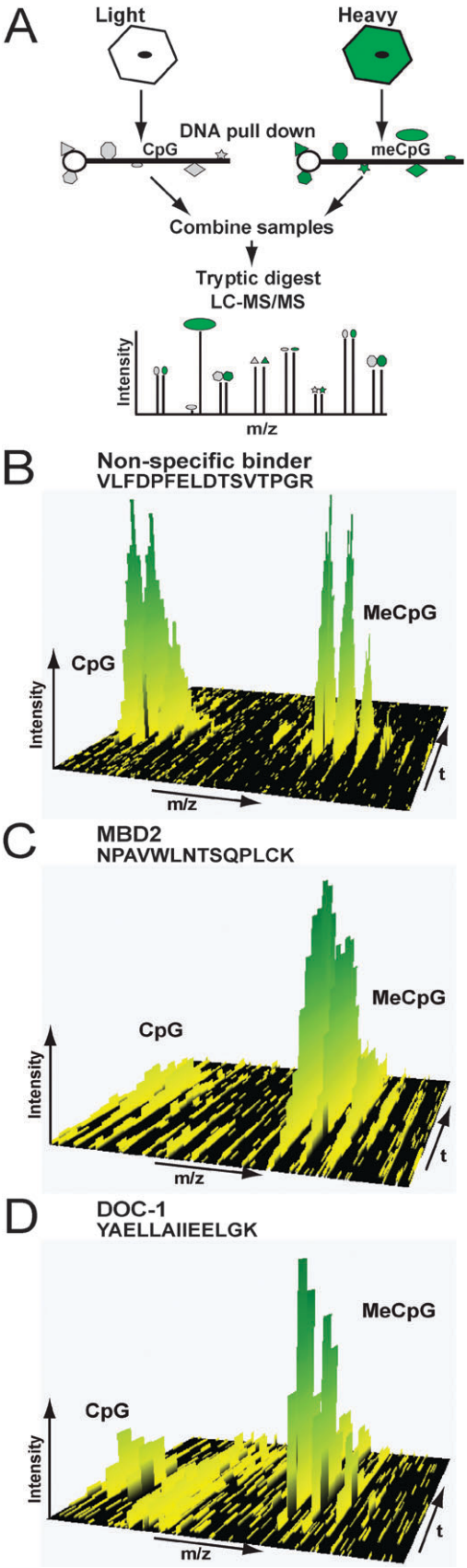

Fig. 2 DOC-1 and MBD2 are specifically recovered on methylated DNA *in vitro*. (**A**) Schematic representation of the experimental approach. (**B–D**) SILAC labeled nuclear extracts from U937 cells were incubated with non-methylated and methylated DNA immobilized on streptavidin conjugated dynabeads. Shown in the figures is the three dimensional representation of the MS signal for the indicated peptides and their relative binding to methylated *versus* non-methylated DNA. Note that for a background protein equal binding to methylated *versus* non-methylated DNA is observed (B), whereas for MBD2 (C) and DOC-1 (D), preferential methyl DNA binding is observed.

SILAC-based quantitative proteomics (Fig. 2A).[23,24] Methylated and non-methylated DNA bound to beads was incubated with heavy or light SILAC labeled U937 nuclear extracts, respectively. Following the pull-down and washes, beads from both pull-downs were combined and bound proteins were separated by one dimensional SDS PAGE. Proteins were subsequently digested with trypsin and peptide mixtures were measured by high-resolution LC-MS on an LTQ-Orbitrap hybrid mass spectrometer. Proteins that interact with DNA irrespective of DNA methylation or bind non-specifically to the beads are equally abundant in the light and heavy state and these proteins therefore show a one to one ratio in the mass spectrometer (Fig. 2B). In contrast, proteins specifically interacting with the meCpGs are more abundant in the heavy form and have a heavy/light ratio higher than one. As a validation of the approach and consistent with previous observations, MBD2, one of the five "classic" proteins containing a meCpG binding domain (MBD),[25] was identified as a specific meCpG binding protein in our quantitative DNA pull-down experiment (Fig. 2C and Supplementary Table 2‡). In agreement with our hypothesis we also identified DOC-1 as a meCpG interactor in our pull-down (Fig. 2D and Supplementary Table 2‡). MBD3, which does not bind specifically to methylated DNA[7,25] was not identified in this experiment. These results indicate that DOC-1 binds to methylated DNA *in vitro* as part of the MBD2/NuRD complex.

DOC-1 is a repressor of transcription and co-localizes with MBD2 *in vivo*

To further functionally characterize the DOC-1 protein, we performed luciferase reporter gene assays using a DOC-1-Gal4 containing construct (Fig. 3A). An expression construct containing the Gal4 DNA binding domain was used as a control and reveals the basal activity of the luciferase gene. Gal4-MBD2 and Gal4-Ash2L constructs were used as additional controls for repressive and activating activities, respectively. Consistent with previous observations and in line with its role in activation of transcription,[26] Gal4-Ash2L potentiated reporter gene activity.[27] In contrast to this and in agreement with its known biological function, Gal4-MBD2 was a repressor of transcription in this experimental set-up (Fig. 3B). DOC-1-Gal4 also conferred repression to the reporter gene in a dose dependent manner comparable to Gal4-MBD2, indicating that in this luciferase reporter assay, DOC-1 is a potent repressor of transcription.

To further study the DOC-1/Mi-2-NuRD interaction *in vivo* we performed immunofluorescence experiments. Mouse

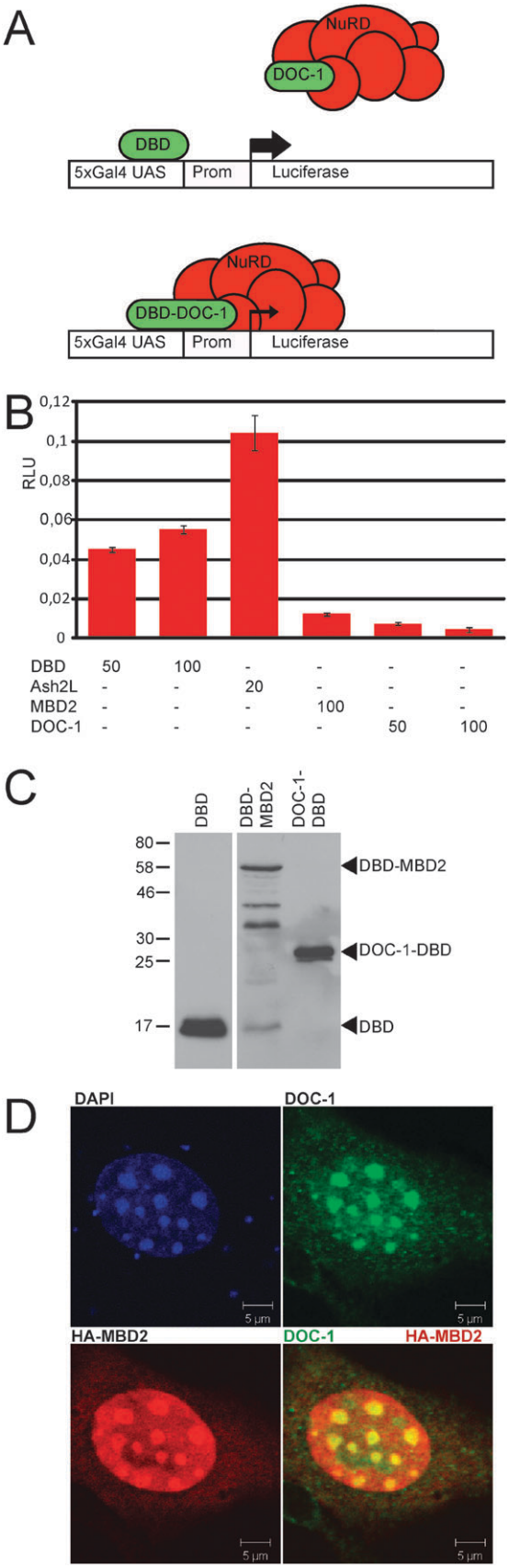

Fig. 3 DOC-1 is a repressor of transcription and co-localizes with MBD2 *in vivo*. (**A**) Schematic representation of the Gal4-luciferase assay. Gal4-DBD (DNA binding domain) binds to the Upstream Activating Sequence (UAS) in front of the reporter gene. Gal4-fusion proteins and their associated proteins can therefore be recruited to the TK promoter to exert their function. (**B**) Luciferase reporter gene assays with the indicated constructs. Transfection amounts are given in nanograms. (**C**) Anti-Gal4 western blot to confirm expression of the Gal4 fusion proteins that were used in Fig. 3B. (**D**) Confocal microscopy of NIH-3T3 cells that were transiently transfected with HA-MBD2 reveals co-localization of endogenous DOC-1 and HA-MBD2 in DAPI-dense regions in the nucleus.

NIH-3T3 cells were transfected with an HA tagged MBD2 construct, and a combination of a mouse monoclonal HA antibody with a rabbit polyclonal antibody against endogenous DOC-1 was used to visualize the proteins in the cells. Both proteins were predominantly found in DNA dense regions in the nucleus and showed substantial overlap, indicating that MBD2 and DOC-1 co-localize in mammalian nuclei *in vivo*. These DNA dense regions in mouse nuclei are known to be enriched for major and minor satellite repeats that are heterochromatic and transcriptionally silent.[28] It should be noted that DOC-1 interacts with both MBD2/NuRD and MBD3/NuRD, which are two distinct complexes. This may explain why MBD2 and DOC-1 do not co-localize completely.

Collectively, the biochemical and cell biological assays presented in this paper reveal that CDK2AP1/DOC-1 is a subunit of the Mi-2/NuRD complex and a repressor of transcription.

Discussion

In this paper we have provided compelling evidence that CDK2AP1/DOC-1 is a bona fide subunit of the Mi-2/NuRD complex. DOC-1 was first reported as a protein that is deleted in oral cancer and was subsequently described as a cyclin dependent kinase 2 associated protein. Our quantitative mass spectrometry data did not reveal an association between CDK2 and DOC-1 in nuclear extracts. Although we cannot exclude that DOC-1 interacts with CDK2 in specific physiological conditions in the cytoplasm, western blotting as well as confocal microscopy revealed that DOC-1 is predominantly nuclear and our quantitative mass spectrometry data show that in the nucleus it exclusively associates with the Mi-2/NuRD complex.

Interestingly, deletion of the Mi-2/NuRD subunit MBD2 in mice protects these mice from intestinal tumors.[29] This is in contrast to the pathology of DOC-1; reduced DOC-1 expression appears to be an inducer of malignant transformation.[16,30] In agreement with this, over-expression of DOC-1 in 293T cells results in a partial G1/S arrest,[17] whereas over-expression of MBD2 in 293T cells enhances cell proliferation (Xavier Le Guezennec and MV, unpublished data). Whether these observations are indicative of antagonistic functions for DOC-1 and MBD2 in the Mi-2/NuRD complex or hinting towards a cytoplasmic DOC-1 function related to CDK2 remains unclear at this point. To further study the potential interplay between DOC-1 and MBD2 in tumorigenesis it would be informative to cross MBD2 deficient mice with a DOC-1 knock-out strain and look at survival rates in polyposis challenge experiments. Alternatively, immortalized MBD2 deficient MEFs could be subjected to DOC-1 siRNA in colony formation assays to look at their proliferation.

Given its apparent general presence in both the MBD2/NuRD and MBD3/NuRD complexes, it is surprising that DOC-1 has not been identified by mass spectrometry previously in Mi-2/NuRD complex purifications. However, given the small size of DOC-1, the protein was not visualized by silver or coomassie stainings prior to LC-MS/MS analyses in a number of studies[3–6] and therefore may have escaped identification. Although our study has clearly established DOC-1 as a Mi-2/NuRD subunit, future research is required to elucidate the molecular function of the protein within the complex, its putative association with methylated promoters as a component of the MBD2/NuRD complex and its link to carcinogenesis.

Materials and methods

Cell culture

HeLa Kyoto, NIH-3T3 and HEK 293 cells were cultured in DMEM containing 10% Fetal Calf Serum, 2 mM Glutamine and 100 U/ml of Penicillin/Streptomycin (BioWhittaker), whereas U937 cells were cultured similarly in RPMI. The DOC-1-GFP BAC line was cultured in the presence of 400 μg ml^{-1} geneticin (G418) (Life Technologies/Gibco). For SILAC labeling experiments, DOC-1-GFP, wild-type HeLa and wild-type U937 cells were cultured in the presence of light and heavy lysine ($^{13}C_6{}^{15}N_2$, Isotec) (GFP pull-down) or light and heavy lysine and arginine ($^{13}C_6{}^{15}N_2$ and $^{13}C_6{}^{15}N_4$, Isotec) (DNA pull-down) for >8 doublings to ensure full incorporation of the heavy isotope prior to preparation of nuclear extracts.

GFP pull-downs

Nuclear extracts (prepared essentially as described in ref. 31) derived from DOC-1-GFP and wild-type HeLa cells (200–300 μg for western blot analyses and 1 mg for mass spectrometric analysis) were incubated with 10 μl of GFP nanotrap beads (Chromotek) for 90 min at 4 °C in binding buffer (PBS, 0.25% NP40, 0.5 mM DTT, 50 μg ml^{-1} ethidium bromide and complete protease inhibitors–EDTA (Roche)). Beads were washed extensively with binding buffer after which proteins were eluted using SDS PAGE loading buffer for western blot analyses or acidic glycine (0.1 M, pH 2.0) for subsequent mass spec analyses. The following antibodies were used for western blotting: MBD2 (Everest Biotech, EB07538); MBD3 (IBL, 3A3); RbAp46 (Abcam, 72457-100); RbAp48 (Abcam, 74188-100); HDAC1 (Santa Cruz Biotechnology, H51 sc-7872); HDAC2 (Santa Cruz Biotechnology, ACII sc-7899 -54); Gal4-DBD (Santa Cruz Biotechnology, RK5C1); GFP (Roche, 11814460001). A rabbit polyclonal antibody against recombinant full length DOC-1 was generated in-house.

Generation of anti-DOC-1 antibodies

A GST-DOC-1 fusion construct was created by ligating a DOC-1 cDNA-clone (IRATp970A0640D, RZPD-clone) into pGEX-2T. The DOC-1 cDNA was PCR-amplified using the following oligos: 5′CGCggatccATGTCTTACAAACCGAA-CTTGGC3′ (forward) and 5′CCGgaattcCTAGGATCTG-GCATTCCGTTC3′ (reverse). The amplified product was ligated into pGEX-2T using BamHI/EcoRI restriction sites. GST-DOC-1 protein was produced in *E. coli* BL21 (DE3) and purified using Glutathione Sepharose 4B-beads (GE Healthcare) according to standard procedures. The GST-tag was removed by thrombin cleavage and DOC-1 was subsequently isolated from preparative SDS PAGE gel and used for immunization of rabbits.

Co-immunoprecipitation

2 μl of DOC-1 antiserum or 2 μl pre-immune serum was immobilized on 30 μl protein A Dynabeads slurry (Invitrogen). Beads were then incubated with 50 μl HeLa nuclear extract (~5 mg ml^{-1}) in 150 μl binding buffer for 2.5 h at 4 °C. Beads were washed extensively with binding buffer after which bound proteins were eluted in SDS PAGE loading buffer and analyzed by western blotting for the presence of DOC-1, MBD2 and MBD3.

DNA pull-down

The following oligos were used for preparation of pull-down DNA: 5′aagcagacactggcaggtttCGGCGGGAGTCCGCGGG-ACCCTCCAGAAGAGCGG***CCGG***CGCCGTGACctaaggc-taaggctcata3′ (forward) and 5′tttatgagccttagccttagGTCACG-GCG***CCGG***CCGCTCTTCTGGAGGGTCCCGCGGACTC-CCGCCGaaacctgccagtgtctgc3′ (reverse), containing a sequence derived from the *GSTP1* CpG-island (in capitals), sites for primer annealing, and a methylation-sensitive restriction site (bold italic). PAGE purified oligos were annealed, phosphorylated and ligated, resulting in fragments with lengths ranging from 85 to 600 bp. Subsequently, biotinylation was performed by incorporation of biotin-14-dATP (Invitrogen) at the 3′end of the forward strand using Klenow Fragment (3′–5′exo-) (New England Biolabs). For the meCpG pull-down, DNA was methylated by M.SssI (New England Biolabs) and methylation was checked by methylation-sensitive digestion followed by quantitative PCR. 75 μl of Dynabeads MyOne Streptavidin C1 (Invitrogen) were incubated with 10 μg of DNA for 1 h at RT in DNA binding buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.1% NP40). After washing, the beads with coupled DNA were incubated with 400 μg U937 nuclear extract and 10 μg poly(dI-dC) competitor DNA (Sigma) for 2 h at 4 °C in protein binding buffer (150 mM NaCl, 50 mM Tris pH 8.0, 0.25% NP40, 0.5 mM DTT, and complete protease inhibitors–EDTA (Roche)). Beads were washed extensively and bound proteins were eluted in SDS PAGE loading buffer and processed for mass spec analyses.

Mass spectrometry

Proteins eluted from the GFP-nanotrap beads were neutralized using Tris (pH 8.5) and subsequently digested with LysC (Wako) using the FASP protocol.[32] Proteins captured during the DNA pull-down were separated by SDS PAGE and subjected to in-gel trypsin digestion as described.[27] Collected peptides were desalted using StageTips[33] and measured on an LTQ-Orbitrap mass analyzer essentially as described.[27] Raw data were analyzed using the MaxQuant software package.[22] The DOC-1-GFP pull-down ratio *vs.* ratio plot was generated using the open software package R.

Cloning

To generate a DOC-1-Gal4-DBD construct, the stop-codon between the HindIII cleavage site and the transcription start site in plasmid pCMV-DBD[34] was mutated into a glycine codon using primers 5′CCAAGCTTCC***G***GAAAGATGA-AGC3′ (forward) and 5′AGGTGACACTATA3′ (reverse). The point mutation in the forward primer is bold italic. The PCR product was ligated into the backbone vector. Full-length DOC-1 was amplified from a pCMX-DBD vector using primers(5′CCCAAGCTTATGTCTTACAAACCGAACTTG3′) and (5′CCCAAGCTTGGGATCTGGCATTCCGTTCC3′). This fragment was then ligated into the mutated pCMV-DBD vector, to obtain a C-terminal Gal4-DBD-fusion.

Luciferase assay

HEK293 cells were seeded in 12-well plates on day 1 and transfected on day 2 (when confluency was ~30–40%). Transfection was done in triplicates, using 1.5 μl Fugene6 reagent (Roche), 15 ng pCMV-Renilla, 200 ng 5xGal4-TK-luciferase and 50/100 ng pCMV DBD; 50/100 ng pCMV DOC-1-DBD; 100 ng pCMX DBD-MBD2 or 20 ng pGal4-Ash2L per well. Cells were lysed by applying 150 μl 1× Passive lysis buffer (Dual-luciferase assay kit (Promega)) and incubation for 20 min at RT. 50 μl lysate was used for measurement in a 96-well Berthold LB96V MicroLumat Plus luminometer.

Confocal immunofluorescence microscopy

NIH-3T3 cells were seeded on coverslips in 12-well plates. At ~40% confluency, cells were transfected with 1 μg stII-3HA-MBD2 plasmid[7] using PEI (Polysciences). At ~80% confluency cells were fixed with 4% v/v paraformaldehyde. Permeabilization was performed by incubation with 0.2% Triton X-100 in PBS for 5 min at RT. Cells were then blocked with 1% Bovine Serum Albumin (Sigma) in PBS supplemented with 0.1% Triton X-100 for 30 min and subsequently incubated with the primary antibodies (DOC-1 and HA, (Santa Cruz Biotechnology, 12xA5)) in blocking buffer for at least 1 h. This was followed by incubation with secondary antibodies (GαR Alexa 488 and GαM Alexa568 (Invitrogen)) for 1 h in blocking buffer. DNA was stained using 10 μg ml^{-1} DAPI (4′-6-diamidino-2-phenylindole). A Zeiss 510 Meta confocal microscope with a 63X/1.4 Oil DIC Plan-ApoChromat objective was used for microscopic analysis.

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References

1 P. B. Becker and W. Horz, ATP-dependent nucleosome remodeling, *Annu. Rev. Biochem.*, 2002, **71**, 247–273.
2 L. Ho and G. R. Crabtree, Chromatin remodelling during development, *Nature*, 2010, **463**, 474–484.
3 P. A. Wade, P. L. Jones, D. Vermaak and A. P. Wolffe, A multiple subunit Mi-2 histone deacetylase from Xenopus laevis

cofractionates with an associated Snf2 superfamily ATPase, *Curr. Biol.*, 1998, **8**, 843–846.
4 Y. Zhang, G. LeRoy, H. P. Seelig, W. S. Lane and D. Reinberg, The dermatomyositis-specific autoantigen Mi2 is a component of a complex containing histone deacetylase and nucleosome remodeling activities, *Cell*, 1998, **95**, 279–289.
5 J. K. Tong, C. A. Hassig, G. R. Schnitzler, R. E. Kingston and S. L. Schreiber, Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex, *Nature*, 1998, **395**, 917–921.
6 Q. Feng and Y. Zhang, The MeCP1 complex represses transcription through preferential binding, remodeling, and deacetylating methylated nucleosomes, *Genes. Dev.*, 2001, **15**, 827–832.
7 X. Le Guezennec, M. Vermeulen, A. B. Brinkman, W. A. Hoeijmakers, A. Cohen, E. Lasonder and H. G. Stunnenberg, MBD2/NuRD and MBD3/NuRD, two distinct complexes with different biochemical and functional properties, *Mol. Cell. Biol.*, 2006, **26**, 843–851.
8 S. A. Denslow and P. A. Wade, The human Mi-2/NuRD complex and gene regulation, *Oncogene*, 2007, **26**, 5433–5438.
9 Y. Wang, H. Zhang, Y. Chen, Y. Sun, F. Yang, W. Yu, J. Liang, L. Sun, X. Yang, L. Shi, R. Li, Y. Li, Y. Zhang, Q. Li, X. Yi and Y. Shang, LSD1 is a subunit of the NuRD complex and targets the metastasis programs in breast cancer, *Cell*, 2009, **138**, 660–672.
10 J. Kim, S. Sif, B. Jones, A. Jackson, J. Koipally, E. Heller, S. Winandy, A. Viel, A. Sawyer, T. Ikeda, R. Kingston and K. Georgopoulos, Ikaros DNA-binding proteins direct formation of chromatin remodeling complexes in lymphocytes, *Immunity*, 1999, **10**, 345–355.
11 S. M. Lauberth and M. Rauchman, A conserved 12-amino acid motif in Sall1 recruits the nucleosome remodeling and deacetylase corepressor complex, *J. Biol. Chem.*, 2006, **281**, 23922–23931.
12 W. Hong, M. Nakazawa, Y. Y. Chen, R. Kori, C. R. Vakoc, C. Rakowski and G. A. Blobel, FOG-1 recruits the NuRD repressor complex to mediate transcriptional repression by GATA-1, *EMBO J.*, 2005, **24**, 2367–2378.
13 C. P. Tan and S. Nakielny, Control of the DNA methylation system component MBD2 by protein arginine methylation, *Mol. Cell. Biol.*, 2006, **26**, 7224–7235.
14 J. Kehle, D. Beuchle, S. Treuheit, B. Christen, J. A. Kennison, M. Bienz and J. Muller, dMi-2, a hunchback-interacting protein that functions in polycomb repression, *Science*, 1998, **282**, 1897–1900.
15 R. Todd, J. McBride, T. Tsuji, R. B. Donoff, M. Nagai, M. Y. Chou, T. Chiang and D. T. Wong, Deleted in oral cancer-1 (doc-1), a novel oral tumor suppressor gene, *FASEB J.*, 1995, **9**, 1362–1370.
16 Z. Yuan, T. Sotsky Kent and T. K. Weber, Differential expression of DOC-1 in microsatellite-unstable human colorectal cancer, *Oncogene*, 2003, **22**, 6304–6310.
17 S. Shintani, H. Ohyama, X. Zhang, J. McBride, K. Matsuo, T. Tsuji, M. G. Hu, G. Hu, Y. Kohno, M. Lerman, R. Todd and D. T. Wong, p12(DOC-1) is a novel cyclin-dependent kinase 2-associated protein, *Mol. Cell. Biol.*, 2000, **20**, 6300–6307.
18 A. M. Deshpande, Y. S. Dai, Y. Kim, J. Kim, L. Kimlin, K. Gao and D. T. Wong, Cdk2ap1 is required for epigenetic silencing of Oct4 during murine embryonic stem cell differentiation, *J. Biol. Chem.*, 2009, **284**, 6043–6047.
19 Y. Bao and X. Shen, SnapShot: chromatin remodeling complexes, *Cell*, 2007, **129**, 632.e1.
20 I. Poser, M. Sarov, J. R. Hutchins, J. K. Heriche, Y. Toyoda, A. Pozniakovsky, D. Weigl, A. Nitzsche, B. Hegemann, A. W. Bird, L. Pelletier, R. Kittler, S. Hua, R. Naumann and M. Augsburg, *et al.*, BAC TransgeneOmics: a high-throughput method for exploration of protein function in mammals, *Nat. Methods*, 2008, **5**, 409–415.
21 U. Rothbauer, K. Zolghadr, S. Muyldermans, A. Schepers, M. C. Cardoso and H. Leonhardt, A versatile nanotrap for biochemical and functional studies with fluorescent fusion proteins, *Mol. Cell. Proteomics*, 2008, **7**, 282–289.
22 J. Cox and M. Mann, MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification, *Nat. Biotechnol.*, 2008, **26**, 1367–1372.
23 G. Mittler, F. Butter and M. Mann, A SILAC-based DNA protein interaction screen that identifies candidate binding proteins to functional DNA elements, *Genome Res.*, 2009, **19**, 284–293.
24 F. Butter, D. Kappei, F. Buchholz, M. Vermeulen and M. Mann, A domesticated transposon mediates the effects of a single-nucleotide polymorphism responsible for enhanced muscle growth, *EMBO Rep.*, 2010, **11**, 305–311.
25 B. Hendrich and A. Bird, Identification and characterization of a family of mammalian methyl-CpG binding proteins, *Mol. Cell. Biol.*, 1998, **18**, 6538–6547.
26 A. Shilatifard, Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation, *Curr. Opin. Cell Biol.*, 2008, **20**, 341–348.
27 M. Vermeulen, K. W. Mulder, S. Denissov, W. W. Pijnappel, F. M. van Schaik, R. A. Varier, M. P. Baltissen, H. G. Stunnenberg, M. Mann and H. T. Timmers, Selective anchoring of TFIID to nucleosomes by trimethylation of histone H3 lysine 4, *Cell*, 2007, **131**, 58–69.
28 M. Guenatri, D. Bailly, C. Maison and G. Almouzni, Mouse centric and pericentric satellite repeats form distinct functional heterochromatin, *J. Cell Biol.*, 2004, **166**, 493–505.
29 O. J. Sansom, J. Berger, S. M. Bishop, B. Hendrich, A. Bird and A. R. Clarke, Deficiency of Mbd2 suppresses intestinal tumorigenesis, *Nat. Genet.*, 2003, **34**, 145–147.
30 M. G. Choi, T. S. Sohn, S. B. Park, Y. H. Paik, J. H. Noh, K. M. Kim, C. K. Park and S. Kim, Decreased expression of p12 is associated with more advanced tumor invasion in human gastric cancer tissues, *Eur. Surg. Res.*, 2009, **42**, 223–229.
31 J. D. Dignani, R. M. Lebovitz and R. G. Roeder, Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei, *Nucleic Acids Res.*, 1983, **11**, 1475–1489.
32 J. R. Wisniewski, A. Zougman, N. Nagaraj and M. Mann, Universal sample preparation method for proteome analysis, *Nat. Methods*, 2009, **6**, 359–362.
33 J. Rappsilber, M. Mann and Y. Ishihama, Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips, *Nat. Protoc.*, 2007, **2**, 1896–1906.
34 C. G. Zwartjes, S. Jayne, D. L. van den Berg and H. T. Timmers, Repression of promoter activity by CNOT2, a subunit of the transcription regulatory Ccr4-not complex, *J. Biol. Chem.*, 2004, **279**, 10848–10854.

