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DNA binding of the p21 repressor ZBTB2 is inhibited by cytosine hydroxymethylation

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ABSTRACT

Recent studies have demonstrated that the modified base 5-hydroxymethylcytosine (5-hmC) is detectable at various rates in DNA extracted from human tissues. This oxidative product of 5-methylcytosine (5-mC) constitutes a new and important actor of epigenetic mechanisms. We designed a DNA pull down assay to trap and identify nuclear proteins bound to 5-hmC and/or 5-mC. We applied this strategy to three cancerous cell lines (HeLa, SH-SY5Y and UT7-MPL) in which we also measured 5-mC and 5-hmC levels by HPLC-MS/MS. We found that the putative oncoprotein Zinc finger and BTB domain-containing protein 2 (ZBTB2) is associated with methylated DNA sequences and that this interaction is inhibited by the presence of 5-hmC replacing 5-mC. As published data mention ZBTB2 recognition of p21 regulating sequences, we verified that this sequence specific binding was also alleviated by 5-hmC. ZBTB2 being considered as a multifunctional cell proliferation activator, notably through p21 repression, this work points out new epigenetic processes potentially involved in carcinogenesis.

1. Introduction

5-Methylcytosine (5-mC) is one of the most extensively studied epigenetic marks [1]. One of its oxidation products, 5-hydroxymethylcytosine (5-hmC), was identified in the 1950s in bacteriophages [2]. This modified base led to a few publications before 2009 when two groups reported the presence of 5-hmC in mouse DNA extracted from embryonic stem cells [3] and brain [4]. Both studies opened the way for an impressive amount of research demonstrating that 5-hmC levels regulation plays a role in stem cell pluripotency, embryo development or carcinogenesis [5].

Some of the key regulation mechanisms involving DNA elements containing 5-mC and 5-hmC are governed by their interactions with proteins [1,6]. As an example, the TET2 (Ten Eleven Translocation 2) oxygenase recognizes 5-mC and converts it to 5-hmC. It is also well known that hypermethylation of CpG islands located in gene promoter regions is associated with transcription abrogation. This can be explained by 5-mC directly inhibiting DNA binding of transcription factors. Another process is the intervention of methyl-CpG binding proteins (MBPs) which recruit chromatin modifying proteins such as histone deacetylases also blocking transcription. The first identified MBP was MeCP2 [7]. A wide range of data about protein interactions with methylated DNA is available [6]. By contrast, elements about protein interactions with 5-hmC are still scarce. It has been demonstrated that DNA binding of some MBPs such as MeCP2 [8] or ZFP57 [9] is inhibited by the presence of 5-hmC unlike Uhrf1. This DNA methylation maintenance factor is indeed able to recognize DNA sequences containing 5-mC as well as those containing 5-hmC [10].

The aim of this work is to identify new DNA interacting proteins sensitive to the hydroxymethylation status of CpG sequences. We first controlled the presence of 5-hmC in our cellular models. Its level was measured by HPLC coupled to tandem mass spectrometry (HPLC–MS/MS) subsequently to DNA extraction and digestion. We next developed a pull down assay to trap proteins associated to 5-mC and/or 5-hmC which were then identified by proteomics. Results were checked by Western-blotting and, finally, the selected protein was produced, purified and used along with modified oligodeoxynucleotides (ODNs) of biologically relevant sequences to check the interaction by ElectroMobility Shift Assay (EMSA).

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2. Materials and methods

2.1. Cellular models

SH-SY5Y, UT7-MPL and HeLa extracts were prepared in our laboratory. Cells were grown at 37 °C under 5% CO_2 in MEM Eagle medium supplemented with 2 mM L-glutamine, 10% (v/v) fetal calf serum (FCS), 50 μg/ml penicillin–streptomycin (Invitrogen) and 5 ng/ml GM-CSF (Sigma) for UT7-MPL cells; RPMI 1640 medium supplemented with 2 mM L-glutamine, 10% (v/v) FCS and 800 μg/ml geneticin (Invitrogen) for HeLa cells; 1:1 mixture of EMEM (Sigma–Aldrich) and Ham F12 (Invitrogen) with 10% (v/v) FCS, 25 μg/mL penicillin–streptomycin (Invitrogen) and 1% MEM non essential amino acids (Invitrogen) for SH-SY5Y cells. Cells were passaged or harvested at 80% confluence. Commercial HeLa nuclear protein extracts from CIL Biotech were also used.

2.2. 5-mdC and 5-hmdC quantification

HPLC coupled through electrospray ionization to MS/MS (TSQ Quantum Ultra, Thermo Fisher Scientific) [11] was used to detect and quantify simultaneously 2′-deoxycytosine (dC), 5-methyl-2′-deoxycytosine (5-mdC) and 5-hydroxymethyl-2′-deoxycytosine (5-hmdC) in the so-called multiple reaction monitoring mode (MRM) using transitions, 228 → 112, 242 → 126 and 258 → 142, respectively (Supporting information – Fig. S1). Conditions for DNA extraction, digestion and HPLC separation were similar to those described in details previously [12]. 5-hmdC was synthesized by photosensitization of 5-mdC as reported [13]. Quantification was performed by external calibration.

2.3. Trapping of proteins bound to 5-mC or 5-hmC

2.3.1. Probe synthesis

The procedure for the production of ODNs containing modified cytosines was derived from [14]. Probe synthesis, probe control and fixation on magnetic beads are described as Supporting Information.

2.3.2. DNA pull down (adapted from [15])

Nuclear extracts were prepared as previously described [16]. Two experimental conditions named DPD1 and DPD2 were used. Protein binding buffer (buffer A-DPD1 (40 mM HEPES-KOH pH 7.8, 50 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT) or buffer A-DPD2 (50 mM NaCl, 50 mM Tris–HCl pH 8.0, 0.25% NP40, 0.5 mM DTT and complete protease inhibitors-EDTA (Roche)) was used to equilibrate functionalised beads. Nuclear extracts (in exception of the commercial one) were dialysed against protein binding buffer using amicon-Ultra-0.5, 3 kDa (Millipore). In a 100 μL final volume, 80 μg of functionalised beads, 120 μg of fish sperm DNA (Roche), and 100 μg of nuclear extracts were mixed in protein binding buffer. Samples were incubated under shaking for 45 min at 37 °C (DPD1) or 2 h at 4 °C (DPD2). Supernatant was eliminated using a magnetic tray, and beads were rinsed twice with 100 μL of buffer B-DPD1 (150 mM NaCl, 20 mM HEPES-KOH pH 7.8, 5 mM $MgCl_2$, 0.025% Nonidet-P-40) or buffer A-DPD2. Proteins were finally released in 20 μL of Laemmli buffer (Tris–HCl pH 6.8 25 mM, SDS 2%, glycerol 4%, β-mercaptoethanol 5%, bromophenol blue 0.0008%) by heating at 90 °C for 10 min.

2.4. Proteomic analyses

Protein separation and digestion as well as peptide analysis using nano-liquid chromatography coupled to MS/MS (LTQ-Orbitrap velos) were performed as previously described [17]. Peptides were identified using Mascot software (v.2.4, Matrix Science) and the Uniprot database (*Homo sapiens* taxonomy). The IRMa software [18] was used to filter the results: conservation of rank 1 peptides, peptide identification FDR < 1% (as calculated by employing the reverse database strategy), and minimum of 1 specific peptide per identified protein group. Identified protein sets were then compared using hEIDI software, as described in [17].

2.5. Western blot

Proteins obtained by pull down were separated by SDS–PAGE, transferred to a nitrocellulose membrane and analyzed by Western blot using the rabbit polyclonal antibody anti-ZBTB2 (Abnova PAB16678). Secondary antibody was a monkey anti-rabbit coupled to horseradish-peroxydase (GE Healthcare). ECL Plus and Hyperfilm ECL (GE Healthcare) were used to detect the bands.

2.6. Production of recombinant proteins

2.6.1. ZF-ZBTB2 recombinant protein

To prepare recombinant HIS-ZF-ZBTB2, the plasmid PUC-HIS-ZFZBTB2 containing the cDNA fragment encoding the zinc fingers (amino acids 254–468) domain of ZBTB2 in fusion with an N-terminal MAHHHHHHGHHHQLENLYFQG tag containing a tobacco etch virus (TEV) protease cleavage site was synthesised by Eurogentec.

2.6.2. MeCP2 recombinant protein

The plasmid pet30bhMECP2 (76–167) used to purify the MBD domain of MeCP2 was a gift from Bird [19].

Details about ZBTB2 and MeCP2 production and purification are provided in Supporting information.

2.7. EMSA

Two probes were used: the "p21 probe" (as described previously [20]) (5′-GAT**C**GGG**C**GGGG**C**GGTTGTATATCA-3′) which corresponds to Sp1 response element on the p21 gene proximal promoter and the "DPD probe" (5′-TATATTAACGTTACGT ATACGTTTA-3′) which corresponds to a fragment of the pull down sequence. Each probe contains either Cytosine (p21-C and DPD-C), 5-mC (p21-mC and DPD-mC) or 5-hmC (p21-hmC and DPD-hmC). Please see Supporting information for synthesis and labelling procedures.

Binding reaction between recombinant protein (ZF-ZBTB2 or MeCP2) and radiolabeled probes were carried out for 30 min at 25 °C in a final volume of 20 μL. The binding buffer was 20 mM HEPES pH 7.9, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 30 mM KCl, 0.5 mM DTT, 0.01% Tween 20 and 10 μg/ml polydIdC (only for the complex with ZF-ZBTB2). Complexes were resolved by non-denaturing electrophoresis on a 4% (ZF-ZBTB2) or 12% (MeCP2) polyacrylamide-Tris-acetate-EDTA gel.

3. Results

3.1. Measurement of 5-mC and 5-hmC

Screening for DNA binding proteins was performed in nuclear extracts isolated from 3 cell lines: HeLa (cervical cancer), SH-SY5Y (neuroblastoma) and UT7-MPL (megakaryoblastic leukemia). The level of cytosine, 5-mC and 5-hmC (actually their nucleosides) was quantified by HPLC-MS/MS ($n = 4$ independent measurements for HeLa and UT7-MPL cell lines, $n = 2$ for SH-SY5Y cells). The amount of 5-mC represented 1.46 ± 0.16%, 2.06 ± 0.09% and 1.80 ± 0.24% of cytosines in HeLa, SH-SY5Y and UT7-MPL cells,

respectively. The levels of 5-hmC were respectively of 1.2 ± 0.2, 2.1 ± 0.1 and 0.9 ± 0.1 modifications per 10^6 cytosines in HeLa, SH-SY5Y and UT7-MPL cells. These experiments confirmed that 5-hmC is present at quantifiable rates in these cellular models even if levels are low in comparison with rates obtained using similar methods in human brain tissues (over 1000 5-hmC per 10^6 cytosines) [21].

3.2. Pull down of proteins interacting with 5-mC or 5-hmC

We next optimized a ligand fishing tool to pull down proteins bound to 5-mC or 5-hmC sequences. Associated to proteomic analysis, this strategy has already been applied to find proteins recognizing epigenetic modifications [22–24] or DNA lesions such as oxidative damage or cisplatin induced crosslinks [15,25,26]. The principle of our system is depicted in Fig. 1. The first step consists in synthetizing modified DNA probes. We produced them using PCR (Polymerase Chain Reaction) by introducing dCTP (2′-deoxycytidine 5′-triphosphate), 5-mdCTP (5-methyl-2′-deoxycytidine 5′-triphosphate) or 5-hmdCTP (5-hydroxymethyl-2′-deoxycytidine 5′-triphosphate) to obtain 3 baits containing respectively cytosine, 5-mC or 5-hmC [14]. These PCR products were amplified using biotinylated primers allowing their grafting on streptavidin coated magnetic beads. We used PCR products in order to include several DNA modifications within a reasonably long probe. Indeed, shorter ODN baits containing few modified cytosines would retain lower quantities of proteins hence affecting the sensitivity of our method. This system was then exposed to nuclear extracts. Two incubation buffers and conditions (including different salt concentrations, temperatures, incubation times) were tested in order to enlarge the panel of retained proteins. Four distinct subproteomes were obtained for each experiment: proteins released from beads without DNA probe (to exclude proteins non specifically adsorbed) and proteins released from C, 5-mC and 5-hmC containing baits. Candidates were identified using shotgun proteomics and comparison of proteins identified in the different analyzed sample types. The most significant result was the reproducible identification of ZBTB2 (Zinc finger and BTB domain-containing protein 2) trapped on C and 5-mC containing probes, while systematically missing on 5-hmC baits (Table 1). This was observed for the three cell lines and did not vary with the different conditions of baits exposure to nuclear extracts. We confirmed this proteomics identification of ZBTB2 by analyzing proteins isolated from the same traps using Western-blot (Fig. 2). Other MBPs were observed using this pull down strategy although they were not identified in all cell lines and experimental conditions. MeCP2 was trapped on the 5-mC bait in UT7-MPL cells and we confirmed in HeLa cells that uhrf1 is able to bind 5-mC as well as 5-hmC containing DNA (Table 1). Differences in the identity of trapped proteins throughout the different cell extracts can, at least partly, be explained by differences in protein expressions in our cellular models. The expression of MeCP2 is for example low in HeLa cells [27].

3.3. Study of ZBTB2 interactions with 5-mC and 5-hmC by EMSA

The aim of these gel shift assays was to further validate results previously obtained by DNA pull down, proteomics and Western-blot. ZBTB2 is a potential cell cycle modulator through a negative regulation of the ARF-HDM2-p53-p21 pathway [20]. One of the mechanisms may involve *p21* transcription repression by ZBTB2 binding on the proximal promoter GC-box 5/6 which should normally be recognized by the transcription factor Sp1. We therefore designed two types of probes: ODNs mimicking one portion of DNA baits used in the pull down assay (later referred to "random ODNs") and ODNs containing a sequence taken from the GC-box 5/6 found in the p21 promoter region (later referred to "*p21* ODNs"). Each ^{32}P labeled probe was synthetized into 5 versions: ODNs containing only C, ODNs containing 1 or 3 5-mC and ODNs containing 1 or 3 5-hmC. The protein that was produced and purified to perform EMSA was the N-terminal zinc finger domain of ZBTB2. In order to compare ZBTB2 EMSA results with those of a known MBP, the methyl-CpG binding domain (MBD) of MeCP2 was also produced and submitted to the same gel shift assays.

EMSA confirmed that the zinc finger domain of ZBTB2 is able to bind ODNs containing C and 5-mC, this interaction being inhibited by the introduction of 5-hmC (Fig. 3). Electrophoretic profiles were slightly different according to the sequence context: binding of ZBTB2 on probes containing three 5-mC was demonstrated on the *p21* ODNs and on random ODNs whereas interaction with sequences containing only C was only visible with *p21* ODNs. We can draw the hypothesis that the affinity of ZBTB2 for the *p21* sequence is stronger than for the random sequence, trapping on this latter DNA fragment (in EMSA and pool down assays) being explained by its high content in CpG dinucleotides. Parallel experiments performed with MeCP2 MBD showed as previously published that this protein recognizes DNA substrates containing

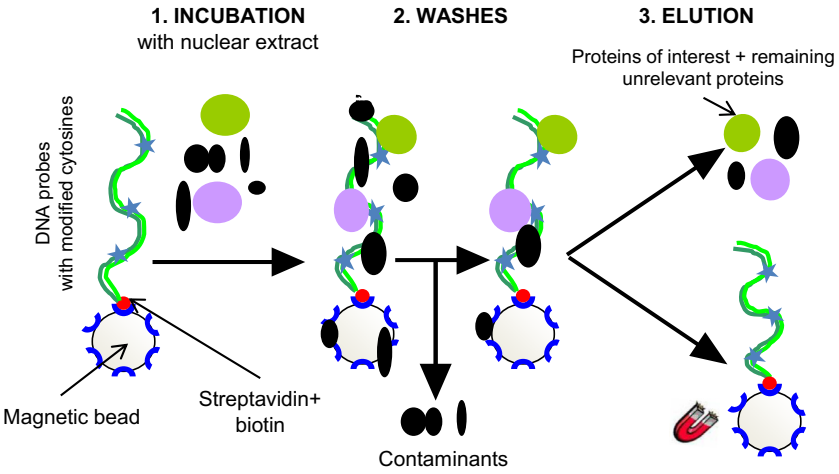

Fig. 1. Pull down principle. DNA probes contain C, 5-mC or 5-hmC are immobilized on magnetic beads through biotin/streptavidin interactions. After incubation with nuclear extracts and washing steps, proteins are eluted directly in Laemmli buffer before analysis by proteomics or Western-blot. Control conditions using DNA without epigenetic modifications and beads without probes limit the identification of unrelevant proteins.

Table 1
Analysis of proteins obtained through pull down. Selection of proteins identified by nanoHPLC-MS/MS. ZBTB2 has systematically been found on C and 5-mC baits but was absent from 5-hmC containing DNA. Proteomic screening with HeLa extracts was performed twice, 2 Mascot scores and peptide numbers are therefore indicated.

	DNA pull down probes							
	Beads w/o DNA		C		5-mC		5-hmC	
Protein name	Mascot score	Peptides number	Mascot score	Peptides number	Mascot score	Peptides number	Mascot score	Peptides number
Commercial HeLa nuclear extract; experimental conditions DPD1								
ZBTB2	–	–	249–186	5–4	102–215	3–4	–	–
Commercial HeLa nuclear extract; experimental conditions DPD2								
ZBTB2	–	–	390–732	7–11	309–513	6–7	–	–
MBD2	–	–	–	–	183–188	3–3	–	–
UHRF1	–	–	–	–	236	4	672	11
HeLa nuclear extract; experimental conditions DPD2								
ZBTB2	Not analysed		Not analysed		573	9	–	–
SH-SY5Y nuclear extract; experimental conditions DPD2								
ZBTB2	–	–	527	8	88	2	–	–
UT7-mpl nuclear extract; experimental conditions DPD2								
ZBTB2	–	–	750	11	932	12	–	–
MeCP2	–	–	–	–	143	3	–	–

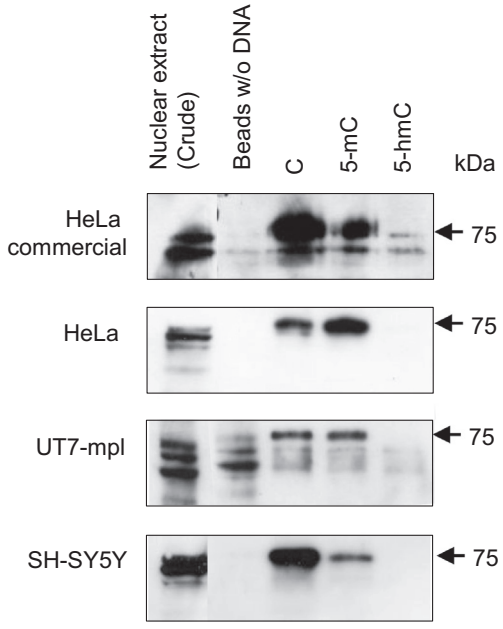

Fig. 2. Analysis of proteins obtained through pull down. Western-blot confirmation of proteomics regarding ZBTB2: inhibition of DNA binding related to the presence of 5-hmC in DNA probes.

5-mC with a stronger affinity than those containing 5-hmC [8,10,28] (a weak shift can be observed with random ODNs containing 5-hmC). In contradiction to this observation, a significant binding of MeCP2 on 5-hmC has recently been highlighted in brain [29]. This interaction may vary according to cellular models. This discrepancy may also be due to different MeCP2 fragments used for *in vitro* gel shift studies. We also observed that the shift using MeCP2 MBD was obtained using lower quantities of DNA and protein in comparison with ZBTB2 zinc finger domain. These data showed that the affinity of ZBTB2 for the tested DNA sequences is weaker than the affinity demonstrated by MeCP2.

4. Discussion

The goal of this study was to identify DNA binding proteins sensitive to cytosine hydroxymethylation. Our tool consisted in associating proteomics to a pull down assay based on beads grafted with PCR products containing C, 5-mC or 5-hmC. When interpreting data extracted from these strategies, one has to keep in mind that they can not provide an exhaustive list of all proteins in close contact with DNA modifications. Indeed, the identity of trapped proteins can vary according to parameters such as buffers stringency, crosslinking of proteins on DNA probes, DNA sequence context, temperature, duration of DNA/extract contact or nature of the biological model [26]. However, even if not exhaustive, these approaches demonstrated their usefulness by highlighting new interactions between proteins and DNA modifications. As an illustration, these assays demonstrated that the complex PTW/PP [15] was able to bind cisplatin induced DNA crosslinks. More recently, this strategy was applied to 5-mC, 5-hmC and its further oxidation products 5-formylcytosine and 5-carboxylcytosine. Biological models were mouse embryonic stem cells (mESC), neuronal progenitor cells and adult mouse brain tissues [24]. In our hands, screening experiments performed in human cancer cells pointed out ZBTB2. We decided to focus our attention on this candidate because: (i) this protein was the only one reliably identified in all experimental conditions tested as being able to bind 5-mC rich sequences but not their 5-hmC rich counterparts, (ii) it has recently been characterized as a regulator of *p21* transcription that may imply an interesting role in tumor biology [20].

ZBTB2 belongs to the BTB/POZ (Broad complex, Tramtrak, Bric à brac/Pox virus and zinc finger) family of transcription factors. To date, 3 members of this family have already been recognized as MBPs able to block transcription: Kaiso/ZBTB33 [30], ZBTB4 and ZBTB38 [31]. In addition to their N-terminal BTB/POZ domain involved in protein/protein interactions, these 3 proteins contain in their N terminal parts Kruppel-like zinc fingers known to bind a consensus Kaiso binding site and methylated DNA. Published data about ZBTB2 are limited to few studies indicating its capacity to repress the ARF-HDM2-p53-p21 pathway [20]. One of the mechanisms is a Sp1/ZBTB2 binding competition for GC-boxes 5/6 contained in p21 regulating sequences. ZBTB2 is also able to compete with p53 for p53 binding elements found in p21 regulating sequences. ZBTB2 also affects cell cycle control by directly interacting with Sp1 and p53 proteins. It was recently postulated that *ZBTB2* acts as an oncogene in human gastric cancers and that its repressor microRNA miR-149 may be considered as a tumor suppressor [32]. Finally, *ZBTB2* may play a role in the development of chronic myeloid leukemia as a genome-wide association study highlighted a chromosomal locus corresponding to this gene [33].

According to our results, ZBTB2 association with DNA is weakly affected by cytosine methylation but rather by its further oxidation

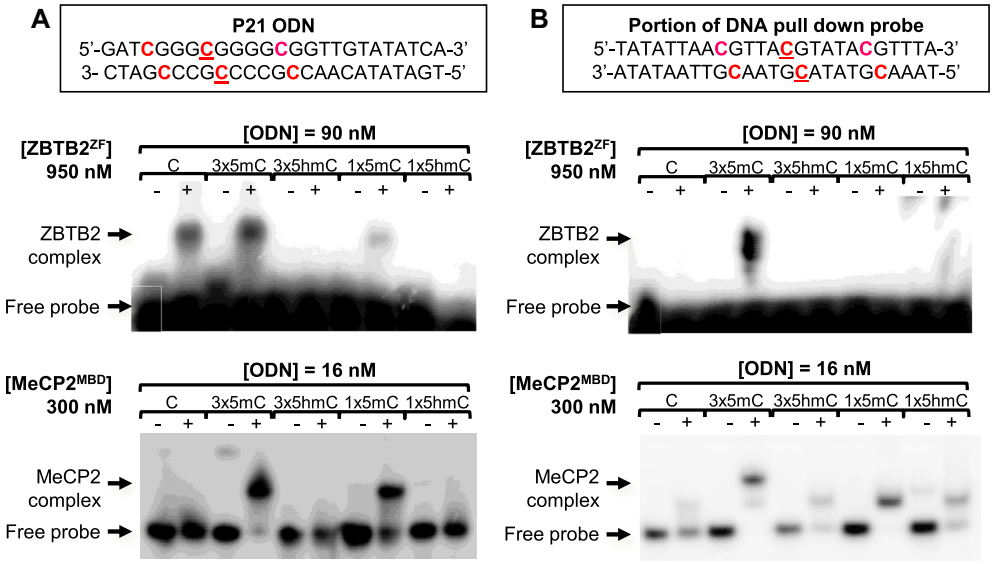

Fig. 3. Interaction of ZBTB2ZF and MeCP2 with ODNs containing C, 5-mC or 5-hmC. Cytosines in red are replaced by 5-mC or 5-hmC in EMSA probes containing 3 methylated or hydroxymethylated positions. Underscored cytosines are replaced by 5-mC or 5-hmC in probes containing only one methylated or hydoxymethylated position. (A) Results obtained with an ODN containing a sequence taken from the GC-box 5/6 found in the *p21* promoter region. (B) Results obtained with an ODN containing one portion of probes used in pull down assays.

into 5-hmC. In mESC, another pull down study showed a different result, ZBTB2 binding being abolished by 5-mC in comparison with sequences containing cytosines [24]. As mentioned in this article, this may be due to significant variations of epigenetic DNA interactomes observed between different cell types. It is worth noting that mechanisms of ZBTB2 binding to methylated DNA are probably different from those of Kaiso, ZBTB4 and ZBTB38. Indeed, ZBTB2N-terminal zinc finger sequence does not match with Kaiso-like zinc fingers. Additionally, ZBTB2 recognizes un-methylated GC-box 5/6 which sequence is not containing Kaiso binding sites.

Published experiments seem to indicate that ZBTB2 can be considered as an oncoprotein, for example through cell proliferation stimulation by p21 repression. If hydroxymethylation inhibits this ZBTB2 deleterious property, one may assume that 5-hmC protects the cell from this carcinogenic process. This type of mechanism may improve our understanding of IDH1/2 inhibitors mode of action [34]. These drug candidates, which are potentially interesting for IDH1/2 mutated patients, could increase oxoglutarate availability and TET enzymes activity, therefore enhancing site specific hydroxymethylation including at *p21* regulatory sites. Hypothesis mentioned here of course need studies exploring 5-hmC impact on DNA binding proteins competing with ZBTB2 such as Sp1 for example. This example highlights that 5-hmC as well as 5-mC has to be taken into account when studying epigenetic regulation of key cellular mechanisms involved in carcinogenesis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.122.

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