A Genetically Encoded FRET Probe to Detect Intranucleosomal Histone H3K9 or H3K14 Acetylation Using BRD4, a BET Family Member

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ABSTRACT: Acetylation is a well-characterized histone modification, which plays important roles in controlling epigenetic gene expression, and its malfunction is tightly associated with cancer. By taking advantage of the specific binding of BRD4 to acetylated lysine residues, we developed a FRET-based probe for visualizing histone H3 acetylation in living cells. BRD4, a protein known to be involved in acute myeloid leukemia and nuclear protein in testis midline carcinoma, recognizes the acetylation of histone H3 via its bromodomains. The probe exhibited a significant change in FRET signaling that was dependent on histone H3 acetylation. Mutagenesis studies revealed that an increase in the emission ratio reflected the acetylation of either K9 or K14 of histone H3 within the probe. Since BRD4 has increasingly drawn attention as a new anticancer drug target, we demonstrated that the developed fluorescent probe will also serve as a powerful tool to evaluate BRD4 inhibitors in living cells.

BRD4 belongs to the bromodomain and extra C-terminal domain (BET) protein family (which includes BRD2, BRD3, BRD4, and BRDT) that binds to acetylated histone and functions in coordinating the cell cycle and transcription. Recent research has established that BRD4 is a potential therapeutic target in nuclear protein in testis (NUT) midline carcinoma (NMC) and acute myeloid leukemia (AML). NMC has been characterized as an aggressive form of human squamous carcinoma driven by a chromosomal translocation, t(15;19). Such translocations result in the tandem fusion of the BRD4 bromodomains on chromosome 19 to the NUT gene on chromosome 15. The resultant BRD4-NUT oncoprotein plays an essential role in maintaining the characteristic proliferation of NMC cells. In an AML mouse model, an advanced RNAi screening approach revealed that BRD4 may be a promising target for AML. These results suggest that inhibitors of BRD4 have potential as therapeutic agents for the treatment of NMC and AML.

Histone modifications, such as acetylation and methylation, have been implicated in regulating diverse biological processes, including gene expression, DNA replication, and genome stability. The acetylation of lysine residues within core histones is tightly controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs). CBP and p300, which belong to the p300/CBP family, are examples of well-studied HATs. While p300 selectively acetylates histones at H3K14, H3K18, H4K5, and H4K8, CBP specifically acetylates H3K14, H3K18, H3K23, H4K8, and H4K12 residues. Deletion of GCN5/PCAF in cells dramatically reduces the acetylation of histone H3K9. The detection of site-specific
histone acetylation is principally mediated by bromodomain proteins. 

FRET has previously been used for detecting dynamic intracellular protein modifications. There are other strategies for detecting dynamic protein modifications in living cells. Bimolecular fluorescence or split luciferase complementation can be used to detect protein–protein interactions in living cells and mice. This method is used for the high-throughput screening of protein–protein interaction inhibitors; however, the reversibility of the signal cannot be necessarily assured. An imaging method using antigen binding fragment (Fab)-conjugated chemical fluorescent dyes has also been reported. Using this method, protein modifications for which modification-binding proteins have not yet been identified can be detected. However, the method is unsuitable for evaluating inhibitors of interactions between a protein modification and its cognate “reader” receptor protein.

To examine when, where, and how histone acetylation is “written” or “erased” by HATs or HDACs in living cells, we developed two novel genetically encoded FRET-based probes, Histac and Histac-K12, which can image histone H4 acetylation on K5/K8 and K12, respectively. In this study, we developed a FRET-based probe to monitor the acetylation of histone H3 based on the specific binding of acetylated histone H3 to its “reader” protein, BRD4. BRD4 has two bromodomains, BRD4 BD1 and BD2, which recognizes multiple acetylation sites, H3K9/K14, H4K5/K8, and H4K5/K12. The FRET-based probe using histone H3 and BRD4 is assumed to specifically respond to acetylation of H3K9/K14, since the intramolecular interaction enhances selectivity and affinity.

We generated a FRET-based probe consisting of a five-part tandem fusion including Venus, the bromodomain of histone H3 and BRD4 (residues 51–466), a flexible linker (two repeats of GGGGS), histone H3, and CFP (Figure 1A). Treatment of COS7 cells expressing the probe with Trichostatin A (TSA), a potent HDAC inhibitor, for 3 h induced a significant change in the emission ratio of the fluorescent probe (Figure 1B). A time course of the emission ratio change showed the acetylation kinetics of the histone H3 domain upon TSA treatment (Figure 1C and D). After an increase in the emission ratio during TSA treatment, the emission ratio decreased after the removal of TSA from the culture medium. Immunoblot analysis revealed that the acetylation and deacetylation kinetics of the histone H3 domain in the probe were similar to those of the endogenous histone H3 (Figure 1E).

To investigate whether the TSA-induced emission ratio change reflected acetylation at specific histone H3 sites within the probe, we generated mutants of the acetylation sites in the probe’s histone H3 domain. A single replacement of lysine with arginine in the probe did not affect the TSA induced emission ratio change (Figure 2A). Replacement of both K9 and K14 with arginine significantly decreased the probe’s response to...
TSA treatment as compared to the probe containing wild-type histone H3 (Figure 2B). On the other hand, the change in emission ratio was not affected by double mutations of other pairs of acetylation sites. These results suggest that the increase in the emission ratio reflected the acetylation of either K9 or K14 on histone H3 within the probe. Thus, we named this probe Histac-H3K9/K14. NMR spectroscopy and peptide pulldown assays have shown that recombinant BRD4 bromodomains specifically bind to peptides with diacetylated H3K9 and H3K14 or diacetylated H4K5 and H4K12,18,20 suggesting that diacetylation is required for strong BRD4 binding. However, our present results suggest that acetylation at a single site, either K9 or K14, was sufficient to maintain the intramolecular interactions between histone H3 and the BRD4 bromodomain.

To ascertain whether the interaction of BRD4 with the acetylated H3 was required for FRET responses, we next examined the effect of mutations of the acetylation-binding domain within Histac-H3K9/K14, which contains two bromodomains of BRD4, BD1 and BD2. Either of the single mutations Y97F in BD119 or Y390F in BD218 completely abolished the response to TSA (Figure 2C), indicating that each single mutation of BRD4 bromodomains severely impaired the binding properties.

When Histac-H3K9/K14 was introduced into COS7 cells, it localized in the nucleus (Figure 1C). To investigate whether Histac-H3K9/K14 could be incorporated into chromatin, we examined the confocal micrographs of Histac-H3K9/K14 by FRAP analysis. The fluorescence of Histac-H3K9/K14 exhibited no recovery after photobleaching, similar to the result obtained with CFP-H4, which is incorporated into the chromatin (Figure 3A). Since Venus was freely diffusible, its fluorescence was not completely bleached and almost entirely recovered within 1 s.

To further confirm that Histac-H3K9/K14 was incorporated into the nucleosomes, COS7 cells expressing Histac-H3K9/K14, ECFP-H4, or monomeric Venus, and recovery of fluorescence was monitored over time. The data represent the means ± standard deviation; n ≥ 3. (B) The cytosolic fraction (Cy), nuclear fraction excluding chromatin (Nu), and chromatin fraction (Ch) of COS7 cells expressing the probe were analyzed by immunoblotting using antibodies against α-tubulin, HDAC2, or histone H3. Each fraction was immunoprecipitated with an anti-GFP antibody and then analyzed by immunoblotting using the anti-GFP antibody.

To further confirm that Histac-H3K9/K14 was incorporated into the nucleosomes, COS7 cells expressing Histac-H3K9/K14 were fractionated into cytoplasmic extracts (Cy), and chromatin fraction (Ch) of COS7 cells expressing the probe were analyzed by immunoblotting using antibodies against α-tubulin, HDAC2, or histone H3. Each fraction was immunoprecipitated with an anti-GFP antibody and then analyzed by immunoblotting using the anti-GFP antibody.
Histac-H3K9/K14 (Supporting Information Figure S1e). In contrast, the half-lives of the effect of the HDAC inhibitors after removal were not identical. Removal of TSA, SAHA, and LBH589 by washing with HDAC inhibitor-free medium resulted in rapid decreases of acetylation states (Supporting Information Figure S1a,b,c). On the other hand, the half-life of FK228 was $7.74 \pm 2.18$ h, which was significantly longer than those of other HDAC inhibitors (Supporting Information Figure S1d,f). FK228 is known to be converted into a reduced and activated form by the intracellular reducing system when it is introduced into living cells. In an in vitro study, HDAC1 activity, which was inhibited by the reduced FK228 treatment, almost entirely recovered after removal of FK228, suggesting that reduced FK228 is noncovalently bound to the enzyme.22 However, the half-life of FK228 after removal was approximately 6-fold longer than that of TSA in living cells (Supporting Information Figure S1f). This result is in agreement with the notion that FK228 might be able to penetrate the cell membrane better than reduced FK228 due to its hydrophobic nature.22

Although BRD4 is a therapeutic target for the treatment of AML and NMC,2,3 it is difficult to evaluate compounds that inhibit the capacity of the BRD4 bromodomain to interact with acetylated histone H3 in living cells. For instance, FRAP analyses have been used to examine whether bromodomain inhibitors can compete with chromatin to bind bromodomains in living cells.2,23 Therefore, we validated whether Histac-H3K9/K14 had the potential to monitor the intracellular activity of a BRD4 bromodomain inhibitor. When COS7 cells expressing Histac-H3K9/K14 were treated with the bromodomain inhibitor (+)-JQ12 for 1 h, the TSA-induced increase in the emission ratio was significantly suppressed (Figure 4). By contrast, treatment with (−)-JQ1, an inactive isomer of (+)-JQ1, did not significantly decrease the emission ratio. These results indicated that Histac-H3K9/K14 can serve as a useful tool to evaluate the cellular activity of compounds that inhibit interactions between the BRD4 bromodomain and acetylated histone H3.

In summary, we have developed a FRET-based imaging tool to detect histone H3K9ac or H3K14ac in living cells. Histac-H3K9/K14 was incorporated into chromatin and reversibly responded to HDAC inhibitor treatment. In addition, we demonstrated that Histac-H3K9/K14 can monitor the effect of compounds that inhibit interactions between acetylated histone H3 and BRD4 in living cells. Thus, Histac-H3K9/K14 will provide a powerful tool to enable the spatiotemporal analysis of histone H3 acetylation or its binding to the BRD4 bromodomain in various cellular systems.

**METHODS**

**Plasmid Construction, Cell Culture, and Transfection.** We isolated a cDNA clone encoding the BRD4 bromodomain from a human brain cDNA library. The amino acid sequence of the flexible linker consisted of two repeated GGGGS sequences. Each cDNA was subcloned into the KpnI–Xhol site of the mammalian expression vector, pcDNA3.1(+) (Invitrogen). COS7 cells were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C under 5% CO2. COS7 cells were transfected with the FuGENE HD transfection reagent (Roche) and then cultured for 24 h at 37 °C under 5% CO2.

**Cell Imaging.** After transfection, the culture medium was replaced with phenol red-free growth medium to facilitate imaging. Cells were observed at 37 °C under 5% CO2 using an Olympus IX81 microscope with a UIC-QE cooled charged-coupled device camera (Molecular Devices) and an Olympus IX83 microscope with an ORCA-FLASH4.0 CMOS camera (Hamamatsu). Images were collected using MetaFluo and MetaMorph (Universal Imaging) with a 440AF21 excitation filter, a 455DLF dichroic mirror, and two emission filters (480AF30 for GFP and 535AF26 for Venus).

**Gel Electrophoresis and Immunoblot Analysis.** Mononucleosome core particles were purified as described by Sasaki et al.15 The fractions were immunoprecipitated using an anti-GFP antibody. Antibodies that recognize histone H3, histone H3K9ac, and histone H3K14ac were purchased from Abcam. The anti-HDAC2 and anti-α-tubulin antibodies were obtained from Sigma. The proteins were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). After the membranes were incubated with the primary and secondary antibodies, the immune complexes were detected with ECL Western Blotting Detection Reagents (GE Healthcare Bio-Science Corp.), and luminescence was analyzed with a LAS4000 image analyzer (Fujifilm).

**FRAP Analysis.** COS7 cells were transfected with constructs encoding Venus, ECFP-H4, and Histac-H3K9/K14. A nuclear region with a diameter of 1 μm was bleached with a focused high-intensity laser in one cell within each field. Images were acquired using an FLUOVIEW FV1000 confocal microscope (Olympus) with an objective lens (UPLSAPO 60XW NA 1.20).

**ASSOCIATED CONTENT**

**Supporting Information**

Figure S1: Biological half-life of HDAC inhibitors after washout. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b00146.

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**Notes**

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REFERENCES