Published in final edited form as:

Nat Chem Biol. 2016 December ; 12(12): 1111–1118. doi:10.1038/nchembio.2218.

Selective Recognition of Histone Crotonylation by Double PHD Fingers of MOZ and DPF2

Xiaozhe Xiong¹, Tatyana Panchenko⁴, Shuang Yang¹, Shuai Zhao¹, Peiqiang Yan^{1,2},
Wenhao Zhang^{2,3}, Wei Xie^{2,3}, Yuanyuan Li^{1,3}, Yingming Zhao⁵, C David Allis⁴, and Haitao
Li^{1,3,6,*}

¹MOE Key Laboratory of Protein Sciences, Beijing Advanced Innovation Center for Structural Biology, Department of Basic Medical Sciences, School of Medicine, Tsinghua University, Beijing 100084, P.R. China

²School of Life Sciences, Tsinghua University, Beijing 100084, P.R. China

³Tsinghua-Peking Joint Center for Life Sciences, Tsinghua University, Beijing 100084, P.R. China

⁴Laboratory of Chromatin Biology and Epigenetics, The Rockefeller University, New York, NY 10065, USA

⁵Ben May Department of Cancer Research, The University of Chicago, Chicago, IL 60637, USA

⁶Collaborative Innovation Center for Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, P.R. China

Abstract

Recognition of histone covalent modifications by “reader” modules constitutes a major mechanism for epigenetic regulation. A recent upsurge of newly discovered histone lysine acylations, such as crotonylation (Kcr), butyrylation (Kbu), and propionylation (Kpr), greatly expands the coding potential of histone lysine modifications. Here we demonstrate that the histone acetylation-binding double PHD finger (DPF) domains of human MOZ (a.k.a. KAT6A) and DPF2 (a.k.a. BAF45d) accommodate a wide range of histone lysine acylations with the strongest preference for Kcr. Crystal structures of the DPF domain of MOZ in complex with H3K14cr, H3K14bu, and H3K14pr

*To whom correspondence should be addressed: Haitao Li, Medical Science Building C228, Tsinghua University, Beijing 100084, P.R.China, Tel: (+) 86-10-6277-1392, lht@tsinghua.edu.cn.

Accession codes

The atomic coordinates and structure factors have been deposited into Protein Data Bank under accession codes 5B75 for MOZ^{DPF}-H3_{1–25}K14bu, 5B76 for MOZ^{DPF}-H3_{1–25}K14cr, 5B77 for MOZ^{DPF}-H3_{1–25}K14pr, 5B78 for MOZ^{DPF}^{DM}-H3_{1–25}K14cr, and 5B79 for DPF2^{DPF}, respectively.

Referenced accessions

Protein Data Bank

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Author contributions

H.L. conceived and designed the study; X.X. designed and performed most of the experiments under the guidance of H.L. Y.L. discovered the crotonyllysine reader activity of DPF domain. T.P. performed the designer nucleosome pulldown assay under the guidance of C.D.A.. S.Y., P.Y., S.Z. Y.L. helped with binding and crystallographic experiments. W.Z., W.X., Y.Z., and C.D.A. provided expertise and critical feedback; H.L. and X.X. wrote the manuscript with inputs from other authors.

The authors declare no competing financial interest.

Supplemental information

Supplemental information includes three tables and six figures.

peptides reveal that these non-acetyl acylations are anchored in a hydrophobic “dead-end” pocket with selectivity for crotonylation arising from intimate encapsulation and amide-sensing hydrogen bonding network. Immunofluorescence and ChIP-qPCR show that MOZ and H3K14cr colocalize in a DPF-dependent manner. Our studies call attention to a new regulatory mechanism centered on histone crotonylation readout by DPF family members.

Keywords

Histone modification; Double PHD finger; MOZ/KAT6A; DPF2/BAF45d; Crystal structure; Epigenetic regulation

Covalent histone modifications carry critical epigenetic information for gene regulation and cell fate decisions¹. These modifications are recognized by a plethora of histone-binding “reader” modules, such as bromodomain, chromodomain, and plant homeodomain finger (PHD), in a type- and site-specific manner, and transduced into functional outcomes²⁻⁴. Histone lysine acetylation (Kac) was first discovered in the early 1960s with a primary function associated with active transcription. The enzymatic generation and removal, selective readout and dynamic regulation of histone Kac are implicated in many cellular functions, and have been extensively studied in the past twenty years⁵. Thanks to an integrated and mass spectrometry-based proteomic approach, an array of non-acetyl histone lysine acylations, such as lysine propionylation (Kpr), butyrylation (Kbu) and crotonylation (Kcr), have been identified in cells^{6,7}, posing a new challenge in the field to characterize the corresponding enzymatic machineries and downstream effectors.

The crotonyl group is a four-carbon, planar chemical moiety containing a C=C bond. Histone Kcr is evolutionarily conserved from yeast to human and twenty-eight Kcr sites have been identified in human histones⁸. Histone Kcr is particularly enriched at promoters and enhancers of actively transcribed genes. Histone Kcr but not Kac preferentially marks “escapee genes” during post-meiotic sex inactivation in mouse testis, suggesting a unique role for Kcr⁹. The level of histone Kcr is intimately correlated with the availability of cellular crotonyl-CoA, thus coupling cellular metabolism to epigenetic regulation¹⁰. Identification of effector proteins for Kcr is indispensable for elucidating how Kcr functions in active transcription.

Bromodomain, Double PHD finger (DPF) and YEATS are three major classes of acetyllysine reader modules¹¹⁻¹⁴. Recently, we and other groups reported that the Kac-binding YEATS domains but not the bromodomains are preferential Kcr readers¹⁵⁻¹⁷. Notably, our cell-based functional studies on human AF9 YEATS show that histone Kcr potentiates expression of inflammatory genes in a YEATS-dependent manner¹³. The DPF domains of MOZ/MORF (a.k.a. KAT6A/KAT6B) and DPF1/2/3 (a.k.a. BAF45b/d/c) have been reported to recognize histone H3K14 acetylation (H3K14ac) and regulate gene transcription^{12,13,18-20}. MOZ and MORF are two paralogous histone acetyltransferases implicated in leukemogenesis²¹⁻²⁴, whereas DPF1/2/3 are non-catalytic subunits of the BAF ATP-dependent chromatin remodeling complexes that carry diverse functions in development and cancer²⁵.

Here we report that DPF domains of MOZ and DPF2 engage a wide range of acylations at H3K14 with the highest affinity for crotonylation. Crystal structures of MOZ DPF domain in complex with H3K14cr, H3K14bu, and H3K14pr peptides reveal that these histone acylations are trapped in a β 2 hydrophobic pocket with the Kcr preference originating from the intimate encapsulation and crotonylamide-sensing hydrogen bonding. Immunofluorescence and ChIP-qPCR studies suggest colocalization of MOZ and H3K14cr in a manner dependent on DPF domain. Our work highlights a new regulatory mechanism of transcription control centered on readout of histone Kcr by DPF family members.

RESULTS

DPF domains are favorable readers of crotonyllysine

In order to explore the responsiveness of the Kac-binding DPF domains to non-acetyl histone acylations, we synthesized a series of acylated histone H3₁₋₂₅K14 peptides for binding studies. These include reported acylations such as formylation (fo), acetylation (ac), propionylation (pr), butyrylation (bu), crotonylation (cr), succinylation (su), 2-hydroxyisobutyrylation (hib), and β -hydroxybutyrylation (bhb) (Fig. 1a)^{6,26}. We recombinantly prepared DPF domains of MOZ and DPF2 (abbreviated MOZ_{DPF} and DPF2_{DPF}, Fig. 1b), and performed isothermal titration calorimetry (ITC) assays using the aforementioned histone H3₁₋₂₅K14 peptides. Remarkably, compared to H3K14ac (K_D = 22.9 μ M), MOZ_{DPF} displays 2.1-, 2.3- and 4.0-fold increase in affinity for H3K14pr, H3K14bu and H3K14cr peptides, respectively. By contrast, shortened (Kfo), hydroxylated (Kbhb), branched (Khib) or acidified (Ksu) acylations cause 7.1-, 4.8-, 5.8-fold decrease or a complete loss of binding affinity compared to Kac (Fig. 1c). DPF2_{DPF} exhibits a similar binding profile but with globally enhanced affinity (Fig. 1d). Notably, H3K14cr stands out as the most favorable mark among all the lysine acylations tested for both MOZ_{DPF} and DPF2_{DPF}. We measured a binding K_D of $\sim$ 5.8 μ M for “MOZ_{DPF}-H3K14cr” and of $\sim$ 85 nM for “DPF2_{DPF}-H3K14cr”, which is about 4- and 8-fold enhancement of affinity compared to H3K14ac, respectively.

Next, we generated “designer” nucleosomes bearing H3K9ac, H3K9cr, H3K14ac, H3K14cr, H3K18ac, H3K18cr, H3K27ac and H3K27cr marks and performed nucleosome pulldown using HeLaS3 cell nuclear extract. Western blot shows that MOZ and DPF2 are best retrieved by the nucleosomes bearing H3K14cr, testifying to the site- and type-specificity of MOZ and DPF2 in the readout of H3K14cr using a more physiologically relevant substrate (Fig. 1e,f).

Collectively, the above *in vitro* ITC titrations and *semi-in vivo* pulldowns assays establish these two DPF domains as selective readers of H3K14cr among a repertoire of histone H3K14 acylations.

Molecular recognition of H3K14cr by MOZ_{DPF}

To elucidate the molecular basis for H3K14cr readout by DPF domains, we solved the crystal structure of MOZ_{DPF} in complex with the H3₁₋₂₅K14cr peptide at 1.65 Å resolution (Supplementary Results, Supplementary Table 1). In this structure, the full length of the H3

peptide can be traced over an integrated double PHD finger surface based on the electron density map (Supplementary Fig. 1a). Both H3_{4–11} fragment and H3_{17–25} fragment form induced α -helices (Fig. 2a), with the latter unobserved in previous structural studies^{18,20}. Structural analysis reveals three signatures in the recognition of H3_{1–25}K14cr peptide (Fig. 2a, b; Supplementary Fig. 1b). Firstly, H3 R2 and K4 are inserted into two acidic pockets respectively at the β 1 surface of the second PHD finger (PHD2), and stabilized by electrostatic and hydrogen-bonding interactions as described previously^{18,20}. Secondly, H3K14cr is anchored into a hydrophobic pocket at the β 2 surface of the first PHD finger (PHD1). Thirdly, H3 L20 and K23 within the H3_{17–25} α -helix bestride the hydrophobic “I208–L213” saddle pair of MOZ PHD1 and contribute to MOZ_{DPF}-H3 interaction (Fig. 2c). The importance of the hydrophobic saddle pair is supported by ITC titrations using I208A and L213A MOZ_{DPF} mutants, which lose 3.8- and 6.5-fold binding affinity respectively in engaging H3_{1–25}K14cr (Supplementary Fig. 1c). In addition, using the dually crotonylated H3_{1–25}K14crK23cr peptide, we measured a binding K_D of 3.76 μ M (Supplementary Fig. 1c), which is about 1.6-fold tighter than the singly crotonylated H3_{1–25}K14cr peptide. Such an affinity enhancement is presumably due to increased hydrophobicity at H3K23 following crotonylation, underscoring the role of hydrophobic contacts around the saddle pair for H3 recognition.

The crotonylamide group of H3K14cr is planar due to π -electron conjugation. We observed a deep and snug insertion of the crotonylamide group into the reader pocket of PHD1 (Fig. 2d, e). This reader pocket is located next to the β 2 strand and the second zinc finger motif of PHD1. It consists of residues N235–G237 of β 2 strand, I228–C230 of β 1 strand, and S210, F211, L242, W257, C259, I260, E261 around the zinc finger coordination sphere (Fig. 2f). Sequence and structural alignment reveal that a glycine residue (G237) within the β 2 strand is critical for the β 2 pocket formation owing to its side-chain free feature (Fig. 2g, h). In most classical histone H3K4-binding PHD fingers (such as BPTF), this glycine residue is substituted by a bulky residue such as tyrosine or phenylalanine, which fills up the β 2 pocket and blocks Kcr insertion (Fig. 2h).

The four-carbon side chain of the crotonyl group is stably positioned at the bottom of the confined hydrophobic pocket (Fig. 2f). Interaction analysis reveals four pairs of hydrophobic contacts, four water-mediated hydrogen-bonding interactions involving side chains of S210, N235, and C259 as well as main chains of I260–E261 (Supplementary Fig. 2a). Collectively, our structure shows that MOZ_{DPF} PHD1 makes use of a hydrophobic β 2-pocket for Kcr-specific readout through tight encapsulation, which is further strengthened by additionally organized hydrogen-bonding network.

Comparison of histone H3K14 acylation recognition

To elucidate the acylation-type selectivity of MOZ_{DPF}, we also solved the crystal structures of MOZ_{DPF} in complex with H3K14pr or H3K14bu peptides at 1.55 Å and 1.7 Å, respectively (Supplementary Table 1). The two new structures along with the reported H3K14ac complex structure¹⁶ allow a systematic comparison of the recognition modes among different H3K14 acylations by MOZ_{DPF}.

In general, MOZ_{DPF} adopts the same β 2 pocket to recognize different H3K14 acylations. The Kac reader pocket is rather compact and leaves no room for water molecules (Fig. 3ai; PDB code: 4LLB); by contrast, the pocket is widened with additionally introduced 4–5 water molecules upon the insertion of bulkier K14pr, K14bu and K14cr acyl groups (Fig. 3a_{ii-iv}). Particularly, H3K14ac takes on a crouched conformation and its acetylamine group is encapsulated by residues S210, F211, C233, N235, W257, C259, I260 and E261; and structural alignment shows that these residues shift outwards following Kpr, Kbu and Kcr insertion to achieve a more open pocket (Fig. 3b). Except for the amide carbonyl flip observed for H3K14bu, the acyl chains of K14pr, K14bu and K14cr are extended and properly aligned to point their tips straight down to the pocket center (Fig. 3b). Interestingly, the insertion of longer acyl chains does not result in a deeper pocket, instead the H3 backbones around K14pr, K14bu and K14cr are uniformly lifted up by ~3 Å compared to K14ac as an adaptation to chain extension (Fig. 3b). Hydrogen bonding analysis reveals that recognition of K14ac is facilitated by one direct hydrogen bond between K14ac acetylamine and I260 main chain (Fig. 3c); by contrast, following pocket widening, water-mediated hydrogen bonding network dominates the recognition of the amide groups of K14pr, K14bu, and K14cr (Fig. 3c, Supplementary Fig. 2a–d). However, we did not detect a clear difference in the numbers of hydrogen bonds for K14pr, K14bu and K14cr readout by MOZ_{DPF}.

MOZ_{DPF} shows about twofold binding preference for K14cr over K14bu and K14pr by ITC. Selectivity for Kcr is achieved by aromatic- π stacking in the cases of the YEATS domains¹⁵. However, the reader pocket of MOZ_{DPF} for Kcr is largely hydrophobic and lack sandwiching aromatic residues (Fig. 3b), suggesting different mechanisms for recognizing Kcr. The four-carbon crotonyl group is characterized by a planar and alkene feature and lacks two hydrogen atoms, which renders crotonyl flat and slim in shape compared to the bulky butyryl group (Fig. 4a). Our complex structure reveals a snug anchoring of the crotonylamide group within the MOZ_{DPF} reader pocket (Fig. 4b-i). The reader pocket is highly size-selective. As the butyryl group contains branched methylene hydrogens, a simple substitution of crotonyl by butyryl reveals steric clashes between the butyryl group and the pocket residues F211, C259 and I260 (Fig. 4b-ii). As a result, the K14bu side chain has to flip to the opposite side to fit into the binding pocket, which further triggers an adaptive side-chain shift of N235 (Fig. 4b-iii; 4c). Propionyl is one carbon shorter in length and is thus less constrained by the size-selective reader pocket. Therefore, K14pr could fit into the pocket by slight positional adjustment without side chain flipping (Fig. 4b-iv; 4c). Thermodynamic parameter analysis (Fig. 4d) reveals that K14 acylation readout by MOZ_{DPF} is driven by both enthalpy and entropy, consistent with the utilization of a hydrophobic pocket for binding. Intriguingly, H3K14cr displays an enthalpy change (ΔH) of -4.5 kcal/mol, which is about 2.5-fold of H3K14pr (ΔH = -1.7 kcal/mol) and H3K14bu (ΔH = -1.8 kcal/mol). As the overall binding modes among all three complexes are essentially the same (Supplementary Fig. 2e), the pronounced enthalpic contribution in the case of K14cr readout reflects a state of optimal Kcr encapsulation. Conceivably, suboptimal shape-complementarity or steric repulsion likely lead to compromised binding affinities in the cases of K14ac, K14pr and K14bu readout.

H3K14cr readout by MOZ_{DPF} double mutant that mimics DPF2

DPF2_{DPF} binds H3K14cr ($K_D = 85$ nM) much tighter than MOZ_{DPF} ($K_D = 5.78$ μ M). Despite our efforts to co-crystallize DPF2_{DPF} with H3₁₋₂₅K14cr peptide, we were only able to obtain the apo structure of DPF2_{DPF} at 2.6 Å (Supplementary Table 1). The structures of DPF2_{DPF} and MOZ_{DPF} are very superimposable with a C α root-mean-square deviation (RMSD) of 1.25 Å; the composition and spatial layout of K14cr-binding pocket residues are essentially the same except for residues D274 and R300 of DPF2_{DPF} (S210 and N235 in MOZ_{DPF}) (Fig. 5a; Supplementary Fig. 3). We then generated a MOZ_{DPF} S210D/N235R double mutant (MOZ_{DPF}^{DM}) to mimic DPF2_{DPF}. ITC titration measures a K_D of 1.88 μ M between MOZ_{DPF}^{DM} and H3₁₋₂₅K14cr peptide, enhanced by threefold compared to that of wild-type MOZ_{DPF} (Fig. 5b). A similar MOZ-mimicking double mutant (D274S/R300N) of DPF2_{DPF} displays a dramatic decrease of binding from 85 nM to 16.9 μ M (Fig. 5b). Collectively, our mutagenesis studies suggest that pocket residues D274 and R300 of DPF2 are essential for the high-affinity engagement of H3K14cr.

We went on to solve the structure of MOZ_{DPF}^{DM} in complex with H3K14cr at 1.4 Å (Supplementary Table 1). In this structure, K14cr is similarly anchored into the hydrophobic pocket as with wild-type MOZ_{DPF} but is stabilized by reorganized hydrogen-bonding network (Fig. 5c). Particularly, the acidic S210D side chain forms a direct hydrogen bond to “NH” of the crotonylamide group, which triggers a rotational shift of K14cr side chain and the occurrence of three reorganized water molecules around the carbonyl group of crotonylamide. In addition, N235R flips its side chain outwards to form hydrogen bonding pairs with S210D and H3 A21 main chain, followed by partial disruption of the induced H3₁₇₋₂₅ helix in the MOZ_{DPF}:H3₁₋₂₅K14cr complex (Fig. 5c). Collectively, the newly formed hydrogen-bonding network introduced by S210D/N235R mutations explains the observed threefold binding-enhancement for MOZ_{DPF}^{DM}, a mechanism likely adopted by DPF2_{DPF} for high-affinity binding of H3K14cr.

MOZ associates with histone H3K14cr *in vivo*

To confirm the occurrence of H3K14cr in cells, we performed Western blot (WB) analysis of acid extracted histone samples from K562, 293T and HeLa cell lines. Peptide dot-blot assays show that the anti-H3K14cr antibody specifically recognizes H3K14cr, but not other modification types or sites including unmodified H3 (unH3), H3K14ac, H3K14pr, H3K14bu, H3K4cr, H3K9cr, and H3K18cr (Supplementary Fig. 4a). Noteworthy, WB shows clear H3K14cr signal in K562 and 293T cell lines with or without crotonate treatment, whereas, HeLa cell line displays clear signal only after crotonate treatment (Supplementary Fig. 4b). In sum, our WB results indicate that histone H3K14cr exists *in vivo*, and its levels may vary depending on cell type or the intracellular metabolic environment.

To further assess the association of MOZ_{DPF} with H3K14cr *in vivo*, we performed mutagenesis, immunofluorescence (IF), and ChIP-qPCR studies. Firstly, we designed two mutants: S210M that affects the H3K14cr pocket and D282A that affects the H3R2 pocket. Circular dichroism analysis suggests that both mutants are well-folded (Supplementary Fig. 4c), and ITC assays show that both S210M and D282A disrupt H3₁₋₂₅K14cr binding by

MOZ_{DPF} (Fig. 6a). We then performed IF imaging to explore their colocalization in 293T cells transiently transfected with wild-type (WT) or mutant FLAG-tagged full-length MOZ. The anti-H3K14cr antibody is validated by peptide competition IF imaging (Supplementary Fig. 4d). As shown in Fig. 6b, FLAG-tagged MOZ (green) and H3K14cr (red) display relatively diffused distribution pattern in the nucleus, and their colocalization is supported by merged yellow signals and a high Pearson coefficient (r) of 0.72; by contrast, this colocalization is markedly reduced in the S210M ($r = -0.07$) and D282A ($r = -0.07$) mutants (Fig. 6c). Interestingly, we noticed an enhanced localization of H3K14cr to the nuclear periphery in the H3 binding deficient mutants (Fig. 6b), which indicates a role of H3K14cr recognition by MOZ in promoting H3K14cr spreading from nuclear periphery to nuclear plasma. Given the multi-faceted roles of nuclear periphery in gene expression control²⁷, it remains an intriguing topic to explore the cellular function of H3K14cr in nuclear periphery, its enzymatic generation and dynamic regulation.

We next performed ChIP-qPCR studies to investigate the co-enrichment of MOZ and H3K14cr at the MOZ target genes in 293T cells. Consistent with the previous report¹⁸, we detected clear recruitment of the ectopically expressed FLAG-tagged full-length MOZ to the promoter regions of HOXA9, HOXA7 and HOXA5 but not to the promoter regions of HOXB1 and HOXD12; such enrichment is dramatically reduced in H3 binding deficient mutants despite the similar protein expression levels of WT and mutant MOZ (Fig. 6d). Likewise, ChIP-qPCR using anti-H3K14cr antibody reveals similar enrichment profile in 293T cells, in which the H3K14cr level is several-fold higher in MOZ target genes (HOXA9, HOXA7, and HOXA5) compared to non-MOZ targets (HOXB1, HOXD12) (Fig. 6e).

Collectively, the above colocalization studies support a physiological association of MOZ with histone H3K14cr in cells in an H3K14cr recognition-dependent manner.

Discussion

A variety of non-acetyl histone lysine acylations have been identified, yet their cellular function and their functional distinction from acetylation are still largely unknown. In the case of Kcr, p300/CBP are effective “writers” of H3K18cr¹⁰, and several Sirtuin family members (e.g. SIRT1–3) can serve as crotonylation “erasers”^{28,29}. Evidence about Kcr-specific downstream readers that link histone Kcr to particular biological outcomes is still lacking. Recently, we and others reported that the YEATS family proteins are preferential “readers”¹⁵⁻¹⁷ of histone Kcr. With ITC assays, designer nucleosome pulldown, structural and colocalization studies, we demonstrate here that the DPF domains of MOZ and DPF2 are novel Kcr-favorable readers and integrate these two proteins into the “write/read/erase” regulatory loop centered on Kcr.

Two induced α -helices (H3₄₋₁₁ and H3₁₇₋₂₅) contribute to the recognition of H3K14cr by MOZ_{DPF}. Helix H3₄₋₁₁ spans and engages the PHD1-PHD2 junction and may facilitate sampling of histone modification status between H3 K4 and T11 by MOZ_{DPF}²⁰. Helix H3₁₇₋₂₅ forms hydrophobic contacts with a saddle pair next to the β 2 pocket (Fig. 2a–c). To our knowledge, the induced formation of H3₁₇₋₂₅ helix has not been reported previously, highlighting a unique mode of recognition by MOZ_{DPF}. Additional crotonylation at H3K23

enhances the binding of the peptide by 1.6-fold, indicative of a minor avidity effect. In sum, the engagement of the entire H3₁₋₂₅ tail by MOZ_{DPF} highlights a feature of multivalent readout³⁰, and holds intriguing potential for sophisticated epigenetic regulation by sensing versatile patterns or cross-talks of histone modifications on H3 tail.

The hydrophobic β 2 pocket within MOZ_{DPF} PHD1 is size-selective for Kcr (Fig. 2f). It is interesting to note that PHD fingers are able to make use of distinct surfaces for recognizing partners³¹. In the case of MOZ_{DPF}, two PHD fingers are highly integrated to form a compact folding unit, which utilizes the β 1 surface of PHD2 for recognizing H3 R2 and K4, and the β 2 pocket of PHD1 for readout of H3K14 acylation (Fig. 2a). Notably, a side-chain free glycine residue (G237 of MOZ_{DPF}) within the β 2 strand is critical for the pocket generation among acyllysine-binding PHD fingers (Fig. 2g, h). In the classical H3K4-binding PHD fingers, such as those of BPTF, ING2, BHC80 and the second PHD fingers of MOZ_{DPF} and DPF2_{DPF}, the glycine residue is substituted by a bulky residue (e.g. F or Y), which occupies the β 2 pocket and blocks acyllysine insertion (Fig. 2h). By contrast, this side-chain free glycine residue is conserved among the PHD1 domains of human MOZ, MORF, DPF1, DPF2, DPF3, and PHF10 (Fig. 2g), suggesting their common acyllysine-reading activity.

Crotonylation differs from other acylations in its rigidity and planar configuration. Compared to Kbu, the alkenyl side chain of Kcr lacks two hydrogens and is flat and slim in shape (Fig. 4a). We observed a snug fitting of Kcr into the hydrophobic β 2 pocket and optimal shape-complementarity between them, which result in high-affinity binding. Strikingly, the β 2 pocket is so size-selective that the bulkier Kbu cannot achieve such a high-affinity mode of encapsulation, but has to flip its butyramide group and adopt a different conformation instead to avoid steric clashes caused by the branched butyryl hydrogens (Fig. 4b). Such size-selectivity is reminiscent of the mono- and di-methylation state-specific readout by L3BMTL1, in which a narrow and deep aromatic pocket acts as a size-selective filter to discriminate against the bulkier and more branched trimethyllysine group^{2,32}. In sum, the design of acylation-reader pocket can be so exquisite that a size difference as minor as a pair of hydrogens can be sensed for preferable readout of Kcr.

In addition to β 2 pocket encapsulation, a water-mediated hydrogen-bonding network around the acylamide group further contributes to the readout of H3 K14cr, K14bu, and K14pr by MOZ_{DPF} (Fig. 3c). Interestingly, such a hydrogen-bonding network is not observed for H3K14ac due to a deeper insertion of the acetylamide group into the β 2 pocket (Fig. 3b). Notably, residues S210 and N235 of MOZ_{DPF} are located at the rim of the β 2 pocket and help to stabilize the hydrogen-bonding network. S210 and N235 are two signature residues that distinguish MOZ/MORF from the DPF1/2/3 subfamilies (Supplementary Fig. 3), S210 and N235 in the former are substituted by D and R in the latter, respectively (Fig. 5a). DPF2_{DPF} displays a higher H3K14cr-binding affinity than MOZ_{DPF}, and S210D/N235R double mutation in MOZ_{DPF} enhances binding of H3K14cr by threefold. Direct hydrogen bond forms between S210D and the NH of crotonylamide in the structure of H3K14cr in complex with MOZ_{DPF}^{DM}, which likely accounts for the observed binding enhancement of MOZ_{DPF}^{DM} over wild-type MOZ_{DPF}, and partly explains the high-affinity binding of H3K14cr by DPF2_{DPF}. In sum, the molecular design of crotonylamide-sensing pocket is

divergent between the two DPF domains in this report, and the *in vivo* functional significance of this divergence remains to be explored.

Bromo, DPF and YEATS domains are three major classes of histone lysine acylation readers with different binding capacity and recognition mechanisms for various lysine acylations^{14,15}. In general, the bromodomains adopt a “side-open” pocket for readout of acyllysines with a binding preference of Kac>Kpr>Kbu»Kcr (Supplementary Fig. 5a). Most bromodomains do not recognize Kcr due to steric hindrance and only a few members such as TAF1 and BRD9 bind Kcr with compromised affinity^{15,33}. By contrast, the YEATS domains harbor an “end-open” aromatic sandwich pocket for readout of acyllysines with a binding preference of Kcr>Kpr~Kbu>Kac (Supplementary Fig. 5b)^{15,16}. In the case of DPF domains in this report, a “dead end” hydrophobic pocket is utilized for Kcr-specific recognition (Supplementary Fig. 5c). Although the DPF domains here display similar Kcr preference as YEATS, the origin of selectivity for Kcr is different. Aromatic- π stacking between the crotonylamide plane and aromatic sandwiching residues account for Kcr-specificity in the case of YEATS domains¹⁵⁻¹⁷; whereas, intimate hydrophobic encapsulation and coordinated hydrogen bonding are exploited for the selective readout of Kcr by DPF domains. Given the prevalence of histone acylations, the evolution of different reader pockets for Kcr recognition suggests more readers or effector modules await discovery for the functional readout of, but not limited to, histone acylations.

In summary, here we demonstrate that the DPF domains of MOZ and DPF2 are specific readers of H3K14cr. MOZ (KAT6A) is a transcriptional co-activator that acetylates histone H3 (K14, K9) and H4 (K5, K8, K12, K16) *in vitro*, and acts as a key epigenetic regulator of hematopoiesis^{23,34}; while the ubiquitously expressed DPF2 (BAF45d) is required for the expression of genes associated with hematopoietic stem cells^{35,36}. Given the important functions of MOZ and DPF2 in gene regulation, the observed 4- and 8-fold enhancement in their binding of H3K14cr over H3K14ac suggest regulatory mechanisms in which Kcr is a better potentiator for transcription control than Kac. MOZ_{DPF} and DPF2_{DPF} are also well responsive to H3K14pr and H3K14bu, which likely play a role in gene regulation depending on the availability of the corresponding acyl-CoA. Often the level of each acyl-CoA is closely related to intracellular metabolic conditions^{10,15,26}. Overall, the readout of histone Kcr by DPF domains calls attention to a new regulatory mechanism that links cellular metabolism to epigenetic regulations associated with chromatin modification and remodeling.

Materials and methods

Protein production

Recombinant wild-type and mutant MOZ_{DPF} domains (residues 194–323) were expressed in a pSUMOH10 vector (modified based on pET28b) that contains an N-terminal 10XHis-SUMO tag. Briefly, both wild-type and mutant MOZ_{DPF} were expressed in *E. coli* strain BL21 (DE3) (Novagen) with 0.4 mM IPTG induction at 16°C in LB media supplemented with 0.1 mM ZnCl₂. After cell lysis by an Emulsiflex C3 (Avestin) high-pressure homogenizer and centrifugation, the supernatant was applied to a HisTrap (GE Healthcare) nickel column and the bound protein was eluted with a linear imidazole gradient from 20

mM to 500 mM. After overnight ULP1 digestion and removal of the cleaved His-SUMO tag, MOZ_{DPF} was further polished by a HiLoad 16/60 Superdex 75 (GE Healthcare) gel filtration column. All the MOZ_{DPF} proteins were concentrated to about 10 mg/ml in buffer of 100 mM NaCl, 20 mM Tris, pH 7.5, and stored in -80°C freezer for future use. Recombinant human DPF2_{DPF} (270–391) was expressed and purified in essentially the same procedures as described for MOZ_{DPF}.

Crystallization, data collection, and structure determination

Crystallization was performed via the sitting or hanging drop vapor diffusion method under 18°C by mixing equal volumes (0.2–1.0 µl) of the DPF sample and the reservoir solution. Prior to crystallization, wild-type MOZ_{DPF} were mixed with histone H3K14 acylation peptides (H3_{1–25}K14pr, H3_{1–25}K14bu or H3_{1–25}K14cr) in 1:2 molar ratio at a concentration of 6–8 mg/ml. Crystals were grown from the reservoir condition: 35% (w/v) polyethylene glycol 4000, 0.2 M lithium sulfate, 0.1 M Tris, pH 8.5, and 3% (w/v) trimethylamine N-oxide. The complex crystal of MOZ_{DPF} S210D/N235R mutant with H3_{1–25}K14cr was obtained under the essentially the same condition. Despite extensive efforts, we were not able to obtain a complex crystal of MOZ_{DPF} bound to H3_{1–25}K14ac peptide. The crystals were briefly soaked in cryoprotectant solution containing the reservoir solution supplemented with 10% glycerol and flash-frozen in liquid nitrogen for data collection. The diffraction data set was collected at the beamline BL17U of the Shanghai Synchrotron Radiation Facility.

The free form DPF2_{DPF} crystal was grown by mixing 8 mg/ml protein solution with reservoir solution: 2.0 M ammonium citrate tribasic, 0.1 M Bis-Tris propane, pH 7.0. The crystal was soaked in cryoprotectant solution containing the reservoir solution supplemented with 20% glycerol and flash-frozen in liquid nitrogen for data collection. The diffraction data set was collected at Tsinghua University using a Rigaku Saturn944+ diffractometer system at 1.5415 Å.

All diffraction images were indexed, integrated, and merged using HKL2000 (ref. ³⁷). The structure was determined by molecular replacement using MOLREP³⁸ with the MOZ-H3K14ac structure (PDB ID: 4LLB) as the search model (for both MOZ_{DPF} and DPF2_{DPF}). Structural refinement was carried out using PHENIX³⁹, and iterative model building was performed with COOT⁴⁰. Ramachandran Plot analysis revealed no outliers in backbone dihedral angle distribution for all the refined structures; About 97.9%, 96.4%, 97.8% 97.9% and 95.7% backbone dihedral angles are located to the favored regions for the MOZ_{DPF}-H3_{1–25}K14cr, MOZ_{DPF}-H3_{1–25}K14bu, MOZ_{DPF}-H3_{1–25}K14pr, MOZ_{DPF}^{DM}-H3_{1–25}K14cr, and DPF2_{DPF} complexes, respectively. Detailed data collection and refinement statistics are summarized in Supplementary Table 1. Structural figures were created using the PYMOL (<http://www.pymol.org/>) program.

Isothermal titration calorimetry (ITC)

ITC measurement was performed as previously described¹³. Briefly, synthetic pre-modified histone H3 peptides (SciLight Biotechnology, LLC) and the recombinant DPFs proteins were extensively dialyzed against ITC buffer: 50 mM NaCl, 20 mM Tris, pH 7.5 for

MOZ_{DPF}, and 100 mM NaCl, 20 mM Tris, pH 7.5 for DPF2_{DPF} samples. Protein and peptide concentrations were measured based on UV absorption at 280 nm and 205 nm, respectively. The titration was performed using MicroCal iTC200 system (GE Healthcare) at 25°C for all DPF samples. Each ITC titration consisted of 17 successive injections with 0.4 μ l for the first and 2.41 μ l for the rest. Usually, H3 peptides at 1.0–1.2 mM were titrated into DPFs proteins at 0.07–0.1 mM. The resultant ITC curves were processed using the Origin 7.0 software (OriginLab) according to the “One Set of Binding Sites” fitting model. Detailed thermodynamic parameters of each titration were summarized in Supplementary Table 2.

Circular dichroism spectroscopy

Purified wild-type MOZ_{DPF} and its S210M and D282A mutants were diluted to 0.75 mg/ml in buffer: 20 mM Tris, pH 7.5, 100mM NaCl. Circular dichroism spectra were recorded under room temperature using an Applied Photo-physics Chirascan plus spectropolarimeter with a 1 mm path-length cell and a bandwidth of 1 nm. Spectra were scanned from 190 to 260 nm with a step-size of 1 nm and were repeated three times. Each reported circular dichroism curve was the average of three scans after the subtraction of buffer control and smoothed. Protein concentrations were determined with their 280 nm absorbance.

Generation of pre-modified histones by amber suppression

All histones without site-specifically modified lysines were expressed and purified as previously described⁴¹. For site specific incorporation of crotonylated lysine, amber suppression system developed by Peter Schultz’s lab was used⁴². Briefly, an orthogonal *Methanosarcina barkeri* tRNA/pyrrolysyl-tRNA synthetase (Mb-PylRS) pair was used where Mb-PylRS was mutated to accept crotonylated lysine⁴². The crotonylated lysine was synthesized as described⁴³. Crotonylated lysine was then used to supplement bacterial culture at 5 mM in the presence of 20 mM nicotinamide. H3 was expressed from a plasmid containing an amber stop codon “TAG” at either lysine 9, 14, 18 or 27. Similarly, an amber suppression system developed by the Jason Chin lab was used in order to generate site specifically acetylated histones. Here an Mb-PylRS was mutated to accept acetylated lysine⁴⁴. N- ϵ -Acetylated lysine was purchased from Sigma and was used to supplement bacterial culture at 5 mM in the presence of 20 mM nicotinamide. H3 was expressed from a plasmid containing an amber stop codon “TAG” at either lysine 9, 14, 18 or 27.

Nucleosome assembly

Octamers were reconstituted as described⁴¹. The 601 nucleosome positioning sequence was used for nucleosome reconstitution⁴¹. The DNA was amplified by PCR using primers containing a biotin tag on the 5’ end to produce 189 bp linear DNA and purified using QIAEXII kit (Qiagen). Nucleosomes were assembled using the standard step-wise dialysis method⁴⁵.

Colocalization analysis

293T cells were transfected with plasmids encoding the wild type or mutant FLAG-MOZ S210 and FLAG-MOZ D282A using the lipofectmine 2000 reagent (Invitrogen). 24h after transfection, 293T cells were fixed with 4% (w/v) paraformaldehyde in PBS for 15 min,

permeabilized with 0.5% Triton X-100 at room temperature for 10 min and washed with PBS. Cells were incubated with primary antibodies against histone H3K14cr and FLAG tag, diluted in 3% BSA overnight at 4 °C, washed with PBS and incubated with Cy5-conjugated goat anti-rabbit and Alexa Fluor® 488-conjugated goat anti-mouse secondary antibodies for 1 h at room temperature. Nuclei were counterstained with DAPI Fluoromount-G (SouthernBiotech, 0100-20). We counted and achieved 50 cells immunofluorescent images each sample. 50 cells for each IF sample generated a *P* value < 0.0001 by t-test, which is significant enough to draw a valid conclusion. Immunofluorescent images were taken as Z-stacks with an Olympus spinning disk confocal laser scanning microscope system. Projected images were generated by Volocity Demo software and quantification was performed with Imaris (Bitplane). Statistical analyses, unpaired t-test and standard derivation were performed with GraphPad Prism 6.0 (GraphPad Software, LaJolla, CA, USA). No images of any sample were excluded from the analysis.

Chromatin immunoprecipitation and quantitative PCR (ChIP-qPCR)

Cells were collected and cross-linked by 1% formaldehyde. Cells were suspended in lysis buffer A (5 mM PIPES pH 8.0, 85mM KCl, 0.5% NP-40), incubated on ice for 10 min followed by centrifugation at 220xg for 10 min at 4°C. Supernatant was removed and precipitates were suspended in lysis buffer B (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 0.3% SDS, freshly added protease inhibitors) for 10 min on ice before sonication. 1 mg beads (Invitrogen, Cat. 11201D) were washed with PBS + 5 mg/ml BSA for three times and incubated with 5 µg antibody at 4°C for 6–8 h before adding chromatin. Sonicated chromatin was diluted in dilution buffer (16.7 mM Tris-HCl pH8.0, 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl, freshly added protease inhibitors), and was incubated with pre-treated beads and antibodies by rotation at 4°C overnight. Then beads were washed with washing buffer (50 mM HEPES pH 8.0, 1% NP-40, 0.7% DOC, 0.5 M LiCl, freshly added protease inhibitors) for 5 times followed by wash with TE buffer once. 100ul elution buffer (50 mM Tris-HCl pH 8.0, 1mM EDTA, 1% SDS) was added and beads were incubated in Thermo mixer at 65°C for 30 min with max speed. Supernatant was collected and treated with protease K at 55°C for 2 h, then purified with DNA purification kit (Tiangen, Cat. DP203-02). RNA was removed by treatment of RNase at 37°C for 1 hour. DNA was then purified by AMPure Beads (Beckman, Cat. A63881) and subjected to qPCR identification. Primer information for qPCR is listed in Supplementary Table 3.

Antibodies and cell lines

For western blot: anti-MOZ (ab41718, Abcam); anti-DPF2 (A303-596A-1, Bethyl); anti-H3K14cr (PTM-535, PTM Biolabs)

For colocalization: anti-FLAG tag (F1804, Sigma); anti-H3K14cr (PTM-535, PTM Biolabs); Cy5-conjugated goat anti-rabbit (111-175-144, Jackson ImmunoResearch); goat anti-mouse (A-11029, Life Technologies)

Cell lines: HeLa S3 from ATCC #CCL-2.2; HeLa from ATCC #CCL-2; 293T from ATCC #CRL-3216; K562 from ATCC #CCL-243. All cell lines were no mycoplasma contamination tested by PCR.

Statistical analysis

All data are expressed as mean $\pm$ s.d. Comparison of two groups was made by two-sided unpaired Student's t-test. Difference with $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Z. Chen for critical comments. We thank the staff members at beamline BL17U of the Shanghai Synchrotron Radiation Facility and Dr. S. Fan at Tsinghua Center for Structural Biology for their assistance in data collection and the China National Center for Protein Sciences Beijing for providing facility support. This work was supported by grants from the Ministry of Science and Technology of China (2016YFA0500700 and 2015CB910503), the National Natural Science Foundation of China (91519304), and the Tsinghua University Initiative Scientific Research Program to H.L. We also acknowledge supports from The Rockefeller University to C.D.A. and the National Institute of General Medicine to C.D.A. (GM040922) to Y.Z. (GM105933, DK107868, and GM115961), and T.P. (GM112365).

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Figure 1. Identification of MOZ_{DPF} and DPF2_{DPF} as favorable readers of Kcr

(a) Chemical structures of known histone lysine acylations (abbreviations and numbering correspond to data shown in (c and d) and are used throughout text with emphasis on Kcr). (b) The domain architectures of MOZ/MORF and DPF1/2/3 proteins (top). The sequence of H3₁₋₂₅ tail with K14 colored in red (bottom). (c and d) ITC fitting curves of MOZ_{DPF} (c) and DPF2_{DPF} (d) titrated with a series of H3₁₋₂₅ peptides containing different K14 acylations shown in (a). (e and f) MOZ_{DPF} (e) and DPF2_{DPF} (f) have a greater affinity for nucleosomes bearing Kcr than those bearing Kac. Direct Blue staining of the membrane shows comparable inputs. The streptavidin monomer co-migrates with histone H4. Nucleosome IP was done in two replicates. The blotting was confirmed by 3 independent blots. Full images of the gels in (e) and (f) are shown in Supplementary Fig. 6a–d.

Figure 2. Molecular details for H3K14cr readout by MOZ_{DPPF}

(a) Overall structure of MOZ_{DPPF} bound to H3₁₋₂₅K14cr (yellow). The PHD1 and PHD2 subdomains are colored blue and pink, respectively; Key H3 residues are shown as sticks; 1 and 2 denote two core β -strands; N and C denote amino- and carboxyl-termini. Grey spheres, zinc ions. (b) Electrostatic surface view of the MOZ_{DPPF}-H3K14cr complex. Electrostatic potential is expressed as a spectrum ranging from -10 kT/e (red) to +10 kT/e (blue). (c) Hydrophobic contacts between helix H3₁₇₋₂₅ and the “I208–L213” saddle pair. (d) Insertion of K14cr into the MOZ_{DPPF} reader pocket. K14cr is depicted as space-filling spheres with yellow for carbon, blue for nitrogen and red for oxygen. (e) Cut-away view of K14cr in the MOZ_{DPPF} reader pocket. K14cr is represented as yellow stick with the crotonylamide group shown as cyan surface. (f) Composition of the K14cr-binding β 2 pocket. K14cr is shown as yellow stick with its extended two-hydrocarbon group highlighted

as green dots; small red balls, water molecules; orange spheres, zinc ions; Cyan dashes, hydrogen bonds. (g) Sequence alignment of the $\beta 2$ motif among different histone-binding PHD fingers, the zinc-coordinating residues are shaded blue. Key pocket-forming glycine (G) residues are shaded orange. (b) Structural alignment of MOZ PHD1 (orange) and BPTF PHD (blue) domains. Key residues are shown as sticks. Arrow head points to the position of MOZ G237 and BPTF Y2885. Note the bulky feature of Y2885 side chain that occupies the K14cr pocket.

Figure 3. Recognition of different histone H3K14 acylations by MOZ_{DPF}

(a) Insertion of K14ac (pink), K14pr (green), K14bu (cyan) and K14cr (yellow) into the MOZ_{DPF} reader pocket. Water molecules are shown as red balls, and hydrogen bonding interactions are indicated by red dashes. (b) Stereo view of H3K14 acylation readout by MOZ_{DPF} reader pockets. H3K14ac (pink), H3K14pr (green), H3K14bu (cyan) and H3K14cr (yellow) peptides are superimposed for comparison. Note the outwards shift of key Kcr-binding residues (yellow sticks and grey ribbons). For clarity, key pocket residues of Kac and Kcr complexes are shown here. (c) Hydrogen bonding network involving H3K14 acylations and MOZ_{DPF} reader pocket. H3K14 acylations and water molecules are covered by the simulated annealing Fo-Fc omit map counteracted at 2.5 σ level. Key residues are shown as sticks.

Figure 4. Size-selective recognition of H3K14cr by MOZ_{DPF}

(a) Comparison of lysine crotonyllysine (Kcr, yellow) and butyryllysine (Kbu, cyan) side chains. Hydrogen, nitrogen, and oxygen atoms are colored grey, blue and red, respectively. Half-transparent surfaces are shown to highlight a slim feature of Kcr and a bulgy feature of Kbu. (b) The MOZ_{DPF} β2 pocket is highly size-selective for K14cr. Crotonyl- (yellow), butyryl- (cyan) and Propionyl- (green) amide groups are showed as space-filling dots. The plates (highlighted by arrow heads) indicate steric clashes. Key residues are highlighted with sticks with hydrogen atoms colored grey. (c) Superimposition of K14pr, K14bu and K14cr in the MOZ_{DPF} reader pocket. Key residues and zinc ions are showed as sticks and spheres. K14pr, K14bu and K14cr complexes are colored green, cyan and yellow, respectively. (d) Thermodynamic parameters of MOZ_{DPF} titrated with the annotated H3K14 acylation peptides. Detailed fitting values are listed in Supplementary Table 2.

Figure 5. Recognition of H3K14cr by DPF2-mimicking MOZ_{DPF}

(a) Structural comparison of the free DPF2_{DPF} (green) with MOZ_{DPF} (pink) bound to H3K14cr (grey) peptide. The two amide-sensing residues are shown as sticks and labelled in the close-up window. (b) ITC fitting curves of MOZ_{DPF} and MOZ_{DPF}^{DM} (S210D/N235R) (top), and of wild type DPF_{DPF} and DPF_{DPF}^{DM} (D274S/R300N) (bottom) titrated with H3₁₋₂₅ K14cr peptides. (c) Crystal structure of MOZ_{DPF}^{DM} bound to H3K14cr (yellow) peptide. The wild type MOZ_{DPF}-H3K14cr complex (grey) is overlaid for comparison. Key residues are shown in stick representation. Small red balls, water molecules; cyan dashes, hydrogen bonds.

Figure 6. Mutagenesis and colocalization studies

(a) ITC fitting curves comparing wild type and mutant MOZ_{DPF} titrated with H3₁₋₂₅K14cr peptides. (b) IF of 293T cells reacted with antibodies against FLAG (green) or H3K14cr (red). FLAG-tagged WT, S210M, and D282A mutant full-length MOZ were transformed into 293T cell for IF imaging (left). Scale bar: 3 μ m. (c) Quantification of the FLAG signal colocalized with H3K14cr (counts are based on 50 cells) (right). **** $P < 0.0001$ by unpaired t-test with Welch's correction, compared to FLAG-tagged WT-K14cr group. Standard derivation of every sample group < 0.1 . (d) ChIP-qPCR analysis using anti-FLAG antibody and IgG control to probe the enrichment of FLAG-tagged WT or mutant MOZ in the promoter regions of indicated HOX genes in 293T cells. A Western blot with anti-FLAG and anti-tubulin antibodies shows that all of the constructs are expressed at appropriate levels. Full image of the gel is shown in Supplementary Fig. 6e. Standard derivation < 0.3 . (e) ChIP-qPCR analysis using anti-H3K14cr antibody and IgG control to probe the enrichment of H3K14cr in the promoter regions of indicated HOX genes in 293T cells (w/o MOZ).

overexpression). standard derivation < 0.1 . In (d) and (e), an average of three real-time PCR replicates of a representative experiment is displayed as a function of percentage of input signal.

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