Bromodomains in living cells participate in deciphering the histone code

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The bromodomain, a module of ~110 amino acids, is found in several chromatin-associated proteins, including histone acetyltransferases and chromatin-remodeling factors, and can bind to acetylated lysines. Such post-translational modifications occur mainly in the N-terminal tail of the histone proteins and, in combination with other modifications, are thought to participate in defining a histone code. Recent findings provide a model for how bromodomain-containing proteins participate in the recognition of acetylated histones.

Post-translational modifications are important in regulating protein–protein interactions. In the case of histones, defined patterns of modifications, including acetylation, phosphorylation and methylation, can function as a recognition code for the recruitment of different factors [1–3]. This is known as the 'histone code' hypothesis (Box 1). However, for this to occur, specific protein domains that recognize these modifications are required. Support for this hypothesis comes, in part, from structural studies revealing that bromodomains are modules that can bind to acetylated lysine [4–8]. This article will illustrate how recent studies *in vivo* [9] provide further insights that support this hypothesis, focussing on the recognition of acetylated histones.

Bromodomains: families and protein structure

Bromodomains are found in several chromatin-associated proteins. Their name comes from the *Drosophila* protein 'Brahma', which was the first bromodomain-containing protein to be identified, and the ATPase subunit of the Brahma chromatin-remodeling complex. The proteins can be divided into three families: (i) histone acetyltransferases (HATs), including Gcn5, p300 cAMP-response-element-binding (CREB)-binding protein-associated factor (PCAF) and TATA-binding-protein-associated factor $(TAF)_{II}250$ – a subunit of the transcription factor $TF_{II}D$; (ii) ATP-dependent chromatin-remodeling complexes, including Brahma, Swi2, Snf2 and Brg1; and (iii) the less-characterized BET (bromodomain and ET domain) family, which is a novel class of transcriptional regulators that includes Bdf1, Bdf2, Bdr4 and Brd2.

The bromodomain is composed of four left-handed helix bundles (α_Z, α_A, α_B, and α_C), with a long loop (ZA) connecting helices Z and A oriented against the small loop (BC) connecting helices B and C (Figure 1). These loops are organized to form an accessible hydrophobic pocket in which the interaction with the acetylated lysine residue occurs (for review, see Ref. [10]).

Bromodomain function

Interactions between bromodomains and acetylated histones are thought to mediate several effects, including transcriptional activation, memory of the transcriptionally activated chromosomal regions and anti-silencing. The role of the interactions between bromodomains and acetylated histone in transcriptional activation is exemplified by the viral activation of the gene interferon β. This results in a unique pattern of histone modifications, particularly acetylation, at the promoter of the gene that can be used as a signal to induce the recruitment of the transcriptional machinery [11]. The sequential recruitment of two bromodomain-containing proteins is then observed: first, Brg1, which is a component of the SWI–SNF complex and, second, the transcription factor $TF_{II}D$ through $TAF_{II}250$. Their recruitment is mediated by interactions with acetylated (Ac) Lys8 on histone H4 (AcK8 H4), and acetylated Lys9 and acetylated Lys14 on histone H3 (AcK9 and AcK14 H3), respectively [11].

In addition, memory of activated states is illustrated *in vitro* by the interaction of the chromatin-remodeling complex SWI–SNF and the HAT complex SAGA (Spt–Ada–Gcn5–acetyltransferase) with the promoters, in the absence of activators. These associations occur through the bromodomain-containing proteins Swi2 or Snf2 and Gcn5, respectively, after the promoter has been acetylated by activator-recruited SAGA [12]. Interestingly, only Swi2 or Snf2, and Gcn5 mediate the association, independent of the presence of several other bromodomain-containing proteins in both complexes. These data highlight the specificity and selectivity within bromodomains.

An anti-silencing role has been assigned to the BET family bromodomain-containing protein Bdf1, through its association with acetylated histone H4 [13]. Chromatin immunoprecipitation assays show that Bdf1 can bind to

Box 1. The histone code

Histones are post-translationally modified (e.g. by acetylation, phosphorylation, methylation and ubiquitination) on specific residues that are located mostly in their N-terminal tail [18]. The histone code hypothesis proposes that defined patterns of modifications, possibly acting in combination, can be recognized by specific factors. In turn, this recognition can induce the recruitment of particular effectors, thereby translating the histone-modification pattern into a particular chromatin state.

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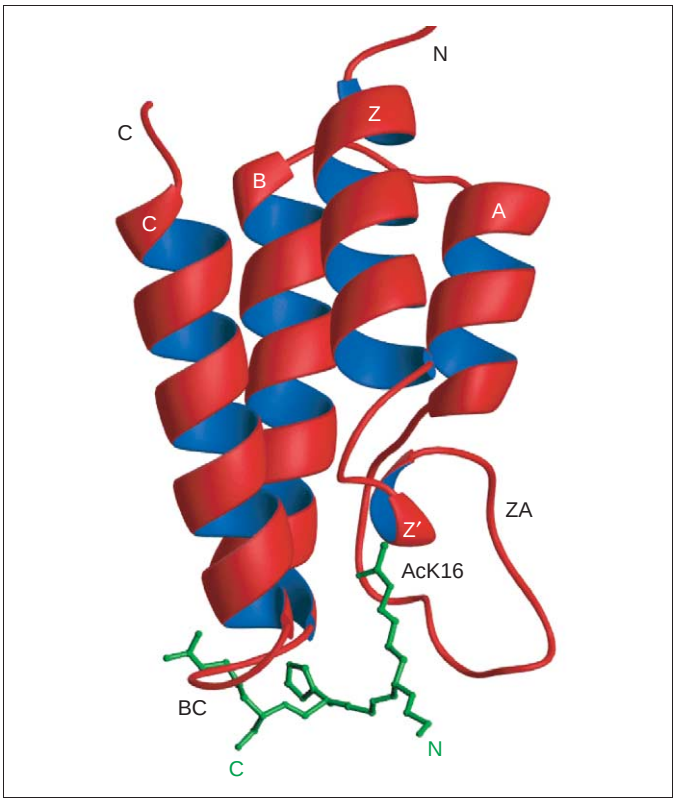

Figure 1. Structure of the Gcn5 bromodomain in complex with an acetylated (Ac) histone H4 peptide. The crystal structure of the Gcn5 bromodomain (red and blue) is shown associated with the AcK16 histone H4 peptide (residues 14–28) (green). The bromodomain structure is composed of four helices (labeled in white: A, Z, B and C) and two loops (ZA and BC). The interaction with the acetylated peptide occurs in a hydrophobic pocket formed by the two loops. Z′ represents an additional α-helix juxtaposed to the Z helix. Reproduced, with permission, from Ref [10].

heterochromatin boundaries found next to telomeres and mating-type loci, through its association with acetylated histone H4. Mutations in *Bdf1* result in the spreading of silencing proteins (SIRs) outside heterochromatic regions. Moreover, Bdf1 and Sir2 compete with each other for binding to acetylated H4, which suggests that Bdf1 acts as a heterochromatic barrier [13]. Interestingly, Bdf1, like many of the BET family of bromodomains, remains associated to chromosomes during mitosis [14]. Therefore, it can be speculated that heterochromatin boundaries might be inherited epigenetically through the interaction between Bdf1 and acetylated H4.

Using FC-FRET to study the role of bromodomains *in vivo*

Until now, interaction analyses have been restricted to *in vitro* experiments or localized associations *in vivo*. In an elegant recent study, Kanno *et al.* [9] shed light on this by investigating the general patterns of association between bromodomain-containing proteins and histones in living cells. The authors used flow cytometry-fluorescence resonance energy transfer (FC-FRET), which is a technique developed recently for studying cell surface receptors [15,16] (Figure 2). Fluorescence resonance energy transfer (FRET) signals are based on the ability of a donor fluorophore of high energy to transfer energy directly to an acceptor fluorophore of lower energy. Because FRET between two fluorophores requires proximity at the Angstrom level, this technique enables specific protein–protein interactions to be detected, although it should be noted that the absence of FRET does not necessarily mean an absence of interaction. The exact conformation of the tagged proteins might not be appropriate for energy transfer to occur.

In FC-FRET, the energy transferred is quantified by flow cytometry. Consequently, thousands of cells can be analyzed rapidly in each experiment, giving statistical robustness to the results. The proteins to be analyzed are fused to green fluorescent protein variants, and the protein-expression vector is transfected into the cell (Figure 2). After 24–48 h, the cells are harvested and processed immediately by flow cytometry [16].

Kanno *et al.* analyzed the *in vivo* histone specificity of three bromodomain-containing proteins – Brd2, $TAF_{II}250$ and PCAF – using several techniques, including FC-FRET, immunoprecipitation, competition assays, mutagenesis and bromodomain-swapping experiments [9]. The results show that different bromodomains have different specificities for binding to acetylated histone (Figure 3). Accordingly, the authors found that, in living cells, the Brd2 bromodomain binds to AcK12 H4; the $TAF_{II}250$ bromodomain binds to AcK8, AcK12, AcK16 H4 and AcK14 H3; and the PCAF bromodomain binds to acetylated histones H3 and H4. Compared with *in vitro* binding studies using peptides or chromatin immunoprecipitation [6,11], despite a general agreement, some differences in the specificity of the acetylated residues recognized are present *in vivo*. This can be explained by the fact that the studies by Kanno *et al.* in living cells included modulations due to the cellular environment and the presence of additional factors. Therefore, *in vitro* results, which do not incorporate these additional constraints, provide one important part of the view but should, at some point, be integrated with the additional cellular components to provide a complete understanding of how the cell can use histone modifications.

An interesting observation is that the specificity of bromodomain binding is a property of the bromodomain

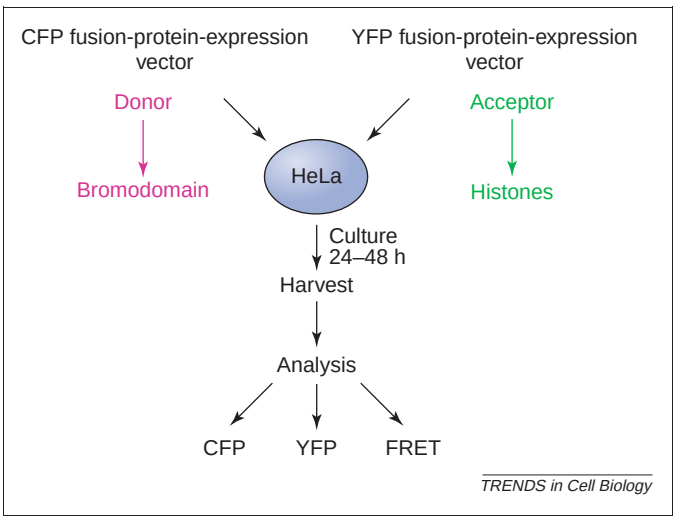

Figure 2. An outline of the experimental design for flow cytometry-fluorescence resonance energy transfer (FC-FRET). The cells are transfected with cyan fluorescent protein (CFP) bromodomain-expression vector and yellow fluorescent protein (YFP) histone-expression vector. The cells are analyzed by flow cytometry (using a FACSvantage™ cytometer) after 24–48 h [16].

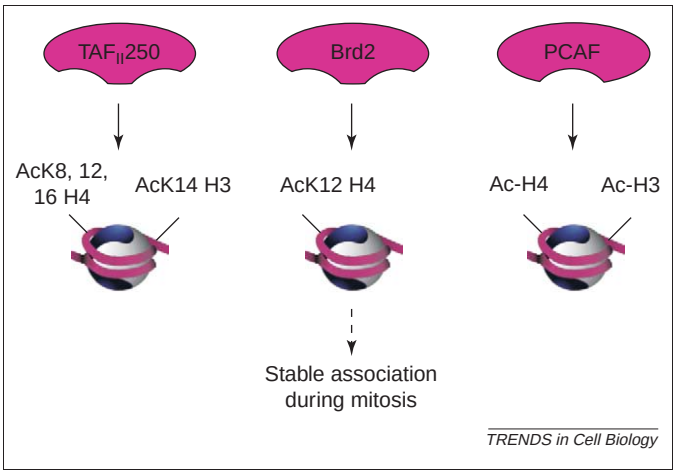

Figure 3. Selectivity of bromodomains in living cells. Three bromodomain-containing proteins – TATA-binding-protein-associated factor $(TAF)_{II}250$, Brd2 and p300 cAMP-response-element-binding (CREB)-binding protein-associated factor (PCAF) – are shown with the number of pockets corresponding to the number of bromodomains that they contain. Specificity for acetylated (Ac) nucleosomal histones in the context of the living cells is indicated. $TAF_{II}250$ interacts with AcK14 H3, and AcK8, AcK12 and AcK16 H4; Brd2 interacts with AcK12 H4; and PCAF has a broader selectivity, interacting with Ac-H3 and Ac-H4. Because Brd2 remains associated to chromosomes during mitosis, additional components might be required, as indicated by the broken arrow.

per se, because transferring the $TAF_{II}250$ bromodomain to bromodomain-deleted Brd2 protein can convey $TAF_{II}250$ specificity. This is perhaps not surprising because, although the bromodomain structure is conserved, the amino acid sequence is less conserved at the loops where the interaction occurs. Future studies should focus on this particular region to determine which amino acids are crucial for providing specificity. The FC-FRET approach might also help us to understand specificity in the natural environment because the presence of other components is likely to modulate the affinity and specificity of bromodomains and acetylated histones.

Kanno *et al.* also observed that Brd2 remains mitotically associated with chromatin, suggesting a role in the inheritance not only of the histone modification from one cell generation to the next but also of its specific binding partner [9]. Because Brd2 belongs to the BET family of bromodomains, its retention within chromosomes during mitosis is expected, as seen for other BET members including Bdf1 and Brd4 [14,17]. This is an exciting finding, considering that only members of the BET family, and neither HAT nor ATP-dependent chromatin-remodeling complexes, associate with mitotic chromosomes, even though they are all bromodomain-containing proteins. Therefore, an intriguing question that remains to be answered is: what determines the anchoring of the BET family of bromodomains to chromatin during mitosis? In addition, what is the role of this anchoring?

Concluding remarks

In conclusion, the study by Kanno *et al.* [9] addresses two important issues. First, it demonstrates that protein-containing bromodomains can recognize different patterns of histone acetylation. This provides further support to the histone code hypothesis; that is, different modifications recruit different factors in a highly specific manner. Second, it offers a novel approach to studying protein–protein interactions in living cells. FC-FRET is a feasible and convenient means of examining interactions in large numbers of cells. This technique also presents a wide range of possibilities for studying the other interactions that comprise the histone code and for establishing correlations with the structural properties of the proteins.

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