

Association of bromodomain BET proteins with chromatin requires dimerization through the conserved motif B

Pablo Garcia-Gutierrez, Maria Mundi and Mario Garcia-Dominguez*

Stem Cells Department, Centro Andaluz de Biología Molecular y Medicina Regenerativa and Consejo Superior de Investigaciones Científicas (CABIMER-CSIC), Av. Americo Vespucio, Spain

*Author for correspondence (mario.garcia@cabimer.es)

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Summary

BET (bromodomain and extra terminal domain) family proteins are unique among bromodomain-containing proteins in that they not only associate with acetylated chromatin in interphase, but also remain attached to chromosomes during mitosis. Although the two tandem bromodomains are essential to display this behaviour, they do not suffice. In this work we report that a small conserved domain, motif B, is also required. A deletion mutant of this domain dissociates from mitotic chromosomes. However, inhibition of histone deacetylases alleviates dissociation. We also show that motif-B-dependent association with chromosomes is not restricted to mitosis. Interestingly, our results indicate that motif B constitutes a surface for homo- and hetero-dimerization between BET proteins. Finally, linked to the prominent role BET proteins play in cell proliferation, we report that ectopic expression of the family member Brd2 interferes with neuronal differentiation in P19 cells and in the vertebrate neural tube, probably because of preservation of adequate levels of cyclin A2 and cyclin D1. By contrast, a deletion mutant of motif B fails to perform in this way, highlighting the relevance of this domain for Brd2 function.

Key words: BET proteins, Brd2, Dimerization, Cell cycle, Bromodomain, Acetyl-histone

Introduction

Acetylation and other histone modifications control essential transcriptional regulatory processes in the cell. Combinatorial display of these modifications modulates the docking characteristics of chromatin to recruit a huge variety of effector proteins that induce changes in its structure and thereby in gene transcription (Jenuwein and Allis, 2001). Precise recognition of these modifications by specialized modules in effector proteins is critical for accurate transcriptional control. Recognition of histone acetylation by the bromodomain illustrates this scenario. Bromodomains are present in a number of chromatin-associated proteins, which include histone acetyltransferases (HATs), ATP-dependent chromatin remodeling factors and proteins of the BET (Bromodomain and Extra Terminal domain) family (Jeanmougin et al., 1997; Yang, 2004).

The BET family of proteins in mammals comprises Brd2, Brd3, Brd4 and Brdt. Except the last, which is restricted to the male germ line, the rest of the members are widely expressed in all tissues. Vertebrate BET proteins are characterized by the presence of two tandem bromodomains at the N-terminus and an exclusive Extra Terminal (ET) domain at the C-terminus. The ET region includes the NET (N-terminal Extra Terminal) domain, and the C-terminal SEED motif (Florence and Faller, 2001). While the function of bromodomains has been well characterized, the function of other domains has been poorly investigated (Denis et al., 2000; Rahman et al., 2011). It is well established that bromodomains of BET proteins bind to acetylated chromatin. While Brd2 seems to preferentially bind histone H4 at the mono-

acetylated state (acetyl-K12), Brd4 and Brdt more efficiently recognize di-acetylated histone H4, at K5/K12 and K5/K8 positions, respectively (Dey et al., 2003; Ito et al., 2011; Kanno et al., 2004; LeRoy et al., 2008; Morinière et al., 2009; Sasaki et al., 2009). In addition, binding of BET members to acetylated proteins, other than histones, has also been reported (Gamsjaeger et al., 2011; Huang et al., 2009; Lamonica et al., 2011). Acetylation of histones correlates with gene activation (Hebbes et al., 1988), and in this respect, BET proteins have been described as transcriptional activators with a prominent role in the control of cell-cycle-associated genes. Brd2 has been implicated in controlling the expression of cyclins A2 and D1 (LeRoy et al., 2008; Sinha et al., 2005). Brd4 has been involved in controlling expression of cyclin D1 and other G1-associated genes required for progression to the S phase (Mochizuki et al., 2008; Yang et al., 2008). A major determinant of Brd4 function is its association with the positive transcription elongation complex P-TEFb (Yang et al., 2005). In contrast to Brd2 and Brd3, Brd4 presents an additional C-terminal domain (CTD) that is essential for this function (Bisgrove et al., 2007). Analysis of knockout mice has revealed that *Brd2* and *Brd4* are essential for embryonic development. Fibroblasts derived from both *Brd2*- and *Brd4*-deficient animals proliferate more slowly than control ones (Houzelstein et al., 2002; Shang et al., 2009). *Brd4* heterozygous mice display pre- and postnatal growth defects associated with a reduced proliferation rate, while nullizygous embryos die shortly after implantation (Houzelstein et al., 2002). On the other hand, most *Brd2*-deficient embryos

die by embryonic day 11.5 (Gyuris et al., 2009; Shang et al., 2009).

A striking characteristic of BET proteins is that they remain attached to chromosomes during mitosis, suggesting they play a role in the transmission of transcriptional memory across cell division (Dey et al., 2003; Kanno et al., 2004). It has been reported that integrity of the bromodomains is required for this ability (Dey et al., 2003; Kanno et al., 2004). However, other bromodomain-containing proteins do not associate to mitotic chromosomes (Kruhlik et al., 2001), suggesting that special and/or additional structural features in BET proteins should be involved in displaying this behavior. Supporting this hypothesis, we found that a conserved region of 47 amino acids located between the second bromodomain and the ET region of BET proteins, and previously designated as motif B (Paillisson et al., 2007), is required for attachment to mitotic chromosomes. Our results demonstrate that motif B constitutes a dimerization domain, and that function of Brd2 is compromised in its absence.

Results

A small conserved motif in BET proteins is involved in their association with mitotic chromosomes

Bromodomain-containing proteins associate to the chromatin through acetylated histones. During mitosis, most proteins, including many bromodomain-containing proteins, dissociate from chromatin (Kruhlik et al., 2001). However, proteins from the BET family remain attached to mitotic chromosomes (Dey et al., 2003; Kanno et al., 2004). Therefore, it is reasonable to speculate that additional structures, others than the bromodomains, are implicated in BET-attachment to mitotic chromosomes. To investigate this hypothesis we have analyzed the localization of different Flag-tagged constructs derived from the mouse BET protein Brd2 on mitotic chromosomes of P19 cells (Fig. 1A). As occurs for Brd4 (Dey et al., 2003), deletion of bromodomain 1 (BD1) displaces Brd2 from mitotic chromosomes (Fig. 1B),

indicating that integrity of the bromodomains is essential for this function. Similarly, a double Brd2 mutant with point mutations Y152K and N428A in BD1 and BD2, respectively, previously shown to affect Brd2 functionality (Huang et al., 2007; Nakamura et al., 2007), did not associate to mitotic chromosomes (Fig. 1B). However, both bromodomains are not sufficient to promote association to mitotic chromosomes, as a construct encompassing both intact bromodomains also failed to attach to them (Fig. 1B). Serial deletions from the C-terminus of Brd2 indicated that a truncated Brd2 protein lacking from amino acid 612 remained attached to mitotic chromosomes (Fig. 1C). However, deletion from amino acid 473 made the protein to appear dissociated (Fig. 1C). The region between amino acids 473 and 612 contains an uncharacterized motif of 47 amino acids, designated as motif B (mB), which is highly conserved among BET proteins (Paillisson et al., 2007).

To investigate more precisely the involvement of motif B in mitotic chromosomes attachment we tested a Brd2 construct lacking this domain (Δ mB). As shown in Fig. 1C, this construct appeared dissociated from chromosomes. By contrast, deletions of similar length, N- or C-terminal to motif B, resulted in no alteration of Brd2 localization (Fig. 1C). Finally, we analyzed the effect of motif B deletion in the ability of Brd3 and Brd4 to associate to mitotic chromosomes. Similarly to Brd2 mutant, Brd3 and Brd4 mutants were also affected in chromosome localization (supplementary material Fig. S1A).

Taken together, our results strongly indicate that the conserved motif B, together with the integrity of the bromodomains, is required for attachment of BET proteins to mitotic chromosomes.

Efficient recognition of acetylated chromatin by Brd2 requires motif B

A detailed analysis of the Brd2 Δ mB construct through the mitosis revealed that while this mutant was completely dissociated from chromosomes at prophase and metaphase,

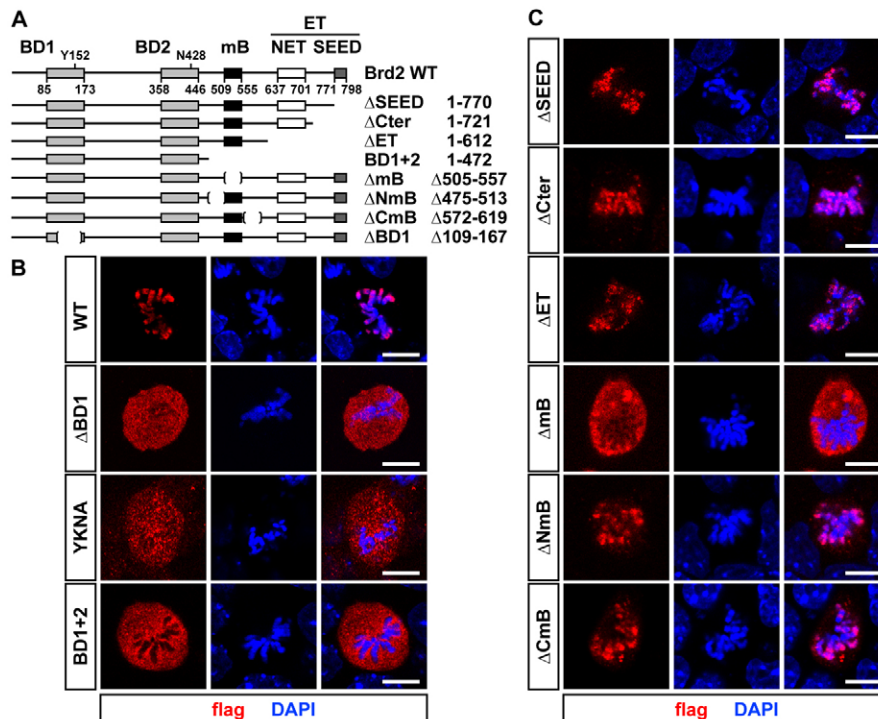


Fig. 1. A small conserved motif is involved in attachment of Brd2 to mitotic chromosomes.

(A) Diagram of the different constructs of Brd2 analyzed in this work. Numbering underneath top construct indicates position of the relevant domains, whereas numbering on the right indicates amino acids comprised or deleted (Δ) in the different constructs. (B) Localization of transfected Flag-tagged constructs of Brd2 in P19 cells harboring the indicated deletions or point mutations was visualized by immunofluorescence with anti-Flag antibodies (red). Images correspond to cells in prophase to metaphase. DNA was counterstained with DAPI (blue). (C) Constructs with serial deletions of Brd2 from the C-terminus or with deletions around motif B were analyzed as in B. BD1, bromodomain 1; BD2, bromodomain 2; mB, motif B; YKNA, Brd2 Y152KN428A mutant. Images are representative of more than 90% of 14–25 cells analyzed per construct. Scale bars: 10 μ m.

significant reassociation was observed at late mitosis, as anaphase proceeds (Fig. 2A). This dual behavior of the Δ mB construct indicates that mitosis progresses in a biphasic manner in relation to the recognition of mitotic chromosomes by Brd2. In this respect, it has been reported that significant deacetylation of the chromatin takes place at the onset of mitosis (Kruhlak et al., 2001). Indeed, real-time imaging experiments have indicated that acetylation of K5/K8 on histone H4 decreases in mitosis (Sasaki et al., 2009). However, not all acetylation marks are lost, since acetylation of K12 on histone H4 is maintained (Ito et al., 2011). To investigate how acetylation affects association of the Δ mB construct to chromosomes we made use of trichostatin A (TSA), which promotes hyperacetylation of chromatin by inhibiting histone deacetylases (HDACs). As shown in Fig. 2B, we observed chromosome association of the Δ mB protein under these conditions. The same result was obtained with Δ mB constructs of Brd3 and Brd4 (supplementary material Fig. S1B). As a control, the Δ BD1 construct remained dissociated in the presence of TSA (Fig. 2B). Titration of TSA indicated significant Brd2 reassociation at 75 ng/ml and almost complete reassociation at 150 ng/ml (supplementary material Fig. S1C).

Next, we asked whether the relevance of motif B in Brd2 association to the chromatin was restricted to mitosis. In order to elucidate that, we decided to compare extraction of transfected WT and mutant Brd2 with increasing salt concentrations from exponentially growing cell cultures, which normally contains $94 \pm 3\%$ ($n=602$) cells in interphase. While WT Brd2 required high salt concentrations (520 mM) to be completely extracted, most Δ mB was extracted at 100 mM NaCl, comparable to the control bromodomain mutant Y152KN428A (Fig. 2C), indicating that requirement of motif B for association to the chromatin is not restricted to mitosis.

We next investigated the direct involvement of motif B in acetyl-histone recognition by in vitro pull-down experiments with protein extracts from cells transfected with different Brd2 constructs. It has been previously reported that Brd2 precipitates acetyl-K14 histone H3 (LeRoy et al., 2008). Thus, for pull-down experiments we used a di-acetylated histone H3 peptide that includes this mark. As observed in Fig. 2D, the Δ mB construct failed to bind to the acetylated peptide in comparison with the wild type, although it retained some binding capacity compared to the negative control Y152KN428A (Fig. 2D).

These results indicate that efficient recognition of the chromatin by Brd2 requires motif B, and that this is particularly appreciable under hypoacetylation conditions, as it occurs during early mitosis.

Conserved motif B constitutes a dimerization domain

To get insights into the role of motif B, we performed a yeast two-hybrid screening with a bait construct encompassing motif B of Brd2. We screened an 11-day-old mouse embryo cDNA library and analyzed about 2×10^6 transformants. Among them, 150 showed positive growth on selective medium, and interestingly, 141 corresponded to a prey construct encompassing motif B of Brd3 (Fig. 3A). As prey and bait constructs included additional sequences besides motif B, we precisely mapped the interaction surfaces and found that motif B of Brd2 interacted with motif B of Brd3 (Fig. 3B). Self-interaction of the Brd2 motif B and other combinations of the different BET motifs B also resulted positive in the yeast assay (Fig. 3B; supplementary material Fig. S2A), suggesting that motif B constitutes a domain for homo- and hetero-dimerization between BET proteins. Direct evidence of the interaction was obtained by pull-down assays with purified recombinant proteins,

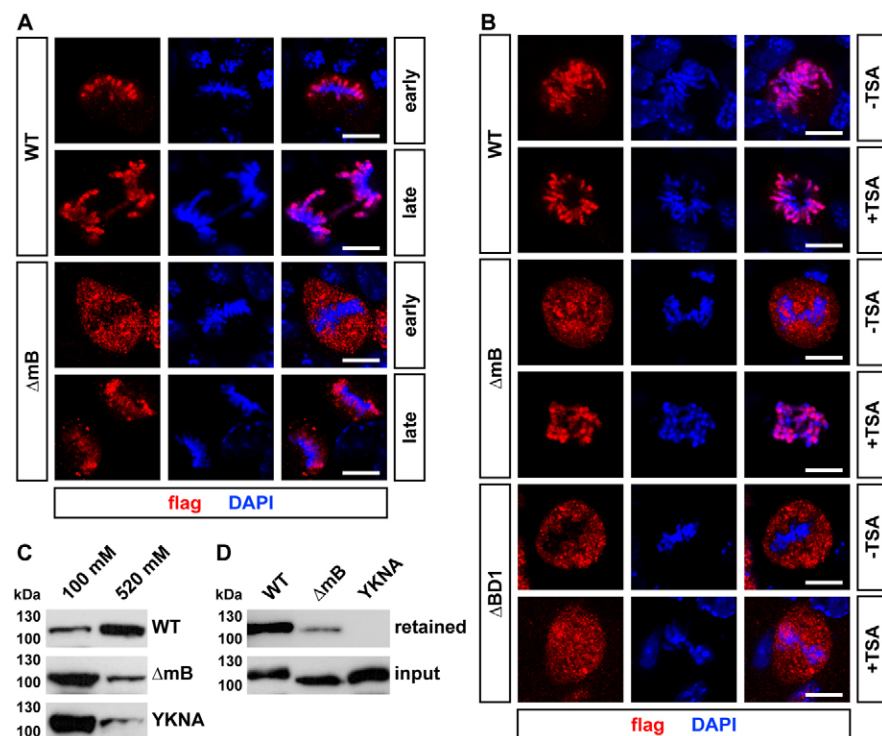


Fig. 2. Motif B is required for proper association of Brd2 to the acetylated chromatin.

(A) Flag-tagged versions of Brd2 WT and Δ mB were transfected and visualized in P19 cells as indicated in Fig. 1 during prophase/metaphase (early mitosis) or anaphase/telophase (late mitosis). (B) P19 cells were transfected with the indicated Flag-tagged constructs and treated (+) or not (-) with TSA. Localization of the constructs was analyzed as in A. Images are representative of more than 90% of 14–25 cells analyzed per construct. Scale bars: 10 μ m. (C) Flag-tagged versions of the indicated constructs were expressed in P19 cells. Then, proteins were consecutively extracted with the indicated NaCl concentrations. The presence of Flag-tagged proteins in the different extracts was detected by western blot. (D) Pull-down experiments were carried out with immobilized diacetylated histone H3 tail peptide and protein extracts from P19 cells transfected with Flag-tagged versions of the indicated constructs. Precipitated Flag-tagged proteins (retained) were detected by western blot. 20% of input protein is also shown.

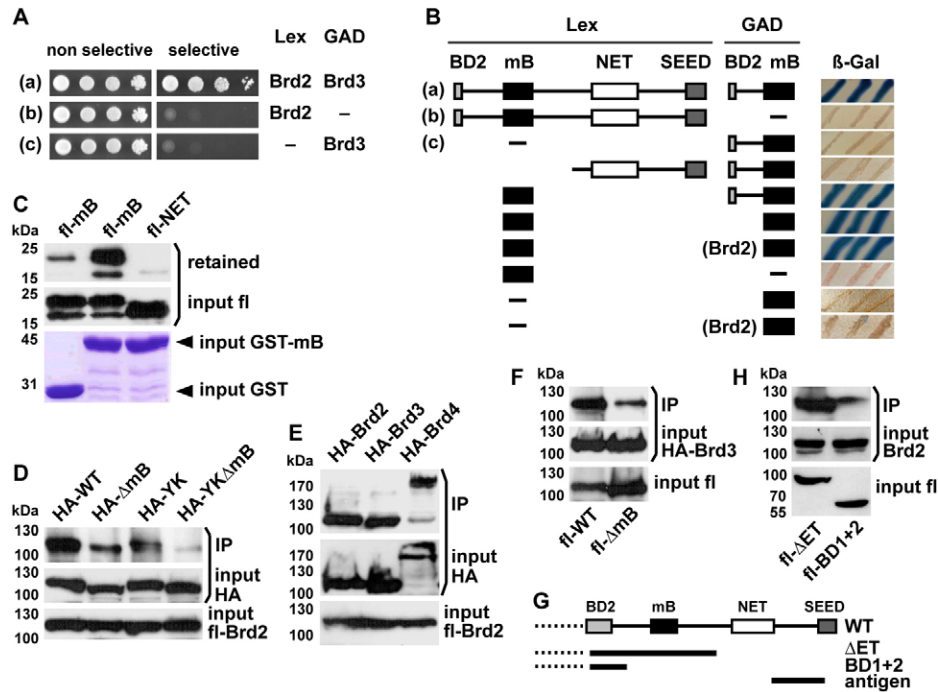


Fig. 3. BET proteins dimerize through the conserved motif B. (A) Growth on non-selective and selective media of yeast harboring bait construct used for two-hybrid screening with *Brd2* (pLexA-N vector, Lex), positive clone corresponding to a fragment of *Brd3* (pGAD-HA vector, GAD), or empty vectors (–), as indicated. Constructs used are depicted in B and indicated as a, b, c. (B) Interaction between the indicated bait (Lex) and prey (GAD) constructs was visualized by blue color in yeast assayed for β -galactosidase activity. All bait constructs correspond to *Brd2*, and all prey constructs to *Brd3*, except where indicated. (C) Pull-down experiments were carried out with immobilized purified glutathione S-transferase (GST) or a GST-motif B (GST-mB) fusion and Flag-tagged motif B (fl-mB) or Flag-tagged NET (fl-NET) peptides purified from bacteria. Precipitated Flag-tagged proteins (retained) were detected by western blot. 20% of Flag-tagged input protein detected by western blot and 100% of GST-derived input protein revealed by Coomassie Blue stain is also shown. (D) Extracts from 293T cells transfected with Flag-tagged *Brd2* (fl-*Brd2*) and the indicated hemagglutinin (HA)-tagged *Brd2* wild-type or mutant constructs were immunoprecipitated with anti-Flag antibodies and co-immunoprecipitated proteins (IP) were visualized by western blot with anti-HA antibodies. YK, *Brd2* Y152K mutant. (E) Extracts from 293T cells transfected with Flag-tagged *Brd2* (fl-*Brd2*) and the indicated HA-tagged *Brd* constructs were immunoprecipitated with anti-Flag antibodies and co-immunoprecipitated proteins (IP) were visualized by western blot with anti-HA antibodies. (F) Extracts from 293T cells transfected with Flag-tagged WT (fl-WT) or Δ mB (fl- Δ mB) *Brd2* constructs and a HA-tagged *Brd3* construct were immunoprecipitated with anti-Flag antibodies and co-immunoprecipitated proteins (IP) were visualized by western blot with anti-HA antibodies. 10% of Flag- or HA-tagged input proteins is also shown in D–F. (G) Schematic representation of *Brd2* constructs analyzed in H and peptide (antigen) used for generation of anti-*Brd2* antibodies. (H) Extracts from P19 cells transfected with the indicated Flag (fl)-tagged *Brd2* deletion constructs were immunoprecipitated with anti-Flag antibodies. Co-immunoprecipitated endogenous *Brd2* was monitored by western blot with anti-*Brd2* antibodies produced in rabbit. 10% of Flag-tagged or endogenous *Brd2* input proteins is also shown.

which showed that a GST-mB construct efficiently retained Flag-mB but not a Flag-NET peptide (Fig. 3C).

We next analyzed the relevance of motif B in *Brd2* self-interaction by immunoprecipitation assays. We included in our analysis a full *Brd2* protein with point mutation Y152K, previously shown to affect dimerization of truncated BD1 (Nakamura et al., 2007). As shown in Fig. 3D, Flag-tagged *Brd2* was efficient in precipitating HA-tagged *Brd2*, less efficient in precipitating the Y152K and Δ mB proteins, and almost ineffective in precipitating the double Y152K Δ mB mutant, indicating that motif B was as necessary as the BD1 for *Brd2* dimerization. Our analysis revealed that *Brd2* precipitated *Brd3* and *Brd4* as well (Fig. 3E). Other BET protein combinations also showed homo- and hetero-dimerization between BET proteins (supplementary material Fig. S2B). We next confirmed the relevance of motif B for *Brd2*–*Brd3* interaction by immunoprecipitation (Fig. 3F). To investigate motif-B-mediated dimerization of endogenous *Brd2* we generated rabbit antibodies against amino acids 698 to 780 at the C-terminus of *Brd2* (Fig. 3G). Using these antibodies we analyzed precipitated endogenous *Brd2* in immunoprecipitation experiments with two *Brd2* constructs

lacking the C-terminus, one of them also lacking motif B. Results revealed that only the construct harboring motif B was efficient in precipitating endogenous *Brd2* (Fig. 3H). Finally, to get evidence of BET homo- and hetero-dimerization in the cell, we analyzed interaction of *Brd2* with itself and with *Brd3* by FRET, and significant FRET signal was observed for GFP-*Brd2*/*Brd2*-RFP and GFP-*Brd3*/*Brd2*-RFP combinations, compared with the negative control Y152K Δ mB (Fig. 4A,B), indicative of protein interaction. Interestingly, deletion of just motif B was sufficient to abrogate the positive FRET signal for *Brd2* self-interaction (Fig. 4A,B).

Our results suggest that dimerization of BET proteins is required for efficient recognition of chromatin. Then, we reasoned that overexpression of a truncated construct containing just the *Brd2* motif B should interfere with chromatin recognition by WT *Brd2*. Hence, we analyze attachment to mitotic chromosomes of WT *Brd2* in the presence of a GFP-motif B construct, and interestingly we found dissociated *Brd2* in prophase/metaphase (Fig. 4C).

Together, our results indicate that homo- or hetero-dimerization of BET proteins through the conserved motif B permits efficient recognition of acetylated chromatin.

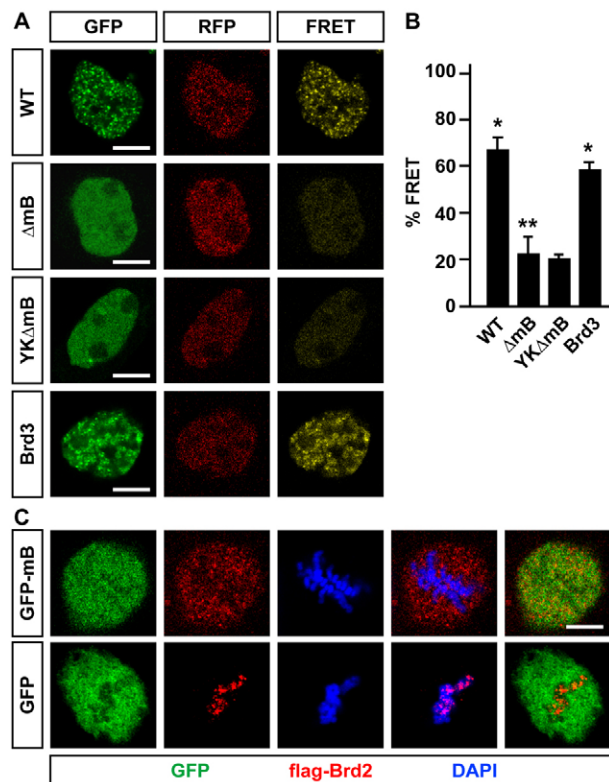


Fig. 4. Dimerization of BET proteins in the cell. (A) FRET analysis in P19 cells expressing Brd2-RFP and GFP fusions of the indicated constructs. YK, Y152K point mutation in Brd2. (B) Quantification of FRET. Data correspond to means \pm s.d. from five independent experiments. Statistical significance was analyzed using the Student's *t*-test. Values for the differences respect to the control YK Δ mB construct: * P <0.001, ** P =0.6. (C) Localization of transfected Flag-Brd2 (red) in P19 cells expressing GFP (green) or a GFP-motif B (mB) fusion protein visualized by immunofluorescence. DNA was counterstained with DAPI (blue). Images are representative of seven out of eight cells analyzed. Scale bars: 10 μ m.

Deletion of motif B impairs Brd2-mediated stimulation of the cell cycle

It has been previously reported that ectopic Brd2 stimulates progression of the cell cycle through cyclin A2 activation (Sinha et al., 2005). Since cyclin D1 has also been reported to be regulated by Brd2 (LeRoy et al., 2008), we have investigated how transfection of WT and Δ mB constructs affects expression levels of cyclin A2 and D1. We found that while WT or Δ NmB Brd2 have a positive effect in cyclins expression, the Δ mB protein has no effect, similar to the Y152KN428A mutant (Fig. 5A). These results correlated with those from chromatin immunoprecipitation (ChIP) experiments involving cyclins A2 and D1 promoters (Fig. 5B). Thus, ChIP experiments showed a similar presence of WT or Δ NmB protein in the promoters, while Δ mB or Y152KN428A were absent. Addition of retinoic acid (RA) to P19 cells induces cell cycle arrest (Pao et al., 2011). In agreement with results described in Fig. 5A,B, we have observed that, while adding RA to control P19 cells or to cells transfected with the Δ mB or the Y152KN428A construct leads to downregulation of cyclins A2 and D1, transfection of the WT or the Δ NmB construct prevents downregulation (Fig. 5C). By analyzing cell cycle profiles we remarked an increase of cells in

the G1 phase upon RA treatment, irrespective of the presence of the Δ mB protein or the Y152KN428A control (Fig. 5D,E). Thus, ratio of cells in G1 respect to cells in the S, G2 and M phases increased from about 0.7 to near 1.5, indicating that cells in G1 increased from 40% to 60%. By contrast, the presence of the WT or the Δ NmB protein impaired these RA effects, resulting in a profile similar to that of non-treated cells (Fig. 5D,E). These results indicate that the Δ mB protein fails to stimulate the cell cycle and to interfere with RA-induced arrest of the cell cycle, as WT Brd2 does.

Based in our results, it is tempting to speculate that Brd2 overexpression, by stimulating the cell cycle, might impair cell differentiation. To investigate this hypothesis we choose a neuronal differentiation model. A standard approach to study neuronal differentiation consists in transfecting P19 cells with E12 and NeuroD2 expression constructs to evaluate the presence of the neuronal marker β III-tubulin in transfected cells (Farah et al., 2000; Seo et al., 2005). Results indicated that transfection of the Δ mB construct had little effect in neuronal differentiation, compared with the significant reduction (42% decrease) mediated by WT Brd2 (Fig. 6A,C). Finally, we decided to confirm these results in an animal model, and without forcing differentiation. Thus, we turned to the chick embryo and used the technique of electroporation to transfect half of the neural tube with WT and Δ mB constructs of Brd2. As Fig. 6B,C indicates, 30 h after electroporation, about 35% of transfected proliferating neuroblasts in the ventricular zone (VZ), which express a GFP control construct, have naturally exited the cell cycle and migrated to the neural tube margin, or mantle layer (ML), where they install to differentiate into neurons. While electroporation of WT Brd2 severely interfered with ML migration of transfected neuroblasts, indicative of neuronal differentiation impairment, the Δ mB construct had no effect (Fig. 6B,C). Thus, these results indicate that the conserved motif B is required for Brd2-mediated interference of neuronal differentiation.

Discussion

Since many bromodomain containing proteins dissociate from chromatin during mitosis, association of BET proteins to mitotic chromosomes constitutes an intriguing feature. The presence of two tandem bromodomains might explain this ability. However, other factors with tandem bromodomains dissociate from mitotic chromosomes [for instance TAFII250 (Kruhlak et al., 2001)], suggesting that additional structural features in BET proteins might account for this behavior. In this regard, we have identified motif B as a dimerization domain involved in association to mitotic chromosomes and proper recognition of acetylated chromatin by BET proteins.

Role of motif B in chromatin recognition

While single bromodomains from Brd2 have been shown to attach to histone peptides in vitro (Huang et al., 2007; Nakamura et al., 2007; Umehara et al., 2010a; Umehara et al., 2010b), we have observed chromosome dissociation of a construct encompassing both intact bromodomains in the cell, highlighting the relevance of other structural characteristics in the context of the full-length protein for chromatin association in vivo. In this regard, motif B seems to be specifically required for chromosome association, since deletions of similar extent, N-terminal or C-terminal to motif B, do not affect Brd2 behavior or function. It is

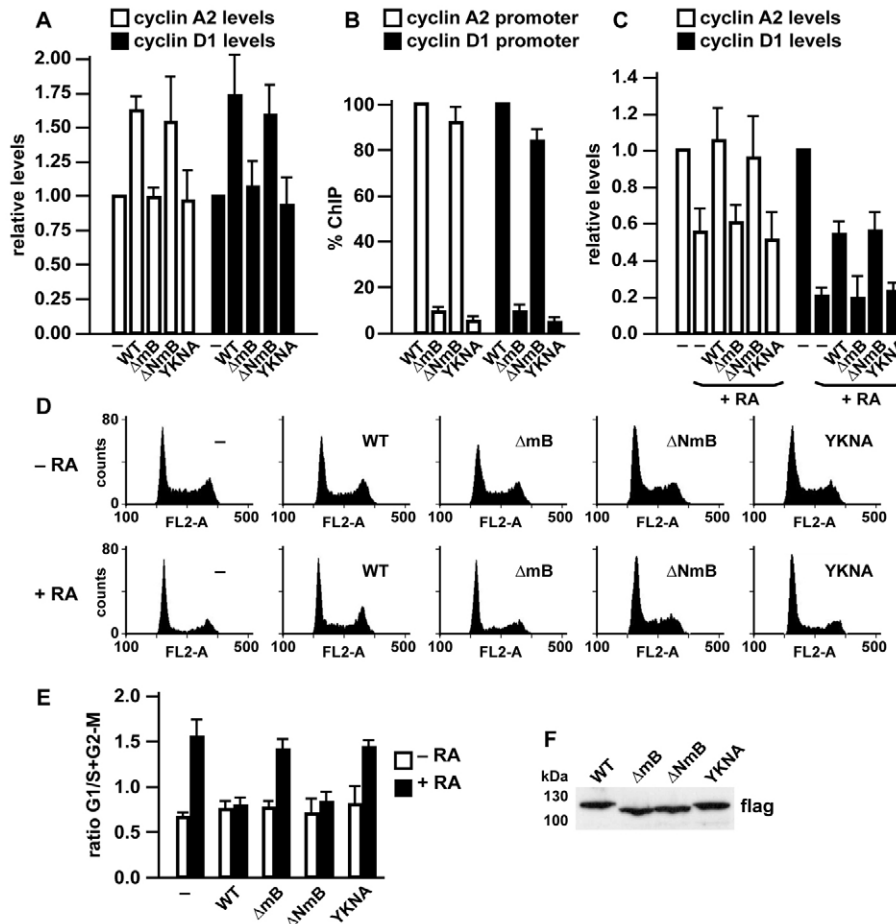


Fig. 5. Deletion of motif B impairs effects of ectopic Brd2 on the cell cycle. (A) Relative levels of expression of the *CcnA2* and *CcnD1* genes as determined by real time PCR in P19 cells transfected with the indicated constructs and subjected to serum stimulation. Levels were normalized to cells transfected with empty vector (-). (B) Levels of association of the indicated Flag-tagged constructs to the *CcnA2* and *CcnD1* promoters in P19 cells determined by chromatin immunoprecipitation (ChIP) with anti-Flag antibodies. Levels were normalized to cells transfected with WT Brd2. (C) Relative levels of expression of the *CcnA2* and *CcnD1* genes determined as in A in P19 cells transfected with the indicated constructs and treated (+) or not for 18 h with all-trans retinoic acid (RA). Levels were normalized to non-treated cells transfected with empty vector (-). (D) Cell cycle profiles determined by flow cytometry of P19 cells transfected with the indicated constructs or empty vector (-) and treated or not with RA as in C. (E) Ratio of cells in G1 respect to cells in the S, G2 and M phases from cell cycle profiles has been represented. Values are means \pm s.d. from three to five independent experiments in triplicate (A-C) or means \pm s.d. from four independent experiments (E). YKNA, Brd2 Y152KN428A mutant. Statistical significance was analyzed using the Student's *t*-test: (A) Differences between control (-) and WT or Δ NmB constructs, $P \leq 0.01$; control vs WT or Δ mB or YKNA, $P \geq 0.45$. (B) WT vs Δ mB or YKNA, $P < 0.001$; WT vs Δ NmB, $P \geq 0.5$. (C) RA-treated vs non-treated control cells, $P < 0.005$; control vs WT or Δ NmB in the presence of RA, $P < 0.02$; control vs Δ mB or YKNA in the presence of RA, $P \geq 0.4$. (E) Owing to RA treatment of control (-) and Δ mB, $P < 0.02$; owing to RA treatment of WT, $P = 0.58$; for the differences between control, WT and Δ mB before RA treatment, $P \geq 0.3$; WT vs control or Δ mB after RA treatment, $P < 0.02$; control vs Δ mB after RA treatment, $P = 0.35$. (F) Western blot showing expression levels of the different Flag-tagged constructs used in A-E transfected in P19 cells; 10 μ g of total protein were loaded per lane.

surprising that the Δ mB protein dissociates from chromosomes at early mitosis but significantly reassociates at the end. Interestingly, previous reports have shown a generalized deacetylation of the chromatin at the onset of mitosis and a progressive reacylation at late mitosis (Kruhlak et al., 2001; Sasaki et al., 2009). Altogether, this suggests that the requirement of motif B might rely on the acetylation status of the chromatin. TSA-mediated reassociation observed at early mitosis supports this idea and highlights the relevance of motif B under hypoacetylation conditions, as it occurs during mitosis. However, despite chromosome reassociation of the Δ mB construct observed at late mitosis our data indicate that motif B is required for efficient recognition of the chromatin independently of the cell cycle phase. The easy extraction of the protein from non-synchronous cells at low salt concentrations, its

absence at cyclin A2 and cyclin D1 promoters, the impaired association to acetylated histone peptides *in vitro* and the lack of activity of the Δ mB construct in functional analysis all support this notion. Apparent discrepancy between chromosome association of this construct at late mitosis or in the presence of TSA at early mitosis and impaired binding to fully acetylated histone peptides may be explained by the different experimental approaches. Then, we have set up *in vitro* pull down conditions (mainly salt concentration) to discriminate small differences in binding capacities of wild type and mutant Brd2. More important, although we observe reassociation of the Δ mB protein to chromosomes in immunofluorescence experiments, affinity or architecture of associated Δ mB might quite differ from that of the wild type protein, as suggest ChIP and differential salt extraction

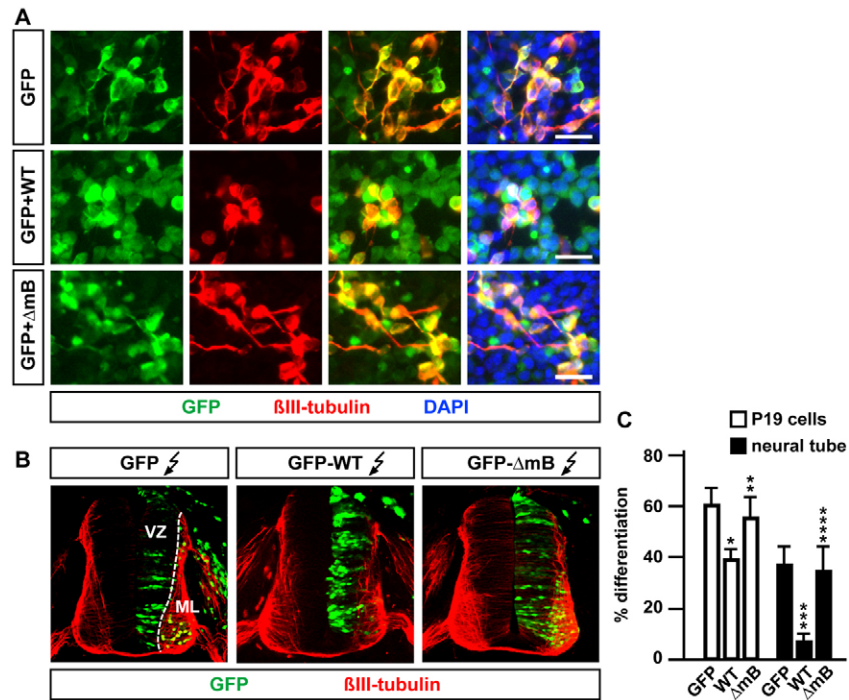


Fig. 6. Motif B is required for Brd2-mediated interference of neuronal differentiation. (A) P19 cells were transfected with NeuroD2 and E12 expression constructs, with a GFP reporter and the indicated Brd2 constructs, and were tested for expression of the neuronal marker β III-tubulin. Neurogenesis was evaluated as the percentage of transfected cells [GFP positive (green)], which are β III-tubulin positive (red). DNA was counterstained with DAPI (blue). Scale bars: 50 μ m. (B) The neural tube of chick embryos at stage HH17 was electroporated on the right side with the indicated constructs and neurogenesis was evaluated by immunofluorescence after 30 h on transverse sections of the spinal cord. In this case, neurogenesis was evaluated as the percentage of transfected cells (GFP positive), which have left the proliferative or ventricular zone (VZ) and have migrated to the differentiation compartment or mantle layer (ML), where the neuronal marker β III-tubulin (red) is expressed. Frontier between VZ and ML is indicated by a dashed line in the electroporated side of the GFP control experiment (left panel). (C) Representation of the percentage of neurogenesis determined as indicated in A (P19 cells) and B (neural tube). Values are means \pm s.d.; two to three areas from four independent experiments were analyzed for P19 cells and three to four sections from five independent experiments for the neural tube. Representative images are displayed. Statistical significance was analyzed using the Student's *t*-test: values compared with control GFP were * P <0.01, ** P =0.37, *** P <0.02, **** P =0.75.

experiments in non-synchronous cells. As our data suggest, improper reassociation of Brd2 lacking motif B to the chromatin might have a detrimental impact in transcriptional memory. Thus, motif B should be relevant for right functional conformation of BET proteins rather than just for physical recognition of acetylated chromatin.

In contrast to Brd4 and Brdt, which preferentially bind histone H4 at the di-acetylated state (Dey et al., 2003; Morinière et al., 2009; Sasaki et al., 2009), Brd2 seems to more efficiently bind mono-acetylated histone H4 at K12 (Ito et al., 2011; Kanno et al., 2004). However, additional acetylation marks have been reported to be recognized by Brd2, for instance acetyl-K5 and acetyl-K8 on histone H4 (Kanno et al., 2004; Umehara et al., 2010b). It is worth noting that these marks decrease during mitosis (Kruhlak et al., 2001; Sasaki et al., 2009). In addition, here we show binding of Brd2 to a histone H3 peptide acetylated at K9/14. Although several reports suggest binding specificities of BET proteins for different acetylation marks, some discrepancies are observed in the literature, probably due to different experimental approaches (Dey et al., 2003; Ito et al., 2011; Kanno et al., 2004; LeRoy et al., 2008; Morinière et al., 2009; Sasaki et al., 2009; Umehara et al., 2010b). Thus, binding specificities have been determined either by in vitro pull-down experiments, SPR binding assays or real-time imaging in living cells, and using

overexpressed proteins, either full-length fusion constructs, chimeric proteins or truncated fragments including just the bromodomains (Dey et al., 2003; Huang et al., 2007; Ito et al., 2011; Kanno et al., 2004; Morinière et al., 2009; Nakamura et al., 2007; Sasaki et al., 2009; Umehara et al., 2010a; Umehara et al., 2010b). Moreover, as discussed below, our data suggest that BET proteins heterodimerize, raising the question about the real binding specificities of the endogenous proteins.

Motif-B-mediated dimerization of BET proteins

Several pieces of evidence support a role of motif B in Brd2 dimerization: (1) self-interaction of motif B by two-hybrid assay; (2) pull-down of recombinant motif B by a GST-motif B fusion protein; (3) motif-B-dependent immunoprecipitation of wild type Brd2; and (4) significant FRET signal in self-interaction experiments. Function of many transcription factors relies on a dimer structure (Amoutzias et al., 2008). It has been previously reported that the N-terminal bromodomain (BD1) of Brd2 is involved in Brd2 dimerization (Nakamura et al., 2007). However, our results indicate that motif B is as necessary as the BD1 for Brd2 self-interaction. In fact, we show motif B is crucial for FRET signal. Thus, both BD1 and motif B might cooperate to display the appropriate functional configuration of Brd2 for efficient recognition of specific acetylation marks on the chromatin by

the bromodomains. Moreover, our results indicate that motif B mediates not only homodimerization but also heterodimerization between BET proteins. Immunoprecipitation experiments, two-hybrid assays and FRET analysis substantiate this notion. It is tempting to speculate that homo- and heterodimerization between the different BET proteins might be involved in modulating transcriptional activity associated to these proteins. In fact, the absence of functional redundancy between BET proteins despite overlapping patterns of expression (Gyuris et al., 2009; Houzelstein et al., 2002; LeRoy et al., 2008; Shang et al., 2009), should argue in favor of a relevant role for the heterodimerization of BET proteins.

The sequence of motif B is highly conserved at the amino acid level among different vertebrate BET proteins (Fig. 7A). Analysis of the Brd2 motif B sequence with the Jpred3 tool (<http://www.compbio.dundee.ac.uk/www-jpred/>) indicates that this region is not disordered, and predicts a coiled coil structure. High score for a coiled coil structure in this region was confirmed with the COILS tool (Lupas et al., 1991). Thus, a score >96% was obtained for this region with a scanning window of 21 residues, in contrast to the low score (<1%) obtained for surrounding regions or other domains in the protein previously shown not to arrange in a coiled coil structure, for instance the bromodomains (Huang et al., 2007; Nakamura et al., 2007).

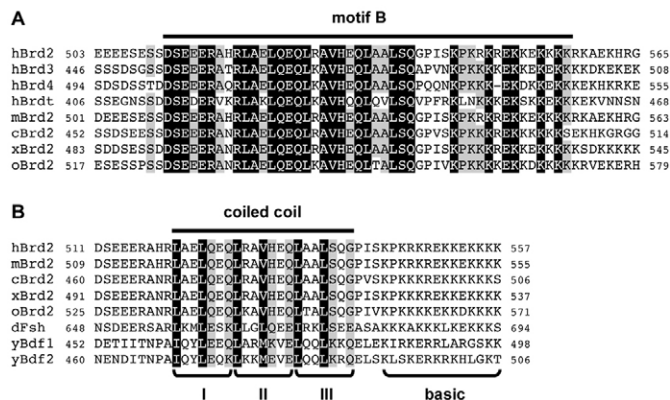


Fig. 7. The conserved motif B contains a putative coiled coil. (A) Amino acid sequence of different BET proteins from vertebrates, corresponding to a region located between the bromodomains and the ET domain, were aligned. Amino acids conserved in all the sequences are boxed in black, whereas amino acids conserved in six of the eight sequences are labeled in gray. The indicated conserved region constitutes the motif B (Paillisson et al., 2007). (B) Motif B of different Brd2 proteins from vertebrates, and BET proteins from *Drosophila* and yeast are aligned. A putative coiled coil structure with a three-heptad repeat (I, II, III) is marked. Black boxes indicate relevant non-polar residues in the heptad repeat, whereas gray boxes indicate putative solvent-exposed residues in the heptad repeat (Grigoryan and Keating, 2008). The conserved basic region at the C-terminus of motif B is also indicated. Alignments were performed with the ClustalX 2.0.11 application. Coiled-coil prediction was performed with the COILS tool (http://www.ch.embnet.org/software/COILS_form.html). Amino acid positions of the aligned sequences are indicated at left and right of each sequence. Accessions and abbreviations are as follows: human Brd2 (hBrd2), NM_001113182; human Brd3 (hBrd3), NM_007371; human Brd4 (hBrd4), NM_014299; human Brdt (hBrdt), NM_001242805; mouse Brd2 (mBrd2), NM_001204973; chicken Brd2 (cBrd2), NM_001030674; Xenopus Brd2 (xBrd2), BC084758; Medaka Brd2 (oBrd2), AB183488; *Drosophila* Fsh (dFsh), NM_078523; yeast Bdf1 (yBdf1), NM_001182287; yeast Bdf2 (yBdf2), NM_001180129.

Moreover, prediction for motif B was very similar to that for established coiled coils, as the classical leucine zipper in the yeast protein GCN4 (>99%) (O'Shea et al., 1991). COILS analysis and alignment of motif B from vertebrate and non-vertebrate BET proteins indicated the conservation of the putative coiled coil through the different species analyzed (Fig. 7B). Coiled coils consist of a heptad repeat (abcdefg) with hydrophobic residues at positions a and d, and polar solvent-exposed residues at e and g (Grigoryan and Keating, 2008). Thus, putative coiled coil in motif B showed a three heptad repeat (Fig. 7B). As coiled coils usually mediate dimerization of proteins (Grigoryan and Keating, 2008), this finding strongly supports a role of motif B in dimerization. However, we cannot rule out the possibility that other sequences in motif B, for instance the well-conserved basic region C terminal to the coiled coil, could be also relevant for dimer formation (Fig. 7B).

Requirement of motif B for Brd2 function

Knocking down experiments have demonstrated that BET proteins are necessary for cell cycle progression and cyclin D1 expression (LeRoy et al., 2008; Mochizuki et al., 2008; Yang et al., 2008). We have shown that ectopic expression of Brd2 is able to sustain elevated levels of cyclins A2 and D1 transcripts, which correlates with reduced neuronal differentiation and impaired RA-induced arrest of the cell cycle. Similarly, overexpression of cyclin D1 in skin keratinocytes has been previously reported to interfere with cell differentiation (Burnworth et al., 2006). An essential aspect of our results is that all the observed effects associated to the ectopic expression of Brd2 are highly impaired in the absence of motif B, indicating that this domain is essential for Brd2 function. The prominent role displayed by BET proteins in cell cycle progression tightly links them to tumor development (Crawford et al., 2008; Filippakopoulos et al., 2010; French, 2012; French et al., 2003; French et al., 2008; Greenwald et al., 2004; Zuber et al., 2011). In fact, it has been very recently shown that interfering with association of BET proteins to the chromatin by synthetic drugs mimicking specific acetylation marks, efficiently counteract cancer progression in mice models (Dawson et al., 2011; Delmore et al., 2011; Filippakopoulos et al., 2010; Mertz et al., 2011; Prinjha et al., 2012; Zuber et al., 2011). In this context, a precise knowledge of structural features involved in association of BET proteins to the chromatin will contribute to the design of more efficient and specific drugs.

Materials and Methods

Plasmid constructs and yeast two-hybrid

All transfection constructs except GFP- and RFP-based constructs were derived from vector pAdRSV-S (Giudicelli et al., 2003), with Flag or HA tags. GFP- and RFP-based constructs were derived from pEGFP-C2 and pDsRed-monomer-N1 vectors (Clontech), respectively. The GFP-mB construct was designed with amino acids T463 to I565 of Brd2. Mouse *Brd2*, *Brd3* and *Brd4* cDNAs were obtained by RT-PCR with RNA isolated from P19 cells. Deletion constructs of Brd2 were performed by standard PCR techniques and were as follows: WT, amino acids M1-G789; ΔSEED, M1-R770; ΔCter, M1-L721; ΔET, M1-K612; BD1+2, M1-L472; ΔmB, deletion (Δ) of amino acids S505 to K557; ΔNmB, Δ S475-E513; ΔCmB, Δ K572-K619; ΔBD1, Δ L109-T167. Y152K and N428A mutations were also performed by standard PCR. Yeast two-hybrid and X-gal assays were performed in the DUALhybrid Kit (Biotech) system, using the pLexA-N bait vector and a day 11 p.c. whole mouse embryo cDNA library cloned in the pGAD-HA vector (Biotech), according to manufacturer instructions. Bait construct for screening encompassed amino acids T463 to G789 of Brd2, while ET and mB constructs encompassed amino acids G607-G789 and T463-I565, respectively. Most positive Brd3 clones corresponded to amino acids K402 to K501. Brd3 and Brd4 mB constructs encompassed amino acids V432 to K501 and P483 to H552, respectively.

Protein production and purification and pull-down assays

Production of proteins was performed in the *E. coli* BL21 strain and purification as GST fusions was achieved by incubation with Glutathione Sepharose 4B matrix (GE Healthcare). GST was excised with PreScission protease (GE Healthcare) when required. GST-mB and GST-Flag-mB constructs encompassed amino acids E482 to A595 of Brd2, while GST-Flag-NET encompassed amino acids T620 to G720. Pull-down experiments with immobilized GST fusion protein or acetylated histone peptides were conducted as previously described (García-Domínguez et al., 2008). For peptide pull-down we used biotinylated acetylhistone H3 (acetyl-K9/K14, Upstate Millipore) and streptavidin-coupled magnetic beads (DynaBeads M-280 Streptavidin, DYNAL, Invitrogen).

Cell culture, transfections and flow cytometry

Human 293T and mouse P19 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and α -modified Eagle's medium supplemented with 7.5% calf and 2.5% fetal bovine sera (PAA), respectively. Transfections were performed with Lipofectamine 2000 (Invitrogen) 36 h before harvesting cells. Except when indicated, trichostatin A (TSA) was used at 500 ng/ml for 4 h. For cyclin analysis under normal growth conditions, P19 cells were deprived of serum for 48 h and harvested 18 h after serum re-addition, as previously described (Sinha et al., 2005). All *trans* retinoic acid (RA) was used at 0.5 μ M for 18 h. For transfection monitoring, the green fluorescent protein expression vector pEGFP-N1 (Clontech) was used. Cell cycle profiles of propidium-iodide-treated cells were analyzed using a flow cytometry FACScalibur apparatus (BD Biosciences).

Differential salt extraction and immunoprecipitation

For differential salt extraction, P19 cells were first extracted with buffer A [50 mM Tris-HCl, pH 7.5, 1% Triton X-100, Complete protease inhibitor cocktail (Roche)] supplemented with 100 mM NaCl. Then, pellet was extracted with the same buffer containing 520 mM NaCl. For immunoprecipitation, 9×10^5 293T cells were extracted with 250 μ l of buffer A containing 150 mM NaCl. Then, NaCl concentration was increased to 600 mM dropwise. Supernatant was recovered and 3-fold diluted with buffer B [50 mM Tris-HCl, pH 7.5, Complete protease inhibitor cocktail (Roche)] for overnight incubation with anti-Flag M2 Affinity Gel (SIGMA). After washing, proteins were eluted with 20 μ l of SDS-containing Laemmli buffer and analyzed by western blot.

Antibody generation and western blot

Antibodies against Brd2 were produced in rabbit after immunization with a peptide corresponding to amino acids 698–780 of the mouse Brd2 protein. Western blot was conducted on PVDF membrane (Bio-Rad) according to the manufacturer's instructions. Antibodies: rabbit anti-Brd2 (1:500), mouse anti-Flag M2 (1:2000, SIGMA), rat monoclonal anti-HA (1:2000, Roche), goat anti-rabbit, anti-rat and anti-mouse HRP-conjugated antibodies (1:10,000, Sigma).

Chromatin immunoprecipitation and quantitative PCR

A total of 10^7 cells fixed in 1% formaldehyde for 10 min at 37°C were used in each experiment. The chromatin was immunoprecipitated with the anti-Flag M2 Affinity Gel (Sigma). Quantitative PCR was used for analysis of the *CcnD1* and *CcnA2* promoters and determination of gene expression levels. Total RNA was isolated with the RNeasy kit (Qiagen). Retrotranscription of RNA was performed with the Superscript III enzyme (Invitrogen). Real time PCR reactions were performed with the SensiMix SYBR Low-ROX kit (Bioline) in the Applied Biosystems 7500 FAST Real-Time PCR System. Endogenous *GAPDH* and transfected *GFP* were used for normalization. Algorithms for calculation of relative units and normalization of values according to primer efficiency have been previously described (Pfaffl, 2001). Sequence of primers was as follows: *CcnA2*-F, 5'-CTTGCTGCACCAACAGTAA-3'; *CcnA2*-R, 5'-AGCAATGAGTGAAGG-CAGGT-3'; *CcnD1*-F, 5'-TCAAGACGGAGGAGACCTGT-3'; *CcnD1*-R, 5'-CTCCTCTCGCACTTCTGCT-3'; *GAPDH*-F, 5'-AACTTTGGCATTGTGGAA-GG-3'; *GAPDH*-R, 5'-GGATGCAGGGATGATGTCT-3'; *GFP*-F, 5'-CAAG-ATCCGCCACAACATCG-3'; *GFP*-R, 5'-GTCCATGCCGAGAGTGATCC-3'; *A2*prom-F, 5'-CCAGCGTTTCCCTATGTTGT-3'; *A2*prom-R, 5'-CTAGGCA-GGACGATGATGAT-3'; *D1*prom-F, 5'-GGAGGACCCTCTTAGGAAA-3'; *D1*prom-R, 5'-CGGACTGCTTCTCTCAAAC-3'.

In ovo electroporation, immunofluorescence and FRET

Electroporation, preparation of embryos and immunofluorescence on neural tube sections or P19 cells were conducted as described previously (Farah et al., 2000; García-Domínguez et al., 2003). For electroporation monitoring, the green fluorescent protein expression vector pEGFP-N1 (Clontech) was used at a concentration of 0.3 μ g/ μ l. Antibodies: mouse anti-Flag M2 (1:250, SIGMA), rabbit anti-neuron specific β III-tubulin (1:500, Abcam), donkey anti-mouse and anti-rabbit DyLight-549 (1:800, Jackson ImmunoResearch). Cell nuclei were exposed by DAPI staining. Fluorescent images were acquired on a Leica confocal microscope TCS SP5. FRET analysis conducted in P19 cells was based on green

and red fluorescent proteins (GFP, RFP) and analyzed with the Leica Application Suite Advanced Fluorescence Software. An RFP-Brd2 construct derived from plasmid pDsRed-monomer-Hyg-N1 (Clontech) was assayed together with different GFP-based constructs derived from plasmid pEGFP-C2 (Clontech). A HCX PL APO lambda blue 63 \times 1.4 Oil objective was used and the following wavelengths were recorded: the green channel (donor excitation/donor emission, 488 nm/496–526 nm, AOTF 21%), the red channel (acceptor excitation/acceptor emission, 543 nm/579–661 nm, AOTF 40%) and the FRET channel (donor excitation/acceptor emission, 488 nm/535–577 nm). A ratiometric calculation of FRET and donor signals was obtained.

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