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RESEARCH ARTICLE

Pleiotrophin antagonizes Brd2 during neuronal differentiation

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ABSTRACT

Bromodomain-containing protein 2 (Brd2) is a BET family chromatin adaptor required for expression of cell-cycle-associated genes and therefore involved in cell cycle progression. Brd2 is expressed in proliferating neuronal progenitors, displays cell-cycle-stimulating activity and, when overexpressed, impairs neuronal differentiation. Paradoxically, Brd2 is also detected in differentiating neurons. To shed light on the role of Brd2 in the transition from cell proliferation to differentiation, we had previously looked for proteins that interacted with Brd2 upon induction of neuronal differentiation. Surprisingly, we identified the growth factor pleiotrophin (Ptn). Here, we show that Ptn antagonized the cell-cycle-stimulating activity associated with Brd2, thus enhancing induced neuronal differentiation. Moreover, Ptn knockdown reduced neuronal differentiation. We analyzed Ptn-mediated antagonism of Brd2 in a cell differentiation model and in two embryonic processes associated with the neural tube: spinal cord neurogenesis and neural crest migration. Finally, we investigated the mechanisms of Ptn-mediated antagonism and determined that Ptn destabilizes the association of Brd2 with chromatin. Thus, Ptn-mediated Brd2 antagonism emerges as a modulation system accounting for the balance between cell proliferation and differentiation in the vertebrate nervous system.

KEY WORDS: Brd2, Ptn, Neuronal differentiation, Chromatin

INTRODUCTION

Proper coordination between cell proliferation and differentiation is crucial for development and growth of living organisms. Accurate control of these processes is a challenge for regenerative medicine when applying cell therapies. Members of the bromodomain and extra terminal (BET) family of transcriptional co-regulators have essential roles in cell cycle progression (Belkina et al., 2014; LeRoy et al., 2008; Mochizuki et al., 2008; Yang et al., 2008). Interestingly, some BET members have been additionally associated with cell differentiation. In this regard, the family member bromodomain-containing protein 2 (Brd2), besides interacting with the transcription factor E2F and controlling expression of cyclin A2 and D1 in proliferating cells (Belkina et al., 2014; Denis et al., 2000; Garcia-Gutierrez et al., 2012; LeRoy et al., 2008; Sinha et al., 2005), is expressed in differentiating neurons (Crowley et al., 2004), which might be linked to the neural tube closure defects observed in *Brd2*-knockout mouse embryos and to the association of mutations in the *BRD2* locus with human juvenile myoclonic epilepsy (Gyuris et al., 2009; Shang et al., 2009; Velíšek et al., 2011).

Four BET proteins have been described in vertebrates, Brd2, Brd3, Brd4 and Brdt, which are broadly expressed with the exception of Brdt, where expression is restricted to the male germ line (Crowley et al., 2004; Rhee et al., 1998; Shang et al., 2004; Taniguchi et al., 1998). BET proteins, by means of two N-terminal bromodomains, recognize and associate with acetylated histones on chromatin (Kanno et al., 2004). In contrast to most proteins, including other bromodomain-containing proteins, some of the BET family members have the ability to remain attached to the chromosomes during mitosis (Dey et al., 2003), suggesting they have an epigenetic function. Although binding of BET proteins to the chromatin depends on the integrity of the bromodomains (Kanno et al., 2004), we have recently reported that dimerization through motif B is also required (Garcia-Gutierrez et al., 2012). Knockout mice of BET family members have highlighted the relevance of these proteins for development and survival. Thus, *Brd4* and *Brd2* mutant mice die at early post-implantation and E11.5 stages, respectively (Houzelstein et al., 2002; Shang et al., 2009). Mouse embryonic fibroblasts derived from *Brd4* and *Brd2* mutant embryos display proliferative defects, which, to a lesser extent, are also observed in heterozygous cells (Houzelstein et al., 2002; Shang et al., 2009). In addition, knockdown of Brd4 in cultured cells leads to cell cycle arrest (Mochizuki et al., 2008; Yang et al., 2008). Interestingly, reduced expression of Brd2 in mice produces a distinct hypomorphic phenotype with extreme obesity and hyperinsulinemia, but enhanced glucose tolerance and low blood glucose (Wang et al., 2010).

In the developing neural tube, proliferation occurs in the ventricular zone, close to the lumen, and neuronal differentiation requires proliferating progenitors to exit the cell cycle and, subsequently, migrate to the pial surface, or mantle layer, to accomplish differentiation. Besides being expressed in neural progenitors, Brd2 is also detected in differentiating neurons of the mantle layer in the developing spinal cord (Crowley et al., 2004). It has been reported that Brd2 stimulates cell cycle progression (Sinha et al., 2005), and in fact, we have shown that overexpressing Brd2 impairs neuronal differentiation, as it results in elevated expression levels of cyclin D1 and A2 (Garcia-Gutierrez et al., 2012). Thus, the prominent role Brd2 displays during cell proliferation raises the question about its presence in differentiating neurons. To reconcile these observations and to better understand Brd2 function we have looked for Brd2-interacting proteins. From a previous two-hybrid screening (Garcia-Gutierrez et al., 2012), we identified pleiotrophin (Ptn) as a Brd2-interacting protein, and chose to study it because it is expressed in P19 cells following induction of neuronal differentiation (Brunet-de Carvalho et al., 2003).

Ptn is abundantly expressed in the developing nervous system, and together with its homologue midkine (Mdk), comprise a family of secreted heparin-binding growth factors (Kadomatsu and Muramatsu, 2004). Its neuroprotective properties, together

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with its capacity to promote neurite outgrowth, are important for nerve regeneration (Jin et al., 2009). In this work, we describe Ptn–Brd2 interaction during neuronal differentiation. Our results indicate that Ptn enhances induced neuronal differentiation by antagonizing Brd2 cell-cycle-stimulating activity. Thus, we propose that Ptn-mediated antagonism accounts for the occurrence of neuronal differentiation in the presence of Brd2.

RESULTS

Ptn interacts with Brd2

To obtain insight into the function of Brd2 and in particular, to begin to explain how the role of Brd2 in regulating the cell cycle can be understood in light of its presence in differentiating neurons, we previously had performed a yeast two-hybrid screening with an 11-day-old mouse embryo cDNA library (Garcia-Gutierrez et al., 2012). At this stage, development of the neural tube is advanced and contains both neural progenitors and differentiating neurons. Among the Brd2 partners we identified was Ptn, previously shown to be expressed following retinoic acid (RA)-mediated induction of neuronal differentiation in mouse teratocarcinoma P19 cells (Brunet-de Carvalho et al., 2003). Interaction in yeast was confirmed by growth in selective medium and β-galactosidase staining of positive clones (Fig. 1A,B). To analyze interaction in mammalian cells we co-expressed HA- and Flag-tagged versions of Ptn and Brd2, respectively, in 293T cells. Immunoprecipitation with anti-Flag antibodies demonstrated co-precipitation of Ptn–HA only when Flag–Brd2 was expressed (Fig. 1C). To analyze direct interaction between both proteins we conducted *in vitro* pulldown experiments by incubating a fragment of Brd2, expressed and purified from *E. coli* as a histidine tail fusion protein, with either purified GST or a GST–Ptn fusion protein. Results revealed Brd2 co-precipitation only in the presence of GST–Ptn, indicating direct interaction between these proteins (Fig. 1D). As Ptn shows a substantial degree of conservation with the other family member Mdk, we asked whether Brd2 could also interact with Mdk. Immunoprecipitation analysis indicated that Brd2 also precipitated Mdk (Fig. 1E).

Next, we wished to map the regions of interaction between Brd2 and Ptn. Given that the C-terminal region of Ptn has been revealed as a functional domain (Bernard-Pierrot et al., 2001; Zhang et al., 1999), we analyzed the interaction between Brd2 and Ptn protein lacking this domain by yeast two-hybrid assays. Our results indicated that the C-terminus of Ptn is required for this interaction (Fig. 1F). Because this domain is rich in basic amino acids, we speculated about the involvement of an acidic region of Brd2 in the interaction. We therefore divided our two-hybrid Brd2 construct into two fragments: the small acidic region and rest of the protein (Fig. 1A). Analysis in yeast two-hybrid assays indicated that the acidic region was necessary and sufficient to mediate interaction with Ptn (Fig. 1F). Interestingly, the acidic region found in Brd2 seems to be absent from other BET family members, which prompted us to investigate the interaction of Ptn with other BET proteins. Yeast two-hybrid analysis indicated that Ptn did not interact with Brd3 or Brd4, indicating that Ptn interaction was specific to Brd2 (Fig. 1F). To confirm these results, we analyzed interaction in 293T cells by immunoprecipitation, and found that Ptn–HA was efficiently precipitated by Flag–Brd2 but not by Flag–Brd3 or Flag–Brd4 (Fig. 1G). In agreement with these results, a deletion mutant of Brd2 lacking the acidic region (Brd2Δacid) was also unable to precipitate Ptn–HA (Fig. 1G).

Finally, we wished to determine whether there was an interaction between endogenous Brd2 and Ptn proteins. For that we turned to P19 cells, where expression of Ptn has been reported following RA treatment (Brunet-de Carvalho et al., 2003). Co-immunoprecipitation of endogenous Ptn was observed when lysates were immunoprecipitated using an anti-Brd2 antibody but not with the corresponding pre-immune serum (Fig. 1H), demonstrating interaction of the endogenous proteins.

Brd2 and Ptn are coexpressed in P19 cells that are differentiating into neurons

We next analyzed the expression levels of Brd2 and Ptn in P19 cells under normal growth conditions, in which cells are proliferating, and after 2 or 4 days of RA treatment, which leads to cell cycle exit prior to neuronal differentiation. Immunoblot analysis revealed Ptn expression only after RA treatment, peaking at 2 days, in agreement with previous reports (Fig. 2A; Brunet-de Carvalho et al., 2003). In contrast, no changes in Brd2 expression were observed throughout the differentiation protocol (Fig. 2A). The pluripotency marker Oct-3/4 (also known as POU5F1) and the neuronal marker βIII-tubulin were used as controls for differentiation. Rapid downregulation of Oct-3/4 was observed at day 2 of treatment and accumulation of βIII-tubulin was observed at day 4, whereas no changes were detected in expression of the housekeeping marker α-tubulin (Fig. 2A). Thus, under proliferating conditions, Brd2 and Ptn are not co-expressed in P19 cells, but they are following RA treatment. We next analyzed their expression by immunofluorescence, observing again coexpression under differentiation conditions (Fig. 2B). Ptn was only detected in differentiating cells, both in the cytoplasm and the nucleus, whereas Brd2 was detected in both conditions: clearly restricted to the nucleus in proliferating cells and partially localized to the cytoplasm in differentiating cells. To confirm cellular localization of Brd2 and Ptn, cells were subjected to biochemical fractionation followed by immunoblotting. As controls we analyzed the nuclear protein BAF155, a SWI/SNF complex subunit also known as SMARCC1, and α-tubulin, which is essentially cytoplasmic. As shown in Fig. 2C, biochemical fractionation confirmed the results observed in immunofluorescence analyses shown in Fig. 2B. As the reported Brd2 functions are mostly associated with the nucleus, we wished to determine whether Ptn was present in the nucleus of differentiating P19 cells, and, to support functional consequences of the Ptn interaction on Brd2 activity, whether the endogenous proteins interacted in the nucleus. For that, we isolated nuclei from RA-treated P19 cells and prepared nuclear extracts to analyze coprecipitation of Ptn with Brd2. As shown in Fig. 2D, co-immunoprecipitation experiments confirmed Ptn–Brd2 interaction in the nucleus. To have an idea of the Ptn:Brd2 ratio in the cell we compared relative levels of Ptn and Brd2 in protein extracts from P19 cells treated for 2 days with RA. Levels were determined from immunoblotting quantification of samples loaded together with known amounts of purified Ptn and Brd2 proteins. Analysis was performed on both whole-cell and nuclear extracts. Results indicate that in whole-cell extracts, levels of Ptn were ~4-fold those of Brd2; however, in the nucleus the ratio was close to 1:1 (Fig. 2E).

Ptn stimulates neuronal differentiation by antagonizing Brd2

The detection of βIII-tubulin in P19 cells transfected with expression constructs for the neurogenic factors NeuroD2 and E12 (also known as Tcf3) represents a way to evaluate neuronal differentiation (Farah et al., 2000). Using this system we have previously reported inhibition of neuronal differentiation by

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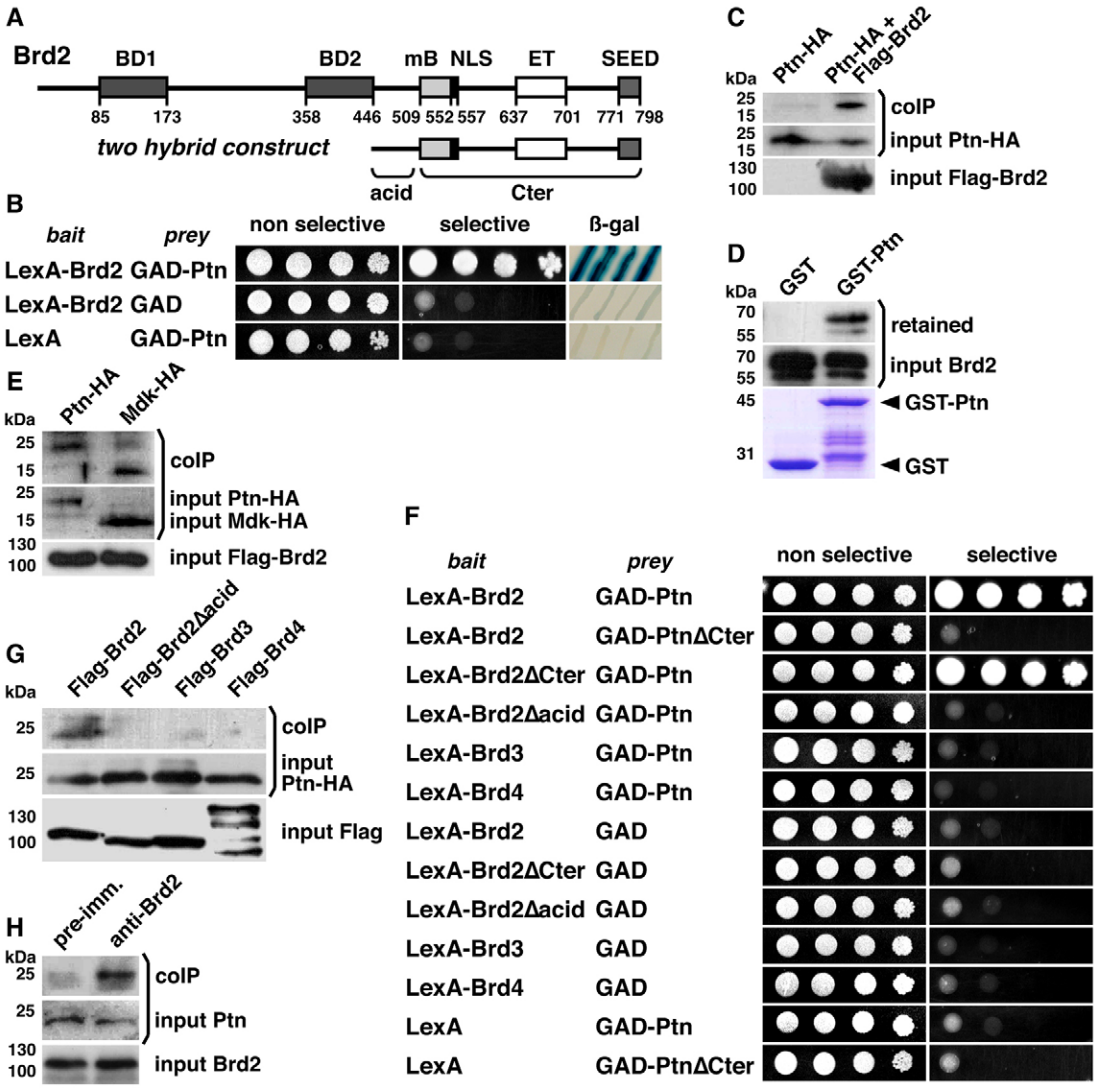

Fig. 1. Brd2 interacts with Ptn through a unique acidic domain. (A) Diagram of the Brd2 bait construct used in the two-hybrid screening. Numbering indicates amino acid position of relevant domains. BD1, bromodomain 1; BD2, bromodomain 2; mB, motif B; ET, extra terminal domain; NLS, nuclear localization signal (as described in Guo et al., 2000); SEED, domain rich in acidic/phosphorylatable amino acids. The acidic domain N-terminal to the motif B (acid) and C-terminal region (Cter) analyzed in F and G are also indicated. (B) Growth on selective and non-selective media of yeast harboring the indicated bait and prey constructs or empty vectors (LexA and GAD, respectively) as indicated. Interaction was also visualized by blue color in yeast assayed for β-galactosidase activity. (C) Extracts from 293T cells transfected with hemagglutinin (HA)-tagged Ptn (Ptn–HA) and Flag-tagged Brd2 (Flag–Brd2) constructs or empty vector were immunoprecipitated with anti-Flag antibodies and the co-immunoprecipitated proteins (coIP) were visualized by immunoblotting with anti-HA antibodies. The input shows 10% of Flag- or HA-tagged input proteins. (D) Pulldown experiments carried out with immobilized purified GST or a GST–Ptn fusion, and a 6×His-tagged Brd2 fragment (Brd2) purified from bacteria. Precipitated Brd2 (retained) was detected by immunoblotting; 20% of Brd2 input protein and 100% of GST-derived input proteins were detected by immunoblotting and Coomassie Blue staining, respectively. (E) Extracts from 293T cells transfected with Flag–Brd2, and Ptn–HA or Mdk–HA constructs were immunoprecipitated with anti-Flag antibodies and the co-immunoprecipitated proteins (coIP) were visualized by immunoblotting with anti-HA antibodies. The input shows 10% of Flag- or HA-tagged input proteins. (F) Growth on selective and non-selective media of yeast harboring bait and prey constructs or empty vectors as indicated. Deletions (Δ) refer to Brd2 acidic region (acid) and to Brd2 and Ptn C-terminal domains (Cter). (G) Extracts from 293T cells transfected with Ptn–HA, and the indicated Flag-tagged constructs were immunoprecipitated with anti-Flag antibodies and the co-immunoprecipitated proteins (coIP) were visualized by immunoblotting with anti-HA antibodies. The input shows 10% of Flag- or HA-tagged input proteins. (H) Extracts from P19 cells treated with RA for 48 h were immunoprecipitated with anti-Brd2 antibodies or pre-immune sera, and co-immunoprecipitated endogenous Ptn was monitored by immunoblotting with anti-Ptn antibodies. The input shows 10% of endogenous input proteins.

ectopic Brd2 expression (Garcia-Gutierrez et al., 2012). As Ptn interacts with Brd2 and is detected upon induction of neuronal differentiation in P19 cells, we wished to explore the functional consequences of the Ptn–Brd2 interaction in this process. We therefore co-transfected different Brd2 and Ptn constructs together with expression constructs for NeuroD2, E12 and GFP in P19 cells, and quantified neurogenesis as the percentage of transfected cells (monitored by GFP expression) expressing βIII-tubulin at 3 days after transfection. As expected, expression of Brd2 impaired neuronal differentiation (Fig. 3A,B), but, interestingly, coexpression of Ptn neutralized the effects of Brd2. Surprisingly, expression of Ptn alone increased neuronal differentiation. Expression of a fragment of Brd2 encompassing the motif B, previously shown to interfere with chromatin binding of Brd2

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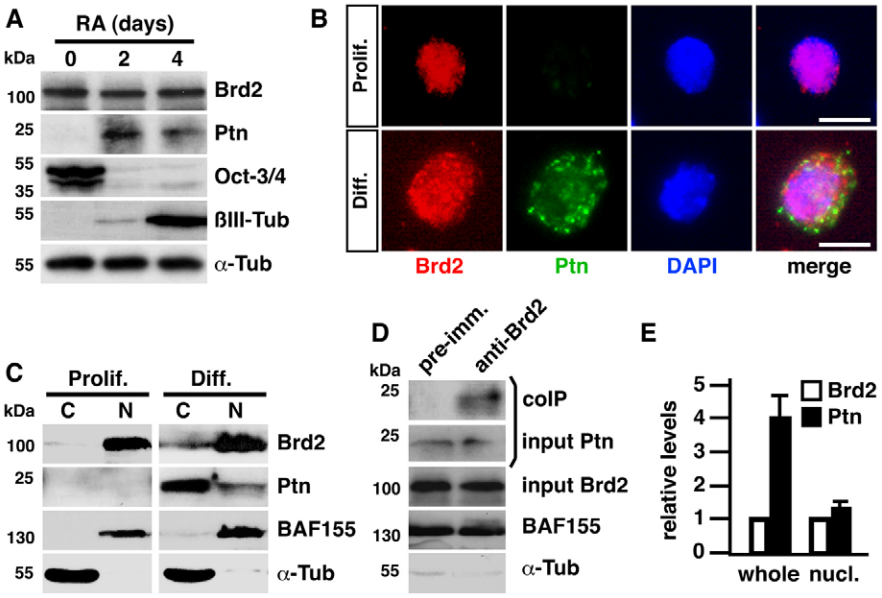

Fig. 2. Brd2 and Ptn are co-expressed in differentiating P19 cells. (A) P19 cells were treated with RA for the indicated times and then collected. Total lysates were analyzed by immunoblotting using antibodies against the indicated proteins. βIII-Tub, βIII-tubulin; α-Tub, α-tubulin. (B) Localization of endogenous Brd2 and Ptn in P19 cells under normal growing conditions (Prolif.) or after treatment with RA for 48 h (Diff.) were visualized by immunofluorescence with anti-Brd2 (red) and anti-Ptn (green) antibodies. DNA was counterstained with DAPI (blue). Scale bars: 10 µm. (C) P19 cells under normal growing conditions (Prolif.) or after treatment with RA for 48 h (Diff.) were subjected to biochemical fractionation and proteins from cytoplasmic (C) or nuclear (N) fractions were analyzed by immunoblotting with the indicated antibodies. (D) Co-immunoprecipitation experiments of endogenous Brd2 and Ptn proteins were conducted as described in Fig. 1H but using nuclear extracts obtained from isolated nuclei of P19 cells treated with RA for 48 h. The input shows 10% of endogenous input proteins. As controls for the purity of the nuclear extract preparation, the levels of the nuclear protein BAF155 and cytoplasmic protein α-tubulin (α-Tub) were also determined from 10% of the extract. (E) Relative levels of endogenous Ptn and Brd2 proteins were determined from immunoblotting quantification of whole and nuclear (nucl.) extracts (60 and 30 µg of total protein, respectively) from RA-treated P19 cells, analyzed together with known amounts of purified Ptn and Brd2 proteins (50, 10 and 2 ng) as described in Fig. 1D. Brd2 levels were normalized to 1. Values are means±s.d. of three independent determinations.

(Garcia-Gutierrez et al., 2012), also led to enhanced differentiation. Thus, antagonizing endogenous Brd2 enhances neuronal differentiation, suggesting that Ptn acts by antagonizing Brd2. In agreement with this possibility, knockdown of endogenous Ptn (Fig. 3C) resulted in reduced differentiation (Fig. 3A,B). We also analyzed Brd2 mutants lacking the small acidic region involved in Ptn interaction (Brd2Δacid). Deletion of this region did not affect the anti-neurogenic activity of Brd2, but, interestingly, Ptn was unable to antagonize it. We next considered whether overexpression of Brd3, a BET family member highly related to Brd2 but naturally lacking the acidic region, would affect neurogenesis. We found that Brd3 also impaired neuronal differentiation, but, similar to the Brd2Δacid molecule, it was resistant to Ptn antagonism. Ptn, when overexpressed together with Brd3 or Brd2Δacid, is probably neutralizing endogenous Brd2, which should result in enhanced neurogenesis. However, as ectopic Brd3 and Brd2Δacid are insensitive to Ptn antagonism, cell-cycle-stimulating activity prevails, resulting in impaired neurogenesis. To estimate Brd2 and Ptn overexpression in these experiments we analyzed protein levels by immunoblotting in cells transfected with NeuroD2 and E12 expression constructs, either alone or together with Brd2 and Ptn expression constructs (Fig. 3D). Quantification indicated a 2.5-fold increase of both Brd2 and Ptn when transfecting cells with their expression constructs. However, as endogenous Brd2 is expressed in all the cells regardless of being transfected or not, we corrected this value according to transfection efficiency (65%), and estimated a 3.3-fold increase in transfected cells. Correction was not necessary for Ptn, because endogenous Ptn is only detected in transfected cells induced by NeuroD2 and E12 expression. Finally, we analyzed localization of overexpressed proteins in differentiating P19 cells. For that we transfected tagged versions of Brd2 and Ptn together with NeuroD2 and E12 expression constructs, and proteins were visualized by immunofluorescence. As observed in Fig. 3E localization was quite similar to that observed for endogenous proteins after 2 days of RA treatment (Fig. 2B).

Ptn-mediated Brd2 antagonism in the developing neural tube

To analyze the Ptn-mediated Brd2 antagonism in an animal model we turned to the developing neural tube, where both proteins, or the corresponding mRNAs, have been previously reported to be abundantly expressed (Crowley et al., 2004; DiBenedetto et al., 2008; Mitsiadis et al., 1995; Mittapalli et al., 2009). For the analysis, we chose the technique of electroporation of the neural tube in chick embryos, which allows transfection of neural progenitors in one half of the neural tube with expression constructs of interest. We have previously reported that overexpression of Brd2 impairs naturally occurring neuronal differentiation (Garcia-Gutierrez et al., 2012). Here, we show that Brd2 overexpression also impairs forced differentiation induced by expression of the neurogenic factor neurogenin 2 (Ngn2, also known as Neurog2). As observed in Fig. 4A,B, Ngn2-transfected cells (GFP positive) localized to the mantle (βIII-tubulin-positive region), indicative of neuronal differentiation. However, ectopic expression of Brd2 impaired the Ngn2-mediated effect. Interestingly, when Ptn was also transfected, a significant

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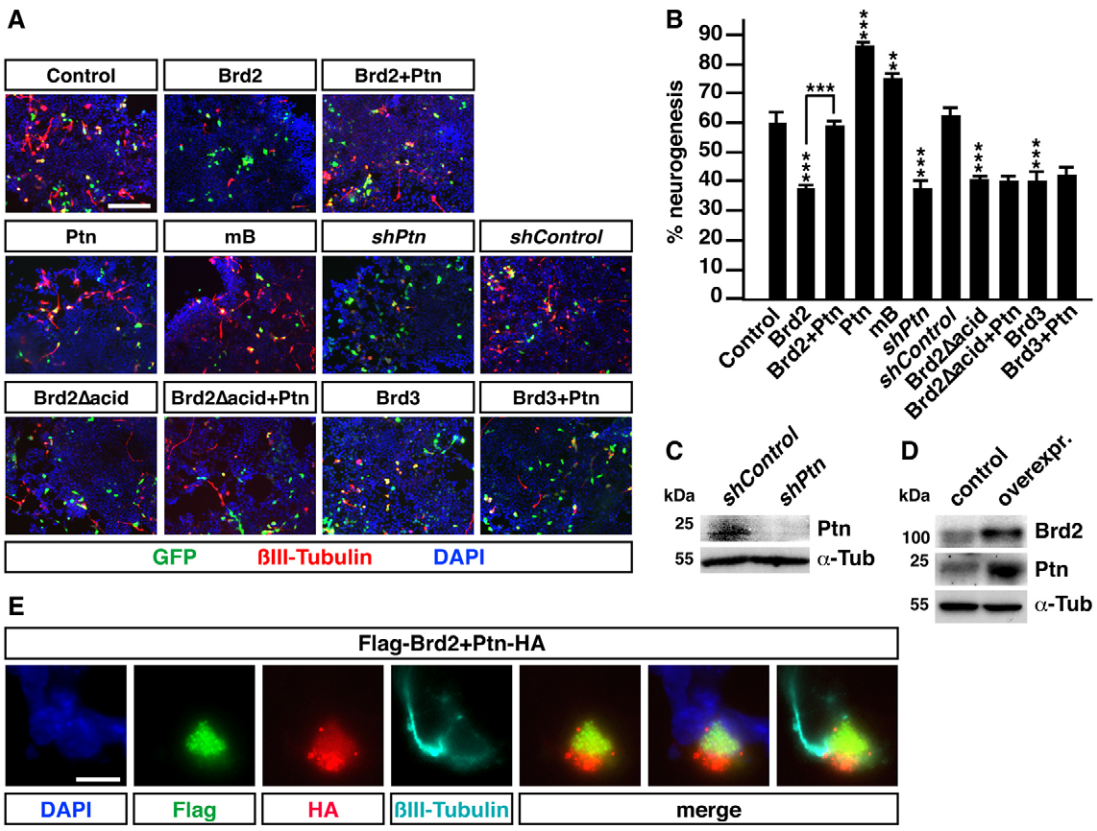

Fig. 3. Ptn stimulates neuronal differentiation by antagonizing Brd2. (A) P19 cells were transfected with NeuroD2 and E12 expression constructs, together with a GFP reporter plasmid and the indicated expression constructs, to be tested for expression of the neuronal marker βIII-tubulin. Control corresponds to empty vector and sh denotes interference by small hairpin RNA molecules. Neurogenesis was evaluated as the percentage of transfected cells (GFP positive, green) that are βIII-tubulin positive (red). Representative images are displayed. Scale bar: 100 μm. (B) Representation of data from A. Values are mean±s.d. from three areas of four independent experiments. Statistical significance was assessed by ANOVA, followed by Bonferroni's post test. Asterisks on top of columns refer to significance comparing with the corresponding control. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$. (C) P19 cells were transfected with shPtn or shControl constructs and treated with RA for 48 h. Cell lysates were analyzed by immunoblotting with anti-Ptn and anti-α-tubulin (α-Tub) antibodies. (D) Protein extracts from cells transfected with NeuroD2 and E12 expression constructs, either alone (control) or together with the Brd2 and Ptn expression constructs used in A (overexpr.), were analyzed by immunoblotting with antibodies against the indicated proteins. (E) P19 cells were transfected with NeuroD2 and E12 expression constructs, together with Flag-tagged Brd2 (Flag–Brd2) and HA-tagged Ptn (Ptn–HA) expression constructs, and analyzed by immunofluorescence with anti-Flag (green), anti-HA (red) and anti-βIII-tubulin (cyan) antibodies. DNA was counterstained with DAPI (blue). Images shown are representative of at least 70% of the analyzed cells. Scale bar: 10 μm.

recovery in Ngn2-driven neurogenesis was observed, indicative of Ptn-mediated Brd2 antagonism. Ptn expression, in the absence of Ngn2, had no effect (Fig. 4A,B), indicating that Ptn is not a neurogenic factor but stimulates neurogenesis.

Because Brd2 and Ptn are also expressed in neural-crest-associated structures (for instance in dorsal root ganglia and dorsal neural tube) (Crowley et al., 2004; Dibenedetto et al., 2008; Mitsiadis et al., 1995; Mittapalli et al., 2009), we decided to also study effects of Brd2 and Ptn misexpression in neural crest migration. Migrating neural crest cells originate from the dorsal-most region of the neural tube and undergo an epithelial-to-mesenchymal transition that enables them to delaminate and migrate into the adjacent mesoderm to create a variety of structures during development (Dupin and Sommer, 2012). The ventrolateral pathway at trunk level gives rise to dorsal root ganglia, among other structures. Electroporation of the dorsal neural tube in embryos with a reporter construct allows tracing of streams of migrating neural crest cells; thus, it has been established that reporter-positive cells in the mesoderm that locate ventrally to the electroporated dorsal neural tube correspond to migrating neural crest cells (Atkins et al., 2000). By using this approach, using a *lacZ* reporter construct followed by β-galactosidase staining, we observed, 30 h after electroporation, that ectopic Brd2 expression had no observable consequences on neural crest migration (Fig. 5A). However, Ptn overexpression drastically perturbed normal neural crest migration (Fig. 5A). As Ptn antagonizes Brd2, we hypothesized that Ptn-mediated interference of neural crest migration might be a consequence of antagonizing endogenous Brd2 function, which might be required for neural crest migration. As Brd2 knockdown (Fig. 5B) or competition with the peptide encompassing the motif B led to impaired neural crest migration (Fig. 5A), we conclude that this process requires Brd2. Finally, we reasoned that if Ptn-mediated impairment of neural crest migration is due to antagonism of endogenous Brd2, overexpressing Brd2 should reverse the effects of Ptn. As shown in Fig. 5A, Brd2 overexpression led to a significant recovery in neural crest migration.

Ptn destabilizes association of Brd2 with chromatin

As our results indicate that Ptn interferes with Brd2 function, we wished to explore the mechanisms involved. To this end, we analyzed the effect of Ptn on the strength of Brd2 attachment to

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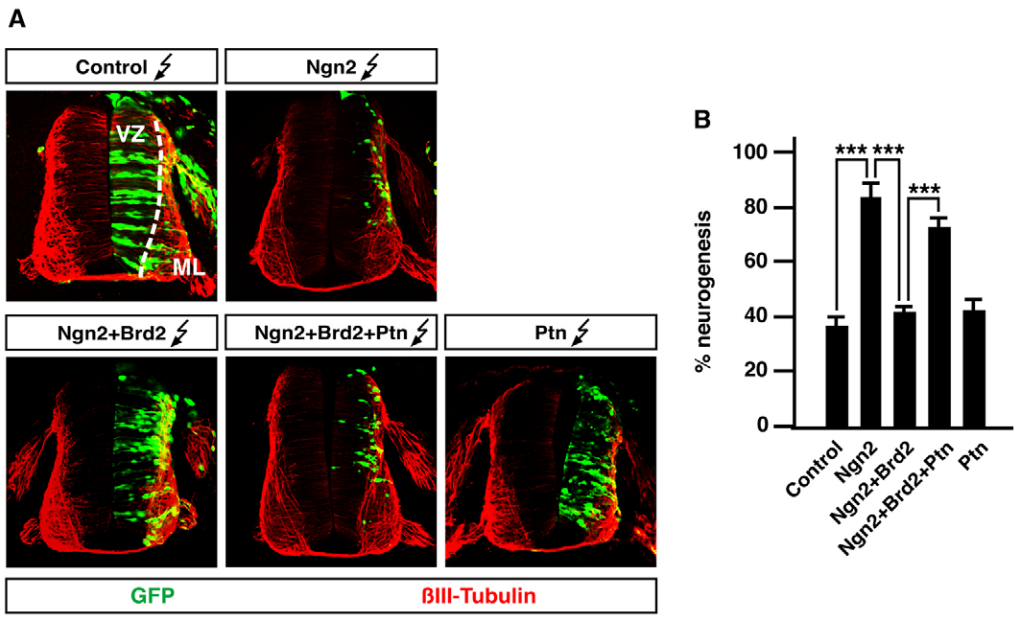

Fig. 4. Ptn antagonizes Brd2 during neuronal differentiation in the developing neural tube. (A) The neural tube of chick embryos at stage HH17 was electroporated on the right side with the indicated constructs and neurogenesis was evaluated by immunofluorescence after 30 h on transverse sections of the spinal cord as the percentage of transfected cells (GFP positive, green) that localize to the mantle layer (ML), the differentiation compartment, where the neuronal marker βIII-tubulin (red) is expressed. Representative images are displayed. The frontier between the proliferative ventricular zone (VZ) and ML is indicated by a dashed line in the electroporated side of the GFP control experiment (left upper panel). (B) Representation of data from A. Values are mean±s.d. from three sections of four independent experiments. Statistical significance was assessed by ANOVA, followed by Bonferroni's post test. ***$P \leq 0.001$.

chromatin in P19 cells. It has been previously shown that high salt concentrations are required to extract Brd2 from chromatin (Denis and Green, 1996; Garcia-Gutierrez et al., 2012). Therefore, cells transfected with a Ptn expression construct or empty vector were subjected to sequential protein extraction with 100 mM and 520 mM NaCl. Most of the endogenous Brd2 in control-transfected cells required 520 mM NaCl to be extracted in solution (Fig. 6A). However, in the presence of Ptn, most of the endogenous Brd2 was extracted with 100 mM NaCl, indicating destabilization of its association with chromatin.

To confirm this observation, we analyzed the occupancy of Brd2 in the promoter regions of the cyclin A2 and D1 genes,

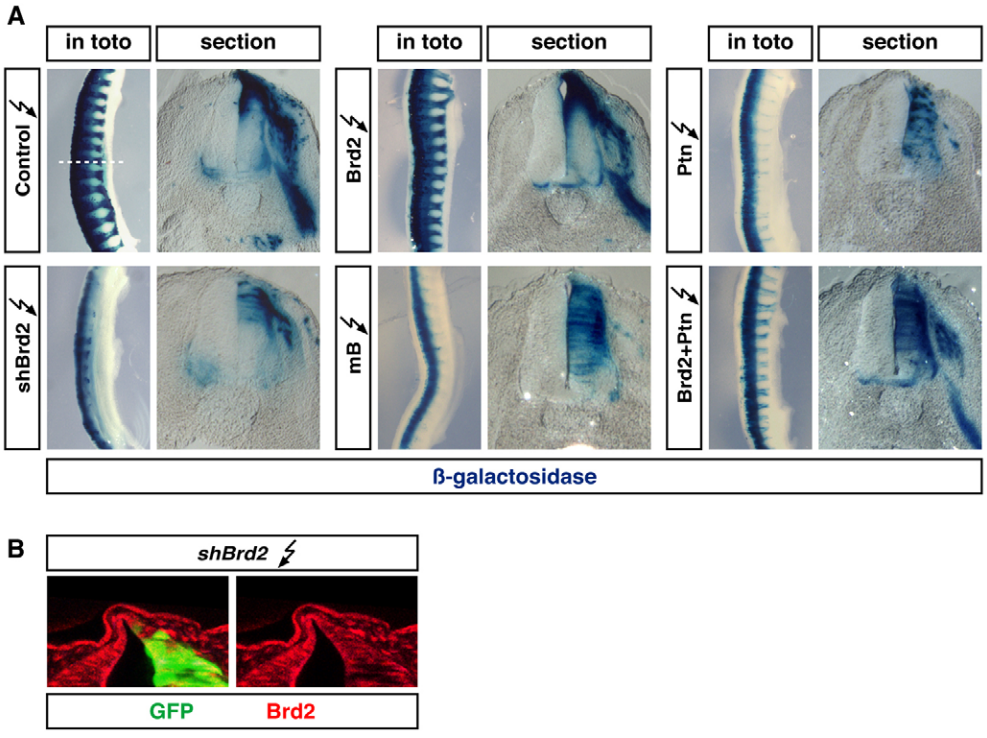

Fig. 5. Ptn-mediated Brd2 antagonism in neural crest formation. (A) The dorsal neural tube of chick embryos at stage HH17 was electroporated on the right side with a *LacZ* reporter construct and the indicated expression constructs, and migration of neural crest cells was evaluated by blue staining of embryos assayed for β-galactosidase activity after 30 h. Representative lateral images from the embryo trunk (in toto) and transverse sections at the level of one of the migratory streams (dashed line) are shown. (B) The neural tube of chick embryos at stage HH17 was dorsally electroporated on the right side with an shBrd2 construct together with a GFP reporter plasmid, and expression of endogenous Brd2 (red) was evaluated in GFP positive cells (green) by immunofluorescence using anti-Brd2 antibodies after 30 h on transverse sections of the spinal cord.

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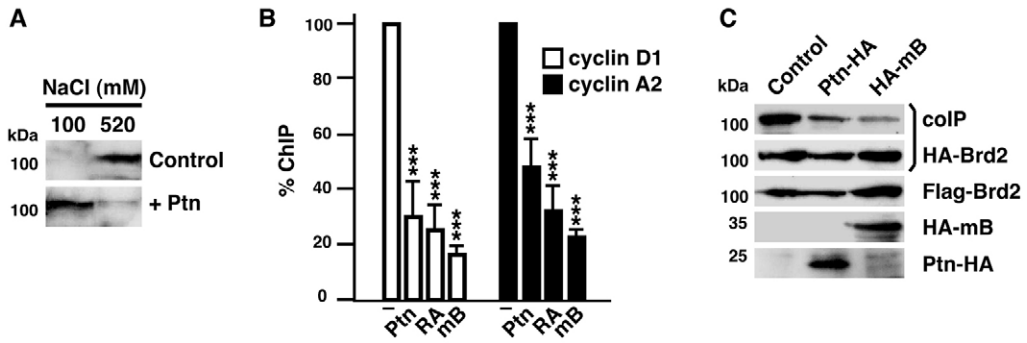

Fig. 6. Ptn destabilizes Brd2 association with chromatin. (A) P19 cells were transfected with a Ptn expression vector (+ Ptn) or the empty vector (Control) and proteins were consecutively extracted with the indicated NaCl concentrations. The presence of endogenous Brd2 protein in the different extracts was detected by immunoblotting. (B) Association of endogenous Brd2 protein with the *Ccnd1* (cyclin D1) and *Ccna2* (cyclin A2) promoters in P19 cells was determined by chromatin immunoprecipitation (ChIP) with anti-Brd2 antibodies. P19 cells were transfected with Ptn or motif B (mB) expression constructs or were treated with RA for 48 h (RA) as indicated. Levels were normalized to non-treated cells transfected with empty vector (−). Values are mean±s.d. from three independent experiments. Statistical significance was assessed by ANOVA, followed by Bonferroni's post test. Asterisks on top of columns refer to significance compared with the control (−). ***$P \leq 0.001$. (C) Extracts from 293T cells transfected with Flag-tagged Brd2 (Flag–Brd2) and HA-tagged Brd2 (HA–Brd2) constructs together with HA-tagged Ptn (Ptn–HA) or motifB (HA–mB) constructs as indicated were immunoprecipitated with anti-Flag antibodies and co-immunoprecipitated proteins (coIP) were analyzed by immunoblotting with anti-HA antibodies. The input shows 10% of Flag- and HA-tagged input proteins.

which have been previously shown to be regulated by Brd2 (Belkina et al., 2014; Garcia-Gutierrez et al., 2012; LeRoy et al., 2008; Sinha et al., 2005), by chromatin immunoprecipitation (ChIP). RA treatment of P19 cells, which induces Ptn expression, resulted in Brd2 dissociation from promoters (Fig. 6B). In fact, expression of Ptn in non-treated cells also caused dissociation, comparable to that driven by a protein fragment encompassing the dimerization motif B, previously shown to dissociate Brd2 (Garcia-Gutierrez et al., 2012). Given that the Ptn interaction domain in Brd2 is close to motif B, we analyzed Ptn interference on Brd2 dimerization by co-immunoprecipitation of Flag- and HA-tagged Brd2 proteins molecules in the presence of Ptn. As illustrated in Fig. 6C, similar to the motif B peptide, the presence of Ptn protein substantially reduced the ability of Brd2 to dimerize.

DISCUSSION

The chromatin adaptor Brd2 is an essential regulator associated with the cell cycle and broadly expressed during development (Crowley et al., 2004; Denis et al., 2000; Garcia-Gutierrez et al., 2012; LeRoy et al., 2008; Rhee et al., 1998; Shang et al., 2009; Sinha et al., 2005). Despite the clear role of Brd2 in cell cycle progression (Denis et al., 2000; Garcia-Gutierrez et al., 2012; LeRoy et al., 2008; Shang et al., 2009; Sinha et al., 2005), expression is also detected in differentiating neurons (Crowley et al., 2004). To shed light on this apparent paradox, we have tried to identify new Brd2 partners. Surprisingly, when looking for Brd2-interacting proteins during neuronal differentiation, we found the growth factor Ptn. Secreted Ptn has been linked to a variety of biological processes, and receptor-mediated functions have been well characterized (Kadomatsu and Muramatsu, 2004). However, intracellular mechanisms of action directly involving Ptn are poorly understood. In this study, we have assigned an intracellular function to Ptn in neuronal differentiation as an antagonist of the cell-cycle-stimulating factor Brd2. In our model, expression of Ptn at the onset of neuronal differentiation leads to Brd2 antagonism, which favors differentiation versus proliferation. To perform a detailed functional analysis, we have taken advantage of the P19 cell line, as only Ptn, but not Mdk, is detected in neuron-like cells following RA treatment (Brunet-de Carvalho et al., 2003). An additional analysis has been conducted in the neural tube, where the scenario becomes more complicated owing to the partially overlapping expression patterns shown by Ptn and Mdk, which might be linked to functional redundancy (Mitsiadis et al., 1995). In fact, the severe phenotype associated with the double *Ptn*/*Mdk* mutation in mice, in contrast to mild phenotypes in the single mutants, has been explained on the basis of functional redundancy and compensation (Muramatsu et al., 2006). We have shown Ptn-mediated Brd2 antagonism in three different systems: neuronal differentiation in P19 cells, neurogenesis in the neural tube and neural crest migration. Unexpectedly, we have also revealed that Brd2 is required for the last process. An explanation for this might be found in two observations: Brd2 is required for cyclin D1 expression (LeRoy et al., 2008) and cyclin D1 has been implicated in neural crest migration (Shoval et al., 2007).

Our model includes several observations and predictions. First, there are observations and predictions with regard to protein expression. As previously indicated, Brd2 is detected in proliferating neuronal progenitors as well as in differentiating neurons (Crowley et al., 2004). We have also shown that Brd2 levels remain constant during neuronal differentiation of P19 cells. By contrast, Ptn is detected in P19 cells after RA addition, its expression peaking at day 2 of treatment, as previously described (Brunet-de Carvalho et al., 2003), indicating that Ptn expression follows induction of neuronal differentiation. Expression of Ptn has also been documented in the developing neural tube of vertebrates (Mitsiadis et al., 1995; Mittapalli et al., 2009). Expression of antagonists (i.e. Ptn) of cell-cycle-associated factors (i.e. Brd2) in postmitotic neurons should prevent the resumption of the cell cycle, ensuring progression of the differentiation process.

Second, there are observations and predictions with regard to protein localization. We have observed partial localization of Brd2 to the cytoplasm in differentiating P19 cells. Similarly, cytoplasmic localization of Brd2 has been previously described in differentiating neurons of the mouse embryo (Crowley et al., 2004). In addition, Brd2 delocalization was originally reported in mouse 3T3 cells following serum deprivation (Guo et al., 2000) and has been also described in the mouse mammary epithelia during pregnancy (Crowley et al., 2002). Regardless of whether cytoplasmic localization of Brd2 is a consequence of Ptn

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interaction, what appears relevant to our hypothesis is that Ptn antagonizes Brd2 nuclear function, that is, the Brd2 cell-cycle-stimulating activity, which opposes to neuronal differentiation. Thus, Ptn antagonism should depend on its nuclear localization, and this aspect has previously been demonstrated for human endothelial cells (Koutsioumpa et al., 2012) and here revealed for P19 cells. Moreover, by co-immunoprecipitation experiments with nuclear extracts we have demonstrated that Ptn and Brd2 interact in the nucleus of RA-treated P19 cells. It is also worth noting that in the nucleus of differentiating cells we have determined that Ptn levels are similar to those of Brd2.

Third, there are observations and predictions with regard to chromatin association. We have previously reported strong association of Brd2 with chromatin in proliferating P19 cells (Garcia-Gutierrez et al., 2012). Our results from ChIP experiments and differential salt extraction demonstrate that Ptn destabilizes the association of Brd2 with chromatin as a consequence of impaired dimerization. Either expressing Ptn or competing Brd2 by expression of a Brd2 fragment encompassing its dimerization motif B has similar effects on Brd2 association with cyclin promoters, and both approaches concomitantly show the same positive effect on induced neuronal differentiation. Interestingly, Brd3, which appears to be insensitive to Ptn antagonism, interferes with neuronal differentiation in a similar manner to Brd2, raising the question about how Ptn stimulates differentiation by selectively acting on Brd2. Although we have not investigated Brd3 in detail, it is possible that just interfering with Brd2 might displace the balance towards differentiation. In fact, Brd2 knockdown has been shown to promote adipogenic differentiation in 3T3-L1 pre-adipocytes (Wang et al., 2010). Alternatively, we have previously demonstrated heterodimerization between different BET family members and postulated functional consequences linked to this (Garcia-Gutierrez et al., 2012), suggesting that interfering with one of the proteins might have broad consequences on BET family functions. Our demonstration of Ptn-mediated interference with the dimerization of Brd2 supports this idea. On the other hand, we have calculated a Ptn:Brd2 ratio of about 1:1 in the nucleus, what might suggest complete antagonism of Brd2 by Ptn. However, we needed to overexpress Ptn to obtain the highest differentiation rates, indicating that the endogenous Ptn pool modulates, rather than fully inhibits, Brd2 activity. Reported interactions of Brd2 with several chromatin co-regulators through the ET domain (Rahman et al., 2011) raise the question of how these factors may alter Ptn function or interaction. Nevertheless, partial inhibition of Brd2 activity by Ptn appears to have an effect in facilitating neurogenesis, since knockdown of Ptn reduces differentiation. In agreement with this, it has been shown that half the dose of Brd4 or Brd2 in heterozygous mouse cells has an impact in cell proliferation (Houzelstein et al., 2002; Shang et al., 2009). Thus, our results support the idea that relative levels of Brd2 may influence transcription or signal transduction, as previously suggested in (Wang et al., 2010).

Finally, there are observations and predictions with regard to the consequences of Brd2-chromatin destabilization. The release of BET proteins from chromatin has a negative effect on cell proliferation. In fact, competing BET association with chromatin through the synthetic drug JQ1, which mimics specific histone acetylation marks, has been successfully used to arrest tumor progression in mice models (Delmore et al., 2011; Filippakopoulos et al., 2010). It would be of great interest to investigate the possible application of this synthetic drug as a novel strategy to enhance neurogenesis following induction of neuronal differentiation. However, the proliferative defects associated with knockout of *Brd2* (Shang et al., 2009) might have largely masked evidence for subsequent involvement of Brd2 in differentiation processes. Our results do not exclude specific roles of Brd2 during the neuronal differentiation program because, as mentioned above, Brd2 protein, besides being detected in neural progenitors in the developing neural tube, also appears in differentiating neurons along the dorsoventral axis, including interneurons, motoneurons and sensory neurons (Crowley et al., 2004). Although several reports have suggested an involvement of Brd2 in the process of neuronal differentiation (Gyuris et al., 2009; Tsume et al., 2012; Velíšek et al., 2011), specific conditional knockout mice are required to accurately assess this issue. Finally, despite functional redundancy between

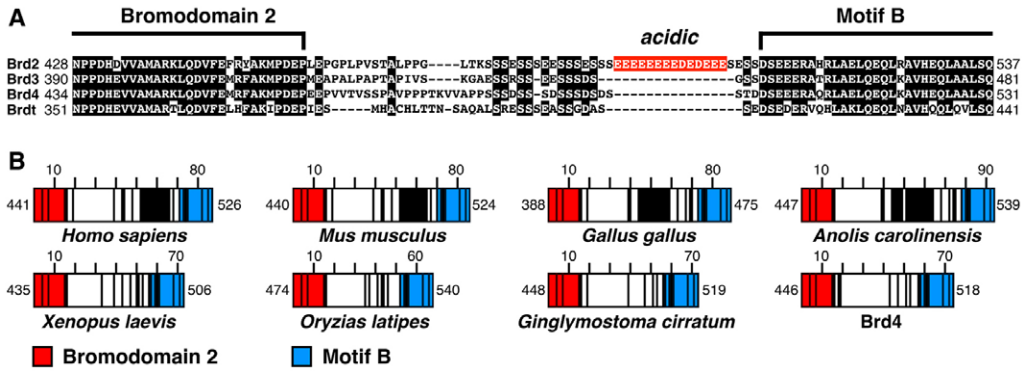

Fig. 7. The acidic stretch of Brd2 is conserved in higher vertebrates. (A) Amino acid sequences of different BET proteins from mouse, corresponding to a region located between the bromodomain 2 and the motif B, were aligned. Amino acids conserved in at least three of the four sequences are shown on a black background. Acidic stretch in Brd2 is shown on a red background. Numbering indicates amino acid positions. (B) The presence of acidic amino acids (Asp or Glu) in a fragment of Brd2 proteins from the indicated vertebrate species or mouse Brd4, corresponding to a region located between the bromodomain 2 (red) and the motif B (blue), was analyzed using the DNA Strider 1.4f6 application. Numbering at both sides indicates amino acid positions flanking each fragment and numbering above indicates positions within each fragment. Acidic amino acids are represented by vertical black lines. Accession numbers are as follows: mouse Brd3, NP_001107045; mouse Brd4, NP_065254; mouse Brdt, NP_473395; mouse Brd2 (*Mus musculus*), NP_034368; human Brd2 (*Homo sapiens*), NM_001113182; chick Brd2 (*Gallus gallus*), NM_001025845; green anoles Brd2 (*Anolis carolinensis*), XP_003229792; *Xenopus* Brd2 (*Xenopus laevis*), NP_001128282; Medaka fish Brd2 (*Oryzias latipes*), BAD93258; nurse shark Brd2 (*Ginglymostoma cirratum*), AEC13624.

Ptn and Mdk, an interesting developmental phenotype associated with the single *Ptn* mutation in mice supports our model: absence of Ptn results in prolonged proliferation and therefore in impaired neurogenesis in the cerebral cortex (Hienola et al., 2004). Similarly, we have shown that Ptn knockdown in P19 cells impairs induced neuronal differentiation.

Thus, Ptn-mediated Brd2 antagonism seems to modulate the balance between proliferation and differentiation in neural progenitors, accounting for appropriate progression into differentiation at early neurogenesis. Consistently, proteomic analysis has revealed Brd2 association with proteins known to play a role in the switch between proliferation and differentiation (Denis et al., 2006), and it has been suggested that Brd2 is a candidate protein to regulate 'stemness' and proliferation during hematopoiesis (Belkina et al., 2014). Intriguingly, the exclusive acidic region in Brd2 that mediates its interaction with Ptn (Fig. 7A) seems to be restricted to higher vertebrates (Fig. 7B), suggesting an evolutionary emergence of the Ptn–Brd2 interaction in higher vertebrates. This is of interest in that neurogenesis notably differs from higher to lower vertebrates, given that in lower vertebrates the neuroectodermal primary neurogenesis takes place before neural tube closing and accumulation of neuronal progenitors.

MATERIALS AND METHODS

Plasmid constructs and yeast two-hybrid analysis

All transfection constructs, except pEGFP-N1, were derived from vector pAdRSV-Sp (Giudicelli et al., 2003), with Flag or HA tags. Mouse *Brd2*, *Brd3*, *Brd4*, *Ptn* and *Mdk* cDNAs were obtained by RT-PCR using total RNA isolated from P19 cells. Brd2 constructs were made by standard PCR techniques and were as follows: Δacid, deletion (Δ) of amino acids 475–513; mB, amino acids 463–565. Yeast two-hybrid and X-gal assays were performed in the DUALhybrid Kit (Dualsystems Biotech, Schlieren, Zurich, Switzerland) system, using the pLexA-N bait and pGAD-HA prey vectors, as previously described (Garcia-Gutierrez et al., 2012). Yeast two-hybrid constructs were generated by standard PCR techniques and were as follows: Brd2, amino acids 437–798; Brd2Δacid, amino acids 515–798; Brd2ΔCter, amino acids 437–517; Brd3, amino acids 393–726; Brd4, amino acids 437–724; PtnΔCter, deletion (Δ) of amino acids 143–168. Expression vectors for small hairpin RNA molecules (shRNA) were based on vector pSuper (OligoEngine, Seattle, WA, USA). Target sequences were: shPtn, 5′-GGAGAAT-GGCAGTGGAGTG-3′; shControl, 5′-CCATCAAGACTCATAGATG-3′; and shBrd2, 5′-GATCTTCCTGCAGAAGGTG-3′.

Protein production and pulldown assays

Production of proteins was conducted in the *E. coli* BL21 strain. Purification of the GST–Ptn fusion protein was achieved by incubation with glutathione–Sepharose 4B matrix (GE Healthcare, Buckinghamshire, UK) and purification of His-tagged Brd2 protein was achieved by incubation with HisTrap matrix (GE Healthcare). The GST–Ptn construct encompassed amino acids 1–168 of Ptn, whereas His-tagged Brd2 encompassed amino acids 50–651 of Brd2. Pull-down experiments with immobilized GST fusion protein were conducted as previously described (Garcia-Dominguez et al., 2008).

Cell culture and transfections

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, respectively. Transfections were performed with Lipofectamine 2000 (Invitrogen, Life Technologies, Paisley, UK). All-trans retinoic acid (RA) was used at 1 µM for 48 or 96 h, as indicated. For transfection monitoring, the green fluorescent protein expression vector pEGFP-N1 (Clontech, Mountain View, CA, USA) was used. shPtn or shControl transfections for *Ptn* knockdown were performed 24 h before RA treatment.

Cell extracts 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, Mannheim, Germany)] supplemented with 100 mM NaCl. The pellet was then extracted with the same buffer containing 520 mM NaCl. The procedure to obtain nuclear and cytoplasmic extracts was as described by Alfonso-Perez et al. (Alfonso-Pérez et al., 2013). Whole-cell extracts were obtained by lysing cells in urea buffer (10 mM Tris-HCl pH 8.0, 8 M urea). Protein concentration was determined by the Bradford reactive assay (Bio-Rad, Hercules, CA, USA). Extracts were separated by SDS-PAGE and analyzed by immunoblotting. For immunoprecipitation, 9×10^5 cells were extracted with 250 µl of buffer A containing 150 mM NaCl. Then, NaCl concentration was increased to 600 mM drop wise. Supernatant was recovered and diluted 3-fold with buffer B [50 mM Tris-HCl pH 7.5, Complete protease inhibitor cocktail (Roche)] and incubated overnight with the respective antibodies: anti-Flag M2 Affinity Gel (Sigma-Aldrich, St Louis, MO, USA), anti-Brd2I rabbit antibodies (see below) or pre-immune serum. Rabbit antibodies were precipitated with rProtein A Sepharose4 Fast Flow matrix (GE Healthcare). After washing, proteins were eluted with 20 µl of SDS-containing Laemmli buffer and analyzed by immunoblotting.

Antibody generation and immunoblotting

Antibodies against Brd2 were produced in rabbit after immunization with a peptide corresponding to amino acids 698–780 (anti-Brd2C) (Garcia-Gutierrez et al., 2012), 50–651 (anti-Brd2N) or 164–274 (anti-Brd2I) of the mouse Brd2 protein or amino acids 628–713 of the chick Brd2 protein. Immunoblotting was conducted on PVDF membranes (Bio-Rad) using the chemiluminescent ECL system (GE Healthcare), according to the manufacturer's instructions. A ChemiDocXRS apparatus (Bio-Rad) was used for chemiluminescence measurement. Antibodies were: rabbit anti-Brd2C (1:500), rabbit anti-Brd2I (1:500), mouse anti-Flag M2 (1:2000, Sigma-Aldrich), mouse anti-His (1:1000, GE Healthcare), mouse anti-α-tubulin (1:1000, Sigma-Aldrich), goat anti-Ptn C-19 (1:500, sc-1394, Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-HA (1:2000, Roche), goat anti-Baf155 R-18 (1:1000, sc-9746, Santa Cruz Biotechnology) rabbit anti-βIII-tubulin (neuron specific, 1:500, Abcam, Cambridge, UK), rabbit anti-Oct3/4 H-134 (1:1000, sc-9081, Santa Cruz Biotechnology), and horseradish peroxidase (HRP)-conjugated goat anti-rabbit Ig, goat anti-rat Ig, goat anti-mouse Ig and rabbit anti-goat Ig antibodies (1:10,000, Sigma-Aldrich).

Chromatin immunoprecipitation and quantitative PCR

A total of 10^7 P19 cells fixed in 1% formaldehyde for 10 min at 37℃ were used in each experiment. The chromatin was immunoprecipitated with anti-Brd2N antibodies (1:50) and rProtein A Sepharose4 Fast Flow (GE Healthcare). Quantitative PCR was used for analysis of the *Ccnd1* and *Ccna2* promoters. Real-time PCR reactions were performed with the SensiMix SYBR Low-ROX kit (Bioline, London, UK) in the Applied Biosystems (Carlsbad, CA, USA) 7500 FAST Real-Time PCR System. Precipitation with pre-immune serum was used for normalization. Algorisms for calculation of relative units and normalization of values according to primer efficiency were as previously described (Pfaffl, 2001). Sequences of primers were: *Ccna2*-F 5′-CCAGCGTTTCCCTA-TGTTGT-3′; *Ccna2*-R 5′-CTAGGCAGGAGCGTATGGAT-3′; *Ccnd1*-F 5′-GGAGGACCCTCTTAGGGAAA-3′; *Ccnd1*-R 5′-GGAGGACCCTC-TTAGGGAAA-3′.

In ovo electroporation, immunofluorescence and β-galactosidase assay

Electroporation, preparation of embryos and immunofluorescence on neural tube sections or P19 cells were conducted as described previously (Farah et al., 2000; Garcia-Dominguez et al., 2003). All animal experiments were performed according to approved guidelines.

For electroporation monitoring, the green fluorescent protein expression vector pEGFP-N1 (Clontech) was used at a concentration of 0.3 µg/µl. Antibodies were: mouse anti-Flag M2 (1:250, Sigma-Aldrich),

rat anti-HA (1:250, Roche), rabbit anti-Brd2C (1:200), rabbit anti-chick Brd2 (1:200), rabbit anti-neuron specific βIII-Tubulin (1:500, Abcam), goat anti-Ptn C19 (1:200, sc-1394, Santa Cruz Biotechnology), donkey anti-rat Ig and anti-rabbit Ig conjugated to DyLight-549 (1:800, Jackson Immunoresearch, Suffolk, UK), donkey anti-goat Ig and anti-mouse Ig conjugated to DyLight-488 (1:800, Jackson Immunoresearch), and donkey anti-rabbit Ig conjugated to DyLight-649 (1:800, Jackson Immunoresearch). Cell nuclei were exposed by DAPI staining. Fluorescent images of cells and embryo sections were acquired on Leica Microsystems GmbH (Wetzlar, Germany) epifluorescence DM6000 and confocal TCS SP5 microscopes, respectively. Images were from a single focal plane in both cases, and were processed with Leica LAS AF and Adobe Photoshop software. For β-galactosidase detection, the embryos were fixed for 20 min in 4% paraformaldehyde and stained with X-gal as previously described (Schneider-Maunoury et al., 1993).

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Competing interests
The authors declare no competing interests.

Author contributions
P.G.-G. designed and performed experiments, F.J.-V. performed experiments, D.J.W. designed experiments and corrected the manuscript, M.G.-D. conceived the project, designed and performed experiments and wrote the manuscript.

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