Lyar-mediated recruitment of Brd2 to the chromatin attenuates *Nanog* downregulation following induction of differentiation

Noelia Luna-Peláez, Mario García-Domínguez

Andalusian Center for Molecular Biology and Regenerative Medicine-CABIMER

CSIC-Universidad de Sevilla-Universidad Pablo de Olavide

Av. Américo Vespucio 24, 41092, Seville, Spain

Corresponding author:

Mario García-Domínguez, CABIMER, Av. Américo Vespucio 24, 41092, Seville, Spain. Tel: +34 954468201, Fax: +34 954461664, E-mail: mario.garcia@cabimer.es

Abbreviations used: RA, retinoic acid; WT, wild type; ZF, zinc finger; ChIP, chromatin immunoprecipitation; ESCs, embryonic stem cells; qPCR, quantitative PCR.

Abstract

During development, cellular differentiation programs need of tight regulation for proper display of the activity of multiple factors in time and space. Chromatin adaptors of the BET family (Brd2, Brd3, Brd4 and Brdt in vertebrates) are transcription co-regulators tightly associated with the progression of the cell cycle. A key question regarding their function is whether they work as part of the general transcription machinery or, on the contrary, they are precisely recruited to the chromatin through specific transcription factors. Here, we report the selective recruitment of Brd2 to the chromatin by the transcription factor Lyar. We show that Lyar downregulation results in Brd2 dissociation from a number of promoters studied. On the contrary, dissociation of BET proteins from the chromatin has no effect on Lyar occupancy. Under differentiation conditions, the absence of Lyar leads to impaired downregulation of the pluripotency gene *Nanog*, with concomitant reduction in the upregulation of differentiation markers. Interestingly, following the induction of differentiation, Brd2 depletion exhibits the same effects as expressing a truncated Lyar molecule lacking the Brd2 interacting domain. Both approaches result in stronger *Nanog* repression, indicating that Lyar-mediated recruitment of Brd2 moderates *Nanog* downregulation when differentiation is triggered. Moreover, expression of truncated Lyar leads to impaired differentiation and increased apoptosis. Thus, Lyar-mediated recruitment of Brd2 would participate in preserving a proper timing for *Nanog* silencing ensuring the appropriate establishment of the differentiation program.

Keywords: transcription regulation; BET proteins; protein-protein interaction; pluripotency markers; retinoic acid

Introduction

BET family of bromodomain and extra terminal domain factors comprise Brd2, Brd3, Brd4 and Brdt proteins in vertebrates. Excluding the male germ line-restricted member Brdt, these proteins are widely expressed in both the developing embryo and the adult organism. BET proteins are key transcriptional co-regulators that recognize acetyl-lysine groups on histones and other proteins through their bromodomains. These proteins have been classically associated with cell cycle progression [1-4], although they have recently been involved in differentiation as well [5, 6]. In agreement with their positive effect on cell cycle-associated genes, aberrant overexpression of BET family members, especially Brd4, has been observed in many types of cancer. Moreover, novel drugs mimicking the acetyl-lysine group efficiently interact with the bromodomain, dissociating BET proteins from the chromatin, and being an effective treatment for a variety of tumors in animal models [7, 8].

A key question on BET function is whether the different members of the family display global chromatin effects through their interaction with general transcription complexes or, on the contrary, they act in a more specific manner through their interaction with particular transcription factors. The major function attributed to Brd4, the most studied BET protein, is in transcription elongation, and is carried out in association with the transcription elongation complex P-TEFb [9]. In addition, Brd4 has also been associated with the Mediator complex and with ATPases of chromatin remodeling complexes, such as SWI/SNF ATPases and CHD4 [10, 11]. Similar to Brd4, Brd2 has been linked to the Mediator complex [12] and to chromatin remodeling machines [13], and several studies have contributed to establish its role in transcription activation [1, 3, 14]. Despite an overlapping pattern of expression of BET genes, animal studies have proved the absence of functional redundancy among them, since knocking out either Brd2 or Brd4 leads to embryonic lethality

[15-17]. In addition, a number of functions have been selectively attributed to one or the other member of the family [15, 18-23], as well as differential association with specific factors and complexes. For instance, as previously indicated, Brd4, but not Brd2, associates with the P-TEFb complex [9], and conversely, Brd2, but not Brd4, associates with Pleiotrophin or CTCF [20, 24]. This functional segregation of Brd2 and Brd4 is thought to be due to differential occupancy in the chromatin. As an example, Brd4 is mostly related to enhancers while Brd2 is related to promoters and insulators [20, 25-28].

Besides interaction with general transcription complexes, a variety of reports also indicate that the activity of specific transcription factors is exerted through direct recruitment of BET proteins. Examples of this are the direct recruitment of Brd4 by Oct4, RelA and Twist [29-31] and the direct recruitment of Brd3 by acetylated transcription factor GATA1 [22, 32]. However, the molecular mechanism implicated in Brd2 recruitment to the chromatin is less known [20]. Thus, how BET proteins, and in particular Brd2, are recruited to the chromatin needs to be further explored.

In this context, we have performed a two-hybrid screening looking for proteins interacting with Brd2 [33]. Among the identified Brd2 partners we found the Ly-1 antibody reactive protein (Lyar). This protein was initially identified as a nucleolar protein with zinc finger DNA-binding motifs [34]. Indeed, it has been recently reported that Lyar behaves as a transcription factor recognizing the consensus sequence GGTTAT to regulate the human fetal globin gene [35]. The nucleolar localization of Lyar has been associated with a role in accelerating the processing of preribosomal RNA [36]. On the other hand, Lyar plays an important role in maintaining embryonic stem cells (ESCs) identity, being highly expressed in undifferentiated ESCs and contributing to self-renewal and differentiation of these cells [37].

Here we show that Lyar interacts strongly with Brd2 but poorly with Brd4. Knockdown experiments indicate that Lyar recruits Brd2 to the chromatin, while Brd2 is dispensable for chromatin localization of Lyar. Notably, the interaction of Brd2 with Lyar modulates *Nanog* downregulation following the induction of cell differentiation.

Results

Brd2 interacts with Lyar

We have previously described a two-hybrid screening aimed to identify proteins interacting with the BET family member Brd2 [33]. One out of the 150 clones isolated from this screening using a Brd2 bait construct lacking the bromodomains (Fig. 1a) corresponded to the Ly-1 antibody reactive protein (Lyar) gene. This interaction between Brd2 and Lyar was verified by two-hybrid assays using serial dilutions of yeast on selective and non-selective media (Fig. 1b). Surprisingly, no significant yeast growth was observed when constructions encompassing other BET members, like Brd3 or Brd4 were employed, indicating that Lyar interacts specifically with Brd2. This specific interaction was confirmed in immunoprecipitation experiments with transfected HA-tagged Lyar and Flag-tagged Brd2 or Flag-tagged Brd4 constructs in HEK293T cells (Fig. 1c). Mapping Brd2 interaction surfaces either by two-hybrid assays or by immunoprecipitation (Figs. 1b and 1c) indicated that Brd2 region containing the motif B (mB) was responsible for the interaction with Lyar. Neither the acidic stretch upstream of the mB nor the ET domain at the C-terminus seems to be relevant for the interaction. Mapping Lyar domains involved in the interaction pointed to the implication of the C-terminal region, downstream of the N-terminal zinc finger motif and a coiled-coil structure (Fig. 1d).

To analyze the interaction of endogenous proteins we used the murine P19 cell line. Since Lyar has been associated with self-renewal and differentiation of embryonic stem cells

(ESCs) [37], we took advantage of the capacity of P19 cells to differentiate into neurons after retinoic acid (RA) treatment to analyze Lyar-Brd2 interaction in the context of both proliferation and differentiation. Although changes in transcription are already observed immediately after RA addition, in a standard differentiation protocol, P19 cells are treated for 4 days in non-adherent dishes with RA and then seeded on adherent surface in the absence of RA for 3 to 7 additional days. Thus, we decided to perform a comparative analysis of proliferating cells (without RA treatment) with cells at day 7 of the differentiation protocol. Interestingly, despite the absence of significant differences in the expression levels of Brd2 and Lyar under both conditions, their interaction was notably impaired under differentiation conditions (Fig. 1e).

Taken together, these data indicate that among BET family members Lyar specifically interacts with Brd2, being the mB region of Brd2 and the C-terminal region of Lyar, involved in this interaction. The interaction between Lyar and Brd2 appears to be more stable under proliferation conditions than in differentiating cells.

Lyar mediates Brd2 recruitment to pluripotency genes

The previously reported involvement of Lyar in self-renewal of ESCs [37] prompted us to initially investigate the interaction with Brd2 in the context of pluripotency genes. We first analyzed in P19 cells the recruitment of Brd2 to the promoters of a selection of pluripotency genes under Lyar knockdown conditions (Fig. 2a). For this purpose we used small hairpin RNA molecules (shRNA) to interfere with Lyar expression. We designed two shRNA molecules targeting different regions in the Lyar gene, which proved to perform similarly (about 90% knockdown, Fig. 2b) and gave similar results in preliminary experiments, so we selected one of them for subsequent experiments (see materials and methods). Chromatin immunoprecipitation (ChIP) experiments using a Brd2 antibody

indicated that Lyar knockdown led to a reduced Brd2 occupancy in all promoters analyzed: *Pou5f1* (*Oct4*), *Nanog*, *Prdm14*, *Sox2* (Fig. 2a). As a control, we analyzed the γ-globin promoter, since Lyar has been shown to bind to this promoter [35]. Similar to the pluripotency genes promoters, Brd2 localized to the γ-globin promoter and occupancy was reduced after Lyar knockdown (Fig. 2a). Finally, we also analyzed by ChIP Brd4 occupancy to these regions. Our results indicated that in contrast to Brd2, Brd4 occupancy in the analyzed promoters was mostly unaltered by Lyar knockdown (Fig. 2a).

A significant proportion of Brd2 is tightly bound to the chromatin, so severe extraction conditions are needed to fully extract it [33]. Thus, in view of the former results we wondered whether Lyar knockdown might affect strength of Brd2 association to the chromatin. Then, we decided to prepare fractionated protein extracts under different conditions. Fractionation experiments showed that under normal conditions most Brd2 eluted in the chromatin fraction (Fig. 2c). However, under Lyar knockdown conditions, we observed a significant increase in Brd2 soluble fraction, indicating that Lyar downregulation weakens Brd2 association with the chromatin, at least partially (Fig. 2c). As a control we used the JQ1 drug, which efficiently detached most Brd2 from the chromatin, essentially eluting in the soluble fraction (Fig. 2c).

The previous results indicate that Brd2 is recruited to a number of chromatin sites in a Lyar-dependent manner but also suggest the absence of 100% overlap between Brd2 and Lyar. Thus, we manage to look for examples supporting this idea. The housekeeping actin gene *Actb* has been shown to be regulated by BET proteins to some extent and Brd2 has been abundantly detected at the *Actb* promoter region [38]. Our ChIP experiments confirmed this fact and indicated that Lyar was absent from this region (Fig. 2d). Another gene occupied by Brd2, and whose expression is dependent on Brd2 is the *Elk3* gene [38, 39]. Similar to *Actb*, we detected Brd2 but not Lyar on *Elk3*. Finally, we identified the *Pnma3* promoter as devoid

of both Brd2 and Lyar. As expected, depletion of Lyar did not alter Brd2 occupancy at these promoters. As a negative control we performed ChIP assays with normal rabbit IgG (Fig. 2d).

These results suggest that in certain promoters Brd2 is recruited to the chromatin in a Lyar-dependent manner. We then analyzed whether the inverse was also true, that is, if Brd2 dissociation from the chromatin affects Lyar occupancy. As shown in Fig. 2e, ChIP experiments revealed that JQ1-mediated detachment of Brd2 in a selection of promoters had no effect on Lyar occupancy at these promoters.

Altogether, these results indicate that Lyar participates in Brd2 recruitment to the chromatin, while association of Lyar to the chromatin seems to be independent of Brd2.

Lyar knockdown alters the expression of *Nanog* and differentiation markers under retinoic acid-mediated differentiation conditions

To investigate the role of Lyar on the expression of those genes co-occupied by Lyar and Brd2, we first monitored gene expression upon depletion of Lyar under normal growth conditions. Under these conditions we did not observe variations in gene expression of *Pou5f1* and *Sox2*, but interestingly, *Nanog* and to a lesser extent *Prmd14*, were upregulated after Lyar knock down, indicating that Lyar acts as a repressor for these two genes (Fig. 3a, upper panels). We also checked dependence of gene expression on BET association to the chromatin. The JQ1 drug visibly impaired expression of *Pou5f1, Nanog* and *Prmd14*, while effect on *Sox2* expression was limited (Fig. 3a, lower panels). Thus, at least *Nanog* expression appears to be controlled by Lyar together with BET proteins, so this gene was selected for subsequent analyses.

Next, we studied the role of Lyar on *Nanog* expression in the context of neuronal differentiation. To do so, we first performed a time course to determine the changes in *Nanog* expression during the differentiation protocol with RA. Sixteen hours after RA addition we

detected a 44% repression of *Nanog* expression, that reached 88% repression by 24 h. Forty-eight hours after RA addition *Nanog* was almost completely repressed (98% repression, Fig. 3b). We subsequently analyzed the presence of Lyar and Brd2 at *Nanog* promoter after RA treatment. While Brd2 occupancy was strongly reduced, no changes in Lyar occupancy were observed (Fig. 3c). Finally, we analyzed the consequences of Lyar knockdown on the expression of *Nanog* and several differentiation markers following RA-mediated differentiation. As observed in Fig. 3d, in the absence of Lyar *Nanog* expression was not properly downregulated by RA treatment. In correlation with the impaired downregulation of *Nanog* we observed aberrant expression of the differentiation markers *Nes (Nestin)*, *Pax6* and *Sox6*, which were not properly upregulated in response to RA (Fig. 3d).

Taken together, the previous results indicate that Lyar behaves as a repressor of *Nanog*, remaining associated to the *Nanog* promoter under differentiation conditions, despite Brd2 clearance. Under these conditions, depletion of Lyar leads to impaired *Nanog* downregulation, with concomitant hampered upregulation of differentiation markers.

Interfering with Lyar-mediated recruitment of Brd2 to *Nanog* leads to stronger *Nanog* downregulation following induction of differentiation

Finally, in order to examine the role of Lyar-mediated recruitment of Brd2 to the chromatin on *Nanog* expression we investigated the consequence of knocking down Brd2 as well as the effects of the expression of a truncated Lyar variant lacking the Brd2 interacting domain.

To deprive P19 cells of Brd2 we targeted it with both, siRNA and esiRNA molecules (see materials and methods). Since both approaches performed similarly in preliminary experiments, we chose the esiRNA for subsequent analyses. As shown in Fig. 4a, 87% reduction in Brd2 protein levels was achieved in knockdown experiments. Under normal

growth conditions we observed no significant differences in *Nanog* expression in the presence or the absence of Brd2. To study *Nanog* expression in the context of cell differentiation we chose to analyze expression 16 h after RA addition, since we have seen that at this time point, *Nanog* has not been deeply downregulated (Fig. 3b) and appreciable expression levels, suitable for our analysis, are still detected. Our results indicated that Brd2 depletion resulted in stronger *Nanog* repression after RA addition (Fig. 4b).

Next, we generated a truncated Lyar molecule unable to interact with Brd2 but keeping the ability to interact with the DNA and with other proteins. To do so, we deleted the C-terminal portion responsible for the interaction with Brd2, but preserved the Zn finger DNA-binding motif and a coiled-coil structure downstream of the Zn finger previously reported to interact with the methyltransferase PRMT5 [35]. This variant, LyarΔC, did not interact with Brd2 in co-immunoprecipitation experiments (Fig. 1d). In addition, LyarΔC was able to associate with *Nanog* promoter similarly to the wild type (WT) protein, and in contrast to a construct lacking the Zn finger DNA-binding motif (LyarΔZF), which showed to be impaired in promoter association (Fig. 4c). We then conducted ChIP experiments to analyze the effect of the different Lyar constructions on Brd2 association to the *Nanog* promoter. While no differences in Brd2 association to the *Nanog* promoter were detected after transfection of WT or LyarΔZF constructs in comparison with non-transfected cells, significant impairment in Brd2 association was observed after transfection of the LyarΔC construct (Fig. 4d, left panel). As a control, we confirmed no effect of LyarΔC on Brd4 association to the *Nanog* promoter (Fig. 4d, right panel). Transfection of the LyarΔC construct under normal growth conditions showed no significant alterations of *Nanog* expression levels (Fig. 4e). However, after RA addition, the expression of the LyarΔC protein led to stronger repression of *Nanog* in comparison with the control (Fig. 4e).

Since former results suggest that LyarΔC displays a dominant negative effect, instead of depleting cells of proteins, we decided to focus on this truncated protein for further characterization of the Lyar-Brd2 interaction. To have an idea about the proportion of cells differentiating following expression of LyarΔC and RA treatment, we conducted immunofluorescence experiments to register the expression of the differentiation marker Pax6. From these experiments we estimated the percentage of neurogenesis, which indicated that expression of LyarΔC results in reduced number of differentiating cells (Fig. 4f). These results prompted us to analyze whether the differentiation program was altered. Thus, we monitored by quantitative PCR the expression of several differentiation markers under these conditions. Results indicated that LyarΔC expression leads to a reduced expression of differentiation markers, denoting that neurogenesis was altered (Fig. 4g). As altered differentiation usually leads to cell death, we measured apoptosis following expression of truncated Lyar and RA treatment. In fact, an increase in cell death was observed upon LyarΔC expression (Fig. 4h).

Therefore, either expressing a truncated Lyar variant unable to interact with Brd2 but able to associate with the chromatin, as well as depleting the cells of Brd2, leads to similar effects on *Nanog* downregulation following RA addition. Both approaches result in stronger *Nanog* repression after RA addition. Moreover, expression of truncated Lyar leads to impaired differentiation and increased apoptosis.

Discussion

In this work we describe Lyar-mediated recruitment of a BET member to particular positions in the chromatin. Lyar-Brd2 interaction illustrates how co-regulators frequently associated with the general transcription machinery can be locally recruited in a selective manner through specific transcription factors. Lyar acting as a transcription factor has been

previously reported [35] and is supported by our data. In agreement with this report, our data indicate that Lyar behaves as a repressor, since Lyar depletion leads to *Nanog* upregulation under normal growth conditions and to impaired downregulation under differentiation conditions.

We initially identified the interaction of Lyar with Brd2 in a two-hybrid screening using Brd2 as a bait. In addition, mapping of the interaction surfaces revealed that the highly conserved motif B region of Brd2 was involved in this interaction. The implication of this highly conserved region of Brd2 may suggest the possible interaction of Lyar with other members of the BET family. However, two-hybrid experiments and immunoprecipitation assays surprisingly showed that, among BET proteins, Lyar specifically interacted with Brd2. Previous studies have described a selective interaction of Pleiotrophin with Brd2 among BET family members [24]. However, this interaction was mediated by an acidic stretch located N-terminal of the motif B region in Brd2, absent in other BET proteins. Interestingly, this acidic stretch was not implicated in Lyar-Brd2 interaction, since a Brd2 variant lacking the acidic stretch did not impair Lyar interaction. Despite the high similarity of motif B along BET proteins, special and distinctive features in this region of Brd2 should account for the exclusive interaction with Lyar. In agreement with this specific interaction with Brd2, ChIP experiments demonstrated that Lyar depletion led to Brd2 dissociation from a number of promoters, while Brd4 occupancy remained unaltered. Therefore, Lyar recruits Brd2 to the chromatin in a selective manner in relation to other BET proteins. Noteworthy, unaltered occupancy of Lyar on promoters following dissociation of BET proteins by JQ1 treatment, excludes a role of Brd2 in recruiting or stabilizing Lyar to the chromatin. Other examples of selective recruitment of BET members to the chromatin through particular transcription factors have been described: the interaction of Brd3 with the transcription factor GATA1 or

the interaction of Brd4 with the transcription factors Oct4, p53, NFkB and Twist [22, 29-32, 40].

In a recent report, Brd2, together with CTCF, was implicated in the establishment of transcriptional boundaries at insulators [20], but the presence of Brd2 at promoters has been profusely documented [25, 26, 28]. In the five promoters initially tested in this study we detected Brd2, and observed that Lyar downregulation resulted in Brd2 dissociation. This raises the question of how general is Lyar-mediated recruitment of Brd2 to the chromatin. Our fractionation experiments indicate that Lyar downregulation significantly dissociates Brd2 from the chromatin, suggesting that Lyar-mediated Brd2 recruitment is not limited to a reduced set of genes. However, the absence of Lyar from Brd2-occupied promoters like *Actb* and *Elk3* promoters also excludes full overlap between Brd2 and Lyar on the chromatin. We chose to analyze in detail the Brd2-Lyar interaction in the context of pluripotency genes due to the previously reported involvement of Lyar in ESCs self-renewal [37]. Despite Brd2 clearance from promoters of all pluripotency genes tested following depletion of Lyar, altered expression of these genes was only observed in the case of *Nanog* and *Prdm14*. These data indicate that local cues, which may involve redundant mechanism for transcriptional control, dictate particular transcriptional outputs from each individual promoter. Otherwise, the use of JQ1 drug demonstrated that BET proteins were required for expression of at least *Pou5f1*, *Prdm14* and *Nanog*, in agreement with previously described regulation of *Nanog* expression by Brd4 [41, 42].

To study Lyar-Brd2 interaction in the context of differentiation we took advantage of RA treatment, as an efficient method to promote neuronal differentiation in P19 cells. Noticeably, the response to RA has been linked to BET proteins, since Brd4 has been reported to act as a transcriptional co-activator of nuclear RA receptors [43]. An alternative method to RA treatment also promoting differentiation in P19 cells consists in depriving cells

from serum, so it would be of interest for future research to explore how Brd2 and Lyar behave in relation to serum starvation and re-stimulation or even in response to different growth factors. The consistent upregulation of *Nanog* in the absence of Lyar prompted us to focus on this gene to further study Brd2-Lyar interaction. Our results indicate that *Nanog* is efficiently downregulated in P19 cells upon induction of RA-mediated differentiation, and that Lyar depletion leads to impaired *Nanog* downregulation. Lyar depletion was also accompanied by impaired upregulation of differentiation markers, in agreement with previous report in a different cellular model [37]. This altered expression of differentiation markers might be explained, at least in part, by the abnormal high levels of *Nanog* (4.5-fold increase) under differentiation conditions. Early after RA addition, when *Nanog* expression presents mid-downregulation, Brd2 depletion results in deeper repression, attributing a role to Brd2 in transcription activation, consistent with the general assumption of the major role of Brd2 in transcription [14, 39]. Interestingly, the same effect was observed when a truncated Lyar variant, lacking the Brd2 interaction domain, was expressed. Altogether, these results point to the absence of the interaction with Brd2 as the cause of the observed effects. Thus, truncated Lyar behaves as a dominant negative molecule. Several observations support this idea: the effects on *Nanog* expression, the absence of interaction with Brd2, and the association of truncated Lyar to the *Nanog* promoter with concomitant dissociation of Brd2. According to our results, truncated Lyar retains repression activity, which seems logical as the interaction domain for the repressor PRMT5 was preserved [35]. Interestingly, under normal growth conditions, neither truncated Lyar nor Brd2 depletion altered *Nanog* expression, which may be explained by the concurrence of redundant mechanisms of transcriptional control that ensure full *Nanog* expression regardless of Brd2. Indeed, Brd4 participation in the control of *Nanog* has been previously reported [41, 42]. Thus, Brd2 and its recruitment to the *Nanog* promoter acquire relevance for *Nanog* control only under differentiation conditions. As

previously reported, depletion of Lyar compromises both self-renewal and differentiation in ESCs, which leads to increased apoptosis [37]. We show that depletion of Lyar under differentiation conditions impairs *Nanog* downregulation, while expression of truncated Lyar leads to stronger *Nanog* repression, in addition to altered differentiation and increased apoptosis. Thus, under differentiation conditions, either Lyar depletion as expression of truncated Lyar, having opposite impact on *Nanog* expression, causes similar effects in P19 cells. Such a mechanism, during stem cell proliferation and development, should ensure cell death in case of altered transcriptional program, preserving the embryo from uncontrolled cell proliferation and aberrant differentiation.

Despite the demonstrated interaction of Brd2 with Lyar, our ChIP experiments indicate that 48 h after RA addition, a significant proportion of Brd2 is dissociated from *Nanog* promoter, while Lyar occupancy remains unaltered, suggesting that under these conditions one or the other protein is blocked for mutual interaction. Reduced co-immunoprecipitation of Lyar with Brd2 under differentiation conditions supports this idea. In this context, it is worth noting that Pleiotrophin has been described to be expressed following induction of differentiation to antagonize Brd2 by protein-protein interaction through the motif B [33], which might be related to our observations.

Therefore, at the onset of differentiation, Lyar, working as a repressor, should retain Brd2, which will function as a transcriptional activator, for gradual downregulation of *Nanog*. This mechanism may preserve the appropriate downregulation timing of key transcription factors once differentiation is triggered. Abrupt downregulation of such factors would otherwise lead to misregulation of other relevant factors resulting in severe alterations of the differentiation program. Future research, comprising genome-wide analysis aimed at identifying relevant genes regulated by alliance between Brd2 and Lyar, would help to shed light on the mechanisms directing the transition from proliferation to differentiation.

Materials and methods

Plasmids, siRNA and yeast two-hybrid

All expression constructions were derived from vector pAdRSV-Sp [44], with Flag or HA tags. Yeast two-hybrid was performed in the DUALhybrid Kit system (Dualsystems Biotech, Schlieren, Zurich, Switzerland), using the pLexA-N bait and pGAD-HA prey vectors, as previously described [33]. Full-length cDNA corresponding to mouse *Lyar* was obtained by RT-PCR using total RNA isolated from P19 cells and cloned into the pAdRSV-Sp vector with an N-terminal HA tag for expression or in the pGAD-HA prey vector for two-hybrid analysis. Deletion constructions were generated by standard PCR techniques and were as follows: LyarΔC, amino acids 1-227; LyarΔZF, amino acids 169-388. Other expression or two-hybrid constructions have been described previously [24]. Expression vectors for *small hairpin RNA* molecules (*shRNA*) were based on vector pSuper (OligoEngine, Seattle, WA, USA). Target sequences were: *Lyar* 5'-CGAGATATCAGTCAAGAAG-3' and 5'-CAGAGATGCCGATCACTAA-3' (we chose this latter for the results presented). For *Brd2* knockdown we used either a predesigned siRNA (5'-GTGACTACCGGGATGCACA-3') or the MISSION esiRNA EMU067621 from Sigma-Aldrich, St Louis, MO, USA (we chose this latter for the results presented). Control shRNA was previously described [24]. Control siRNA was derived from the *Luciferase* sequence (5'-CGTACGCGGAATACTTCGA-3').

Cell culture and transfection

Human HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Sigma-Aldrich). This line was used as a tool for tagged protein expression in immunoprecipitation experiments, so they were checked for this ability. Mouse P19 cells were directly purchased from ATCC (LGC Standards,

Barcelona, Spain) as authenticated and were cultured in α-modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 7.5% calf serum (HyClone) and 2.5% fetal bovine serum. Absence of mycoplasms was verified for both lines. For RA treatment of P19 cells, media was changed to 5% fetal bovine serum. RA (Sigma-Aldrich) was used at 1 μM for the indicated times. JQ1 drug (Sigma-Aldrich) was used at 0,2 μM at the indicated times. Transfections were performed with Lipofectamine 2000 (Invitrogen, Life Technologies, Paisley, UK) for 24, 48 or 72 hours for plasmids, shRNA or siRNA molecules, respectively.

Cellular fractionation, immunoprecipitation and blotting

For cellular fractionation [45] P19 cells were extracted in hypotonic buffer A [10mM Hepes, 10mM KCl, 1.5mM $MgCl_2$, complete protease inhibitor cocktail (Roche)] and then subjected to three cycles of short freeze-thaw with liquid N_2. After 5 min of centrifugation at 1300g the supernatant (S1) and pellet (P1) fractions were obtained. S1 fraction was centrifuged 5 min at 12000g, giving rise to the cytoplasmic fraction (S2). P1 fraction was resuspended in buffer B [3mM EDTA, 0.2 mM EGTA, 1mM DTT, complete protease inhibitor cocktail (Roche, Mannheim, Germany)] and incubated 30 min on ice. After 5 min of centrifugation at 1700g the nucleoplasmic soluble fraction (S3) and the chromatin precipitated fraction (P3) were obtained. Fraction P3 was resuspended in Laemmli buffer and fractions S2 and S3 were mixed to obtain the soluble fraction. For immunoprecipitation, cells were extracted with IP buffer [150mM NaCl, 50mM Tris-HCl pH 7.5, 1% Triton X-100, complete protease inhibitor cocktail (Roche)]. Protein amount was determined by the Bradford reactive assay (Bio-Rad, Hercules, CA, USA) to incubate 0.5 mg of protein overnight at 4ºC in rotation with 3 μg of the appropriate antibody, which was later precipitated after 2h of incubation at 4ºC in rotation with protein A or G Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) in the case antibodies were raised in rabbit or mouse, respectively. After washing, proteins were eluted

from beads with 20 µL of Laemmli buffer and boiled before immunoblotting analysis. For this purpose, proteins were separated in SDS gels and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK) for antibody blotting. Signal was revealed with a chemiluminescence ECL system (Bio-Rad) and a ChemidDoc XRS apparatus (Bio-Rad). Antibodies used were: mouse anti-Flag M2 (1:2000, Sigma-Aldrich), rat anti-HA (1:2000, Roche), mouse anti-α-tubulin (1:10000, Sigma), rabbit anti-Lyar (1:3000, AB-4344, Millipore, Burlington, MA, USA), rabbit anti-Brd2C (1:1000, [33]) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, anti-rat IgG and anti-rabbit IgG (1:10000, Sigma-Aldrich).

Chromatin immunoprecipitation, RNA isolation and quantitative PCR

Chromatin immunoprecipitation (ChIP) samples were prepared from a total of 10^7 P19 cells crosslinked in 1% formaldehyde for 10 min at room temperature. Nuclei were isolated using lysis buffer 1 [5 mM Pipes pH 8, 85Mm KCl, 0.5% NP40, complete protease inhibitor cocktail (Roche)] and were lysed using lysis buffer 2 [1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.1, complete protease inhibitor cocktail (Roche)]. Chromatin was sheared into an average size of 500 pb by eight 30s pulses at 4ºC in a waterbath sonicator Bioruptor (Diagenode, Liège, Belgium). Thirty µg of chromatin were incubated overnight at 4ºC after 1:10 dilution in IP buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl] with the corresponding antibody. Protein A Dynabeads (Thermo Fisher Scientific) were used to capture antibodies. After washing, complexes were eluted from beads and de-crosslinked overnight at 65ºC. Samples were treated with proteinase K (Roche) for 1h at 37ºC and DNA purified using the ChIP DNA Clean and Concentrator kit (Zymo Research, Irvine, CA, USA). Antibodies used were: 20 µL of anti-Brd2N serum [24], 4 µg of anti-Brd4 antibody (A301-985A100, Bethyl Laboratories, Montgomery, TX, USA), 4

µg of anti-Lyar antibody (AB-4344, Millipore). Normal rabbit IgG (Sigma-Aldrich) was used as a control. For gene expression analysis total RNA was extracted using the NZYTech Total RNA isolation kit (NZYTech, Lisbon, Portugal) and was retro-transcribed using the iScript cDNA Synthesis kit (Bio-Rad). Quantitative PCR was performed with Power SYBR Green (Applied Biosystems, Carlsbad, CA, USA) in the 7500 Fast Real-Time PCR System (Applied Biosystems). The *RpLp* gene was used as a reference gene to analyse relative expression and data were normalized according to [46]. Primers used for ChIP and expression analyses are detailed in Table1.

Immunofluorescence and determination of apoptosis

For immunofluorescence, cells were grown on coverslips and fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline (PBS). Cells were permeabilized in 0.5% Triton X-100 in PBS for 10 min and blocked in 10% donkey serum in PBS for 30 min prior to antibody incubation. Antibodies: mouse anti-Pax6 (1:50 from supernatants, Developmental Studies Hybridoma Bank, Iowa, IA, USA), donkey anti-mouse cy3 (1:800, Jackson Immunoresearch, Suffolk, UK). Cell nuclei were exposed by DAPI (Sigma-Aldrich) staining. Fluorescent images were acquired on a Leica Microsystems GmbH (Wetzlar, Germany) epifluorescence DM6000 microscope. For apoptosis determination cells (10^5/well) were transfected in 6-well plates as indicated in figure legends. After transfection, hypodiploid apoptotic cells (subG1 fraction) were detected by flow cytometry. For that, cells were washed with phosphate buffered saline (PBS), fixed in cold 70% ethanol and then stained with 10 µg/ml propidium iodide (Sigma-Aldrich) while treated with RNAse A (Sigma-Aldrich). Quantitative analysis of subG1 cells was carried out in a FACSCalibur cytometer using the Cell Quest software (Becton Dickinson, Mountain View, CA, USA).

Statistical analysis

For statistical significance of differences between two conditions, data were analyzed using the Student's t-test. Statistically significant *p*-values were as follows: * < 0.05, ** < 0.01, *** < 0.001.

Acknowledgements

We are grateful to Pablo Garcia-Gutierrez for his contribution to preliminary results related to this work and to Petra I. Lorenzo for critical reading of the manuscript. The Pax6 antibody, developed by A. Kawakami, was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. N. L.-P. was the recipient of a FPU fellowship from Ministerio de Educación, Spain. This work was supported by Ministerio de Economía y Competitividad (MINECO), Spain (grant BFU2012-37304/BFI to M. G.-D.).

References

[1] G. LeRoy, B. Rickards, S.J. Flint, The double bromodomain proteins Brd2 and Brd3 couple histone acetylation to transcription. Mol. Cell, 30 (2008) 51-60.

[2] K. Mochizuki, A. Nishiyama, M.K. Jang, A. Dey, A. Ghosh, T. Tamura, H. Natsume, H. Yao, K. Ozato, The bromodomain protein Brd4 stimulates G1 gene transcription and promotes progression to S phase. J. Biol. Chem., 283 (2008) 9040-9048.

[3] A. Sinha, D.V. Faller, G.V. Denis, Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A. Biochem. J., 387 (2005) 257-269.

[4] Z. Yang, N. He, Q. Zhou, Brd4 recruits P-TEFb to chromosomes at late mitosis to promote G1 gene expression and cell cycle progression. Mol. Cell. Biol., 28 (2008) 967-976.

[5] A.J. Stonestrom, S.C. Hsu, K.S. Jahn, P. Huang, C.A. Keller, B.M. Giardine, S. Kadauke, A.E. Campbell, P. Evans, R.C. Hardison, G.A. Blobel, Functions of BET proteins in erythroid gene expression. Blood, 125 (2015) 2825-2834.

[6] M. Tsume, C. Kimura-Yoshida, K. Mochida, Y. Shibukawa, S. Amazaki, Y. Wada, R. Hiramatsu, K. Shimokawa, I. Matsuo, Brd2 is required for cell cycle exit and neuronal differentiation through the E2F1 pathway in mouse neuroepithelial cells. Biochem. Biophys. Res. Commun., 425 (2012) 762-768.

[7] A.C. Belkina, G.V. Denis, BET domain co-regulators in obesity, inflammation and cancer. Nat. Rev. Cancer, 12 (2012) 465-477.

[8] J. Shi, C.R. Vakoc, The mechanisms behind the therapeutic activity of BET bromodomain inhibition. Mol. Cell, 54 (2014) 728-736.

[9] Z. Yang, J.H. Yik, R. Chen, N. He, M.K. Jang, K. Ozato, Q. Zhou, Recruitment of P-TEFb for stimulation of transcriptional elongation by the bromodomain protein Brd4. Mol. Cell, 19 (2005) 535-545.

[10] Y.W. Jiang, P. Veschambre, H. Erdjument-Bromage, P. Tempst, J.W. Conaway, R.C. Conaway, R.D. Kornberg, Mammalian mediator of transcriptional regulation and its possible role as an end-point of signal transduction pathways. Proc. Natl. Acad. Sci. U. S. A., 95 (1998) 8538-8543.

[11] S. Rahman, M.E. Sowa, M. Ottinger, J.A. Smith, Y. Shi, J.W. Harper, P.M. Howley, The Brd4 extraterminal domain confers transcription activation independent of pTEFb by recruiting multiple proteins, including NSD3. Mol. Cell. Biol., 31 (2011) 2641-2652.

[12] T.E. Crowley, E.M. Kaine, M. Yoshida, A. Nandi, D.J. Wolgemuth, Reproductive cycle regulation of nuclear import, euchromatic localization, and association with components of Pol II mediator of a mammalian double-bromodomain protein. Mol. Endocrinol., 16 (2002) 1727-1737.

[13] G.V. Denis, M.E. McComb, D.V. Faller, A. Sinha, P.B. Romesser, C.E. Costello, Identification of transcription complexes that contain the double bromodomain protein Brd2 and chromatin remodeling machines. J. Proteome Res., 5 (2006) 502-511.

[14] G.V. Denis, C. Vaziri, N. Guo, D.V. Faller, RING3 kinase transactivates promoters of cell cycle regulatory genes through E2F. Cell Growth Differ., 11 (2000) 417-424.

[15] A. Gyuris, D.J. Donovan, K.A. Seymour, L.A. Lovasco, N.R. Smilowitz, A.L. Halperin, J.E. Klysik, R.N. Freiman, The chromatin-targeting protein Brd2 is required for neural tube closure and embryogenesis. Biochim. Biophys. Acta, 1789 (2009) 413-421.

[16] D. Houzelstein, S.L. Bullock, D.E. Lynch, E.F. Grigorieva, V.A. Wilson, R.S. Beddington, Growth and early postimplantation defects in mice deficient for the bromodomain-containing protein Brd4. Mol. Cell. Biol., 22 (2002) 3794-3802.

[17] E. Shang, X. Wang, D. Wen, D.A. Greenberg, D.J. Wolgemuth, Double bromodomain-containing gene Brd2 is essential for embryonic development in mouse. Dev. Dyn., 238 (2009) 908-917.

[18] A.C. Belkina, W.P. Blanton, B.S. Nikolajczyk, G.V. Denis, The double bromodomain protein Brd2 promotes B cell expansion and mitogenesis. J. Leukoc. Biol., 95 (2014) 451-460.

[19] C.S. Choi, S.H. Hong, S. Sim, K.S. Cho, J.W. Kim, S.M. Yang, S.J. Jeon, J.S. You, C.Y. Shin, The Epigenetic Reader BRD2 as a Specific Modulator of PAI-1 Expression in Lipopolysaccharide-Stimulated Mouse Primary Astrocytes. Neurochem. Res., 40 (2015) 2211-2219.

[20] S.C. Hsu, T.G. Gilgenast, C.R. Bartman, C.R. Edwards, A.J. Stonestrom, P. Huang, D.J. Emerson, P. Evans, M.T. Werner, C.A. Keller, B. Giardine, R.C. Hardison, A. Raj, J.E. Phillips-Cremins, G.A. Blobel, The BET Protein BRD2 Cooperates with CTCF to Enforce Transcriptional and Architectural Boundaries. Mol. Cell, 66 (2017) 102-116 e107.

[21] E. Korb, M. Herre, I. Zucker-Scharff, R.B. Darnell, C.D. Allis, BET protein Brd4 activates transcription in neurons and BET inhibitor Jq1 blocks memory in mice. Nat. Neurosci., 18 (2015) 1464-1473.

[22] J.M. Lamonica, W. Deng, S. Kadauke, A.E. Campbell, R. Gamsjaeger, H. Wang, Y. Cheng, A.N. Billin, R.C. Hardison, J.P. Mackay, G.A. Blobel, Bromodomain protein Brd3 associates with acetylated GATA1 to promote its chromatin occupancy at erythroid target genes. Proc. Natl. Acad. Sci. U. S. A., 108 (2011) E159-168.

[23] E. Shang, H.D. Nickerson, D. Wen, X. Wang, D.J. Wolgemuth, The first bromodomain of Brdt, a testis-specific member of the BET sub-family of double-bromodomain-containing proteins, is essential for male germ cell differentiation. Development, 134 (2007) 3507-3515.

[24] P. Garcia-Gutierrez, F. Juarez-Vicente, D.J. Wolgemuth, M. Garcia-Dominguez, Pleiotrophin antagonizes Brd2 during neuronal differentiation. J. Cell Sci., 127 (2014) 2554-2564.

[25] L. Anders, M.G. Guenther, J. Qi, Z.P. Fan, J.J. Marineau, P.B. Rahl, J. Loven, A.A. Sigova, W.B. Smith, T.I. Lee, J.E. Bradner, R.A. Young, Genome-wide localization of small molecules. Nat. Biotechnol., 32 (2014) 92-96.

[26] K.L. Cheung, F. Zhang, A. Jaganathan, R. Sharma, Q. Zhang, T. Konuma, T. Shen, J.Y. Lee, C. Ren, C.H. Chen, G. Lu, M.R. Olson, W. Zhang, M.H. Kaplan, D.R. Littman, M.J. Walsh, H. Xiong, L. Zeng, M.M. Zhou, Distinct Roles of Brd2 and Brd4 in Potentiating the Transcriptional Program for Th17 Cell Differentiation. Mol. Cell, 65 (2017) 1068-1080 e1065.

[27] R. Di Micco, B. Fontanals-Cirera, V. Low, P. Ntziachristos, S.K. Yuen, C.D. Lovell, I. Dolgalev, Y. Yonekubo, G. Zhang, E. Rusinova, G. Gerona-Navarro, M. Canamero, M. Ohlmeyer, I. Aifantis, M.M. Zhou, A. Tsirigos, E. Hernando, Control of embryonic stem cell

identity by BRD4-dependent transcriptional elongation of super-enhancer-associated pluripotency genes. Cell Rep., 9 (2014) 234-247.

[28] L.E. Surface, P.A. Fields, V. Subramanian, R. Behmer, N. Udeshi, S.E. Peach, S.A. Carr, J.D. Jaffe, L.A. Boyer, H2A.Z.1 Monoubiquitylation Antagonizes BRD2 to Maintain Poised Chromatin in ESCs. Cell Rep., 14 (2016) 1142-1155.

[29] B. Huang, X.D. Yang, M.M. Zhou, K. Ozato, L.F. Chen, Brd4 coactivates transcriptional activation of NF-kappaB via specific binding to acetylated RelA. Mol. Cell Biol, 29 (2009) 1375-1387.

[30] J. Shi, Y. Wang, L. Zeng, Y. Wu, J. Deng, Q. Zhang, Y. Lin, J. Li, T. Kang, M. Tao, E. Rusinova, G. Zhang, C. Wang, H. Zhu, J. Yao, Y.X. Zeng, B.M. Evers, M.M. Zhou, B.P. Zhou, Disrupting the interaction of BRD4 with diacetylated Twist suppresses tumorigenesis in basal-like breast cancer. Cancer Cell, 25 (2014) 210-225.

[31] T. Wu, H.B. Pinto, Y.F. Kamikawa, M.E. Donohoe, The BET family member BRD4 interacts with OCT4 and regulates pluripotency gene expression. Stem Cell Reports, 4 (2015) 390-403.

[32] R. Gamsjaeger, S.R. Webb, J.M. Lamonica, A. Billin, G.A. Blobel, J.P. Mackay, Structural basis and specificity of acetylated transcription factor GATA1 recognition by BET family bromodomain protein Brd3. Mol. Cell. Biol., 31 (2011) 2632-2640.

[33] P. Garcia-Gutierrez, M. Mundi, M. Garcia-Dominguez, Association of bromodomain BET proteins with chromatin requires dimerization through the conserved motif B. J. Cell Sci., 125 (2012) 3671-3680.

[34] L. Su, R.J. Hershberger, I.L. Weissman, LYAR, a novel nucleolar protein with zinc finger DNA-binding motifs, is involved in cell growth regulation. Genes Dev., 7 (1993) 735-748.

[35] J. Ju, Y. Wang, R. Liu, Y. Zhang, Z. Xu, Y. Wang, Y. Wu, M. Liu, L. Cerruti, F. Zou, C. Ma, M. Fang, R. Tan, S.M. Jane, Q. Zhao, Human fetal globin gene expression is regulated by LYAR. Nucleic Acids Res., 42 (2014) 9740-9752.

[36] N. Miyazawa, H. Yoshikawa, S. Magae, H. Ishikawa, K. Izumikawa, G. Terukina, A. Suzuki, S. Nakamura-Fujiyama, Y. Miura, T. Hayano, W. Komatsu, T. Isobe, N. Takahashi, Human cell growth regulator Ly-1 antibody reactive homologue accelerates processing of preribosomal RNA. Genes Cells, 19 (2014) 273-286.

[37] H. Li, B. Wang, A. Yang, R. Lu, W. Wang, Y. Zhou, G. Shi, S.W. Kwon, Y. Zhao, Y. Jin, Ly-1 antibody reactive clone is an important nucleolar protein for control of self-renewal and differentiation in embryonic stem cells. Stem Cells, 27 (2009) 1244-1254.

[38] G. LeRoy, I. Chepelev, P.A. DiMaggio, M.A. Blanco, B.M. Zee, K. Zhao, B.A. Garcia, Proteogenomic characterization and mapping of nucleosomes decoded by Brd and HP1 proteins. Genome Biol., 13 (2012) R68.

[39] J. Hnilicova, S. Hozeifi, E. Stejskalova, E. Duskova, I. Poser, J. Humpolickova, M. Hof, D. Stanek, The C-terminal domain of Brd2 is important for chromatin interaction and regulation of transcription and alternative splicing. Mol. Biol. Cell, 24 (2013) 3557-3568.

[40] H.J. Stewart, G.A. Horne, S. Bastow, T.J. Chevassut, BRD4 associates with p53 in DNMT3A-mutated leukemia cells and is implicated in apoptosis by the bromodomain inhibitor JQ1. Cancer Med., 2 (2013) 826-835.

[41] G.A. Horne, H.J. Stewart, J. Dickson, S. Knapp, B. Ramsahoye, T. Chevassut, Nanog requires BRD4 to maintain murine embryonic stem cell pluripotency and is suppressed by bromodomain inhibitor JQ1 together with Lefty1. Stem Cells Dev., 24 (2015) 879-891.

[42] W. Liu, P. Stein, X. Cheng, W. Yang, N.Y. Shao, E.E. Morrisey, R.M. Schultz, J. You, BRD4 regulates Nanog expression in mouse embryonic stem cells and preimplantation embryos. Cell Death Differ., 21 (2014) 1950-1960.

[43] S. Flajollet, C. Rachez, M. Ploton, C. Schulz, R. Gallais, R. Metivier, M. Pawlak, A. Leray, A.A. Issulahi, L. Heliot, B. Staels, G. Salbert, P. Lefebvre, The elongation complex components BRD4 and MLLT3/AF9 are transcriptional coactivators of nuclear retinoid receptors. PLoS One, 8 (2013) e64880.

[44] F. Giudicelli, P. Gilardi-Hebenstreit, F. Mechta-Grigoriou, C. Poquet, P. Charnay, Novel activities of Mafb underlie its dual role in hindbrain segmentation and regional specification. Dev. Biol., 253 (2003) 150-162.

[45] J. Wysocka, P.T. Reilly, W. Herr, Loss of HCF-1-chromatin association precedes temperature-induced growth arrest of tsBN67 cells. Mol. Cell. Biol., 21 (2001) 3820-3829.

[46] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res., 29 (2001) e45.

Legends to figures

Fig. 1. Lyar selectively interacts with Brd2 among BET proteins. (a) Schematic representation 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; ac, acidic region; mB, motif B; ET, extra terminal domain; SEED, domain rich in Ser, Asp and Glu. (b) Growth on selective and non-selective media of yeast harboring the indicated bait and prey (LexA and GAD, respectively) constructions or empty vectors. (c) Extracts from HEK293T cells transfected with HA-Lyar and the indicated Flag-tagged constructions were immunoprecipitated with anti-Flag antibodies, and the co-immunoprecipitated proteins were visualized with anti-HA antibodies. Black and white arrowheads point to Lyar and IgG, respectively. (d) Extracts from P19 cells transfected with the indicated HA-tagged constructions were immunoprecipitated with anti-Brd2 antibodies, and the co-immunoprecipitated proteins were visualized with anti-HA antibodies.

Arrowheads point to the different Lyar (HA-tagged) constructions. (e) Extracts from P19 cells treated with RA for the indicated days were immunoprecipitated with anti-Brd2 antibodies, and the co-immunoprecipitated endogenous Lyar was monitored by immunoblotting with anti-Lyar antibodies. (c-e) Inputs of the indicated proteins correspond to 10% of the different cell extracts.

Fig. 2. Lyar recruits Brd2 to the chromatin. (a) Association of Brd2, Brd4 and Lyar to the *Pou5f1* (*Oct4*)*, Nanog, Prdm14, Sox2* and *Hbb-y* (γ-globin) promoters in P19 cells was determined by chromatin immunoprecipitation (ChIP). P19 cells were transfected with an shRNA against Lyar (shLyar) or a control shRNA for 48h. Levels were normalized to cells transfected with control shRNA. (b) P19 cells were transfected with shLyar or control shRNA for 48h. Cell lysates were analyzed by immunoblotting with anti-Lyar antibodies. Anti-α-tubuline antibody was used as a loading control. 20 µg of total protein were loaded per lane. (c) P19 cells were transfected with shLyar for 48h, treated with the JQ1 drug for 3h, or kept untreated (control). After fractionation, the presence of the endogenous Brd2 in the soluble and chromatin fractions was detected by immunoblotting. (d) Association of Brd2 and Lyar to the *Actb, Elk3* and *Pnma3* promoters in P19 cells transfected with shLyar or control shRNA for 48h was determined by ChIP. Levels were normalized to Brd2 levels in the *Actb* promoter in control cells. ChIP experiments with normal rabbit IgG were included as negative controls. (e) Association of Brd2 and Lyar to the *Pou5f1, Nanog* and *Prdm14* promoters in P19 cells treated with JQ1 drug or DMSO (vehicle) for 16h was determined by ChIP. Levels were normalized to DMSO-treated cells (control). (a, d, e) Values are means ± s.d. from three independent experiments analyzed in triplicate. Statistical significance of the differences in relation to controls (a, d, e) and between IgG and Brd2 or Lyar antibodies in

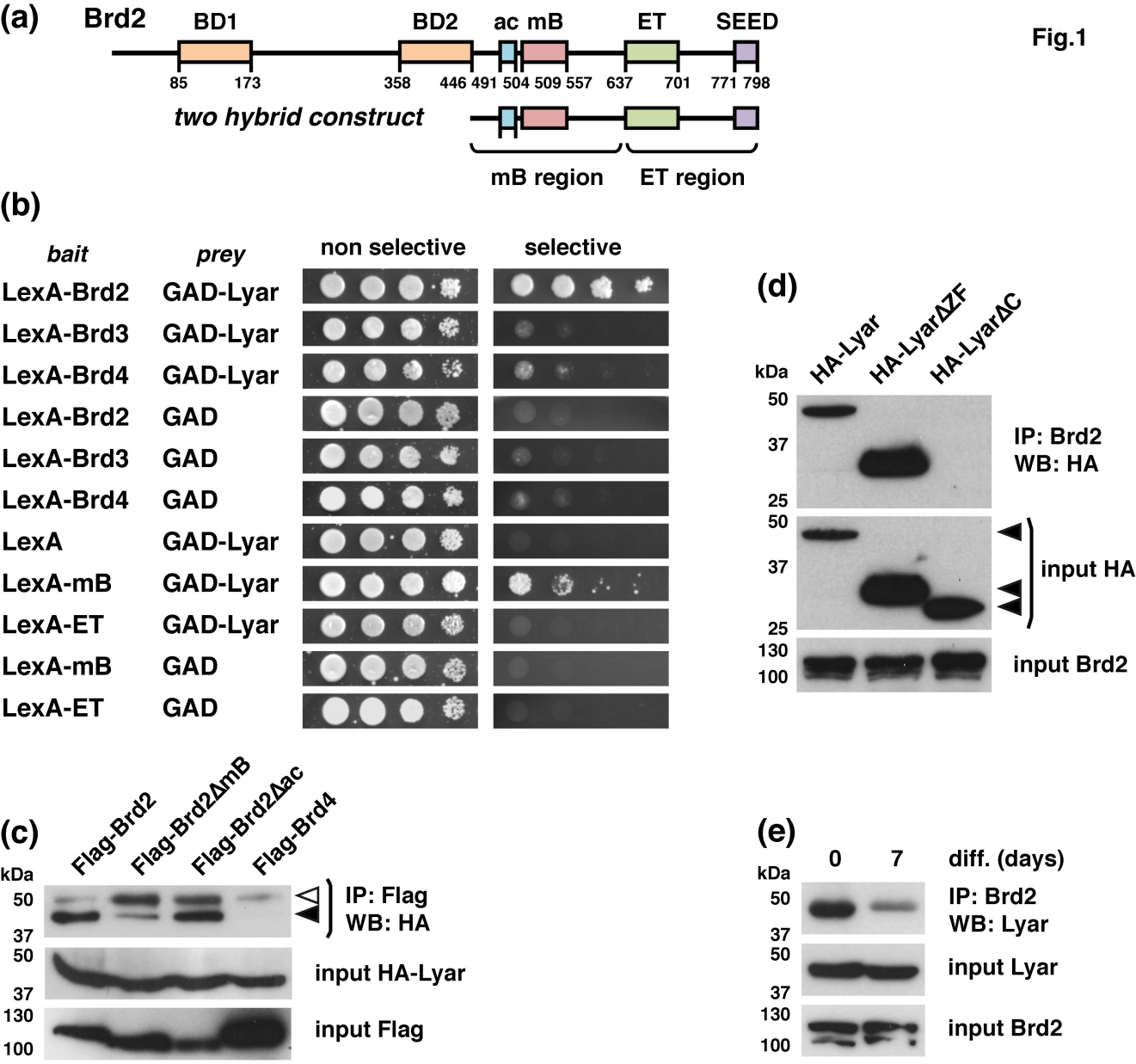
control transfections (d) were analyzed by the Student's t-test. *p*-values: * < 0.05, ** < 0.01, *** < 0.001.

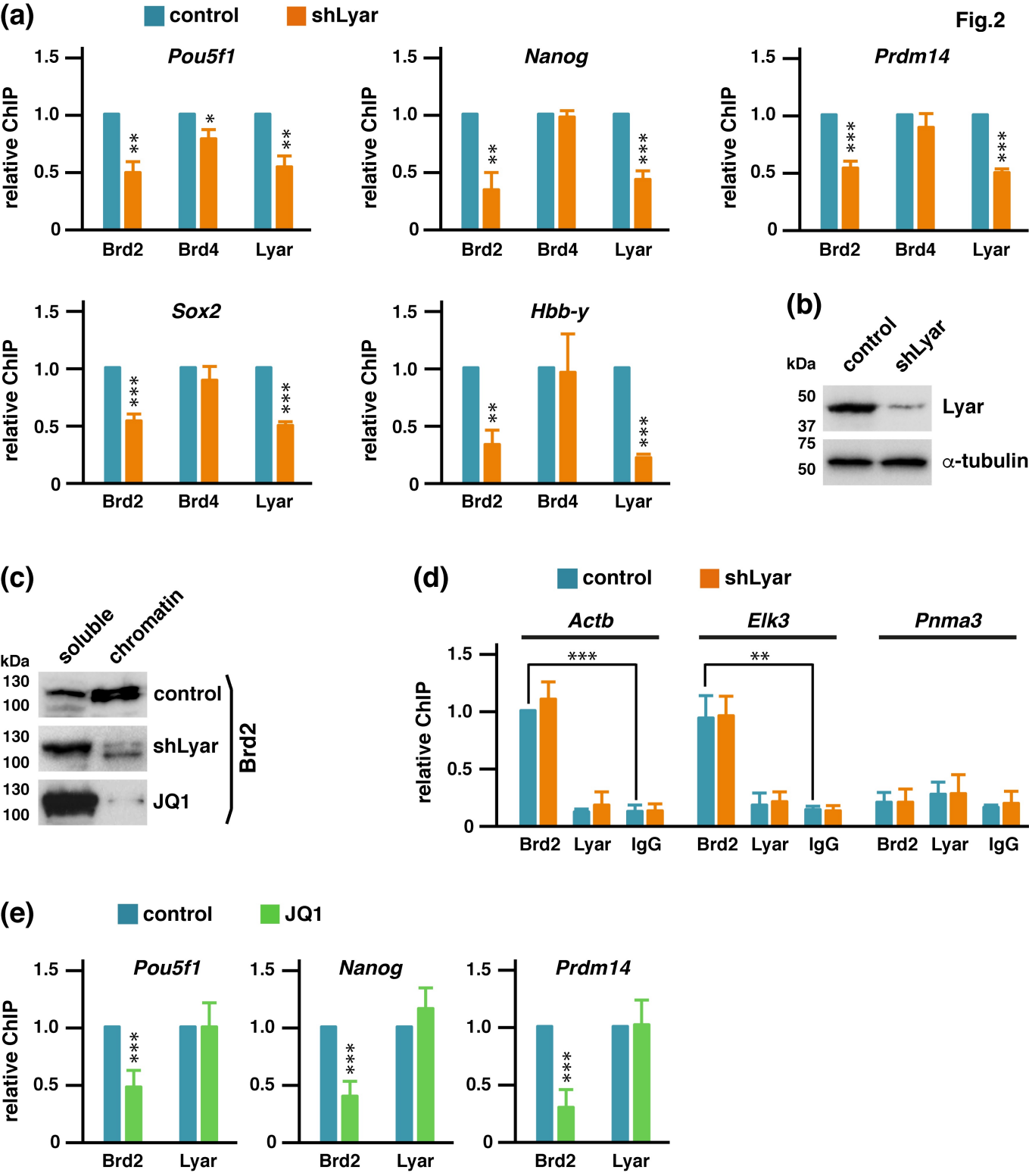
Fig. 3. Lyar acts as a repressor of Nanog. (a) Relative levels of expression of the *Pou5f1, Nanog, Prdm14* and *Sox2* genes determined by quantitative PCR in P19 cells transfected with an shRNA against Lyar (shLyar, shLy) or a control shRNA (control, shC) for 48h (upper panels) or treated with JQ1 drug or DMSO as vehicle (control, –) for 24h (lower panels). Levels were normalized to cells transfected with control shRNA or treated with DMSO, respectively. (b) Relative levels of expression of the *Nanog* gene determined by quantitative PCR in P19 cells treated with RA for the indicated times in hours (h). Levels were normalized to non-treated cells (0 h). (c) Association of Brd2 and Lyar to the *Nanog* promoter in P19 cells treated with RA for 48h was determined by ChIP. Levels were normalized to non-treated cells (control). (d) Relative levels of expression of the *Nanog, Nes (Nestin), Pax6* and *Sox6* genes determined by quantitative PCR in P19 cells transfected with shLyar (shLy) or control shRNA (shC) for 48h and subsequently treated with RA for 48h or kept untreated (control). Levels were normalized to non-treated cells transfected with control shRNA (control). (a-d) Values are means ± s.d. from three independent experiments analyzed in triplicate. Statistical significance of the differences in relation to controls (a, c) or between the indicated conditions (b, d) were analyzed by the Student's t-test. *p*-values: * < 0.05, ** < 0.01, *** < 0.001.

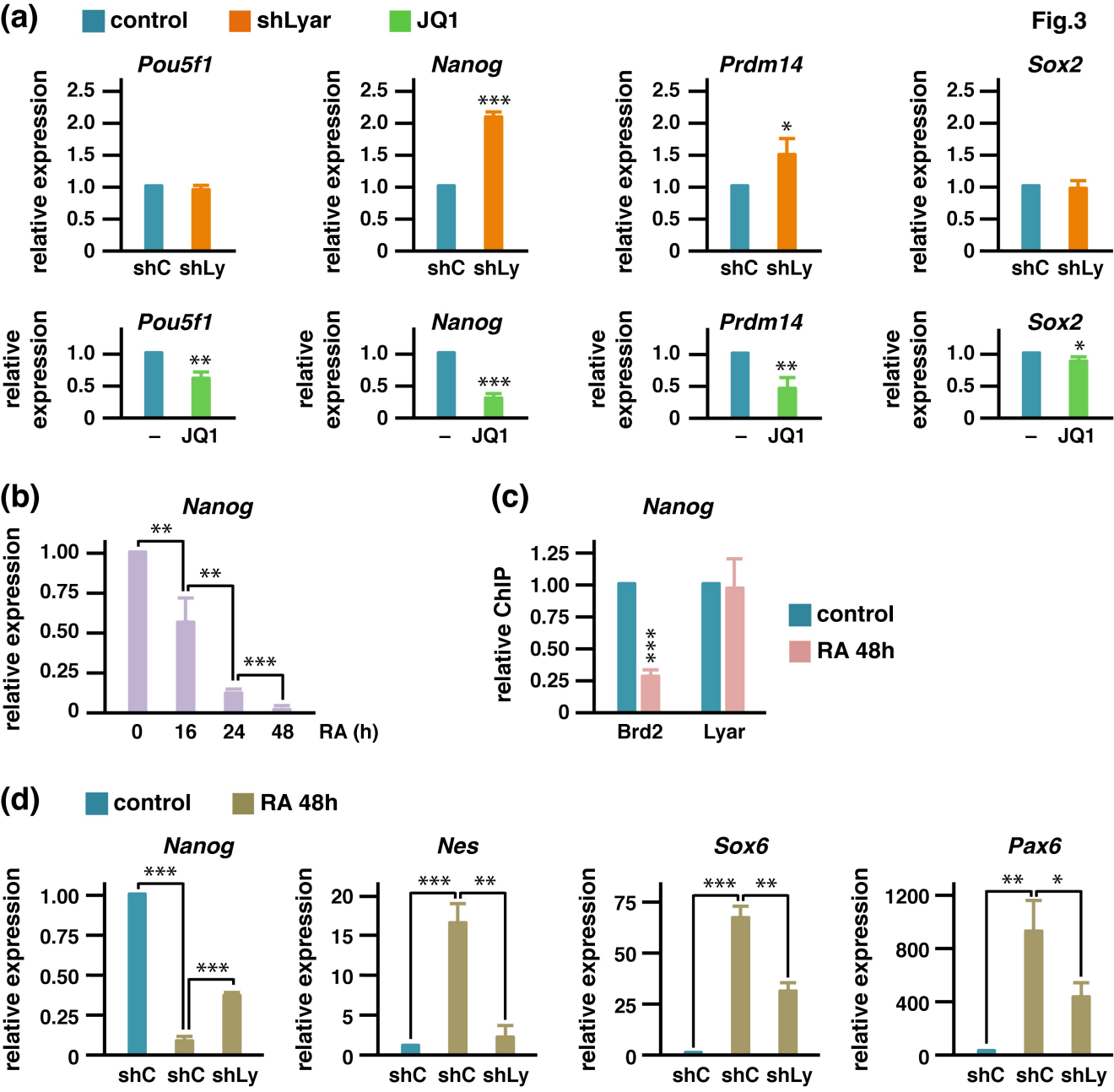
Fig. 4. The interaction of Brd2 with Lyar controls *Nanog* downregulation following induction of differentiation. (a) P19 cells were transfected with an esiRNA against Brd2 (siBrd2) or control siRNA (siControl) for 72h and cell lysates were analyzed by immunoblotting with anti-Brd2 and anti-α-tubuline antibodies. 20 µg of total proteins were loaded per lane. (b)

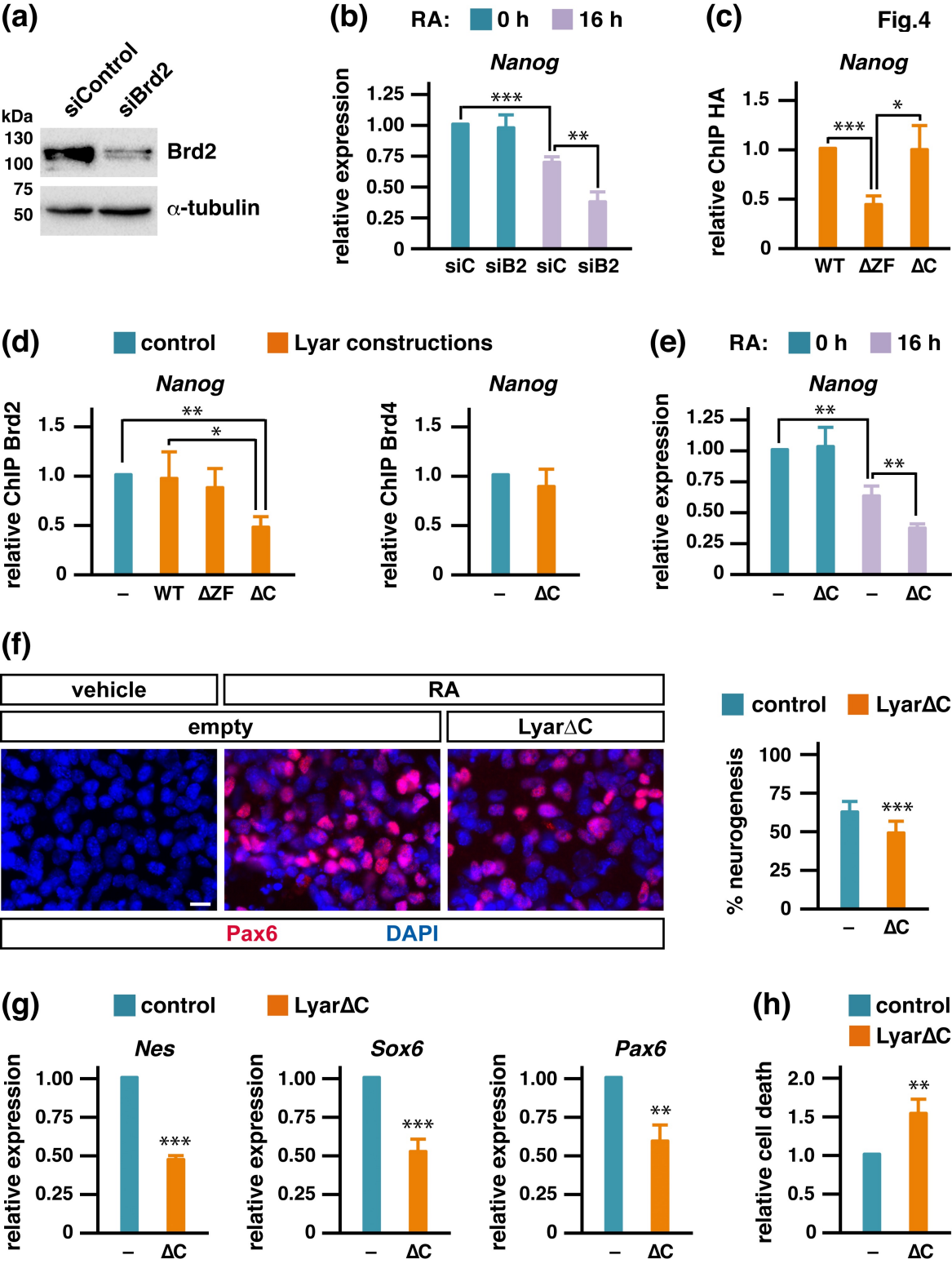
Relative levels of expression of *Nanog* determined by quantitative PCR in P19 cells transfected with siBrd2 (siB2) or control siRNA (siC) for 72h and subsequently treated with RA for 16h or kept untreated (0h). Levels were normalized to untreated cells transfected with control siRNA. (c) Association of different HA-tagged constructions of Lyar to the *Nanog* promoter in P19 cells transfected for 24h was determined by ChIP with anti-HA antibodies. Levels were normalized to cells transfected with the wild type (WT) construct. ΔZF, Lyar lacking the zinc finger DNA-binding domain; ΔC, Lyar lacking the C-terminal Brd2 interacting domain. (d) Association of Brd2 (left panel) and Brd4 (right panel) to the *Nanog* promoter in P19 cells transfected with the indicated HA-tagged Lyar constructions described in (c) or empty vector (control, –) for 24h was determined by ChIP. Levels were normalized to cells transfected with empty vector. (e) Relative levels of expression of *Nanog* determined by quantitative PCR in P19 cells transfected with the LyarΔC construct (ΔC) or empty vector (–) for 24h and subsequently treated with RA for 16h or kept untreated (0h). Levels were normalized to those of untreated cells transfected with empty vector. (f) Pax6 was revealed by immunofluorescence in cells transfected with empty vector and treated with ethanol (vehicle) for 48h and in cells transfected with empty vector or the LyarΔC construct and treated with RA for 48h (left panels). Scale bar 20 μm. The percentage of neurogenesis was estimated as the proportion of cells expressing Pax6 related to total number of cells (right panel). (g) Relative levels of expression of the differentiation markers *Nes*, *Sox6* and *Pax6* determined by quantitative PCR in P19 cells transfected with the LyarΔC construct (ΔC) or empty vector (control, –) for 24h and subsequently treated with RA for 48h. Levels were normalized to those of control cells. (h) Apoptosis was evaluated by cytometer measurement of the subG1 population of P19 cells transfected with LyarΔC (ΔC) or empty vector (control) and subsequently treated with RA for 48h. Levels were normalized to those of control cells. (b-e, g) Values are means ± s.d. from three independent experiments analyzed in triplicate. (f,

right panel) Values are means ± s.d. of counting ≥ 2800 cells in 22 fields from 9 samples from three independent experiments. (h) Values are means ± s.d. from four independent analyses. Statistical significance of the differences in relation to controls or between the indicated conditions were analyzed by the Student's t-test. *p*-values: * < 0.05, ** < 0.01, *** < 0.001.


(a)
Brd2
BD1
BD2
ac mB
ET
SEED
85
173
358
446
491
504
509
557
637
701
771
798
two hybrid construct
mB region
ET region
Fig.1
(b)
bait
prey
non selective
selective
LexA-Brd2
GAD-Lyar
LexA-Brd3
GAD-Lyar
LexA-Brd4
GAD-Lyar
LexA-Brd2
GAD
LexA-Brd3
GAD
LexA-Brd4
GAD
LexA
GAD-Lyar
LexA-mB
GAD-Lyar
LexA-ET
GAD-Lyar
LexA-mB
GAD
LexA-ET
GAD
(c)
Flag-Brd2
Flag-Brd2ΔmB
Flag-Brd2Δac
Flag-Brd4
kDa
50
37
50
37
130
100
IP: Flag
WB: HA
input HA-Lyar
input Flag
(d)
HA-Lyar
HA-LyarΔZF
HA-LyarΔC
kDa
50
37
25
50
37
25
130
100
IP: Brd2
WB: HA
input HA
input Brd2
(e)
0
7
diff. (days)
kDa
50
37
50
37
130
100
IP: Brd2
WB: Lyar
input Lyar
input Brd2


(a)
control
shLyar
Pou5f1
Nanog
Prdm14
Sox2
Hbb-y
relative ChIP
1.5
1.0
0.5
0
Brd2
Brd4
Lyar
(b)
kDa
control
shLyar
50
37
75
50
Lyar
α-tubulin
(c)
soluble
chromatin
kDa
130
100
control
shLyar
JQ1
Brd2
(d)
control
shLyar
Actb
Elk3
Pnma3
relative ChIP
Brd2
Lyar
IgG
(e)
control
JQ1
Pou5f1
Nanog
Prdm14
relative ChIP
Brd2
Lyar


(a)
control
shLyar
JQ1
Fig.3
Pou5f1
Nanog
Prdm14
Sox2
relative expression
shC shLy
–
JQ1
(b)
Nanog
relative expression
0 16 24 48 RA (h)
(c)
Nanog
relative ChIP
Brd2
Lyar
control
RA 48h
(d)
control
RA 48h
Nanog
Nes
Sox6
Pax6
shC shC shLy


(a)
siControl
siBrd2
kDa
130
100
75
50
Brd2
α-tubulin
(b)
RA:
0 h
16 h
Nanog
relative expression
1.25
1.00
0.75
0.50
0.25
0
***
**
siC
siB2
siC
siB2
(c)
Fig.4
Nanog
relative ChIP HA
1.5
1.0
0.5
0
***
*
WT
ΔZF
ΔC
(d)
control
Lyar constructions
Nanog
relative ChIP Brd2
1.5
1.0
0.5
0
**
*
–
WT
ΔZF
ΔC
Nanog
relative ChIP Brd4
1.5
1.0
0.5
0
–
ΔC
(e)
RA:
0 h
16 h
Nanog
relative expression
1.25
1.00
0.75
0.50
0.25
0
**
**
–
ΔC
–
ΔC
(f)
vehicle
RA
empty
LyarΔC
Pax6
DAPI
control
LyarΔC
% neurogenesis
100
75
50
25
0
***
–
ΔC
(g)
control
LyarΔC
Nes
relative expression
1.00
0.75
0.50
0.25
0
***
–
ΔC
Sox6
relative expression
1.00
0.75
0.50
0.25
0
***
–
ΔC
Pax6
relative expression
1.00
0.75
0.50
0.25
0
**
–
ΔC
(h)
control
LyarΔC
relative cell death
2.0
1.5
1.0
0.5
0
**
–
ΔC