The Genetic and Biochemical Analysis of the Chromatin-Targeting Protein BRD2 in Mammalian Development

by

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Brown University, Providence, RI
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Graduate Research

Studied the roles of the chromatin-targeting protein BRD2 in both *in vivo* and *in vitro* systems. Developed and characterized mice with both global and tissue specific ablation of *Brd2*, uncovering significant defects in both global growth regulation and midbrain-hindbrain development. Designed and conducted biochemical assays to further understand the mechanisms and complexes in which BRD2 protein guides transcriptional machinery to engage developmental pathways. Significant experience with immunohistochemistry, fluorescence imaging, immunoprecipitation, quantitative real-time PCR, cloning, transfection and tissue culture. Animal experience includes transgene design, cardiac perfusion, embryonic dissection and dosing animals by subcutaneous and intraperitoneal injection, as well as by oral gavage.

Dana-Farber Cancer Institute / Brigham and Women's Hospital, Boston, MA
Center for Molecular Oncologic Pathology
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Research Assistant

CMOP explores innovative technologies and methods to analyze genetic markers in an attempt to provide prognostic information, predict response to therapy and identify novel targets for drug development. Established the cytogenetic laboratory within the Center and developed custom genetic assays for specific gene targets using fluorescence *in situ* hybridization (FISH) in frozen and paraffin embedded tissue sections, as well as whole nuclei. Trained laboratory staff in techniques, assay development and fluorescence microscopy.

Brigham and Women's Hospital, Boston, MA
Developmental Genome Anatomy Project, Department of Pathology
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Research Assistant

Identified genes involved in human development by determining genes disrupted or dysregulated by balanced chromosomal translocations in patients with congenital phenotypes. Mapped translocation breakpoints by FISH and cloned breakpoints by Southern blot analysis and suppression PCR. Streamlined protocols, leading to a significant increase in productivity while incurring marginal costs. Assisted in writing grant renewals and developed budgets for FISH mapping projects. Supervised and trained personnel, including post-doctoral fellows, graduate and undergraduate students. Maintained and managed BAC collections, patient cell lines and research data via a custom Microsoft Access database.

Beth Israel Deaconess Medical Center, Boston, MA
Department of Molecular Medicine
2000 – 2002
Research Assistant

Characterized the regulation of the Egr-1, Flt-1 and vWF promoters, both *in vivo* and *in vitro*. Experience with transient and stable transfection of cell lines, luciferase and β-galactosidase assays, DNA and RNA manipulation, PCR-based site directed mutagenesis, Northern, Southern, and Western blotting. Created and used transgenic mice as experimental models and developed a Microsoft Access database to maintain records.

Massachusetts Institute of Technology, Cambridge, MA
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Assisted graduate students in manipulating levels of lysine production in *Corynebacterium glutamicum* by genetically and metabolically engineering precursor expression constructs.

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2010 – Present Member, American Association for the Advancement of Science
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SELECTED ABSTRACTS

Donovan DJ, Seymour K, Freiman RN. "The Molecular Mechanisms and Developmental Roles of the Chromatin Targeting Protein Brd2." Presented at the Keystone Symposium on the Molecular Basis for Chromatin Structure and Regulation, Taos, NM, Jan 17-22, 2010.

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Chapter 1: Introduction

Early Epigenetic Phenomena

When Waddington first described “causal interactions between genes and their products, which bring the phenotype into being” and later coined the term epigenetics, little was known about the intricacies of genetic inheritance or chromatin function throughout differentiation and development [1,2]. Epigenetic phenomena were typified by changes in a cell’s or organism’s phenotype which were heritable, but did not rely on DNA mutation. These changes were binary, switch-like phenomena: with distinct “on” and “off” states, rather than anything with a graded response. These changes remained heritable, even if the conditions which caused the switch disappeared. Early examples were seen across myriad systems and model organisms: the lysis/lysogeny switch in lambda bacteriophage [3,4], pili switching in uropathogenic E. *coli* [5,6], position effect variegation in Drosophila [7,8], and prion diseases [9,10] and X chromosome inactivation in mammals [11,12]. In each of these instances, the primary sequences of DNA remain unchanged while the observable phenotypes can be remarkably different. Much speculation surrounded what elements of control must be in place to assert such changes—could there be information packaged in the proteins which interacted with the DNA, might there be distinct *trans* effects from proteins which had only transient interactions with genes, or could there possibly be another more complex mechanism to describe these phenomena? Whichever mechanisms regulate these changes, they are heritable to future generations of cells and organisms without changes in DNA sequence, can be rapidly induced, and are fully reversible in certain conditions. As such, a

better understanding of these mechanisms is crucial to our understanding of genetic regulation.

Chromatin Structure and Function

In the late 19th and early 20th century, the cumulative works of many scientists had set the stage for the investigation of chromosomes and organismal inheritance. Early work by the German biologists Theodor Boveri and Walther Flemming posited that chromosomes were the heritable cellular component and that the protein-rich chromatin unravels to a single thread [13-16]. Over the ensuing century, these earliest observations of cell morphology and behavior have been proven to be astonishingly accurate and a great deal more has been learned about the cell's structure and genetic components. The most basic units of chromatin, originally termed *v* bodies, were originally identified in preparations of nuclei isolated from the rat thymus, rat liver and chicken erythrocytes [17]. Unbranched strands of DNA were visualized from ruptured nuclei, and spherical particles were found to be dispersed at irregular intervals along the strands themselves. At some points, the strands appeared to be thickened, which was believed to be clusters of *v* bodies, and these early observations led to the description of DNA and protein particles to be like "particles on a string". These particles, which were then speculated to be composed of at least one—and perhaps two—of each histone protein, were also believed to be responsible for the significant packing of DNA into higher order structure. Early work delved into chromatin structure and determined that it is made up of repeating units, and that

these units are composed of histone proteins around which DNA is wound [18-22]. Chemical evidence suggested that approximately 140-160 base pairs of DNA were wound around each nucleosome particle, which is composed of a histone octamer, which is made up of two units each of histones H2A, H2B, H3 and H4 [23-26] (Figure 1).

Post-Translational Modifications and Consequences on Transcription

Projecting out from each of the core histones is a histone “tail”, a loosely structured protein domain containing a number of residues which can undergo post-translational modification (PTM). Research across many species has identified a number of these PTMs and found them to be critical for transcriptional regulation [27,28] (reviewed in [29,30]). Euchromatic regions are more loosely packed and are most evident in transcriptionally active interphase cells, although these regions can be seen throughout the cell cycle. Heterochromatic regions, on the other hand, are composed of tightly compressed nucleosomes and are generally found to coincide not only with gene-poor regions, but to also have patterns unique to cell lineage and differentiation status.

Generally, histone PTMs can be grouped into two classes: the addition of small chemical groups such as acetylation, phosphorylation and methylation, and the addition of large peptides, such as ubiquitination and sumoylation. How these changes affect the regulation of gene expression has been hypothesized to act through one or more of three distinct pathways. The first of which, operating in *cis*, is the theory that by affecting the physical structure of chromatin itself, these

modifications prevent critical contacts between proteins which are required to produce specific conformations or give rise to higher order chromatin structure surrounding given regions of DNA. This alteration of conformation would then lead to differential access by members of the transcriptional machinery and consequently differential gene expression. Both of the remaining two hypotheses suggest that the PTMs operate in *trans*. One hypothesis is that these modifications lead to a specific disruption of protein binding sites within the histones themselves, disallowing the appropriate control mechanisms to assert their influence. The opposite situation also holds true, that these alterations in histone proteins may themselves provide novel binding surfaces which may attract other effector proteins. It is this last mechanism of PTMs affecting gene regulation which has been best characterized to date.

Although the tails are composed of a number of different amino acid residues, lysine (K), serine (S), threonine (T), and arginine (R) are the predominately altered residues and thus ample research has been done into their significance in gene expression [29]. In general, acetylated lysine, phosphorylated serine, and methylated arginine are associated with transcriptional activation, while sumoylated lysine is indicative of repressed states. The methylation of lysine can promote transcription in some contexts (H3K4, H3K36, H3K79), while inhibiting it in others (H3K9, H3K27, H4K20) and also has the added complexity in that between one and three methyl moieties can be added to each amino acid. The chemistry of these modifications is well

understood, and several different protein complexes which catalyze such reactions have been identified [31].

Chromatin Modifying and Remodeling Complexes

The primary goals of chromatin remodelers are to package the genome after replication and to properly regulate access to the DNA for repair, recombination and gene transcription, while the main objectives of general modifying complexes are to appropriately mark nucleosomes for targeting by remodeling and transcriptional machinery. Together, these two classes of cellular machinery serve as the master regulators of gene expression and provide the cell with vast and dynamic opportunities for differentiation, adaptation and survival.

There are four major families of remodeler complexes, each of which requires ATP hydrolysis to drive changes in chromatin conformation and acts in different biological contexts [32-35]. The INO80 (inositol requiring 80) complex is primarily recruited to sites of DNA repair and is known to be involved in the replacement of canonical histones for rare variants [32]. Members of the ISWI (imitation switch) family typically shift nucleosome spacing to favor transcriptional repression [36-38], while members of the CHD (chromodomain helicase DNA binding) family acts in a context dependent manner, at times both favoring transcription and repression [33]. In contrast, members of the SWI/SNF family of remodelers, whose components have been shown to interact with the Bromodomain containing protein 2 (BRD2), has been noted to efficiently remove

nucleosomes from DNA, aiding in transcriptional progression [39,40]. Intriguingly, SWI/SNF family members also contain bromodomain motifs similar to those found in the Bromodomain and Extra Terminal domain (BET) family of proteins, which suggests that the two families may act in similar capacities to activate transcription.

Bromodomain Identification and Characterization

The bromodomain was originally identified in a *Drosophila* screen which aimed to highlight similarities between the *Drosophila* developmental regulator, *brahma (brm),* and the SNF2/SWI2 yeast transcriptional activator complex [41]. This comparison uncovered four highly conserved domains, including a 77 amino acid motif which was also found in the *Drosophila* homeotic gene *female sterile homeotic (fsh)*. Conserved among eukaryotes, the bromodomain module (BRD) has been identified in at least a dozen yeast proteins, and greater than 60 unique BRDs have been recognized in at least 46 human proteins [41,42].

As the only protein motif known to interact with ε-N-acetylated lysine residues, bromodomains are poised to be a critical 'reader' of epigenetic marks [41,43]. These conserved motifs, which were later defined as consisting of approximately 110 amino acids, bromodomains are contained within multiple classes of proteins, including histone acetyltransferases (HATs) such as GCN5 and PCAF [44], transcriptional regulators such as TAF1 [45], and the bromodomain and extra-terminal (BET) family of proteins [43]. Despite variations in amino acid sequence, all BRDs have a characteristic fold of four α-helices [46],

which flank the binding site for acetylated lysine residues [47,48] and each BRD appears to have a unique, context dependent binding affinity for specific residues, allowing for cooperation among bromodomains in binding to particularly modified nucleosomes [49-51]. These innate preferences in acetylated lysine binding, when coupled with the variety of protein classes which have bromodomain containing proteins, suggest that BRDs may be critical in interpreting the histone code and translating it into changes in transcription.

The BET (Bromodomain and Extra Terminal Domain) Protein Family

Members of the bromodomain and extra terminal domain (BET) family of proteins are characterized by tandem amino-terminal bromodomains linked to an extra-terminal (ET) domain at the C-terminus and have been implicated in the regulation of transcription in many different contexts [43]. In mouse and humans, there are four major BET proteins: BRD2, BRD3, BRD4, and BRDT [52-55], each of which is highly conserved between the two species. Research has demonstrated that each pair of bromodomains binds preferentially to its own set of histone modifications, and it is presumed that the ET domains may also confer unique binding sites for partner proteins [50,56,57].

Remarkably, the BET bromodomain has great potential as a target for therapeutic inhibitors, and several small molecule drugs have shown promise [58,59]. Benzodiazepines and benzotriazepines are drugs commonly used to regulate the function of gamma-amino butyric acid (GABA) receptors and are frequently used to treat a range of disorders including, seizures, sleep

disturbances, and anxiety. Structural data indicate that these classes of drugs may have significant interaction with BET bromodomains, providing a clinically approved drug as a starting point for the development of more potent inhibitors [58]. Similarly, the study of a small molecule bromodomain inhibitor, JQ1, demonstrated the compound's ability to compete with acetylated lysine residues for BRD binding in both BRD4-dependent cell lines and xenografts of human tumors with chromosomal rearrangements involving BRD4 [59]. As a result of this competitive binding disruption, the BRD4 fusion protein separates from chromatin, and changes in nuclear localization were demonstrated, suggesting that the protein's downstream transcriptional effects may also be altered [59]. Most recently, the same JQ1 compound has been used to target the bromodomains of the testis specific BET, BRDT, in mice as a form of male contraception [60]. Structurally, JQ1 was found to engage the BRD residues within the acetylated lysine binding pocket, and could competitively inhibit binding. *In vivo,* male mice were given daily intraperitoneal doses of JQ1 and assayed for markers of fertility: size of seminiferous tubules, sperm counts and motility, and changes in hormone levels. Remarkably, JQ1 was found to efficiently cross the blood-testis barrier, and its administration resulted in a significant and reversible reduction in fertility. However, as JQ1 binds to all BET BRDs with significant affinity, the molecule would need to be modified to prevent off-target effects which would be expected, given the critical roles other BET family members play in the cell. This series of experiments highlights the

importance of BET proteins in critical developmental pathways and emphasizes how they are attractive targets for pharmaceutical interventions.

BRD2 Orthologs in *Drosophila melanogaster* and *Saccharomyces cerevisiae*

In an early attempt to characterize genes in *Drosophila melanogaster* which were responsible for sex-linked development, X-linked chromosome mutations were induced by EMS mutagenesis, females were screened for reproductive phenotypes, and 95 individual mutants were isolated [61]. One of these mutants was termed *fs(1)h*, or *female sterile homeotic*, as the mutation had largely maternal and temperature sensitive effect on the offspring, resulting in the appearance of homeotic transformations of the bithorax and metathoracic structures [62]. From these studies, it was hypothesized that the origins of such abnormal developmental phenotypes were reliant on two separate components: the underlying genes responsible for development and the *trans* element which acted upon them, which was first proposed by Waddington nearly thirty years previously. From their fastidious genetic mapping studies, it was speculated that *fs(1)h* had the potential to be one such long range gene regulatory component.

Continuing studies in *Drosophila* refined the role of *fs(1)h* in development, showing that mutations in the zygotic gene caused either lethality or sterility, while maternal mutations were responsible for a number of novel transformations of the head and tail regions [63]. Additional work linking *fs(1)h*'s transcriptional regulation to the Ras signaling pathway supported the earlier theories that the

gene could act as a wide-range transcriptional activator, and further examination of different mutant alleles of *fsh* suggested that the protein may act in a modular fashion, with different effects seen when changes were made to only long or short isoforms. One of the next challenges came in determining the range of transcriptional targets which could be affected by this novel regulator. The homeotic gene *Ultrabiothorax* was one of the first transcriptional targets identified for the FSH-S protein, and its regulation was dependent on the presence of functional bromodomains [64].

Sequence analyses have shown that *fsh* had significant homology with two double bromodomain proteins in yeast, Bdf1 and Bdf2, which are an associated component of the TFIID complex in *cerevisiae* [65]. While their characterization suggested that these two proteins had overlapping and redundant roles in regulating RNA synthesis and meiotic division, they had distinctly different histone binding affinities [66]. Bdf1, which corresponds to the mammalian $TAF_{II}250$ subunit of TFIID, has a binding preference for acetylated H4, while the highly similar Bdf2 had no such binding affinity [65,67]. Mutants who lacked both proteins had significant growth defects, lending further evidence to the hypothesis that double bromodomain proteins are an essential part of underlying transcriptional mechanisms.

Identification of Mammalian *Brd2*

Originally identified in a T-cell library as RING3 (Really Interesting New Gene 3), through a chromosome walk of the human MHC region from 6p22.1 to

6p21.3, *Brd2* was found to have significant homology with the *Drosophila* regulator *fsh* and is considered an ortholog [55]. The gene itself spans approximately 10kb of DNA and is comprised of approximately 12 coding exons, which may be differentially spliced to give rise to alternate transcripts. The homologous mouse gene has been mapped to a similar MHC locus on chromosome 17qB1, and its gene structure and the resultant protein sequence are highly conserved between the species. *Brd2* gene expression is upregulated during proliferation in multiple cell lines, including proliferating endothelial cells, as well as in several tissues, including the brain, heart, kidney, liver and lung [68,69]. One study also suggested that BRD2 could autophosphorylate and may have nuclear kinase activities, although a presumptive catalytic residue for kinase activity was identified in the human protein (K547A), there are still a number of questions regarding its accuracy and specificity [70].

Mammalian BRD2 Protein Architecture

Murine BRD2 is a 798 amino acid protein with a predicted molecular mass of 110 kDa and is composed of three separate functional domains, the two N-terminal bromodomains (BD1 and BD2) and the carboxy-terminal extra-terminal (ET) domain (Figure 2). BRD2 shares greater than 80% homology in each domain with BET members BRD3 and BRD4, and greater than 70% homology with BRDT, suggesting that the proteins may serve similar, yet distinct, functions within the cell (Figure 3). Because of the significant homology BRD2 BD1 has with the bromodomain found in the histone acetyltransferase GCN5, it was the

initial focus for structural investigation. Through the initial characterization of BRD2's bromodomains, it was discovered that BD1 forms a dimer, with two accessible acetylated lysine binding pockets and forming an additional presumptive binding pocket at the dimer interface [71]. Further characterization suggested that BD1 alone is sufficient to bind to full-length BRD2 both *in vitro* and *in vivo,* and a number of residues which are critical for the maintenance of this interaction were identified, including the point mutations Q78A, Y153K, E170A, L174E, V177E, and the double mutant M142A/Q143A. Additional mutants were shown to weaken the dimerization interaction, in support of the theories suggested by the structural analyses.

The crystal structures of the second bromodomain of human BRD2 complex with a histone H4 tail peptide which is acetylated at both K5 and K12, with different modules of the dimer seeing each independent modification [48,72]. Similarly, the N-terminal bromodomain of human BRD2 recognizes both H4K5ac and H4K12ac independently and in combination, and it has been noted that the direction of the BRD2 protein backbone binding to the residues is antiparallel to GCN5's backbone, such that it is possible that both proteins could retain the ability to interact with the same histone tail simultaneously [47]. While it is known that both proteins interact with modified histones, their interaction with each other does not appear to be dependent upon the BRD2 bromodomains, as will be discussed in detail in Chapter 3. Interestingly, H4K8 and H4K16, in addition to H3K9 and H3K14, are targets of GCN5's histone acetyltransferase activity,

suggesting that BRD2 target loci are not dependent upon this particular HAT activity (Figure 4).

While much investigation has been done on the structure and the function of both BD1 and BD2, there is little direct evidence for the function and structure of the BRD2 ET domain. Structural predictive software does not readily identify the ET domain as having any specific secondary conformation, and thus far the crystal structure has not been solved. Because of this non-specific nature of the ET domain and the overall variability in the carboxy-terminal tails of the BET family members, it has been hypothesized that the ET domain may act in a scaffolding capacity, bridging interactions between specifically marked histone residues and proteins of other chromatin remodeling or transcriptional machinery.

BRD2 Interacts with Chromatin Remodeling and Transcriptional Machinery

Through developing an understanding of the dynamic protein complexes which contain bromodomain-containing proteins, a more complete picture of the mechanisms underlying gene regulation can be drawn. Denis and colleagues, using IP-Mass spec, identified a number of protein interactions in 293T cells including each of the core histones, components of Swi/Snf and E2Fs, highlighting the significance BRD2 plays in the coordination of large multi-protein complexes in transcription (Table 1) [40,73]. Additionally, E2F proteins were identified in BRD2/RING3 containing complexes isolated from HeLa cell nuclear extracts, and this interaction was dependent upon the 93 amino acid residues at the carboxy terminus of the protein [70]. This suggests that BRD2 interacts with

E2Fs transcription factors through its ET domain, lending evidence to the hypothesis that BRD2 acts as a scaffolding protein, using the ET domain to bridge many of the protein-protein interactions. Supporting data from neuronal cell cycle studies suggest that *Brd2* is required for neuronal E2F1 pathway dependent differentiation in the mouse, putting a developmental perspective on the protein interaction [74].

BET proteins are also known to interact with each other in a cellular context. For example, BRD2 and BRD7 demonstrably interact through the C-terminal end of BRD2, and colocalize in COS7 cells [75]. Given BRD7's ability to bind to histones in addition to BRCA1 [76], reports of BRD2 and BRD4 colocalization [77], and the presence of both BRD2 and BRD3 at specific loci [39], there are seemingly endless combinations in which BET family members could guide transcription on a temporal and tissue restricted basis. Additional interactions between BRD2 and the latency-associated nuclear antigen 1 (LANA-1) of the Kaposi's sarcoma associated herpesvirus and the mouse gammaherpesvirus 68 (MHV-68) provide additional insight into the universal interactions BRD2 may mediate [78,79].

BRD2 in Transcriptional Activation

There is increasing evidence that BRD2 plays a strong role in transcriptional regulation. Localization to the nucleus during proliferation and preferential binding to active histone modifications suggests a role in transcriptional activation rather than repression. Association with components of

chromatin remodeling machinery and RNA polymerase components strengthens this argument, but the lack of direct target genes makes it difficult to form definitive conclusions. One of the few pieces of evidence of BRD2 directly influencing gene expression comes from a study of the cyclin A promoter. As a gene intimately linked to the cell cycle, cyclin A is under both negative regulation by promoter-bound RB family members and positive control of E2Fs. In one study, the overexpression of BRD2 coupled with the ectopic expression of ras resulted in significantly increased *de novo* protein levels of cyclin A, as visualized by autoradiography of radiolabeled protein extracts [80]. Further analysis by chromatin immunoprecipitation revealed the presence of BRD2 at the cyclin A promoter, as well as clear evidence of an associated HAT activity, although no specific HAT could be identified.

Building on the evidence that BRD2 is bound to acetylated histones, additional studies using FLAG-tagged BRD2 and BRD3 constructs have demonstrated that it is bound specifically to genes which are actively transcribed, rather than to areas which are not transcriptionally active but still retain these chromatin marks. By analyzing extracts from cultured 293 cells expressing tagged BRD2 and BRD3 protein, it was shown that these BET proteins were preferentially bound to transcribed genes encoding *geminin*, *β-actin*, *cyclin D1*, *RPL7* and *RPS28*, but were not found bound to DNA containing the transcriptionally repressed *β-globin* or *BMP4* [39]. Attempts to determine the relative abundance of BRD2 in the promoter and coding regions of *cyclin D1* was inconclusive; BRD2 protein was found deposited along the entire length of

the gene, and the low recovery of the presumably nucleosome-depleted promoter region made a direct comparison unreliable[39]. Interestingly, this study did determine that the hyperacetylated chromatin to which BET proteins preferentially bind is not restricted to the promoter and can be found along the length of the gene body, suggesting a possible role of BET proteins not only in transcriptional initiation but also in progression. Other studies support this theory that BRD2, in some instances, is responsible for post-initiation transcriptional progression. Using a Tet-On/Tet-Off system with a fluorescently labeled transcriptional activator, the order of protein recruitment to the transcription start site in a human derived osteosarcoma cell line could be monitored with single cell precision [77]. BRD2 recruitment was dependent on intact acetyl-lysine binding motifs within the bromodomains and lagged behind that of BRD4 and RNA Pol II, suggesting that hyperacetylation of chromatin is required for BRD2 binding after transcription initiation. Taken together, these studies implicate several direct, complementary, roles for BRD2 in mammalian transcription.

Subcellular Localization of BRD2

As a chromatin binding protein involved in transcriptional regulation during proliferation, BRD2 is expected to localize to the nucleus. In cultures of exponentially growing HeLa cells, human BRD2 protein is consistently found in the nuclear compartment, both by cell fractionation and by immunolocalization [81]. In contrast, BRD2 shows a distinct diffuse and peri-nuclear localization pattern throughout the cell in serum starved NIH3T3 fibroblasts, suggesting that

the mitogenic signals in the serum could induce the protein's nuclear translocation [82]. Further analysis using the carboxy terminal portion of BRD2 tagged with GFP revealed that BRD2's mitogenically stimulated shift to a predominately nuclear localization was dependent upon a presumptive nuclear localization signal (NLS), located between the bromodomains and the ET domain, while mutations which inhibited the protein's presumptive kinase activity (K574A) had no effect on subcellular localization. This work provided an interesting insight into the role BRD2 may play in the development of human cancers as possibly a master switch controlling a number of cell cycle and pro-growth genes.

Despite strong evidence of changes in subcellular localization of BRD2, it was not known if it would be reflected in the intact animal. *Brd2* mRNA transcripts had been readily detected in a number of mouse tissues, including high expression in the developing embryonic neural tissues and in the adult gonads [83]. Given the strong association of nuclear BRD2 protein with cells in active growth phases, it was hypothesized that BRD2's localization may change upon terminal differentiation, as seen during neuronal differentiation. To investigate this, the subcellular localization of BRD2 was determined at several time points throughout embryonic development, both before and after precursor cells exited the cell cycle upon differentiation [84]. Immunostaining of thin prep sections of mouse embryo clearly demonstrated that in regions where neural tissue was proliferating, such as the E9.0 neural tube and E11.5 dorsal root ganglia, and interneuron precursors, BRD2 remained largely in the nucleus. However, later in

development, the localization of BRD2 shifted to the cytoplasm, as the cells differentiated into motor, sensory, and interneurons within the spinal cord. This evidence further strengthens the belief that BRD2's most significant contribution to the cell is to mediate the transcription of a subset of growth related genes. The potential role of BRD2 in non-dividing cells has not yet been identified.

The study of the localization of BRD2 in mouse mammary epithelium, which undergoes significant physiological and transcriptional changes during pregnancy and lactation, would yield significant results. There is a notable increase of *Brd2* mRNA throughout the mammary epithelial layer during pregnancy and lactation, which suggests that BRD2 is imported into the nucleus in a regulated fashion [85]. Correspondingly, BRD2 staining of euchromatin is significant in these transcriptionally active nuclei.

In each of these scenarios—the *in vitro* serum starvation, the differentiation of embryonic neurons, and in the hormonally stimulated mammary ducts— BRD2 protein localizes to the nucleus during the growth phase and loses such localization when the cells dip into states of lower transcriptional activity. Whether this shift is due to post translational modifications of the BRD2 protein itself, BRD2's ability to find and bind to other protein complexes, or the specific affinity of BRD2's bromodomain for chromatin motifs remains unclear, and understanding the mechanisms which govern the protein's behavior could yield significant insight into how chromatin associated complexes such as remodelers and modifiers are recruited to specific sites throughout the cell cycle.

BRD2 in Development

A protein essential for mammalian development [86], BRD2 is well conserved among species, and its orthologous *Drosophila* protein, female sterile homeotic 1 (*fsh1*), is also required for successful embryogenesis [61,63,64]. *Brd2*'s orthologs in yeast, Bdf1 and Bdf2, have also been shown to have critical roles in properly interpreting histone modifications, as well as guiding transcription by associating with the general transcription factors in TFIID [65,67,87]. Structural studies have determined that human BRD2, which has greater than 90% overall homology to murine BRD2 and shares identical bromodomains, binds preferentially to acetylated histone H4 at K5 and K12 specifically [47,48,71].

Although it was initially identified in the mouse as an atypical nuclear kinase, BRD2 acts as an integrator by providing bridging interactions among acetylated histones and components of Mediator, Swi/Snf and TFIID complexes, as well as E2F proteins [40,70,80,85]. Studies of the expression pattern of *Brd2* have shown that it is widely expressed during development as well as in the adult animal, but it has the highest levels of expression in the developing neural tissue during embryogenesis and in the adult gonads [83,86,88]. BRD2's essential role in embryogenesis was made clear by several studies which demonstrated its requirement for proper neural tube closure and overall embryonic growth [74,86,89], as embryos lacking BRD2 protein suffer from significant developmental delay and hindbrain exencephaly, which will be discussed in further detail in Chapter 2.

Roles for *Brd2* in Mammalian Disease

The most confounding aspect of human genetic research is the vast amount of conflicting data when multiple genes are believed to influence disease states and incomplete penetrance is commonplace. Positive associations between genotype and phenotype can be indicative of gene involvement, but without knowing the etiology of the disease in question, there is ample room for speculation and discord among researchers and clinicians alike. Patients in whom the genetic lesion is present but the disease phenotype is not may erroneously detract from the significance of the gene-disease association, as these patients may have other protective genetic, environmental or developmental aspects which prevent full disease manifestation.

Idiopathic generalized epilepsy (IGE) is a complex group of epilepsy disorders characterized by a lack of anatomical abnormalities and a significant genetic component. In humans, *BRD2* promoter mutations have been intimately linked to an increased susceptibility to the seizure disorders juvenile myoclonic epilepsy and photoparoxysmal response [90-92]. Patients with such a diagnosis display myriad clinical phenotypes and variable responses to medical intervention, and further understanding of the genetic components behind the disorder will hopefully lead to personalized, genetics-backed, tailored approaches to both disease-risk assessment and pharmaceutical intervention.

More significantly, the overexpression of BRD2 in murine lymphoid cells has been shown to cause B-cell lymphoma and leukemia [93,94], lending further credibility to the argument that BRD2 is an indispensable regulator of the

pathways responsible for appropriate growth in both embryonic and adult animals. Additionally, recent human genetic studies have suggested a role for differential levels of *Brd2* expression in men with defects in spermatogenesis [95], and a corresponding use of BET inhibitors in the mouse has shown promise as a form of male contraception [60].

While the complete absence of BRD2 protein on mouse development is discussed in detail in Chapter 2, additional findings from a mouse line with low *Brd2* expression have implicated the gene in the development of obesity and insulin resistance. In this study, mice with hypomorphic alleles of *Brd2* rapidly became obese, but had remarkably enhanced levels of glucose tolerance, low blood glucose levels. In a striking deviation from standard animal models of obesity, these mice did not develop type II diabetes, leading to speculation that BRD2 may play a role linking insulin resistance to the inflammatory response [96-98] and direct critical chromatin and transcriptional regulation key to adipogenesis [99].

Identifying Roles of BRD2 in Development and Chromatin Remodeling

Many components of the molecular machinery which regulates gene expression have been identified and characterized, including the BET family of proteins and the chromatin-associated protein BRD2. The significant impact that subtle changes in histone post-translational modification has on cell fate has been well established, and the histone code hypothesis of interrelated modification “writers” and “readers” has gained widespread acceptance.

However clear these hypotheses may be, the underlying reality is that the molecular mechanisms of such control are not yet well understood, and no direct interactions between chromatin modifying readers and writers have yet been characterized in a developmental context.

One common objective of research in both chromatin biology and developmental genetics is to understand how these simple patterns of histone modifications, in combination with subsets of chromatin regulatory proteins, affect transcription with exceptional precision. By studying the role of BRD2 in the context of the mouse embryo, we hope to be able to identify specific gene regulatory networks affected by the protein itself and how these alterations affect the overall development of the organism. In contrast, by identifying additional interacting components and pathways both *in vivo* and in cell culture based assays, we will be building a more comprehensive understanding of the underlying molecular interactions chromatin readers. Taken together, our *in vivo* and cell culture studies will provide unique insights into how the combinatorial complexity of histone modifications and chromatin binding proteins guides transcriptional regulatory networks in the developing mouse embryo.

Research Aims

Given the global expression pattern of murine *Brd2*, we hypothesize that it plays a critical role in embryogenesis. The tandem bromodomain motifs and demonstrated binding preferences for acetylated histone H4 lead us to additionally hypothesize that BRD2 acts to read histone marks and recruit additional members of the chromatin remodeling or transcriptional machinery to given loci. As such, this thesis aims to develop a better understanding of the genetic and biochemical roles BRD2 plays in mammalian development. The two main methods utilized in this work are the characterization of mouse line containing a null allele of *Brd2* to address the role of the protein throughout embryonic development and the use of *in vitro* techniques to identify functional significance of individual protein domains.

<u>Research Aims:</u>

1) Characterize the global role of BRD2 in mouse development
2) Identify BRD2-dependent transcriptional networks *in vivo*
3) Characterize the *in vivo* and *in vitro* interactions between BRD2 and the histone acetyltransferase GCN5

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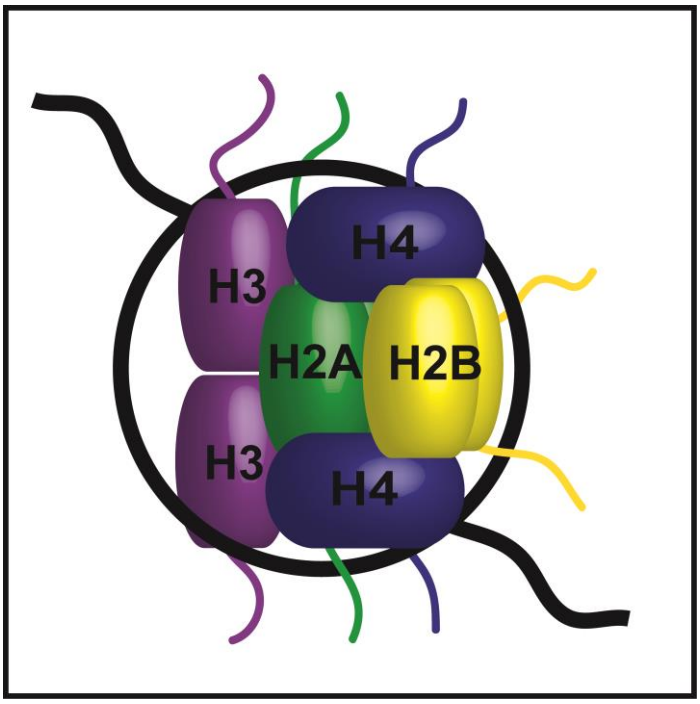

Figure 1: Schematic diagram illustrating nucleosome structure
A schematic diagram of histone octamer organization, including associated DNA (black line). Unstructured histone tails extend from the nucleosome core, which consists of globular protein domains.

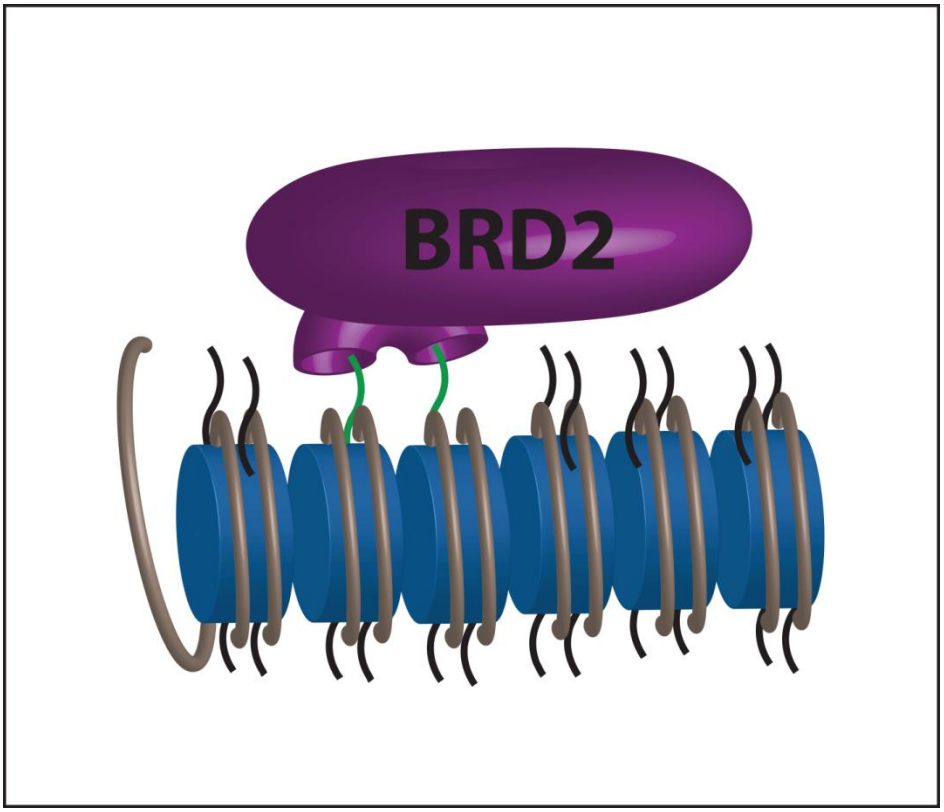

Figure 2: Schematic diagram illustrating BRD2 protein structure
A schematic diagram of BRD2 protein (purple) and associated chromatin template (blue) and DNA (grey). Unstructured histone tails extend from the nucleosome cores (black), while acetylated H4K5 and H4K12 tails are represented by green.

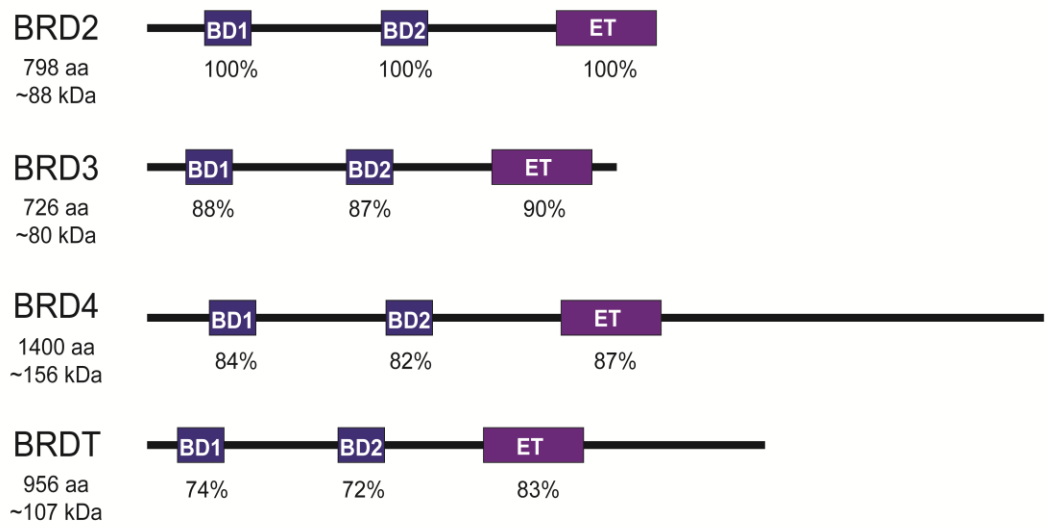

Figure 3: The domain structure of murine BET proteins

The BET protein family is characterized by dual N-terminal bromodomains (blue) and a C-terminal extra-terminal (ET) domain (purple). Percentage homology of each protein relative to BRD2, is noted below each structural domain.

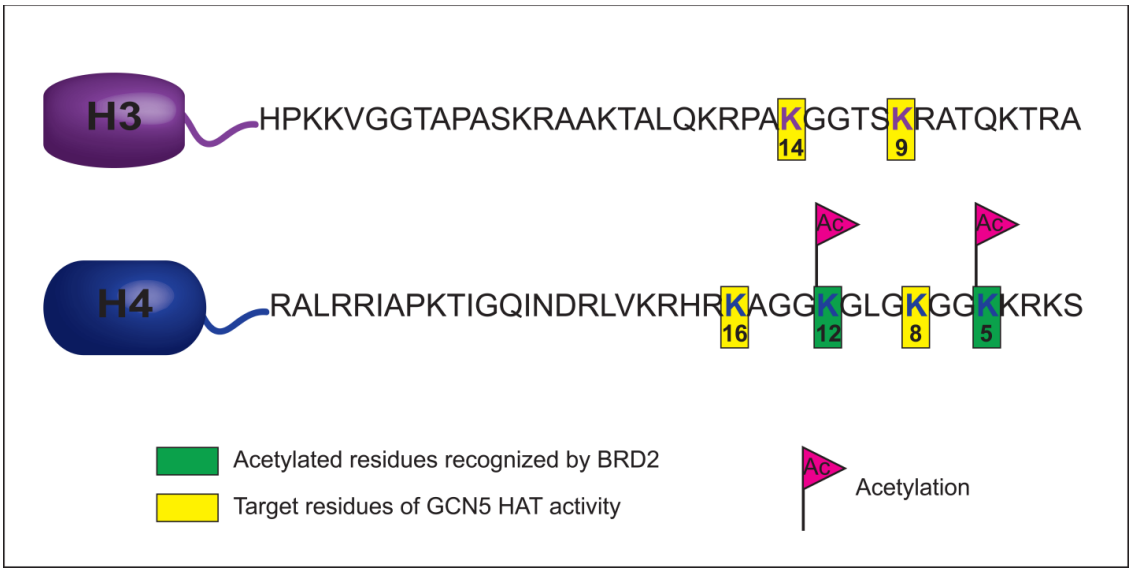

Figure 4: Sites of histone tail modifications

The amino-terminal tails of histones H3 and H4 are illustrated to show relevant covalent modification sites. Residues recognized by BRD2 and modified by GCN5 are as identified.

Table 1: BRD2 Interacting Proteins	
Chromatin Components	Histone H2A
	Histone H2B
	Histone H3
	Histone H4
	CAF1- β (Chromatin assembly factor 1 β)
	NAP1 (Nucleosome assembly protein 1)
Chromatin Remodeling	BAF155 (BRG1-associated factor, Swi/Snf p155)
	ARID1A (Swi/Snf p270)
	BRG1 (brahma, Snf2β)
	HDAC11 (Histone deacetylase 11)
Coactivating Complexes	MED1 (CRSP1, TRAP220)
	MED6 (Mediator subunit 6)
	CDK8 (Cyclin dependent kinase 8)
Transcriptional Regulators	TAF1 (TAFII250)
	TAF7 (TAFII55)
	BTAF1 (TAFII170)
	TBP (TATA-box Binding Protein)
	RNA Polymerase β
	E2F-1 (RPB3, transcription factor)

Chapter 2:
The Chromatin-Targeting Protein BRD2 Is Required for Neural Tube Closure and Embryogenesis

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The chromatin-targeting protein BRD2 is required for neural tube closure and embryogenesis

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Abstract

Chromatin modifications are essential for directing transcription during embryonic development. Bromodomain-containing protein 2 (Brd2; also called RING3 and Fsrg1) is one of four BET (bromodomain and extra-terminal domain) family members known to selectively bind acetylated histones H3 and H4. Brd2 associates with multiple subunits of the transcriptional apparatus including the mediator, TFIID and Swi/Snf multiprotein complexes. While molecular interactions of Brd2 are known, the functions of Brd2 in mammalian embryogenesis remain unknown. In developing a mouse model deficient in Brd2, we find that Brd2 is required for the completion of embryogenesis and proper neural tube closure during development. Embryos lacking *Brd2* expression survive up to embryonic day 13.5, soon after mid-gestation, and display fully penetrant neurulation defects that largely result in exencephaly of the developing hindbrain. In this study, we find that highest expression of *Brd2* is detected in the developing neural tube, correlating with the neural tube defects found in Brd2-null embryos. Additionally, embryos lacking *Brd2* expression display altered gene expression programs, including the mis-expression of multiple genes known to guide neuronal development. Together these results implicate essential roles for Brd2 as a critical integrator of chromatin structure and transcription during mammalian embryogenesis and neurogenesis.

1. Introduction

Bromodomains are conserved chromatin-targeting modules found in many eukaryotic transcriptional regulatory proteins and have been shown to bind specifically to acetylated histones H3 and H4 [1-5]. Brd2 belongs to the BET subfamily of bromodomain proteins that contain two tandem N-terminal bromodomains (B) and a single C-terminal extra-terminal (ET) domain [6]. Epigenetic modifications of chromatin structure, such as histone acetylation and methylation, are known to have important consequences in the regulation of gene transcription [7]. Therefore, understanding the roles of murine Brd2 in interpreting combinatorial histone modification of chromatin is a critical part of investigating the regulation of transcription during mammalian development.

Brd2 structure is conserved among plants, animals and fungi [6]. The yeast orthologs of Brd2, TFIID-associated components bromodomain factors 1 and 2 (Bdf1 and Bdf2), have been shown to be required for anti-silencing functions at subtelomeric regions of the yeast genome and for correctly interpreting histone modifications [2,8,9]. In *Drosophila*, Brd2 is most closely related to female sterile homeotic 1 (*fsh1*), a trithorax-group gene required for proper gene expression and fly embryogenesis [10-12]. Three of the four mammalian BET proteins—Brd2, Brd3 and Brd4— are broadly expressed, while the fourth, Brdt, is selectively expressed in the germline [13,14]. In the mouse, the ubiquitously expressed Brd2 has the highest levels of expression during embryogenesis as well as in the adult testis, ovary and brain [13-16]. Brd2 was initially identified as a nuclear kinase in human cells that is involved in guiding the

expression of cell cycle genes through its binding to multiple E2Fs [17-19]. In addition, Brd2 has been shown to be associated with several multiprotein regulators of transcription, including the mediator, TFIID, and Swi/Snf complexes [16,20]. These widespread interactions implicate Brd2 in targeting critical components of the transcriptional machinery to precisely modified regions of the eukaryotic genome.

While distinct interactions and expression patterns of the mammalian BET proteins have been described, little is known about the potential function of Brd2 in normal mammalian development. Disruption of the Brd2-related paralog, Brd4, in the mouse leads to early post-implantation lethality *in vivo* and an inability to maintain the inner cell mass *in vitro* [21]. Recently, a single bromodomain of the testis-specific Brdt has been shown to be required for male germ cell differentiation [22]. These studies suggest that although the basic structure of related BET family members is conserved, their expression patterns and functions in mammalian development are diverse. A number of studies in mammalian cells have implicated Brd2 function in the positive control of cell proliferation. Brd2 has been shown to bind several E2F cell cycle transcriptional activators and its exogenous expression was shown to help activate the cyclin A promoter [18,19]. Moreover, specific over-expression of *Brd2* in the lymphoid lineage was shown to result in B cell lymphoma and leukemia [23]. Several studies have documented the nuclear accumulation of Brd2 during diverse proliferation events in cultured cells and in neural and reproductive tissues *in vivo* [16,23,24]. In an embryonic development study of the mouse, expression of *Brd2*

mRNA peaked between E8.5 and E12.5 and was prominently detected in the developing CNS [24]. In humans, mutations in the promoter of the *BRD2* gene have been linked to increased susceptibility to juvenile myoclonic epilepsy (JME), an adolescent-onset generalized epilepsy [25]. *BRD2* has also been genetically linked to photoparoxysmal response (PPR), a related seizure disorder in humans [26].

Given the current known biochemical functions of Brd2 and its potential role in neural development and disease, we disrupted the *Brd2* gene in the mouse to investigate its biological function during mammalian development. Here, we show that Brd2-deficient embryos deviate from normal developmental programs at embryonic day 9.0 (E9.0), when they exhibit delayed development, later growth retardation and fail to survive after E13.5. Strikingly, as neural development progresses, Brd2-null embryos consistently manifest neural tube closure defects that most commonly appear as exencephaly of the hindbrain. Moreover, deregulation of transcription at E9.0 may underlie the developmental defects observed in the Brd2-null embryos before they become apparent. Together, these data indicate essential roles for Brd2 in regulating chromatin structure and transcription during mammalian development.

2. Materials and methods

2.1. ES cells and generation of Brd2-null mice

The ES cells RREO50, which carry a gene-trap construct in between the first and second coding exons of *Brd2* (BayGenomics), were grown on mitotically inactive feeder layers until 90% confluent and were dissociated by trypsinization before injection. E3.5 blastocysts were derived from C57BL/6-Tyr$^{c\text{-}Brd}$ female mice and injected with 12–20 ES cells. The injected blastocysts were implanted into the uteri of day 2.5 pseudo-pregnant females, with eight to ten embryos implanted per uterine horn. The resulting male chimeras were mated with C57BL/6-Tyr$^{c\text{-}Brd}$ females to obtain F1 progeny. The strain carrying the germline transmitted allele (named *Brd2*^{Gt1RFr} and hereafter referred to as minus allele) was maintained on a mixed C57BL/6-129Ola background. Heterozygous animals were intercrossed to produce *Brd2*$^{+/+}$, *Brd2*$^{+/-}$ and *Brd2*$^{-/-}$ embryos. A second gene-trapped ES cell line from EUCOMM (OTTMUSG00000017279) was used to derive a second disrupted *Brd2* mouse line (named *Brd2*^{Gt2RFr} and hereafter referred to as the *LacZ* allele). All breeding and procedures were carried out according to institutional regulations at Brown University Animal Facility and NIH Guide for the Use and Care of Laboratory Animals.

2.2. PCR genotyping

To differentiate wild type (+) from mutant (−) alleles of *Brd2*, a 551 bp amplicon of the mutant allele was PCR-amplified using Brd2For5-

GTTCCCTGAGGTCAAGATGCTG and βGalRev7-ACCCCTTCCTCCTACATAGTTGGC and subsequently sequenced to identify the junction between the endogenous sequence and the insertion cassette. This analysis uncovered that 417 bp at the 5′-end of the gene trap was lost when it incorporated into the genome. However, the splice acceptor essential for the function of this disruption vector was retained. To identify the wild type allele, a pair of primers, Brd2For1-GCTGAGCGGCGGCGGTTCCC proximal to Brd2For5 and Brd2IntRev93-CGGAACGCCGCCCCCCAACC downstream of the insertion cassette junction, were used to generate an amplification product of 106 bp. When resolved on 2% agarose-TAE gels stained with ethidium bromide, these two PCR products allowed us to identify genomic DNA of all three genotypes. Genomic DNA was isolated from either adult mouse-tail biopsies or yolk sacs of developing embryos using the DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions.

2.3. Embryo dissection and RNA preparation

Female mice of *Brd2* heterozygous intercrosses were checked every morning for the presence of a copulation plug. The day on which the copulation plug was observed was designated as E0.5. Dissections took place on subsequent days of development. Females were sacrificed and the uterine horns were excised and placed in sterile PBS. Decidual swellings corresponding to embryos were dissected individually, and yolk sacs retained for PCR genotyping. Resultant embryos were imaged using a Zeiss Discovery V8 stereomicroscope

equipped with an Axiocam MRc camera and Axiovert software. Immediately after imaging, embryos were dounced in Trizol (Invitrogen) to preserve RNA quality, and RNA was isolated using manufacturer's protocols.

2.4. Microarray

Total RNA from nine E9.0 embryos (3 *Brd2*$^{+/+}$, 3 *Brd2*$^{+/-}$, 3 *Brd2*$^{-/-}$) was obtained as described above and further purified using micro RNeasy columns (Qiagen). RNA quality was checked using a Bioanalyzer, and concentration determined using a Nanodrop. 100 ng of each RNA sample were used in the Affymetrix Whole-transcript Sense Target Labeling Assay (Rev 3) followed by hybridization to a GeneChip® Mouse Gene 1.0 ST Array. Nine GeneChips were used to provide biological triplicates of each genotype. The Affymetrix Expression Console (v 1.1) was used to normalize data and determine signal intensity (RMA-Sketch). Analysis was performed in Microsoft Excel. Transcripts with two-fold or greater changes in *Brd2*$^{-/-}$ embryos are reported in Supplemental Table 1.

2.5. Quantitative RT-PCR

RNA was isolated from E9.5 embryos collected from timed heterozygous matings as described earlier. RNA concentrations were determined by Nanodrop (Thermo Scientific), and 0.5 to 1 µg of total RNA was used to prepare 20 µl of cDNA using the iScript Select kit (BioRad). Real-time PCR reactions were performed in triplicate using 1 µl of cDNA template, SYBR green PCR master-mix (ABI) and gene-specific primers for *Brd2*, *Med26*, *Brachyury*, *NeuroD1*,

NeuroD4, *Olig3*, *SlitRK6*, and *18S* rRNA (Invitrogen) in the ABI 7300 Real Time PCR System, according to manufacturers' protocols. Primer sequences can be found in Supplemental Table 2. Relative mRNA expression levels were determined using ΔCt values and were normalized to *18S* rRNA levels to correct for minor variations in starting RNA concentrations.

2.6. β-galactosidase, phosphorylated-H3 and TUNEL staining

β-galactosidase staining of whole E13.0 embryos was performed using standard protocols. Freshly harvested embryos were fixed for 20 min at room temperature in 0.2% glutaraldehyde, washed three times in 0.1 M phosphate buffer and incubated in 1 mg/ml X-gal (Invitrogen) overnight at room temperature. For immunostaining and TUNEL, freshly harvested embryos were fixed overnight at 4 °C in 4% PFA, cryopreserved through serial incubation in 15% and 30% sucrose at 4 °C, frozen in OCT blocks in liquid nitrogen and sectioned at 10 μm. For P-H3 antibody staining, slides were blocked for 1–2 h in 10% goat serum and 10% BSA in PBT. Slides were then incubated at 1:100 dilution anti-P-H3 antibody (Cell Signaling, 9701S) in 10% goat serum, 10% BSA in PBT at 4 °C overnight. The slides were washed three times in PBS at room temperature and incubated with a 1:100 dilution of goat anti-rabbit Alexa Fluor 594 (Molecular Probes, A11012) in 10% goat serum, 10% BSA in PBT for 2 h at room temperature. For fluorescent analysis, the slides were washed three times in PBS, mounted with Vectashield plus DAPI (Vector Labs) and imaged on a Zeiss ImagerM1 fluorescence microscope using Axiovision software. For TUNEL

staining, a Fluorescence In-Situ Cell Death Detection Kit (Roche) was utilized. Embryo sections were permeabilized for 6 min on ice in 0.1% Sodium Citrate, 0.1% Triton X-100 and rinsed twice in PBS. TUNEL substrate was mixed according to the manufacturer's protocol (Roche). Slides were covered, placed in dark, humidified chambers and incubated at 37 °C for 1 h, rinsed three times in PBS, mounted with Vectashield plus DAPI (Vector Labs) and imaged on a Zeiss ImagerM1 fluorescence microscope.

3. Results

3.1. Gene-trap-mediated disruptions of the mouse *Brd2* gene

To ascertain the functions of Brd2 in mouse development, a heterozygous embryonic stem (ES) cell line (RRE050) with a gene-trap vector insertion in between the first two coding exons (Fig. 1A) of the mouse *Brd2* gene was obtained from BayGenomics [27,28]. The β-geo cassette of the gene-trap cassette contains a splice acceptor site which functions with the splice donor site of coding exon 1 of *Brd2* to produce a truncated *Brd2* transcript. This ES cell line was used to derive founder *Brd2* heterozygous (*Brd2*$^{+/-}$) mice. Initial genotyping of germline-transmitting founder *Brd2*$^{+/-}$ intercross progeny yielded viable wild type (*Brd*$^{+/+}$) and heterozygous (*Brd2*$^{+/-}$) progeny. However, intercrosses of heterozygous *Brd2* males and females failed to produce any *Brd2*$^{-/-}$ mice at weaning age (Fig. 1B). Thus, this disruption of *Brd2* results in apparent embryonic or early postnatal lethality. Interestingly, the numbers of *Brd2*$^{+/-}$ progeny at weaning were lower than the expected Mendelian ratio of 2:1 (Fig. 1B, $p < 0.0001$). Therefore, having only a single functional copy of *Brd2* may have some deleterious effects on embryonic development, resulting in partially penetrant haploinsufficiency of heterozygous offspring.

3.2. Disruption of *Brd2* expression leads to embryonic lethality

The lack of viable *Brd2*$^{-/-}$ offspring suggested that Brd2 is required to complete embryogenesis. To characterize the role of Brd2 during

embryogenesis, timed matings of *Brd2*$^{+/-}$ males and females were used to recover embryos across multiple developmental time points. PCR genotyping of yolk sac derived genomic DNA reliably detected embryos of all three *Brd2* genotypes in litters ranging from E8.5 to E13.5 from heterozygous intercrosses. To verify *Brd2* disruption by the insertion of the gene-trap vector, quantitative RT-PCR (qPCR) was used to detect and quantify transcripts of *Brd2* and the β-galactosidase transgene in E9.5 in littermate embryos of all three *Brd2* genotypes. *Brd2* mRNA was undetectable in *Brd2*$^{-/-}$ embryos, while the relative *Brd2* mRNA level in *Brd2*$^{+/+}$ embryos was approximately two-fold higher than in *Brd2*$^{+/-}$ embryos (Fig. 1C). Accordingly, *Brd2*$^{-/-}$ embryos were shown to have approximately two-fold higher expression of the β-galactosidase transgene relative to the *Brd2*$^{+/-}$ embryos. *Brd2* mRNA levels were normalized to *18S* rRNA levels to account for slight variation in total RNA yield from each individual embryo, and these experiments were repeated on multiple litters (data not shown). To assess the relative time of lethality, the progeny of multiple timed heterozygous intercrosses from E8.5 to E13.5 were recovered and genotyped. Of the 166 embryos recovered, 38 *Brd2*$^{-/-}$ embryos (23%) were identified between E8.5 and E11.5. In contrast, only 5 *Brd2*$^{-/-}$ embryos from a total of 59 recovered between E12 and E13 have been detected (8%; Table 1). At developmental stages E12 and later, uterine evidence of embryonic lethality was observed, which likely represents resorption of non-viable *Brd2*$^{-/-}$ embryos. These data indicate that only a fraction of the *Brd2*$^{-/-}$ embryos progress past E12, with a

continuum of embryonic lethality occurring through several days (E8.5 to E11.5) during mid-gestation.

3.3. Developmental delay and neural tube closure defects of Brd2-null embryos

Phenotypic analysis of multiple embryonic litters derived from *Brd2* heterozygous intercrosses revealed fully penetrant severe growth retardation and defects in neural tube closure for *Brd2*$^{-/-}$ embryos compared to matched wild type littermates. Representative whole embryo images of wild type and *Brd2*$^{-/-}$ littermates from E9.0 to E13.5 are shown (Fig. 2). Comparison of wild type and *Brd2*$^{-/-}$ embryos reveals a reduction in the overall size of the *Brd2*$^{-/-}$ embryos. Severe growth retardation and developmental delay are most obvious at E9.0, when some *Brd2*$^{-/-}$ embryos are significantly smaller than their wild type counterparts and have not undergone axial rotation (Fig. 2A and C). This lag in development was observed in 60% of Brd2-null embryos recovered at this early time point. Since most *Brd2*$^{-/-}$ embryos fail to develop past E12 (Table 1), it is likely that this severe developmental delay has lethal consequences for most embryos that fail to express *Brd2*. As development proceeds, all of the *Brd2*$^{-/-}$ embryos display cranial defects in neural tube closure (Fig. 2E–P). Most *Brd2*$^{-/-}$ embryos do complete axial rotation, and the most common neurodevelopmental defect observed is exencephaly in the developing hindbrain region (Fig. 3). The neural folds in the rhombencephalon are large, thickened, and splayed open to the outside of the embryo (Fig. 2, M–P and Fig. 3). Additionally, multiple *Brd2*$^{-/-}$

embryos display a curved caudal neural tube with frequent openings within the neural region (Fig. 4). In contrast, other aspects of development such as limb, heart and somite development appear relatively intact in the older *Brd2*$^{-/-}$ embryos (Fig. 2, M–P). The neural tube defects of the *Brd2*$^{-/-}$ embryos are fully penetrant, as a *Brd2*$^{-/-}$ embryo with proper neurulation (n = 43) has not been detected; however, the defects are pleiotropic in nature. In addition, several heterozygous *Brd2*$^{+/-}$ embryos have been identified with similar neural tube defects, although variable in nature (Supplemental Fig. 1). Taken together, these data indicate that Brd2 function is necessary for proper neural tube closure during embryonic development and suggest that Brd2 may play an essential role in the regional specification of the developing rhombencephalon.

3.4. High expression of *Brd2* in the developing neural tube

To address the localization of Brd2 function during mouse embryonic development, the expression of a β-galactosidase reporter gene in a second targeted *Brd2* strain (Fig. 1A), derived from an EUCOMM ES cell line, was used to examine the endogenous expression pattern of *Brd2* in developing embryos. *Brd2*^{Gt2RFr} mice demonstrated embryonic β-galactosidase activity in genotypically *Brd2*$^{+/lacZ}$ versus matched wild type *Brd2*$^{+/+}$ control embryos. Much of the light blue β-galactosidase staining is readily detectable on the inside of the E13.0 embryos (Fig. 5). Strikingly, β-galactosidase staining is most readily detected in the developing brain and spinal cord, precisely in the neural tube that fails to close properly in the *Brd2*$^{-/-}$ embryos. This neural-specific expression pattern is

consistent with an RNA in situ pattern reported in a previous study of *Brd2* expression during mouse embryogenesis [24]. The heightened and localized expression of *Brd2*, together with the developmental defects in null mutants reported here, suggests a critical role in the development of the mouse CNS.

3.5. Proliferation and apoptosis in the developing Brd2-null neural folds

A number of diverse cellular processes are known to underlie proper neural tube closure in rodents and humans [29,30]. To begin to assess the cellular etiology of the neural tube defects associated with loss of Brd2, phosphorylated histone H3 (P-H3) and terminal deoxynucleotidyl transferase (TUNEL) staining were performed to detect proliferation and apoptosis, respectively. Cell proliferation in the thickened neural folds of the *Brd2*$^{-/-}$ embryo is evidenced by the nuclear staining of P-H3 on the ridges of the neural folds. The extent and localization of staining in the mutant, whose neural folds are spread apart, is similar, although not identical, to the wild type control, where the neural folds are bending towards each other (Fig. 6A and B). Similarly, little difference in apoptosis is observed between the *Brd2*$^{+/+}$ and *Brd2*$^{-/-}$ embryos at this E11.5 time point (Fig. 6C and D). Together, these data indicate that there are no significant defects in cell proliferation and apoptosis in this *Brd2*$^{-/-}$ embryo at this time point; however, such cellular defects may arise in more severely affected embryos that are more difficult to assay or at different time points in development.

3.6. Gene expression profiling and quantitative RT-PCR reveal changes in neuronal gene expression

To assess global gene transcription in the *Brd2*$^{-/-}$ embryos, the expression profiles of *Brd2*$^{+/+}$, *Brd2*$^{+/-}$and *Brd2*$^{-/-}$ embryos were compared at E9.5 by microarray analysis. The transcript levels of a number of genes were found to differ, with two-fold or greater enrichment found in 46 genes and two-fold or greater reduction seen in only 10 transcripts (Supplemental Table 1). A subset of the reduced transcripts in the *Brd2*$^{-/-}$ embryos encodes several key neuronal regulators such as the neurogenic differentiation factors 1 and 4 (NeuroD1 and NeuroD4) and the oligodendrocyte transcription factor 3 (Olig3; Table 2). The ephrin receptor A3 (EphA3) and the integral membrane protein SLITRK6, which shares homology with the neurotrophin receptor family, were also found to be expressed at two to three fold lower levels in the *Brd2*$^{-/-}$ embryos by expression array (Table 2). The reduced expression of these genes was confirmed by quantitative RT-PCR of RNA extracted from additional *Brd2*$^{+/+}$ and *Brd2*$^{-/-}$ littermates, demonstrating a three fold reduction in NeuroD4 and SLITRK6 and a four fold reduction in NeuroD1 and Olig3 in the *Brd2*$^{-/-}$ embryos compared to *Brd2*$^{+/+}$ littermates (Fig. 7A). To confirm that these expression changes were not due simply to differences in embryonic stage of development, additional quantitative RT-PCR was performed on *Brd2*$^{+/+}$ and *Brd2*$^{-/-}$ embryos with 37–39 somite pairs. Similar fold reductions between *Brd2*$^{+/+}$ and *Brd2*$^{-/-}$ embryos were observed (Fig. 7B). All gene expression levels were normalized to *18S* rRNA.

4. Discussion

Spina bifida is one of the most common birth defects worldwide, whereas juvenile myoclonic epilepsy (JME) is much less common; however, both may have links to *Brd2* deregulation. Spina bifida involves a posterior opening of the spinal cord. Brd2 may play an indirect or direct role in this neural development defect. The *curly tail* mouse has been an extensively studied model of spina bifida, and recent progress has implicated the reduced expression of the transcription factor encoding gene *Grainy-head-like-3* (*Grhl3*) as being responsible for the opening of the posterior neuropore in this mutant [31-33]. As spina bifida is only partially penetrant in the *curly tail* strain, a number of *curly tail* modifier genes have been mapped in the mouse genome. Strikingly, one of these *curly tail* modifiers, *Mct1*, has been mapped to the HLA region of mouse chromosome 17, in close proximity to the *Brd2* gene [34]. Based on the neural tube defects of the Brd2-null embryos presented here, Brd2 and Grhl3 may collectively coordinate precise transcriptional events required for proper neural tube closure.

Mutations in the promoter of the human *BRD2* gene have been linked to increased susceptibility to juvenile myoclonic epilepsy (JME), an adolescent-onset generalized epilepsy [25]. In addition, *BRD2* has also been genetically linked to photoparoxysmal response (PPR), a related seizure disorder in humans [26]. It is worth noting that differences between null mutations in the mouse that elicit striking neurodevelopmental defects (this study) and more subtle regulatory mutations of the human *BRD2* promoter may have profoundly different

consequences. The human mutations are predicted to alter the relative levels of *BRD2* expression in certain individuals and are not predicted to alter the full-length protein product [25]. This hypothesis posits a threshold model of *BRD2* expression and susceptibility to JME. Given the striking neural tube closure defects of the Brd2-null embryos, it is possible that subtle changes in *BRD2* expression may result in viable offspring with neurodevelopmental changes consistent with an increased susceptibility to seizures.

Given Brd2's diverse molecular interactions and its relevance to human neural developmental defects, a functional investigation of Brd2 in mammalian development was warranted. Using reverse genetics to establish a Brd2-null mouse line, we demonstrate that the disruption of the *Brd2* gene causes embryonic lethality. Brd2-null embryos deviate from normal developmental programs at embryonic day 9.0 (E9.0) where they exhibit developmental delay and generalized growth retardation. As development progresses, *Brd2*$^{-/-}$ embryos consistently manifest neural tube closure defects that most commonly appear as exencephaly of the hindbrain. This observation correlates with a high expression of *Brd2* in the developing CNS.

Consistent with the notion of Brd2's involvement in cell proliferation, we find an overall reduction in the growth potential of the *Brd2*$^{-/-}$ embryos compared to Brd2-containing embryos (Fig. 2). However, in contrast to Brd4-null embryos which fail much earlier in development, Brd2-null embryos have traversed many cell division cycles to reach these time points [21]. Here, we observed similar

neural epithelial proliferation in a *Brd2*$^{-/-}$ embryo compared to a matched wild type control and conclude that significant differences in proliferation are not apparent (Fig. 6). However, we cannot rule out the possibility that subtle differences in proliferation may accumulate over time and detract from normal neuronal development. Defects in coordinated specification and proliferation of early neural tissue may be associated with the neural tube defects of the Brd2-null embryos [29,30,35]. Expression of *Brd2* mRNA peaks between E8.5 and E12.5, and is prominently detected in the developing CNS [24]. Thus, at approximately E8.5 when neuronal proliferation is initiated, Brd2 may be required to promote neurogenesis by regulating the gene expression networks required to drive the expansion of newly born neuronal cell types [29]. The nuclear accumulation of Brd2 in multiple proliferating neuronal cell types is consistent with this notion [24]. Thus, the inability of neural folds to fuse might reflect the inability of the Brd2-null embryos to produce enough neuronal precursor cells during early CNS development. Alternatively, *Brd2*$^{-/-}$ embryos may be unable to execute the correct amount of neuronal apoptosis, as Brd2 has been previously shown to be induced during apoptosis in PC12 cells and in neurons; this function may be important also during early neurogenesis [36]. Thus, in the absence of Brd2, a subtle loss in the balance of proliferation and apoptosis may help establish an unmanageable expansion of neural precursor cells, which may result in an inability to correctly fuse the neural folds. Additionally, Brd2 function may be required outside of the developing neural ectoderm. This result would be similar to neural tube closure defects in *Twist* knockout mice in which head

mesenchyme or neural crest derivatives are the root of neural tube closure defects [37,38]. Future studies will aim to distinguish these diverse, yet related, possibilities.

Other models which provide insight into *Brd2* function include the *Drosophila Brd2* ortholog, *fsh1,* and murine *Gcn5* mutants. A recent report indicates that *fsh1* mutants undergo homeosis of the head and tail region that may be similar in nature to the neuronal defects of the Brd2-null mouse embryos [39]. In this regard, genes that are known to be critical regulators of midbrain–hindbrain specification and regionalization during early neuronal development may also be targets of Brd2 function [40,41]. In a recent report by Bu et al., cranial neural tube closure defects, similar to that observed in the Brd2-null embryos, are described in homozygous *Gcn5* mutants, which contain a single point mutation in the catalytic core of the histone acetyltransferase (HAT) domain [42]. Disruption of a related HAT, *p300,* leads to similar hindbrain exencepahly as in the Brd2-null embryos [43]. In addition to HATs, disruptions of *de novo* DNA methyltransferases *Dnmt3b* and *Dnmt1o* in the mouse result in neural tube closure defects [44,45]. As histone acetylation and DNA methylation are functionally linked in epigenetic regulation, it is possible that Brd2 might play a central role in stabilizing methylation marks on the developing mammalian genome required for proper neurulation. Thus, phenotypic variation in the Brd2-null mutants may reflect mosaic methylation patterns between individual embryos [44]. Future studies using conditional alleles of Brd2 will focus on the molecular

mechanism of Brd2 in regulating the specification and regionalization of the developing mouse brain.

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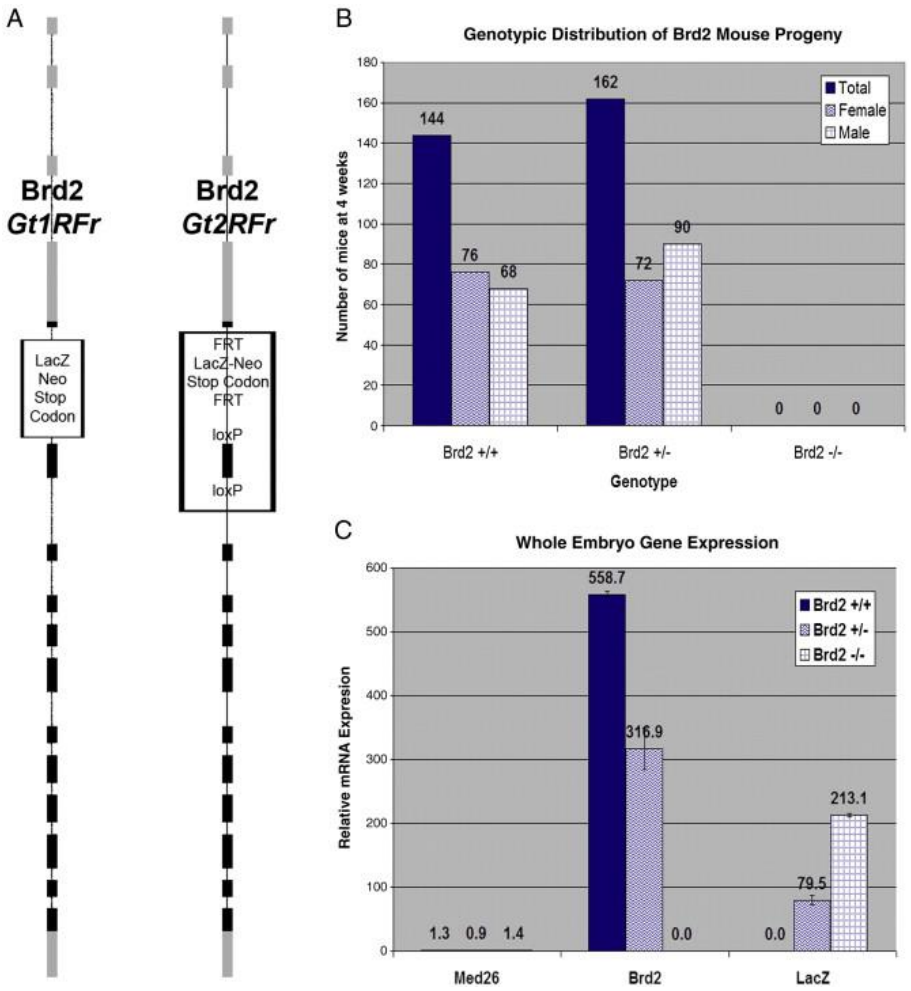


Figure 1: Identification of Brd2-null embryos

(A) Schematic of genomic *Brd2* loci with insertions of two independent gene-trap constructs between the first and second coding exons. (B) *Brd2* genotypic distribution of viable mice at 4 weeks of age born to *Brd2* heterozygous intercrosses. Total number of mice and sex-distribution of *Brd2* genotypes are shown with wild type ($Brd2^{+/+}$), *Brd2* heterozygote ($Brd2^{+/-}$) and null homozygous *Brd2* mutants ($Brd2^{-/-}$). (C) Quantitative RT-PCR analysis of mRNA derived from whole E9.5 embryos across the three *Brd2* genotypes. Relative levels of *Brd2* mRNA and mRNA from the *LacZ* transgene are graphed from a wild type embryo (+/+), a *Brd2* heterozygous embryo (+/−) and a homozygous *Brd2* mutant embryo (−/−) derived from the same litter. Relative mRNA expression levels were determined using ΔCt values and were normalized to *18S* rRNA levels to correct for minor variations in starting RNA concentrations. The absence of signal in the $Brd2^{-/-}$ embryo indicates that this embryo is devoid of wild type *Brd2* transcript and represents a Brd2-null embryo. *LacZ* expression was only detected in the *Brd2* heterozygous embryo (+/−) and the homozygous *Brd2* mutant embryo (−/−) and *Med26* expression was assayed as a control. Numbers at the top of each column are relative expression values for each gene set.

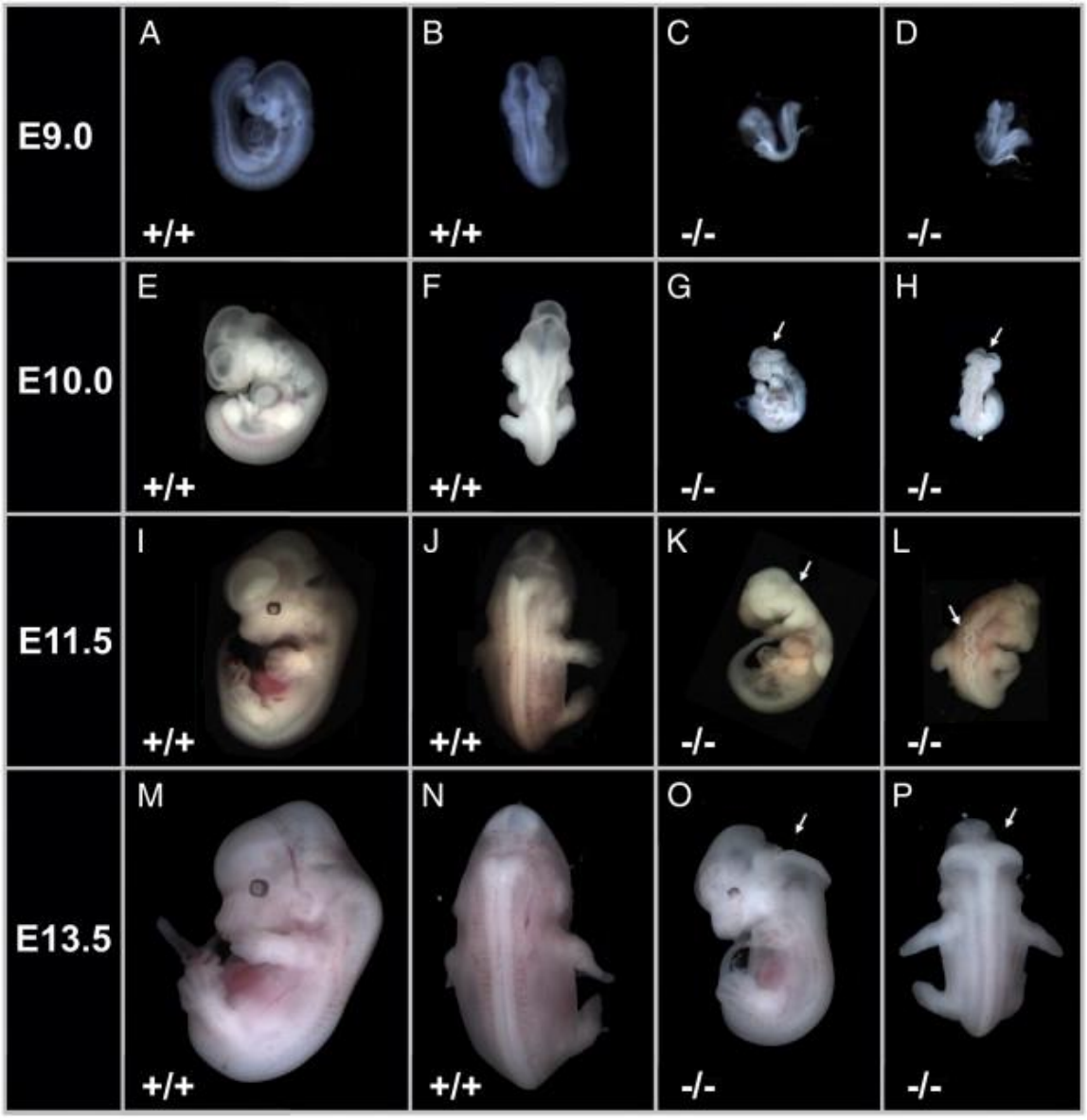


Figure 2: Growth retardation and hindbrain exencephaly of Brd2-null embryos

Whole-mount analysis of matched wild type and Brd2-null embryos from E9.0–E13.5. Each embryo is represented by a side-by-side comparison of lateral and dorsal images. The genotypes of the embryos are as follows: (A, B, E, F, I, J, M, N) wild type (+/+) embryos and (C, D, G, H, K, L, O, P) Brd2-null (−/−) embryos. Note the marked decrease in overall size and the open neural tube (white arrows) at the level of the mesencephalon (G, H) and rhombencephalon (K, L, O, P) of the Brd2-null embryos. The four images at each time point are shown at identical magnification.

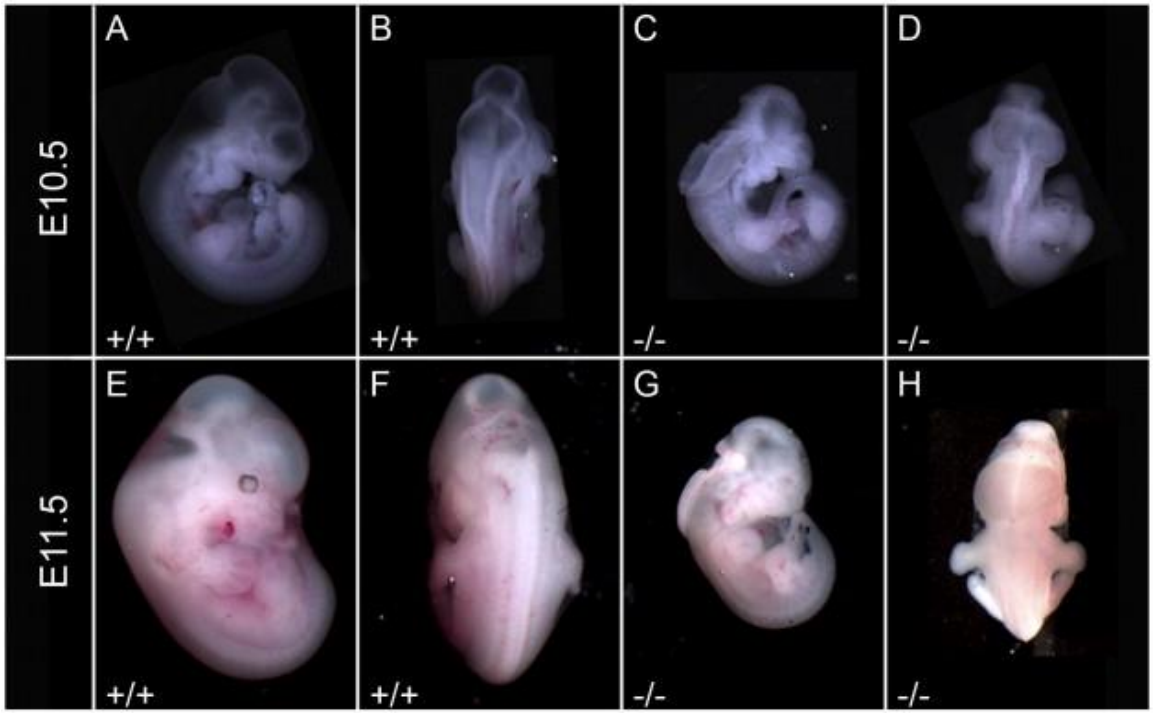


Figure 3: Hindbrain exencephaly of *Brd2*$^{-/-}$embryos at E10.5 and E11.5

Whole-mount analysis of littermate *Brd2*$^{+/+}$and *Brd2*$^{-/-}$ embryos demonstrate the open hindbrain region and thickening of the edges of the open neural tube. Note the wavy, thickened neural tube in the *Brd2*$^{-/-}$E10.5 embryo. The four images at each time point are shown at identical magnification to illustrate relative size differences.

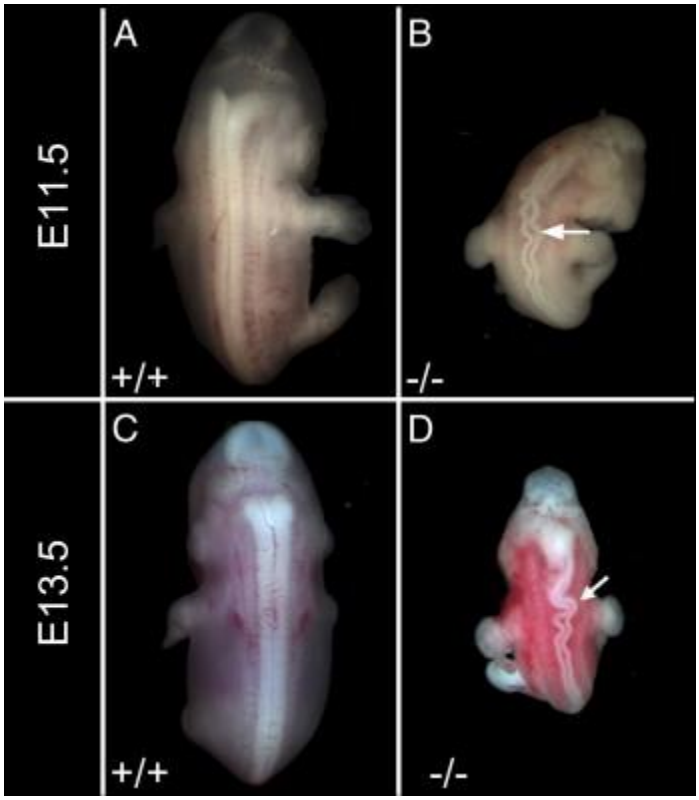


Figure 4: The caudal neural tubes of *Brd2*$^{-/-}$ embryos display incomplete closure and frequent malformations

Whole-mount analysis of E13 *Brd2*$^{+/+}$ and *Brd2*$^{-/-}$ littermates from two separate heterozygous crosses illustrate the closure defects frequently found in Brd2-null embryos. Note the wavy, open neural tubes of the null embryos (white arrows) compared to wild type littermates.

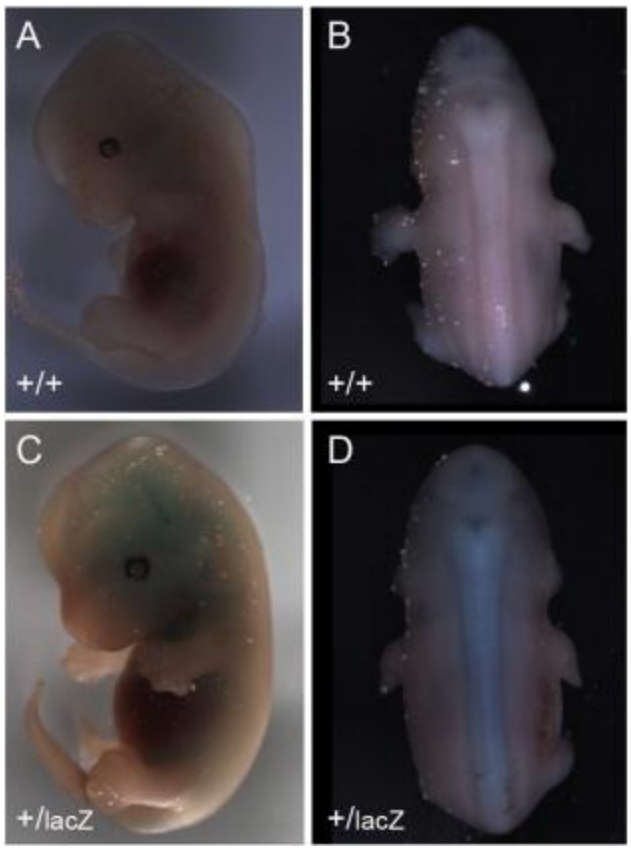


Figure 5: Embryonic *Brd2* expression in the developing neural tube

A matched wild type (+/+, A, B) embryo and *LacZ*-containing *Brd2* heterozygous (+/*LacZ*, C, D) embryo from the same E13.0 litter is shown after β-galactosidase staining. Lateral (A, C) and dorsal (B, D) views are presented to highlight the expression of this *Brd2* reporter gene in the entire developing neural tube.

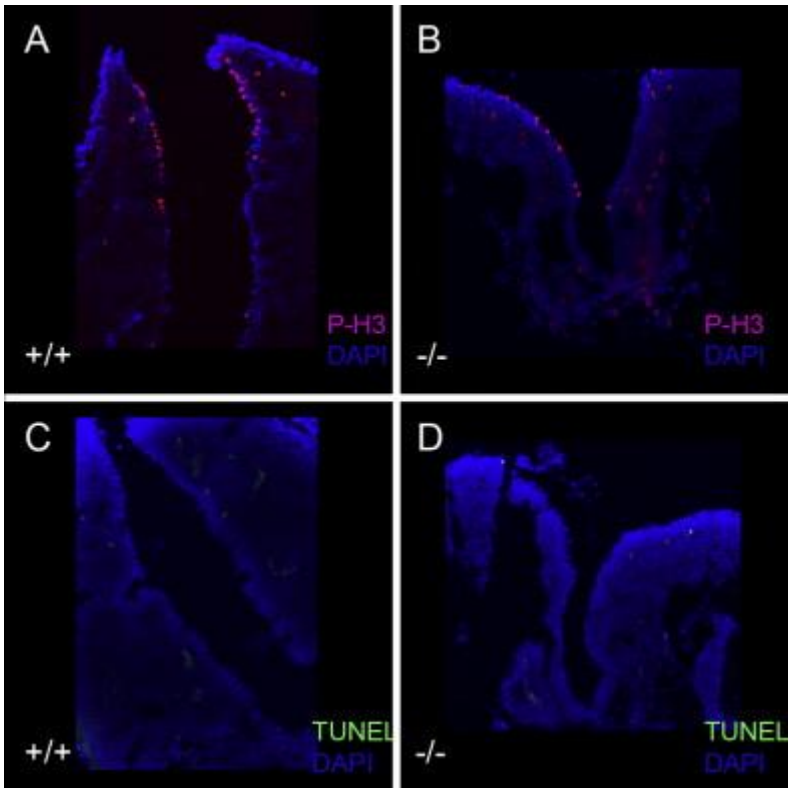


Figure 6: Proliferation and apoptosis in the Brd2-null embryos

Sections from a matched wild type control (+/+, A, C) embryo and Brd2-null (−/−, B, D) embryo from the same E11.5 litter are shown after anti-phosphorylated histone H3 (A, B) and TUNEL staining (C, D). Phosphorylated histone H3 staining appears pink, TUNEL staining appears light green, and DAPI is shown in blue.

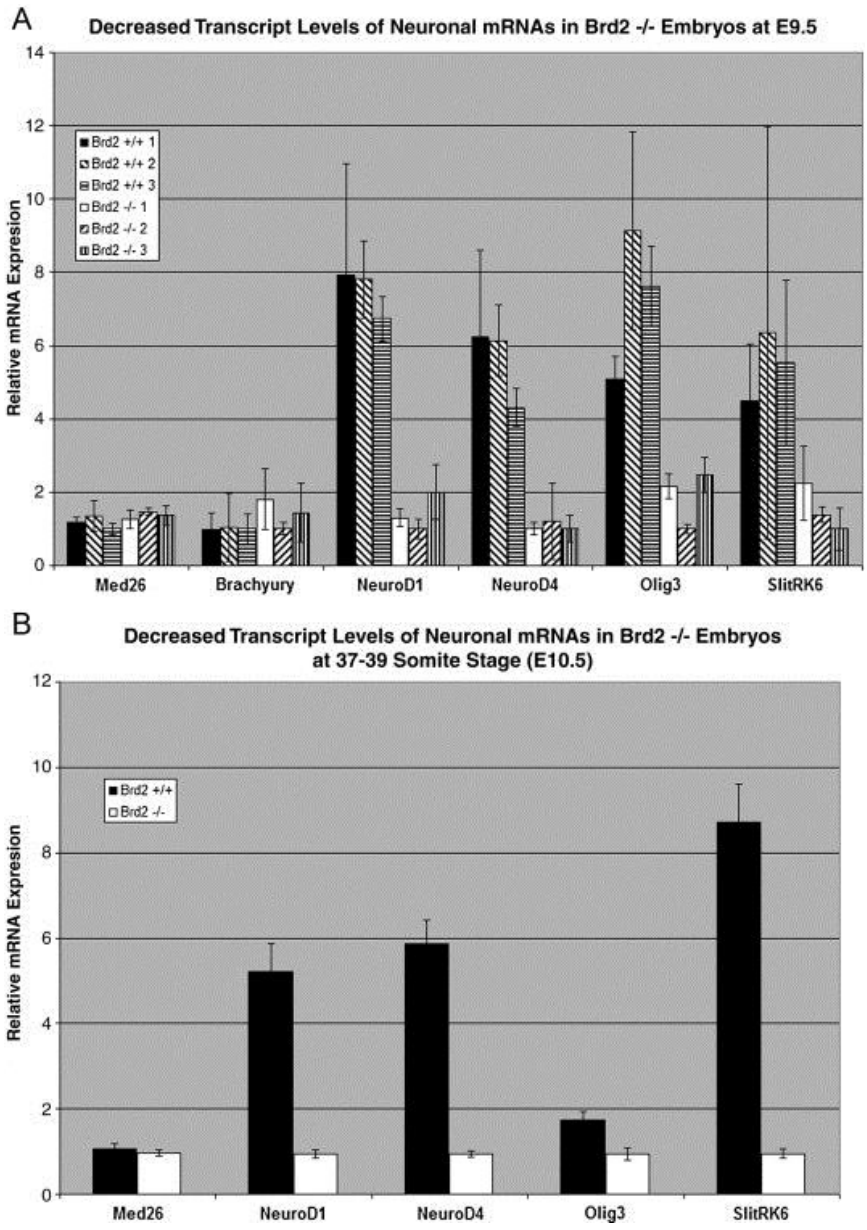


Figure 7: Decreased transcript levels of neuronal mRNAs in *Brd2*$^{-/-}$ embryos

(A) Quantitative RT-PCR for mediator component *Med26* and mesodermal marker *Brachyury* (controls) show little relative difference between *Brd2*$^{+/+}$ and *Brd2*$^{-/-}$ embryos at E9.5, while mRNA levels of genes known to be involved in neuronal development *NeuroD1*, *NeuroD4*, *Olig3* and *SlitRK6* show a marked (3–4 fold) decrease in transcript level. (B) Expression of neuronal mRNAs in *Brd2*$^{+/+}$ and *Brd2*$^{-/-}$ embryos with 37–39 somites. Relative mRNA expression levels were determined using ΔCt values and were normalized to *18S* rRNA levels to correct for minor variations in starting RNA concentrations.

Table 1: Recovery of Brd2-null embryos during development

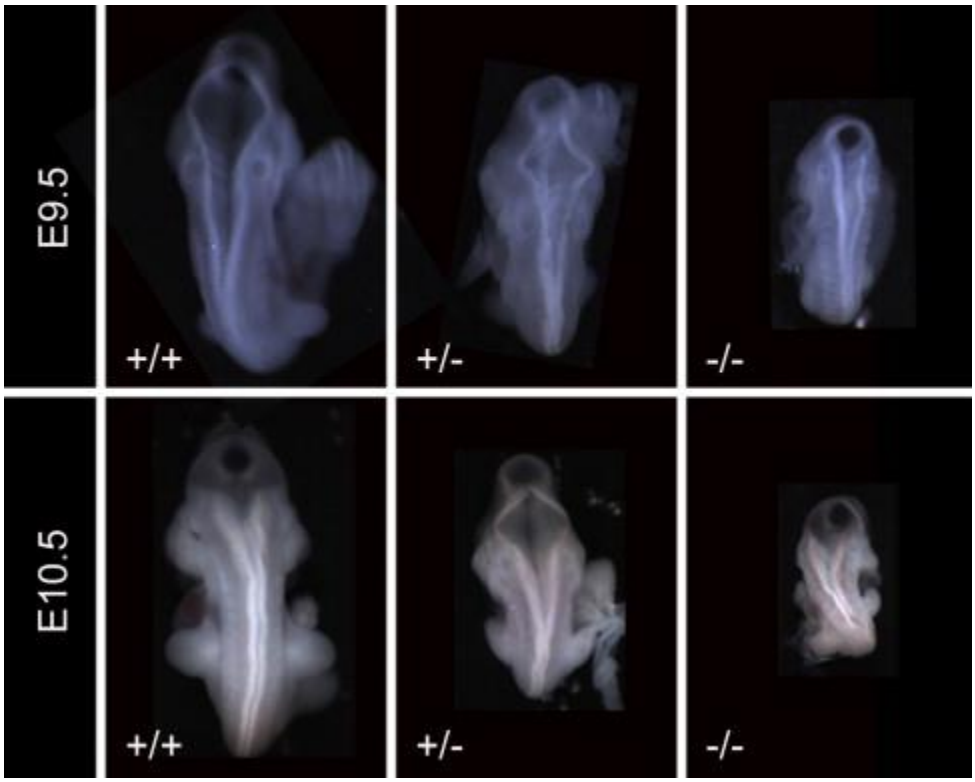
Age	# Litters	*Brd2*$^{+/+}$	*Brd2*$^{+/-}$	*Brd2*$^{-/-}$
E8–8.5	2	**4** (3)	**6** (6)	**2** (3)
E9–9.5	11	**29** (23)	**42** (46)	**20** (23)
E10–10.5	4	**5** (5)	**10** (11)	**7** (5)
E11–11.5	4	**6** (10)	**26** (21)	**9** (10)
E12–12.5	3	**11** (6)	**11** (12)	**1** (6)*
E13–13.5	3	**9** (9)	**23** (18)	**4** (9)*
Total	27	**64** (56)	**118** (114)	**43** (56)

Numbers of recovered mouse embryos are shown in bold and expected numbers of embryos based on Mendelian ratios are shown in parentheses. Asterisks denote litters where reduced numbers of *Brd2*$^{-/-}$ embryos were recovered and increased numbers of resorbed embryos were detected.

Table 2: Genome-wide expression profiling reveals changes in global gene expression

Gene	Description	Average signal intensity			Fold change
		Brd2$^{+/+}$	*Brd2*$^{+/-}$	*Brd2*$^{-/-}$	
Brd2	Bromodomain containing 2	1435	948	17	− 81.7
Neurod1	Neurogenic differentiation 1	78	71	23	− 3.33
Epha3	Eph receptor A3	105	91	32	− 3.26
Slitrk6	SLIT and NTRK-like family, member 6	81	66	29	– 2.74
Olig3	Oligodendrocyte transcription factor 3	141	162	59	– 2.4
Neurod4	Neurogenic differentiation 4	96	112	40	– 2.35

Microarray analysis revealed average expression values in biological triplicate embryos of each *Brd2* genotype. Each of these genes was expressed at statistically significant ($p < 0.05$) reduced levels in the *Brd2*$^{-/-}$ and *Brd2*$^{+/-}$ embryos versus their age-matched wild type embryos. Fold change of these genes by microarray in the *Brd2*$^{-/-}$ embryos versus the wild type embryos is shown in the rightmost column.



Supplemental Figure 1: Whole -mount analysis of *Brd2+/+*, *Brd2+/−*-, and *Brd2−/−*- littermates at E9.5 and E10.5 show graded effects of *Brd2* copy number.

Note the minor growth retardation in the heterozygotes compared to wild- type and Brd2-null littermates, as well as the less severe delay in neural tube closure as compared to the Brd2-null mice. The intermediate phenotypes seen in the *Brd2+/−*- mice suggest partial haploinsufficiency. All images at each time point are shown at identical magnification for size comparison.

Supplemental Table 1: Gene transcripts enriched or reduced two-fold or greater in *Brd2*$^{-/-}$ embryos

Decreased Transcript in Brd2$^{-/-}$			
Gene	**Fold Change**	**Gene**	**Fold Change**
Zfy1	10.54	Olig3	2.4
Cxcl4	7.62	Wdr69	2.39
Fabp7	3.83	Neurod4	2.35
Hemgn	3.5	Fez1	2.31
Nppa	3.34	Nap1l2	2.23
Neurod1	3.33	Heph	2.22
Epha3	3.26	Nat2	2.19
Pklr	3.16	Ms4a4d	2.18
Serpinf1	2.99	N6amt1	2.18
Cp	2.96	Gap43	2.18
Ptx3	2.92	Col3a1	2.17
Slitrk6	2.74	Rhd	2.16
1810011O10Rik	2.72	Dnm3os	2.13
3830403N18Rik	2.72	Ifit2	2.07
Dcn	2.65	Sgce	2.06
Olfr1199	2.61	Plunc	2.06
Pamci	2.55	Dlx1	2.04
V1ra1	2.48	Cpa2	2.04
Svs3b	2.44	Lactb	2.03
Sparcl1	2.42	Ubxd6	2.02
Asb17	2.41	Mbnl3	2.02
Hbb-y	2.41	Olfr1477	2
Ifna12	2.41	Apobec3	2

Increased Transcript in Brd2$^{-/-}$	
Gene	**Fold Change**
H2-K1	0.32
Olfr1167	0.39
Olfr107	0.4
4930583H14Rik	0.42
Olfr829	0.44
Nepn	0.46
Adm	0.47
H2-Q7	0.48
Idi2	0.49
Dnahc3	0.49

Supplemental Table 2: Primer sequences used for quantitative RT-PCR assays

Transcript	Forward Primer 5'-3'	Reverse Primer 5'-3'
Brachyury	CTCACCAACAAGCTCAATGG	GGTCTCGGGAAAGCAGTGGC
Brd2	GCTGAGCGGCGGCGGTTCCC	GTAAAGCTGGTACAGAAGCC
LacZ	CTGGCGTAATAGCGAAGAGG	GACAGTATCGGCCTCAGGAA
Med26	GACTCCCAGAGCAACATCCG	CCCTAGTCGTGTCTCCTCCAG
NeuroD1	GGGGTCCCAAAAAGAAAAG	TGGGTCTTGGAGTAGCAAGG
NeuroD4	ATTCAGGGCTCGAAGAGTCA	TTCCTTGCCAGTCGAAGAGT
Olig3	AGGTGTTAGCGAAGGGGACT	AAACTCCACTGATCCCATCG
SlitRK6	CCATCACGACCTTTCCACTT	CAAGGTGTATTGAAAGGGCATT
18S rRNA	CCGCGGTTCTATTTTGTTGG	GGCCGTCCCTCTTAATCATG

Chapter 3:

The *In Vitro* and *In Vivo* Association of the BET Protein BRD2 with the Histone Acetyltransferase GCN5

The following chapter is a manuscript in preparation for submission. The original 3x-FLAG Brd2 construct was made by Nathaniel Smilowitz (Freiman Lab, Brown University), and the ET-Only construct was cloned by Eric Gustafson (Freiman Lab, Brown University). I performed all experiments in this chapter except for the ES cell co-immunoprecipitation, which was performed by Brian A. Rowley (Dent Lab, MD Anderson Cancer Center).

The *in vitro* and *in vivo* association of the BET Protein BRD2 with the histone acetyltransferase GCN5

(Brd2 association with GCN5)

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Abstract:

Given the combinatorial and dynamic nature of histone modifications, deciphering the histone code poses a significant challenge. To simplify this landscape, a number of reader and writer chromatin proteins have been posited to work with one another to stabilize specific chromatin states. However, how these reader and writer proteins communicate with each other remains largely unknown. Previously, we identified similar neural tube defects in loss of function mouse mutants targeted for disruption of the reader protein Brd2 and the writer histone acetyltransferase GCN5. To test the hypothesis that these two proteins interact, we identified the association of these two proteins in several diverse contexts. In vivo, BRD2 and GCN5 colocalize in the developing neuroepithelium and reciprocally co-immunoprecipitate each other in mid-gestation whole mouse embryo extracts. In mouse ES cell extracts, purification of GCN5-containing complexes revealed the co-association of BRD2. Finally, in cultured N2A cells, the C-terminal ET domain of BRD2 is shown to be dispensable for its association with GCN5 but important for the it correct nuclear localization. Taken together, these results suggest that BRD2 may be acting to integrate the reading and the writing of chromatin modifications by guiding the histone acetyltransferase activity of GCN5.

1. Introduction

A significant obstacle in deciphering the histone code is correlating each of the epigenetic readers or writers with changes in chromatin state, gene transcription and developmental outcomes. Much is already known about the roles of chromatin modifying and remodeling complexes, as well as the influence the local chromatin state has on transcriptional regulation [1,2]. Still, a considerable number of questions remain about how epigenetic states are integrated and linked to measurable outcomes in complex mammalian tissues. By systematically dissecting the functions and mechanisms of reader and writer proteins, a clearer picture of epigenetic regulation will be revealed.

As the only protein motif known to interact with ε-N-acetylated lysine residues, the bromodomain (BRD) module is found in a number of chromatin-associated proteins conserved among eukaryotes and is posited to be a critical 'reader' of epigenetic marks [3,4]. A conserved motif consisting of approximately 110 amino acids, bromodomains are contained within multiple classes of proteins, including histone acetyltransferases (HATs) such as GCN5 and PCAF [5], transcriptional regulators such as TAF1 [6], and the bromodomain and extra-terminal (BET) family of proteins containing BRD2, 3, 4 and BRDT [4]. Despite variations in amino acid sequence, all BRDs have a characteristic fold of four α-helices, which flank the binding site for an acetylated lysine residues [7,8] and each BRD appears to have its own unique binding affinity for specific residues, allowing for cooperation among bromodomains in binding to particularly modified nucleosomes [9,10]. These innate preferences in acetylated lysine binding, when

coupled with the variety of protein classes which have bromodomain containing proteins, suggests that BRDs may be critical in interpreting the histone code and translating it into changes in gene transcription.

One member of the BET family of bromodomain proteins, which is characterized by tandem amino-terminal bromodomains linked to an extra-terminal (ET) domain at the C-terminus, is Bromodomain-containing protein 2 (BRD2; formerly FSRG1 and RING3). BRD2 is well conserved protein that we have shown is essential for mammalian development [11]. The orthologous *Drosophila* protein, female sterile homeotic 1 (*fsh1*), is also required for successful embryogenesis [12-14]. BRD2's orthologs in yeast, Bdf1 and Bdf2, have also been shown to have critical roles in properly interpreting histone modifications, as well as guiding transcription by associating with the general transcription factor TFIID [15-17]. Structural studies have determined that human BRD2, which has greater than 90% overall identity to murine BRD2, shares identical bromodomains and binds to acetylated histone H4 at K5 and K12 specifically [7,8,18].

BRD2's essential role in mouse embryogenesis was revealed by several studies that demonstrated its requirement for proper neural tube closure and overall embryonic growth [11,19,20]. Embryos lacking BRD2 protein suffer from significant developmental delay and hindbrain exencephaly. The histone acetyl transferase GCN5 protein has been shown to share several key features with BRD2; both proteins have similar bromodomain motifs, demonstrate high levels of expression in the developing neural tube and are essential for embryonic

development [21-24]. There is a striking similarity between the *Brd2*-null embryos and embryos lacking the GCN5 HAT function; both have significant hindbrain exencephaly, marked by characteristic outgrowths of the neural plate.

To determine the significance of this developmental overlap, we examined the potential interaction of these proteins in several cellular and developmental contexts. In vivo, BRD2 and GCN5 colocalize in the neuroepithelium of the developing hindbrain and reciprocally co-immunoprecipitate each other in E11.5 whole mouse embryo extracts. In mouse ES cell extracts, purification of GCN5-containing complexes revealed the co-association of BRD2. Finally, in cultured N2A cells, the C-terminal ET domain of BRD2 is shown to be dispensable for its association with GCN5 but is required for the correct nuclear localization of BRD2. Together, these data confirm the novel association of BRD2 and GCN5 and indicate that reader-writer chromatin proteins are more intimately associated with each other than previously appreciated.

2. Materials & Methods:

2.1 Construct and Cell Line Generation

The full length Brd2 coding sequence was isolated by PCR amplification from a mouse brain cDNA library and ligated into the TOPO-TA cloning vector. Subsequently, it was excised using the XbaI and BamHI sites introduced during the initial amplification and ligated into the multiple cloning site of the 3xFLAG-CMV-9 expression vector (Catalog #E9783, Sigma-Aldrich). The resulting construct, named 3x-FLAG-Brd2 and referred to as Full Length, was verified by restriction digest and sequencing, and used as template for the generation of additional mutants. Each of the point mutations, BD1-Y112F, BD2-Y385F, and Early Stop, was made by site directed mutagenesis using the Quikchange II Site-Directed Mutagenesis Kit (Catalog #200523, Agilent Technologies), according to manufacturer's instructions. The ET-Only construct was made by PCR amplifying the C-terminal fragment of the 3xFLAG-Brd2 construct and ligating it into the MCS of the 3xFLAG-CMV-9 backbone using BamHI and EcoRI. All primer sequences can be found in Supplemental Table 1. The identity of each cloned or mutated construct was verified by sequencing before further use (GeneWiz).

A vial of mouse neuroblastoma Neuro-2A (N2A) cells was purchased from ATCC (Manassas, VA, Catalog #CCL-131) and maintained in MEMα with GlutaMAX (Invitrogen), supplemented with 10% fetal bovine serum (FBS) and 1% Pen-Strep solution. Transient transfections were performed using a 3:1 Fugene 6 reagent to DNA ratio (Roche Applied Sciences, Indianapolis, IN).

Stable cell lines were isolated after transient transfection by dilution and subsequent selection with X mg/ml Geneticin G418 (Invitrogen). Construct expression was verified in each line by Western blotting with α-FLAG and α-Brd2 antibodies and indirect immunofluorescence.

2.2 Brd2-null Mouse Model and Embryo Dissection

The Brd2-null mice used in this study were previously described [11]. To obtain appropriately aged embryos, heterozygous (*Brd2*$^{+/-}$) males and females were mated and checked daily for the presence of a copulation plug, which would be noted as embryonic day E0.5. Pregnant dams were then monitored routinely for maintenance of pregnancy and were sacrificed between E10.5 and E12.5. The uterine horns were excised into cold, sterile PBS, and each decidual swelling was dissected independently under a Zeiss Discovery V8 stereomicroscope, and embryos were stored in PBS on ice. Yolk sacs were retained for PCR genotyping, as previously described [11]. All animal colony maintenance and procedures were approved by and performed in compliance with the IACUC standards of care set forth by the Brown University Animal Facility and the NIH Guide for the Use and Care of Laboratory Animals.

2.3 Anti-BRD2 Antibody Generation

Anti-BRD2 peptide antibodies were custom generated by YenZym (South San Francisco, CA) to specifically detect BRD2 among the highly homologous BET proteins. The first 17 amino acids of mouse BRD2 protein were used to

raise Peptide Antibody N1. The Peptide Antibody M556 was raised against amino acids #556-575, and is located in the hinge region between BRD2's second bromodomain and the ET domain. Crude rabbit anti-serum was affinity purified using specific peptides and used for all experiments.

2.4 Co-Immunoprecipitation and Western Blot Analysis

To prepare whole protein extracts, flash frozen or freshly dissected embryos were cleaned of their membranes and any residual maternal tissue and were subsequently dounce homogenized in extraction buffer (200 mM KCl, 100 mM Tris pH 8.0, 0.2 mM EDTA, 0.1% NP-40, 10% glycerol, 1 mM PMSF). The extraction mix was then incubated on ice for one hour and centrifuged for 10 minutes at 4°C to pellet cell debris. To prepare protein from N2A cells, the culture medium was removed from plates achieving ~75% confluence and the adherent cell layer was washed twice with cold, sterile PBS. The cells were then scraped into an additional 1 ml of PBS and pelleted by centrifugation. The supernatant was aspirated and the pellets resuspended in extraction buffer and treated as above. Protein concentrations were determined by Bradford assay, and equivalent amounts of protein were used for Western blot analysis. Following separation by SDS-PAGE, proteins were transferred onto nitrocellulose membranes and blocked in TBS-T with 5% milk. Blots were probed with primary antibodies as described in Supplementary Table 2 and with Li-COR IRDye 680 and 800 channel secondary antibodies diluted 1:5000. Images were obtained from and analyzed by a Li-COR Odyssey CLx Imaging system.

To isolate complexes containing BRD2 and GCN5, at least 300 µg of whole embryo or N2A cell line extracts were incubated with 20 µl of washed Protein A/G beads (GE Healthcare) and either: 20 µl α-GCN5 antibody (Santa Cruz), 20 µl α-BRD2 M556, or 1 µl α-FLAG antibodies (Sigma), respectively, in extraction buffer containing protease inhibitors. Control immunoprecipitations comprised of cell extracts and beads or normal IgG were performed in parallel with each experiment. Reactions were allowed to mix overnight on an end-over-end shaker at 4°C. Beads were pelleted by centrifugation and the supernatant discarded. The pellets were then washed three times in cold IP wash buffer (200 mM KCl, 100mM Tris pH 8.0, 0.5mM EDTA, 0.05% NP-40) and resuspended in 30 µl of 2x SDS loading buffer. Samples were then boiled and loaded onto SDS-PAGE gels for Western blot analysis.

2.5 Immunofluorescence

For immunofluorescence on embryonic tissue, embryos were dissected from timed matings, as previously described, and washed in cold, sterile PBS. Individual unfixed embryos were then oriented in wells containing OCT embedding medium, and the blocks frozen in a liquid nitrogen bath. Cryostat sections were made at a tissue depth of 10 µm, affixed to slides and stored with dessicant at -80°C until use. N2A cells were grown on glass coverslips in 6-well tissue culture dishes until ~75% confluent. Medium was then gently removed, and the coverslips washes twice with cold, sterile PBS to remove traces of medium and cellular debris.

Coverslips and slides were fixed with 4% paraformaldehyde for 20 minutes at room temperature, then permeabilized for an additional 30 minutes in PBS-T. Each was incubated in blocking solution (5% BSA, 10% serum in PBS-T) for one hour and probed for one hour each with primary and secondary antibodies, respectively. All incubations were carried out at room temperature and antibody dilutions can be found in Supplementary Table 2. Slides and coverslips were washed three times in PBS-T and mounted in Vectashield with DAPI (Vector Labs). Samples were analyzed using a Zeiss ImagerM1 fluorescence microscope and images obtained and analyzed with the Axiovision software suite.

3. Results:

3.1 *Brd2*$^{-/-}$ embryos lack BRD2 protein expression

Our previous study reported the absence of Brd2 mRNA in *Brd2*$^{-/-}$ embryos, but were not able to demonstrate a lack of protein due to the lack of suitable mouse-specific antibodies against BRD2 [11]. To determine how much, if any, BRD2 protein was present in *Brd2*$^{-/-}$ embryos, peptide antibodies were raised against amino acids #1-17 (Peptide Antibody N1) and amino acids #556-575 (Peptide Antibody M556), which is located in the hinge region between the second bromodomain and the ET domain (Figure 1A). Affinity-purified antibodies were then used to detect the presence of BRD2 in both whole embryo extracts and in tissue sections of embryonic tissues. Western blotting of *Brd2*$^{+/+}$ and *Brd2*$^{+/-}$ demonstrated a robust and specifc signal at approximately 110 kDa that

was deficient in the *Brd2*$^{-/-}$ embryos assayed (Figure 1B). To confirm these data at the tissue level, we used α-Brd2 M556 on tissue sections from both *Brd2*$^{+/+}$ and *Brd2*$^{-/-}$ embryos, demonstrating a robust nuclear signal in the wild type and lack of signal in the *Brd2*$^{-/-}$ embryos (Figure 2C, 2D). These support our prior findings that Brd2 mRNA is not produced in the *Brd2*$^{-/-}$ embryos, and indicates the absence of the BRD2 protein leads to mid-gestational embryonic lethality. In addition, it provides us with a critical reagent to detect protein-protein interactions with endogenous BRD2 that was previously unavailable.

3.2 BRD2 and GCN5 interact *in vitro*, independent of the ET domain

Given the striking similarities between the *Brd2*$^{-/-}$ and HAT mutant GCN5 embryos, we hypothesized that the two proteins may associate during development. To first determine if BRD2 and GCN5 interact in cell extracts derived from cultured N2A cells, a series of FLAG-tagged wild type and mutant BRD2 expression plasmids were constructed (Figure 2A) and stable N2A cell lines expressing exogenous FLAG-BRD2 proteins at variable levels (Figure 2B).

To begin to test the hypothesis that GCN5 and BRD2 associate with one another, we performed co-immunoprecipitation experiments using whole cell extracts from N2A cells expressing wild type and mutant forms of FLAG-BRD2. As demonstrated in Figure 2C, co-immunoprecipitations of native GCN5 from N2A protein extracts was detected with FLAG-tagged Full Length BRD2 and several of the BRD2 mutants including Early Stop, BD1 Y112F, BD2 Y385F, and BD1 Y112F & BD2 Y385F. In contrast, the BRD2 ET-only construct, in which all

but the C-terminal ET domain of the protein had been deleted, failed to recover GCN5. These data indicate that (1) a robust interaction between full length exogenous BRD2 and endogenous GCN5 is detected in N2A cells and that (2) this association is not dependent on the ET domain, which has been previously reported to act as an interaction scaffolding domain for other transcription factors [25,26].

3.3 The ET domain of BRD2 is required for its nuclear localization

A previous study has shown native BRD2 protein to be largely localized within the nuclei of actively proliferating cells, while they are found only in the cytoplasmic compartment of fully differentiated neurons [27]. To determine which, if any, domain of BRD2 is required for its proper subcellular localization, we subsequently screened this series of stable cell lines by fluorescence microscopy. Both endogenous BRD2 and ectopic full length FLAG-BRD2 were exclusively localized in the nucleus as were all the FLAG-tagged full length bromodomain point mutants (Figure 3, A-D'). In contrast, the FLAG-tagged early stop BRD2 protein, deleting the c-terminal ET domain was largely detected in the cytoplasm (Figure 3, E'-J'). This suggests that the ET domain plays a pivotal role in either initial translocation or persistence of the BRD2 protein in the nucleus during active growth phases.

3.4 BRD2-dependent mislocalization of endogenous GCN5 to the cytoplasm

Given the interaction between BRD2 and GCN5 in N2A cells, we hypothesized that the mis-localization of the BRD2 Early Stop mutant may affect the subcellular localization of GCN5. To address this question, we analyzed actively proliferating cultures of each stable N2A cell line for the localization of endogenous GCN5 with respect to ectopic FLAG-BRD2 localization. For each of the lines which had nuclear localization of FLAG-Brd2 (Full Length, BD1 Y112F, BD2 Y385F, BD1 & BD2, and ET-Only), endogenous GCN5 was predominantly localized in the nuclear and perinuclear compartments (Figure 4, A-D, I-X). In contrast, in the Early Stop line, endogenous GCN5 was concentrated largely in the cytoplasm (Figure 4, E-H), along with the truncated BRD2 protein. Taken together, these data suggest that in the absence of the ET domain, FLAG-BRD2□ET sequesters endogenous GCN5 in the cytoplasm, thereby perturbing its normal nuclear localization and function.

3.5 BRD2 and GCN5 interact *in vivo* during mammalian embryogenesis

Despite robust evidence that Brd2 and GCN5 interact *in vitro* and colocalization studies in mouse embryos, there has yet been any direct evidence that they interact *in vivo*. To address this issue, we conducted reciprocal co-immunoprecipitation experiments of endogenous BRD2 and GCN5 in E11.5 whole embryo extracts. When extracts were immunoprecipitated using the α-BRD2 M556 antibody, GCN5 protein was recovered (Figure 5A). In the complimentary control experiments, no GCN5 was recovered when beads alone or beads with normal rabbit IgG antibodies were used, despite relatively

abundant levels of GCN5 detected in all Brd2 mutant embryo genotypes (Figure 5C). Additionally, the reverse experiment yielded similarly significant results; in embryo extracts immunoprecipitated with α-GCN5 antibodies, BRD2 protein was also detected by western blot; none was recovered when only beads or normal mouse IgG with beads were used as negative controls (Figure 5B). These data indicate that during embryonic development BRD2 and GCN5 are associated with one another and suggest that their related functions in neuronal development are dependent upon this close association. This is further supported by the colocalization of BRD2 and GCN5 in the embryonic neural tube (Figure 6).

Discussion

While significant progress has been made in unraveling the intricacies of global chromatin modifications, our knowledge of the underlying mechanisms has been lacking. Here, we show that in the intact mouse embryo, ES cells and N2A neuroblastoma cell cultures, the chromatin adaptor protein BRD2 associates with the histone acetyltransferase GCN5. This interaction may lead to a cascading effect, where regions of the genome may rapidly become accessible to the general transcriptional machinery. The BRD2:GCN5 interaction is mediated through the amino- terminal bromodomains of BRD2 rather than the scaffolding carboxy-terminal ET domain of the protein. In contrast, the observation that it is the ET domain of BRD2 which is required for the protein's subcellular localization in actively growing cells suggests that while the bromodomains alone are

sufficient to recognize and bind select nucleosomes, their affinity alone is not sufficient to anchor the protein in the nucleus. Rather, BRD2's association with additional proteins or multiprotein complexes through the ET domain is likely necessary for the protein to enter or remain anchored in the nucleus where it can associate with GCN5.

GCN5 is a crucial component of the SAGA (Spt-Ada-Gcn5 acetyltransferase) complex, a large multi-protein complex which is conserved between yeast and humans [28]. SAGA has been largely characterized in several species, and it is known to contain at least four functional modules, of which two confer specific enzymatic activity and are responsible for both the acetylation and deubiquitination of proteins, including histones [28,29]. Most notably, GCN5 is the catalytic subunit of the histone acetyltransferase module of SAGA, and its major targets of acetylation were identified as mononucleosomal and free H3 (H3K9 and H3K14) as well as H4K5 and H4K12 [30]. The SAGA complex has been implicated in regulating tissue-specific gene expression, and different mechanisms for each functional domain are believed to target the complex to gene-specific promoters [31]. The loss of Gcn5 acetyltransferase activity alone leads to significant defects in neural tube closure in the mouse, leading to hindbrain exencephaly [21]. These defects largely mirror those seen in the *Brd2*-null embryos, suggesting that the two proteins may play related roles in development.

Interestingly, BRD2 has not been identified as a component of the SAGA complex, although both BRD2 and GCN5 have both been shown to interact with

similar components of Swi/Snf, Mediator, and the transcriptional machinery [26,28]. Given the lack of prior identification of BRD2 in SAGA, the interaction of BRD2 and GCN5 can be explained by one of two likely scenarios: BRD2 is a previously unidentified, perhaps tissue-specific, component of the SAGA complex that aids in targeting the complex to gene-specific loci based on their underlying chromatin context, or that BRD2 and GCN5 operate in concert in an non-SAGA-dependent fashion, forming a novel chromatin reader-writer complex. As such, this new class of chromatin reader-writer complexes could couple chromatin targeting adapter proteins, such as BET family members, with histone modifying proteins, such as the acetyltransferases GCN5, p300 and PCAF, in a combinatorial fashion, creating small, efficient, and chromatin context dependent mechanisms for directing euchromatic regions for gene expression. Further work characterizing the BRD2-GCN5 interaction should reveal more about the nature of these protein complexes.

Because of the transient and indirect nature of the association between chromatin binding proteins and the genome, there is a significant challenge in finding direct gene targets. Few direct target genes have been posited for BRD2, such as cyclin A, but additional potential targets have been identified, such as the ephrin receptor A3, NeuroD1 and NeuroD4 [11,25]. Previous work on GCN5 has uncovered an overlapping gene regulatory network between GCN5 and the N-myc transcriptional cofactor in neural stem cells, suggesting that both proteins may be involved in maintaining the neural progenitor-like cell state [32]. Given the profound and similar roles BRD2 and GCN5 play during embryonic

development, understanding their interrelated functions is of paramount importance, and finding similar transcriptional network overlap between BRD2 and GCN5 is key to understanding the role each play in the developing embryo and nervous system. Future studies attempting to uncover both global and locus specific changes in histone acetylation and methylation states in cells with mislocalized, over-expressed, or absent BRD2 protein will significantly advance our mechanistic understanding of how chromatin targeting and modifying complexes link directly to transcriptional regulation and direct complex programs of neural development.

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Tables & Figures:

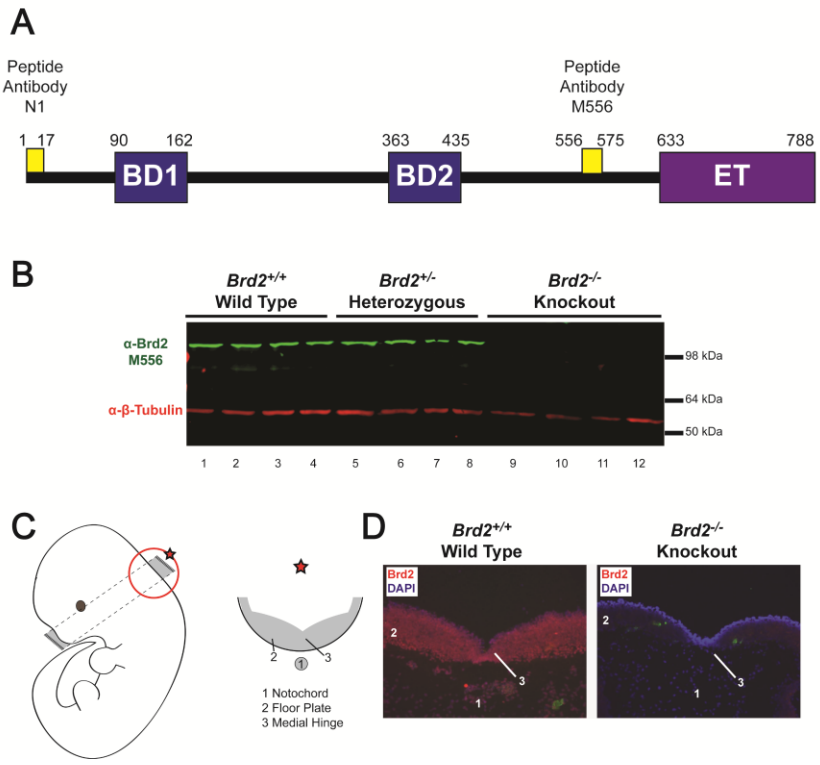

Figure 1: The Brd2$^{-/-}$ embryo is devoid of BRD2 protein

(A) The 788 amino acid protein BRD2 is characterized by dual N-terminal bromodomains (blue) and a C-terminal Extra-Terminal (ET) domain (purple). The yellow boxes indicate the recognition motifs of antibodies generated and used throughout these experiments. (B) Western blot on whole cell extracts from E11.5 embryos. Membrane was simultaneously probed for BRD2 using the peptide antibody M556 and an anti-β-tubulin antibody. Images were captured and analyzed using a LI-Cor Odyssey imaging system and are representative of results seen from at least 15 embryos of each genotype. (C) Schematic showing plane of sectioning for (D). (D) Immunofluorescent staining of *Brd2* wild type and knockout embryos demonstrates lack of protein expression in knockout neural plate. Transverse sections through the developing neural tube of E11.5 littermates stained with α-BRD2 M556 antibody (red) show clear, robust nuclear localization in the wild type embryo but no detectable amounts in the knockout embryo.

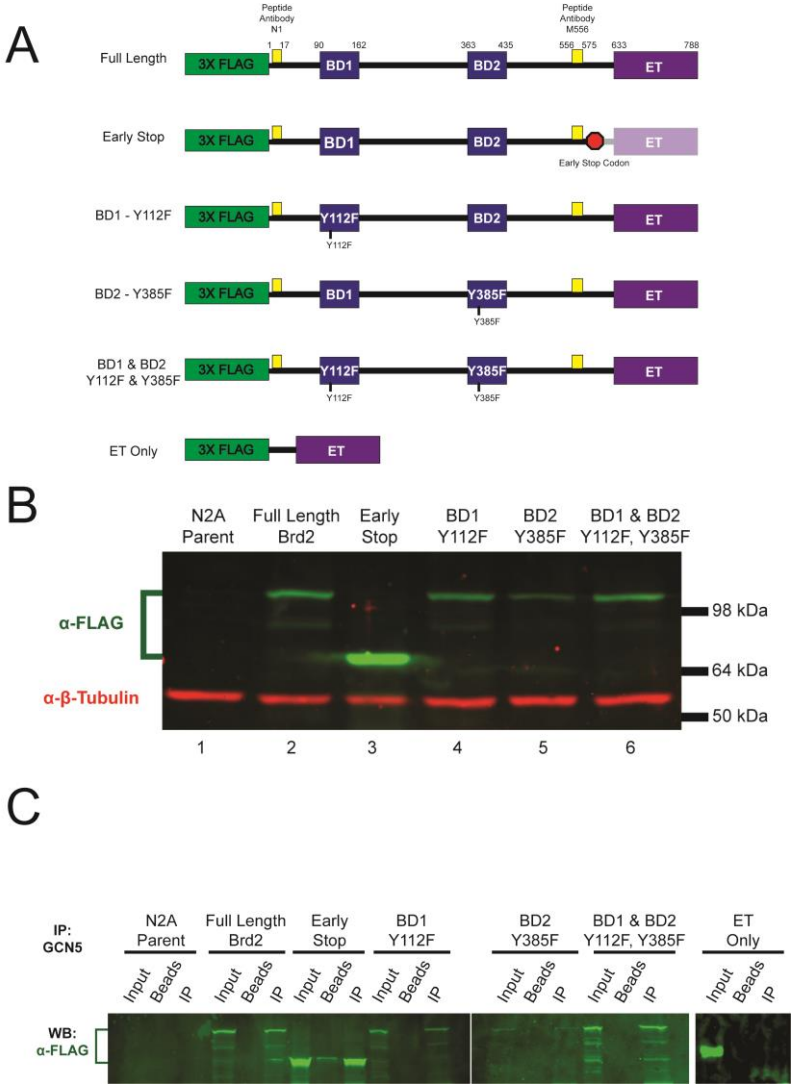

Figure 2: BRD2 and GCN5 interact *in vitro,* independent of the ET domain of BRD2

(A) Schematic of FLAG-tagged BRD2 constructs used. (B) Whole cell extracts from stable N2A cell lines were probed with anti-FLAG and anti-β-tubulin antibodies. (C) Whole cell extracts were immunoprecipitated with an anti-GCN5 antibody and probed with anti-FLAG antibodies.

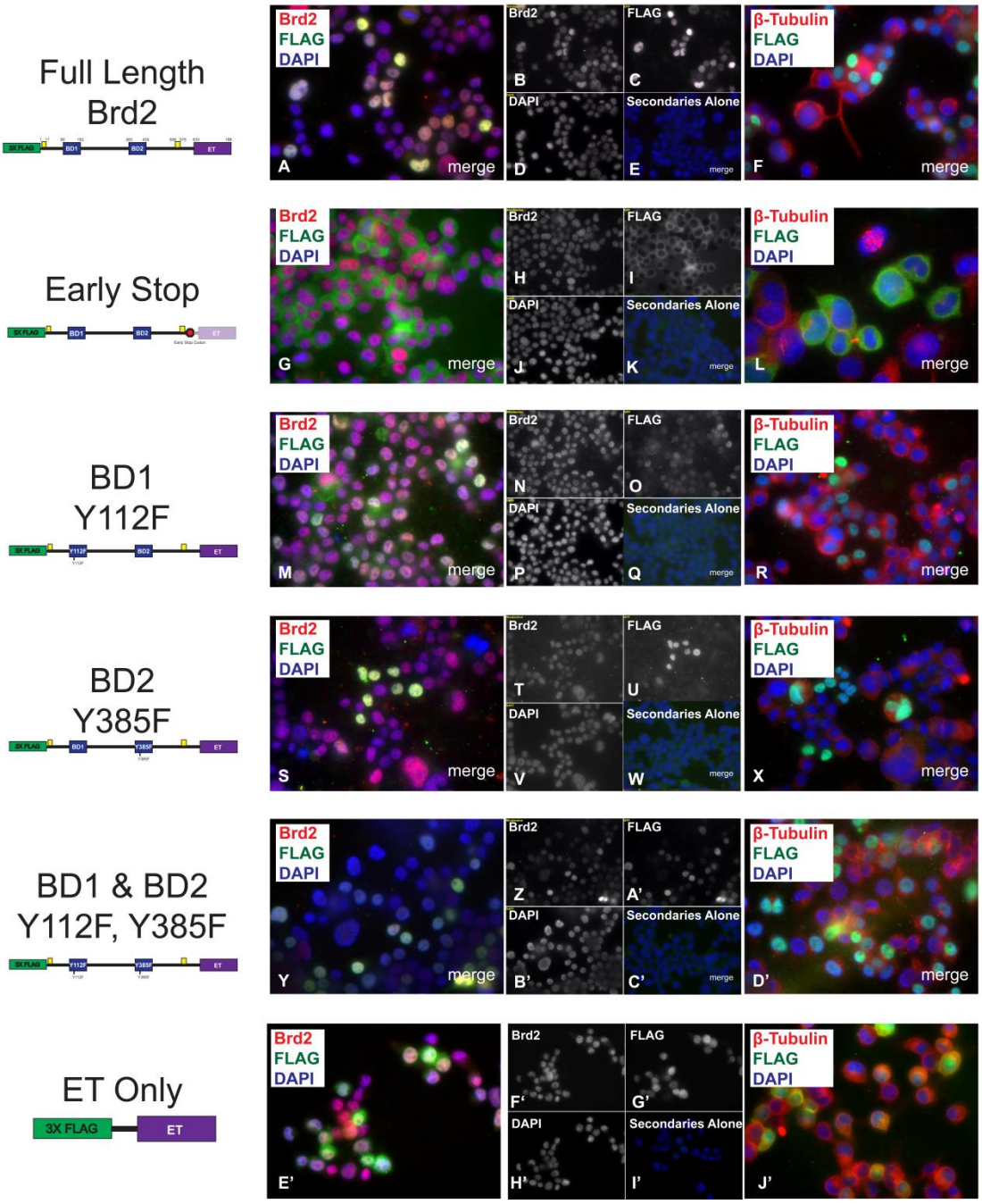

Figure 3: The ET domain of BRD2 is required for nuclear localization

The localization of FLAG-tagged BRD2 constructs in stable N2A cell lines was determined by immunofluorescence. Endogenous BRD2 (red) and FLAG tagged BRD2 (green) co-localize to the nucleus for Full Length, BD1 Y112F, BD2 Y385F and BD1 & BD2 Y112F, Y385F mutant lines, but BRD2 lacking the ET domain (Early Stop) localizes to the cytoplasm (Panels A-D, G-J, M-P, S-V, Y-B', E'-H'). Cytoplasmic localization was verified by co-staining for FLAG (green) and β-tubulin (red) (Panels F, L, R, X, D', J'). Controls with secondary antibodies alone show negligible background (Panels E, K, Q, W, C', I').

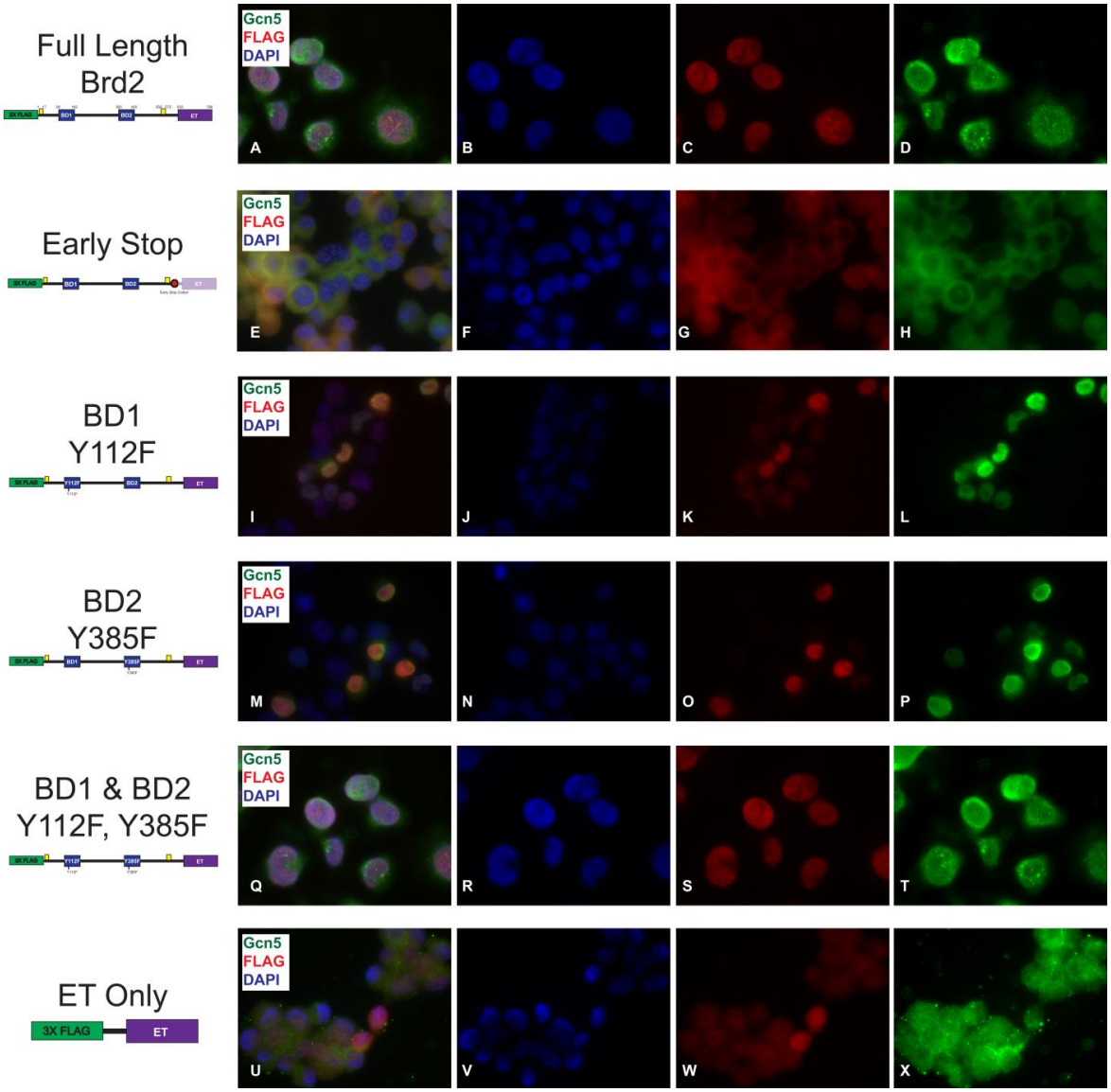

Figure 4: GCN5 protein is dependent on the BRD2 ET domain for maintenance of nuclear localization

FLAG-tagged BRD2 (red) and GCN5 (green) co-localize in the nucleus when BRD2 contains its ET domain (panels A-D, I-X) but co-localize in the cytoplasm in its absence (panels E-H).

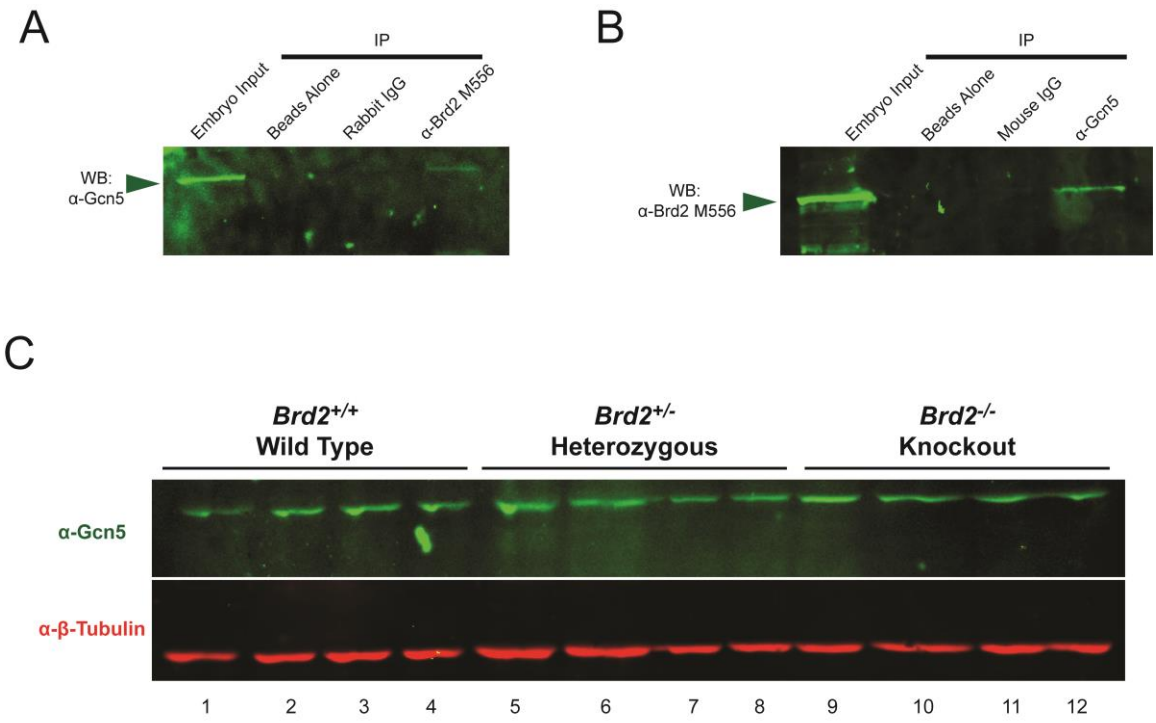

Figure 5: BRD2 and GCN5 interact *in vivo* during mammalian embryogenesis

Whole cell extract from wild type E11.5 embryos was used in reciprocal immunoprecipitations. (A) Embryo extract was immunoprecipitated with the α-BRD2 M556 antibody; Western blot using α-GCN5 antibodies detects a robust band. (B) Embryo extract was immunoprecipitated with an α-GCN5 antibody; Western blot using α-BRD2 M556 detects a robust band in the α-GCN5 lane and significantly weaker background bands in the control beads and mouse IgG lanes. (C.) Western blot for GCN5 shows equivalent protein expression in wild type, heterozygous and knockout embryos.

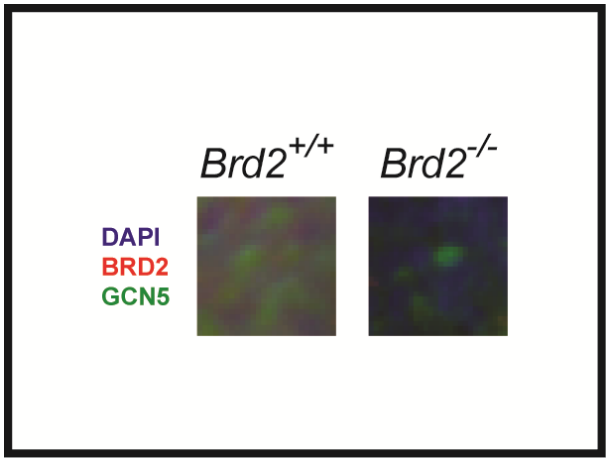

Figure 6: BRD2 and GCN5 Colocalize in the Nucleus of the Developing Neural Tube During Embryogenesis

Frozen sections from wild type *Brd2*$^{+/+}$ and knockout *Brd2*$^{-/-}$ E11.5 littermate embryos were probed with antibodies against BRD2 (red) and GCN5 (green), demonstrating co-localization in the nucleus.

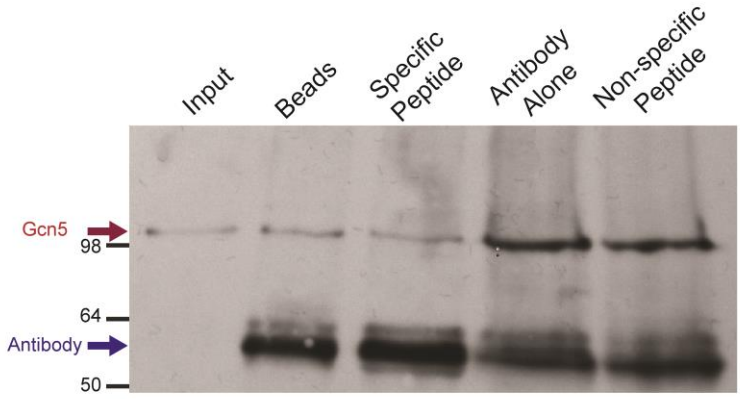

Figure S1: The interaction between BRD2 and GCN5 is specific

Whole cell extract from wild type E11.5 embryos was used for immunoprecipitation with the α-BRD2 M556 antibody; Western blot using α-GCN5 antibodies detects a robust band. Addition of the M556 specific peptide reduced the interaction seen to background levels, while the addition of a non-specific peptide did not alter the protein interaction

Supplemental Table 1: Primers used to generate Brd2 mutant constructs

Construct or Mutation	Forward Primer 5'-3'	Reverse Primer 5'-3'
Full Length Cloning	GCTCTAGACTGCAAAACGTGACTCCCCA	CGCGGATCCTTAGCCCGAGTCTGAATCGC
ET Only Cloning	TCAAAGAATTCTAATGCTACTACACTCAGCCATCCTGG	TCTTAGGATCCGCCCGAGTCTGAATCGCTGGTGTC
Early Stop Mutation	GGTGGTGGGGGTAGCTAGGCTACTACACTCAGC	GCTGAGTGTAGTAGCCTAGCTACCCCCACCACC
BD1 – Y112F	TATAATTTTGTGAAAATCCGGCAAACCCAGCTTCACAGC	GCTGTGAAGCTGGGTTTGCCGGATTTTCACAAAATTATA
BD2 – Y385F	CTTCTGCTCTTGGCCTTCATGATTTCCATGACATCATTAAA	TTTATTGATGTCATGGAAATCATGAAGGCCAAGAGCAGAAG

Supplemental Table 2: Antibodies Used for Western Blotting, Immunoprecipiation, and Immunohistochemistry

Protein & Assays	Antibody	[IF]	[WB]
3xFLAG – IF, IP, WB	Sigma, F3165	1:1500	1:5000
3xFLAG – IF, WB	Sigma, F2555	1:1000	1:5000
GCN5 – IF, IP, WB	Santa Cruz, sc-365321	1:100	1:300
β-tubulin – WB	ThermoFisher, RB-9249	1:1500	1:5000
BRD2 M556	Yenzym	1:100	1:300

Chapter 4:

The Roles of BRD2 in Development and Chromatin Associated Complexes

BRD2 in Development

These studies have underscored the importance of components of the chromatin targeting machinery throughout mammalian development. Given BRD2's broad expression pattern and diverse molecular interactions, a study of its function *in vivo* was crucial to understanding the role of the protein in development. Through the characterization of the *Brd2*-null mouse, we have established a model describing its role in development. Consistent with prior works which indicate that BRD2 plays a significant role in cell proliferation and cell cycle progression, we found that *Brd2*-null mice had a significant global growth defect as well as specific deficits in neuronal development [1-3]. While the global growth retardation can be ascribed to more broad deficits in cell cycle regulation or to proliferation as a whole, the specificity of the neural tube defects seen in null embryos does not fit this paradigm.

To better explain the hindbrain exencephaly and the failure of neural plate fusion in the knockout embryo, we propose a model in which the loss of BRD2 in the developing neural epithelium causes a deviation in gene expression from the normal pattern of development (Figure 1). In the normal developing embryo, the bromodomains of BRD2 bind to histone H4, acetylated at the H4K5 and H4K12 residues. This binding event may protect these residues from deacetylation, effectively stabilizing their modification state. Alternately, BRD2 may act as a histone code "reader" in recognizing these motifs and in turn recruit components of the chromatin remodeling or transcriptional machinery to promote gene expression. BRD2's recognition of these specific chromatin marks may be

coupled with its potential to mediate additional protein-protein interactions, ultimately acting to recruit or anchor additional histone code “writers” such as histone acetyltransferases to these specific loci and effectively induce the rapid spread of heterochromatic regions.

Previous work determining the binding affinities of the BRD2 bromodomains supports the notion of histone-modification specific binding of BRD2, while additional studies identifying and describing multiple binding partners of BRD2 protein suggest that it may act as a protein scaffold, bringing members of a number of different protein complexes in close proximity to specific gene loci [4-9]. Similarly, the work presented here in Chapter 2 links the loss of BRD2 protein in the developing mouse embryo to distinct changes in the formation of neural structures as well as significant changes in neuronal gene expression [10]. Furthermore, the data presented in Chapter 3, which demonstrate the interaction between BRD2 and the histone acetyltransferase GCN5 give justification to the idea that BRD2 binding to chromatin may modulate global changes in histone modification and secondary structure.

To attempt to separate the role of BRD2 in the developing neural tube from the evident global growth defects, additional studies were done using mice carrying a conditional allele of *Brd2,* in which the second coding exon is flanked by loxP sites (Figure 2A). The *Brd2*-null allele had undetectable levels of transcription, as well as undetectable levels of full length or truncated protein products, resulting in the complete loss of BRD2 (Figure 2B). Experiments using a global driver of Cre recombinase (Figure 2C, *Rosa-CreER*) proved useful to

determine that the conditional allele could readily recombine and result in a similar loss of both mRNA transcript and protein, as detected by qPCR, Western blotting and immunohistochemistry. When recombination was induced between E8.5 and E12.5, these mice displayed remarkably similar phenotypes to the *Brd2*-null mice, despite losing BRD2 protein at later stages in development, suggesting that BRD2 plays a role in regulating both early and late transcriptional events. Mice which lost *Brd2* in the Engrailed-2 expressing tissues (Figure 2E) developed normally and were born at appropriate Mendelian ratios. These mice had no apparent developmental, cognitive, or reproductive deficits and thus were not further examined.

Initial results using the *Wnt1*-CreER^{T2} mouse line were promising, showing similar hindbrain deformities and some growth retardation, but significant complications arose with regard to Tamoxifen administration. Despite careful titration of the Tamoxifen dosage, no dams were able to carry a litter to term and deliver live offspring, regardless of genotype. As such, the viability of these conditional knockout mice remains unknown. Additionally, studies examining the embryonic phenotypes were complicated by both the precision of Tamoxifen administration relative to the gestational age of the embryos as well as the variability seen within each genotype. Another confounding factor in these experiments was that fairly often entire litters of embryos, regardless of genotype, would display growth retardation or appear to be too young for the gestational age as determined by the presence of a copulation plug. This could be attributed to at least two factors. First, the Tamoxifen administration may

disrupt normal endocrine or hormonal functions which help maintain a healthy pregnancy. Additionally, while the presence of a copulation plug is good evidence of copulation, it is not ultimately indicative of successful fertilization, given variations in the timing of the female's estrus cycle among other factors.

However, several key pieces of data were identified through these studies. First, the conditional allele of *Brd2* which was generated does recombine to give a null allele, producing neither a coding mRNA transcript nor protein. The removal of *Brd2* at later points in development demonstrates that while the gene is expressed even in embryonic stem cells, it is required throughout the different stages of development. Early expression of the gene does not compensate for loss of the gene later in development, which suggests a continual role of BRD2 protein in transcriptional regulation. Additionally, while the loss of BRD2 in all tissues results in a severe growth retardation, this was seen to a less significant degree in the embryos lacking BRD2 in the *Wnt-1* expressing tissues, suggesting that conditional deletion of *Brd2* in such tissues or additional developmentally restricted domains without the complication of Tamoxifen toxicity may yield robust, reproducible results.

BRD2's Subcellular Localization

Although previous studied demonstrated a difference in BRD2 subcellular localization in proliferating neuronal precursors and terminally differentiated, quiescent neurons in the mouse spinal cord, little is known about the precise timing, mechanism, or significance of this change [11,12]. In our efforts to

understand the roles of each of BRD2's functional domains, we have identified a novel role of the ET domain in nuclear localization.

Bioinformatic analysis of the BRD2 protein sequence suggests a putative nuclear localization signal (NLS) in the hinge region between the second bromodomain and the C-terminal ET domain (Figure 3A). While this has not been empirically tested, the removal of this domain may affect BRD2's ability to dimerize, but whether this is dependent upon the aberrant localization of the protein or the specific sequence itself has not been investigated [13]. Previous works have described the localization of native BRD2 protein to be largely nuclear, with a presumably functional NLS (Figure 3B). Experimental data suggest that in terminally differentiated, quiescent neurons of the dorsal spinal cord, this localization changes significantly, with the majority of the BRD2 protein accumulated in the cytoplasm (Figure 3C). Whether the exclusion of BRD2 protein from the nucleus is causative of the change in proliferation state or the change in proliferation state results in the removal of BRD2 protein from the nucleus has not been further studied. Additionally, no nuclear export signal has been determined by either experimentation or bioinformatics studies.

In our proliferating cultures of N2A cells, FLAG-tagged Full Length BRD2 and Early Stop BRD2 protein was readily expressed in both transient transfections as well as in stable cell lines. In all cases, the full length protein was abundant in the nuclei, with minimal localization in the surrounding cytoplasm. This result mimics the localization of endogenous BRD2 protein, and confirms that our transgenic protein can interact appropriately within the cell (Figure 3D).

In contrast, both the transiently transfect and stably expressed FLAG-Early Stop BRD2 protein accumulates in the cytoplasm of these undifferentiated, actively proliferating cells (Figure 3E). This result is in direct contradiction to what would be predicted, given prior experimental evidence. The ability of the N2A cells to continue to grow unhindered by the nuclear exclusion of the truncated BRD2 protein is not unexpected; this cell line contains endogenous levels of BRD2 protein, whose nuclear localization remains unchanged. This suggests that an active proliferation state alone is not sufficient to import truncated BRD2 protein into the nuclei. In at least this instance, there are crucial regulatory elements in the structure of the ET domain.

There are several possible mechanisms by which truncated BRD2 is sequestered in the cytoplasm. For example, it is possible that the loss of the ET domain prevents nuclear import (Figure 3F). This could be accomplished by several independent mechanisms. The most straightforward hypothesis is that ET domain could contain a novel, cryptic nuclear localization signal, upon which BRD2 localization is ultimately dependent. This could be probed experimentally by producing an additional number of mutant constructs which express smaller portions of the ET domain. If any of these constructs confer the ability to localize to the nucleus, they can then be rigorously examined for NLS ability by attaching them to a reporter protein such as GFP to determine if the sequence is sufficient to bring an artificial protein to the nucleus. The identification of a novel nuclear localization signal would have significant impact on the study of nuclear proteins and import/export pathways.

Another possibility for the cytoplasmic sequestration of Early Stop BRD2 is that the structural portion of the ET domain is required to prevent the masking of the putative NLS. To test this hypothesis, additional structural elements, such as a GFP fusion protein, could be added at the C-terminus of the protein to determine if this results in alterations in the subcellular localization of the protein. If the masking of the putative NLS is what drives BRD2 out of the nucleus, identifying potential conformational changes in the protein or which additional proteins bind to block the nuclear localization signal upon differentiation would be crucial to understanding the regulation of BRD2 protein mechanics.

Additionally, the loss of the ET domain may not result in the inability of BRD2 to locate to the nucleus, but instead may affect its ability to accumulate there. This model suggests that Early Stop BRD2 is imported into the nucleus, but lacks the ET domain, which would effectively act as an “anchor” to integrate the protein into complexes which result in its nuclear retention (Figure 3G). As such, what is seen as nuclear exclusion is the result of an imbalance in import/export pathways, with BRD2 which is unable to form the necessary complexes in the nuclei being more rapidly removed or degraded than the ability of the cell to import additional molecules. To test this hypothesis, additional experiments could be performed using inhibitors of nuclear export, such as leptomycin B. Leptomycin B is an inhibitor of CRM1-dependent nuclear export, which requires the presence of a nuclear export signal. In such an experiment, the FLAG- Early Stop BRD2 cell lines would be treated with low concentrations of leptomycin B and then analyzed to determine how much, if any, truncated BRD2

protein has accumulated in the nuclei. If more protein is present in the nuclei than is present in the cytoplasm, it could be concluded that there is no predominant defect in nuclear import, but there is a significant defect in nuclear retention. Additional studies blocking nuclear export could be performed, using BRD2 constructs with additional portions of the ET domain, to determine which, if any features of the ET domain are able to confer the ability of the protein to be retained in the nucleus.

BRD2, GCN5 and the SAGA Complex

The identification of the novel interaction between the chromatin "reader" BRD2 and the "writer" histone acetyltransferase GCN5 could have significant impact on our understanding of how the histone code is interpreted and reproduced in a cellular context. However, the context in which these two proteins interact is so far unknown. As mentioned previously, BRD2 has been shown to interact with numerous proteins, including members of the Swi/Snf family of chromatin remodelers, the massive transcriptional coactivating Mediator complex, and members of the transcriptional apparatus including E2F, TAF1 and the TATA binding protein [9]. Similarly, GCN5 is one of the core components of the SAGA (Spt-Ada-Gcn5 Acetyltransferase) complex, a transcriptional coactivator complex which aids RNA polymerase II transcriptional initiation. Whether the interaction between BRD2 and GCN5 is accompanied by any or all of these other complexes is as yet unknown.

To test the context in which BRD2 and GCN5 interact, several lines of investigation could be undertaken. Protein isolates from tissue culture cells, whole embryos, or specific adult tissues could be subjected to co-immunoprecipitation by α-BRD2 antibodies, α-GCN5 antibodies, or both together and resulting complexes separated by SDS-PAGE. The remaining protein isolates could then be probed by panels of antibodies to test for interactions with each of the proteins known to take part in either context. The benefit of this approach is that it could be performed without access to additional lab equipment or experimental expertise, as the only de facto requirements are suitable protein input and sufficient antibodies for the proteins of interest. However, there are several drawbacks to this approach. The first limitation is that the only interactions that will be identified are ones which are either already likely, due to known interactions between the protein being probed and either BRD2 or GCN5. While this would be sufficient to help better classify the interaction into one or more complex contexts, it would be unable to resolve any novel complexes which may contain these two chromatin associated proteins. Additionally, these studies are further restricted to the availability of suitable antibodies for Western blotting and immunoprecipitation. Indeed, initial studies using this method were done to try to identify the presence of additional SAGA members. Unfortunately, they were not successful, largely due to antibodies which were not sufficiently stringent to identify mouse SAGA components.

A more unbiased approach to identifying whether BRD2 and GCN5 interact in a SAGA-dependent or SAGA-independent manner would be to

undertake a proteomics-based experimental approach. In this fashion, protein preparations and immunoprecipitations would be carried out as previously described. These isolates could then be screened by mass spectrometry to identify which proteins immunoprecipitated in complexes with BRD2 and GCN5. The benefits of this approach are that it would be able to identify proteins whose interaction with either BRD2 or GCN5 has not yet been identified and that it is only dependent on the quality of the input protein preparations and the ability of the antibodies used for immunoprecipitation. However, this type of experiment would require specialized equipment and expertise, as well as additional time and resources to analyze the resulting data.

If either approach is used, it is possible that the data may suggest that BRD2 is an alternate member of the SAGA complex, likely in a tissue-specific manner. As such, we would hypothesize that additional experiments to find binding partners of both proteins would reveal a large number of canonical SAGA components, such as Spt3 or ADA2 (Figure 4, upper). Alternately, the interaction between BRD2 and GCN5 could be in a SAGA-independent manner, identifying a new context for GCN5 histone acetyltransferase function. In this case, we would anticipate finding a number of novel interacting proteins, whose interactions with BRD2 and GCN5 have not yet been fully vetted, or we would identify a subset of the known BRD2 interacting proteins and complexes (Figure 4, lower).

Independent or Interdependent Recruitment to Chromatin

Another key component to understanding the roles BRD2 and GCN5 play in a cellular context is identifying the order in which they are recruited to their respective chromatin binding sites. One possibility is that GCN5 initially binds chromatin, forming its associated protein-protein interactions with members of the SAGA complex or additional transcriptional regulators. In this model, BRD2 is subsequently recruited to the site and is able to bind the hyperacetylated chromatin surrounding the GCN5 complex. This recruitment eventually leads to transcriptional activation, as BRD2 is able to recruit further proteins to the site, such as TBP (Figure 5A). It is equally likely that the reverse situation would occur: BRD2 recognizes its chromatin target motifs and binds, bringing along some of its associated factors, such as Swi/Snf or Mediator components. BRD2, in turn, recruits GCN5 to the locus (Figure 5B). Upon recruitment to the site, GCN5 can then rapidly hyperacetylate the surrounding chromatin and aid in the transcriptional activation of the gene of interest.

A third likely scenario is that BRD2 and GCN5 each find and bind their respective chromatin modifications, each bringing with them a specific subset of their interacting proteins (Figure 5C). This model allows for the highest degree of flexibility as it does not preclude BRD2 and GCN5 acting in an independent fashion. In the instances where both BRD2 and GCN5 are present at a given locus, the additional recruited factors from both proteins are able to guide chromatin modification or remodeling to allow efficient transcription of the locus. In instances where only one of the proteins is present, they would be able to

contribute to the remodeling or activation in a separate, distinct manner, which could rely on the associations with as yet unidentified complexes to help guide, speed, or regulate gene activation.

To test this order of addition, several approaches could be undertaken. Fluorescently tagged BRD2 and GCN5 proteins could be introduced into cell lines and the order of recruitment to bait loci could be monitored in live cells in real time. While this would be technically challenging, it could readily indicate which, if either, protein is required to recruit the other to a given locus. However robust the data, this would not necessarily mean that the order of assembly at a given locus is representative of all loci. It is not only possible but also likely that each of these three possible recruitment scenarios occur with some level of probability, with none being mutually exclusive of the others.

In addition to diversity in the order of assembly, the novel association of BRD2 and GCN5 opens the possibilities of other chromatin “reader” and “writer” interactions. BRD2 could interact in other tissue-specific contexts with other histone acetyltransferases, such as P300 or PCAF. It is also likely that each of the other BET family members, BRD3, BRD4, and BRDT each interact with their own chromatin remodeling proteins, including other histone acetyltransferases, or histone methyltransferases. In this scenario, the precise combination of chromatin “readers” and recruited “writers” could effectively guide not only the speed with which transcription is initiated, but also modulate the rate at which it occurs. This combinatorial effect of multiple independent proteins assembling at

a given site could be key to understanding the precise control over gene expression which governs much of the cell cycle.

BRD2 in Chromatin Modifying Complexes

Through these studies, the requirement of BRD2 protein in normal mammalian embryonic development has been demonstrated, and suggestive data from *in vitro* cell culture experiments point to critical roles in the maintenance of chromatin state. Previously, several members of the BET family of proteins have been implicated in modulating chromatin and transcriptional states. Brdt, the testis-specific variant, is required for the organization of the chromocenter and is essential for germ cell differentiation [14-16]. Similarly, BRD4 associates with acetylated chromatin and plays a crucial role in maintaining the higher order structure, while also acting as an atypical kinase to phosphorylate RNA polymerase II [17-19]. Additional research has shown that BRD4's ET domain is essential for transcriptional activation of the elongation factor pTEFb, a feature which is dependent upon ET-mediated interactions with protein complexes [20]. Given the diverse interactions of the other BET family members, a better understanding of BRD2's network of interactions is critical to understanding its roles in cell growth and mammalian development.

This work has demonstrated that BRD2's interaction with GCN5 is not dependent upon the ET domain, which is unique among the known BET family member interactions, which largely depend upon the "scaffolding" ET domain. This interaction of a BET family member and the key member of the SAGA complex may result in a rapid change in chromatin state, with resulting changes

in transcription. In stark contrast to the ET domain of Brd4, the ET domain of BRD2 is required to maintain the protein's nuclear localization, and identifying and understanding its interactions with multiprotein complexes is of the highest importance in order to understand how chromatin readers and writers are linked to transcriptional outcomes.

BRD2 and Human Disease

One of the most crucial applications of basic biological research is the application of the findings to further the understanding of normal human development and the onset and progression of disease states. In the human population, *BRD2* may play multiple roles in clinical diagnoses. Several single nucleotide promoter mutations found in human *BRD2* alleles have been linked to an increase in susceptibility to juvenile myoclonic epilepsy (JME), a seizure disorder that frequently has an onset during adolescence [21]. Similar mutations in regulatory elements have been associated with the photoparoxysmal response (PPR), a related seizure disorder in which seizures are triggered by light stimulation [22]. While the knockout mouse does not mirror the disorders seen in cases of human mutation, the mouse model does provide insight into a more severe defect. Whereas the human mutations are believed to result in subtle, graded changes in gene expression or possibly aberrant tissue expression, the striking defects seen in the mouse neuronal tissues result from a complete absence of protein. The differences in severity of disease state between patients with slight aberrations in gene expression levels and the drastic defects seen in

the knockout mice coincide with the level of severity of phenotype; the patients are largely normal, with some neurological involvement while the null mice have severe, lethal defects. This supports the model that the expression levels of *BRD2*, alone or in combination with other factors, can increase the incidence of seizure disorders.

The prominent neural tube defects of the *Brd2*-null mouse described here also suggest that BRD2 may play a role in the development of one of the most common birth defects, spina bifida. This congenital disorder has a peak incidence of 1 in 1000 live births in some populations, and carries a 0.7 in 1000 incidence on average in the United States [23]. As expected, open neural tube defects such as spina bifida occur due to an incomplete closure of the embryonic neural tube, which may allow portions of the spinal cord to extend beyond the sacral and lumbar vertebrae. Accordingly, children born with these defects suffer from a wide range of complications, including paralysis, neurological deficits and learning delays. While there have been some successes at prenatal intervention through the MOMS trial (Management of Myelomeningocele Study) at the Children's Hospital of Philadelphia and the University of California at San Francisco, surgical repair carried a significant risk to both mother and fetus, and is a complex procedure which is not readily available in many areas [24]. Primarily, there is a focus on prevention by increasing the mother's intake of folic acid throughout her early pregnancy.

Although there is a strong genetic component to the development of neural tube defects, few genes, such as *VANGL1,* which plays roles in both the

planar cell polarity and convergent extension pathways, have been identified as having a causal role in the development of subsets of these disorders [25]. A large number of mouse models of neural tube defects have been characterized, including the spina bifida model, *curly tail*, whose defect is potentially a result of low expression of the transcription factor *Grainy-head-like-3* [26-28]. One of the modifiers of the *curly tail* gene has been mapped to the region in which the murine *Brd2* gene is located [29]. Similarly, mouse embryos which lack proper expression of *GCN5* share a similar phenotype, and the work described here demonstrates that both BRD2 and GCN5 interact during mouse embryonic development [30,31]. Taken together, these data further strengthen the argument that *Brd2* may play a critical role in neural development and neural tube defects such as spina bifida.

In conclusion, the experiments described here provide a both an in depth analysis of BRD2's role in mammalian development as well as a framework for understanding how chromatin readers and writers could potentially interact in a combinatorial fashion to give precise control over transcriptional regulation. Future biochemical studies will allow us to compose a more complete picture of how tissue specificity and protein domain structure work together to drive the cell toward an appropriate cell fate, while additional genetic and developmental analyses will allow us to gain insight into the contributions of BRD2 in different cell populations result in both normal development and the onset of disease states.

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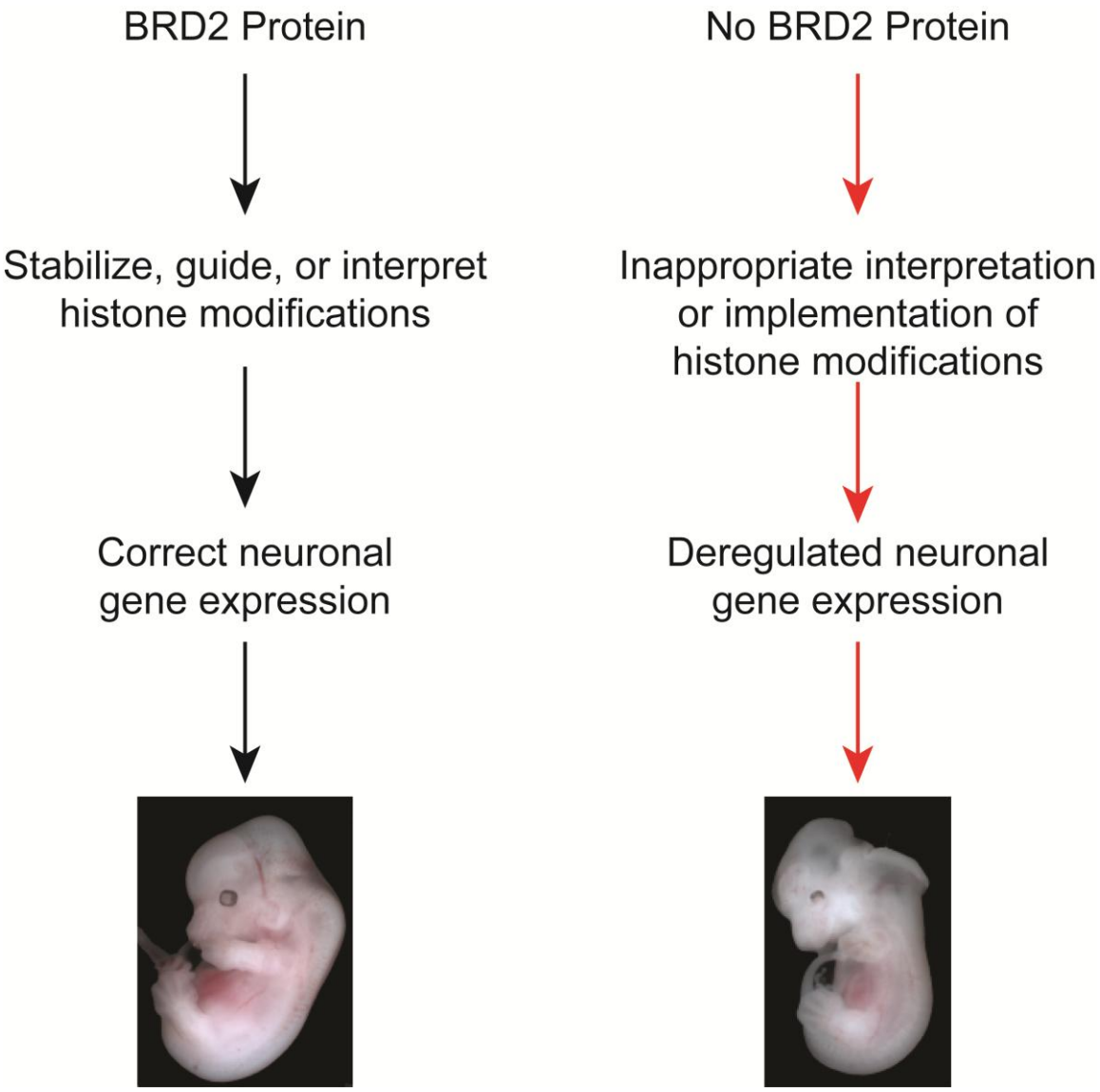


Figure 1: Model of Neural Tube Defects in *Brd2*-null Embryos

(*Left*): A probably role of BRD2 protein in normal mouse development

(*Right*): A likely model in which the loss of BRD2 protein in the *Brd2*-null embryos results in the inappropriate interpretation or implementation of histone modifications. This ultimately results in changes in gene expression of those genes responsible for the continued development of the neural tube.

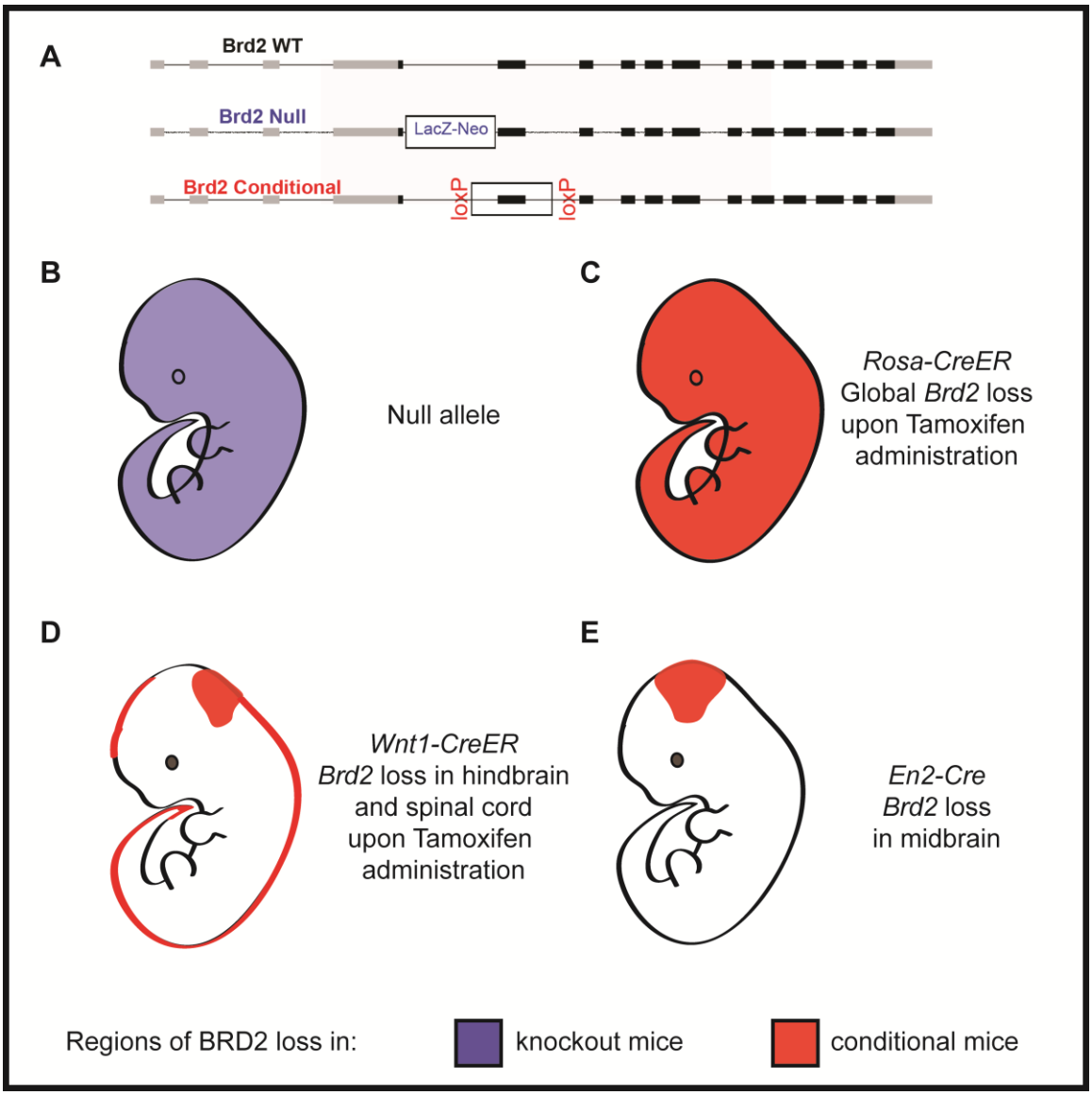
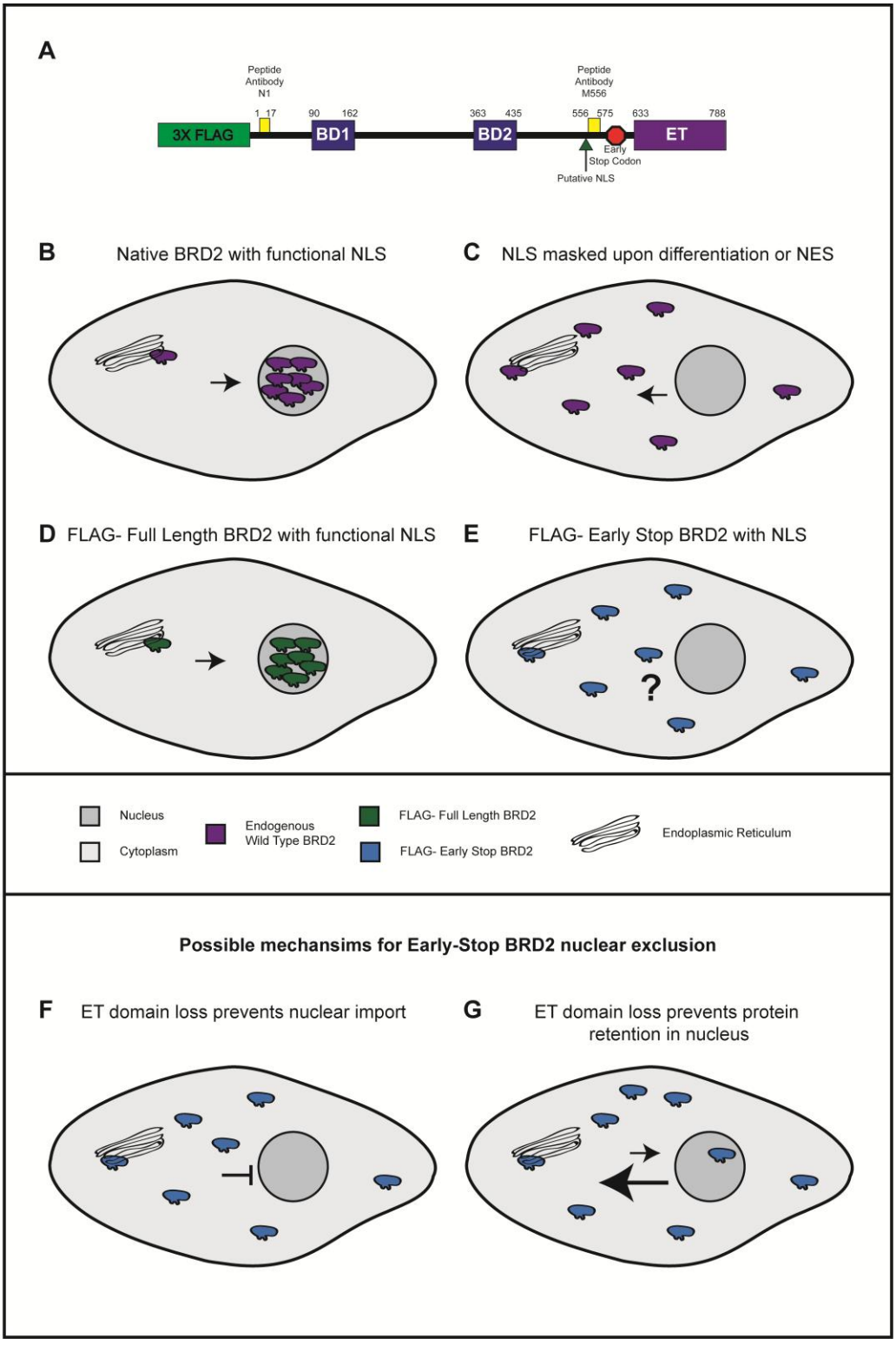


Figure 2: Preliminary Data from the *Brd2* Conditional Allele

A. Schematic representation of the three *Brd2* alleles used in these studies. Coding exons are depicted by black boxes, while non-coding exons are gray. B-E. Illustrations of mouse embryos with regions of BRD2 loss for each mouse line highlighted. Blue regions denote loss from the null allele, while red regions denote regions of expected loss from the conditional allele.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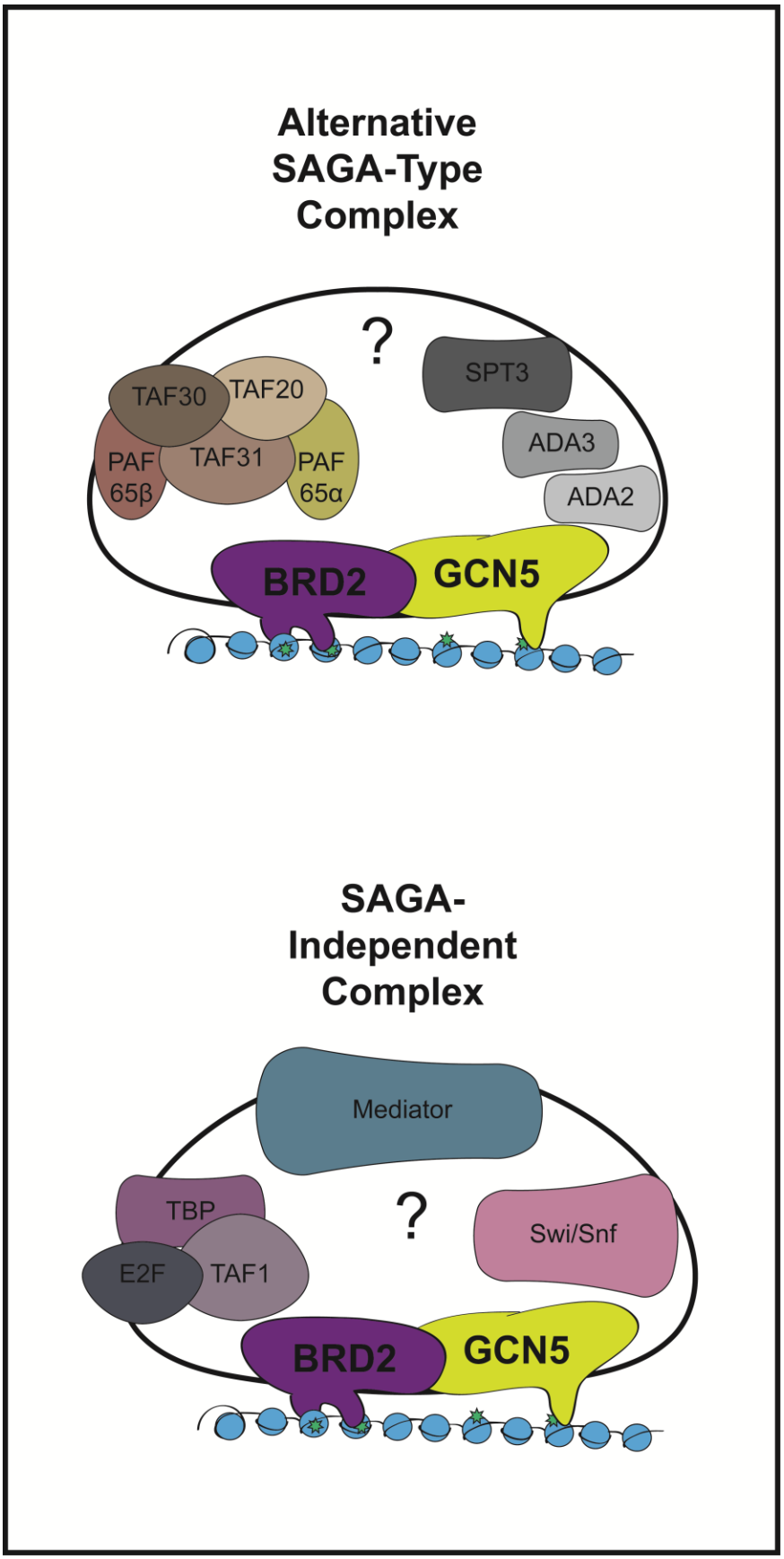
Peptide Antibody M556
1 17
90 162
363 435
556 575
633 788
3X FLAG
BD1
BD2
ET
Early Stop Codon
Putative NLS
B Native BRD2 with functional NLS
C NLS masked upon differentiation or NES
D FLAG- Full Length BRD2 with functional NLS
E FLAG- Early Stop BRD2 with NLS
?
Nucleus
Cytoplasm
Endogenous Wild Type BRD2
FLAG- Full Length BRD2
FLAG- Early Stop BRD2
Endoplasmic Reticulum
Possible mechansims for Early-Stop BRD2 nuclear exclusion
F ET domain loss prevents nuclear import
G ET domain loss prevents protein retention in nucleus

Figure 3: The subcellular localization of BRD2 is dependent up on cell state and the ET domain

A. Schematic representation of the FLAG-BRD2 proteins expressed in N2A cell lines. The domain structure, in addition to the locations of the antibody recognition motifs, putative NLS, and early stop codon are noted. B. Previous experiments have demonstrated that native BRD2 protein accumulates in the nuclei of actively growing cells, including developing neural tissue. C. In contrast, BRD2 protein has been found in the cytoplasm of differentiated neurons, although the mechanism of its sequestration has not been determined. D. Full length FLAG-BRD2 protein behaves in a manner similar to the native BRD2 protein. E. In contrast, the truncated Early Stop FLAG-BRD2 protein, which lacks the ET domain, is largely excluded from nuclei in proliferating cell cultures. F&G: Possible mechanisms for the nuclear exclusion of FLAG-Early Stop BRD2 protein from nuclei. F. The loss of the ET domain removes additional, necessary, nuclear import signals or protein interactions, or causes conformational changes which mask the putative NLS, preventing or reducing active import. G. The loss of the ET domain does not affect the active transport of the protein to the nucleus, but prevents binding interactions which effectively anchor the protein within it. As a result, Early Stop BRD2 is exported from the nucleus at a rate greater than the import rate, resulting in significant retention of BRD2 in the cytoplasm.


Alternative
SAGA-Type
Complex
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SPT3
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ADA3
PAF
65β
TAF31
PAF
65α
ADA2
BRD2
GCN5
SAGA-
Independent
Complex
Mediator
TBP
?
Swi/Snf
E2F
TAF1
BRD2
GCN5

Figure 4: BRD2 and GCN5 May Act in Alternate Complexes

The interaction between BRD2 and GCN5 may be in either a SAGA-dependent context (above), which suggests that BRD2 could act as a component of SAGA, possibly in a tissue- or development- restricted state. As depicted here, some of the other core components of the SAGA complex, such as the core members ADA2, ADA3, and SPT3, would play a role in guiding GCN5 function. Alternately, BRD2 and GCN5 may interact in a SAGA-independent manner (below). In such an instance, the complex may include any or all of the known BRD2 interacting proteins, such as components of Mediator, Swi/Snf, or the transcriptional regulators TBP, TAF1, and E2F.

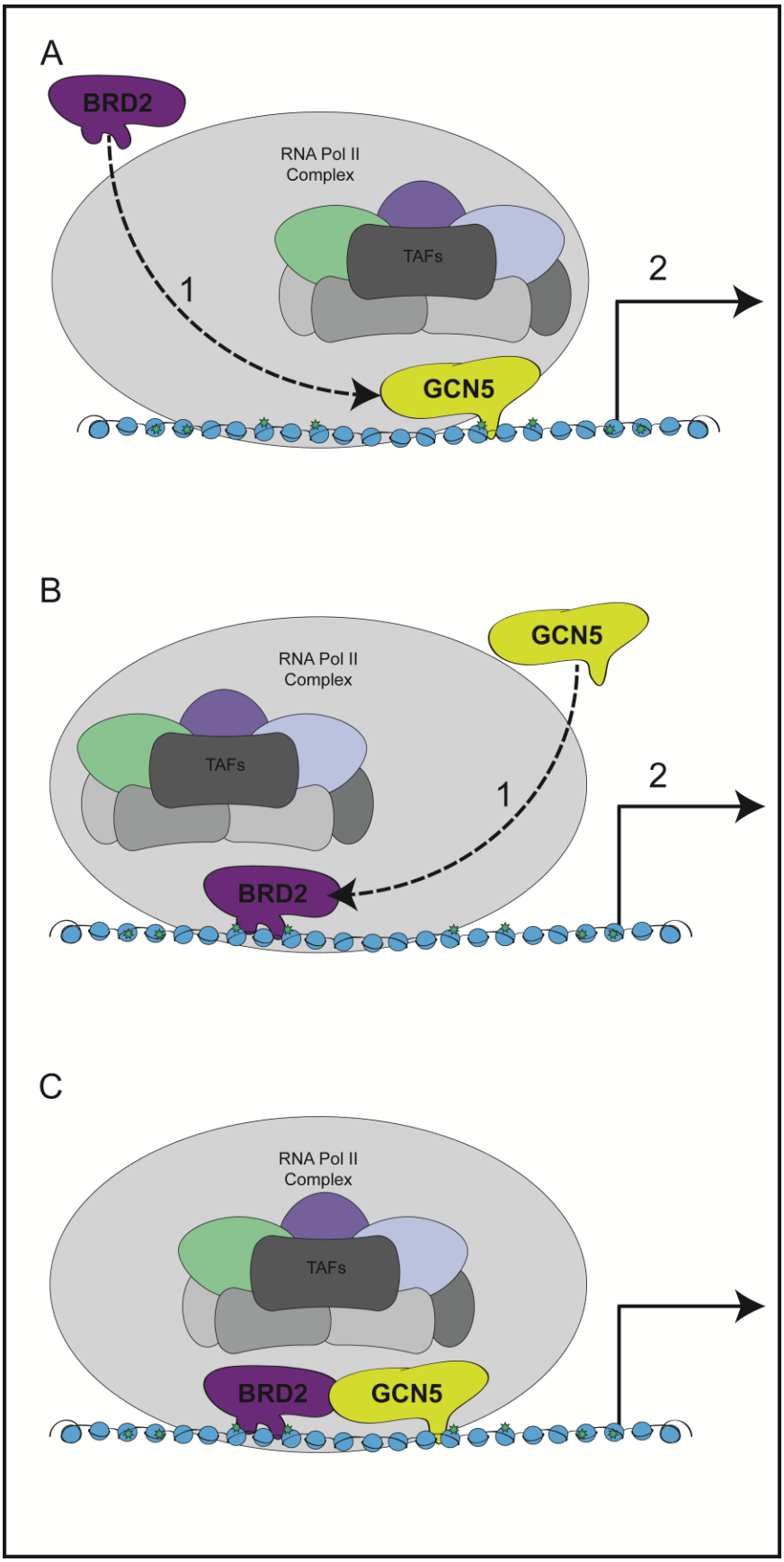
A
BRD2
RNA Pol II
Complex
TAFs
1
2
GCN5
B
GCN5
RNA Pol II
Complex
TAFs
1
2
BRD2
C
RNA Pol II
Complex
TAFs
BRD2
GCN5

Figure 5: Models for the order of transcriptional activation

Three possible models for the order of recruitment of the transcriptional machinery to BRD2 and GCN5 recognition sites. A. GCN5 binding to chromatin and multi-protein complexes recruits BRD2 to its recognition motifs and allows for BRD2 to modulate additional protein interactions, resulting in transcriptional activation. B. BRD2 binding to chromatin and multi-protein complexes brings GCN5 to its chromatin recognition motif, allowing GCN5 HAT activity to acetylate nearby residues, resulting in transcriptional activation. C. BRD2 and GCN5 independently bind to their respective histone modifications and together recruit appropriate chromatin remodeling and transcriptional machinery.