FUNCTION OF BROMODOMAIN AND EXTRA-TERMINAL MOTIF PROTEINS (BETs) IN GATA1-MEDIATED TRANSCRIPTION

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ABSTRACT

FUNCTION OF BROMODOMAIN AND EXTRA-TERMINAL MOTIF PROTEINS (BETs) IN GATA1-MEDIATED TRANSCRIPTIONAL ACTIVATION

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Bromodomain and Extra-Terminal motif proteins (BETs) associate with acetylated histones and transcription factors. While pharmacologic inhibition of this ubiquitous protein family is an emerging therapeutic approach for neoplastic and inflammatory disease, the mechanisms through which BETs act remain largely uncharacterized. Here we explore the role of BETs in the physiologically relevant context of erythropoiesis driven by the transcription factor GATA1. First, we characterize functions of the BET family as a whole using a pharmacologic approach. We find that BETs are broadly required for GATA1-mediated transcriptional activation, but that repression is largely BET-independent. BETs support activation by facilitating both GATA1 occupancy and transcription downstream of its binding. Second, we test the specific roles of BETs BRD2, BRD3, and BRD4 in GATA1-activated transcription. BRD2 and BRD4 are required for efficient transcriptional activation by GATA1. Despite co-localizing with the great majority of GATA1 binding sites, we find that BRD3 is not required for GATA1-mediated transcriptional activation. However, exogenous BRD3 efficiently compensates for BRD2 loss, suggesting that BRD2 and BRD3 function redundantly. Third, we tested the role of BETs in mitotic bookmarking. We present evidence that mitotic binding by BRD4, the BET most strongly implicated in preserving transcriptional state during mitosis, is extensively remodeled during this phase of the cell cycle. Additionally, disruption of mitotic BET occupancy has no measurable impact on post-mitotic gene reactivation, calling into question the role of BETs in mitotic bookmarking. These results elucidate new factors critical for GATA1-mediated erythropoiesis. In addition to furthering understanding of

the mechanisms underlying BET function, these findings have important consequences for the rational development of BET inhibitors.

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CHAPTER 1. INTRODUCTION

Transcriptional regulation

Lineage-specific gene expression

High-throughput technologies have made comprehensive description of gene expression patterns routine. The resulting wealth of information being generated holds great promise both in understanding basic biology and in directing rational therapeutic intervention. Fulfillment of this promise will require a better understanding of the mechanisms responsible for the gene expression patterns being cataloged. Research over the past several decades has revealed a number of the factors involved.

Lineage-specific master transcription factors drive gene expression programs. These proteins regulate transcription through association with specific DNA sequences in genomic regulatory regions. At promoter regions adjacent transcription start sites, their binding can lead to recruitment of general transcription factors and RNA polymerase II to activate gene expression. However, the DNA sequence motifs recognized by transcription factors occur at many more sites in the genome than are actually bound, and most transcription factors can both activate and repress transcription. Transcription factor specificity and activity depends critically on additional co-activator and co-repressor proteins and the local chromatin environment.

Much of chromatin structure involves the wrapping of DNA around nucleosome octamers of histone proteins. Both histones and DNA are regulated in large part by covalent modification. The number of possible combinations of modifications on even a single histone tail greatly exceeds the number of genes in the genome and provides a vast number of regulatory modes. Regulation can be dynamic throughout the cell cycle, adding further regulatory complexity. The combination of all these factors creates an elaborate chromatin language controlling gene expression in each cell (Berger, 2007; Strahl & Allis, 2000). This dissertation focuses on the role of the ubiquitous Bromodomain and Extra-Terminal motif protein (BET) cofactors in the gene

expression program induced by the master erythroid transcription factor GATA1. Through binding to acetylated histones and acetylated GATA1, BETs integrate transcription factor binding with chromatin structure and regulate cellular transcription.

Acetylation of histones and transcription factors

Histone lysine acetylation is associated with transcriptionally active regions of the genome (Allfrey, 1966; Hebbes, Thorne, & Crane-Robinson, 1988). Acetylation may contribute to activation by altering nucleosome structure or through signaling to recruit other factors. Structural alterations may result from neutralization of positive charges or through steric effects. Recent work has supported the possibility that acetylation of lateral histone surfaces contacting DNA may destabilize nucleosomes (Tropberger et al., 2013). However, the most well-characterized histone modifications are on the tails of these proteins where the primary mode of action is likely signaling. Histone acetylation is written by histone acetyltransferases (HATs) and erased by histone deacetylases (HDACs). Rapid turnover of acetylated lysines makes this modification ideal for temporally precise transcriptional control. While the best characterized HAT substrates are histones, these enzymes modify thousands of proteins and regulate virtually every cellular process (Choudhary et al., 2009; Kouzarides, 2000). Notably, acetylation of transcription factors regulates stability and activity (Soutoglou, Katrakili, & Talianidis, 2000), and many HATs self-regulate through auto-acetylation (Blanco-García, Asensio-Juan, la Cruz, & Martínez-Balbás, 2009; P. R. Thompson et al., 2004). HDACs often mediate transcriptional repression as part of large multi-protein co-repressor complexes. Interestingly, pharmacologic HDAC inhibitors have found in clinical use in cancer and other settings demonstrating the feasibility of therapeutic targeting of such general chromatin mechanisms. HDAC inhibitors support cellular reprogramming during the creation of induced pluripotent stem cells, emphasizing the importance of acetylation in transcriptional identity (Huangfu et al., 2008; Kazantsev & Thompson, 2008). Acetyl-lysine marks are recognized largely by bromodomains, although other histone acetyl-lysine

binding domains have been described (Ishizaka et al., 2012; Marmorstein & Zhou, 2014). Bromodomains are often found in proteins with HAT activity or in multiples, such as the tandem bromodomains in Bromodomain and Extra-terminal motif proteins (BETs). Recognition of acetylated lysines by bromodomains is required for the association of BETs with chromatin, but may not be sufficient (Filippakopoulos & Knapp, 2014; Filippakopoulos et al., 2012). Large gaps remain in our understanding of how interpreters of acetylation such as bromodomain proteins interact with histones and transcription factors to create and maintain cellular transcription patterns.

Mitotic bookmarking: maintenance of transcriptional state through cell division

Once established in development, the transcriptional identity of individual cells needs to be maintained while they continue to cycle. Both DNA replication and mitosis challenge the maintenance of the information required to maintain cellular identity. During mitosis transcription is silenced, chromatin is condensed, and transcription factors are removed (Martínez-Balbás, Dey, Rabindran, Ozato, & Wu, 1995; PRESCOTT & Bender, 1962; Fangwei Wang & Higgins, 2013). Recent work examining the three dimensional structure of the mitotic genome show that it is grossly effaced (Naumova et al., 2013). Likely because of these features, mitotic nuclei are reprogrammed far more efficiently than interphase nuclei (Halley-Stott, Jullien, Pasque, & Gurdon, 2014). This has led to the proposal that mitosis represents both a challenge to cellular identity as well as an opportunity for lineage change. The remnants of structure preserved during this period must be sufficient for resumption of lineage-appropriate transcription at the beginning of the next cell cycle.

Patterns of nuclease sensitivity are to a large degree maintained in mitotic chromatin (Gazit, Cedar, Lerer, & Voss, 1982; Hsiung et al., 2014; Martínez-Balbás et al., 1995). This observation suggests that certain transcription factors, "mitotic bookmarks", might remain bound to mitotic chromatin to allow newborn cells to remember which genes were active during the

previous cell cycle (John & Workman, 1998; Michelotti, Sanford, & Levens, 1997). DNA methylation and some histone modifications remain largely unchanged during mitosis and may also function in mitotic memory of transcriptional state (Campos, Stafford, & Reinberg, 2014; Ghenoiu, Wheelock, & Funabiki, 2013). However, neither of these features easily explains the nuclease patterns observed. A likely explanation is provided by the growing number of transcription factors found to remain associated with mitotic chromatin (Kadauke & Blobel, 2012).

Several studies have provided evidence that mitotic bookmarking could be important for preservation of cell identity through mitosis. Depletion of some mitotically-bound transcription factors have been shown to delay post-mitotic transcriptional reactivation in several settings (Blobel et al., 2009; Caravaca et al., 2013; Dey, Nishiyama, Karpova, McNally, & Ozato, 2009; Sarge & Park-Sarge, 2009). While intriguing, it is difficult to directly attribute inefficient transcriptional reactivation to mitotic bookmarking directly in these studies because factor depletion occurred throughout the cell cycle. A major step toward mitosis-specific factor manipulation was recently made by the use of mitotic degron-transcription factor fusion proteins that cause mitosis-specific degradation (Kadauke et al., 2012). While a significant improvement over cell-cycle independent depletion, this strategy is also imperfect in that the targeted factor needs to be resynthesized following mitotic destruction. Further studies applying more mitosis-specific methods are needed to better understand the mitotic roles of bookmarks. This work examines mitosis-specific functionality of BETs using pharmacologic inhibitors to test their role in mitotic memory.

Bromodomain and Extra-Terminal motif proteins (BETs)

The BET family

Bromodomain and extra-terminal motif proteins (BETs) are chromatin regulators that associate with acetylated histones and transcription factors (Belkina & Denis, 2012). The eponymous sequence features of the BET family are tandem homologous bromodomains and a

shared extra-terminal motif (Figure 1.1). The bromodomains are responsible for association with chromatin, while the extra-terminal motif and other conserved regions mediate interaction with other proteins and may allow BET oligomerization (Dey, Chitsaz, Abbasi, Misteli, & Ozato, 2003; Garcia-Gutierrez, Mundi, & Garcia-Dominguez, 2012; Y. Liu et al., 2008; Rahman et al., 2011; Z. Yang et al., 2005). BETs are widely implicated in transcription, particularly at the level of elongation (discussed further in the context of individual BETs) (Jang et al., 2005; Kanno et al., 2014; Z. Yang et al., 2005). BETs also interact with and regulate the occupancy of prominent acetylated transcription factors including p53, RelA, and GATA1 (B. Huang, Yang, Zhou, Ozato, & Chen, 2009; Lamonica et al., 2011; S.-Y. Wu, Lee, Lai, Zhang, & Chiang, 2013). The association of BETs with chromatin likely reflects a combination of their association with acetylated histones and with acetylated transcription factors.

BETs are found only in eukaryotes. Vertebrate genomes encode four BET homologs: BRD2, BRD3, BRD4, and BRDT. In mammals the expression of BRDT is restricted to the testis and ovary, while the other BETs are expressed ubiquitously. Functional similarity among BETs is suggested by strict conservation of their bromodomains, association with many of the same regulatory complexes, and overlapping genomic binding profiles (Anders et al., 2014; Asangani et al., 2014; Dawson et al., 2011). Additionally, common functional themes unite the BETs within and between species. Many reports demonstrate roles of BETs in pathologic growth. Depletion of BRD2, BRD3, and BRD4 have each been shown to slow the growth of diverse tumors (Asangani et al., 2014; Y. Tang et al., 2014). Interestingly, chromosomal translocations of *Brd3* or *Brd4* with the *NUT* gene cause histopathologically indistinguishable NUT midline carcinoma (French et al., 2003; 2008). These translocations involve the BET bromodomains but not their C-terminal domains. Roles in the inflammatory response are also common to BETs. Depletion of either BRD2 or BRD4 attenuates inflammation in different circumstances (Belkina, Nikolajczyk, & Denis, 2013; Nicodème et al., 2010; Wienerroither et al., 2014). BRD2, BRD3, and BRD4 have also each been linked to nucleosome remodeling in the context of transcription (Kanno et al., 2014;

LeRoy, Rickards, & Flint, 2008), and BRDT in histone exchange during spermatogenesis (Gaucher et al., 2012). Yeast BETs Bdf1 and Bdf2 may similarly maintain heterochromatin boundaries through a mechanism involving histone turnover (Ladurner, Inouye, Jain, & Tjian, 2003; Jiyong Wang et al., 2013). Interestingly, Bdf1 and Bdf2 are redundantly required for growth suggesting that BETs have overlapping functions in yeast. The molecular basis for the distinctions between these proteins remains unclear, and large gaps remain in our understanding of their individual roles.

BETs in all species are distinguished by their ability to remain associated with chromatin through mitosis, a feature that sets them apart from the great majority of transcription factors and chromatin regulators examined. Knockdown and inhibitor studies have suggested BRD4 in particular may have a role in transcriptional reactivation in newborn cells immediately following mitosis (Dey et al., 2009; R. Zhao, Nakamura, Fu, Lazar, & Spector, 2011). In these studies BETs were depleted or inhibited in post-mitotic cells as well as mitotic cells and the functional role of mitotic association not directly addressed. However, speculation that BETs function as mitotic bookmarks in this regard is widespread. Clarification of the nature and importance of mitotic BET association is needed.

Short BETs: BRD2 and BRD3

BRD2 and BRD3 lack extended C-terminal domains and are the "short" members of the vertebrate BET family. Mice deficient for BRD2 have severe developmental defects (Fangnian Wang et al., 2009), and BRD2 ablation is incompatible with life (Shang, Wang, Wen, Greenberg, & Wolgemuth, 2009). Deficiency causes a number of developmental deficits and an attenuated inflammatory response (Belkina et al., 2013; Fangnian Wang et al., 2010). BRD2 knockdown or deficiency is associated with altered cell cycle and growth characteristics (Denis, Vaziri, Guo, & Faller, 2000), and overexpression promotes hematologic malignancies in mice (Belkina, Blanton, Nikolajczyk, & Denis, 2014). BRD3 was discovered by homology to BRD2 and remains much less

studied. Interestingly, relative mRNA expression of *BRD2* and *BRD3* correlates across a wide range of human tissues (Thorpe et al., 1997). Where BRD3 knockdown has a mild anti-growth effects in several cancer models, the effects are less dramatic than for those of *Brd2* and *Brd4* depletion (Asangani et al., 2014; Y. Tang et al., 2014). A BRD3 knockout mouse has yet to be reported. Large gaps remain in our understanding of the functional roles of BRD2 and BRD3.

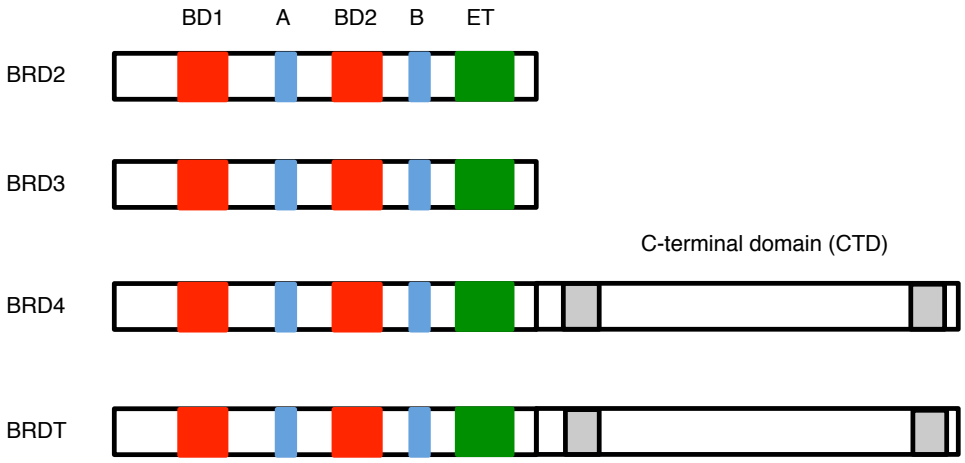


Figure 1.1 BET structure. Mammalian BETs. BD1 - Bromodomain 1. A - Motif A. BD2 - Bromdomomain 2. B - Motif B. ET- Extra-terminal domain. CTD - C-terminal domain. Grey bars show regions of sequence similarity between BRD4 and BRDT in their CTDs.

BRD4

BRD4 is the BET most consistently linked to transcription, oncogenesis, and mitotic bookmarking. BRD4 ablation causes early embryonic lethality in mice (Houzelstein et al., 2002). BRD4 can be expressed either as a short isoform similar in length to BRD2 and BRD3, or a long isoform with an extended C-terminal domain (CTD). The long form of BRD4 is the dominant transcript expressed in most tissues (Shang et al., 2004). Its CTD is unrelated to other mammalian proteins, although there are short regions of amino acid sequence overlap with the

CTD of BRDT. BRD4 also interacts with, and may activate, CDK9, the kinase subunit of the elongation complex pTEFb (Bisgrove, Mahmoudi, Henklein, & Verdin, 2007; Jang et al., 2005; Z. Yang et al., 2005). BRD4 has also been reported to itself be a kinase capable of directly phosphorylating RNA polymerase II (Devaiah et al., 2012). The BET extra-terminal motif has also been proposed to have a major role in stimulating pTEFb-independent transcription, perhaps by recruiting the histone methyltransferase NSD3 (Rahman et al., 2011). Interestingly, changes in DNA structure are visible at the levels of both immunofluorescence and DNase sensitivity upon BRD4 depletion in mammalian cells supporting a role in global chromatin structure (R. Wang, Li, Helfer, Jiao, & You, 2012).

BRD4 is connected to cancer in several settings. Robust BRD4 expression is required for transcription of oncogenic drivers in a number of cancers and its depletion variably slows growth, induces apoptosis, and promotes differentiation in diverse malignancies (Ott et al., 2012; Schwartz et al., 2011; Zuber et al., 2011). The precise oncogenes implicated seem to be tumor-specific, with *Myc* being a major target in some cancers but not in others (Mertz et al., 2011). *MYCN, AR, IL17R, GLI*, and *TWIST* and among the other oncogenes targeted in particular settings (Asangani et al., 2014; Ott et al., 2012; Puissant et al., 2013; Jian Shi et al., 2014; Y. Tang et al., 2014). Additionally, direct amplification of BRD4 has been observed in breast cancer (Kadota et al., 2009).

While all mammalian BETs have been observed on mitotic chromatin, BRD4 specifically has been implicated in post-mitotic transcriptional reactivation (Dey et al., 2009; Voigt & Reinberg, 2011). Interest in BRD4 as a mitotic bookmark has been amplified by the observation that its depletion causes some tumor cells to differentiate (Filippakopoulos et al., 2010; Schwartz et al., 2011; Zuber et al., 2011). This has led to speculation that BRD4 helps cancer cells remember they are cancer (Bradner, 2011). Further examination of the mitotic role of BRD4 specifically, and BETs in general, will be critical to understanding the role of mitotic bookmarking in their transcriptional functions.

BET inhibitors

Small molecule BET inhibition has generated intense interest as an emerging therapeutic modality (Baud et al., 2014; Dawson, Kouzarides, & Huntly, 2012; Filippakopoulos & Knapp, 2014). Oncologic applications are the most advanced and multiple clinical trials have been launched with these drugs for the treatment of diverse malignancies (Belkina & Denis, 2012; Dawson et al., 2012; Junwei Shi & Vakoc, 2014). The major class of thienodiazepine BET inhibitors were discovered by scientists at Yoshitomi Pharmaceuticals, who subsequently noted their potential both as anti-inflammatory and anti-cancer agents ("Thienotriazolodiazepine compounds and medicinal uses thereof," 1998). However, these molecules remained virtually unknown to the general scientific community prior to their adoption and optimization into the molecule JQ1 and its use in NUT midline carcinoma (Filippakopoulos et al., 2010). At the same, an unrelated drug development effort to find mimics of histone lysine acetylation converged on similar small molecules and published them along with their anti-inflammatory properties (Nicodème et al., 2010). Prior to these discoveries, the protein-protein interaction between bromodomains and acetylated lysines was considered impossible to pharmacologically target (Bradner, 2011). However, since this time several chemically unrelated molecules have been found that can target the same binding site in BET bromodomains (Picaud et al., 2013). The current generation of BET inhibitors do not distinguish between BET family members, and the development of additional BET inhibitors with distinct specificities remains an important goal (Filippakopoulos & Knapp, 2014; Nicodème et al., 2010; Picaud et al., 2013).

Many tumors are significantly more sensitive to BET inhibitors than is NUT Midline Carcinoma, despite this disease being driven by a BET bromodomain-containing fusion oncogene. Some genes with critical roles in cancer progression, such as *Myc* in some hematologic cancers, are exquisitely sensitive to BET inhibition for largely unknown reasons (Mertz et al., 2011; Zuber et al., 2011). A number of ChIP-seq studies examining BET protein

occupancy have been reported, but the binding patterns observed have disappointingly little ability to predict which genes are or are not sensitive to BET inhibition. Large genomic regions termed "super-enhancers" (SEs) have been proposed as regions that are particular sensitive to BET inhibition. However, when JQ1 sensitivity at SE-associated genes has been shown to be at most 15-20% more on average than at regular enhancer-associated genes (Chapuy et al., 2013; Lovén et al., 2013). Prediction of the transcriptional targets of BET inhibitors remains a major challenge.

BET inhibitors may be useful in applications outside of cancer, particularly as anti-inflammatories. BET inhibition prevents death in mouse models of sepsis, attenuates autoimmunity, and lessens damage from overactive inflammatory responses in the lung (Bandukwala et al., 2012; Mele et al., 2013; Nicodème et al., 2010; X. Tang et al., 2013). Pre-clinical studies have also demonstrated efficacy in applications that would require chronic administration such as heart failure (Anand et al., 2013). Additional studies of BETs in physiologic contexts will be critical to understanding the range of feasible applications of these molecules.

The erythroid transcription factor GATA1

Erythropoiesis

Erythropoiesis occurs in the bone marrow by differentiation of hematopoietic stem cells (HSCs). Lineage-specific transcription factor expression successively commits HSCs to the myeloid lineage, the megakaryocyte-erythroid lineage, and into committed erythroblasts. Following an initial expansion phase, erythroblasts mature through a process of cellular and nuclear condensation, and subsequently nuclear ejection (Figure 1.2). These final steps are accompanied by massive activation of genes involved in hemoglobin synthesis (globin genes and heme biosynthetic enzymes) and those encoding components of the specialized erythrocyte membrane. Transcriptional regulation by GATA1 drives this conversion from proliferative progenitor cells into specialized oxygen carriers.

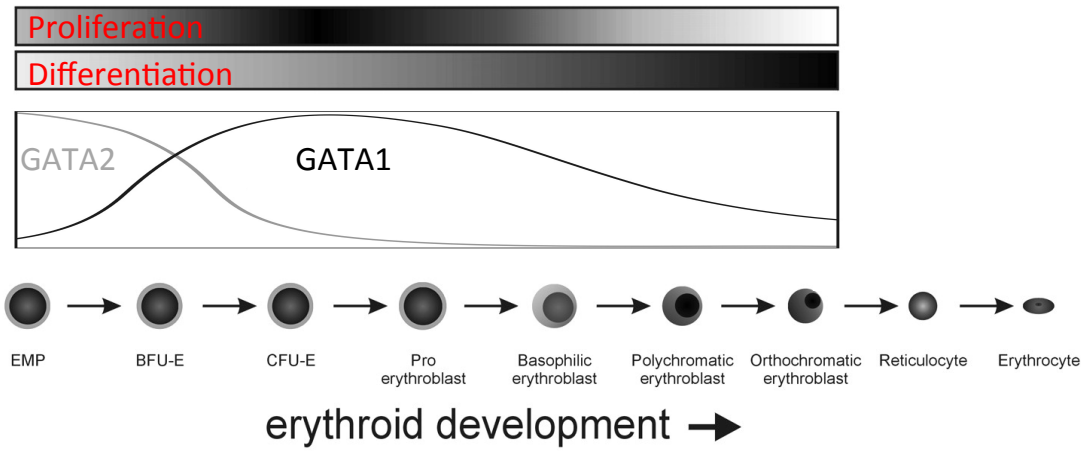


Figure 1.2 GATA1 expression and function in erythropoiesis. EMP - Common erythoid-myeloid progenitor. BFU-E - Blast forming unit erythroid. CFU-E - Colony forming unit erythroid.

Transcriptional activation and repression by GATA1

GATA1 was initially described as an erythroid transcription factor that activates the beta-globin gene (Evans, Reitman, & Felsenfeld, 1988). It is now recognized as part of the GATA family of zinc finger transcription factors that control diverse developmental processes (Patient & McGhee, 2002). GATA1 mutant mice are unable to form mature erythroid cells, and several types of congenital anemias in humans are associated with GATA1 mutations (Campbell, Wilkinson-White, Mackay, Matthews, & Blobel, 2013; Fujiwara, Browne, Cunniff, Goff, & Orkin, 1996; Nichols et al., 2000). During erythroid maturation, GATA1 activates nearly all erythroid-specific genes while silencing genes associated with the immature proliferative state (Cheng et al., 2009; Welch et al., 2004). In addition to being required for erythrocyte development, GATA1 is also essential for normal differentiation of megakaryocytes, mast cells and eosinophils (Crispino, 2005).

Study of GATA1 has been greatly facilitated by the GATA1-null erythroblast (G1E) cell line (Weiss, Yu, & Orkin, 1997). G1E cells are an erythroblast line derived from embryonic stem cells obtained from GATA1 null mice. These cells proliferate rapidly in culture, but upon introduction of GATA1 by transfection or by activation of an estrogen-responsive GATA1 allele (G1E GATA1-ER cells) mature following a pattern of gene expression changes mirroring those observed in maturing primary erythroblasts (Welch et al., 2004; M. Yu et al., 2009). Studies examining transcriptome changes in G1E cells as cell as primary erythroblasts found evidence that GATA1 activation is associated with activation and repression of a similar number of genes (Cheng et al., 2009; Welch et al., 2004; M. Yu et al., 2009). Importantly these studies examined gene expression changes in relation to total RNA content, a standard called into question by the dramatic changes in cell size and morphology that accompany erythroblast maturation (Dacie & White, 1949). This dissertation re-examines global transcription in this system by using external RNA spike-in controls which allow quantification relative to cell number rather than assuming a cellular RNA content to be constant.

Activation and repression by GATA1 is strongly influenced by cofactor proteins (M. Yu et al., 2009). The importance of GATA1 cofactors is underscored by the localization of many disease-causing GATA1 mutations to cofactor binding sites (Campbell et al., 2013; Nichols et al., 2000). Friend of GATA-1 (FOG1) was the first described GATA1 co-factor (Tsang et al., 1997). FOG1 associates with co-repressor complexes and histone deacetylases, but is still recruited along with GATA1 to both activated and repressed genes (Miccio et al., 2010). Mutations in GATA1 that impair FOG1 binding variably compromise GATA1 occupancy across the genome, suggesting that FOG1 may function at the level of GATA1 occupancy as well as by recruiting transcriptional effectors (Letting, Chen, Rakowski, Reedy, & Blobel, 2004; Pal et al., 2004). Another important GATA1 cofactor is the acetyltransferase CBP (Blobel, Nakajima, Eckner, Montminy, & Orkin, 1998). GATA1 is acetylated by CBP and p300 on lysines that are conserved

across species and across GATA-family proteins (Hayakawa et al., 2004; Hung, Lau, Kim, Weiss, & Blobel, 1999; Yamagata et al., 2000).

The cofactor CBP also has a major role in GATA1 function by acetylating it at conserved lysine-rich motifs (Hung et al., 1999). Mutations that impaired GATA1 acetylation had no effect on binding to naked DNA templates or plasmids, but did impair the genomic occupancy of GATA1 *in vivo* (Lamonica, Vakoc, & Blobel, 2006). This finding prompted a search for GATA1 cofactors that might bind to acetylated GATA1 and support its occupancy on chromatin. Peptide affinity pull-down using an acetylated GATA1 peptide in murine erythroleukemia (MEL) nuclear lysates recovered the BETs BRD2, BRD3, and BRD4 while the same unmodified peptide did not (Lamonica et al., 2011). This made the BETs leading candidates for the identity of the GATA1 cofactor responsible for *in vivo* occupancy.

Association of GATA1 with BETs

While BRD2, BRD3, and BRD4 were recovered in initial efforts to identify acetyl-GATA1 binding partners, several lines of evidence made BRD3 appear to be the most important BET in the context of erythropoiesis. First, early array studies suggested that BRD2 was not expressed in G1E cells (Welch et al., 2004), making it seem less likely to be important as erythroid maturation occurs efficiently in these cells. Second, while peptides from GATA1 associated with both BRD3 and BRD4, their interaction with BRD3 required acetylation while their interaction with BRD4 did not. Third, genomic occupancy of BRD3 correlated extremely well with genomic occupancy of GATA1 and BRD4 was absent from from a number of important GATA1-target sites. These observations strongly supported a relationship between GATA1 and BRD3. Because of this, the finding that nonspecific BET inhibition prevented transcriptional changes induced by GATA1 was interpreted as being due to BRD3 inhibition.

Recently, several findings led us to reconsider the question of whether BRD2 and BRD4 might have a role in GATA1-mediated erythropoiesis. More recent expression profiling studies

(Cheng et al., 2009; Paralkar et al., 2014) reported expression of BRD2 in G1E cells, and we confirmed this at both RNA and protein levels (this work). Additionally, more recent examination of the genomic occupancy of BRD4 suggested that it is present at a greater fraction of GATA1 sites than previously thought. Further exploration of the association and functional relationship between individual BETs and GATA1 is presented in Chapter 3. Another intriguing avenue of research is suggested by recent work demonstrating that GATA1, like BETs, binds to mitotic chromatin (Kadauke et al., 2012). Together with the proposed roles of BETs in bookmarking, this led us to hypothesize that these factors form a functional mitotic bookmarking complex. This is examined in Chapter 4.

Specific Aims

The dissertation explores the transcriptional roles of BETs in GATA1-mediated erythropoiesis. The central hypothesis of this work is that BRD2, BRD3, and BRD4 support GATA1-mediated transcriptional activation in interphase and mitosis. Specifically, we aimed to:

1. Evaluate the functional role of the BET family in GATA1-mediated transcription. To test the role of BETs in GATA1-mediated activation and repression, we pharmacologically inhibit them with JQ1 and measure transcriptome changes during GATA1-mediated erythropoiesis. We subsequently examine the requirement of BETs in transcriptional activation at the level of GATA1 occupancy and at the level of downstream transcription.

2. Test the individual contributions of BETs in GATA1-activated transcription. We characterize the genomic occupancy patterns of BRD2, BRD3, and BRD4 in the absence and presence of GATA1. We then test the functional requirement of each BET by depleting them using shRNA and CRISPR/Cas9.

3. Examine the role of BETs in mitotic bookmarking during erythroid maturation. We examine genomic association of BRD4, the BET most implicated in mitotic bookmarking, during mitosis. To directly test the functional role of BETs in mitotic bookmarking, we disrupt BET binding specifically during mitosis and measure post-mitotic transcriptional reactivation.

Together these studies aim to elucidate general mechanisms of BET function in the context of erythropoiesis.

CHAPTER 2. EXPERIMENTAL METHODS

Standard methods

Cell culture

Culture of GATA1- erythroblast (G1E) cells and G1E cells expressing a conditionally-active estrogen receptor-GATA1 fusion protein has been described(Tripic et al., 2009). G1E cells were grown in Iscove's Modified Dulbecco's Medium (IMDM) with 15% fetal bovine serum (FBS), 2% penicillin- streptomycin, 0.6% kit-ligand (KL) conditioned medium (CM), 45 uM monothioglycerol (MTG), and 2 U/mL Epoetin alpha (EPO). GATA1 was activated in G1E GATA1-ER cells by addition of 100nM estradiol for 24 hours (+GATA1). Where applicable, JQ1 was added to a final concentration of 250nM (unless explicitly stated otherwise) and was diluted in media from 10mM in DMSO carrier.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described (Letting, Rakowski, Weiss, & Blobel, 2003) with modifications as follows: Protein-DNA crosslinking was with 1% formaldehyde for 10 minutes at room temperature. 1M glycine was added to quench the reaction. Nuclei were extracted by lysis of outer cell membranes in cell lysis buffer (10 mM Tris pH 8.0, 10 mM NaCl, 0.2% Nonidet P-40) for 10 minutes on ice, and nuclei lysed in nuclei lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS). Nuclear lysates were sonicated for 30 minutes using a Bioruptor (Diagenode) to lower the range of chromatin fragment sizes. Chromatin was diluted in IP dilution buffer (20 mM Tris pH 8.0, 2 mM EDTA, 150mM NaCl, 1% Triton X-100, 0.01% SDS), pre-cleared with IgG and Protein A/G beads. Chromatin incubated with 10 ug of each antibody bound to Protein A/G Sepharose for each antibody IP, and 200ul of pre-cleared input chromatin from each sample saved separately. Following incubation overnight, beads were washed once with IP wash buffer 1 (20 mM Tris pH 8.0, 2 mM EDTA, 50 mM NaCl, 1% Triton X-100, 0.1% SDS), twice with high salt buffer (20 mM

Tris pH 8.0, 2 mM EDTA, 500 mM NaCl, 1% Triton X-100, 0.01% SDS), once with IP wash buffer 2 (10 mM Tris pH 8.0, 1 mM EDTA, 0.25 M LiCl, 1% Nonidet P-40, 1% sodium deoxycholate), and twice with TE (10 mM Tris pH 8.0, 1 mM EDTA). Immunoprecpitated complexes were eluted with 100ul of IP elution buffer (0.1 M $NaHCO_3$, 1% SDS). Eluates were incubated with RNaseA (0.02 mg/mL) for 30 minutes at 37 degrees, and then proteinase K (0.06 mg/mL) and NaCl (0.3 M) were added and samples placed at 65 degrees to reverse crosslinks overnight. DNA was purified on Qiagen Mini-prep columns and eluted in 60ul of water for (DNA) or 133.3 ul water (input DNA). Antibodies used for IPs were: GATA1 (sc265-N6; Santa Cruz), BRD2 (A302-583A; Bethyl), BRD3 sera (see (Lamonica et al., 2011)), BRD4 (A301-985A; Bethyl), HA (12CA5). Quantitative PCR (qPCR) was run on ViiA7 Real-Time PCR System (Life Technologies) using Power SYBR Green (Invitrogen) and sample DNA content was compared in the linear range of a standard curve generated from serial dilution of input DNA.

RNA isolation and RT-qPCR

RNA was isolated using Trizol or Trizol LS (Life Technologies). Reverse transcriptase (RT)-qPCR was performed with iScript (BIO-RAD) and Power SYBR Green (Invitrogen) using a Viia7 (Applied Biosystems). Mature transcript primers were designed to span exon-exon junctions across introns that are at least 500bp to prevent genomic DNA amplification. Primary transcript primers spanned exon-intron junctions are were placed at least 2kb beyond the transcription start site to prevent measurement of 5' abortive transcripts. qPCR signal was normalized either to spike-in controls or to *Gapdh*, depending on experimental design. For spike-in normalization, *in vitro* transcribed mRuby2 RNA, or Solaris RNA Spike-in(Thermo), was added directly to Trizol in proportion to cell number for a per cell frame of reference for quantification(Lovén et al., 2012; van de Peppel et al., 2003).

Mouse fetal liver erythroblast culture

Primary mouse cell experiments were designed in consultation with Kristen Jahn, and performed by Kristen Jahn. Mouse fetal liver cells from E14.5 pregnant females were dispersed and lineage-depleted using the EasySep Kit (Stemcell; 19756A) which removes cells by depletion of cell populations with the following surface markers: CD5, CD11b, CD19, CD45R, 7-4, Ly-6G/C (Gr-1), TER119. The progenitor-enriched cell population was expanded for three days in StemPro-34 media (GIBCO) supplemented with Epoetin (EPO) (0.5U / ml), Kit Ligand (1% mSCF conditioned media), dexamethasone (1um), monothioglycerol (MTG) (0.1mM), L-glutamine, and penicillin/streptomycin. Cells were differentiated by re-suspension in media without other than EPO. Cells were harvested in Trizol for RNA analysis and analyzed by flow cytometry (stains: Ter119-APC (BioLegend), DAPI (4,6 diamidino-2-phenylindole)). Analysis was performed on an LSR Fortessa flow cytometer (BD Biosciences). All mice were used in accordance with guidelines from the Institutional Animal Care Committee of the Children's Hospital of Philadelphia (IACUC#012-7-660).

Immunoblot

Nuclear extracts were prepared by removal of cytosol by selective permeabilization of cell membranes, centrifugation of nuclei, and nuclear lysis in high salt. Samples were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose (Bio-Rad) or PVDF membranes (Millipore). Signal was detected with chemiluminescence (Thermo). For quantification of BRD3 knockdown, an Odyssey Imager (LI-COR Biosciences) was used with fluorescent-labeled secondary antibodies, and band intensity quantified with ImageJ (Schneider, Rasband, & Eliceiri, 2012). The following primary antibodies were used: ACTIN (A3854; Sigma), BRD2 (A302-583A; Bethyl), BRD3 (2088C3a; Santa Cruz), BRD4 (A301-985A; Bethyl).

Mitotic ChIP

Cells were arrested in pro-metaphase of mitosis using nocodazole. Nocodazole offers high single-drug mitotic purity, but the potential that results obtaining using it reflect effects of microtubule disruption rather than of mitosis itself. Mitotic ChIP was performed with purification based on the phosphorylated mitotic epitope MPM2 (Campbell, Hsiung, & Blobel, 2014b). Briefly, nocodazole-treated cell populations were fixed with 1% formaldehyde for 10 minutes at room temperature. Cellular membranes were permeabilized with cell lysis buffer (see ChIP section), and cells were subsequently stained with anti-MPM2 antibody (Cell Signaling) (2.5ul/ml) for x 2 hours at 4 degrees. A secondary stain with anti-mouse APC was performed for 45 minutes at room temperature, and cell sorted based on this label. Following sorting of APC high population, 400ul was removed for mitotic purity checks by direct microscopy of DAPI-stained nuclei, and the remainder of sorted cells treated per ChIP protocol (above) beginning from nuclear lysis step.

Mitotic arrest-release and measurement of transcriptional reactivation

Assessment of post-mitotic transcriptional reactivation was performed as described (Kadauke et al., 2012). G1E ER-GATA1 cells infected with MigR1/YFP-MD constructs. Cells were arrested in prometaphase of mitosis with nocodazole for 7-12 hours, and released for 0-5 hours by nocodazole washout. Cells were placed on ice and a newborn cell population was sorted as YFP-dim (mitotically degraded YFP) / FSC-low (small size) population on a FACSAria II (BD Biosciences) into TRIZOL LS (Invitrogen). RNA isolation and RT-qPCR was performed as described above.

New methods

CAS9-mediated genetic disruption in G1E cells

This section outlines a simple approach to CAS9-based genome engineering used to create G1E knock-out cell lines. Parts of this section are modified from a write-up of our lab's experience with CAS9 co-authored with Sarah Hsu that was previously shared with labs in the Epigenetics Program at the University of Pennsylvania via the "(Epi)genome Engineering CRISPR CLUB" organized by Professors Jennifer Phillips-Cremins and Roberto Bonasio.

Overall strategy:

The strategy used was to introduce random mutations into the coding region of gene targets and screen for cells in with bi-allelic frame-shift mutations that made no functional protein product. This relies on the non-homologous end-joining (NHEJ) DNA repair pathway so that while the most common mutations observed are small deletions (1 to 50bp), significantly larger deletions and insertions of unexpected sizes are also commonly observed. This strategy can easily be extended to generating larger genomic deletions of other regions such as enhancers and non-coding RNAs, and is currently being applied to these purposes in our laboratory.

Guide RNA design and cloning:

The major factors considered in designing guide RNAs were (1) generation of an optimal target, and (2) minimization of off-target binding. Guide RNA targets were selected early in coding regions so that as little protein would be produced, however the first 100bp of coding sequence was avoided so that use of an alternative start codon could not easily make a functional protein product. Guide RNAS were expressed as sgRNAs, combination guide RNA fusions to structural RNA that attaches the complex to the CAS9 protein (Jinek et al., 2012), from a human U6 promoter with the vector phU6-gRNA(Addgene #53188). A single 'G' nucleotide was added to

guide RNA sequences that did not start with G to initiate transcription. Guide RNA sequences were ordered as sense and antisense versions, and annealed by boiling for 5 minutes followed by air cooling on the bench top to room temperature. Annealed oligos were ligated into BbsI-cut vector and transformed into highly competent *E. coli* DH5-alpha. Constructs were verified by sequencing from the human U6 promoter (sequencing primer: AAGGTCGGGCAGGAAGAGGG).

CAS9-transfection and single cell sorting:

CAS9/GFP and guide RNA/mCherry plasmids were transiently co-transfected into G1E cells using an Amaxa II electroporator (Lonza) with program G-016 and Kit R. Single transfected cells were sorted into a 96-well plate using a FACSAria II cell sorter (BD Biosciences), expanded, and screened by DNA sequencing and immunoblot. Immunoblot must consider the location of the antibody epitope (ideally toward the end of the protein) relative to the position of the mutation being induced. Guide RNA sequences were generated using the CRISPR design tool (http://crispr.mit.edu), and are listed below:

Brd2 (exon 3)	TTAGGACAATATCATCGGT
Brd3#1 (exon 4)	ACCTTGACTACAGGCGGTGT
Brd3#2 (exon 1)	GGGACTGCCGGGATCCCCGT

Mitotic ChIP by ro3306-release

Microtubule-disrupting drugs such as nocodazole are effective in arresting cell in mitosis, however cellular stress responses and the general toxicity of this agent create the potential that results obtained are not generalizable to cells undergoing mitosis normally. Furthermore, these agents limit mitotic study to pro-metaphase of mitosis specifically. Some reports suggest BRD4 may dissociate from chromatin as a result of microtubule disruption with nocodazole but still is able to re-associate with chromatin in late telophase(Nishiyama, Dey, Tamura, Ko, & Ozato, 2012). However, as we observed mitotic BET protein binding by ChIP, it is unclear whether dissociation of BRD4 at the global level of immunofluorescence would be important for

bookmarking if it occurs. Still, nocodazole-independent methods of mitotic purification will be important for verifying observations about BET proteins as well as any mitotic phenomenon observed.

For nocodazole-independent mitotic purification, we used the CDK1 inhibitor ro3306 (Vassilev et al., 2006) to initially enrich G1E cells progressing through mitosis. ro3306 arrested cells in late G2, and following its washout cells were grown in media for 30 minutes at 37 degrees and then harvested for mitotic ChIP by fixation with 1% formaldehyde. Subsequent mitotic ChIP staining, cell sorting, and immunoprecipitation was performed as described for standard nocodazole-based mitotic ChIP. ro3306-purified populations represent cells in a range of different stages of mitosis and anaphase and telophase cells are clearly visible by microscopy using this method. Data from use of this method are presented in chapter 4.

High-throughput data generation and analyses

ChIP-seq library preparation and initial read mapping

ChIP libraries for sequencing were created as described above for ChIP-qPCR. Subsequent Illumina library preparation and sequencing was performed was performed by Cheryl A. Keller and Belinda Giardine at the Hardison Laboratory at Penn State University per manufacturer's recommendations. Libraries were prepared from a 10ng of ChIP DNA. DNA fragments were filled to generate linear blunt-ended double-strand oligos. Fragments were amplified by 18 cycles of PCR following adaptor ligation, and DNA between 200 and 400 bp gel purified and used for sequencing. Sequencing was performed on an Illumina Hi-seq2000. Non-unique reads were considered PCR artifacts and discarded. Reads were mapped to mouse genome assembly mm9 using Bowtie (Langmead, Trapnell, Pop, & Salzberg, 2009). Reads were extended in the 3' direction to 200bp using MACS (Y. Zhang et al., 2008) to generate bigwig format files for browser display. Libraries generated and used are presented in Table 2.1.

Target	Antibody	Condition	# replicates
Libraries generated:			
H3K27ac	Millipore	G1E G1E+GATA1	2 2
GATA1	Santa Cruz (N6) sc265	G1E G1E+GATA1	2 2
BRD2	Bethyl A302-583A	G1E G1E+GATA1	1 1
BRD2	HA	G1E+GATA1	2
BRD4	Bethyl A301-985A	Mitotic G1E+GATA1 (nocodazole) Mitotic G1E+GATA1 (ro3306)	1 2
BRD4	Bethyl A301-985A	G1E G1E+GATA1	2 2
Initial reported analysis of prior libraries:			
BRD3	sera (Lamonica 2011)	G1E G1E+GATA1	1 1

Table 2.1 ChIP-seq libraries generated and analyzed in this dissertation. BRD3 ChIP was performed by Janine M. Lamonica and Stephan Kadauke.

Analysis of ChIP-seq data

Bigwig format files were visualized with the UCSC genome browser (Meyer et al., 2013). Heatmap visualization of multiple tracks at GATA1 locations (Mouse ENCODE Consortium et al., 2012) was performed using the Cistrome (T. Liu et al., 2011) Galaxy (Giardine et al., 2005) analysis tool 'Heatmap'. BRD2, BRD3, BRD4, H3K27ac signal in bigwig format was aligned across 4kb regions centered on GATA1 locations. GATA1 locations were clustered using k-means (k=5) considering only BRD4 signal. Regions of BRD4 high signal were easily identified independent of k in the ranges allowed by 'Heatmap' (k=3-10). Peak calling employed MACS (Feng, Liu, Qin, Zhang, & Liu, 2012; Y. Zhang et al., 2008) (default parameters: $p < 10^{-5}$ threshold, band width = 300, tag size = 25, effective genome size = 2.7×10^9) and SISSRS (maximum fragment length L = 300, genome size = $2.7\ 10^9$, using G1E input DNA as background) (Jothi, Cuddapah, Barski, Cui, & Zhao, 2008; Narlikar & Jothi, 2012). Signal at particular regions promoters and enhancers was measured by counting aligned reads or bigwig

signal as indicated using bedtools (Quinlan & Hall, 2010). Binned scatter plots were generated using the function geom_bin2d in the r package ggplot2 (Wickham, 2009). from each experiment at promoter locations (within 500bp of RefSeq transcription start sites), and enhancer locations (5kb regions that were H3K4me1 enriched, DNase hypersensitive, and promoter excluded (Hsiung et al., 2014)

GATA1 occupancy analyses were conducted using ChIP-seq data performed in two control replicates in parallel with two JQ1 replicates. MACS was used to call peaks in control samples using default parameters, and a high-confidence peak set was defined as peaks called independently in each control replicate. Read density was measured at these locations using bedtools as above. For comparison to array data, the nearest gene within of each high confidence GATA1 peak was located using bedtools. Varying length of associations allowed between peaks and genes of 2kb to 100kb were tested and gave similar results. Gene symbols were matched to array symbols, and binned scatter plots generated plotting (sum GATA1 +JQ1 / sum control GATA1) on the x-axis against fold change in mRNA on the y-axis for each GATA1 peak with ggplot2.

Microarray hybridization and analysis

To allow normalization of results to cell number, ERCC RNA Spike-In Mix (Ambion #4456740) was added directly to Trizol-homogenized samples in proportion to cell number. RNA quality control and subsequent microarray hybridization was performed by the UPENN Molecular Profiling Facility. Details on microarray procedures prior to analysis are summarized from information provided by Molecular Profiling Facility staff who performed these steps. Protocols were performed as described in the Ambion WT Expression Manual and Affymetrix GeneChip Expression Analysis Technical Manual. Total RNA (250ng) was converted to first-strand cDNA with addition of the T7 promoter sequence using reverse transcriptase priming with poly(T) and random oligomers. Second-strand cDNA was synthesized by *in vitro* transcription with T7 RNA

polymerase for linear amplification. Product cRNA was converted to cDNA, fragmented, assessed by Bioanalyzer, and biotinylated by terminal transferase end labeling. Labeled cDNA was added to Affymetrix hybridization cocktails, denatured at 99ºC for 5 minutes, and hybridized for 16 h at 45ºC to Affymetrix Mouse Gene 2.0 ST GeneChips. Arrays were subsequently stained with streptavidin-phycoerythrin (SA-PE). Fluorescence was amplified by adding biotinylated anti-streptavidin and additional SA-PE stain. Fluorescence signal was collected with a GeneChip 3000 7G scanner. Affymetrix Command Console and Expression Console were used to quantify expression levels using default analysis parameters provided by Affymetrix.

Analysis was performed using the Robust Multi-array Analysis (RMA) method (Irizarry et al., 2003) and subsequently normalized to spike-in controls as described (Lovén et al., 2012). Genes whose probe intensity was above the median level of background probes in 2 or fewer replicates (3 replicates were made in each condition) were excluded from further analysis. Differentially expressed genes were defined as those which changed at least 2-fold on average when replicates were averaged with Bonferroni-corrected p-value was <.05 using a linear model of statistical variance calculated by the r package limma (Smyth, 2004).

Public access to data via Gene Expression Omnibus

Data generated as described in this manuscript is accessible via the Gene Expression Omnibus (Edgar, Domrachev, & Lash, 2002) repository under the SuperSeries accession GSE62737 which consists of ChIP-seq accession GSE62709 and microarray accession GSE62736. This data is on pre-publication hold pending acceptance of our manuscript "Function of BET proteins in GATA1-mediated transcriptional activation" by Stonestrom et al. and will be publicly accessible in early 2015.

Primers

Primer design strategy is explained per specific experiment above.

Chip-qPCR primers

CD4 5'TR	CCAGAACATTCCGGCACATT
	GGTAAGAGGGACGTGTTCAACTTT
Eraf promoter	TTAAAGGGTCTGGGCATCATG
	CAACATCTTGGGAGAACGGTC
Hba-a1 promoter	TGACCAAGGTAGGAGGATACTAACTTCT
	TTGCCCGGACACACTTCTTAC
Hba-a1 HS-12	ACCCTGACTCAAAACAACAAAGTAA
	GGTTTCTGAGTTTCCTTATCTGCAA
Hba-a1 HS-31	TTCTGACCTCACCTCAGCTAAGC
	TGTGTGGGCAGAGGACACA
Hbb HS2	GGGTGTGTGGCCAGATGTTT
	CACCTTCCCTGTGGACTTCCT
Hbb-b1 promoter	CAGGGAGAAATATGCTTGTCATCA
	GTGAGCAGATTGGCCCTTACC
Gata2 -2.8	GCCCTGTACAACCCCATTCTC
	TTGTTCCCGGCGAAGATAAT
Gata2 promoter	CCCCTCGAAGTGATGTCGAA
	TCTGGCTGTCTCTCGGTTCC
Kit -114	GCACACAGGACCTGACTCCA
	GTTCTGAGATGCGGTTGCTG
Kit +5	GGCTGGAAACCACTGCCTTA
	AGCCTTGCCTGTGCTTAAAGC
Kit +33	TGGCAGTCCTGGTTGTAGCA
	GCTGCAAGCATGCGATCA
Kit +58	GGAGGAGTTAGGGAATATGTCGATAG
	GCAGTTCTCCAGGTTGAGTCAGA
Klf1 promoter	TCTGCTCAAGGAGGAACAGAGCTA
	GGCTCCCTTTCAGGCATTATCAGA
Klf1 enhancer	CTGGCCCCCCTACCTGAT
	GGCTCCCTTTCAGGCATTATC
Lyl1 promoter	TCAGCATTGCTTCTTATCAGCC
	CGCAGAGGCCAGAGGATG
Lyl1 5' TR	TCCAGGAGCAGCTCACTTTCTC
	CGGAGGGCCTGAGTAGCTT
Lyl1 intron	ACAGCCATCAGATAGGTCACCAGT
	TGAGAGGTCCATGCTGTGGTTTCA
Nfe2 +3.4	GTGATAGCAACCCTTCCCTC
	GGTAAAGGTCCAGTGTCTCC
Runx1 +4	CTCTCACGAGAAAGGGAACAAT
	GCAGGAGGAAACCTGTAACTC
Slc4a1 promoter	CTGAGCAGTCAAGCCTTAGTTCAC
	CCTGTCCAGTCCCTAAGGTCTTT
Slc4a1 intron 1	ATCAGAAGCAACCTAGAGTCCAGC
	TAAGAGTGTAGGACCAGCAGGCAA
Zfpm1 +2	CTTTTCTCCTGCCCAGTCG
	TGCTGTTGCCTCGAACC
Zfpm1 +4.7	AATTGTGCCCCTTATC TCCTG
	CTGGAGTATTATTCACGAGCCG
Zfpm1 intron 1	TGCAAGTCCCATCCTGATAAGA
	GCACGCCAGATAAGATCACAATT

Reverse transcription-qPCR primers

Alas2	TATGTGCAGGCCATCAACTACCCA
	TTTCCATCATCTGAGGGCTGTGGT
Eraf	GCCATGACAGAATTCCAGCAA
	TTTGGACTTCAGAAAGGTCCTGTAT
Klf1	CACGCACACGGGAGAGAAG
	CGTCAGTTCGTCTGAGCGAG
Epb4.9	TGCAGAAGCAACCTCTTACC
	AGATCCTTGTAGCCCAACACC
Hba-a1	CACCACCAAGACCTACTTTCC
	CAGTGGCTCAGGAGCTTGA
Hbb-b1	AACGATGGCCTGAATCACTTG
	AGCCTGAAGTTCTCAGGATCC
FOG1	CCTTGCTACCGCAGTCATCA
	ACCAGATCCCGCAGTCTTTG
Gata2	CACCCCTAAGCAGAGAAGCAA
	TGGCACCACAGTTGACACACT
Gapdh	AGGTTGTCTCCTGCGACTTCA
	CCAGGAAATGAGCTT GACAAAG
Hexim1	TTGTGGAAGAAGCTGGTGAG
	TCCTGCTTGCTCATGTTCTG
Kit	AGCAGATCTCGGACAGCACC
	TGCAGTTTGCCAAGTTGGAG
Lyl1	CCTGACCTGG ACTGACAAACCT
	CACATGGACCCCACGGATA
Myb	TGACTTTCGACACATGGCTCCTCA
	AATGCACTTGGTGCTGCTCTCAAC
Myc	AACCAGAGCTTCATCTGCGATCCT
	AGAAACCGCTCCACATACAGTCCT
Nfe2	TGGAACTGACTTGGCAAGAG
	ACAGGGGCAATATGTTGGAG
Slc4a1	TGGAGGCCTGATCCGTGATA
	AGCGCATCGGTGATGTCA
Spna1	AAAGAGTTCCGCTCTTGCCTGAGA
	TTTCCTCCCTGGATCCACAGCATT
Uros	CAGGCACAATGAAGGTTCTC
	TGACAGCACAGGAATCAGTG

Primary transcript rt-qPCR primers

Hbb-b1	GCCTGCAGTATCTGGTATTTTTG
	TGAAATCCTTGCCCAGGTG
Klf1	TTGGAGGGTGGTACTTACAGC
	AGAAGGGACGATGTCCAGTG
Nfe2	AATTCTGCACGAGGACAACC
	CTCCACAAGCACAAAGGATTC
Tal1	CACAGGGAGAATCCATCTAAGG
	TGTTGGTGAACATGGGGAAG

Hba-a1	TCAGGGTGTCCACTTTGTCTC
	GGCAAGGAA TTTGTCCAGAG
Uros	CCATTCCCACTTCCATTCC
	TAGCTCTTCAGGCTCCCTTG
Pabpc1	TCTGAAGTTCCTGGGATTGG
	TAGCCCTTGGAGCCATTTTC
Gapdh	CTCAGCTCCCCTGTTTCTTG
	AATCTCCACTTTGCCACTGC
cKit	TTACTTCGCCAAGACAGCTC
	CTGATTGTGCTGGATGGATG
Gata2	TTCGCTGAGTTGTGATCCTG
	GCTGTGCAACAAGTGTGGTC
Myb	TGCTGAAGCGTTTCTGTCTG
	AGCCCATCGTAGTCATGGTC
Lyl1	ATGGTCTTTGGCCTTCCTTC
	TTTCTCAGTCATGGCGGAAC

CHAPTER 3. FUNCTION OF BET PROTEINS IN GATA1-MEDIATED TRANSCRIPTIONAL ACTIVATION

Chapter summary

Bromodomain and Extra-Terminal motif proteins (BETs) associate with the master erythroid transcription factor GATA1 and are required for its normal *in vivo* occupancy. Here we test the extent to which BETs regulate the erythroid transcriptome as a family and the individual roles of each BET. Pharmacologic inhibition of the BET family impaired GATA1-mediated transcriptional activation but allowed repression to continue normally. BETs were required both to facilitate the occupancy of GATA1 and support downstream transcription. We subsequently investigated the role of each expressed BET BRD2, BRD3, and BRD4 independently. We identified BRD3 binding at the great majority of GATA1 sites, BRD4 binding at a large subset, and BRD2 binding at relatively few. Surprisingly, depletion of either BRD2 or BRD4 blunted erythroid gene activation, while depletion of BRD3 did not. BRD3 loss only affected erythroid transcription in the setting of BRD2 deficiency. Additionally, exogenous BRD3 expression compensated for BRD2 loss. These results characterize the role of BETs in gene activation during GATA1-mediated erythropoiesis and reveal the individual functions of BRD2 and BRD4 in this process. They further suggest that BRD2 and BRD3 can function redundantly.

Introduction

The mammalian Bromodomain and Extra-Terminal motif proteins (BETs) have drawn widespread interest as pharmacologic targets for the treatment of various diseases, including hematological malignancies and solid tumors (Belkina & Denis, 2012; Dawson et al., 2012; Prinjha, Witherington, & Lee, 2012; Junwei Shi & Vakoc, 2014). Within the BET family BRD2, BRD3 and BRD4 are ubiquitously expressed in mammalian tissues, while

BRDT is testis-specific. These molecules contain two tandem bromodomains that mediate association with chromatin by binding to acetylated histones and transcription factors (Dey et al., 2003; B. Huang et al., 2009; Lamonica et al., 2011; Nishiyama, Dey, Miyazaki, & Ozato, 2006; S.-Y. Wu et al., 2013). BETs function in regulatory complexes that impact mRNA production at multiple steps of the transcription cycle, such as modifying and remodeling chromatin and promoting transcription elongation (Bartholomeeusen, Xiang, Fujinaga, & Peterlin, 2012; Dawson et al., 2011; Itzen, Greifenberg, Bösken, & Geyer, 2014; Jang et al., 2005; LeRoy et al., 2008; Patel et al., 2013; S.-Y. Wu & Chiang, 2007; Z. Yang et al., 2005).

Given the widespread expression of BETs, it was initially surprising that pharmacologic inhibitors, such as JQ1, elicit cell- and gene-specific responses. These BET inhibitors selectively bind BET bromodomains and displace them from acetylated lysine residues on histones and transcription factors (Dawson et al., 2011; Filippakopoulos et al., 2010; Nicodème et al., 2010). Promising results in animal models of malignancy have sparked clinical trials and intensified efforts to better understand BET function (Belkina & Denis, 2012; Dawson et al., 2012; Delmore et al., 2011; Junwei Shi & Vakoc, 2014). The current generation of BET inhibitors do not distinguish between BET family members, and the development of additional BET inhibitors with distinct specificities remains a major goal.

Erythroid maturation is a developmental process driven in part by the erythroid master transcription factor GATA1 which activates essentially all erythroid-specific genes and silences genes associated with the immature proliferative state (Cheng et al., 2009; Welch et al., 2004). Mice lacking GATA1 die *in utero* due to failure to form mature erythroid cells (Fujiwara et al., 1996), and several types of congenital anemias in humans are associated with GATA1 mutations (Campbell et al., 2013; Nichols et al., 2000). GATA1 is acetylated near its zinc finger DNA binding domain, and mutations of acetylated lysines impair the ability of GATA1 to associate with chromatin *in vivo* (Lamonica et al., 2006). Exposure of erythroid cells to BET inhibitors diminishes

GATA1 occupancy at a subset of target genes. The BET protein BRD3 specifically associates with acetylated GATA1 and chromatin immunoprecipitation (ChIP) studies suggest BRD3 in particular is present at most GATA1-occupied sites (Lamonica et al., 2011). Here we expand these initial studies of BET function in the context of GATA1-mediated erythropoiesis by first characterizing BET functions and mechanisms as a family, and second elucidating the roles of individual BETs. We find that BETs facilitate GATA1-mediated transcriptional activation but are largely dispensable for repression. BETs appear to both support the initial occupancy of GATA1 and subsequently activate transcription downstream. BRD2 and BRD4 depletion compromise GATA1-induced erythroid gene activation. Unexpectedly, despite the presence of BRD3 at nearly all GATA1 occupied sites, BRD3 is not required for normal GATA1-activated transcription. However, BRD3 deficiency exacerbates transcriptional defects associated with BRD2 loss and BRD3 expression compensates for BRD2 loss suggesting these proteins have overlapping functions. Together these studies reveal the aspects of the GATA1-mediated transcription program that utilize BETs, and identify the functional role of each BET individually.

Results

BETs are required for efficient GATA1-dependent transcriptional activation but not repression

Pharmacologic inhibition of BETs impaired activation of several GATA1-target genes (Lamonica et al., 2011). To evaluate the contribution of BETs to GATA1-induced gene expression changes genome wide, we performed microarray analysis on G1E cells treated with 250nM JQ1 or DMSO control concurrent with GATA1 activation or in its absence for 24 hours in biological triplicate. As dramatic alterations in cell size and RNA content occur during erythroid maturation, we added external spike-in RNA controls to each sample in proportion to cell number to normalize our results (Lovén et al., 2012; van de Peppel et al., 2003). We initially visualized changes in mRNA levels of expressed genes as a heatmap arranged by hierarchal clustering (Figure 3.1A). A preponderance of genes were most highly

expressed in untreated G1E cells and declined slightly upon JQ1 treatment. A stronger decline in transcripts was observed following GATA1 induction, and transcript levels were lowest upon GATA1 activation in the presence of JQ1. Focusing first on GATA1's impact on gene expression, we plotted all transcripts from most repressed to most activated (Figure 3.1B). 5,094 transcripts decreased while only 220 increased following GATA1 addition using stringent differential expression criteria (2-fold change and Bonferroni-corrected $p<0.05$) (Figure 3.2). The predominance of repression upon GATA1-induction contrasts with prior studies of GATA1-mediated transcriptome changes that were based on internal standards and concluded that the number of activated and repressed genes are similar (Doré, Chlon, Brown, White, & Crispino, 2012; Welch et al., 2004; W. Wu et al., 2011; M. Yu et al., 2009). This highlights the importance of spike-in controls in transcriptome studies in which global mRNA output is dramatically altered.

To evaluate the role of BETs in transcriptional regulation by GATA1, we plotted transcriptional change with GATA1 induction against JQ1 sensitivity for each gene (Figure 3.3A). While significant variation was observed across measured genes, consistent with gene selectivity in JQ1 response (Dawson et al., 2011; 2012; Lovén et al., 2013; Nicodème et al., 2010; Zuber et al., 2011), JQ1 sensitivity trended strongly with increased GATA1-mediated activation. In contrast, transcripts that decreased upon GATA1 induction decreased no more or less on average with concurrent JQ1 treatment. BET inhibition increased mRNA levels at some genes, which could be due to repressive functions of BETs or be indirect. These results are consistent with BETs functioning principally in GATA1-mediated activation and having little role in repression. Our findings were confirmed at canonical erythrocyte genes in independent experiments by RT-qPCR (Figure 3.3B).

To verify the transcriptional effects of BET inhibitors in primary erythroid cells, we measured gene expression in mouse fetal liver erythroblast progenitors differentiated in the presence or absence of JQ1. BET inhibition suppressed surface expression of the erythroid

maturation marker TER-119, but had no overt toxic effect (Figure 3.4A). Similar to results in G1E cells, activation of erythroid gene expression was impaired by JQ1 while gene repression occurred normally (Figure 3.4B). We also noted induction of the transcriptional repressor HEXIM1 upon BET inhibition as has been observed in other cell types (Bartholomeeusen et al., 2012; Chaidos et al., 2014). Together these results support the role of BETs in GATA1-driven transcriptional activation, and further suggest repression may be largely BET-independent.

Role of BETs in GATA1 occupancy genome-wide

We previously reported that GATA1 must interact with BETs in order to bind to a handful of its occupied sites (Lamonica et al., 2011). To evaluate the role of BETs in GATA1 occupancy genome-wide, we performed anti-GATA1 ChIP-seq following 24 hours of GATA1-induction in the absence or presence of JQ1. BET inhibition almost entirely prevented GATA1 binding at some loci (*Hbb*), while having no measurable effect on binding at others (*Zfpm1*) *(*Figure 3.5A). To quantitatively examine the requirement of BETs for GATA1 occupancy we first defined 5,096 high-confidence GATA1 sites using the intersection of MACS peak calls in two control replicates (Y. Zhang et al., 2008). We then compared the maximum read pileup height at these locations in controls compared to JQ1-treated samples (Figure 3.5B). BET inhibition reduced GATA1 occupancy at 89.6% of sites (points below blue line), but did so only partially at the great majority of loci. To evaluate the relationship between inhibition of GATA1 occupancy and inhibition of transcription, we plotted fractional maintenance of GATA1 occupancy against transcriptional sensitivity of presumptive target genes nearby GATA1 sites (Figure 3.5C).

Loss of GATA1 occupancy was not a strong predictor of JQ1-sensitivty at genes adjacent GATA1 OS. However, genes adjacent JQ1-evicted GATA1 sites that also activated by GATA1 were more JQ1-sensitive than those adjacent GATA1 sites that were JQ1-stable

(Figure 3.6). Still, a wide range of transcriptional sensitivities was observed regardless of the stability of GATA1 occupancy at all sites. This included reduction of GATA1 at many sites adjacent genes whose expression was unaffected by BET inhibition. At these OS, GATA1 binding is either not required for transcription or partial occupancy is sufficient for transcriptional activation. GATA1 signal was higher when induction occurred concurrent with JQ1 treatment at a small number of sites. This suggests that BETs might also function in an occupancy-inhibitory manner. The sensitivity of GATA1 sites to JQ1 correlated with TAL1 occupancy, but not with the other mouse ENCODE-defined transcription factors in the same cell type, promoter or enhancer localization, or DNase hypersensitivity (Table 3.1). As TAL1 is associated with transcriptional activation, this is consistent with GATA1-activated genes having an increased need for BETs for full occupancy. Independent experiments confirmed variable JQ1-mediated reductions in GATA1 occupancy similar to those observed by ChIP-seq (Figure 3.7). These results suggest BETs are widely required for maximal GATA1 occupancy at many sites, but that the role of BETs in GATA1-mediated transcription is likely to extend beyond assisting GATA1 in binding to chromatin.

BETs activate transcription subsequent to establishment of GATA1 occupancy

BETs interact with the general transcription machinery and may stimulate transcription directly (Anand et al., 2013; Devaiah et al., 2012; Jang et al., 2005; LeRoy et al., 2008; Patel et al., 2013; Z. Yang et al., 2005). Based on the results above, we tested whether BETs function in GATA1-activated transcription subsequent to the establishment of chromatin occupancy. As GATA1 occupancy might be less sensitive to BET inhibition once established, we examined the short-term effects of BET inhibition on accomplished GATA1 occupancy. Indeed, JQ1 treatment for one hour removed BETs from all sites examined with little effect on GATA1 occupancy (Figure 3.8-9). We next measured primary transcript levels of GATA1 target genes under these conditions. Several GATA1 targets including *Hbb-b1* (***β***-

globin), *Klf1*, and *Nfe2*, were immediately repressed upon BET inhibition, suggesting with transcriptional activity of BETs downstream of GATA1-occupancy (Figure 3.10-11). In contrast, transcription of other genes like *Hba-a1*(***α****-globin*) and *Uros* was unperturbed by short-term JQ1 treatment despite their sensitivity to long term JQ1 exposure and proximity to BET-bound regulatory elements. At these genes BETs might function by predominantly by assisting GATA1 occupancy or by secondary mechanisms. As expected, genes repressed by GATA1, such as *Gata2* and *Kit* remained inactive upon JQ1 treatment. We conclude that at a subset of genes BETs augment GATA1 transcriptional activity subsequent to its chromatin binding.

GATA1 drives global reorganization of BET occupancy

We previously demonstrated that BRD3 and BRD4 directly interact with acetylated GATA1, and BRD3 is recruited to several GATA1 binding sites in GATA1-dependent manner (Lamonica et al., 2011). Early microarray data (Welch et al., 2004) suggested BRD3 and BRD4 were the only BETs expressed in G1E cells, however recent genome-wide data (Cheng et al., 2009; Paralkar et al., 2014) indicated BRD2 was indeed expressed. To better define the role of each individual BET in erythroid maturation, we performed chromatin immunoprecipitation and high-throughput sequencing (ChIP-seq) for BRD2, BRD3, and BRD4 in G1E cells in the absence and presence of GATA1 (Weiss et al., 1997). Although we had previously generated a BRD3 ChIP-seq data set (Lamonica et al., 2011), we repeated this experiment to improve sequencing depth and compare BET profiles generated on the same platform. We initially inspected BET occupancy at model GATA1 target loci including *Hbb* (*beta-globin*) (Figure 3.12A) and observed recruitment of each BET to these sites in similar but distinct patterns. All were recruited to the *beta-globin* gene (*Hbb*), but BRD3 and BRD4 occupied much more of the nearby *locus control region* than did BRD2. We then examined occupancies of these BETs across all 13,123 GATA1 occupied sites (Mouse

ENCODE Consortium et al., 2012; W. Wu et al., 2011). We clustered binding signals for BRD2, BRD3, BRD4, and GATA1 together with acetylated histone 3 lysine 27 (H3K27ac) across each GATA1 sites using k-means (k=5), and visualized this as a heat map (Figure 3.12B). BRD3 was present at nearly all the locations bound by GATA1, while BRD4 was strongly present at only a subset (3,344 sites). BRD4 occupancy correlated strongly with H3K27ac in the vicinity of GATA1 OS but was maximal directly over GATA1 peaks where H3K27ac was relatively lower. The distribution of H3K27ac likely reflects acetylated nucleosomes flanking GATA1 occupied nucleosome-depleted regions. These results support a model of GATA1-dependent recruitment of BETs to GATA1 sites. These results suggest that upon GATA1 induction BRD3 is recruited to nearly all GATA1 sites, BRD4 to a large minority, and BRD2 to the vicinity of some.

We next expanded our analysis of BET binding to include all BET-occupied sites. We used two different peak identification methods (MACS and SISSRS) to define BET-enriched regions across the genome. We compared the binding sites identified for each with predicted regulatory regions and mouse ENCODE-defined transcription factor binding sites in erythroid (G1E and MEL) cells in the absence and presence of GATA1 (Table 3.2). GATA1 induction increased the fraction of BRD3 and BRD4 sites at promoters, and BRD3 also at enhancers, but did not substantially change the fraction of BRD2 sites at these potential regulatory elements. Additionally, the fraction of BRD3 and BRD4 sites at DNase hypersensitive sites increased following GATA1 induction. This is consistent with an increase in DNA association mediated by transcription factors relative to histones. Both peak identification methods demonstrated a much stronger overlap of BRD3 and BRD4 with GATA1 than BRD2. The distinct binding pattern of BRD2 was confirmed by retroviral expression of HA-BRD2 and ChIP-seq using an antibody directed against the HA epitope in independent experiments (Table 3.3). These results show substantial reorganization of BRD3 and BRD4 binding upon GATA1 activation.

To parse the functional contributions of BETs, we re-examined the effects of BET inhibition on GATA1 occupancy and transcriptional activation together with the BET-specific occupancy data generated. To test whether occupancy of a particular BET family member explained the effect of JQ1 on GATA1 occupancy, we plotted BRD2, BRD3, and BRD4 binding against the fractional occupancy of GATA1 upon JQ1 treatment (Figure 3.13). No strong relationship was observed between GATA1 eviction and the presence of the BETs alone or in combination. This suggests that factors other than quantity of BET binding are likely to determine whether GATA1 requires BETs for occupancy at given sites. Other GATA1 cofactors are likely sufficient for stable GATA1 binding to chromatin at many locations. To examine whether a particular BET family member predicted transcriptional response to JQ1, we plotted BRD2, BRD3, and BRD4 read counts at promoters and enhancers against JQ1 response with or without concurrent GATA1 induction (Figure 3.14). BET occupancy was not a strong predictor of JQ1 sensitivity overall, however a weak relationship between JQ1 effects and BRD4 occupancy at promoters was observed. This is consistent with previous reports showing that most BET occupied genes fail to respond to BET inhibitors (Anders et al., 2014; Lovén et al., 2013; Nicodème et al., 2010; Zuber et al., 2011). Regions with high histone acetylation or BRD4 occupancy, referred to as “super-enhancers”, have been suggested to identify genes particularly sensitive to JQ1 (Lovén et al., 2013). We defined these regions using the algorithm ROSE (Rank Ordering of Super-Enhancers) using BRD4 signal and examined JQ1 sensitivity of associated genes (Figure 3.15). Genes linked to super-enhancers were only minimally more JQ1 sensitive than random genes. Activation by GATA1 was a much stronger predictor of JQ1 sensitivity.

Individual roles of BETs

Current knowledge of BET function is largely built on studies using inhibitors that do not distinguish between individual BETs (Asangani et al., 2014; Belkina & Denis, 2012; Lovén et al., 2013; Mele et al., 2013; Nicodème et al., 2010; Junwei Shi & Vakoc, 2014). To dissect the roles

of individual BETs in GATA1-driven erythropoiesis, we initially used a loss-of-function approach combining CRISPR/CAS9-engineered gene disruption (Mali et al., 2013) and shRNA-mediated knockdown. As BRD3 occupies nearly all GATA1 OS, we had speculated that it was the most relevant BET in GATA1-mediated transcription. Surprisingly, cells engineered to produce no detectable BRD3 expressed all examined GATA1 target genes at essentially normal levels upon GATA1 induction (Figure 3.16A), suggesting BRD3 is not essential. In contrast, BRD2 deficient cells failed to transcribe erythroid genes at normal levels following GATA1 activation (Figure 3.16B). However, the effects of BRD2 depletion were less pronounced than those observed with JQ1 treatment implicating additional BETs in GATA1 driven erythroid maturation. Attempts at functional deletion of BRD4 failed, perhaps due to its requirement for cell growth (Houzelstein et al., 2002). However, transient shRNA-mediated depletion of BRD4 significantly decreased GATA1-induced gene expression (Figure 3.16C) supporting its importance in this process. While no growth defects were observed upon BRD3 ablation, cells deficient in BRD2 or BRD4 proliferated more slowly, indicating these genes have additional roles in G1E proliferation (data not shown). These results suggest BRD2 and BRD4 are individually required for normal GATA1-mediated transcriptional activation.

Overlapping functionality between BETs

Given the physical association of BRD3 with acetylated GATA1 and to genome-wide co-localization, the indifference of GATA1-activated transcription to BRD3 depletion was surprising. We therefore tested whether other BETs might compensate for BRD3 loss. To do this we used shRNAs to deplete BRD3 in BRD2-replete and BRD2-deficient cells (Figure 3.17A). As expected, BRD3 knockdown on its own had no significant impact on gene activation. However, BRD3 knockdown exacerbated the consequences of BRD2-deficiency on GATA1-activated gene expression. Hence, BRD2 and BRD3 assist GATA1 in an at least partially overlapping manner. To further test this we compared re-expression of BRD2 to over-expression of BRD3 in BRD2-

deficient cells. GATA1-mediated activation of the **β**-globin gene *Hbb-b1* and *Alas2* were largely rescued by *Brd3* expression (3.17B). *Slc4a1* was not significantly rescued, however this gene also failed to activate normally upon *Brd3* expression in cells with normal *Brd2* expression. This indicates that transcriptional rescue is gene specific. To test rescue at the phenotypic level we examined cell pellet color as an indicator of hemoglobin production following GATA1 activation (Figure 3.17C). BRD2-replete cells turned red following GATA1 activation while BRD2-deficient pellets remained mostly white. As expected, retroviral expression of BRD2 rescued red color changes in these cells and validated knockout specificity. Consistent with functional redundancy between BRD2 and BRD3, retroviral BRD3 expression also rescued red color changes in BRD2 deficient cells. These results strongly suggest functional overlap between BRD2 and BRD3 in GATA1-dependent transcriptional activation.

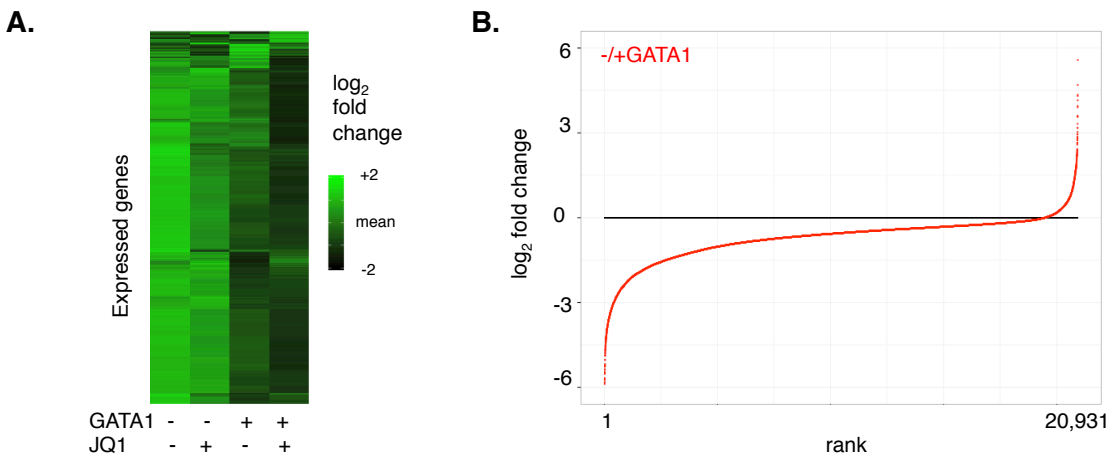
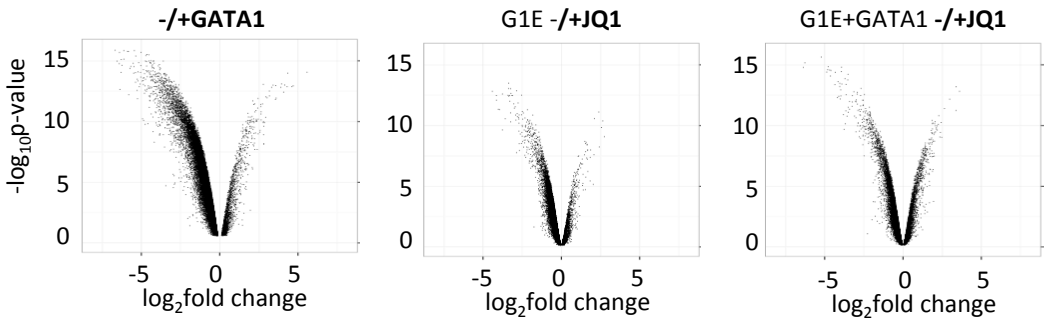


Figure 3.1 Transcriptome changes driven by GATA1 activation and BET inhibition. (A-B) Microarray expression profile of G1E cells -/+ GATA1 induction in the presence or absence of JQ1. Transcript levels were normalized to cell number using external spike-in controls. Data represent the average of three biological experiments with similar results. (A) Heatmap showing relative expression of every expressed transcript in each condition. (B) Distribution of mRNA changes upon GATA1 activation (red points).



# significant genes:			
repressed	5094	494	810
activated	220	25	211

Figure 3.2 Differentially expressed genes. (Top panels) Expression fold change versus p-value of differential expression for genes compared in the conditions in bold. (Bottom) Number of high-confidence up-regulated and down-regulated transcripts for each comparison (Bonferroni-corrected p<.05 and fold change >2).

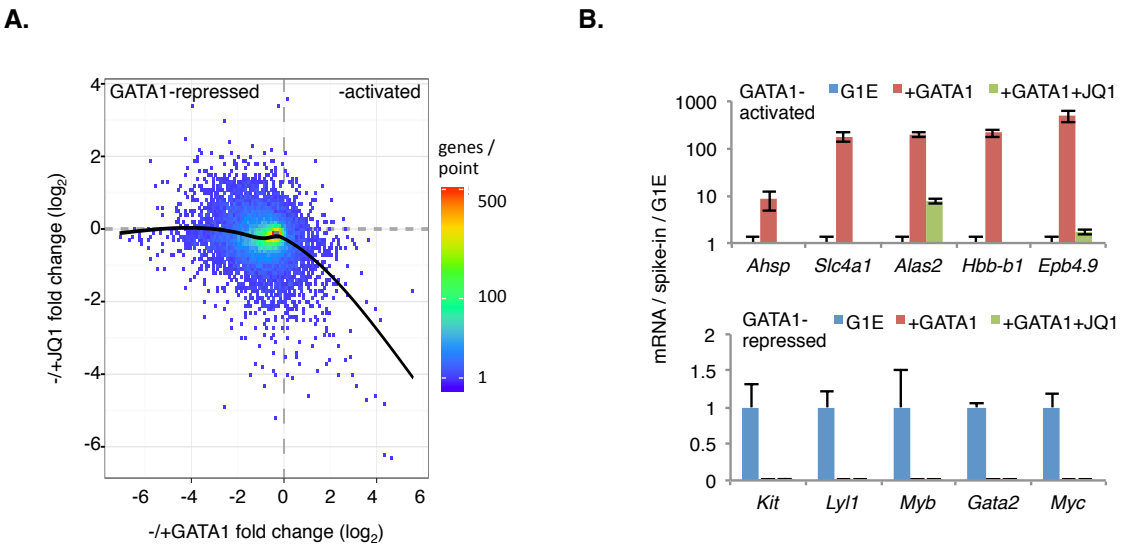


Figure 3.3 BET inhibition preferentially targets GATA1-activated genes during GATA1 induction. (A) Relationship of activation by GATA1 with JQ1 sensitivity in a binned scatter plot where color represents number of genes at a location. Black line shows a Loess regression. (B) GATA1-activated and repressed transcript levels as determined by RT-qPCR. Data were plotted relative to untreated G1E cells normalized to cell number by RNA spike-in controls. Error bars represent SEM of three biological replicates.

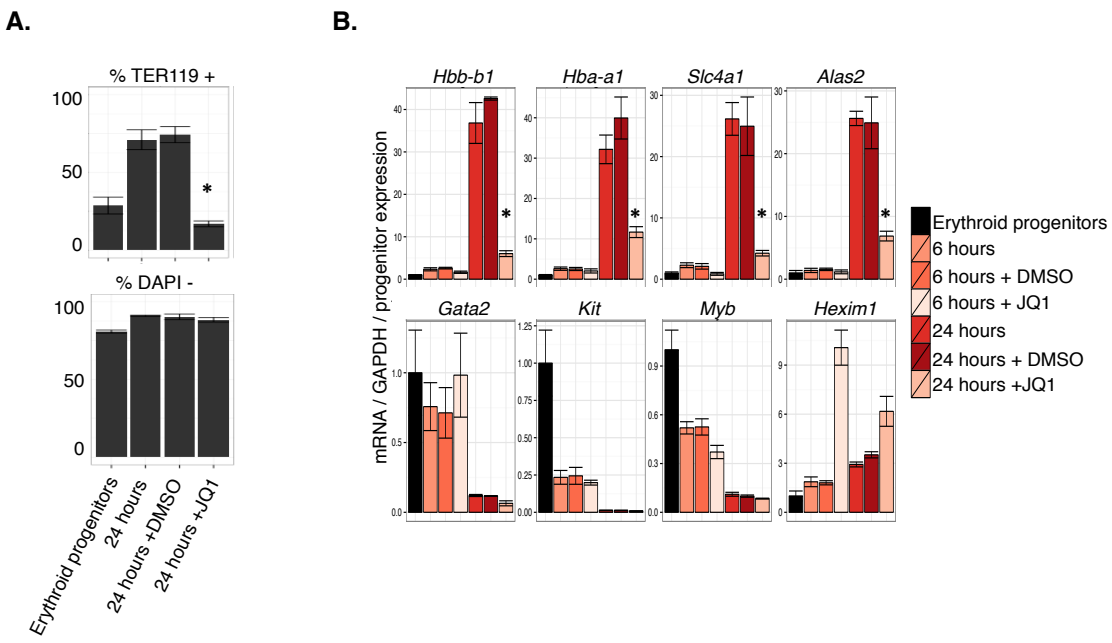


Figure 3.4 BET inhibition prevents erythroid gene activation in primary fetal liver erythroid progenitors. (A) Percentage of fetal liver progenitors expressing TER-119 and percentage viable (DAPI negative) measured by flow cytometry following 24 hours growth in differentiation media in the presence or absence of JQ1 or DMSO carrier. (B) RT-qPCR measurement of erythroid transcripts in differentiating primary fetal liver erythroblasts. Error bars represent SEM, n=3.

* indicates $p < .001$ for JQ1 treatment versus DMSO-treated and untreated samples.

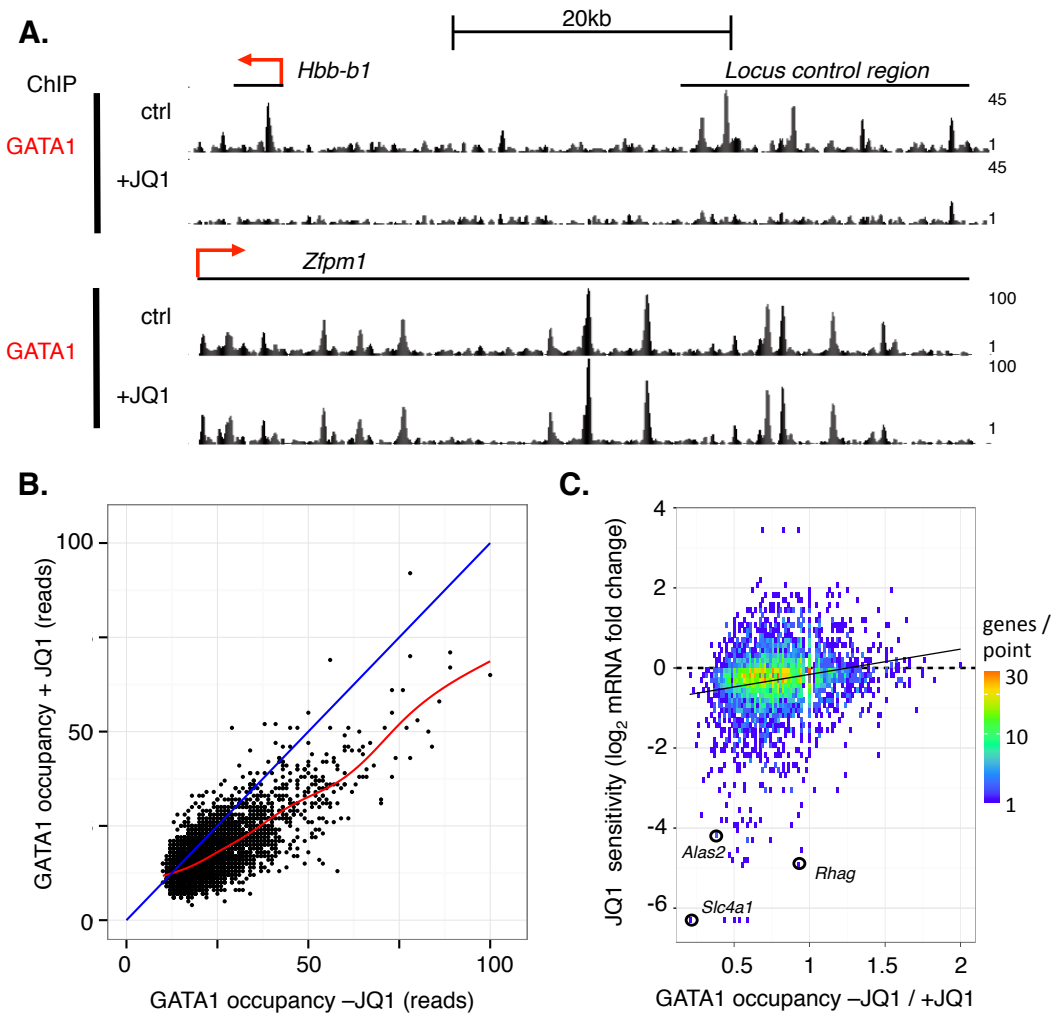


Figure 3.5 Effects of BET inhibition on GATA1 occupancy genome-wide. (A) Genome browser tracks showing GATA1 binding at the *Hbb* and *Zfpm1* loci in the absence and presence of 250nM JQ1. Tracks are from one biological experiment and representative of two with similar results. (B) GATA1 ChIP-seq read counts (read pileup heights) at GATA1 sites following GATA1 induction for 24 hours in the absence versus presence of JQ1. The red line shows a Loess regression, the blue diagonal demarcates no change between control and JQ1 treatment. (C) Binned scatter plot showing the relationship between BET dependence of GATA1 occupancy and transcriptional activation. GATA1 peaks are linked to nearest gene within 5kb. Black line shows a

linear regression. Highlighted examples for varying behaviors are *Alas2* and *Slc4a1* which lose nearby GATA1 peaks and are repressed following JQ1 treatment. In contrast, *Rhag* is repressed upon JQ1 treatment but an adjacent GATA1 peak is maintained.

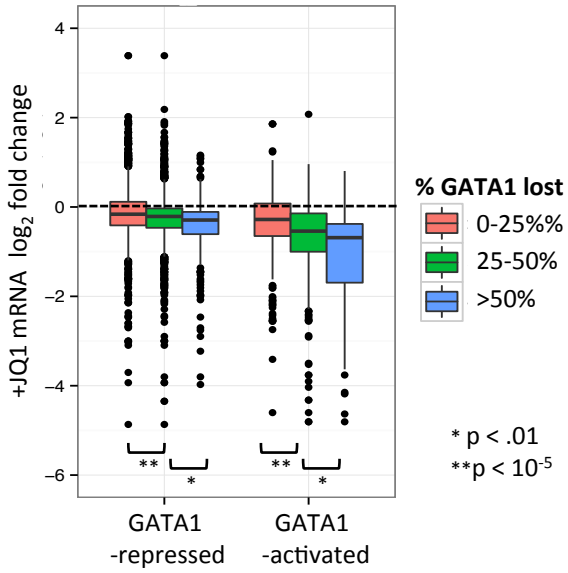


Figure 3.6 GATA1 occupancy is a better predictor of JQ1 sensitivity at GATA1-activated than GATA1-repressed genes. GATA1-repressed and GATA1-activated genes are defined as those changing at least two-fold upon GATA1 induction relative to spike-in control. GATA1 sites were linked to the nearest gene within 5kb. Dotted black line indicates no change upon JQ1 treatment.

	% GATA1 sites overlapping regulatory region or binding site:				
% GATA1 retained	DNase hypersensitive	promotor	enhancer	TAL1	CTCF
<40% (125 sites)	82.4	22.4	59.2	72.0	6.4
40-50% (516 sites)	75.2	20.2	52.5	56.6	8.1
50-60% (1108 sites)	73.2	21.9	51.2	46.2	9.2
60-70% (1312 sites)	76.0	24.4	52.2	44.8	9.8
70-80% (961 sites)	80.6	27.6	55.7	40.2	10.0
80-90% (520 sites)	83.7	25.6	55.6	38.1	11.3
90%+ (97 sites)	88.7	24.7	59.8	32.0	15.5

Table 3.1 Relationship of differentially JQ1 sensitive GATA1 sites to promoters, enhancers, and ENCODE-defined genomic regions. Promoter regions are here defined as within 2.5 kb of transcription start sites. Enhancer regions are defined as H3K4me1-enriched, DNase-hypersensitive regions that do not overlap transcription start sites (Hsiung et al., 2014). ENCODE regions represent all transcription factor regions defined in GATA1-null erythroblasts following GATA1 induction. Enhancers were H3K4me1 enriched, DNase hypersensitive sites at least 500bp away from transcription start sites and defined in G1E cells as in (Hsiung et al., 2014).

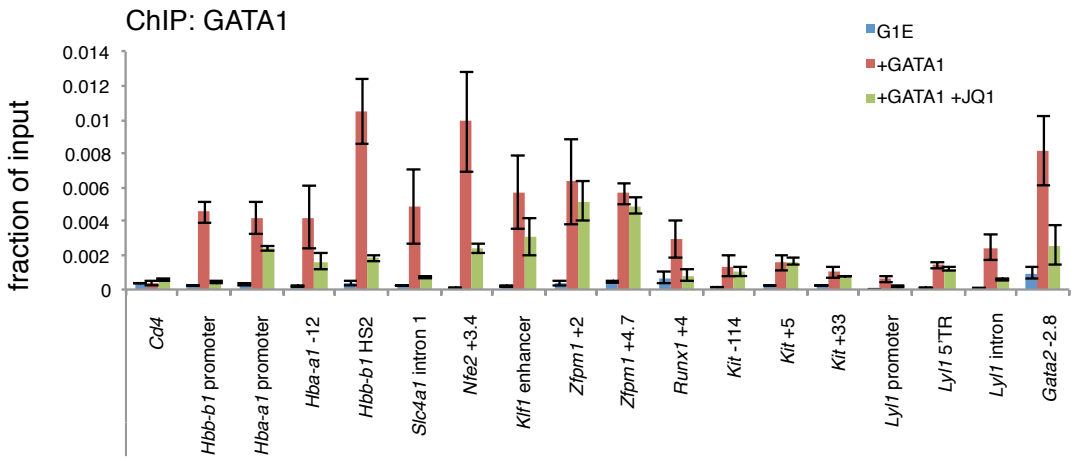


Figure 3.7 Confirmation of variable BET-dependent GATA1 occupancy changes by ChIP-qPCR. GATA1 ChIP performed in the absence of GATA1 (blue bars), following 24 hours of GATA1 induction (red bars), or 24 hours of GATA1 induction with concurrent 250nM JQ1 treatment. *Cd4* is a negative control for GATA1 binding. Error bars represent SEM of three independent biological experiments.

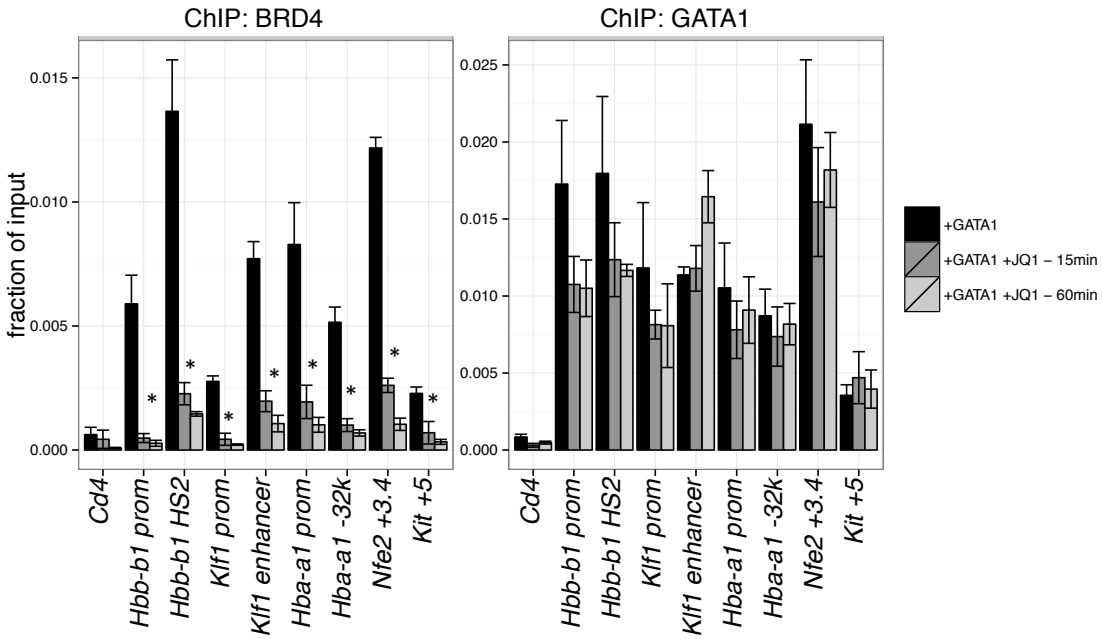


Figure 3.8 Short-term effect of BET inhibition on established GATA1 occupancy. ChIP for BRD4 and GATA1 in G1E GATA1-ER cells with established GATA1 activation treated with 250nM JQ1 for up to 60 minutes. *Cd4* is a negative control for GATA1 occupancy. Error bars represent SEM of three biological replicates. * indicates $p < .05$.

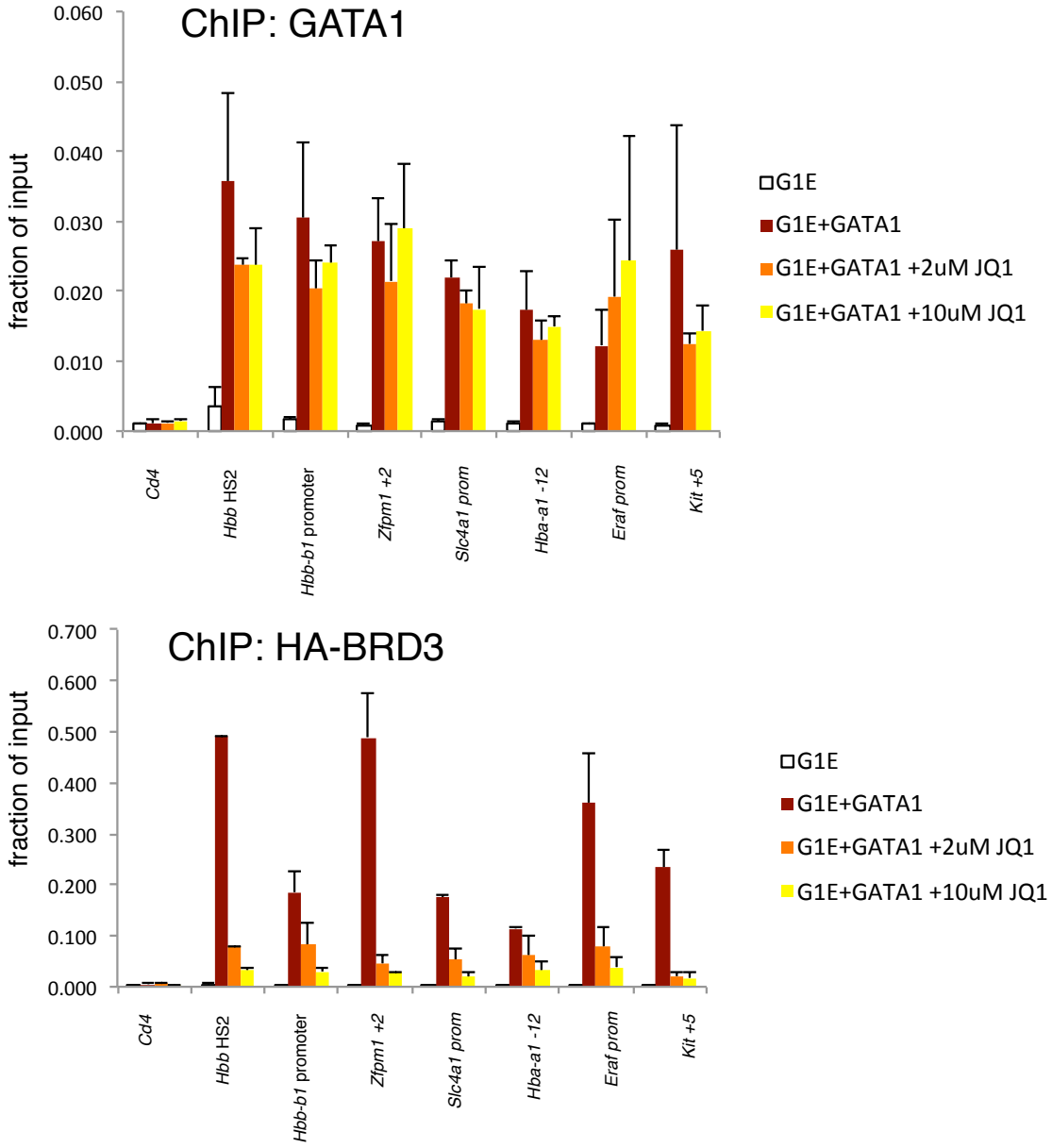


Figure 3.9 Effect of BET inhibition at various doses for one hour on established GATA1 occupancy. G1E ER-GATA1 cells retrovirally expressing HA-BRD3 were induced to activate GATA1 for 24 hours, and subsequently treated with indicated JQ1 concentration for one hour. ChIP-qPCR for (A) GATA1 and (B) HA-BRD3 at GATA1 sites in these cells. Error bars represent SEM of three independent biological experiments.

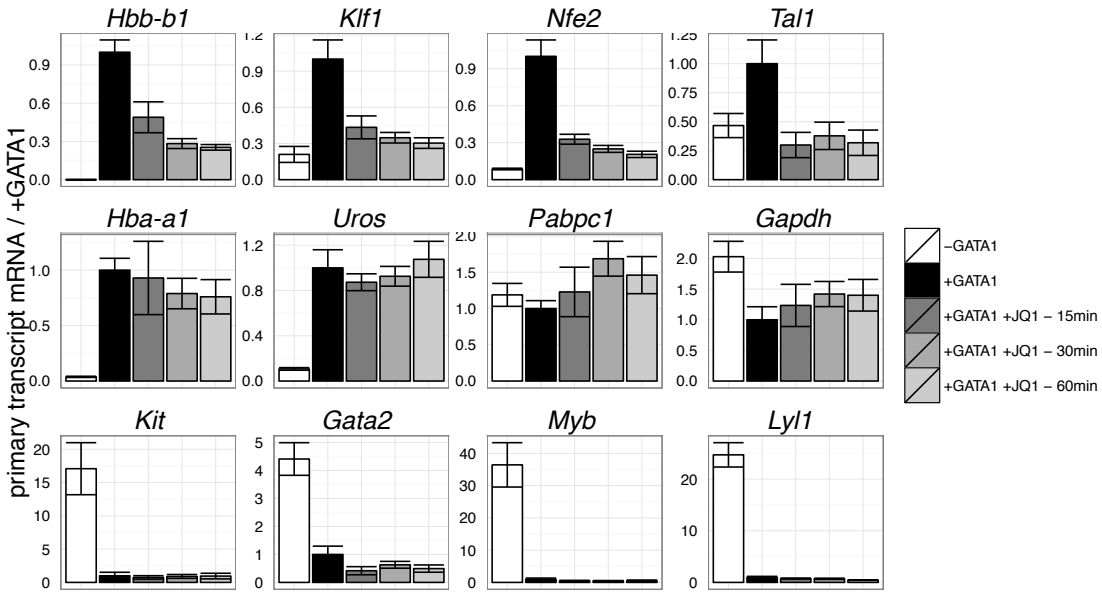


Figure 3.10 Transcriptional requirement of BET proteins following establishment of GATA1 occupancy. Primary transcript RT-qPCR measuring levels of indicated transcripts in response to 250nM JQ1 treatment in GATA1-induced cells. Error bars represent SEM from four biological replicates. This data was generated as part of a dose-response (Figure 3.11).

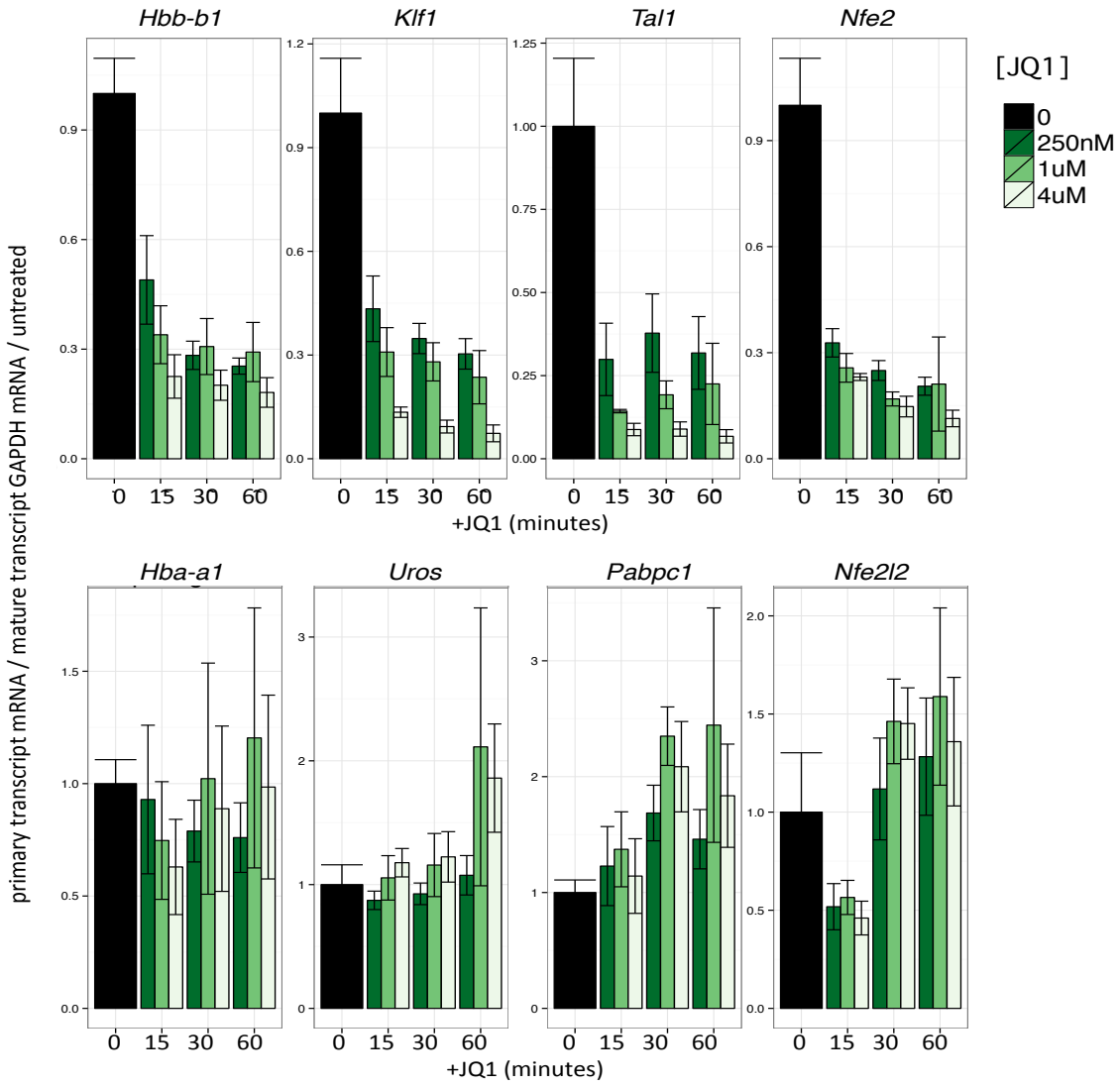


Figure 3.11 Dose-response time course of primary transcript levels in GATA1-activated cells following short-term BET inhibitor treatment. Primary transcript primers span exon-intron boundaries. Results are normalized to mature transcript GAPDH and shown as a fraction of expression in untreated GATA1-activated cells. Error bars represent SEM of 4 biological experiments.

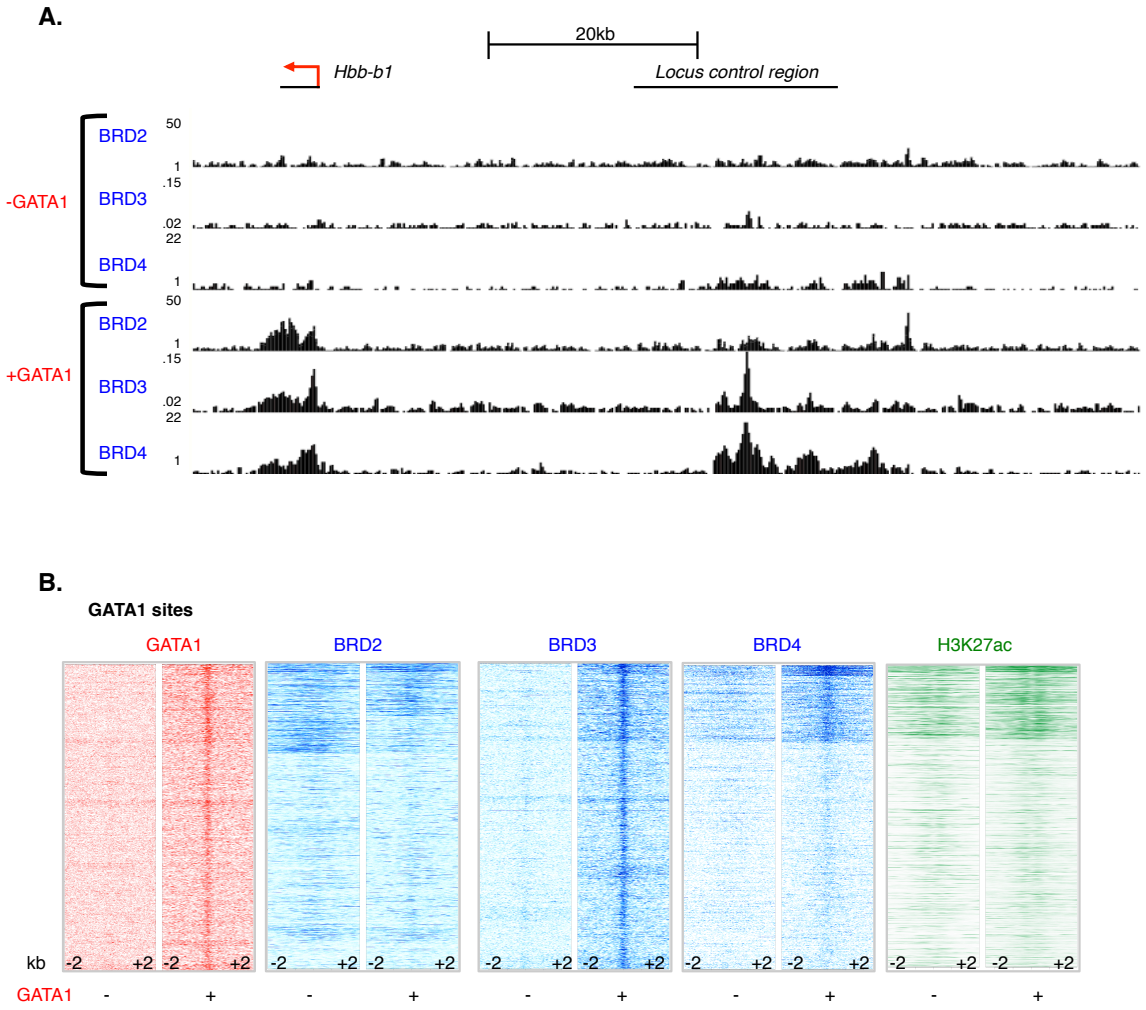


Figure 3.12 BETs associate with GATA1 sites upon GATA1 complementation. (A) UCSC Genome Browser track showing BRD2, BRD3, and BRD4 signal at the *beta-globin (Hbb)* locus in G1E cells in the absence and presence of GATA1. (B) ChIP-seq signal across 4kb regions centered on 13,123 mouse ENCODE GATA1-binding sites in G1E cells clustered by k-means (k=5).

		-GATA1			+GATA1		
Cell line	MACS	% BRD2 (10557 sites)	% BRD3 (4001 sites)	% BRD4 (11293 sites)	% BRD2 (15834 sites)	% BRD3 (9110 sites)	% BRD4 (12328 sites)
G1E	DNase	93.9	29.9	47.2	84.2	70.8	68.6
	promotor	44.4	16.7	21.3	38.0	37.8	44.3
	enhancer	20.9	6.3	21.6	21.3	20.6	23.0
	GATA1	0.1	0.0	0.0	10.2	24.1	33.1
	GATA2	2.9	3.2	2.1	3.6	11.3	2.5
	TAL1	4.6	2.6	13.8	2.9	14.1	17.5
MEL	DNase	94.6	31.6	48.1	96.9	73.2	73.4
	ETS1	7.3	4.3	16.3	5.7	13.4	21.2
	KAT2A	10.8	3.6	2.7	8.7	10.0	8.4
	P300	6.8	3.0	20.7	4.8	14.2	23.6
	MYB	2.6	1.3	6.8	1.9	3.9	4.9
	MYC	16.0	4.3	10.5	12.3	13.5	11.8
	SISSRS	% BRD2 (14208 sites)	% BRD3 (726 sites)	% BRD4 (2663 sites)	% BRD2 (9921 sites)	% BRD3 (3623 sites)	% BRD4 (5945 sites)
G1E	DNase	95.5	51.4	68.9	84.9	92.6	80.7
	promotor	56.6	23.3	29.1	41.6	55.1	51.1
	enhancer	19.2	9.9	31.4	23.0	26.1	26.9
	GATA1	0.0	0.0	0.0	6.3	33.7	43.1
	GATA2	0.6	5.6	2.4	0.7	4.9	2.0
	TAL1	3.8	5.4	19.9	1.2	22.1	24.5
MEL	DNase	96.5	50.4	66.1	98.1	92.3	83.7
	ETS1	8.8	9.6	25.2	5.7	21.9	28.3
	KAT2A	13.3	5.9	2.9	9.7	15.7	7.9
	P300	8.6	3.4	32.0	4.9	21.2	32.0
	MYB	3.5	2.1	10.9	1.9	5.7	5.9
	MYC	22.2	6.3	15.8	12.5	18.2	13.2

Table 3.2 Comparison of BET binding sites to regulatory regions and transcription factor binding sites in the absence and presence of GATA1. DNase hypersensitive sites and transcription factor binding sites were defined by the mouse ENCODE project. Factor binding sites are from G1E or MEL (murine erythroleukemia) cells. Enhancers were H3K4me1 enriched, DNase hypersensitive sites at least 500bp away from transcription start sites and defined in G1E cells as in (Hsiung et al., 2014).

MACS		% of BRD2 sites	% of TF/RR sites
% BRD2 (endog.)	DNase	84.2	13.7
15,834 sites	promotor	38.0	28.7
	enhancer	21.3	11.1
	GATA1	10.2	13.3
	GATA2	3.6	4.3
	TAL1	2.9	9.7
HABRD2 rep 1	DNase	71.6	25.5
34,566 sites	promotor	33.1	44.1
	enhancer	26.8	32.2
	GATA1	19.2	58.2
	GATA2	3.7	10.0
	TAL1	8.8	65.7
HABRD2 rep 2	DNase2	71.7	25.0
32,845 sites	promotor	33.4	43.5
	enhancer	26.9	31.5
	GATA1	19.3	56.7
	GATA2	3.7	9.6
	TAL1	8.8	63.6
SISSRS		**% of BRD2 sites**	**% of TF/RR sites**
% BRD2 (endog.)	DNase	84.9	7.5
9921 sites	promotor	41.6	15.5
	enhancer	23.0	7.2
	GATA1	6.3	4.3
	GATA2	0.7	0.5
	TAL1	1.2	2.4
HABRD2 rep 1	DNase	73.9	25.1
32,965 sites	promotor	34.2	43.7
	enhancer	28.1	32.2
	GATA1	19.8	57.5
	GATA2	2.7	6.6
	TAL1	9.1	65.0
HABRD2 rep 2	DNase2	73.9	24.5
31,350 sites	promotor	34.5	43.0
	enhancer	28.1	31.5
	GATA1	19.9	56.0
	GATA2	2.8	6.4
	TAL1	9.2	63.0

Table 3.3 Comparison of endogenous BRD2 with exogenous HA-BRD2. All comparisons are in G1E cells induced with GATA1. HA-BRD2 is expressed approximately 5-fold higher than endogenous at the mRNA level than endogenous BRD2 (endog.). TF/RR - transcription factor or regulatory region.

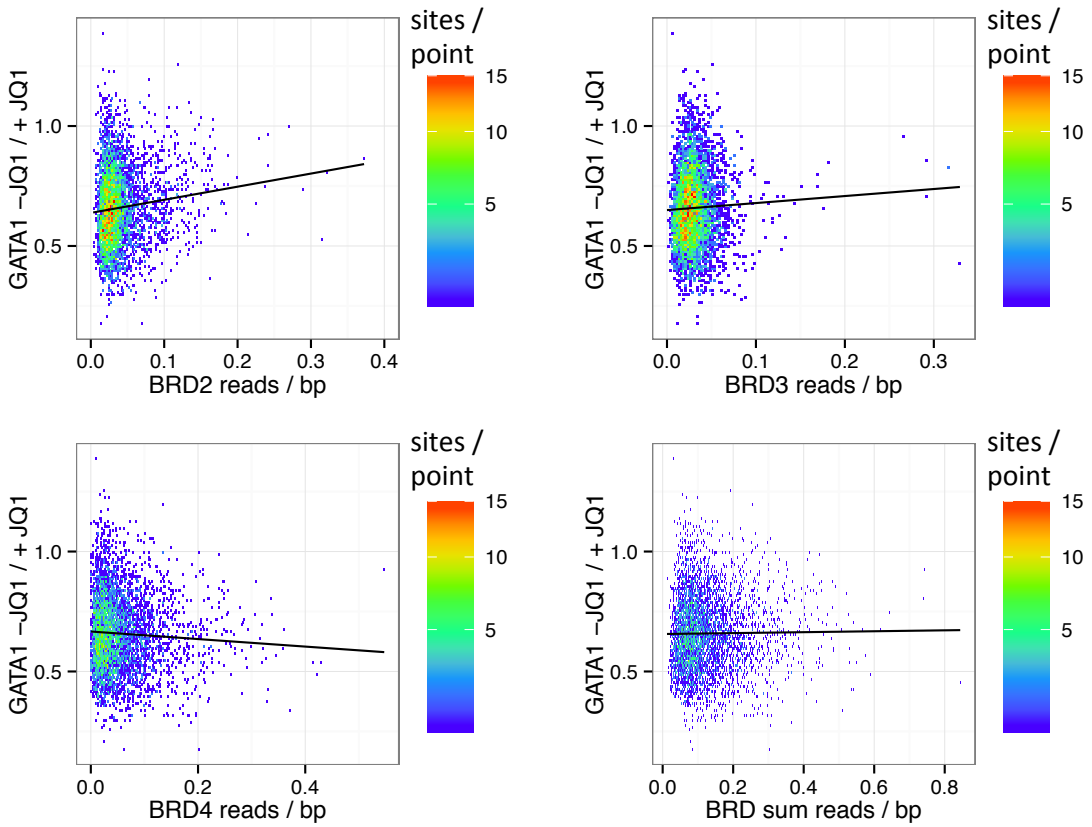


Figure 3.13 BET occupancy and JQ1-mediated GATA1 eviction. Scatter plots show the relationship of BRD2, BRD3, and BRD4 occupancy with fractional GATA1 loss mediated by BET inhibition. Color shows the number of GATA1 sites at each point. Black lines show a linear regression.

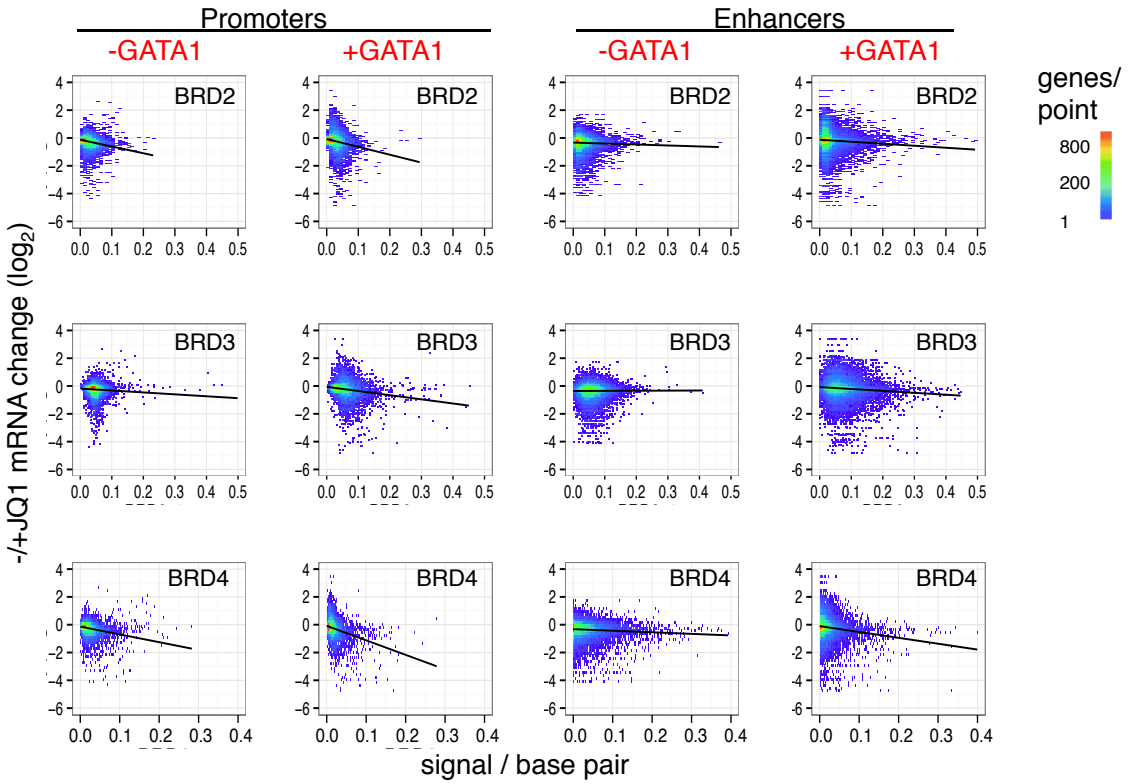


Figure 3.14 Ability of individual BET promoter and enhancer occupancy to predict JQ1 sensitivity at nearby genes. Binned density plots where color indicates number of points showing BRD2 (top row), BRD3 (middle row) or BRD4 (bottom row) occupancy at promoters and enhancers plotted against JQ1-induced transcriptional response of genes associated with those elements. Enhancers are linked to nearest gene within 100kb. Variation of genomic linkage between 10kb and 1mb gave similar results. Black trend-line shows a linear regression, Pearson coefficient $r^2 < .1$ in each plot.

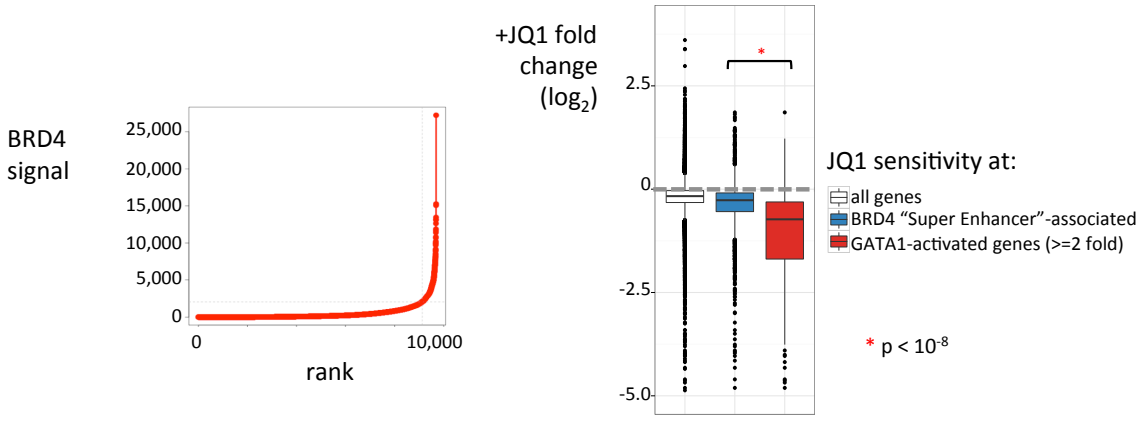


Figure 3.15 Comparison of GATA1 activated genes to BRD4-"super enhancers" in predicting JQ1 sensitivity. (Left panel) Annotation of 564 super-enhancers (SEs) using ROSE (Lovén et al., 2013) and BRD4 ChIP-seq. (Right panel) JQ1 sensitivity of SE-associated genes (linked to nearest gene when not overlapping one) compared to JQ1 sensitivity of GATA1-activated genes.

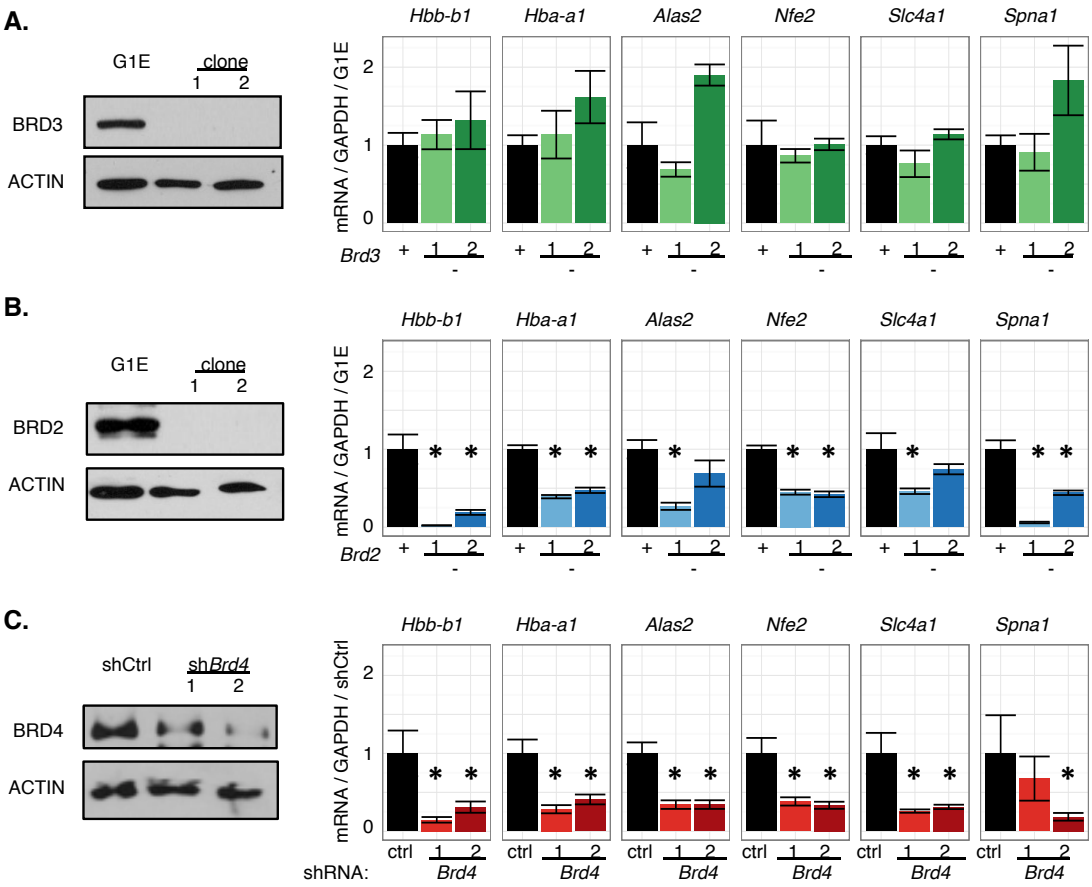


Figure 3.16 Functions of individual BETs in GATA1-activated transcription. (A-C) Left: Western blots with antibodies against listed BET proteins; right: Relative transcript levels following GATA1 activation in cells depleted of (A) BRD3 (B) BRD2, or (C) BRD4. BRD4 reduction was achieved by shRNA-mediated *Brd4* knockdown. (D) shRNA-mediated *Brd3* knockdown in BRD2 replete versus deficient cells. Error bars represent SEM, n=3. * indicates $p < .05$ for BRD2 or BRD4 deficient cells compared to unmodified or control hairpin infected erythroblasts.

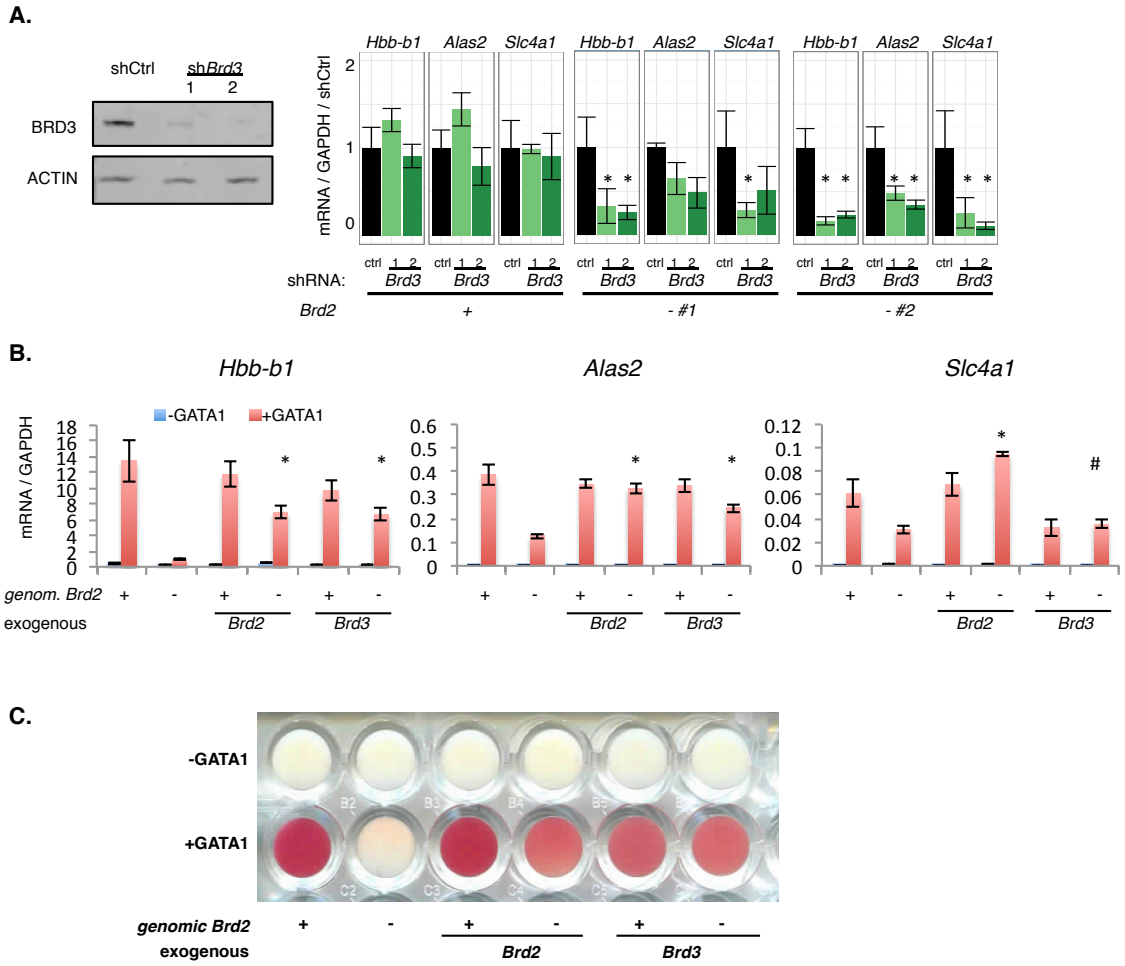


Figure 3.17 Evidence for overlapping functionality between BRD2 and BRD3. (A) Immunoblot showing BRD3 protein level following shRNA-knockdown with ACTIN control (left panels). shRNA-mediated BRD3 knockdown in unmodified G1E GATA1-ER cells or BRD2-deficient cells (right panels). * indicates $p < .05$. (B) qRT-PCR for GATA1-activated genes * indicates $p < .01$ that measured mRNA in *Brd2*- or *Brd3*-expressing *Brd2*-deficient cells is greater than in *Brd2*-deficient cells following GATA1 induction. # indicates $p > .2$. (C) Representative cell pellets before and after GATA1 induction in unmodified cells or BRD2-deficient cells infected or not with retroviruses expressing *Brd2* or *Brd3*. Error bars show SEM and n=3 to 4 for each experiment.

CHAPTER 4. ORGANIZATION AND FUNCTION OF BET PROTEINS DURING MITOSIS

Chapter summary

Most transcription factors are evicted from chromatin during mitosis, yet lineage-specific transcription patterns faithfully reactive at the start of each new cell cycle. Bromodomain and Extra-Terminal motif proteins (BETs) have been implicated in 'bookmarking' previously activated genes for rapid post-mitotic transcription. However, neither genomic BET organization during mitosis nor its functional implications have been examined in depth. Here we test mitotic bookmarking by BETs in the context of GATA1-mediated erythropoiesis. GATA1 recruits BETs in interphase, and is itself implicated in mitotic bookmarking, raising the possibility of functional convergence between GATA1 and BETs at the level of mitotic bookmarking. We define genome-wide mitotic occupancy of BRD4, the BET most commonly implicated in bookmarking. We find that it occupies largely distinct binding sites in mitosis relative to interphase, but remains bound to mitotic chromatin at some loci. To test the functional importance of BET bookmarking during mitosis, we pharmacologically disrupt BET binding during this phase of the cell cycle. This has no measurable effect on post-mitotic transcriptional activation. These results suggest that continuous BET binding during mitosis may not have a major role in preserving the transcriptional state of particular genes.

Introduction

Mitotic chromatin condensation is associated with widespread global ejection of transcription factors from DNA (Martínez-Balbás et al., 1995). The longstanding observation that nuclease hypersensitivity can be retained in the vicinity of active genes led to the proposal that some 'bookmarking' factors bind mitotic chromatin to allow immediate accessibility and transcriptional activation at the beginning of the next cell cycle (Gazit et al., 1982; John & Workman, 1998; Michelotti et al., 1997). This may be a way in which cellular lineage identity is

maintained during cell division (Kadauke & Blobel, 2012; Sarge & Park-Sarge, 2005; Zaidi et al., 2010). While an increasing number of transcription factors have been identified that remain at least partially bound to mitotic chromatin (Blobel et al., 2009; Caravaca et al., 2013; D. Chen, Hinkley, Henry, & Huang, 2002), functional evaluation of bookmarking remains a major challenge. Standard approaches, such as RNAi, deplete factors throughout the cell cycle. Mitosis-specific transcription factor functions remain largely untested.

Bromodomain and extra-terminal motif proteins (BETs) are among the factors most commonly speculated to function as mitotic bookmarks (Devaiah & Singer, 2013; Follmer & Francis, 2011; Voigt & Reinberg, 2011). This is due to their global association with mitotic chromatin in many cell types combined with their ability to support transcriptional reactivation in newborn cells (Dey et al., 2003; 2009; Toyama, Rebbert, Dey, Ozato, & Dawid, 2008; R. Zhao et al., 2011). Additionally, the ability of chromosomal translocations involving BETs to abrogate cellular differentiation (French et al., 2003; Yan, Diaz, Jiao, Wang, & You, 2011), and of pharmacologic BET inhibition to cause differentiation of some cancers (Filippakopoulos et al., 2010; Zuber et al., 2011), implicate BETs in lineage maintenance. BRD4 has been reported to bind to mitotic chromatin at promoters (Dey et al., 2009). BRD4 also has a clear functional role in transcription in many systems, and a defined role in post-mitotic transcriptional reactivation (Dey et al., 2009; R. Zhao et al., 2011). Interestingly, BRD4 expression has been implicated both in preserving lineage and in facilitating its change (Di Micco et al., 2014; Fernandez et al., 2014; W. Liu et al., 2014). Despite repeated observation, the importance of the association of BRD4 with mitotic chromosomes in the cell fate decisions influenced by this protein remains poorly understood.

Recent work has shown that the erythroid lineage factor GATA1 is retained at select sites on mitotic chromatin and may have a functional role in mitotic bookmarking (Kadauke et al., 2012). As GATA1 recruits BETs to its occupied sites, we hypothesized that BETs have a role in bookmarking at GATA1 sites. We first examine the genomic localization of BRD4 and second test

its function through transient removal in GATA1 null erythroblasts following GATA1 complementation (G1E+GATA1). We find that BRD4 binding is extensively reorganized during mitosis. While mitotic retention of BRD4 appears independent of mitotic GATA1 retention, some of the sites it remains bound to are putative GATA1-bookmarked genes. We utilize the kinetic control afforded by small molecule BET inhibitors to specifically disrupt BET binding during mitosis to directly test the hypothesis that BETs function as bookmarks. We find that post-mitotic transcriptional reactivation is insensitive to disruption of BET binding during mitosis. This suggests BET binding during mitosis may not significantly contribute to post-mitotic transcriptional state.

Results

Dynamics of BET occupancy during mitosis

To examine mitotic BET occupancy, we performed ChIP in mitotic G1E cells purified using two independent methods. In the first, cells were prevented from progressing beyond pro-metaphase of mitosis using nocodazole, and mitotic cells further purified by fluorescence-activated cell sorting (FACS) for the mitotic marker phospho-MPM2 (Figure 4.1A) (Campbell, Campbell, Chris, Chris, et al., 2014a; Kadauke et al., 2012). Nocodazole causes microtubule disruption, and has been shown to destabilize BET binding in some settings (Nishiyama et al., 2012). To address the possibility that any binding patterns observed resulted from nocodazole treatment, we developed a parallel method that captured cells progressing through mitosis normally (Figure 4.1B). Cells were arrested immediately prior to mitosis in late G2 using the CDK1 inhibitor ro3306 (Vassilev et al., 2006), released from this block, allowed to enter mitosis, and sorted for phospho-MPM2. Unlike the nocodazole arrest method in which recovered cells were homogenously in pro-metaphase (Figure 4.2A), this method returned cells distributed across all mitotic sub-stages (Figure 4.2B-D).

Initial inspection of BRD4 ChIP-seq data from mitotic cell populations at critical GATA1 occupied-loci demonstrated that ChIP-seq signal from each of the two mitotic populations was overall similar (Figure 4.3). Interestingly, occasional sharp signal peaks were higher in the nocodazole-enriched cells that could represent features specific to pro-metaphase. During mitosis BRD4 was almost entirely lost from the *beta-globin (Hbb)* locus and retained at the *Zfpm1* and *Nfe2* loci. Interestingly, GATA1 also departs from *Hbb* and is retained at *Zfpm1* and *Nfe2* during mitosis. We next defined mitotic BRD4 sites genome-wide independently using two different peak callers (MACS and SISSRS). The nocodazole-arrest BRD4 data was used for this analysis because of its superior signal-to-noise ratio. Interestingly, most mitotic BRD4 sites were specific to this phase of the cell cycle, and largely overlapped GATA1 binding sites (Figure 4.4). We investigated this further using a heatmap to visualize mitotic binding at GATA1 sites. As a control we compared this pattern to association with all DNase hypersensitive sites in G1E cells (mouse ENCODE) (Figure 4.5). Interphase BRD4 binding correlated weakly with mitotic binding, and a subset of GATA1 sites with strong binding in mitosis but little in interphase was apparent using k-means clustering (k=5). We then expanded our analysis to consider mitotic BRD4 binding in the context of additional annotated genomic regions in G1E cells (Table 4.1). In contrast to published work suggesting BRD4 is maintained at promoters during mitosis (Dey et al., 2009), mitotic BRD4 sites were greatly reduced at promoters relative to enhancers (highlighted in orange). These sites were highly enriched for GATA1, TAL1, p300, and ETS1 binding (highlighted in yellow). This could indicate mitotic recruitment by one of these factors.

Role of mitotic BET protein binding in transcriptional reactivation

To directly test the functional role of mitotic BET binding, we undertook a strategy to specifically disrupt mitotic binding using the pharmacologic BET inhibitor JQ1. Remodeling of BET binding occupancy raises the possibility that mechanisms other than bromodomain binding are responsible for their association with particular locations during this time period. To confirm

that JQ1 efficiently removes BETs from mitotic chromatin, we treated mitotically arrested cells with JQ1 and performed mitotic ChIP to measure BRD4 occupancy (Figure 4.6). Endogenous BRD4 was efficiently removed from mitotic chromatin by JQ1 supporting its use in disruption of mitotic BET occupancy. To test the requirement for continuous BET binding to maintain transcriptional state during mitosis, we disrupted BET occupancy specifically during mitosis with JQ1 and measured post-mitotic transcriptional reactivation of erythroid genes (Figure 4.7). To insure that the cell population being assayed had indeed progressed through mitosis, post-mitotic cells were purified by fluorescence-activated cell sorting (FACS) using a combination of small cell size (FSC-low) and low fluorescence intensity of mitotically degraded YFP (Kadauke et al., 2012). Mitosis-specific JQ1 treatment did not significantly alter cell cycle progression in newborn cells (Figure 4.8). Despite the ability of primary transcript qPCR to detect small changes in transcription rate, transient BET disruption during mitosis did not measurably affect reactivation of any erythroid gene measured (Figure 4.9). Importantly the genes assayed included GATA1-activated genes where a bookmarking function has been reported (*Zfpm1, Nfe2, Runx1*), all of which are bound by BRD4 in mitosis and two of which (*Nfe2* and *Runx1*) are very JQ1-sensitive in interphase. Additionally no transcriptional changes were observed in GATA1-repressed genes where bookmarking has been suggested to prevent expression of lineage-inappropriate genes (*Kit, Gata2*). A post-mitotic transcriptional spike was apparent in a number of transcripts (*Nfe2, Runx1, Gata2, Myb*), as has been observed previously in our lab (Hsiung et al., in preparation). This was also unaffected by mitotic BET disruption. Together these results call into question whether BETs need to remain bound in mitosis in order for post-mitotic transcription to reactivate normally.

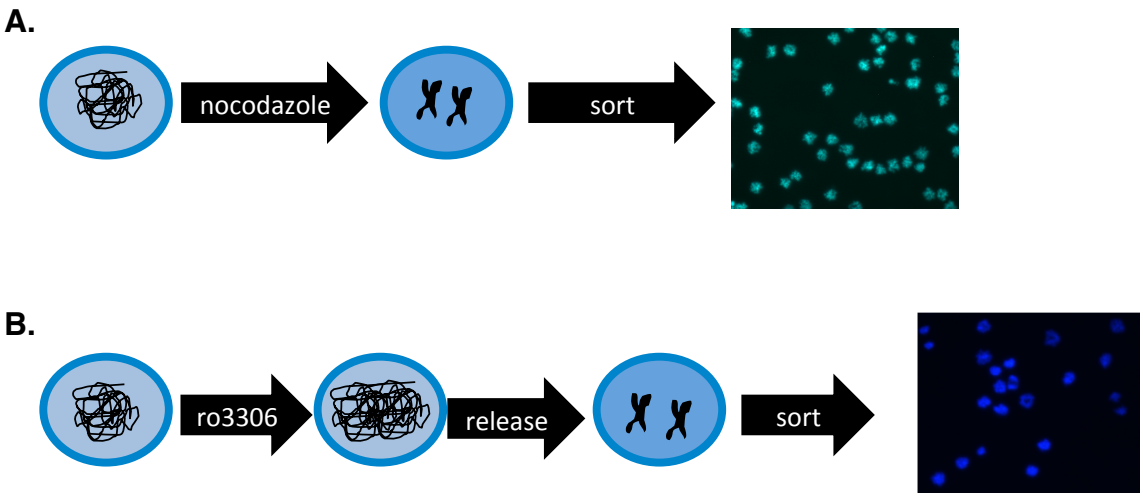


Figure 4.1. Mitotic purification methods. (A) Purification of a pro-metaphase mitotic population by cell sorting from a population of cells treated with nocodazole (Campbell, Hsiung, & Blobel, 2014b). (B) Isolation of mitotic cells from cell synchronized in late G2 with ro3306 (Vassilev et al., 2006), released, and sorted while progressing through mitosis naturally.

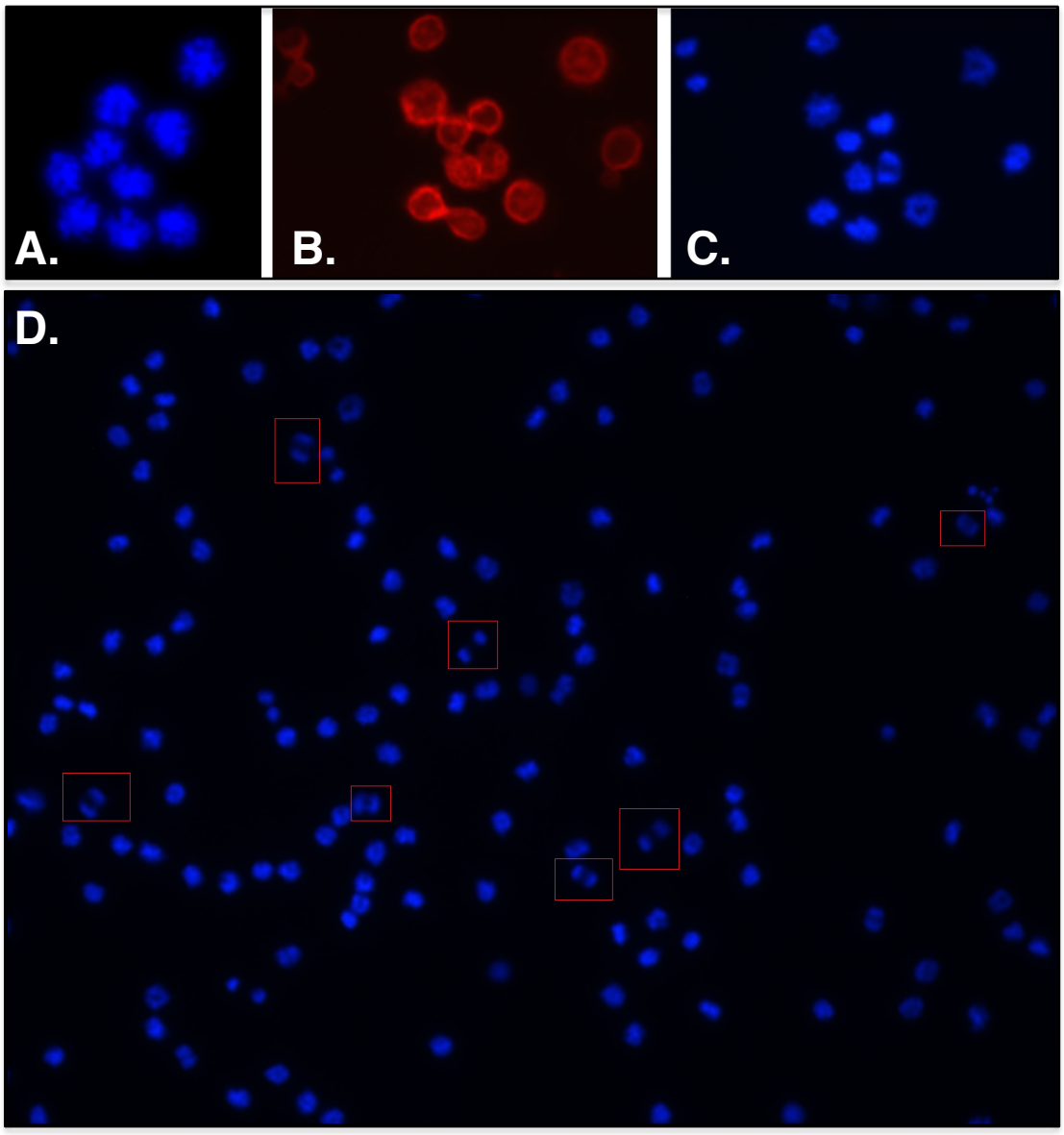


Figure 4.2 Visualization of mitotic cells by fluorescence microscopy. (A) Mitotic cells arrested in pro-metaphase recovered using the nocodazole arrest method. Chromatin is homogenously condensed. (B-D) Mitotic cells recovered using the ro3306 method. (B) Phospho-MPM2 and (C) DAPI stains showing the same cells. (D) A wider field showing the diversity of mitotic cells recovered using ro3306-based synchronization. Red boxes highlight anaphase and telophase cells.

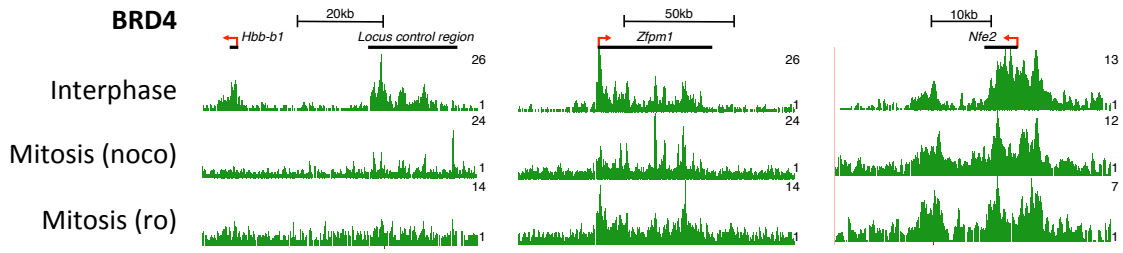


Figure 4.3 BRD4 ChIP-seq at key erythroid genes in interphase and mitosis. Mitotic ChIP using either nocodazole or ro3306 demonstrates loss of BRD4 binding around the *Hbb (beta-globin)* locus (left) and gross retention of BRD4 at the *Zfpm1* (middle) and *Nfe2* loci (right). Browser tracks were displayed using the UCSC Genome Browser.

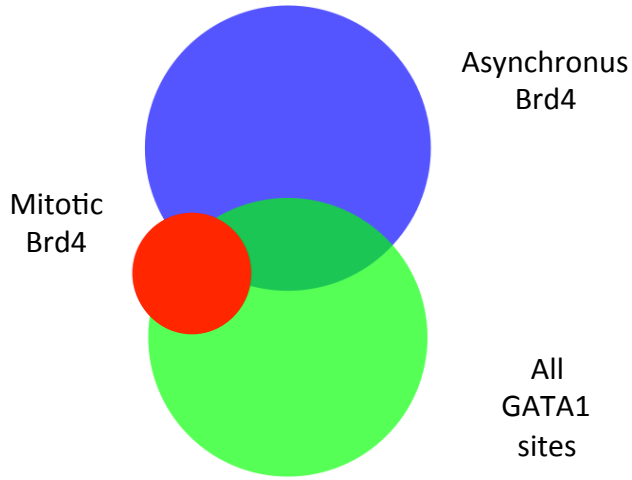


Figure 4.4 Relationship of mitotic BRD4 binding with BRD4 and GATA1 in asynchronous cells. Circles are drawn in proportion to the 2,064 mitotic BRD4 sites, 12,328 asynchronous BRD4 sites, and 13,123 GATA1 sites used in this comparison.

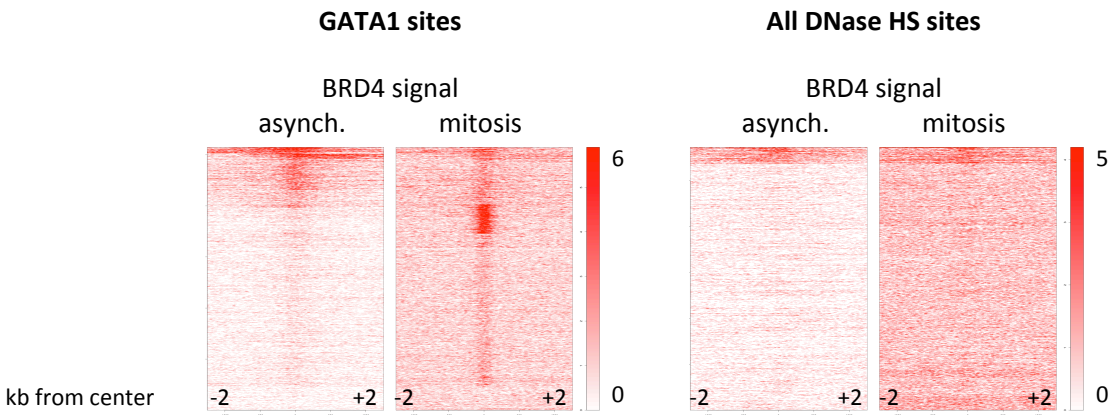


Figure 4.5 BRD4 occupies distinct GATA1 sites in mitosis. Heatmaps show BRD4 signal across four kilobase windows centered on ENCODE-defined GATA1 peaks (left) and DNase hypersensitive sites (right) in G1E+GATA1 cells.

	% of BRD4 sites		
	BRD4 peaks (SISSRS)	asynch BRD4	mitotic BRD4
G1E	DNase	80.7	69.6
	promotor	51.1	10.7
	enhancer	26.9	78.2
	GATA1	43.1	77.5
	GATA2	2.0	0.7
	TAL1	24.5	44.1
MEL	(MEL) GATA1	42.6	91.4
	(MEL) ETS1	28.3	73.4
	(MEL) KAT2A	7.9	1.1
	(MEL) P300	32.0	87.6
	(MEL) MYB	5.9	25.6
	(MEL) MYC	13.2	10.9
	BRD4 peaks (MACS)	asynch BRD4	mitotic BRD4
G1E	DNase	68.6	71.3
	promotor	44.3	15.7
	enhancer	23.0	72.5
	GATA1	33.1	75.1
	GATA2	2.5	1.6
	TAL1	17.5	42.3
MEL	(MEL) GATA1	36.2	88.0
	(MEL) ETS1	21.2	68.4
	(MEL) KAT2A	8.4	1.5
	(MEL) P300	23.6	81.6
	(MEL) MYB	4.9	23.0
	(MEL) MYC	11.8	12.7

Table 4.1 Comparison of BRD4-enriched regions in asynchronous and mitotic cells with genomic regulatory regions. DNase hypersensitive and transcription factor regions were downloaded from mouse ENCODE (Mouse ENCODE Consortium et al., 2012). Promoters are within 2.5kb of Refseq transcription start sites. Enhancers are H3K4me1-enriched, DNase hypersensitive regions that do not overlap transcription start sites (Hsiung et al., 2014).

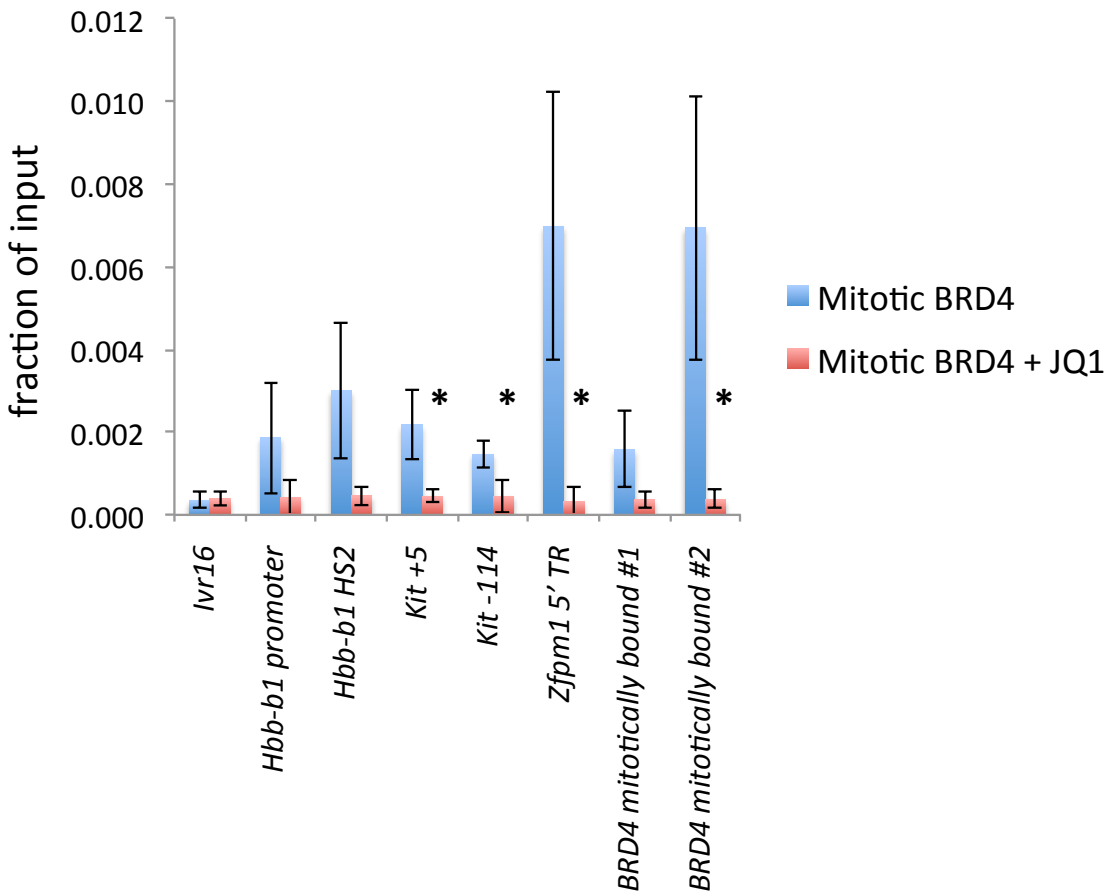


Figure 4.6 Mitotically retained BRD4 remains sensitive to JQ1. Mitotic BRD4 ChIP in nocodazole-treated cells treated or not with 250nM JQ1 for 15 minutes. *Ivr16* is a negative control for BRD4 binding. Sites labeled *BRD4 mitotically bound* were selected from BRD4 mitotic ChIP-seq peaks at GATA1 sites. Error bars represent SEM. n=3 for each condition. * indicates $p < .05$ that JQ1-treated samples are lower than untreated.

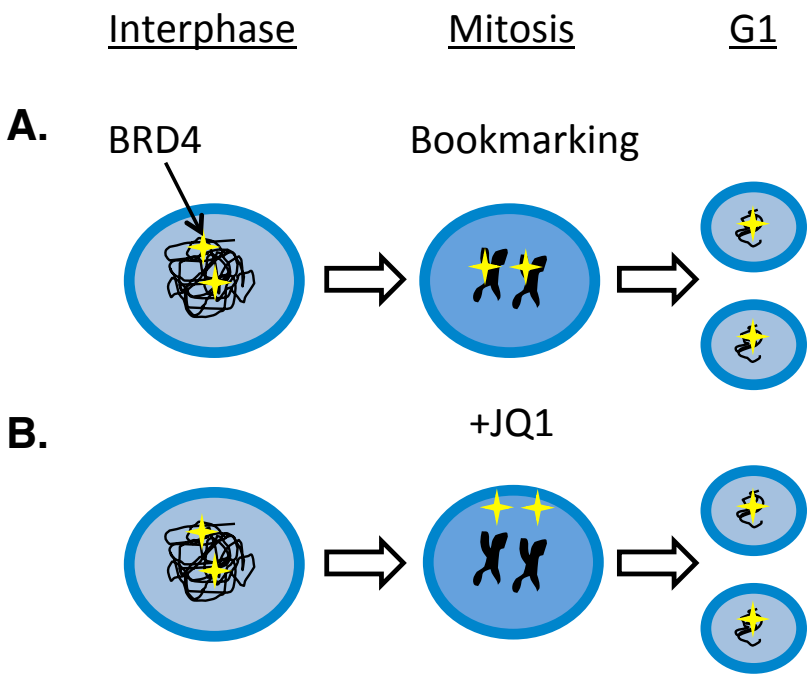


Figure 4.7 Testing the "bookmarking" hypothesis. (A) Mitotic bookmarking implies that stable association of proteins such as BRD4 throughout mitosis has a role transcriptional identity in newborn cells. (B) Disruption of BRD4 occupancy during mitosis using JQ1 perturbs continuous BET occupancy and tests the reversibility of its mitotic removal.

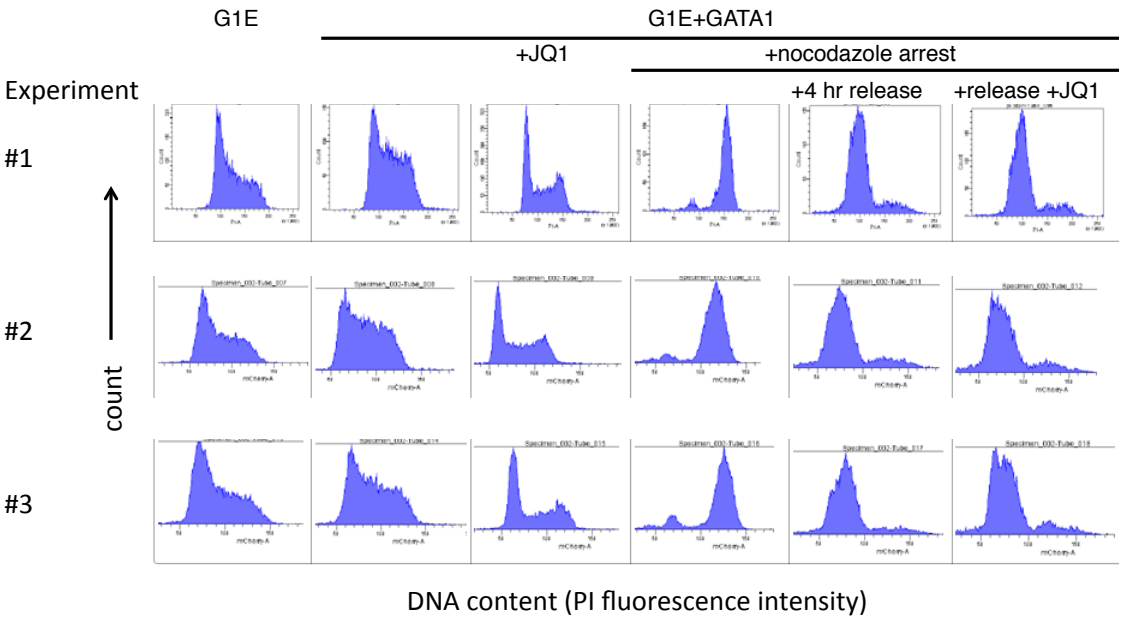


Figure 4.8 Mitotic BET inhibition does not alter cell cycle progression. G1E cells were induced with GATA1, arrested with nocodazole, treated with JQ1 or not beginning in mitosis, and released from nocodazole block. DNA content was measured by propidium iodide (PI) stain measured by flow cytometry (arbitrary linear scale) following EtOH fixation. Each row is an independent biological experiment.

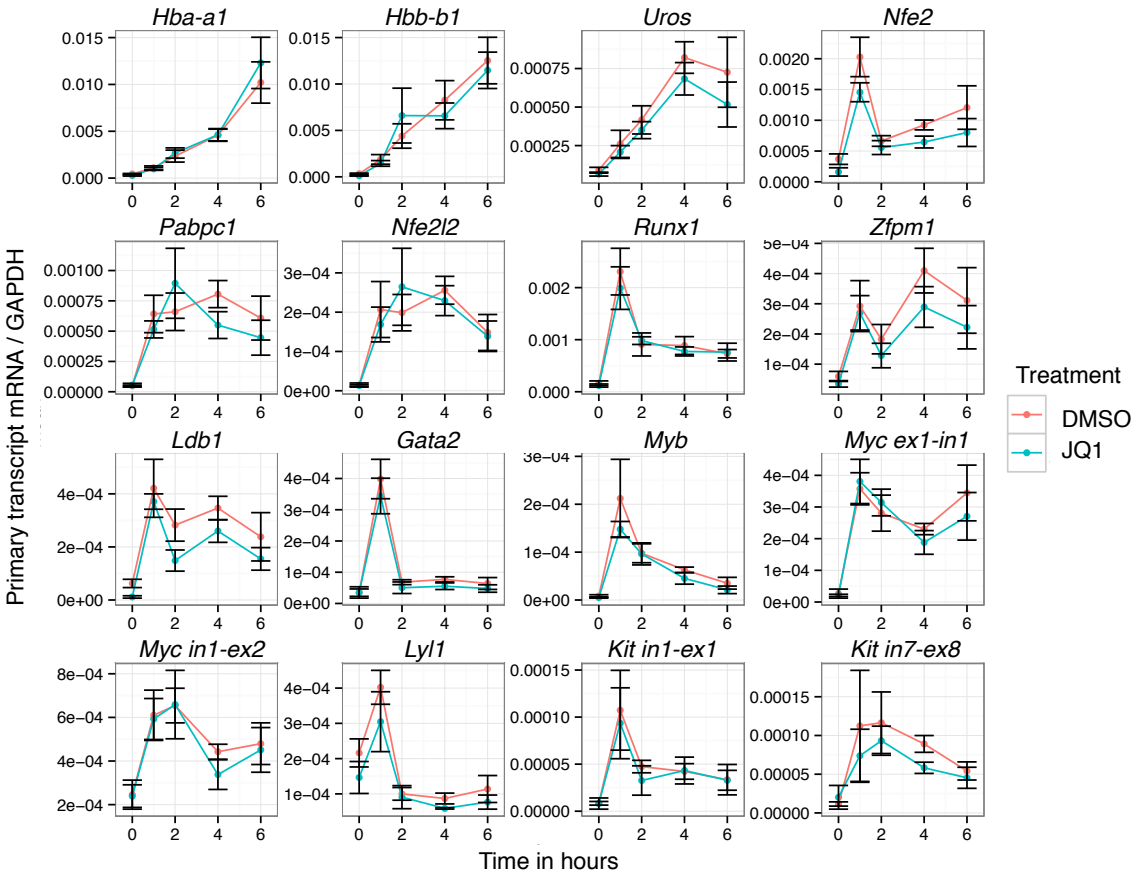


Figure 4.9 Mitotic BET disruption does not significantly affect post-mitotic transcriptional reactivation. G1E+GATA1 cells expressing mitotically degraded YFP were arrested in mitosis with nocodazole, treated with JQ1 (1uM for 15 minutes) or DMSO control, and released into growth media for the indicated times. Newborn cells (YFP-dim, FSC-small) were sorted and analyzed by primary transcript qRT-PCR. Error bars represent SEM. n=6 for each condition.

CHAPTER 5. DISCUSSION

Chapter summary

The advance of BET inhibitors into human clinical trials provides a strong incentive to better understand the role of BETs in normal physiology. The first part of the work (Chapter 3) describes the roles of BETs in erythroid maturation activated by the master transcription factor GATA1 by characterizing their functions as a family and as individuals. BET inhibition prevents GATA1-mediated transcriptional activation by both compromising GATA1 occupancy and downstream transcription. BRD3 occupies nearly all GATA1 sites, BRD4 a significant minority, and BRD2 relatively few. Depletion of BRD2 and BRD4 compromise GATA1-activated transcription, while depletion of BRD3 does not. However, increased BRD3 expression compensates for BRD2 depletion suggesting these proteins have redundant functions. The second part of this work (Chapter 4) addresses the role of BETs in mitotic bookmarking. Genomic binding assays in mitotic erythroblasts suggest that BRD4 occupies a distinct set of binding sites BRD4. BET disruption during mitosis does not affect transcriptional reactivation. This calls into question the role of BETs in mitotic bookmarking. These results are discussed along with their implications and the outstanding questions raised.

GATA1-mediated erythroid maturation involves widespread repression

Lineage transitions can dramatically alter RNA content and limit the reliability of transcriptional comparison relative to cell-intrinsic parameters like total RNA content or housekeeping genes (Lovén et al., 2012; van de Peppel et al., 2003). Erythroid differentiation involves obvious changes in cellular size and morphology (Dacie & White, 1949). Here transcriptome measurements made relative to exogenous RNA spike-in controls revealed that most transcript levels decreased upon GATA1 induction in erythroblasts. These results contrasted with prior studies of the same process which suggested that the number of activated

and repressed genes were similar (Cheng et al., 2009; Doré et al., 2012; Welch et al., 2004; M. Yu et al., 2009). This was critical in defining the global decrease in transcript levels resulting from GATA1 activation, as well as the specific roles of BETs in erythroid maturation.

Several mechanisms are likely to contribute to large-scale transcriptional repression. GATA1 is known to recruit co-repressors such as the FOG1/NuRD complex (Cantor & Orkin, 2005; Tsang et al., 1997). The extent to which GATA1 recruits co-repressors genome-wide has not been examined. It is possible that many, if not most GATA1 sites have roles in the direct repression of specific genes. While difficult to study on a large scale, repression may be more likely to happen at larger genomic distances than activation (Cheng et al., 2009). Inhibition of *Myc*, which has been suggested to function in a rheostat-like manner to dim transcription globally, may also contribute to large-scale repression in this system (Lin et al., 2012; Nie et al., 2012). Additionally, GATA1 could repress many genes by altering the physical arrangement of the erythroid genome to prevent normal enhancer-promoter interaction. In support of this, GATA1 has been reported to form a repressive loop responsible for *Kit* inhibition (Jing et al., 2008). It is possible that similar mechanisms control expression of many genes. Yet another possibility is that indirect repression could occur as a result of redistribution of the factors required for transcription at the small number of genes that are massively activated by GATA1. Mechanisms similar to this have previously been proposed both in the context of GATA1 and other transcriptional activators (Cheng et al., 2009; Step et al., 2014). Importantly, non-transcriptional mechanisms such as RNA degradation may also influence transcript levels and contribute to the erythroid transcriptome changes we observed. However, changes in primary transcript mRNA levels correlated extremely well with total mRNA changes at both activated and repressed genes. This is consistent with transcriptional control being the major factor controlling erythroid mRNA levels but does not preclude significant contributions from other factors.

GATA1-mediated repression is largely BET-independent

Given that JQ1 disrupts GATA1 occupancy at most sites including at known targets of GATA1-mediated repression, it is surprising that GATA1-mediated repression is not significantly mitigated by JQ1 treatment. As repression only involves disruption of the transcriptional process, it is possible that it may occur efficiently despite reduced GATA1 binding. For example, GATA1 may only need to be present often enough to disrupt the combinatorial factor and cofactor binding events that may be needed for productive transcription to occur. Another possibility is that part of the mechanism of repression by GATA1 is BET eviction. Consistent with this BRD2 and BRD4 are detectable at some GATA1 repressed genes like *Kit* and *Gata2* in the absence of GATA1 but are undetectable upon its induction. In these cases, transcription may be crippled by BET removal equally well through full GATA1 binding or through partial GATA1 binding combined with pharmacologic BET inhibition.

GATA1-activated genes are BET dependent

GATA1-mediated activation is a strong predictor of BET inhibitor sensitivity during erythroid maturation. As has been observed in other systems, BET binding near genes is a weak predictor of JQ1 response (Itzen et al., 2014; Lovén et al., 2013). Efforts to improve predictions by quantitatively taking into account BET signal or considering promoters or enhancers specifically did not provide biologically significant predictive power in our hands. "Super-enhancer" regions (SEs) have also been proposed as an explanation for differential JQ1 sensitivity. However, here as well as in the reports describing these regions (Chapuy et al., 2013; Lovén et al., 2013), the magnitude of transcriptional predictions based on these regions were very small and of questionable biological significance. This could be due to failure to link these regions to the genes they regulate. Alternatively, the underlying assumption that a large quantity of ChIP-seq signal necessarily makes a region an instance of a distinct class of biological entity could be false. The

predictive ability of GATA1 in this setting emphasizes its functional connection with BETs. GATA1-mediated activation is, like post-mitotic transcriptional reactivation and activation of inflammatory gene expression, a setting in which the major effect of JQ1 seems to be to prevent the ability of silent genes to be turned on (Khan, Kirkham, Barnes, & Adcock, 2014; Meng et al., 2014; Nicodème et al., 2010). Studies in other cell types will be helpful in understanding how generally inducibility is a useful paradigm in predicting the pharmacologic effects of BET inhibitors.

BETs facilitate GATA1 occupancy

Genome-wide occupancy analysis showed that BET inhibition compromised GATA1 occupancy at least partially at the vast majority of binding sites. BETs might facilitate genomic GATA1 occupancy at the level of initial binding, stability, or both. The activity of BETs in mediating nucleosome exchange suggests a possible mechanism through which BETs might facilitate GATA1 binding to chromatin. However, GATA1 sites are almost uniformly DNase hypersensitive prior to GATA1 binding making nucleosome occupancy less likely to be a barrier to DNA accessibility. However, high H3K27ac signal immediately adjacent to GATA1 binding sites implies adjacent nucleosomes at most binding sites. Speculatively, BETs may stabilize or recruit GATA1 by acting as a scaffold to connect it with adjacent nucleosomes.

BETs facilitate transcription downstream of GATA1 occupancy

Transcriptional effects of BETs in GATA1-mediated transcription were revealed by kinetic isolation of occupancy-independent effects using short-term JQ1 treatment. This is in agreement with observations that BETs have direct transcriptional roles in many other systems. While several mechanisms through which this occurs have been proposed, a consensus understanding of the role of BETs in transcription is lacking. The C-terminal domain of BRD4 is the best studied transcriptional activator and may recruit elongation factors, relieve the effects of inhibitory factors,

or directly phosphorylate RNA polymerase II (Devaiah et al., 2012; Itzen et al., 2014). How the short BET proteins, BRD2, BRD3, and the short isoform of BRD4 promote transcription are less well understood. It is likely that at least some of this activity can be mapped to the extra-terminal (ET) domains of these proteins which have been shown to interact with transcription elongation factors (Dawson et al., 2011). The importance of this domain and others could be investigated genetically in by expression of BRD2 mutants in BRD2 knockout cells generated here. Several reports have suggested that short BETs may facilitate elongation throughout genes by displacing acetylated histones (Kanno et al., 2014; LeRoy et al., 2008). Somewhat counter to this idea is that ChIP-seq studies of different BETs across different cell types have revealed enrichment at promoters and enhancers but not to a large extent in gene bodies. However, BETs do not bind DNA directly and they may still function in these processes despite failing to be detected by ChIP. ChIP detection issues have been previously noted in the context of the GATA1 cofactors, as FOG1 which is poorly detected at GATA1 regions by ChIP using formaldehyde crosslinking but is well-detected when a longer chain crosslinker is used (Zeng, Vakoc, Chen, Blobel, & Berger, 2006).

Relationship of BET occupancy to function in GATA1-mediated transcription

During erythroid maturation BET occupancy is regulated by association with GATA1, other transcription factors, and acetylated histones. Direct recruitment by GATA1 appears to be most important for BRD3, which is present at nearly all GATA1 binding sites. As BRD4 is present at only a subset of GATA1 binding sites other factors may be required to bring BRD4 to these sites. Direct *in vivo* association between BRD4 and GATA1 is supported both by previous immunoprecipitation studies and overlapping genomic occupancy patterns. Despite also being identified as a GATA1 binding partner by mass spectrometry, direct association between GATA1 and BRD2 is less likely to be an important mechanism *in vivo* based on the poor co-localization observed between these factors by ChIP-seq.

The functional effects of depletion of individual BETs on GATA1-mediated transcription were surprising based on their genome-wide binding patterns. The first surprise was that erythroblasts activate GATA1-mediated transcription normally when BRD3 is ablated. This suggests that this factor either is not required for GATA1 to function despite the genomic evidence linking the two, or that other BETs may compensate for its loss. If compensation occurred through increased binding of other BETs to GATA1, this could be measured by ChIP for BRD2 and BRD4 in BRD3-deficient cells. A second surprise was the phenotypic importance of BRD2 loss despite relatively poor genome-wide correlation with GATA1. While BRD2 does not co-localize with GATA1 to the extent that BRD3 or BRD4 does, BRD2 is present in the vicinity of many critical GATA1 activated sites. It may be required for transcriptional activation at these genes through GATA1-indirect mechanisms.

BRD2 and BRD3 have redundant functions in GATA1-mediated transcriptional activation

Despite a high degree of co-occupancy with GATA1, BRD3 depletion did not measurably compromise transcription except in the context of BRD2 deficiency. Additionally, retroviral BRD3 expression compensated for BRD2 deficiency to almost the same degree as BRD2 expression. These findings suggest the ability of these short BETs to execute the same biological functions. Interestingly, *Brd3* mRNA is approximately 4-fold less abundant than *Brd2* mRNA in RNA-seq studies in G1E cells (Paralkar et al., 2014). This raises the possibility that phenotypic differences between these proteins may be due to their different expression levels. However, BRD2 and BRD3 have very different genomic binding patterns in cells expressing both. To gain insight into how this works, we are currently testing whether each can physically take the place of the other when the other is depleted.

An important caveat is that the genetic manipulation of BETs that affected GATA1-mediated transcription also affected erythroblast proliferation. BRD4 may be essential for erythroblast growth as a BRD4 null erythroblast cell line could not be made, and cells depleted of

BRD4 or heterozygous for the *Brd4* gene had proliferation impairments. Similarly, while BRD3 depletion had no measurable effect on growth in BRD2-replete cells it slowed growth in BRD2 null cells, and cells simultaneously lacking both BRD2 and BRD3 could not be generated. This suggests that at least one of these factors for growth. In this context, the pharmacologic BET inhibitor studies we performed examining primary transcript mRNA complements these genetic studies by demonstrating transcriptional compromise upon BET inhibition in the immediate short term. This strongly supports the direct role of BETs in GATA1-mediated transcriptional activation.

Implications for pharmacologic BET inhibition

BRD4 depletion alone is sufficient to slow growth and suppress inflammation in a number of settings. However, in light of the functional overlap of BRD2 and BRD3 it is possible that the combined contribution of these molecules has been underestimated in studies that deplete them individually (Anand et al., 2013; Knoechel et al., 2014; Sahai et al., 2014; Zuber et al., 2011). In erythroblasts, BRD2 deficiency renders BRD3 depletion synthetically lethal. If this relationship generalizes to other cell types, it is likely that inhibition of both BRD2 and BRD3 will have a synergistic effect on growth that could be of similar importance as BRD4 inhibition. Future dissection of the mechanisms through which BETs act distinctly or compensate for each other will be critical to development of BET inhibitors directed against specific members of this family.

While BET inhibitors are a promising class of therapeutics, the ubiquitous importance of their targets is likely to limit the range of practical applications. Anemia has not been observed despite the strong effects of JQ1 on GATA1-dependent erythroid differentiation. Significant effects on the erythroid lineage are expected only with long-term administration of BET inhibitors because of the long lifespan of erythrocytes. While anemia has not been observed, GATA1 is also essential for platelet formation and thrombocytopenia is a consistent side effect of BET inhibition observed in human patients (Dombret, n.d.; Thieblemont, n.d.). Longer-term studies will reveal whether GATA1 function is more generally affected in patients receiving these drugs.

Dose-limiting toxicity could result from functional inhibition of any of the growing number of transcription factors that utilize BETs (Asangani et al., 2014; Nagarajan et al., 2014; Y. Tang et al., 2014).

BETs reorganize during mitosis

The association of BRD4 with specific genomic locations during mitosis remains largely unexamined despite longstanding observation of their broad mitotic association by immunofluorescence (Dey et al., 2003). BRD4 mitotic chromatin immunoprecipitation showed us that BRD4 occupied an almost entirely distinct set of sites during mitosis as during interphase. This results stand in contrast to one report suggesting that BRD4 remains bound to promoter sites during mitosis where chromatin is likely to remain open (Dey et al., 2009). However, a similar pattern of RNA polymerase II retention is also reported on mitotic chromatin calling into question the mitotic purity of the cell population examined. Interestingly, the finding that mitotic occupancy can be largely reorganized at specific sites while remaining globally bound is similar to what has been observed for the proto-oncogene MLL (Blobel et al., 2009) and the hepatocyte transcription factor FoxA1 (Caravaca et al., 2013).

What explains this alteration in genomic occupancy? The association of BRD4 at specific DNase hypersensitive sites occupied by several hematopoietic transcription factors during interphase is consistent with these being critical to its mitotic association with chromatin at specific sites. These sites are mostly enhancers and their transcriptional targets difficult to definitively identify. Increased histone phosphorylation during mitosis may be a major factor in controlling the mitotic distribution of BET binding. BRD4 bromodomains preferentially associate with acetylated histones that are simultaneously phosphorylated on nearby residues (including H3K14acS10p, H3K27acS28p) (Filippakopoulos et al., 2012) and this may increase mitotic binding widely and increase non-specific signal. ChIP-seq spike-in controls have been described and may be useful in measuring changes in background signal intensity in this context

{Orlando:2014be}. Further work is required to understand the functional meaning of the mitotic BRD4 binding pattern described here.

BETs do not function as mitotic bookmarks

Mitotic bookmarks are here defined as factors stably associated with mitotic chromatin that facilitate post-mitotic transcriptional reactivation. An original prediction of the 'bookmarking' hypothesis was that "Extraction of the bookmark (during mitosis) would abolish the distinction between *active* and *inactive* genes" (John & Workman, 1998). While previous studies have reported the role of BRD4 in transcriptional reactivation (Dey et al., 2009; Z. Yang, He, & Zhou, 2008; R. Zhao et al., 2011), the question of whether continuous mitotic binding of BRD4 supports gene reactivation has not been previously addressed. Here, disruption of mitotic BET binding did not affect post-mitotic transcriptional reactivation, calling into question the role of BETs as mitotic bookmarks. Additionally, cell cycle release profiles of cells treated with BET inhibitors in mitosis were not significantly changed suggesting that if BETs have transcription-independent functions in mitosis they are either not critical to normal cycle progression or also do not require continuous BET association. Together these results suggests that BET localization may not be important in mitotic memory of transcription state. Histone modifications and transcription factors which directly bind to DNA may be more likely to fulfill cellular bookmarking needs.

Transcription restarts during telophase of mitosis as the nuclear membrane reforms. This occurs before some nuclear factors can be re-imported into the nucleus (K. V. Prasanth, Sacco-Bubulya, Prasanth, & Spector, 2003). It is possible that non-specific association of transcription factors with mitotic chromatin could avoid the need for import once the nuclear membrane reforms. Indeed, a role for non-specific association of a bookmarking factor in rapid transcriptional restart has been described (Caravaca et al., 2013). The association of transcription factors with mitotic chromatin could regulate transcriptional restart by staying only loosely associated with mitotic chromatin and without 'bookmarking' specific genomic locations.

Conclusions

This work elucidates aspects of BET function in the context of GATA1-mediated transcription. As a family, BETs function predominantly in gene activation by facilitating the occupancy of GATA1 and by promoting downstream transcription. Their continuous association with chromatin was not required for the preservation of transcriptional memory during mitosis. Individual BETs have distinct but overlapping binding patterns that follow the distribution of GATA1 and of histone acetylation to varying degrees. Genomic deletion and knockdown experiments demonstrate that BRD2 and BRD4 are individually required for GATA1 to activate transcription normally, but that BRD3 is not. As ChIP-seq studies showed a tight correlation between BRD3 and GATA1, but not BRD2 and GATA1, these studies highlight the need for functional experimental modalities to complement ChIP-seq in interpreting the meaning of genome-wide binding studies. Importantly, increasing BRD3 expression compensated for BRD2 deficiency suggesting that these proteins can function redundantly. These results provide a number of insights into BET function during erythropoiesis. Future studies are needed to better understand both the mechanisms through which BETs perform the functions described here and to evaluate the generalizability of the mechanisms uncovered to other physiologic contexts.

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