Disrupting the Interaction of BRD4 with Diacetylated Twist Suppresses Tumorigenesis in Basal-like Breast Cancer

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SUMMARY

Twist is a key transcription activator of epithelial-mesenchymal transition (EMT). It remains unclear how Twist induces gene expression. Here we report a mechanism by which Twist recruits BRD4 to direct WNT5A expression in basal-like breast cancer (BLBC). Twist contains a “histone H4-mimic” GK-X-GK motif that is diacetylated by Tip60. The diacetylated Twist binds the second bromodomain of BRD4, whose first bromodomain interacts with acetylated H4, thereby constructing an activated Twist/BRD4/P-TEFb/RNA-Pol II complex at the WNT5A promoter and enhancer. Pharmacologic inhibition of the Twist-BRD4 association reduced WNT5A expression and suppressed invasion, cancer stem cell (CSC)-like properties, and tumorigenicity of BLBC cells. Our study indicates that the interaction with BRD4 is critical for the oncogenic function of Twist in BLBC.

INTRODUCTION

Recruitment and activation of RNA-Pol II at gene promoters are two key steps required for a productive transcription (Zhou et al., 2012). After RNA-Pol II recruitment to a gene promoter, TFIIH phosphorylates serine 5 of the heptapeptide repeats in the C-terminal domain (CTD) of RNA-Pol II, resulting in initial synthesis of short RNA species. However, RNA-Pol II pauses in the proximal promoter and requires a second phosphorylation event on serine 2 of the CTD that is carried out by the pause release factor P-TEFb, a complex composed of CDK9 and cyclin T1/2. Importantly, the recruitment of P-TEFb to RNA-Pol II is mediated, in part, by BRD4 (Jang et al., 2005).

BRD4 is a member of the BET (bromodomain and extra terminal domain) family proteins that are characteristic of two tandem bromodomains (BDs) located in the N terminus. The BDs of BET proteins recognize acetylated-lysine residues in nucleosomal histones (Filippakopoulos et al., 2012), facilitating the

Significance

BLBC is associated with an aggressive clinical history, development of recurrence, distant metastasis, and shorter patient survival. BLBC contains abundant EMT transcription factor Twist and possesses many CSC-like characteristics, suggesting that the Twist-activated EMT program confers growth advantages to BLBC. However, the absence of a clear ligand-binding domain in Twist creates a formidable hurdle toward developing inhibitors that can suppress its function. We found that Twist interacts with and recruits the BRD4/P-TEFb/RNA-Pol II transcription complex to the WNT5A superenhancer for gene activation. BET-specific inhibitors disrupted the Twist-BRD4 interaction and resulted in significant Wnt5a reduction, leading to inhibition of invasion and tumorigenicity of BLBC in vitro and in vivo. Our study indicates that targeting the Twist-BRD4 interaction provides an effective approach for treating BLBC.
recruitment of transcriptional proteins to chromatin. Recent studies have shown that pharmacologic inhibition of BRD4 with BET-specific BD inhibitors effectively blocks MYC expression in multiple myeloma (Delmore et al., 2011), Burkitt’s lymphoma, and acute myeloid leukemia (Dawson et al., 2011; Zuber et al., 2011). However, many mechanistic questions about BRD4 functions as a chromatin regulator in gene transcription are still unanswered, including (1) how BRD4 interacts and works with transcription factors at the target gene promoter and enhancer sites and (2) whether and how the two BDS in BRD4 function differently in gene transcription.

Breast cancer is a heterogeneous disease that can be divided into four major subtypes based on gene expression profiling: luminal A, luminal B, ErbB2, and basal like. Basal-like breast cancer (BLBC) is characterized by the lack of expression of es-
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The absence of effective targeted therapies and poor response to standard chemotherapy often results in a rapidly fatal clinical outcome for this disease. Notably, BLBC has activated the epithelial-mesenchymal transition (EMT) program, which provides cells with increased plasticity and stem-cell-like properties required during embryonic development, tissue remodeling, wound healing, and metastasis (Thiery et al., 2009).

Twist and Snail are two key members of EMT-activating transcriptional factors. During mesoderm development in Drosophila, Snail functions as a transcriptional repressor to prevent expression of genes that belong to ectoderm, whereas Twist serves as a transcriptional activator to induce mesodermal gene expression (Leptin, 1991). A complete loss of all meso-
dermal characteristics occurs only when both Snail and Twist are absent. These results suggest that Snail and Twist work synergistically, controlling distinct sets of genes, to coordinate EMT induction and mesoderm formation (Zeitlinger et al., 2007). We previously showed that Snail interacts with several transcrip-
tional repressive complexes to suppress gene expression. However, the mechanism underlying gene transcriptional activ-
tion by Twist has remained elusive. In this study, we sought to identify Twist-interacting proteins and determine the mecha-
nism by which Twist controls gene transcriptional activation in EMT and BLBC.

RESULTS

BRD4-BD2 Interacts with Lysine-Acetylated Twist

We sought to identify Twist-interacting proteins from a stable HeLa S3 cell line expressing Flag-Twist. Affinity protein purifica-
tion, followed by SDS-PAGE and analysis by mass spectrom-
etry, revealed the presence of BRD4 and TRRAP/EP400 (data not shown). To validate the interaction between Twist and BRD4, we coexpressed hemagglutinin (HA)-Twist and Flag-
BRD4 in HEK293 cells in the presence or absence of the histone deacetylase inhibitor Trichostatin (TSA). After immunoprecipit-
at ing Twist, we detected the associated BRD4, and vice versa (Figure 1A and Figure S1A available online). Although similar amounts of Twist were immunoprecipitated from cells with and without TSA treatment, Twist was more acetylated and interacted with more BRD4 in cells treated with TSA. In addition, immunoprecipitation with a pan-acetylated-lysine (pan-AcK) antibody pulled down Twist and BRD4 in cells treated with TSA. Similar observations were made in Twist-expressing HeLa S3 cells (Figures 1B and S1B). We further confirmed the interaction between the endogenous Twist and BRD4 and acety-
lation of the endogenous Twist in four BLBC cell lines, both of which were substantially enhanced with TSA treatment (Figures 1C and S1C). The Twist-BRD4 interaction is specific because Twist did not associate with other BET members (BRD2, BRD3, and BRD7) or lysine specific demethylase 1 (LSD1), and BRD4 did not associate with TCF4 (Figure S1D). The increased Twist-BRD4 interaction by TSA could not be due to an altered subcellular localization of these two proteins as TSA did not affect their localization (Figure S1E).

We next generated BRD4 deletion constructs and coex-
pressed them with Twist in HEK293 cells. We found that only N-terminal fragments containing both BDS, but not other regions of BRD4, retained the ability to interact with Twist (Figure 1D). When BD1 or BD2 was coexpressed with Twist in HEK293 cells, only BD2WT, but not BD1WT, bound to Twist (Figures 1E and S1F). Mutation of the conserved tyrosine and asparagine resi-
dues in the acetylysine binding pocket of BD2 to alanine (BD2YN) reduced its binding to Twist. The Twist-BRD4 inter-
action was readily disrupted when JQ1, a BET-specific BD inhibi-
tor, was added to the immunoprecipitation reaction (Figures 1F, 1G, S1G, and S1H). Similarly, MS417, a BET-specific BD inhibi-
tor with approximately 10-fold higher binding affinity than JQ1 (Zhang et al., 2012), effectively blocked the Twist-BRD4 inter-
action in four BLBC cell lines (Figures S1I and S1J). These results indicate that the Twist-BRD4 interaction is mediated by the BD2 of BRD4 binding to lysine-acetylated Twist.

Twist Diacetylation at K73 and K76 by Tip60 Is Required for Twist-BRD4 Interaction

The N-terminal half of Twist contains an acidic segment and two lysine/arginine-rich basic motifs that share high sequence simi-
larity to histones H2B and H4, respectively (Figure S2A). We generated Twist deletion constructs DL1 (residues 15–202), DL2 (residues 31–202), and DL3 (residues 47–202) and coex-
pressed them individually with Flag-BRD4 in HEK293 cells. DL1 retained, whereas DL2 and DL3 lost, interaction with Twist (Figures 2A and S2B). Surprisingly, in contrast to DL1, DL2 and DL3 also completely lost acetylation (Figures 2B and S2C). Because the first 30 N-terminal residues in Twist do not contain lysine, the loss of acetylation in DL2 and DL3 suggests that the N-terminal region is critical for Twist acetylation. In our mass spectrometry analysis, the NuA4 histone acetyltransferase complex proteins, including TRRAP and EP400 (Doyon and Côté, 2004), were identified as Twist association partners. We postulated that Tip60, the acetyltransferase of NuA4 complex, was responsible for Twist acetylation. Indeed, when TwistWT, DL1, DL2, and DL3 were coexpressed with Tip60 in HEK293 cells, we found that TwistWT and DL1, but not DL2 or DL3, interacted with Tip60 (Figures 2C and S2D). Endogenous Twist-Tip60 interaction was confirmed in three BLBC cell lines, which was markedly enhanced by TSA treatment (Figure S2E). We further observed that ectopic expression of Tip60 in BT549 and SUM1315 cells resulted in enhanced acetylation of Twist and the association of Twist with BRD4 even in the absence of...
Figure 1. BD2 of BRD4 Is Required for Its Interaction with Acetylated Twist

(A) HA-Twist and Flag-BRD4 were coexpressed in HEK293 cells. After treatment of cells with TSA (2 μM) for 12 hr, Twist, BRD4, and acetylated Twist were immunoprecipitated with HA, Flag, and pan-acetylated-lysine (pan-AcK) antibodies, respectively, and analyzed by western blotting. (B) HeLa cells stably expressing Flag-Twist were treated with TSA as in (A). Flag-Twist, endogenous BRD4, and acetylated Twist were immunoprecipitated and examined by western blotting. (C) Cells were treated as described in (A). Endogenous Twist, BRD4, and acetylated Twist were immunoprecipitated and examined by western blotting. (D) Schematic depiction of the functional domains of BRD4 and deletion constructs used (top). ET, extraterminal domain. Flag-tagged wild-type (WT) or deletion mutants of BRD4 were coexpressed with HA-Twist in HEK293 cells. After being immunoprecipitated with HA or Flag antibody, the bound BRD4 or Twist was examined by western blotting (bottom).
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TSA, whereas knockdown of Tip60 yielded opposite effects even in the presence of TSA (Figures 2D and S2F).

Twist contains five lysine residues (K33, K38, K73, K76, and K77) in its N-terminal region, which are highly conserved among different species (Figure S2A). Point mutation of K33R, K73R, and K76R showed a reduced level of acetylation compared to that of TwistWT (Figures 2E and S2G). TwistWT, K33R, K38R, and K77R showed a similar interaction with BD2, whereas K73R and K76R exhibited clearly weaker binding to BD2 (Figures 2F and S2H). The K73R/K76R double mutant showed an almost complete loss of acetylation and interaction with BD2 (Figures 2G and S2I). We further confirmed the Tip60-mediated Twist acetylation on K73/K76 by mass spectrometry analysis (Figures 2H and S2J). There are 12 nuclear histone acetyltransferases (HATs), divided into three major groups: (1) the GNAT family (e.g., PCAF), (2) the MYST family (e.g., Tip60), and (3) the p300/CBP family (e.g., p300 and CBP) (Rekowski and Giannis, 2010). To examine whether other HATs can also acetylate Twist, we knocked down the expression of p300, CBP, PCAF, or Tip60 individually in BT549 and SUM1315 cells (Figure S2K). We found that knockdown of Tip60, but not p300, CBP, or PCAF, suppressed Twist acetylation at K73/K76. Taken together, these data support our contention that Tip60 is the major HAT responsible for the K73/K76 acetylation on Twist and that diacetylation of Twist is required for its association with BRD4-BD2.

Histone H4 Mimicry in Twist Is Responsible for Its Interaction with BRD4

Diacetylations of the N-terminal tail of H4 at K5 and K8 are often required for the interaction of H4 with BDs of BET family proteins (Filippakopoulos et al., 2012; Morinière et al., 2009). The Twist sequence at K73 and K76 shares high similarity to the N-terminal tail of H4 at K5 and K8 (Figure 3A). To investigate this “histone mimicry” in the interaction between Twist and BRD4, we performed a pull-down study using biotinylated H4 and Twist peptides and lysate of HEK293 cells expressing BRD4-BD2. We observed that biotinylated H4-K5ac/K8ac peptide (residues 1–21) was bound to BD2 and that this interaction was disrupted by nonbiotinylated H4-K5ac/K8ac peptide (Figure 3B, lane 2 versus lane 1). This interaction was also markedly reduced by a Twist-K73ac/K76ac peptide (residues 61–80) but not by the unacetylated corresponding peptide (Figure 3B, lanes 3 and 4 versus lane 1). Similarly, the interaction of a biotinylated Twist-K73ac/K76ac peptide with BD2 was disrupted by a Twist-K73ac/K76ac, but not unacetylated, peptide (Figure 3B, lanes 7 and 8 versus lane 5). Notably, acetylated H4 peptide also disrupted the acetylated Twist and BD2 association (Figure 3B, lane 6 versus lane 5), indicating that diacylated K5/K8 in H4 and diacylated K73/K76 in Twist function similarly as a recognition motif for BRD4-BD2.

We then developed a specific antibody against Twist-K73ac/K76ac. Twist recognition by this antibody was disrupted by a Twist-K73ac/K76ac peptide, but not the corresponding nonacetylated peptide (Figures 3C and S3A). This antibody recognized immunoprecipitated TwistWT, but not TwistK73R/K76R that harbored mutated K73 and K76 (Figures 3D and S3B). In line with our contention that Tip60 acetylates Twist at K73/K76, both Twist and H4 acetylated by purified Tip60 in vitro were recognized by this Twist-K73ac/K76ac antibody and a pan-acetylated antibody (Figure 3E). Furthermore, this antibody readily detected endogenous Twist-K73ac/K76ac that was immunoprecipitated from four BLBC cell lines (Figures 3D and S3B). Although immunoprecipitation of the endogenous Twist by this antibody was weak, it was robustly increased by the addition of JQ1 to the binding buffer, indicating that the K73ac/K76ac site is masked by binding to BRD4 in cells (Figure 3F).

We further characterized the functional importance of this diacetylation-dependent Twist-BRD4 interaction in human mammary epithelial (HMLE) cells. Ectopic expression of TwistWT resulted in an induction of EMT, as indicated by the downregulation of E-cadherin and upregulation of vimentin (Figures 3G and S3C). While localized in the nucleus (Figure S3D), TwistK73R/K76R expression failed to induce EMT, indicating that the interaction with BRD4 is critical for the function of Twist.

Molecular Basis of BRD4 Binding to Lysine-Acetylated Twist

To determine the molecular basis of diacetylation-dependent Twist-BRD4 association, we characterized binding of the two BDs of BRD4 to a series of Twist peptides (residues 68–79) bearing no, single-acetylated, or diacylated lysine at K73 and K76 by nuclear magnetic resonance (NMR) titration. As shown in 2D 1H-15N HSQC spectra (Figure S4A), BRD4-BD2 exhibited substantially more extended chemical shift perturbations upon binding to the single-acetylated, and even more to the diacylated, Twist peptides than those produced by BRD4-BD1, confirming that BRD4-BD2 is largely responsible for BRD4 association with the diacylated Twist. The preferred recognition of Twist-K73ac/K76ac by BRD4-BD2 was supported in a fluorescence anisotropy competition binding study using a fluorescein-labeled H4K5ac/K8ac peptide as an assay probe, yielding a Ki of 800 nM for BRD4-BD2 and 3,000 nM for BRD4-BD1, respectively (Figure S4B). Furthermore, BRD4-BD1 and other BDs, including those from CBP and PCAF, showed almost no interaction with the single- or diacylated Twist peptides (data not shown).

We next solved the 3D structure of BRD4-BD2 bound to Twist-K73ac/K76ac peptide using NMR spectroscopy to determine the molecular basis of this selective interaction (Figures 4A, 4B, and S4C and Table S1). As revealed in the 3D structure, the Twist-K73ac/K76ac peptide is bound in the protein across...
Figure 2. Twist Diacetylation at K73/K76 by Tip60 Is Required for Interaction with BRD4

(A) Schematic diagram showing the domain organization of Twist, with deletion and mutation constructs used (top). HA-tagged WT or deletion mutants of Twist were coexpressed with Flag-BRD4 in HEK293 cells treated with or without TSA. Twist and BRD4 were immunoprecipitated with HA and Flag antibodies, respectively, and analyzed by western blotting (bottom).

(B) HA-tagged WT or deletion mutants of Twist were expressed in HEK293 cells treated with TSA. Twist and acetylated Twist were immunoprecipitated with HA and pan-AcK antibodies, respectively, and analyzed by western blotting.

(C) Flag-tagged WT or deletion mutants of Twist were coexpressed with HA-Tip60 in HEK293 cells treated with TSA. Twist and Tip60 were immunoprecipitated with Flag and HA antibodies, respectively, and analyzed by western blotting.

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an elongated cavity formed between the ZA and BC loops of this left-handed four-helical bundle structure. Specifically, acetylated K73 is bound in the canonical acetyllysine binding site, forming a hydrogen bond between its carbonyl oxygen and the side-chain nitrogen of the conserved Asn433. Acetylated K76 is recognized, next to K73ac, by the BD2 in a small hydrophobic cavity that is lined with Trp374, Val380, Leu385, and Val439. While the overall recognition of the diacetylated K73/K76 in Twist by BD2 is similar to that of the diacetylated K5/K8 in H4 by the BD1 of BRD4, several additional interactions observed in the former complex explain its selectivity. For instance, the imidazole nitrogen atom of His437 of BD2 is within hydrogen bond distance to the backbone carbonyl oxygen of the K73ac (Figures 4A and 4B). Notably, within this highly conserved acetyllysine binding pocket, His437 in BRD4-BD2, which corresponds to Asp144 in BRD4-BD1, is unique. Asp144 was not engaged in any interaction with the H4 peptide as shown in the crystal structure of the BD1/H4-K5ac/K8ac peptide complex (Filippakopoulos et al., 2012), explaining the failed binding of BRD4-BD1 to Twist-K73ac/K76ac. To further examine the role of His437 in BRD4/Twist association, we engineered two point mutants by switching His437 and Asp144 in the two BDs, generating BD2-H437D and BD1-D144H mutants. Remarkably, we found that BD2-H437D almost completely lost its ability to bind to the diacetylated Twist, whereas BD1-D144H gained binding ability for the acetylated Twist (Figure 4C), confirming the important function of His437 in the Twist-K73ac/K76ac recognition.

We observed additional intermolecular interactions in the complex structure that contribute to the selectivity of BRD4-BD/Twist recognition. For instance, the methyl group of Ala70 of Twist interacts with the aromatic side chain of the conserved Tyr432 in BRD4-BD2, whereas side chains of Ser78 of Twist and Glu438 of the BD2 form electrostatic interactions. Importantly, both Ala70 and Ser78 in Twist are located outside the diacetylation GK-X-GK motif and are not conserved in H4. Ala70 has no corresponding residue in H4, whereas the corresponding residue for Ser78 in H4 is Leu10, which could not form a favorable interaction with Glu438 in BD2, or even Asp145 in BD1. Collectively, our structural insights provide a detailed understanding of the molecular basis for the selective recognition of Twist-K73ac/K76ac by BRD4-BD2.

**Histone H4 and Twist Synergistically Interact with BRD4**

Because single BD2 of BRD4 can interact with H4 or Twist, we examined their interactions in a cellular context by expressing Twist or TwistK73R/K76R with single or double BDs of BRD4 in HEK293 cells. After immunoprecipitation of Twist, BDs, or H4 individually, the association and acetylation of the other two molecules were analyzed by western blotting (Figures 4D and S4D). First, we immunoprecipitated Twist and examined the presence of other two molecules (Figure 4D, left panel). We found that TwistK73R/K76R did not associate with any of the BDs (Figure 4D, left panel, lanes 7–9), whereas Twist associated with BD2 and BD1+BD2 but not BD1. Notably, the associated BD1+BD2 also contained H4 (Figure 4D, left panel, lanes 4–6). Second, we immunoprecipitated BD1, BD2, or BD1+BD2 and examined the presence and acetylation of Twist and H4 (Figure 4D, middle panel). BD1 associated with H4 but not Twist, whereas BD2 interacted with both Twist and H4, indicating that Twist and H4 can compete for interaction with BD2 (Figure 4D, middle panel, lanes 4–6). BD1+BD2 also associated with Twist and H4. Intriguingly, the amount of H4 associated with single BD (BD1 or BD2) is similar to that with double BDs in BD1+BD2 (Figure 4D, middle panel, lanes 2 and 3 versus lane 1), suggesting that only one BD in BD1+BD2 binds to H4. In the presence of Twist, the binding of BD1+BD2 to H4 did not alter (Figure 4D, middle panel, lane 4 versus lane 1). Because Twist interacts with BD2 but not BD1, H4 likely only interacts with BD1 when BD1+BD2, Twist, and H4 are all present. Consistent with this contention, the amount of Twist associated with BD1+BD2 was more than that with BD2 (Figure 4D, middle panel, lane 4 versus lane 5), where Twist and H4 competed for the binding to the BD2. Lastly, we immunoprecipitated H4 and examined the association of Twist and BDs (Figure 4D, right panel). We found that levels of BD1, BD2, and BD1+BD2 appeared to be equivalent, suggesting that H4 interacted equally with BD1, BD2, and BD1+BD2 and that only one BD interacted with H4 in BD1+BD2. In the presence of Twist, the immunoprecipitated BD2 was reduced (Figure 4D, right panel, lane 5 versus lane 2), suggesting that Twist and H4 compete for the interaction with single BD2. However, when the double BDs (BD1+BD2) were present, only one BD was engaged in interaction with H4, since the intensity of immunoprecipitated BD1+BD2 was about equal to that of Twist (Figure 4D, right panel, lane 1 versus lane 4). Consistent with the results from immunoprecipitated Twist, the immunoprecipitated BD1+BD2 by H4 contained Twist, reaffirming the association of three protein molecules in cells. Taken together, these results indicate that Twist and H4 can simultaneously interact with the double BDs of BRD4, in which BD1 binds to H4, whereas BD2 associates with Twist. This distinct binding selectivity of the two BDs of BRD4 is supported by our structural analysis.

**Twist-BRD4 Interaction Is Required for WNT5A Expression**

To identify the transcriptional target of the Twist-BRD4 complex, we performed cDNA microarray analysis of HMLE and luminal
Figure 3. K73ac/K76ac Twist and BRD4 Interaction Is Critical for the Function of Twist
(A) Sequence alignment between K5/8 of histone H4 and K73/76 of Twist. H, human; M, mouse; Ch, chimpanzee; Mk, monkey; R, rat; B, bovine; Sh, sheep.
(B) The indicated biotinylated peptides were mixed with lysates from HEK293 cells expressing BD2 without or with indicated nonbiotinylated competing peptides. The bound BD2 was analyzed by western blotting after pull-down of the biotinylated peptides.
(C) HA-Twist was expressed in HEK293 cells treated with or without TSA. The immunoprecipitated Twist was analyzed on western blots using indicated antibody in the presence of Twist-K73/K76 or Twist-K73ac/K76ac peptides.
(D) HA-tagged or endogenous Twist was immunoprecipitated from cells treated with or without TSA using HA and Twist antibodies, respectively, and analyzed by K73ac/K76ac antibody.

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T47D cells that have undergone Twist-mediated EMT (Figures 3G and S5A). We reasoned that genes that are transcriptionally active in Twist/HLME and Twist/T47D cells but are downregulated by JQ1 in these cells but not in vector control cells are likely targets of the Twist-BRD4 complex (Figure 5A). Among the 29 overlapping genes, WNT5A is noted to encode a critical ligand of both canonical (controlling pluriotipivity) and noncanonical (regulating motility and planar cell polarity) Wnt pathways. Upregulation of Wnt5a is correlated with an aggressive phenotype in melanoma, as well as breast, lung, and prostate tumors (Witze et al., 2008). We thus selected WNT5A as an example to characterize the transcriptional mechanism of Twist. We noticed that TwistWT but not TwistK73R/K76R induced Wnt5a expression (Figures 3G and S5C). Similarly, TwistWT induced EMT and Wnt5a expression in T47D cells (Figure S5A). In addition, TWIST expression positively correlates with WNT5A expression in eight microarray data sets from human breast cancer (Figure S5B). Using Twist and Wnt5a antibodies that detect Twist and Wnt5a, respectively, in xenograft tumors derived from SUM1315 cells, which express high levels of Twist and Wnt5a, but not MCF7 cells, which express low levels of Twist and Wnt5a (Figure S5C), we found that Twist is also positively correlated with Wnt5a expression in breast cancer specimens, with both increased expression found predominantly in estrogen receptor negative (ER-) breast cancer (Figure S5D). Further, in 14 breast cell lines (Figure 5B), both the mRNA and protein levels of Twist and Wnt5a were found to be largely correlated, with elevated expressions found in BLBC cell lines. BRD4 expression is relatively constant among normal breast, luminal, and BLBC cell lines (Figure 5B). Consistently, no significant difference in BRD4 mRNA was found between ER+ and ER- breast cancers from a 477 sample microarray data set (Figure S5E).

We generated a clone of SUM1315 cells with stable knockdown of Twist. Twist knockdown reduced the mesenchymal phenotype as these cells were clustered together; cells also gained expression of epithelial markers and reduced the expression of mesenchymal markers (Figures 5C and S5F). Ectopic expression of TwistWT, but not TwistK73R/K76R, restored the mesenchymal phenotype in these cells. Twist knockdown also resulted in suppression of Wnt5a expression. Ectopic expression of TwistWT, but not TwistK73R/K76R, recovered Wnt5a expression (Figure 5C) and restored the invasiveness and mammosphere formation in these cells (Figure S5G). These results are in line with observations in HMLE cells (Figure 3G) and indicate that the Twist-BRD4 interaction is critical in maintaining mesenchymal phenotype characteristics in BLBC cells. Consistently, JQ1 suppressed Wnt5a expression in both Twist/HLME and Twist/T47D EMT cells (Figure S5H). In addition, knockdown of Twist or BRD4 in five BLBC cell lines resulted in reduced Wnt5a expression; double knockdown of these two molecules almost completely abolished Wnt5a expression (Figure 5D). The downregulation of Wnt5a by BRD4-knockdown is specific, because knockdown of other BET members did not alter Wnt5a expression (Figure S5I). In addition, knockdown of BRD4 did not change the expression of either epithelial or mesenchymal markers (Figure S5J). The downregulation of Wnt5a correlated with inhibition of invasiveness in BLBC cells; addition of recombinant Wnt5a partially restored invasiveness (Figure S5K). Collectively, these results indicate that the Twist-BRD4 interaction is most likely conserved in HMLE and BLBC cells for EMT and that this interaction is required for the expression of Wnt5a, which may represent as a bona fide target of Twist for promoting tumorigenicity in BLBC.

**Twist-BRD4 Interaction Is Required for the Recruitment of BRD4 at the WNT5A Super enhancer**

To delineate how the Twist-BRD4 complex activates WNT5A expression, we constructed a Twist-Gal4 fusion protein by fusing Twist N-terminal residues 1–100 to Gal4 DNA-binding domain (DBD). We also generated several N-terminal deletion mutants of Twist fused with Gal4-DBD, including DL1-Gal4, DL2-Gal4, DL3-Gal4, and KR-Gal4 (Figure S6A). When these Twist-Gal4 constructs were coexpressed with the Gal4-luciferase reporter, Gal4-luciferase activity was moderately increased by about 2-fold compared to the control; coexpression of BRD4 with the Twist-Gal4 fusion constructs that contain the N-terminal region required for Tip60-mediated acetylation (i.e., TW and DL1) greatly enhanced luciferase activity to approximately 8-fold, suggesting that the N-terminal half of Twist contains transactivation activity and its interaction with BRD4 boosts this activity.

The WNT5A promoter contains two Twist-responsive E boxes, conserved in human and mouse, and located at −160 bp and −67 bp from transcription start site (TSS) (Figure 6A). We cloned the human WNT5A promoter (−2,000 bp upstream of the translation start site) and generated several deletion and E box mutants of the promoter-luciferase constructs, including Luc1 (−2,000 bp), Luc2 (−760) and LucEM (−760, two E box mutations). As expected, Twist alone induced Luc1 and Luc2 promoter luciferase activity; coexpression of BRD4 further enhanced the Twist-induced Luc1 and Luc2 promoter luciferase activities (Figure 6A, left panel). In addition, mutation of each E box (E1M and E2M) in this region reduced, whereas mutation of both E boxes (EM) completely abolished, Twist-BRD4-mediated activation of the WNT5A promoter luciferase activity, suggesting that both E boxes are required for Twist-BRD4-induced transcriptional activation. TwistK73R/K76R mutant partially decreased WNT5A promoter luciferase activity and was completely insensitive to BRD4-mediated transcriptional activation (Figure 6A, right panel). BRD4-mediated enhancement of Twist transcriptional activity is specific because other BET members did not possess this capability, and treatment with JQ1 or MS417 disrupted this BRD4-mediated enhancement (Figure S6B).

(E) Purified human Twist or histone H4 was incubated with purified Tip60 in the absence or presence of acetyl-CoA. The acetylation of Twist and histone H4 was examined by pan-AcK and anti-K73ac/K76ac antibodies.

(F) K73ac/K76ac Twist was immunoprecipitated with K73ac/K76ac antibody in the presence or absence of JQ1. The bound Twist was examined by western blotting.

(G) HMLE cells expressing the vector or the WT or K73/76R Twist were examined for morphological changes indicative of EMT by phase microscopy and the expression of E-cadherin, vimentin, Wnt5a, and Twist (green) by immunofluorescence staining. Nuclei were stained with DAPI (red). Scale bars, 50 μM. See also Figure S3.
Figure 4. The Structural and Molecular Basis of Twist-K73ac/K76ac Recognition by BRD4
(A) Stereo ribbon diagram of the 3D solution structure of the BRD4-BD2 bound to a diacetylated K73ac/K76ac Twist peptide (yellow). Side chains of key residues engaged at the protein/peptide interactions are depicted and color coded by atom type.
(B) Surface electrostatic potential (left) or space-filled (right) representation of the BRD4-BD2/Twist-K73ac/K76ac complex structure highlights His437 (red) at the acetyllysine binding site that is responsible for the BRD4-BD2 specificity of this molecular recognition.

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Chromatin immunoprecipitation (ChIP) analysis revealed that Twist, BRD4, and acetylated H4 associated at the WNT5A promoter in BT549 and SUM1315 cells, together with P-TEFb and RNA-Pol II (Figure 6B). A recent study indicated that BRD4 preferentially occupied a small subset of superenhancers in transcriptional active key oncogenes that are critical for proliferation and survival of tumor cells (Lovén et al., 2013). Intriguingly, WNT5A is one of these key oncogenes that contain BRD4-associated superenhancer, which covers exon1, promoter, and a region up to 30 kb upstream of the TSS in the WNT5A genomic sequence in chromosome 3. To examine whether Twist and BRD4 also bind the WNT5A superenhancer in BLBC, we designed two sets of ChIP primers that are 22 and 28 kb upstream of the TSS. ChIP experiments indicated that Twist and BRD4 indeed occupied the WNT5A enhancer together with H3K27ac, a mark of active enhancer (Figure 6C). Knockdown of Twist or JQ1 treatment inhibited the association of BRD4 at the WNT5A enhancer (data not shown). Knockdown of Twist or JQ1 treatment also reduced the presence of BRD4, P-TEFb, and RNA-Pol II at the WNT5A promoter (Figure 6D). However, JQ1 treatment did not affect the association of Twist at the WNT5A promoter, suggesting that Twist is required for the recruitment of the BRD4/P-TEFb/RNA-Pol II complex to the WNT5A promoter. Consistent with these observations, TwistK91R, which could not interact with BRD4 and failed to rescue Wnt5a expression (Figure 5C), was unable to recruit the BRD4/P-TEFb/RNA-Pol II complex to the WNT5A promoter (data not shown). In addition, ectopic expression of BRD4 increased Twist interaction with P-TEFb and RNA-Pol II, whereas knockdown of BRD4 reduced the association of Twist with P-TEFb and RNA-Pol II at the WNT5A promoter/enhancer to activate transcription.

The direct transcriptional activation of WNT5A by the Twist-BRD4 complex prompted us to investigate the stimuli responsible for Twist acetylation and WNT5A expression. We found that several stimuli, including TNFα and EGF plus insulin, could induce Twist acetylation at K73/K76 (Figure 6C). TNFα and EGF/insulin treatments greatly enhanced the interaction of Twist with BRD4 and with Tip60, increased K73ac/K76ac of Twist, and promoted Wnt5a expression (Figure 6F, left panel); JQ1 blocked the interaction of Twist with BRD4 and thus suppressed Wnt5a expression (Figure 6F, right panel). Consistent with these findings, TNFα or EGF/insulin treatment greatly enhanced the association of Twist, BRD4, P-TEFb, and RNA-Pol II at the WNT5A promoter (Figure 6D). Knockdown of Twist suppressed the association of BRD4 and Twist at the WNT5A promoter; however, JQ1 treatment did not inhibit the binding of Twist at the WNT5A promoter (Figure 6E). These data suggest that the association of BRD4 at the WNT5A promoter is mediated by Twist.

Although JQ1 was reported to reduce c-Myc expression, we noticed that JQ1 (1 μM) caused a decrease of c-Myc expression only in one of five examined BLBC cell lines (Figure 6G). However, JQ1 reduced Wnt5a expression in all cell lines. The low sensitivity to JQ1 in Hs578T cells is likely due to the remarkably high expression levels of Twist and Wnt5a in this particular cell line (Figure 5B). Increased JQ1 concentration resulted in Wnt5a downregulation in a dose-dependent manner in this cell line (Figure 6F). The downregulation of Wnt5a by JQ1 correlated with its inhibition of invasion and tumorsphere formation of these cells; addition of recombinant Wnt5a could partially restore this inhibitory effect (Figures 6H, S6F, and S6G). Together, these data indicate that the Twist-BRD4 interaction, enhanced by extracellular signals, is required for the recruitment of P-TEFb/RNA-Pol II complex to the WNT5A superenhancer for transcription of WNT5A, which executes, at least in part, the oncogenic function of Twist. JQ1 disrupts this interaction and thereby suppresses WNT5A expression in BLBC.

The Twist-BRD4-Wnt5a Axis Is Critical for Tumorigenicity in Breast Cancer

To further examine the oncogenic role of the Twist-BRD4-Wnt5a axis and explore the therapeutic potential of BET-specific inhibitors for targeting this axis in BLBC in vivo, we established two Wnt5a knockdown clones in SUM1315 cells. Knockdown of Wnt5a inhibited the noncanonical Wnt pathway, exemplified by the downregulation of JNK phosphorylation (Figure 7A). Wnt5a knockdown also suppressed the canonical Wnt/β-catenin pathway, indicated by the downregulation of β-catenin and the suppression of Akt/GSK-3β phosphorylation. These effects were further confirmed by β-catenin reporter assay (Figure S7A). Although Wnt5a knockdown did not alter the expression of epithelial or mesenchymal markers, it did reduce the expression of several pluripotent molecules (CD44, Sox2, and Oct4). Consistently, Wnt5a knockdown suppressed invasion and tumoursphere formation in these cells (Figure 7B).

In vivo studies were performed by injection of SUM1315 vector control cells or Wnt5a knockdown clones into the mammary fat pads of NOD-SCID mice. When control tumors were approximately 100 mm3, mice were divided into three groups to receive daily treatments of JQ1 (50 mg/kg), MS417 (20 mg/kg), or solvent control for 2 weeks. We found that knockdown of Wnt5a completely inhibited tumor growth in SUM1315 cells and that both JQ1 and MS417 treatments significantly inhibited tumor growth (Figure 7C). The growth inhibitory effect of JQ1 and MS417 correlated with suppression of Wnt5a expression and downregulation of proliferative marker Ki67 in these tumors (Figure S7B). These results suggest that Wnt5a is critical for the tumorigenicity of BLBC. BET-specific inhibitors suppress the tumorigenicity of BLBC by inhibiting the Twist-BRD4 interaction and Wnt5a expression.

DISCUSSION

Our study provides several mechanistic insights into how Twist and BRD4 function cooperatively to activate gene transcription in EMT and BLBC. First, we show that Twist uses a unique...
Figure 5. Twist Positively Correlates with Wnt5a Expression in Breast Cancer
(A) Gene expression profiling analysis (left) was used to identify potential Twist target genes. Common Twist target genes between HMLE and T47D cells are shown in the heatmap (right).

(B) The mRNA and protein levels of Twist, Wnt5a, and BRD4 were analyzed by RT-PCR and western blotting.

(C) The effects of stable small hairpin RNA knockdown of endogenous Twist in SUM1315 cells that were transiently transfected with WT or mutant (KR) Twist on the expression of Wnt5a and various molecules was evaluated by western blotting.

(D) Wnt5a expression in five BLBC cell lines with knockdown of Twist and/or BRD4 was analyzed by western blotting. NTC, nontarget control small interfering RNA. See also Figure S5.
Figure 6. The Twist-BRD4 Complex Directly Activates WNT5A Transcription

(A) Schematic depiction of the WNT5A promoter and WNT5A reporter luciferase constructs used (top). Enhancement of Wnt5a luciferase activity by coexpression of Twist and BRD4 in HEK293 cells is shown (bottom).

(B) Twist, BRD4, H4ac4, RNA-Pol II, and P-TEFb (CDK9) association at the WNT5A promoter as assessed by ChIP. SP, specific primer; CP, control primer (5 kb downstream of the TSS).

(C) Twist, BRD4, H4ac4, and H3K27ac association at the WNT5A enhancer as assessed by ChIP. SP, specific primer (22 kb upstream of the TSS).

(D) Twist, BRD4, H4ac4, and P-TEFb association at the promoter as assessed by ChIP. SP, specific primer (5 kb downstream of the TSS).

(E) Twist, BRD4, H4ac4, RNA-Pol II, and P-TEFb association at the promoter as assessed by ChIP. SP, specific primer (5 kb downstream of the TSS).
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mechanism for recruiting BRD4 in gene transcription (Figure 7D). Although BRD4 is the key transcriptional regulator, it lacks specific DNA binding motif. How BRD4 and its associated transcriptional complex are recruited to gene-specific promoters/enhancers remains elusive. We found that Twist contains an “H4-mimic” GK-X-GK motif and becomes diacetylated by Tip60, which also acetylates multiple lysine residues in histone H4 including K5 and K8. By binding to BRD4-BD2 via the K73ac/K76ac motif, Twist recruits BRD4 to target gene promoters/enhancers through the recognition of and interaction with E boxes by its bHLH domain. Once localized in the chromatin, BRD4-BD1 binds with acetylated H4-K5ac/K8ac to facilitate the docking of the BRD4 complex on promoters/enhancers and thereby activates pause release factor P-TEFb to phosphorylate and release RNA-Pol II for WNT5A transcription.

Our study demonstrates that the two BDs of BRD4 have distinct functions and binding specificities for acetylated proteins in transcription. Although a single BD1 or BD2 of BRD4 is individually capable of interacting with acetylated H4 in vitro, only BD1 is engaged in the binding with acetylated H4 in the tandem BD1+BD2. This is consistent with the observation that a single BD1 of Brdt binds to acetylated histone H4 nearly as well as Brdt (full length; contains BD1+BD2) (Morière et al., 2009) and that BRD4-BD1 specifically recognizes acetylation marks on H4, whereas BRD4-BD2 has broad binding specificity for diacetylated substrates (Filippakopoulos et al., 2012). In line with this contention, only BRD4-BD2 interacts with Twist-K73ac/K76ac. We found that charged amino acid residues (D144 in BD1, H437 in BD2) surrounding the acetyllysine-binding pocket of BDs contributed to the binding specificity of BD1 and BD2. Additional residues beyond the diacylation motif further contribute to Twist’s association with BRD4-BD2. Notably, it has recently been reported that BRD4 is phosphorylated by CK2 on several Ser residues in the C-terminal region of BD2 and that these phosphorylations were suggested to affect the interaction of BRD4 with acetylated histones and transcriptional cofactors (Wu et al., 2013). Although a single BD2 of BRD4 can interact with either acetylated H4 or Twist-K73ac/K76ac individually, the tandem BD1+BD2 of BRD4 apparently form a ternary complex with two acetylated proteins in that diacetylated H4 is bound to BD1 and diacetylated Twist is bound to BD2. These results suggest that BRD4 utilizes its tandem BDs as an integration platform to cooperatively interact with H4 and Twist in assembling the integrated transcriptional complex containing P-TEFb and RNA-Pol II at target gene promoters/enhancers. Notably, several transcription-associate proteins that contain tandem binding modules have been shown to engage in combinatorial recognition of different posttranslational modifications (PTMs) in histones for the assembly of transcriptional complexes (Zeng et al., 2010). For example, the tandem PHD-BD module in BPTF specifically recognizes a combination of H3K4me3 and H4K16ac in gene activation (Ruthenburg et al., 2011). Our results not only support this notion, but also extend the functionality of these tandem binding modules in directing gene transcriptional activation as exemplified by the tandem BDs of BRD4 in bridging histone and nonhistone transcription factor.

Notably, these histone-mimic sequences contain lysine/arginine-rich residues, which are often viewed conventionally as a nuclear-localization signal (NLS), as in the case of Twist. However, TwistK2R mutant, which cannot be acetylated but still resides in the nucleus, fails to interact with BRD4 and is unable to induce EMT and WNT5A expression. Our results suggest that these lysine/arginine-rich “potential” NLS motifs in transcription factors may have previously unrecognized histone-mimic functions. Consistent with our findings, histone-mimic sequences are deployed by influenza nonstructural protein 1 (NS1) in inhibiting human transcription elongation complex in the antiviral response and by HP1 in forming HP1-chromatin complex (Canzio et al., 2013; Marazzi et al., 2012). We believe that PTMs on histone-mimic sequences present in nonhistone proteins likely play an important role, via conserved molecular mechanisms as seen with those PTMs in histone, in governing the assembly and function of transcriptional complexes in chromatin.

Second, our study demonstrates that Twist is a transcriptional activator responsible for WNT5A expression in BLBC. Twist has been shown to bind to the E-cadherin promoter to repress transcription in a way similar to that of Snail. However, this contradicts the role of Twist in development, where it acts as a transcriptional activator to upregulate mesoderm-specific genes in Drosophila. When the bHLH domain of Twist was replaced with Gal4-DBD, we found that the Twist-Gal4-DBD fusion was sufficient to activate gene expression, indicating that Twist functions as a transcriptional activator. We further show that Twist recruits BRD4 and the associated P-TEFb and RNA-Pol II to the WNT5A promoter/enhancer to directly activate WNT5A expression, which is required for invasion and the maintenance of CSC-like properties of BLBC. Notably, Wnt5a is induced in epithelial cells during EMT and required for maintenance of CSC-like properties in the resulting mesenchymal cells (Scheel et al., 2011). In addition, Wnt5a expression is required for the loss of cell-cell contacts, allowing cells to migrate to the edge.
Figure 7. The Twist-BRD4-Wnt5a Axis Is Critical for Tumorigenicity In Vitro and In Vivo
(A) Expression of various molecules in SUM1315 cells with Wnt5a knockdown.
(B) Invasion and tumorsphere formation in SUM1315 cells with Wnt5a knockdown. Data are presented as a percentage of vector control values (mean ± SD in three separate experiments in duplicates). Representative pictures of tumorspheres are shown at the bottom. Scale bar, 100 μM.
(C) Vector control and Wnt5a knockdown SUM1315 cells were injected into the mammary fat pad of NOD-SCID mice. When tumors from mice injected with control cells reached 100 mm³, mice were divided into three groups and treated with JQ1 (50 mg/Kg), MS417 (20 mg/Kg), or solvent control, respectively. The size

(legend continued on next page)
of wounds, and is also necessary for intestinal epithelial stem cells to regenerate damage tissues during wound healing and tissue repair (Miyoshi et al., 2012). The correlated expression of Twist and Wnt5a in BLBC supports our contention that the Twist-BRD4-Wnt5a signaling axis plays a critical role in the development and progression of BLBC.

Third, our study indicates that the Twist-BRD4 interaction represents a druggable target for treating BLBC. Although Twist is highly expressed in BLBC, the absence of a clear ligand-bind- ing domain in Twist creates a formidable obstacle toward developing small molecules that inhibit its activity as a transcription factor. We found that BET-specific BD inhibitors disrupted the Twist-BRD4 interaction and resulted in significant Wnt5a reduction, leading to inhibition of invasion and tumorigenicity of BLBC cells in vitro and in vivo. Based on our mechanistic understanding of Twist-BRD4 interaction in gene transcription, we predict that selective chemical inhibition of BRD4/H4 interaction would result in a broad inhibition of BRD4 functions as chromatin regulator in gene transcription, whereas selective inhibition of the BRD4/transcription factor association might affect specific transcription factor’s ability in their target gene activation. BD inhibitors selectively target BD2 over BD1 of BRD4 are needed to address these questions; they will also further functionally validate the effectiveness and therapeutic benefits of targeting BRD4 for treating BLBC.

**EXPERIMENTAL PROCEDURES**

**Protein Purification and Mass Spectrometry Analysis**

We generated a clone of HeLa S3 cells with stable expression of Flag-Twist (Li and Zhou, 2011). After enriching the nuclear extracts from 40 I of suspension culture, we carried out affinity protein purification with Flag affinity columns. The final eluted immunocomplexes were separated on SDS-PAGE, and the bound proteins were excised from the gel and subjected to nano-liquid chromatography-tandem mass spectrometry (nano-LC-MS/MS) analysis (Applied Biosciences). For identification of the acetylated lysine residues on Twist, the acetyl- ylated Twist was digested with trypsin, and the tryptic peptides were analyzed by LC-MS/MS using an LTQ Velos Orbitrap mass spectrometer (Thermo Fisher Scientific) coupled with a nano-LC Ultra/ChiroPLC Nanoflex high-performance liquid chromatography system (Eksigent) through a nanoelectrospray ionization source (Li et al., 2013). MS/MS data were acquired using CID fragmentation of selected peptides during the information-dependent acquisition. The LC-MS/MS results were subjected to protein identification and acetylation sites determination using ProteomeDiscoverer 1.3 software (Thermo Fisher Scientific) and Mascot server.

**Protein Structure Analysis by NMR**

The NMR spectral collection, analysis, and structure determination of the BRD4-BD2 with Twist-K73ac/K76ac were performed as previously reported (Zhang et al., 2012). In brief, NMR samples contained a protein/peptide complex of 0.5 mM in a 100 mM sodium phosphate buffer (pH 6.5) that contains 5 mM piperazine dithiothreitol and 0.5 mM EDTA in H₂O/D₂O (9/1) or D₂O. All NMR spectra were collected at 30°C on NMR spectrometers of 800, 600, or 500 MHz. The 1H, 13C, and 15N resonances of the protein in the complex were assigned by triple-resonance NMR spectra collected with a 3D 13C-15N-labeled and 75% deuterated BRD4-BD2 bound to an unlabeled Twist peptide (Clare and Gronenborn, 1994). The distance restraints were obtained from 3D 13C-NOESY and 15N-NOESY spectra. Protein structures were calculated with a distance geometry-simulated annealing protocol using X-PLOR (Brunger, 1993) that was aided with iterative automated NOE assignment by ARIA for refinement (Nilges and O’Donoghue, 1998). Structure quality was assessed by PROCHECK-NMR (Laskowski et al., 1996). The structure of the protein/ligand complex was determined using intermolecular NOE-derived distance restraints that were obtained from 13C/15N-filtered (F) 3D NOESY spectra.

**Immunoprecipitation, Immunoblotting, Immunofluorescence, Immunohistochemical Staining, RT-PCR, and CHIP**

Detailed methods are provided in the Supplemental Experimental Procedures.

**Tumorigenesis Assay**

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Kentucky College of Medicine and conformed to the legal mandates and federal guidelines for the care and maintenance of laboratory animals. Animals were maintained and treated under pathogen-free conditions. Female NOD-SCID mice (6–8 weeks old, Taconic) were injected with breast cancer SUM1315 (2 × 10⁶ cells/mouse) cells via mammary fat pad, and mice had three groups: vector control and two stable clones with Wnt5a-knockdown expression. Tumor growth was monitored with caliper measurements. When tumors were approximately 1.0 cm in size, mice were euthanized and tumors excised. Data were analyzed by Student’s t test; p < 0.05 was considered significant.

**Statistical Analysis**

Data are presented as mean ± SD. A Student’s t test (two-tailed) was used to compare two groups (p < 0.05 was considered significant) unless otherwise indicated.

**ACCESSION NUMBERS**

Microarray data of Twist expression in HMLE and T47D cells with or without JQ1 treatment were deposited at the Gene Expression Omnibus database with the accession number GSE53222. Structure factors and coordinates for the second bromodomain of BRD4 in complex with K73ac/K76ac diacetylated Twist peptide were deposited at the Protein Data Bank under ID code 2MJV, and the NMR spectral data were deposited at the BioMagResBank (BMRB) under BMRB accession number 19738.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.ccr.2014.01.028.

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