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Twist-BRD4 Complex: Potential Drug Target for Basal-like Breast Cancer

Jian Shi¹, Jingying Cao^{1,2}, and Binhua P. Zhou^{1,*}

¹Department of Molecular and Cellular Biochemistry, Markey Cancer Center, University of Kentucky, College of Medicine, Lexington, KY 40506, USA

²Sun Yat-sen University Cancer Center, State Key Laboratory of Oncology in South China, and Collaborative Innovation Center of Cancer Medicine, Guangzhou 510060, China

Abstract

Twist is an important basic helix-loop-helix (bHLH) transcription factor. It has been reported that Twist is associated with several physiological processes, such as mesodermal development, and pathological processes, for example, Saethre-Chotzen syndrome. During cancer progression, Twist induces epithelial-mesenchymal transition (EMT), potentiating cancer cell invasion and metastasis. Although many studies have revealed its multiple biological roles, it remained unclear how Twist transcriptionally activates targeted genes. Recently we discovered tip60-mediated Twist di-acetylation in the “histone H4-mimic” GK-X-GK motif. The di-acetylated Twist recruits BRD4 and related transcriptional components to super-enhancer of its targeted genes during progression of basal-like breast cancer (BLBC). Here, we review this new advance of regulation and functional mechanism of Twist.

Basal-like breast cancer: a highly malignant subtype of breast cancer

Breast cancer is a highly heterogeneous disease with diverse phenotypes and clinical outcomes (1). Gene expression profiling of breast cancer has identified several subtypes; one of most aggressive subtypes is basal-like breast cancer. Specifically, expression of estrogen receptor (ER), progesterone receptor (PR) and epidermal growth factor receptor 2 (HER2) in BLBC is lost, whereas several basal markers, including cytokeratins (5/6, 14 and 17), Epidermal growth factor receptor (EGFR), caveolin 1 and P-cadherin are highly expressed (2). The concepts of basal-like breast cancer and triple negative breast cancer are overlapping, but different. Although Basal-like breast tumors only account for up to 15% of all breast cancers, owing to its highly aggressive phenotype and poor prognosis as well as its limited response to endocrine therapies (3, 4), effective therapies are deficient extremely. Currently several clinical trials are under development but, in general, the clinical responses for this disease have been poor with a rapidly fatal outcome. Hence, BLBC will be a great challenge to breast cancer researchers in the future.

*To whom correspondence should be addressed: Binhua P. Zhou, MD, PhD, 750 Rose Street, Room 377, Lexington, KY 40536, Phone: 859-323-4475, peter.zhou@uky.edu.

Several well-known oncogenic signaling pathways are hyper-activated in BLBC cells, and these phenomena are critical to oncogenic progression. For example, canonical Wnt signaling is involved in BLBC cell proliferation, invasion and metastasis. In particular, canonical Wnt signaling is linked to maintenance of the cancer stem-like property in BLBC cells (5–8). Prolonged activation of NF- κ B signaling is also reportedly linked to BLBC cell proliferation and capacity for self-renewal (9–13). On the other hand, a few molecules have been identified as tumor suppressors in BLBC, which block oncogenic progression. Alpha-catenin exhibits E-cadherin-independent function and inhibits BLBC cell growth (14). Fructose-1,6-bisphosphatase 1 (FBP1) has been reported to reverse the tumor cell glycolysis process, permitting oxidative phosphorylation; this is associated with a suppressed tumorigenicity in BLBC (15). Notably, basal-like breast cancer cells have a typical mesenchymal phenotype indicating that they may have undergone epithelial mesenchymal transition. This possibility is supported by observations that several EMT inducing transcriptional factors, such as Snail, Slug, Twist and FOXC2 etc. are highly expressed in BLBC cells, whereas typical epithelial markers such as E-cadherin are completely lost (16–19). Snail has been shown to recruit several chromatin modifying enzymes constituted transcriptional repressive complexes to the E-cadherin promoter and repress its transcription (20–22). However, the exact functional mechanisms by which Twist promotes oncogenic progression in BLBC cells remain obscure.

Twist: a transcriptional factor well known to promote cancer progression but with unclear work mechanism

Twist is a member of the basic helix-loop-helix protein family, and a transcription factor that is critical to the induction of EMT. Multiple roles of Twist in cancer progression have been intensively investigated. Several lines of evidence link Twist to acquisition of cancer stem-like property and cells with abundant Twist expression have the CD44⁺CD24[−] phenotype and self-renewal ability (23–25). Twist-induced EMT is well known to contribute to cancer cell invasion and metastasis by activating several signaling pathways such as Mir-10b and Platelet-derived growth factor receptor (PDGFR) (26–28). It has also been reported that Twist plays an important role in counteracting tumor suppressor-induced apoptosis, oncogene-induced pre-mature senescence and acquisition of resistance to chemotherapy (29–33). In comparison with knowledge about the biological roles of Twist, understanding on regulation of Twist is limited; however several advances have occurred related to modifications to Twist protein, especially, phosphorylation. Two residues, Thr125 and Ser127, in the bHLH domain are phosphorylated by protein kinase A (PKA) (34). Protein kinase B (PKB/AKT) allows Ser42 phosphorylated Twist to confer resistance to p53 mediated cell apoptosis in response to DNA damage, and promotes EMT and breast cancer metastasis by enhancing the activity of TGF- β signaling (35, 36). Three mitogen-activated protein kinases, p38, c-Jun N-terminal kinases (JNK), and extracellular signal-regulated kinases1/2 (ERK1/2) were reported to phosphorylate Twist at serine 68 in vitro and prevent its protein degradation as well as promote breast cancer cell invasiveness (37). IL-6 activates casein kinase 2 and phosphorylates Twist at Ser 18 and Ser 20, which stabilizes Twist protein and enhances cancer cell motility (38). Recent studies also show that the stability of

Twist is negatively regulated by GSK-3 β mediated phosphorylation on the WR domain of Twist protein (39) (Figure 1).

Although it is well known that Twist is important for many physiological and pathological processes, the mechanisms by which Twist transcriptionally activates targeted gene expression during these processes remains obscure. Structurally, Twist contains the bHLH DNA binding domain close to COOH terminus which recognizes the DNA sequence known as E-box motif. A majority of mutations which cause Saethre-Chotzen syndrome are located in the bHLH domain demonstrating its functional importance (40). Recently, the WR motif, also known as the Twist box has been identified. Several molecules were reported to associate with WR domain of Twist, and several point mutations in the WR domain are also linked to Saethre-Chotzen syndrome (41–43). To date, no functional motifs have been identified from the first nearly 100 amino acids close to NH₂ terminus of the protein although this region of the Twist protein is highly conserved among different species. Therefore, little was known about exact biological roles of this region. Through sequence comparison, we found one potential motif in this region that shares high similarity with the histone H4 tail. In the corresponding region of the H4 protein two lysine residues, K5 and K8, have been reported to undergo post-translational acetylation modification. At the same time, these two modifications are recognized by many chromatin association factors, especially, those proteins containing bromodomain (44). Interestingly, through mass spectra and immunoblotting, we disclosed K73 and K76 di-acetylation in this H4-like region of Twist protein (45) (Figure 1). Thus, due to the similarity in sequences and modification patterns with histone H4, we speculated this region may be a potential regulatory motif in Twist. Investigations were initiated to uncover the regulation and functional mechanism of this H4-like motif within Twist.

BRD4: a key mediator of gene transcriptional activation

BRD4 is a member of the BET (bromodomain and extra-terminal domain) family (46). It was first described as a mitotic bookmark that constantly bound to chromosomes during mitosis and passed to daughter cells, since the tandem bromodomains of BRD4 recognize specific acetylated lysine marks on H3 and H4 histones tails (47–52). Subsequently, it was determined that papillomavirus E2 hijacks transcriptional machinery in human cells by interaction with BRD4, suggesting that BRD4 may play an essential role in transcriptional regulation (53). In recent years, it has been well demonstrated that BRD4 can activate RNA polymerase II dependent transcriptional elongation by acting as a protein kinase to directly phosphorylate the C-terminal tail (CTD) of RNA polymerase II, or indirectly by recruiting the transcriptional elongation factor, p-TEFb (54–56). The critical roles of recruitment and activation of RNA polymerase II make BRD4 essential to many diverse biological processes.

As a large molecular weight protein, BRD4 usually acts as an adaptor protein. Analysis of its protein structure suggests that BRD4 has several domains that readily associate with multiple molecules. The NH₂ terminal two bromodomain (BD1 and BD2) both recognize specific acetylated lysines on H3 or H4, but BD2 also recognizes acetylated lysine residues in non-histone proteins, such as cyclin T1 and RelA (57, 58). More and more transcriptionally-related proteins such as NSD3 and JMJD6 were found to associate with

extra-terminal (ET) domain of BRD4 (59, 60). The C-terminal region of BRD4, especially the last 100 amino acids, has been identified as the docking site of the papillomavirus E2 protein, and responsible for interacting with p-TEFb (53, 61, 62). Recently p53 was also shown to be a binding partner of BRD4 in casein kinase 2-mediated phosphorylation-dependent manner (63). In all, these findings indicated that BRD4 has the capacity to assemble diverse transcriptional complexes on gene promoters. Recently, BRD4 was identified as a promising target for several cancers because of its preferential occupation on super-enhancer of a set of oncogenic genes, which induces and maintains their high expression levels during cancer progression (64). Hence, a series of small inhibitors of BET bromodomain were screened out and proven therapeutic for several kinds of tumors, including T cell acute lymphoblastic leukemia, malignant peripheral nerve sheath tumors, diffuse large B cell lymphoma, glioblastoma, melanoma, lung adenocarcinoma, medulloblastoma, acute lymphoblastic leukemia and mixed-lineage leukemia (65–72).

Two fundamental questions about BRD4 need urgent attention. First, it has been generally speculated that BRD4 globally recognizes acetylated histones H3 and H4 throughout the genome thus leading to BRD4-dependent recruitment and activation of RNA polymerase II. Although BRD4 is a key transcriptional activator, it lacks DNA binding motif, which enables targeting to specific DNA sequences. Whether and how BRD4 and its associated transcriptional components are recruited to specific gene promoters/enhancers remain poorly known. Second, even though the two bromodomains of BRD4 share very similar sequences, they indeed display quite different functions. For example, their binding specificities for substrates are different; BD1 only recognizes acetylation marks on H3 and H4, whereas BD2 has a few non-histone substrates. Even for their common substrates histone H3 and H4, two bromodomains also show obviously distinct binding affinity. The detailed structural basis of these differences between two the BDs is totally unclear.

Histone H4 mimic motif of Twist recruits BRD4

Recent studies have revealed the existence of histone mimic motifs present in non-histone proteins. A methylated histone H3 tail-like motif has been identified, located in histone methyltransferase G9a, as a mimic, heterochromatin protein 1 (HP1) recognizes this region as methylated of H3 lysine 9 (73). Human PAF1 complex (hPAF1C) is important for the antiviral response; Non-structural (NS1) protein of influenza A H3N2 could target to polymerase associated factor (PAF1) by its histone-like sequence. This recognition represses hPAF1C-mediated transcriptional elongation and suppresses the antiviral response (74). Since our sequence comparison data has suggested a histone H4-like region is present in Twist protein, we wondered whether this histone mimic region functions similarly to that of H4, which would endow Twist with abilities to recruit downstream effectors. If this kind of recognition indeed exists, some chromatin associated proteins might be recruited to a specific gene promoter/enhancer by Twist-mediated DNA-site specific recognition, and may explain how chromatin associated proteins could be targeted to a specific gene locus under specific context. Our recent study indicated that by mimicking K5ac/K8ac of histone H4, the K73ac/K76ac motif of Twist recruits BRD4 and then targets the complex to specific gene promoters/enhancers through the recognition of E-box sequences by bHLH domain of Twist. We showed that K73/76 di-acetylated Twist has much in common with the K5/8 di-

acetylated histone H4: 1) similar amino acid sequence; 2) the same acetylation pattern, 3) the same acetyltransferase, Tip60, 4) the same downstream effector, BRD4. Interestingly, an acetylation antibody could simultaneously recognize these two motifs. These demonstrations confirmed that this K73/76 di-acetylated motif of Twist functions as a natural histone H4 mimic to recruit BRD4 protein (Figure 2). This finding not only proved that histone-mimic sequences present in non-histone proteins do have critical physiological roles, but also provided a novel insight into functional mechanism of transcriptional factors.

BRD4 tandem bromodomains integrate the activated Twist and H4 signaling

A fundamental concern in cell biology is how to maintain the specificity and fidelity of signal transduction. In summary, there are two ways which ensure the accurate signaling transition: one is covalent modification between upstream and downstream signaling molecules; another is modular recognition between signaling and adaptor proteins, which is proved by a conserved interaction between particular modules or between covalent modification and module. In some specific circumstance, continuous and repeated modules are required to guarantee the conservation of signaling transduction. One natural example is the tandem domain. Several kinds of modules exist in tandem including tandem bromodomains, tandem SH2 domains, tandem WW domains and tandem PDZ domains. These tandem domains were also found to act in a manner that was more than synergistic. Analysis of sequences from tandem WW domains (WW3 and WW4) of Suppressor of deltex (Su(dx)) protein showed that there is striking difference between their two substrate binding regions, indicating that they may engage in combinatorial recognition of different motifs for one substrate, or motifs from diverse substrates (75, 76).

Interestingly, we found that even though BRD4 could simultaneously recognize both Twist and H4 due to histone mimicry, tandem bromodomains function distinctly in this process. Although individual BD1 and BD2 of BRD4 both recognizes acetylated lysine on H3 or H4, BD2 is known to have broader binding capabilities, for acetylated substrates besides histone. Consistent with other studies, we found that only BD2, but not BD1, interacts with Twist-K73ac/K76ac, and that a key residue, His 437, was responsible for this selectivity. While BD2 can interact with either acetylated H4 or Twist individually *in vitro*, since the binding affinity of BD2-H4 is much weaker than that of BD1-H4, BD1 competes with acetylated H4 when tandem BD1+BD2 is present; a similar situation exists for BD1 and bromodomain testis-specific protein (Brdt) (77). These data suggest that the tandem bromodomains of BRD4 integrate activated H4 and Twist signaling, and facilitate assembly of a ternary BRD4/Twist/H4 complex at targeted gene super-enhancers with high efficiency (Figure 2). The study clearly demonstrates that, owing to subtle differences of structure, the tandem bromodomains of BRD4 develop distinct functions and binding specificities for acetylated substrates, even they two could recognize two different acetylated marks present in two molecules. This is also the first report illustrating that active histone and transcription factor signaling are connected by the tandem BDs of BRD4.

Twist-activated targeted genes transcription requires BRD4

In the study of EMT, Twist has been long considered as a transcription repressor like Snail, as it has been shown to bind to the E-cadherin promoter to block its transcription. However, in *Drosophila* development, Twist was shown to act as a master transcriptional activator to target and activate a signaling network that governs the expression of mesoderm-specific genes (78, 79). We demonstrated that Twist could recruit the BRD4/pTEFb/RNA polymerase II complex which is critical for transcription elongation, indicating that Twist could activate downstream targeted genes transcription in EMT and cancer progression. To identify transcriptional activation targets of Twist, we performed cDNA microarray analysis of HMLE and luminal T47D breast cells that have undergone Twist-mediated EMT. A total of 1315 and 1713 up-regulated genes were identified from HMLE-twist and T47D-twist cells respectively. 211 and 334 genes respectively, were further identified from these two groups as specific BRD4-Twist targeted genes since their up-regulated expression in Twist overexpressed cells could be repressed by bromodomain inhibitor JQ1. We further showed in detail that Twist recruits BRD4 and the associated P-TEFb and RNA-PolIII to the *WNT5A* super-enhancer. The transcriptional activation of *Wnt5a*, as an excellent example, fully described the unique mechanism of the Twist-BRD4 complex in gene transcriptional activation. In all, our study demonstrated that Twist-mediated genes transcriptional activation requires, in part, BRD4 (45).

Significance of Twist-BRD4 targeting in basal-like breast cancer and strategy of transcriptional factor inhibition

Twist is aberrantly expressed in many types of tumors, including breast cancer, hepatocellular carcinoma, pancreatic cancer, gastric cancer as well as prostate cancer etc. (43), and plays multiple roles during cancer progression. During cancer progression Twist endows cancer cells with abilities of persistent proliferation, enhanced motility, and can help antagonize signals disadvantageous to cancer cell survival that originate from the microenvironment. The intensive involvement of Twist in human cancers suggests that Twist inhibition is a promising strategy to targeting those Twist-driven cancers and relevant for extensive patients. However, Twist encodes a basic bHLH transcription factors without obvious ligand-binding domain or enzymatic domain, which made traditional targeted therapies lose their effectiveness toward developing small inhibitors. In addition, simply destroying the Twist protein will lead to severe side effects since it is pivotal in maintaining several physiological functions in body. We found that BET-specific BD inhibitors disrupted Twist-BRD4 interaction and resulted in significant *Wnt5a* reduction, leading to inhibition of tumorigenicity of BLBC cells *in vitro* and *in vivo* (45). A specific concern is that current BET-specific BD inhibitors cannot discriminate BD1 or BD2; in other words, these inhibitors block the functions of both BD1 and BD2. According to our mechanistic understanding of Twist-BRD4 interaction, inhibition of BD1/H4 interaction would result in large-scale effects on gene transcription, but selective inhibition of the BD2/Twist association might specifically shut down the targeted gene transcriptional activation by Twist. Thus, based on the detailed structural information of BD2/Twist association,

developing specific inhibitors which directly targeting BD2/Twist complex becomes feasible.

Our studies provide new insights for the development of inhibitors/antagonists to oncogenic transcriptional factors as cancer therapeutics. Several transcriptional factors are highly expressed or hyper-activated during cancer progression such as c-myc, HIF-1 α , p65 etc. Unlike kinases or other enzymes, transcriptional factors are always considered “undruggable” since targeting is difficult. Here, we propose a potential strategy for drug design. Similarly to histones, transcriptional factors may also possess diverse modified motifs and corresponding potential effectors, and each pair of those recognitions may have a distinct role in signal transduction. Through effective identification of those associations in specific contexts, researchers could design appropriately-targeted inhibitors, blocking specific activity or recognition, without destroying the protein itself or affecting other recognitions, and this strategy may reduce toxicity. We speculated that this strategy may reveal more therapeutic possibilities for those previously “undruggable” proteins, especially, transcriptional factors.

Conclusion

It was initially assumed that Twist would participate in EMT induction by repressing E-cadherin directly, similar to what has been described for Snail and Slug. Subsequently, it was determined that ectopic expression of Twist also induces the expression of multiple genes, which are related to the EMT process. Questions remained: what causes Twist to switch from a transcriptional repressor to an activator, and what is the transcriptional machinery employed by Twist. Our data provides an explanation as to how Twist could function as transcriptional activator. Extracellular stimuli such as TNF- α or insulin/EGF induce an association between Twist and the acetyltransferase Tip60. Tip60 recognizes the NH₂-terminal of Twist and di-acetylates it at lysine 73 and 76, in a fashion similar to the lysines in histone H4. Although Twist still recognizes the E-box DNA sequence by the bHLH motif, acetylated Twist recruits a pivotal transcriptional activation related protein, BRD4, and recruits the transcription machinery of the pTEFb complex and RNA polymerase II to its target genes, thus allowing transcription activation. Importantly, we found that this mechanism contributes to Twist-mediated activation of a set of critical oncogenes that govern proliferation and invasion of basal-like breast cancer cells. These findings provide novel insights for potential clinical and therapeutic uses. In particular, use of BET inhibitors that significantly disrupt the Twist/BRD4 interaction may be used for Twist overexpression tumors. Despite these developments, a great deal remains unknown about the role of the Twist/BRD4 complex. For instance, are other factors involved in regulating the association of Twist and BRD4? Do endogenous factors exist that can disrupt the BD2/Twist interaction? Is Twist/BRD4 interaction relevant to other physiological or pathological processes in which Twist is already known involved? In addition to *Wnt5a*, are there other downstream targeted genes of Twist/BRD4 that need to be clarified? Finally, additional investigations are required to reveal the specific microenvironment that activates the Twist/BRD4 complex. Efforts to answer all of these questions will favor the development of effective therapeutic strategies for treating Twist related disease.

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Figure 1.

Schematic diagram showing the functional motifs and post-translational modification on Twist. Pink, blue and green rectangles represent the histone H4 mimic, bHLH and WR motif, respectively, in the Twist protein. Blue and yellow ovals identify the phosphorylation and acetylation modifications respectively to the Twist protein.

Figure 2.

Illustration of the role of histone H4 mimicry in Twist on transcriptional regulation. Lysines 73 and 76 of Twist share similar surrounding sequence and acetylation pattern, the same acetyltransferase, TIP60, and the same downstream reader, BRD4, with lysine 5 and 8 of histone H4.