MULTIPLE FUNCTIONS OF BRD4 IN E2-MEDIATED HPV TRANSCRIPTIONAL REGULATION

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DEDICATION

I would like to dedicate my thesis to my family members, whom I love most and thank for everything. I would also like to acknowledge my friends who are my most valuable asset. I would like to thank my mentor, Dr. Cheng-Ming Chiang, and Dr. Shwu-Yuan Wu for their patience and support. Also, I would like to thank my committee members, Drs. Melanie Cobb, Thomas Kodadek, and David Mangelsdorf, for their input and help.

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by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2009

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HPV TRANSCRIPTIONAL REGULATION

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Human papillomaviruses (HPVs) are DNA viruses that cause benign and malignant tumors of epithelial origins. Expression of HPV-encoded E6 and E7 oncoproteins is controlled by the viral E2 protein, which plays a dual role in gene activation and repression. Recently, we identified bromodomain-containing protein 4 (Brd4) as a cellular corepressor for E2-mediated inhibition of HPV transcription. Brd4 contains two bromodomains which function as acetyl-lysine-binding modules that facilitate chromatin targeting via their interactions with acetylated histones.

Although Brd4 has been known to be involved in E2-mediated transcriptional regulation, it was unclear how Brd4 regulates E2 function and whether the involvement

of Brd4 in transactivation and transrepression is common to different types of E2 proteins. Here, we show that Brd4 enhances E2 binding to its cognate sequences through Brd4's bromodomains and the E2-interacting region in chromatin. We further demonstrate that the corepressor function of Brd4 is common to E2 proteins encoded by cancer-inducing high-risk HPV, wart-causing low-risk HPV, and bovine papillomavirus type 1. The general cofactor function of Brd4 on E2-mediated transcription is in part controlled by enhancing the protein stability of E2, which is normally degraded via the ubiquitin-dependent proteasome pathway. These findings indicate that a chromatin adaptor can enhance the binding of a sequence-specific transcription factor to chromatin and further promote the stability of a labile transcription factor via direct protein-protein interaction.

Also, we identify two additional E2-interacting regions of Brd4: the E2-interacting domain (E2ID) and phosphorylation-dependent interacting domain (PDID). While E2ID binds to all different types of E2 proteins, PDID interacts only with high risk E2 (HRE2) in a casein kinase 2 (CK2) phosphorylation-dependent manner. In addition to HRE2-specific interaction, the phosphorylation of PDID also induces intramolecular interaction between PDID and E2ID, which blocks the E2-interaction of E2ID. Finally, we show that the PDID-HRE2 interaction is important for the HRE2-mediated transcriptional activation. Collectively, our data show that the posttranslational modification of the cellular protein Brd4 confers selective recognition of HRE2, thereby providing a unique regulation mechanism for the protein encoded by cancer-inducing HPV.

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ACF - ATP-utilizing chromatin assembly and remodeling factor
Bdf1 - bromodomain factor 1
BE2 - BPV-1 E2
BET - bromodomains and extraterminal domain
BPV-1 - bovine papillomavirus type 1
Brd4 - bromodomain-containing protein 4
Brdt - for bromodomain, testis-specific
Cdk9 - cyclin-dependent kinase 9
ChIP - chromatin immunoprecipitation
CIP - calf intestine phosphatase
CK2 - casein kinase 2
CTM - C-terminal motif
DBD - DNA-binding domain
E2ID - E2-interacting domain
ET - extraterminal
FL - full-length
Fsh - female sterile homeotic
Fshrg1 - female sterile homeotic related gene 1
HAT - histone aceryltransferases
HPV - Human papillomavirus
HRE2 - high risk E2
LRE2 - low risk E2
MCAP - mitotic chromosome-associated protein
MNase - micrococcal nuclease
NE - nuclear extract
NF-κB - nuclear factor kappa-B
NTD - N-terminal domain
Nut - nuclear protein in testis
P/CAF - p300/CBP-associated factor
PDID - phosphorylation-dependent interacting domain
PIC - preinitiation complex
pol II - RNA polymerase II
P-TEFb - positive transcription elongation factor b
RING3 - really interesting new gene 3
TBP - TATA binding protein

URR - upstream regulatory region

CHAPTER I:

INTRODUCTION

BROMODOMAIN-CONTAINING PROTEIN 4 (BRD4)

Brd4 is a ubiquitously expressed chromatin adaptor which binds to acetylated histone H3 and H4. It belongs to the BET protein family which is characterized by an extraterminal domain and two bromodomains. Many reports have implicated Brd4 in cellular and viral activities such as transcription, cell cycle progression, cancerization, and viral genome segreagation. Brd4 null mutant mice are embryonically lethal. The heterozygote displays growth defects before and after birth as well as postnatal lethality, suggesting its pivotal role in fundamental biological processes (Houzelstein et al, 2002).

Bromodomain

The bromodomain is an acetyl-lysine binding module with approximately 110 amino acid residues (Winston & Allis, 1999) and is a highly structured domain with 4 α-helices and 2 helix-connecting loops (Dhalluin et al, 1999). It is broadly found in proteins ranging from yeast to humans. Many chromatin-

associating factors possess this module, implicating its crucial role in chromatin-targeting through the recognition of acetylated histones.

The bromodomain-containing proteins include chromatin-modifying enzymes such as nucleosome remodeling factors and histone acetyltransferases (HATs). Due to the essential roles these proteins play in chromatin dynamics and transcriptional regulation, a number of structures of the bromodomain have been reported as follows: human HAT P/CAF (p300/CBP-associated factor) (Dhalluin et al, 1999; Mujtaba et al, 2002), human transcription factor TAF1 subunit of TFIID (Jacobson et al, 2000), human and yeast HAT GCN5 (Hudson et al, 2000; Owen et al, 2000), human HAT CBP (CREB-binding protein) (Mujtaba et al, 2004), human transcription factor BPTF (bromodomain and PHD domain transcription factor) (Li et al, 2006), human nucleosome remodeling factor Brg1 (Brahma-related gene 1) subunit of SWI/SNF complex (Shen et al, 2007), human remodeling factor Brd7 (Sun et al, 2007), human corepressor KAP1 (KRAB-associated protein 1) (Zeng et al, 2008), and human BET (bromodomains and extraterminal domain) family proteins Brd2 (Huang et al, 2007; Nakamura et al, 2007) and Brd4 (Liu et al, 2008).

BET family protein

Characteristics of BET proteins

While most of the bromodomain-containing proteins only possess one bromodomain, the BET family proteins are characterized by their two tandem bromodomains (BDI and BDII), and an additional conserved extraterminal (ET) domain (Florence & Faller, 2001). In addition to revealing the structures of BDI (Nakamura et al, 2007) and BDII (Huang et al, 2007; Nakamura et al, 2007) of Brd2, and BDII of Brd4 (Liu et al, 2008), the solution structure of the ET domain of Brd4 has been recently resolved (Lin et al, 2008b). However, its function is still obscure. The homology of the two bromodomains, BDI and BDII, within the same protein (~44%) is lower than the homology between each respective bromodomain among other proteins in the same family (Wu & Chiang, 2007). Specifically, BDI, when compared to BDI in another protein, and BDII compared to another BDII in other proteins within the family share >75% identity, suggesting distinct target specificity or function of the two bromodomains. Recently, Motif A, Motif B, and SEED domain have been reported as additional conserved modules among BET proteins through sequence analysis, even though their functional significance has not been revealed yet (McBride et al, 2004; Paillisson et al, 2007; Wu & Chiang, 2007).

BET family proteins are evolutionarily conserved among various species from yeast to higher eukaryotes, and include four mammalian proteins: Brd2, Brd3, Brd4, and Brdt, and proteins from the following non-mammalian sources: *Drosophila* Fsh (female sterile homeotic), and *Saccharomyces cerevisiae* Bdf1

(bromodomain factor 1) and Bdf2 (Figure 1-1). A unique feature of BET proteins is their association with chromosomes during mitosis when most of the other bromodomain-containing factors involved in transcription and chromatin modification are displaced into the cytoplasm because of chromosomal condensation (Dey et al, 2000). Due to this characteristic of mitotic chromosome association, BET proteins have been suggested to play a role in defining a histone code and transmitting epigenetic memory to daughter cells (Dey et al, 2000; Kanno et al, 2004; Loyola & Almouzni, 2004). Except for Brdt (for bromodomain, testis-specific), which is exclusively expressed in testis and ovary as the name indicates, all other mammalian BET proteins, Brd2, Brd3, and Brd4, are ubiquitously expressed in various tissues.

Functions of BET proteins

Yeast Bdf1 was initially identified as a TFIID-interacting protein and an ortholog of the C-terminal half of the higher eukaryotic protein, TAF1, which indicates its function as a general transcription factor (Lygerou et al, 1994; Matangkasombut et al, 2000). More recently, Bdf1 was also found to associate with SWR-C, suggesting its involvement in nucleosome remodeling (Krogan et al, 2003). Bdf1 and its homolog, Bdf2, are genetically redundant because cells are viable without one of the two proteins, but require at least one of the factors for survival (Matangkasombut et al, 2000).

Fsh is known to be involved in the determination of segmental organization and identity in *Drosophila* (Huang & Dawid, 1990). Brd2, initially named RING3 (really interesting new gene 3) or Fshrg1 (female sterile homeotic related gene 1), is a mitogen-activated nuclear kinase (Denis & Green, 1996). It has been implicated in transcriptional regulation through its interaction with E2F1 (Denis et al, 2000). More recently, its role as a histone chaperone has been reported (LeRoy et al, 2008). Brd3, also called ORFX or Fshrg2, is poorly characterized although a recent report suggests it facilitates transcription in the presence of acetylated chromatin (LeRoy et al, 2008). Brdt has been reported to play roles in acetylation-dependent chromatin reorganization (Pivot-Pajot et al, 2003) and male germ line differentiation (Shang et al, 2007). Brd4 (also called MCAP, Fshrg4 or Hunk1) plays versatile roles in cellular proliferation (Houzelstein et al, 2002), DNA replication (Maruyama et al, 2002), transcription regulation of viral and cellular genes (Jang et al, 2009; Jang et al, 2005; Lee & Chiang, 2009; Schweiger et al, 2006; Wu et al, 2006; Yang et al, 2005), viral segregation (You et al, 2004), cell cycle progression (Dey et al, 2000), and gene arrangement in t(15;19)-associated carcinomas (French et al, 2003). More detailed characteristics and functions of Brd4 would be discussed in the next section.

Brd4

Brd4 is a 200-kDa nuclear protein. So far, no DNA sequence specificity or enzymatic activity of Brd4 has been reported, although it possesses a kinase-like motif (Dey et al, 2000). Thus, Brd4 plays versatile roles mainly through protein-protein interactions with various viral and cellular factors and the published interaction results are summarized in Figure 3-1.

Brd4 isoforms

As shown in Figure 1-2, three isoforms of Brd4 have been reported: 1) the wild-type long form, 2) its rare splicing variant short form (French et al, 2001; Houzelstein et al, 2002), and 3) a fusion protein with Nut (nuclear protein in testis), Brd4-Nut (French et al, 2003). The Brd4 short form includes an identical N-terminal region (amino acids 1-719) to the wild-type protein and three additional amino acids encoded by an alternative exon. The expression of this variant is often not detectable, thus its biological function is obscure. Chromosomal translocation t(15;19)(q13, p13.1) results in a fusion oncoprotein, Brd4-Nut, and is found in a highly malignant epithelial neoplasia. Even though the mechanism of how Brd4-Nut leads to oncogenesis has not been fully defined yet, its role in the inhibition of epithelial cell differentiation and maintenance of carcinoma cell growth has been suggested (French et al, 2008).

Chromatin binding of Brd4

Brd4 is a chromatin-associating protein with selective affinity for acetylated lysine 14 on histone H3 and lysines 5/12 on H4 (Dey et al, 2003). During interphase, Brd4 is widely distributed in the nucleus except that it is not found in the nucleoli. In mitotic cells, it exclusively binds to the entire condensed chromosomes except for at the centromere, which constitutes heterochromatin. (Dey et al, 2000). Contrary to histone proteins, which are stable components of chromatin, Brd4 associates with chromosomes in a rapid on and off fashion and preferentially recognizes acetylated chromatin in interphase cells. This suggests that its chromatin binding occurs through protein-protein interaction (binding to the acetylated histone), not tight binding to the DNA (Dey et al, 2003; Dey et al, 2000).

Because of its mitotic chromosome association, Brd4 was originally named as MCAP (mitotic chromosome-associated protein), and related to this feature, many reports have shown that Brd4 can tether viral episomes to the host mitotic chromosome through its interaction with viral proteins such as papillomavirus E2 (You et al, 2004; You et al, 2005), KSHV (Kaposi's sarcoma-associated herpesvirus), and LANA (latency-associated nuclear antigen) (You et al, 2006) to ensure the viral genome segregation during mitosis without the episomes being displaced to the cytoplasm.

Besides the recognition of acetylated histones by Brd4, it has also been suggested that it is involved in the maintenance of acetylated histone codes. Brd4 haploinsufficience of Brd4 +/- cells led to hypoacetylated lysine 14 on histone H3 and lysine 12 on H4, the sites for Brd4 binding, indicating that Brd4 contributes to preserving the acetylation status of its binding sites (Nishiyama et al, 2006).

Role of Brd4 in cell cycle regulation

Brd4 is involved in cell cycle progression through various mechanisms. Depletion of endogenous Brd4 by microinjection of anti-Brd4 antibody into HeLa cells induces G_2 arrest, suggesting an important role of Brd4 in cell cycle progression to mitosis (Dey et al, 2000). Later on, the same group reported that the proper balance between Brd4 and its interacting partner, SPA-1 (signal-induced proliferation-associated protein 1), a Rap GAP (GTPase-activating protein), is important for the G_2/M transition, suggesting that the G_2 arrest caused by Brd4 depletion is due to an imbalance between Brd4 and SPA-1 (Farina et al, 2004). In contrast, ectopic expression of Brd4 inhibited cell cycle progression from G_1 to S by disrupting the stoichiometric balance between RFC-140 (replication factor C-140) and Brd4, which is an inhibitor of RFC, since the functional equilibrium of the two factors is crucial for the entry into S phase (Maruyama et al, 2002). Similarly, overexpression of Brd4-Nut fusion protein

inhibited S phase progression, probably due to the interplay between the fusion protein and RFC (Haruki et al, 2005).

Role of Brd4 in cancer

Apart from its role in cell cycle regulation, Brd4 has been shown to be involved in breast cancer progression and a fundamental mechanism of many metastasis-predictive gene signatures (Crawford et al, 2008a; Crawford et al, 2008b). When metastatic Brd4-expressing cells were implanted into mice, they showed significantly reduced tumor growth and metastasis. Furthermore, the same group found that activation of Brd4 or Brd4-associated pathways in a tumor was an important determinant of disease progression and patient survival. The exact role of Brd4 in the survival and relapse of breast cancer needs to be further explored.

Role of Brd4 in transcription

In early and subsequent studies, Brd4 has been found to be a component of mammalian Mediator, a well-established transcription coactivator for gene-specific activators (Houzelstein et al, 2002; Jiang et al, 1998; Wu & Chiang, 2007). Thus, the involvement of Brd4 in the transcription process has been speculated about, but its function as a part of Mediator has not yet been revealed. The initial report stating that Brd4 is involved in transcription was in a study on

the activation of HIV-1 (human immunodeficiency virus type 1) promoter (Jang et al, 2005; Yang et al, 2005). Brd4 stimulated the activity of HIV-1 LTR (long terminal repeat) promoter via the formation of a positive transcription elongation complex through the interaction with both of the subunits of P-TEFb (positive transcription elongation factor b), cyclin T1 and Cdk9 (cyclin-dependent kinase 9) (Bisgrove et al, 2007; Jang et al, 2005; Yang et al, 2005). More recently, the regulation of cellular gene expression by the P-TEFb/Brd4 complex has been reported. Several Brd4 knock-down studies show that Brd4 binds to the promoters of some G_1 genes and further recruits P-TEFb and pol II (RNA polymerase II), resulting in transcriptional activation of G_1 genes, thereby promoting progression to S phase (Mochizuki et al, 2008; Yang et al, 2008). An additional factor involved in this functional regulatory interaction between Brd4 and P-TEFb has been reported. HTLV-1 (human T-lymphotropic virus type 1)-encoded Tax protein forms a positive transcription elongation complex with P-TEFb via competitively targeting cyclin T1 with Brd4, suggesting that Tax can functionally substitute Brd4 in P-TEFb regulation (Cho et al, 2007).

In addition to the positive regulation of Brd4 on HIV transcription, its involvement in the inhibition of transcription has been reported. Brd4 inhibits Cdk9 kinase activity by inducing its autophosphorylation on threonine 29 in the HIV transcription initiation complex, resulting in transcription inhibition (Zhou et al, 2009). Since the proviral gene expression is suppressed during HIV latency,

the inactivation of Cdk9 by Brd4 has been suggested to contribute to viral latency (Zhou et al, 2009).

Apart from its roles as a complex with P-TEFb, Brd4 has also been implicated in the regulation of viral and cellular promoters' activity through its interaction with multiple factors. The proteins encoded by the open reading frame 73 (orf73) of the gammaherpesviruses, LANA for KSHV and orf73 for MHV-68 (murine gammaherpesvirus 68), bind to the region around the ET domain of Brd4 and this interaction modulates the G_1/S transition by regulating the activity of G1 gene promoters such as CDK2 (You et al, 2006), cyclin E (Ottinger et al, 2006; Ottinger et al, 2009), and cyclin D1, and D2 (Ottinger et al, 2009). ChIP (Chromatin immunoprecipitation) data suggest that Brd4 is preferentially recruited to the EBNA1 (Epstein-Barr virus nuclear antigen 1)-responsive element of the EBV (Epstin-Barr virus) genome through its interaction between Brd4 and EBNA1, resulting in the activation of EBNA1-dependent transcription (Lin et al, 2008a).

Recently, the first report of the recognition of an acetylated non-histone protein by Brd4 was published. The acetylated RelA (lysine 310) subunit of NF-κB (nuclear factor kappa-B), which is a transcriptional regulator that plays a central part in the response to inflammatory signals, recruits Brd4 onto NF-κB-responsive genes' promoters, which further recruits P-TEFb to form a positive

transcription complex, thereby activating acetylated RelA-mediated transcription (Huang et al, 2009).

Brd4 also affects the transcription of genes on the HPV (human papillomavirus) genome. The involvement of Brd4 in papillomavirus E2-dependent transcription regulation will be discussed in the Brd4 and E2 section.

PAPILLOMAVIRUS

Characteristics

Papillomaviruses are non-enveloped, small double-stranded DNA viruses. Approximately 200 types of this family of viruses have been identified (Munger et al, 2004). They have strict host specificity, and thus HPVs (human papillomaviruses) only infect humans, BPVs (bovine papillomaviruses) only infect bovines, and so forth. The viruses infect the epithelial and mucosal lining of the anogenital tract and other areas. Therefore, the life cycle of the virus is closely linked to epithelial differentiation processes of the infected host keratinocyte (Longworth & Laimins, 2004). The virus infection causes either benign or malignant tumors (Steben & Duarte-Franco, 2007), and the virus can be classified into two groups depending on the propensity for malignancy: 1) high

risk HPVs such as HPV-16, -18, -31, -33, -45, -51, -52, 58, etc., which are the cause of over 90% of cervical cancers (Narisawa-Saito & Kiyono, 2007), 2) low risk HPVs such as HPV-6, -11, -15, -20, etc., which induce benign genital or skin warts (zur Hausen, 2000). Almost all women diagnosed with cervical cancer, the second commonest cancer in women, have tested positive for HPV infection and about 50-70% and 7-20% of cases are associated with HPV-16 and HPV-18, respectively (Parkin & Bray, 2006). Virtually 100% of external genital warts are induced by HPV infection and HPV type 6 and 11 are the cause of 90% of these cases (Greer et al, 1995; Steben & Duarte-Franco, 2007). Even though many of the infected viruses are cleared by cellular immune responses, HPV infections are the most commonly diagnosed sexually transmitted disease with an approximate prevalence of 26.8% in sexually active individuals (Dunne et al, 2007; Steben & Duarte-Franco, 2007).

Genome organization of HPV

All different types of HPVs share a very similar genomic organization. Their genomes are approximately 8,000 base pairs in size and can be divided into three parts: 1) an upstream regulatory region (URR or long control region), a non-coding region which contains numerous viral and cellular factor binding sites and regulates the viral replication and gene expression, 2) the early genes including

E1, E2, E4, E5, E6, and E7, and 3) the late genes, L1 and L2. E1 is a helicase and is important for DNA replication. E2 plays a role as a viral transcription factor regulating the expression of oncoproteins, E6 and E7, and as a replication protein through the cooperation with E1. Another early protein, E4, interacts with cytoskeletal proteins and is implicated in viral assembly. E6 and E7 are the major players in the development of HPV-induced cervical cancer. They accomplish this via their binding to and inactivation of the tumor suppressor proteins, p53 and retinoblastoma, respectively. Additionally, L1 and L2 are major and minor capsid proteins, respectively.

PAPILLOMAVIRUS PROTEIN E2

Characteristics of E2 protein

The papillomavirus E2 is a sequence-specific DNA-binding protein and a central regulator of the virus life cycle. It plays versatile roles such as viral transcription regulation (Hou et al, 2002; Wu et al, 2006), DNA replication (Abbate et al, 2004; Stenlund, 2003), viral episome maintenance and genome segregation (Abroi et al, 2004; Botchan, 2004; Ilves et al, 1999; McBride et al, 2004; Skiadopoulos & McBride, 1998; You et al, 2004), cell cycle regulation

(Hwang et al, 1993), apoptosis (Blachon et al, 2005; Blachon & Demeret, 2003; Demeret et al, 2003), and senescence (Goodwin et al, 2001). The E2 protein consists of three parts: 1) the N-terminal domain (NTD) important for transactivation and replication via cooperation with viral protein E1, 2) the flexible hinge region, 3) the C-terminal DNA-binding domain (DBD), which is responsible for DNA binding and dimerization (Figure 1-4). While N- and C-terminal domains are well conserved, the hinge region is relatively divergent. The domain organization is very similar among different types of E2 proteins (Figure 1-4).

E2 stability

The stability of E2 is regulated by polyubiquitination and its subsequent degradation through the proteasome-mediated pathway (Bellanger et al, 2001; Penrose & McBride, 2000). The hinge region of BE2 is phosphorylated by CK2, a serine/threonine kinase, and this modification signals the initiation of a proteasomal degradation pathway (Penrose et al, 2004; Penrose & McBride, 2000). On the other hand, the degradation of 18E2 occurs mainly through its N-terminal transactivation domain (Bellanger et al, 2001), suggesting that different types of E2 might be regulated by distinct mechanisms. Brd4 has been implicated in E2 stabilization when it is bound to the N-terminus of E2 through an unknown

mechanism (Lee & Chiang, 2009). Recently, sumoylation of cellular proteins, and not E2 itself, blocked the proteasomal degradation of 16E2, indicating that the functional modulation of this posttranslational modification might occur on the E2-interacting partners or the enzymes involved in the phosphorylation of E2 (Wu et al, 2009).

Role of E2 in mitotic chromosome targeting

To replicate viral DNA, the E2 protein binds to the viral origin cooperatively with the E1 protein (Howley, 2001). However, this replication does not ensure long-term maintenance of the viral episome. For stable maintenance of the virus, E2-bound viral genomes need to be associated with mitotic chromosomes to make sure that viral genomes are segregated to daughter cells (Skiadopoulos & McBride, 1998). Just recently, You et al. identified Brd4 as an E2-interacting partner, implicating it in mitotic chromosome targeting (You et al, 2004) (detailed results would be discussed in Brd4 and E2 section).

However, several lines of evidence suggest that the association with Brd4 may not be the only mechanism for tethering the papillomavirus episomes to mitotic chromosomes. HPV-11 E2 colocalizes with the mitotic spindle during prophase and metaphase and it relocates to the central spindle microtubules during anaphase, telophase, and throughout cytokinesis (Dao et al, 2006). The C-

terminal DNA binding domain, which does not associate with Brd4, was necessary and sufficient for this localization, indicating that Brd4 independently binds to the spindle apparatus (Dao et al, 2006). Also, DNA helicase ChlR1 has been identified as another cellular E2-binding protein for mitotic chromosome targeting (Parish et al, 2006a). BPV-1 E2 (BE2) mutant W130R, which binds to Brd4, but not to ChlR1, failed to associate with the mitotic chromosome, indicating that the E2/Brd4 interaction is not sufficient for E2 association with mitotic chromosomes (Parish et al, 2006a).

E2 proteins from different types of papillmaviruses have shown that they bind to different regions of the host chromosomes during mitosis (Oliveira et al, 2006). Whereas BE2 bind to all chromosomes in a complex with Brd4, HPV-8 E2 localizes to the pericentromeric regions of chromosomes (Oliveira et al, 2006). The same group further revealed that the pericentromere-localized 8E2 was not associated with Brd4 and it bound to the repeated ribosomal DNA genes found in the pericentromere and colocalized with UBF, the ribosomal transcription factor (Poddar et al, 2009). All these data indicate that different types of E2 may have distinct strategies to target their host's chromosome.

Role of E2 in transcription regulation

The E2 protein regulates viral transcription through binding as a dimer to a palindromic consensus sequence, $ACCN_6GGT$, in the URR (McBride et al, 1991; Sousa et al, 1990). There are four E2-binding sites within the URR. Two are adjacent to the TATA box, and affect early promoter (E6 promoter) function. The other two are located in a further upstream region. At low concentrations, E2 activates the early promoter through the recognition of promoter-distal binding sites (Chong et al, 1991). When at high concentration levels, it occupies promoter-proximal binding sites and represses transcription by blocking the binding of cellular transcription factors and TATA binding protein (TBP), which is important for the initiation complex formation (Dong et al, 1994; Dostatni et al, 1991). Also at high concentrations, it inhibits the preinitiation complex (PIC) assembly at steps after TATA binding by TBP and TFIID (Hou et al, 2000).

Different properties between high risk and low risk E2

The domain organization and three-dimensional structure are very similar among different types of E2, and they have been shown to have conserved activity in transcription (Demeret et al, 1994; Dostatni et al, 1991; Lee & Chiang, 2009; Romanczuk et al, 1990; Tan et al, 1992; Thierry & Howley, 1991) and replication (Desaintes & Demeret, 1996), except for differences in the extent of their DNA

binding affinity (Hou et al, 2002). Nevertheless, some distinct properties of high-risk E2 compared to low risk E2 have been reported.

While low risk E2 proteins (11E2 and 6E2) remain exclusively nuclear, high risk E2 proteins (16E2 and 18E2) are shuttled between the nucleus and the cytoplasm, and this cytoplasmic portion of E2 induces apoptosis through activation of caspase 8 by an unknown mechanism (Blachon et al, 2005). These observations raise an interesting possibility: that disruption of the E2 gene during viral genome integration ensures carcinogenesis not only by upregulating the expression of E6 and E7 viral oncoproteins, but also by avoiding E2-mediated apoptosis (Blachon et al, 2005). Another group also reported that high risk E2 induces apoptosis, but through a different mechanism. High risk E2, but not low risk E2, binds to p53, and this interaction is critical for E2-induced apoptosis in non-HPV-transformed cells, but not in HPV-transformed cells, indicating that HPV-transformed cells might utilize a unique pathway for E2-induced apoptosis (Parish et al, 2006b).

High risk E2, but not low risk E2, expressing cells induce a mitotic block and present abnormal mitotic phenotypes. They also create problems with genomic instability such as polyploidization, chromosomal mis-segregation, and centrosome overduplication, possibly through the specific binding of high risk E2 to Cdc20 and Cdh1, activators of APC (anaphase promoting complex), a ubiquitin ligase (Bellanger et al, 2005). Even though high risk E2 proteins are not

substrates of APC, they stabilize cyclin B2, a substrate of APC, by inhibiting APC activity through delocaliztion of Cdh1 (Bellanger et al, 2005). Thus, high risk HPV E2 proteins have been suggested to participate in HPV's oncogenic potential through inducing genome instability, one of the contributors of the process of cancerization.

BRD4 AND E2

Since Brd4 has been identified as an E2-interacting cellular protein, many reports have implicated Brd4 in various E2-mediated functions. Examples of highly studied functions are its role in viral transcription regulation and viral genome segregation through its mitotic chromosome tethering.

Role of Brd4 in E2-mediated genome segregation

Many DNA viruses have evolved a mechanism to target their host's mitotic chromosome during genome segregation to ensure that viral DNA is not dispersed into the cytoplasm during mitosis (McBride et al, 2004). As mentioned above, viral protein E2 is a central player in chromatin targeting for papillomavirus. However, it has been reported that E2 could interact with mitotic

chromosomes in the absence of viral genomes, and its DNA-binding domain is not necessary for the association with mitotic chromosome. This suggests that E2 may interact with cellular proteins, allowing for its function in chromatin targeting (Skiadopoulos & McBride, 1998). Recently, Brd4 has been identified as a major cellular E2-interacting protein mediating viral genome attachment to mitotic chromosomes (You et al, 2004). The Brd4/E2 interaction is critical for this tethering, since overexpression of Brd4 C-terminal motif (CTM), the BE2-interacting region, inhibits E2 binding to Brd4 and abrogates tethering of the viral genome to mitotic chromosomes (You et al, 2004). Further studies using site-directed mutagenesis created mutants of Brd4, which altered amino acid residues important to binding of BPV-1 E2 showed that the E2/Brd4 interaction correlates with the ability of E2 to bind to the mitotic chromosome (Baxter et al, 2005). These data indicate that E2 tethers the viral genome to cellular chromosomes through its interaction with Brd4. In addition to the interaction during mitosis, E2 also shows colocalization with Brd4 in cells during interphase (McPhillips et al, 2005). Moreover, this protein-protein interaction stabilizes the association of Brd4 with chromosomes in both mitotic cells and during interphase, and the DNA-binding domain of E2 is not required for this function, indicating a more active role of E2 for a stable tethering to cellular chromosomes (McPhillips et al, 2005).

Role of Brd4 in E2-mediated transcription regulation

Function in repression

E2 can function as a transactivator or repressor depending on the context of the promoter. In host cells, HPV E2 mainly functions as a transcription repressor because two of the four E2-binding sites are proximal to the Sp1-binding site and TATA box in URR, and E2 binding to its cognate sequence excludes Sp1 and TBP binding to their binding sites. Consequently, this inhibits transcription initiation (Bernard et al, 1989; Demeret et al, 1997; Dong et al, 1994; Dostatni et al, 1991; Hou et al, 2000; Tan et al, 1994).

Previously, our group showed that the C-terminal domain of E2 is sufficient, for E2-mediated transcriptional repression, and the N-terminal domain is not required, through a reconstituted *in vitro* transcription assay using a DNA template (Hou et al, 2000). However, *in vivo* experiments with virus-infected or transiently transfected HeLa cells indicates that the N-terminal domain of E2 also plays a role in E2-mediated transcriptional repression (Goodwin et al, 1998). This discrepancy led us to hypothesize that the N-terminal domain of E2 may associate with some cellular proteins to accomplish E2-mediated transcriptional repression. Indeed, Brd4 was identified as a novel cellular factor that modulates E2-mediated repression using 293 cells conditionally expressing 11E2 (Wu et al, 2006). As a complex with 11E2, Brd4 inhibits AP-1-activated HPV chromatin transcription in

an E2-binding site-dependent manner (Wu et al, 2006). The cell-based assay further showed that Brd4 knockdown alleviates E2-mediated repression, confirming the role of Brd4 in transcriptional silencing of HPV mRNA (Wu et al, 2006). Coimmnoprecipitation using HeLa cells carrying integrated HPV-18 genome showed that the recruitment of TFIID and RNA polymerase II (polII) to the HPV promoter region is significantly reduced when E2 is expressed, leading us hypothesize that the chromain adaptor, Brd4, binding to acetylated histones stabilizes E2's recognition of its binding sites in a chromatin environment (Wu et al, 2006). This possibility was explored by performing DNAase I footprinting with *in vitro* assembled HPV chromain, showing that Brd4 enhanced E2 binding to its cognate sequences in bromodomain- and E2-interacting domain-dependent manners (Lee & Chiang, 2009). *In vitro* transcription and luciferase assay with C-33A-derived stable Brd4 knock-down cells (Wu et al, 2006) suggests that Brd4 functions as a general corepressor for all the different types E2 tested: high risk HPVs 16E2 and 18E2, low risk 11E2, and BPV-1 E2 (Lee & Chiang, 2009).

Function in activation

When E2 binding sites are situated away from core promoter region, E2 can function as an activator, since there is no competition between E2 and other transcription factors binding to their cognate sequences (Hirochika et al, 1988; Li et al, 1989; Spalholz et al, 1985; Thierry et al, 1990). When a heterologous

promoter with mulitmerized E2 binding sites, which is located 123 bps (base pairs) away from TATA box is used, Brd4 augments E2-mediated transcriptional activation. For this enhancement, the C-terminus of Brd4, which mediates the interaction with E2, is critical (McPhillips et al, 2006; Schweiger et al, 2006; Senechal et al, 2007). The coactivator function of Brd4 is universal for different types of E2 proteins encoded by high-risk and low-risk HPVs as well as by animal papillomaviruses, such as BPV-1, and it requires at least one bromodomain for chromatin targeting and the C-terminal motif for E2-inteaction (Lee & Chiang, 2009).

Recently, a genome-wide ChIP-chip (chromatin immunoprecipitation–on-chip) analysis using C-33A cells showed that BE2 and Brd4 bound to transcriptionally active promoters, which do not possess the consensus E2-binding sequence, since the chromatin-binding profiles of Brd4 and E2 matched the one for pol II and H3K4me3 (trimethylation on lysine 4 of histone 3), which are indicators of active transcription (Jang et al, 2009). Therefore, E2 targeting on transcriptionally active promoters is suggested to be a strategy for the virus to ensure the viral genome remains in transcriptionally active regions of the nucleus, thereby continuing expression of the viral proteins (Jang et al, 2009).

In this thesis, I have characterized the function and the mechanism of the chromatin adaptor Brd4 protein in HPV E2-mediated transcription regulation.

First of all, the molecular mechanism by which Brd4 enhances E2-dependent repression of the HPV promoter was defined, and whether Brd4 plays a dual role in E2-dependent activation and repression for different types of E2 was addressed. Next, the functional regulation of Brd4 by CK2-mediated phosphorylation was investigated. Finally, the Brd4-interacting cellular proteins were identified and their functional modulation through the protein-protein interaction was explored.

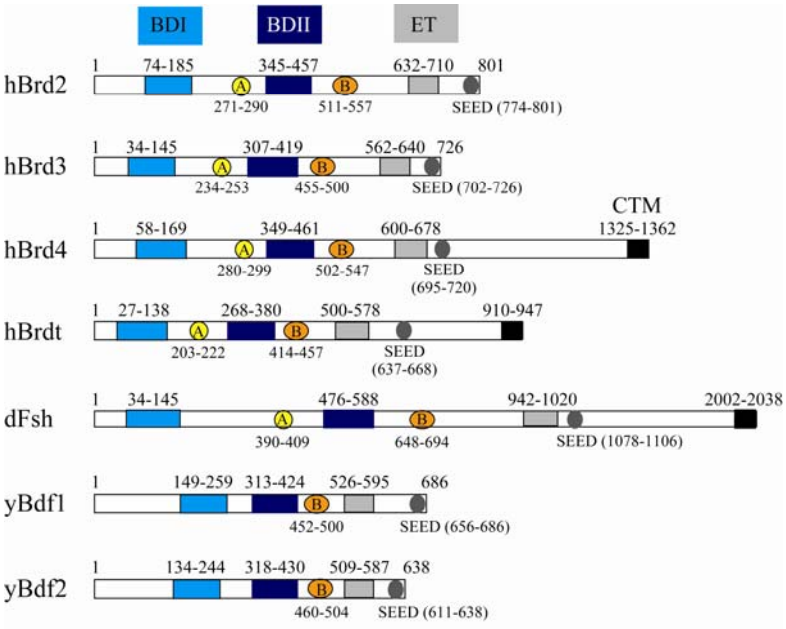

Figure 1-1. BET family proteins
Schematic representation for domain organization of BET family proteins: human proteins (hBrd2, hBrd3, hBrd4, and hBrdt), *Drosophila* Fsh (dFsh), and yeast proteins (yBdf1 and yBdf2). Numbers indicate the positions of amino acid residues or the boundaries of respective protein domains, including bromodomain I (BDI), motif A (A), bromodomain II (BDII), motif B (B), extraterminal (ET) domain, SEED domain (SEED), and the C-terminal motif (CTM).

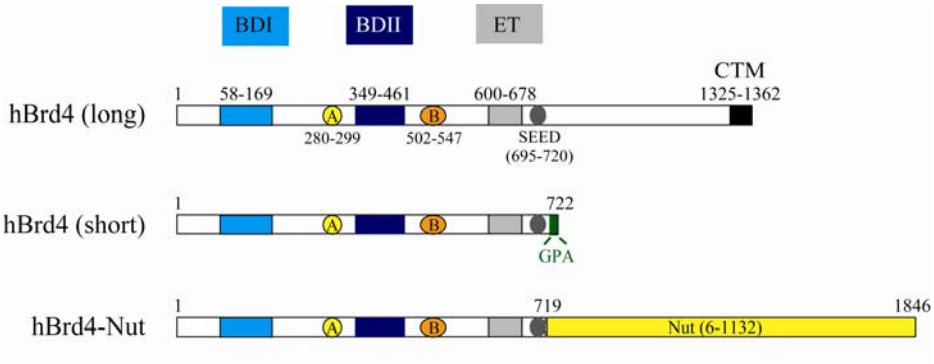

Figure 1-2. Isoforms of human Brd4 protein
Schematic representation of Brd4 isoforms. Numbers indicate the positions of amino acid residues or the boundaries of respective protein domains.

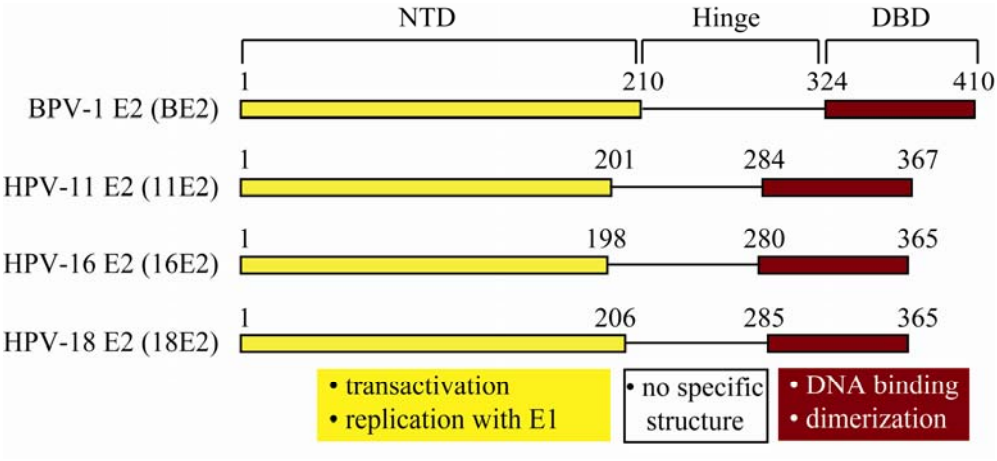

Figure 1-3. Papillomavirus-coding protein E2
The domain organization among different types of E2 proteins is well conserved. It consists of three parts: NTD (N-terminal domain), hinge region, and DBD (DNA-binding domain). Numbers indicate the positions of amino acid residues or the boundaries of respective protein domains.

CHAPTER II:

CHROMATIN ADAPTOR BRD4 MODULATES E2 TRANSCRIPTION ACTIVITY AND PROTEIN STABILITY

INTRODUCTION

E2 encoded by human papillomaviruses (HPVs) is a multifunctional protein regulating viral DNA replication, genome segregation, transcription, cell cycle control, and senescence (Wu et al, 2006). Its primary function relies on the sequence-specific recognition of a 12-nt palindrome, $ACCN_6GGT$, located at the upstream regulatory region (URR) of HPVs and animal papillomaviruses, such as bovine papillomavirus type 1 (BPV-1). The sequence context and the location of E2-binding sites (E2BSs) as well as the nature of E2 proteins all contribute to E2's activity in viral gene regulation. The transcriptional activity of E2 appears to be commonly shared by different types of E2 proteins encoded, for examples, by cervical cancer-inducing HPV type 16 (HPV-16) and 18 (HPV-18), genital wart-associated HPV type 11 (HPV-11), and BPV-1. In general, HPV-16 E2 (16E2) exhibits strongest transcriptional activity, followed by BPV-1 E2 (BE2), HPV-18 E2 (18E2), and HPV-11 E2 (11E2), and correlates well with their corresponding binding affinities to E2-BSs derived from the promoter-proximal regions of

naturally occurring HPV-11, HPV-16, and HPV-18 sequences (Hou et al, 2002). Since two of the four E2BSs in genital HPVs are flanked by an upstream Sp1-binding site and the downstream TATA box of the E6 early promoter, HPV E2 typically functions as a transcriptional repressor by excluding Sp1 and TFIID/TBP from binding to their cognate sequences and thus prevents the assembly of a transcriptional preinitiation complex (Demeret et al, 1997; Dong et al, 1994; Dostatni et al, 1991; Hou et al, 2000; Tan et al, 1994). HPV E2 also exhibits transactivation activity functioning in a heterologous promoter context where multimerized E2BSs are situated away from the TATA box (Hirochika et al, 1988), as seen in several natural BPV-1 promoter regions (Li et al, 1989; Thierry et al, 1990). Like many other cellular transcription factors, E2 has a dual role in gene activation and repression.

Recently, several groups have independently identified cellular bromodomain-containing protein 4 (Brd4) as an E2-interacting protein involved in viral genome segregation (Abbate et al, 2006; Dao et al, 2006; McPhillips et al, 2006; You et al, 2004) and transcriptional control (Ilves et al, 2006; McPhillips et al, 2006; Schweiger et al, 2006; Senechal et al, 2007; Wu & Chiang, 2007; Wu et al, 2006). While Brd4 and other cellular proteins, such as ChlR1 (Parish et al, 2006a), may serve as chromatin adaptors facilitating viral genome segregation during mitosis, Brd4 appears to play a more active role in cell cycle progression (Mochizuki et al, 2008) and cancer development (Crawford et al, 2008a; French et

al, 2008), largely through its ability to modulate gene transcription by recruiting different transcription components to selective target genes. The association of Brd4 with Mediator and positive transcription elongation factor b (P-TEFb) likely accounts for Brd4's coactivating function in Tat-independent stimulation of the human immunodeficiency virus type 1 (HIV-1) promoter (Jang et al, 2005; Wu & Chiang, 2007; Yang et al, 2005). Whether Brd4 is similarly implicated in transcriptional activation by HPV and BPV-1 E2 proteins is somewhat unclear, as only circumstantial evidence based primarily on the inhibition of E2-dependent reporter activity by overexpression of an E2-interacting domain containing the C-terminal 300 amino acids of Brd4 was provided (Ilves et al, 2006; McPhillips et al, 2006; Schweiger et al, 2006). Since, this C-terminal motif (CTM) of Brd4 is conserved among different species of Brd4 and some members of the BET family proteins, including mammalian Brdt and *Drosophila* Fsh (Wu & Chiang, 2007), inhibition of E2 transactivation by overexpression of a CTM peptide in the cell may be due to squelching of a CTM-interacting cellular protein needed for E2-dependent activation, not necessarily reflecting a true requirement for Brd4. Likewise, although the repressing activity of Brd4 has been convincingly demonstrated by *in vitro*-reconstituted HPV chromatin-dependent transcription and cell-based experiments with 11E2 (Wu et al, 2006), it remains undetermined whether the corepressor activity of Brd4 is common to E2 proteins encoded by cancer-inducing HPVs and animal papillomaviruses.

To define the molecular mechanism by which Brd4 enhances E2-dependent repression of the HPV promoter and to determine whether Brd4 indeed plays a dual role in E2-dependent activation and repression seemingly common to different types of E2 proteins, we performed chromatin and DNA footprinting analysis as well as functional complementation experiments by introducing wild-type or mutant Brd4 back to a stable Brd4-knockdown human cell line. We found that the presence of Brd4 significantly enhances site-specific recognition of E2 to both DNA and chromatin with a strict dependence on the CTM but only a selective requirement of Brd4 bromodomains for E2 binding to chromatin but not DNA. Moreover, Brd4 was shown to be a universal coactivator and corepressor for different types of E2 proteins. Interestingly, direct association of Brd4 with E2 significantly enhances the stability of the labile E2 protein, which is normally undetectable in the cell lysate. These activities of Brd4 collectively contribute to E2-regulated transcription and institute the notion that chromatin adaptors, such as Brd4, not only function as nucleosome-binding factors but also play an active role in recruiting sequence-specific transcription factors to their chromatin target sites, thereby modulating gene activity via multiple tiers of regulatory mechanisms.

MATERIAL AND METHODS

Plasmid constructions

The full-length human Brd4 (hBrd4) cDNA encoding amino acids 1-1362 was cloned into a baculovirus transfer vector by a two-step cloning process. First, the coding region for amino acids 1-1043 was amplified by PCR using pcDNA4C-Brd4-FL (You et al, 2004) with an *Nde*I site-containing upstream primer and a *Bam*HI site-containing downstream primer, and cloned into pF:TBP-11d (Chiang et al, 1993), after swapping with the TBP insert between *Nde*I and *Bam*HI sites, to generate pF:hBrd4(1-1043)-11d. The FLAG-tagged N-terminal 1043 amino acid-coding sequence was then isolated from pF:hBrd4(1-1043)-11d and subcloned into pVL1392 (Invitrogen) between *Xba*I and *Bam*HI sites to create pVL-F:hBrd4(1-1043). An *Eco*RI-*Not*I fragment encoding amino acids 645-1362 of hBrd4 was isolated from pcDNA4C-Brd4-FL and cloned into pVL1393 (Invitrogen) at the same enzyme-cutting sites to generate pVL-F:hBrd4(EcoRI-NotI), in which the 3' part of the coding region (amino acids 731-1362), along with the vector sequence, was isolated between *Sma*I and *Xho*I, and ligated to the *Sma*I-*Xho*I fragment of pVL-F:hBrd4(1-1043) containing the coding sequence for the FLAG-tagged N-terminal 731 amino acids of Brd4 to create pVL-F:hBrd4(FL). The mammalian expression plasmid, pcDNA3-F:hBrd4(FL), was generated by cloning the *Not*I-flanked FLAG-tagged hBrd4-coding sequence

into *Not*I-cleaved and calf intestine phosphatase (CIP)-treated pcDNA3 (Invitrogen).

The mammalian expression plasmid pcDNA3-F:hBrd4(1-1223) was constructed by replacing the coding sequence for amino acids 1057-1362 between *Bst*EII and *Xho*I sites with the coding region for 1057-1223, which was prepared by *Bst*EII and *Xho*I digestion of a DNA fragment amplified from pcDNA3-F:hBrd4(FL) with an upstream primer annealing to amino acid 1033 position and a downstream *Xho*I site-containing primer hybridizing to amino acid 1223 position. A *Bgl*II-*Xba*I fragment containing the coding sequence for FLAG-tagged hBrd4 amino acids 1-1223 was then isolated from pcDNA3-F:hBrd4(1-1223) and cloned into pVL1392 between *Bgl*II and *Xba*I sites to generate pVL-F:hBrd4(1-1223).

To generate domain-specific deletion mutants of hBrd4, an intermediate plasmid pF:hBrd4(1-722)-7 was first created by cloning the coding sequence for the N-terminal 722 amino acids of hBrd4, amplified from pcDNA4C-Brd4-FL with an *Nde*I site-containing upstream primer and a *Bam*HI site-containing downstream primer, into pFLAG(S)-7 (Chiang & Roeder, 1993) between *Nde*I and *Bam*HI sites. Inverse PCR was then employed to generate domain-specific deletions of BDI, BDII, and ET, respectively, in pF:hBrd4(1-722)-7 by using 5'-phosphorylated primer pairs flanking the deleted region for outward PCR amplification. The amplified products were then digested with *Dpn*I at 37^{o}C for

one hour to remove the parental template before self-ligation at 16^{o}C overnight. The resulting clones, after bacterial transformation and DNA sequencing, were named pF:hBrd4(1-722)ΔBDI-7, pF:hBrd4(1-722)ΔBDII-7, and pF:hBrd4(1-722)ΔET-7, respectively. The ΔBDI/II deletion plasmid, pF:hBrd4(1-722)ΔBDI/II-7, was similarly constructed by inverse PCR using pF:hBrd4(1-722)ΔBDI-7 as template with 5'-phosphorylated primer pairs flanking the BDII region.

To generate pVL-F:hBrd4ΔBDI, pVL-F:hBrd4ΔBDII, and pVL-F:hBrd4ΔBDI/II for protein expression and purification in insect cells, the *Bgl*II-*Eco*RI fragment containing the coding region for FLAG-tagged N-terminal 645 amino acids of hBrd4 with the deleted bromodomain(s) was released from pF:hBrd4(1-722)ΔBDI-7, pF:hBrd4(1-722)ΔBDII-7, and pF:hBrd4(1-722)ΔBDI/II-7, respectively, and cloned into *Bam*HI/*Not*I-cleaved pVL1393, along with the *Eco*RI-*Not*I fragment containing the coding region for amino acids 645-1362 prepared from pVL-F:hBrd4(FL), for three-piece ligation. Plasmids pcDNA3-F:hBrd4ΔBDI, pcDNA3-F:hBrd4ΔBDII, and pcDNA3-F:hBrd4ΔBDI/II were similarly constructed by three-piece ligation, except using *Bam*HI/*Not*I-linearized pcDNA3.

To construct pVL-F:hBrd4ΔET, a *Sac*I-cleaved fragment spanning the coding region for amino acids 514-706 with the deleted ET domain was first used to replace the corresponding wild-type sequence in pF:hBrd4(1-1043)-11d to

generate pF:hBrd4(1-1043)ΔET-11d, in which the *Xba*I-*Sma*I fragment containing the coding sequence for FLAG-tagged N-terminal 731 amino acids with the deleted ET domain was used to replace the corresponding wild-type sequence in pVL-F:hBrd4(FL). The plasmid pcDNA3-F:hBrd4ΔET was then created by subcloning the FLAG-tagged hBrd4ΔET insert from pVL-F:hBrd4ΔET into pcDNA3 between *Eco*RI and *Not*I sites.

Baculovirus expression plasmids pVL-F:BE2, pVL-F:16E2, and pVL-F:18E2 were constructed by cloning the FLAG-tagged E2 insert, amplified by PCR respectively from pF:BE2-11d, pF:16E2-11d, and pF:18E2-11d (Hou et al, 2002) with a *Not*I site-containing upstream primer (5'-AGAATTCGCGGCCGC CATGGACTACAAAGACGT-3') and a *Bam*HI site-containing downstream primer, into pVL1392 between *Not*I and *Bam*HI sites. Mammalian expression plasmids pCMV-F:11E2, pCMV-F:16E2, and pCMV-F:18E2 were similarly constructed by cloning the FLAG-tagged 11E2, 16E2, and 18E2 cDNA, amplified from pF:11E2-11d (Hou et al, 2002), pF:16E2-11d, and pF:18E2-11d, respectively, into *Not*I/*Bam*HI-linearized pFLAG-CMV-2 (Sigma). The plasmid pCMV-F:BE2 was generated by first isolating the FLAG-tagged BE2 insert from pF:BE2-11d between *Bgl*II and *Eco*RI sites and then cloned into *Bam*HI/*Eco*RI-linearized and CIP-treated pcDNA3. The plasmid pVL-F°:E2, used for 11E2 expression and purification, has been described (Wu et al, 1999).

Protein purification

FLAG-tagged full-length (FL) Brd4 and domain-specific deletion mutants, ΔBDI, ΔBDII, ΔBDI/II, ΔET, and 1-1223, were purified from insect Sf9 cells infected with baculoviruses harboring pVL-F:hBrd4(FL), pVL-F:hBrd4ΔBDI, pVL-F:hBrd4ΔBDII, pVL-F:hBrd4ΔBDI/II, pVL-F:hBrd4ΔET, and pVL-F:hBrd4(1-1223), respectively, following the published protocol (Wu et al, 1999). FLAG-tagged 11E2, 16E2, 18E2, and BE2 were similarly purified from Sf9 cells infected with recombinant baculoviruses carrying pVL-F°:E2, pVL-F:16E2, pVL-F:18E2, and pVL-F:BE2, respectively. Purification of recombinant human AP-1 (Wang et al, 2008), p300, hNAP-1, *Drosophila* ACF, and HeLa core histones (Thomas & Chiang, 2005) has been described.

DNase I footprinting with chromatin and DNA templates

HPV chromatin was assembled *in vitro* using p7072-70GLess/I^+ DNA (Hou et al, 2000) with recombinant hNAP-1, *Drosophila* ACF, and HeLa core histones according to the published protocol (Wu et al, 2006). For DNase I footprinting, a 30-μl reaction containing 2 μl of HPV chromatin or mock-assembled DNA (i.e., without HeLa core histones, hNAP-1 and ACF), with or without 50 ng of hBrd4 and a different amount (10, 30, 100, or 300 ng) of 11E2, was incubated in transcription buffer (Wu et al, 2003) at 30^{o}C for 30 min. The incubated chromatin or DNA (12.5 μl) was digested with 0.25 U (chromatin) or

0.025 U (DNA) of DNase I (Invitrogen) at room temperature for 2 min. The reaction was terminated by adding 15 µl of DNase I stop solution containing 200 mM Tris-HCl (pH 7.5), 50 mM EDTA, 2% SDS, 200 µg/ml of proteinase K, and 250 µg/ml of glycogen, and then treated with 20 µg of proteinase K at 60°C for 30 min, followed by phenol/chloroform extraction. The soluble portion was precipitated with 200 µl of 5 M ammonium acetate, 2 µl of glycogen (10 mg/ml), and 1 ml of 100% ethanol at -20ºC overnight. The precipitated DNA was washed with 70% ethanol and air-dried. The pellet was resuspended in 10 µl of H_2O. The resulting DNA was then amplified by asymmetric PCR at 95ºC for 1 min, 68ºC for 2 min, and 76ºC for 2 min for 2 cycles in a 25-µl reaction containing 10 µl of DNA, 0.4 U of Vent DNA Polymerase (New England Biolabs), 0.1 pmole of the ^{32}P-labeled Gless(AS)-31/55 primer (Wu et al, 2006), 0.2 M dNTP, 40 mM NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$, 0.01% gelatin (Sigma), and 0.1% Triton X-100. The PCR products were mixed with 125 µl of stop solution containing 10 mM Tris-HCl (pH 7.5), 4 mM EDTA, 260 mM sodium acetate (pH 5.2), and 20 µg of tRNA, and then precipitated with 700 µl of 100% ethanol on dry ice for 10 min. The DNA fragments were spun, dried, resuspended in 6 µl of formamide dye (90% formamide, 1x TBE, and 0.02% bromophenol blue/xylene cyanol), and analyzed in a 6% polyacrylamide/7 M urea gel at 75 W for 2 hr. The gel was transferred onto a chromatography paper (Fisher), dried, and exposed in a

PhosphorImager screen. Signals were detected by Typhoon 9200 PhosphorImager (GE Healthcare).

In vitro transcription assay

HPV chromatin, assembled as described above, was used for order-of-addition transcription experiments according to the published scheme (Wu et al, 2006) with minor modifications. Briefly, a 30-µl reaction containing 6 µl of *in vitro*-assembled chromatin, 30 µM acetyl-CoA, and 20 ng of p300 was incubated, in the absence or presence of 40 ng of AP-1, with 30 ng of hBrd4 and 1-15 ng of E2, unless otherwise indicated, in transcription buffer at 30^{o}C for 30 min. Eight µl of HeLa nuclear extract (8-10 mg/ml) and 10 ng of pMLΔ53 DNA were then added and incubated at 30^{o}C for another 30 min. Transcription was initiated by adding [α-^{32}P]CTP and NTP mix, and incubated for another hour. The reactions were terminated and processed as described previously (Wu et al, 2006). Relative transcription (Rel Txn) in each set of reactions was defined as the signal intensity quantified by PhosphorImager from the HPV chromatin relative to that performed in the presence of AP-1 without E2 and Brd4. Fold change in each set of experiments represents the level of Rel Txn relative to that obtained in the absence of E2.

Luciferase reporter gene assay

Human cervical cancer-derived C-33A cells containing a stably integrated Brd4 shRNA-expressing cassette (named #1-13, see Wu et al, 2006) were maintained in DMEM plus 10% fetal bovine serum. For E2 repression assays, #1-13 cells (~80% confluency) grown in 24-well were transfected with 100 ng of pGL7072-161 (Hou et al, 2000), 50 ng of an E2 expression plasmid (pCMV-F:BE2, pCMV-F:11E2, pCMV-F:16E2, or pCMV-F:18E2), and 0.5 μg of pcDNA3-F:hBrd4(FL), pcDNA3-F:hBrd4ΔBDI/II, pcDNA3-F:hBrd4ΔBDI, pcDNA3-F:hBrd4ΔBDII, pcDNA3-F:hBrd4ΔET, or pcDNA3-F:hBrd4(1-1223) using Fugene 6 (Roche) according to the manufacturer's protocol. Four hours later, media was replaced with fresh media containing 5 mM sodium butyrate and incubated for another 20 hours. Cells were then harvested in 1x reporter lysis buffer (Promega) and mixed with assay buffer containing D-Luciferin (Pharmingen). Luciferase activity was measured with the POLARstar OPTIMA plate reader (BMG Labtechnologies). E2 activation assays were similarly performed as described above, except that 100 ng of the p2x2xE2BS-luc reporter (Kovelman et al, 1996) and 10 ng of an E2 expression plasmid were used. Sodium butyrate (5 mM) was added 24 hours posttransfection. After another 24 hours, cells were harvested for luciferase assays. Fold repression or fold activation in each set of reactions was defined as the luciferase activity relative to that performed without Brd4 expression.

Protein detection in transiently transfected cells

For experiments described in Figure 2-7C, #1-13 cells (~80% confluency) grown in one 100-mm plate were transfected with 8 μg of a Brd4 expression plasmid and 1.5 μg of an E2 expression plasmid, both driven by the CMV promoter, using Fugene 6. Four hours later, sodium butyrate was added to final 5 mM. After incubation for another 20 hours, cells were lysed in 200 μl of modified RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 300 mM NaCl, 1 mM EDTA, and 10% glycerol). The lysates were cleared, following sonication and centrifugation at 4^{o}C, 13,000 rpm for 30 min, and adjusted to 150 mM salt by mixing with an equal volume of modified RIPA buffer without NaCl. Ten μl (bed volume) of anti-FLAG M2-agarose beads (Sigma) were then added for an overnight incubation with rotation at 4^{o}C. The immunoprecipitated E2 and Brd4 were analyzed by Western blotting with anti-FLAG M2 monoclonal antibody (Sigma).

For experiments described in Figure 2-9, #1-13 cells grown in a 150-mm plate at 60% confluency were transfected with 4 μg of pCMV-F:11E2 or pCMV-F:16E2, in the absence or presence of 12 μg of a wild-type or mutant Brd4 expression plasmid, using Fugene 6. Forty-two hours posttransfection, cells were either left untreated or treated with DMSO or 40 μM MG132 for 6 hours, and then lysed in 200 μl of RIPA buffer and analyzed by Western blotting with anti-FLAG M2 monoclonal antibody or with anti-β-tubulin antibodies (Santa Cruz).

Generation of DNA sequencing ladder for footprinting marker

Footprinting marker was prepared using Thermo sequenase dye primer manual cycle sequencing kit (USB) following the manufacturer's protocol with some modifications. Briefly, 72 µl of the master mix containing 2 pmole of the ^{32}P-labeled Gless(AS)-31/55 primer, 8.8 µl of RCTN buffer (from kit), 1 µg of p7072-70GLess/I^{+} DNA, and 80 U of thermo sequenase was prepared. 16 µl of reaction was aliquoted into the tubes with 4 µl of each ddNTP (ddATP, ddGTP, ddCTP, or ddTTP) termination mix and was amplified with 30 cycles of following parameters: 95^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 60 seconds. The reaction was stopped by the addition of 100 µl of formamide loading dye.

RESULTS

A bromodomain and the CTM are both crucial for Brd4-enhanced E2 binding to chromatin

To explore our working hypothesis that chromatin adaptor Brd4 binding to acetylated histone H3 and H4 helps stabilize E2 binding to its cognate sequences in chromatin (Wu & Chiang, 2007), DNase I footprinting was performed with HPV-11 chromatin containing the HPV-11 URR spanning nucleotides 7072-7933/1-70 which includes four E2BSs and the TATA box of the E6 promoter linked to a 377-nt G-less cassette (Figure 2-1A). The chromatin template, prepared by incubating the HPV-11 DNA with purified HeLa core histones (C.H.), human NAP-1 (hNAP-1) histone chaperone, and *Drosophila* ACF chromatin assembly/remodeling factor (Figure 2-1B and C), faithfully reproduces the positions of nucleosomes assembled on the HPV genome typically seen *in vivo* (Wu et al, 2006). Formation of regularly spaced nucleosomes on *in vitro*-assembled chromatin was confirmed by micrococcal nuclease (MNase) digestion (Figure 2-1D). The chromatin was digested with DNase I after incubation with 11E2 and full-length (FL) human Brd4 (hBrd4), and the purified DNA product was amplified with isotope-labeled PCR and was visualized by autoradiography (Figure 2-1E). As shown in Figure 2-1F, purified 11E2 alone failed to bind stably to HPV-11 chromatin, as no protection of E2BSs was observed (lanes 2-4). Only

under the condition with the highest amount of E2 was weak binding to E2BSs observed (lane 5). However, in the presence of Brd4, which did not bind stably to chromatin by itself (lane 6), enhanced protection of #2, #3 and #4 E2BSs were readily detected on HPV chromatin (lanes 3-5 vs. lanes 7-9), indicating that Brd4 indeed enhances E2 binding to its cognate sequences in chromatin.

To define which domains of Brd4 are involved in this enhancement, we purified several FLAG-tagged domain-specific deletion mutants of Brd4 from insect cells using a baculovirus expression system. Bromodomains I and II, implicated in binding to acetylated lysine residues, were removed individually or together, along with the deletion of an evolutionarily conserved extraterminal (ET) domain, whose function remains undefined, or the E2-interacting CTM was removed (Figure 2-2A). The FLAG epitope-coding sequence was introduced at the N-terminus of the respective constructs to facilitate protein purification via one-step immunoaffinity purification. Each protein was successfully purified from baculovirus-infected insect Sf9 cells with a small amount of degradation products also detected, inevitably, due to the large size of the expressed protein (Figure 2-2B). Compared to wild-type Brd4, deletion of one bromodomain (ΔBDI or ΔBDII) did not prevent E2 binding to E2BSs (Figure 2-3, lanes 8, 9, 11, 12). However, removing both bromodomains significantly diminished Brd4-enhanced E2 protection (lanes 14 and 15), consistent with *in vivo* chromatin immunoprecipitation (ChIP) data showing that at least one bromodomain is

essential for Brd4 binding to chromatin (Wu et al, 2006). Deletion of the ET domain did not have any effect on E2 footprinting (lanes 17 and 18), indicating that the ET domain is not necessary for Brd4 enhancement of E2 binding to chromatin. Not surprisingly, interaction with E2 is crucial for Brd4 function, since removal of the CTM dramatically abolishes Brd4-enhanced E2 footprinting on HPV chromatin (lanes 20 and 21). It is noted that the hypersensitive sites induced by E2 binding to #3 and #4 E2BSs were not completely eliminated by ΔBDI/II (lanes 14 and 15, asterisk). Perhaps this is caused by Brd4-induced conformational changes via the CTM's interaction with E2 that somehow unmasks the DNA-binding domain of E2 (see below), thereby slightly enhancing E2's cooperativity for binding to juxtaposed E2BSs.

The CTM, but not bromodomains, is essential for Brd4-stabilized E2 binding to its cognate sequences in DNA

To examine whether Brd4 enhancement of E2 binding is unique to the chromatin template, we performed a similar footprinting analysis with the same DNA template without assembled nucleosomes. As shown in Figure 2-4, E2 alone exhibited sequence-specific binding to DNA only at higher concentrations (lanes 1-5), indicating an inherently weak DNA-binding activity of E2. Nevertheless, E2 binds more efficiently to DNA rather than chromatin, as a clear protection of E2BSs could be detected by 100 ng of E2 with the DNA but not

with the chromatin template (compare Figure 2-4, lane 4 with Figure 2-3, lane 3). Somewhat surprisingly, we also observed enhanced E2 footprinting in the presence of Brd4 with the DNA template (lanes 8 vs. 3), suggesting that Brd4-enhanced E2 binding to its cognate sequences is not unique to the chromatin environment. Perhaps, Brd4 association with the N-terminal domain of E2 induces a conformational change that allosterically enhances the C-terminal DNA-binding activity of E2, consistent with the observation that the N-terminal domain of 18E2 negatively regulates its C-terminal DNA-binding activity (Demeret et al, 1997). Since Brd4 could stimulate site-specific binding of E2 to DNA, we further analyzed the domain requirement of Brd4 for E2 binding. Interestingly, we found that while the E2-interacting CTM was absolutely essential for this stimulation (lanes 22 and 23), the bromodomains were not needed at all for Brd4-enhanced E2 binding to DNA (lanes 11, 14, and 17). This suggests that bromodomains are indeed evolved for chromatin targeting by Brd4. As observed with the chromatin template, the ET domain was also dispensable for this protein-DNA interaction (lane 20).

Brd4 promotes E2-mediated repression of HPV chromatin transcription by different types of E2 proteins

The corepressor function of Brd4 was illustrated previously with 11E2 encoded by a benign HPV that causes genital warts (Wu et al, 2006). It is

important to demonstrate whether this corepressor activity of Brd4 could likewise enhance transrepression of E2 encoded by oncogenic HPVs, such as 16E2 and 18E2, as well as by animal papillomaviruses, particularly BE2. To this end, we conducted *in vitro* transcription using an AP-1-dependent chromatin transcription system in which recombinant dimeric c-Jun/c-Fos was first incubated with HPV-11 chromatin, along with p300 histone acetyltransferase and acetyl-CoA, with or without E2 and Brd4 (Figure 2-5A-C). HeLa nuclear extract, which provides all the cellular components necessary for chromatin-dependent transcription, and a DNA control template containing the adenovirus major late core promoter (pMLΔ53) were then included, followed by the addition of [α-^{32}P]CTP and other ribonucleoside triphosphates to initiate transcription (Figure 2-5A and C). Transcripts of approximately 380 and 280 nucleotides derived respectively from HPV chromatin and the internal control template were separated by polyacrylamide gel electrophoresis and quantified by PhosphorImager. As shown in Figure 2-5D, dose-dependent repression of AP-1-stimulated HPV chromatin transcription by BE2 (lanes 3-5 vs. lane 2), 16E2 (lanes 12-14 vs. lane 11) and 18E2 (lanes 21-23 vs. lane 20) was further enhanced by the presence of Brd4 (compare lanes 7-9 and 3-5, lanes 16-18 and 12-14, and lanes 25-27 and 21-23). This chromatin transcription experiment had been repeated several times with similar results. A line graph averaged from two representative experiments

showing Brd4-enhanced repression of AP-1-dependent chromatin transcription by BE2, 16E2 and 18E2 was presented in Figure 2-5E.

To define whether the requirement of a bromodomain and the E2-interacting CTM for enhanced E2 binding to chromatin reflects a functional role of Brd4 for E2-mediated inhibition of HPV chromatin transcription, we analyzed by *in vitro* chromatin transcription experiments different domain-specific deletion mutants of Brd4. As shown in Figure 2-6, Brd4 mutants deficient in both bromodomains (lanes 14-16) or the CTM (lanes 20-22) failed to support E2-mediated inhibition of HPV chromatin transcription (lane 7), whereas removal of the ET domain (lanes 17-19) or only one bromodomain (lanes 8-10 and lanes 11-13) had no effect on E2 repressing activity, thus providing a functional verification of the involvement of the bromodomain and the CTM for E2 targeting to HPV chromatin as revealed by the chromatin footprinting assay (see Figure 2-3). A line graph averaged from two representative experiments was also presented in Figure 2-6B.

A bromodomain and the CTM are both essential for Brd4-enhanced repression by different types of E2 proteins in living cells

Since Brd4 could enhance E2-mediated repression by different types of E2 proteins in chromatin-dependent transcription assays *in vitro*, we further examined whether the corepressor function of Brd4 could be detected *in vivo* and

whether the bromodomain and the E2-interacting CTM of Brd4 are generally required for transrepression by different types of E2 proteins. To address this, we conducted an E2 repression assay by transfecting an HPV-11 URR-driven luciferase reporter (Figure 2-7A), together with an expression plasmid for different types of E2 and either wild-type or a domain-specific deletion mutant of Brd4, into a human cervical carcinoma-derived C-33A cell line (#1-13) that harbors a stably integrated Brd4 shRNA-expressing cassette. An approximate 50-fold reduction of Brd4 protein was observed in #1-13 cells (Figure 2-7B, lanes 1 and 2), in which the reduced level of Brd4 could be efficiently restored by exogenous Brd4 expression to a level slightly (~70%) higher than the endogenous protein (lanes 1 vs. 3). Under this experimental condition, we compared different mutants of Brd4 with the wild-type protein for their ability to support E2-mediated inhibition of E6 promoter activity. As shown in Figure 2-7C, an effective inhibition of E6 promoter activity by BE2, 11E2, 16E2, and 18E2 was observed when full-length, ΔET, ΔBDI or ΔBDII, but not ΔBDI/II and CTM-deleted, Brd4 was cotransfected into #1-13 cells. This result indicates that Brd4 is indeed a general corepressor for different types of E2 proteins and, furthermore, a bromodomain and the CTM are both crucial for Brd4-mediated E2 repression of HPV E6 promoter activity. A slightly reduced repression was reproducibly seen with ΔBDI, ΔBDII, and ΔET for unknown reasons. When the levels of different Brd4 and E2 proteins were monitored in transfected cells, we found, while the

amounts of various Brd4 mutants and E2 proteins remained mostly comparable in these assays, the expression levels of different types of E2 proteins appeared to be reduced when Brd4(1-1223) was coexpressed with E2 in the Brd4-knockdown cells (Figure 2-7C, lanes 6, 12, 18, and 24). Clearly, the bromodomain and the CTM of Brd4 are both important for E2-inhibited HPV transcription, even though the protein stability of E2 is also influenced by its ability to associate with the CTM of Brd4 (see below).

Brd4 also functions as a transcriptional coactivator for different types of E2 proteins with the same bromodomain and CTM requirements as in transrepression

Since most of the transcription cofactors display a dual role in gene activation and repression (Thomas & Chiang, 2006) and circumstantial evidence has implicated Brd4 in E2 transactivation (see Introduction), we were prompted to explore the coactivator function of Brd4 in E2-mediated activation. With the experimental system described above for the study of E2 transrepression, we performed a similar complementation experiment in Brd4-knockdown C-33A cells using a reporter construct containing multimerized E2BSs that are situated at the promoter-distal region (Figure 2-8A). This enhancer configuration of E2BSs allows BE2, 11E2, 16E2, and 18E2 to activate reporter gene activity, again in a bromodomain- and CTM-dependent manner (Figure 2-8B). Clearly, Brd4 is

capable of stimulating E2-depenent activation by different types of E2 proteins and this coactivating activity of Brd4 also depends on its ability to interact with E2 via the CTM and its ability to target chromatin via one of its bromodomains.

Brd4 stabilizes E2 protein via direct protein-protein interaction

Since E2 is a labile protein with an estimated half-life around 1 hour and appears to be ubiquitinated at its N-terminal domain (Bellanger et al, 2001; Penrose et al, 2004), we wondered whether Brd4 association with the N-terminal domain of E2 may stabilize E2 protein by preventing proteasome-mediated degradation and thus accounting for the reduction of E2 in CTM-deleted cotransfection experiments (Figure 2-7C). To explore this, we analyzed the expression levels of low-risk 11E2 and high-risk 16E2 in Brd4-knockdown C-33A cells, with or without coexpressed wild-type or CTM-deleted Brd4. As shown in Figure 2-9A, 11E2 and 16E2 were undetectable in Brd4-knockdown cells (lanes 2 and 6). Interestingly, coexpression of wild-type Brd4 stabilized E2 expression to a level comparable to the addition of the proteasome inhibitor MG132 (Figure 2-9A, compare lanes 2-4 with lane 10, and lanes 6-8 with lane 12). Deletion of the CTM failed to provide the stabilizing effect on 11E2 and 16E2 (Figure 2-9A, compare lanes 4 and 5, and lanes 8 and 9), suggesting that direct protein-protein interaction is essential for Brd4 to stabilize E2 protein in the cell. Additional transfection of an HPV-11 URR-driven reporter plasmid or other

DNA templates together with E2 and Brd4 gave the same results as shown in Figure 2-9A (data not shown), indicating that the Brd4-stabilized E2 effect is not due to conformational changes induced by E2 binding to DNA. This E2-stabilizing effect is unique to the CTM, as coexpression of other domain-specific deletion mutants of Brd4, which all retain the ability to interact with E2, failed to destabilize 11E2 (Figure 2-9B) or 16E2 (Figure 2-9C). We thus conclude that direct association with Brd4 is critical for E2 stabilization in the cell.

DISCUSSION

In this report, we dissected the mechanisms by which chromatin adaptor Brd4 regulates E2-dependent transcription using *in vitro*-reconstituted chromatin and DNA templates for DNase I footprinting analysis and also carrying out chromatin-dependent transcription as well as functional complementation assays with wild-type and domain-specific deletion mutants of Brd4 in stable Brd4-knockdown human C-33A cells. We uncovered several important aspects of Brd4 functions. First, Brd4 enhances E2 binding to both chromatin and DNA templates in a site-specific and E2-dependent manner. Without E2 association, Brd4 binds chromatin/DNA in a transient and more dynamic manner, indicating that cooperative interactions between a sequence-specific transcription factor and a chromatin adaptor are indeed critical for Brd4-regulated transcription as predicted in our previous model (Wu & Chiang, 2007). Second, while bromodomains are crucial for Brd4-enhanced E2 binding to chromatin, they (i.e., BDI and BDII) are dispensable for Brd4-facilitated E2 binding to DNA. This finding suggests that BDI and BDII are evolved for chromatin targeting and the enhanced sequence-specific binding of E2 is potentially caused by Brd4-induced conformational changes, perhaps via its interaction with the N-terminal domain of E2 that allosterically regulates E2 C-terminal DNA-binding activity. Third, Brd4 is an authentic transcription cofactor playing a dual role in E2-mediated activation and

repression, depending on the sequence context of E2BSs, similar to the regulatory properties of general transcription cofactors, such as TFIID, Mediator, p300, and USA-derived positive and negative cofactors (Thomas & Chiang, 2006). Fourth, Brd4 serves as a universal transcription cofactor for different types of E2 proteins encoded by high-risk and low-risk HPVs as well as by animal papillomaviruses, such as BPV-1. Fifth, Brd4 is an important cellular factor stabilizing E2 proteins in the cell, as the normally undetectable level of E2 proteins observed in Brd4-knockdown cells could only be stabilized by wild-type Brd4, but not by a Brd4 mutant unable to interact with E2. It is noted that the growth rate of our human Brd4-knockdown cells slows down with a generation time nearly doubling depending on the extent of knockdown during passage. This is consistent with a recent report indicating that murine Brd4 is critical for G1 progression in both mouse NIH3T3 cells and mouse embryonic fibroblasts (Mochizuki et al, 2008).

The ability of Brd4 to stabilize E2 proteins in the cell provides an additional tier of regulation for E2-dependent activation and E2-dependent repression (Figure 2-10). Under normal circumstances, the effect of Brd4-stabilized E2 may not be observed due to the high amount of endogenous Brd4 present in regular cells. The use of Brd4-knockdown cells, in combination with the functional rescue by exogenously expressed Brd4 proteins, allows us to uncover the role of Brd4 in E2 stabilization. Conceivably, the protein half-life of 18E2 and BE2, previously estimated each to be around 1 hour and degraded

through the ubiquitin-dependent proteasome pathway, will be influenced by the levels of Brd4 in the cell, as no E2 proteins are detectable in Brd4-knockdown cells (Figure 2-9A). It is likely that Brd4 stabilizes E2 by blocking ubiquitination at the E2 N-terminus, preventing proteasome binding, or inhibiting phosphorylation-regulated E2 stability occurring through the hinge region. This is an area requiring further investigation in the future.

It is also important to mention that, although protein stability may partly account for reduced E2 activation and repression by the CTM-deleted Brd4 mutant (see Figures 2-7 and 2-8), chromatin targeting mediated by at least one of the Brd4 bromodomains is essential for both E2-dependent transcription and E2 promoter occupancy (Wu et al, 2006). The dependence of the CTM for E2 transcription activity was observed in low amounts of E2 expression, in which E2 instability was readily detected (Figure 2-7), and also in high amounts of E2 expression, when the levels of E2 remained undiminished with coexpressed CTM-deleted Brd4 (Wu et al, 2006). Thus, the reduced repression observed in cotransfected CTM-deleted Brd4 could not be entirely attributed to a declined level of E2 expression in Brd4-knockdown cells. Evidently, multiple mechanisms are employed by Brd4 to regulate E2-dependent transcription, including promoter-specific chromatin targeting, enhanced E2 binding to chromatin and DNA, and protein stabilization. Another point to note is that the effect of Brd4 on E2 transactivation and transrepression is not unique to viral transcription factors,

as Brd4 is able to interact with multiple cellular transcription components involved in gene regulation, such as P-TEFb and Mediator (Wu & Chiang, 2007). A recent report identifying P-TEFb as another CTM-binding factor (Bisgrove et al, 2007), besides papillomaviral E2 proteins, raises a concern regarding data interpretation based mainly on the use of CTM-containing domains for deducing the function of Brd4 in E2-regulated biological processes. The multifunctional effects of Brd4 have undoubtedly presented a challenge for properly deciphering the molecular mechanisms underlying E2-regulated transcription.

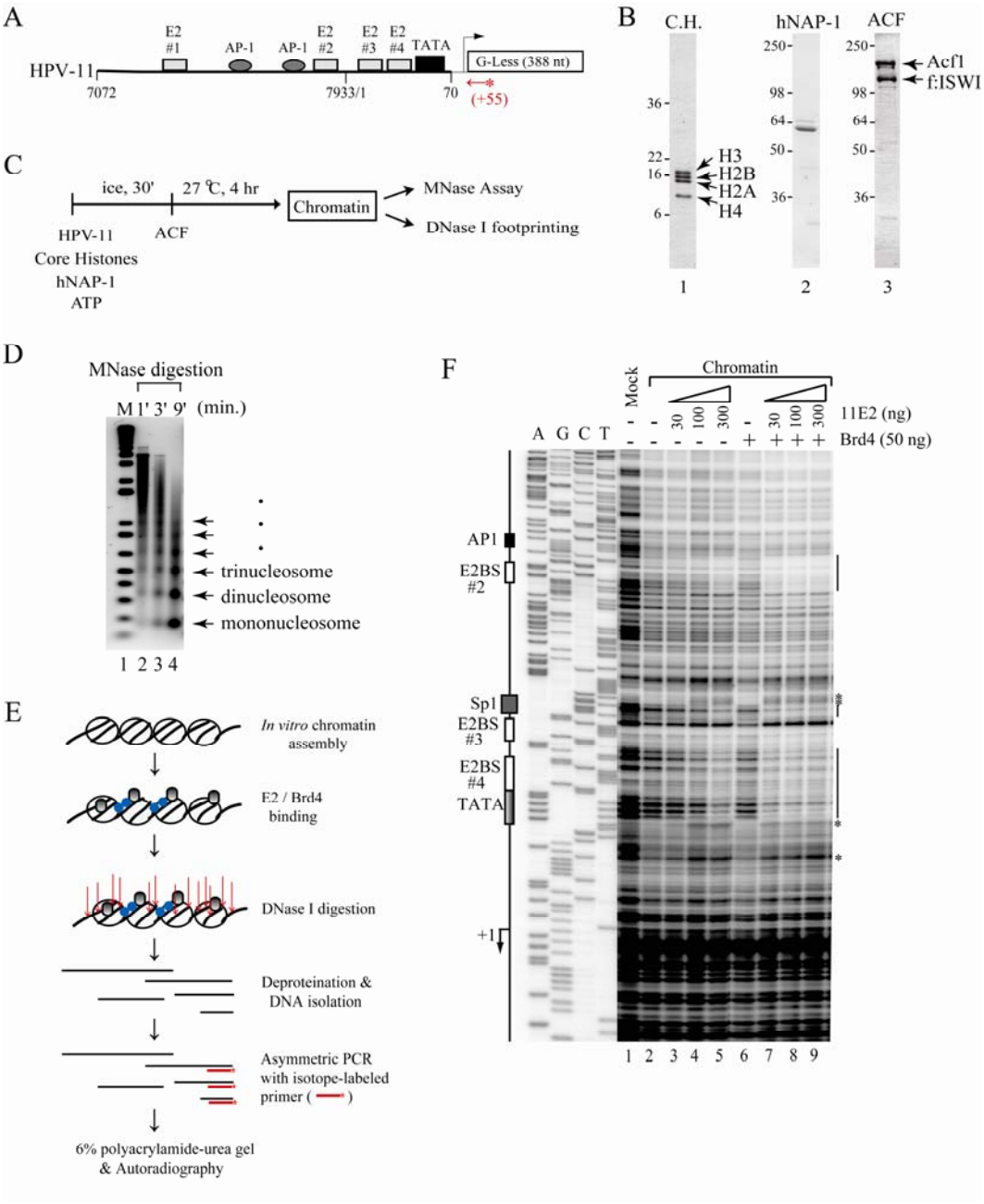
A
E2 #1
AP-1
AP-1
E2 #2
E2 #3
E2 #4
TATA
HPV-11
G-Less (388 nt)
7072
7933/1
70
(+55)
B
C.H.
hNAP-1
ACF
Acf1
f:ISWI
H3
H2B
H2A
H4
1
2
3
C
ice, 30'
27 ℃, 4 hr
Chromatin
MNase Assay
DNase I footprinting
HPV-11
Core Histones
hNAP-1
ATP
ACF
D
MNase digestion
M 1' 3' 9' (min.)
trinucleosome
dinucleosome
mononucleosome
1 2 3 4
E
In vitro chromatin assembly
E2 / Brd4 binding
DNase I digestion
Deproteination & DNA isolation
Asymmetric PCR with isotope-labeled primer
6% polyacrylamide-urea gel & Autoradiography
F
Mock
Chromatin
11E2 (ng)
Brd4 (50 ng)
A G C T
AP1
E2BS #2
Sp1
E2BS #3
E2BS #4
TATA
+1
1 2 3 4 5 6 7 8 9

Figure 2-1. Brd4 enhances E2 binding to its cognate sequence in chromatin (**A**) Schematic representation of the HPV-11 promoter-linked G-less cassette used for chromatin assembly and DNase I footprinting. Numbers below the line indicate the positions of nucleotides in the HPV-11 genome. The transcription start site is marked as a black arrow. The location for primer annealing for asymmetric PCR is indicated in red. (**B**) Purified core histones (C.H.), hNAP-1, and ACF used for chromatin assembly. (**C**) Outline of *in vitro* chromatin assembly for MNase digestion and DNase I footprinting. (**D**) Formation of regularly spaced nucleosomes in *in vitro* reconstituted HPV-11 chromatin analyzed by MNase digestion at different incubation times as indicated. A 1 kb plus ladder (Invitrogen) was used as a size marker (M). (**E**) Flow chart for DNase I footprinting. Experimental details are described under Materials and Methods. (**F**) *In vitro*-reconstituted HPV-11 chromatin was incubated without (-) or with (+) increasing amounts of HPV-11 E2 (11E2) and FL Brd4 protein as indicated. Mock assembly (without proteins) was performed with naked DNA. Chromatin/Mock footprinting was conducted with PCR-amplified and labeled DNA fragments separated by denaturing polyacrylamide/urea gel electrophoresis. PCR products werevisualized following PhosphorImager analysis. Positions of protein factor-binding sites and the transcription start site (+1) are indicated on the left. DNA sequencing ladders were prepared with the same template and primer used for footprinting using Thermo sequenase dye primer manual cycle sequencing kit (USB). The protected regions and hypersensitive sites (*) are marked on the right.

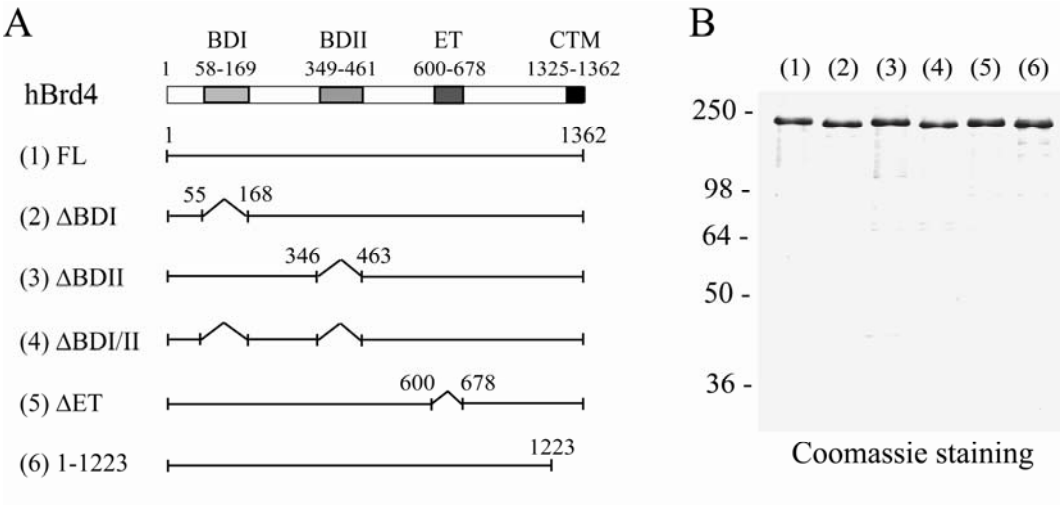

Figure 2-2. Construction and purification of wild-type and mutant human Brd4

(**A**) Schematic representation of protein domains present in wild-type and mutant Brd4 proteins. Numbers indicate the positions of amino acid residues for the boundaries of respective protein domains, including bromodomain I (BDI), bromodomain II (BDII), extraterminal (ET) domain, and the C-terminal motif (CTM). Each protein has an N-terminal FLAG-tag sequence to facilitate protein purification. (**B**) Coomassie blue staining of purified human Brd4 proteins. Recombinant FLAG-tagged full-length (FL) Brd4 and its internally truncated (Δ) or C-terminally deleted mutants were purified from baculovirus-infected insect cells as described under Materials and Methods. Protein size markers (in kDa) are indicated on the left.

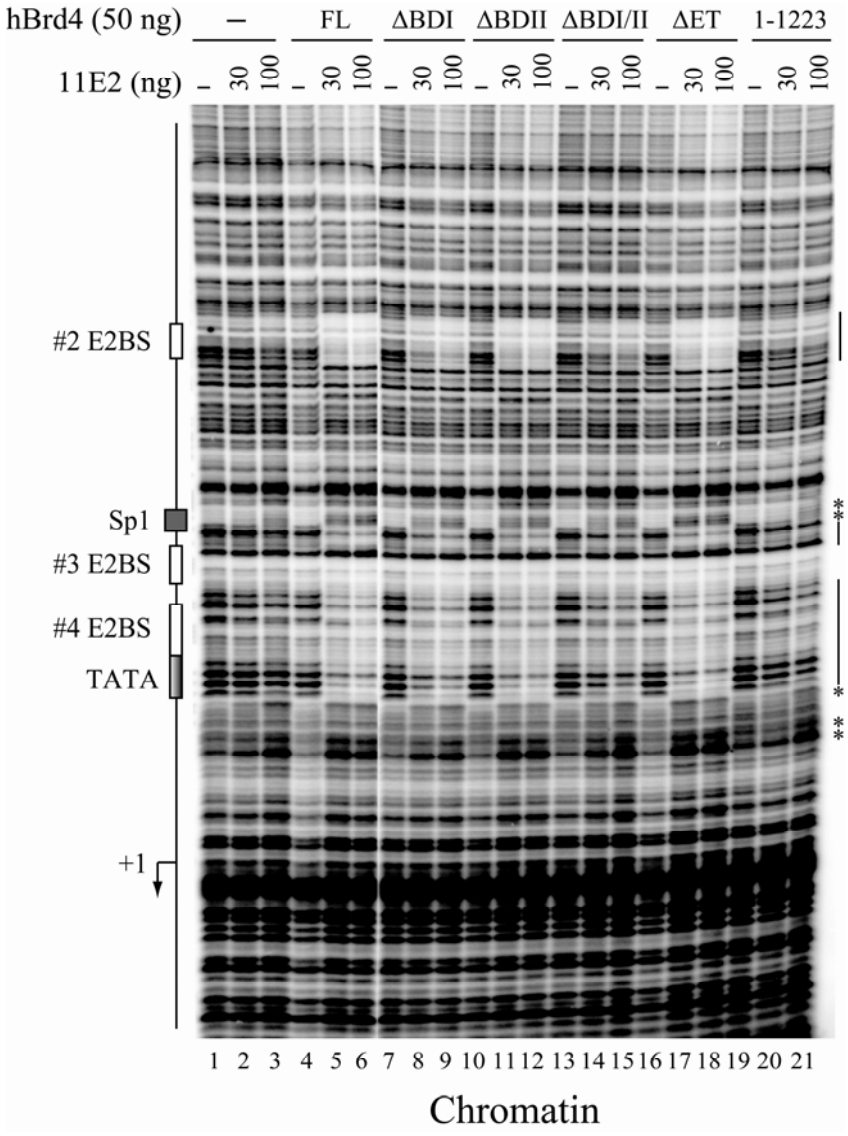

Figure 2-3. Bromodomains and the CTM are both critical for Brd4-enhanced E2 binding to HPV chromatin

In vitro-reconstituted HPV-11 Chromatin was incubated with or without (-) HPV-11 E2 (11E2) and different forms of Brd4 proteins as indicated. Chromatin footprinting was conducted as described under Materials and Methods with PCR-amplified labeled DNA fragments separated by denaturing polyacrylamide/urea gel electrophoresis and visualized by PhosphorImager analysis. Positions of protein factor-binding sites and the transcription start site (+1) are indicated on the left. The protected regions and hypersensitive sites (*) are marked on the right.

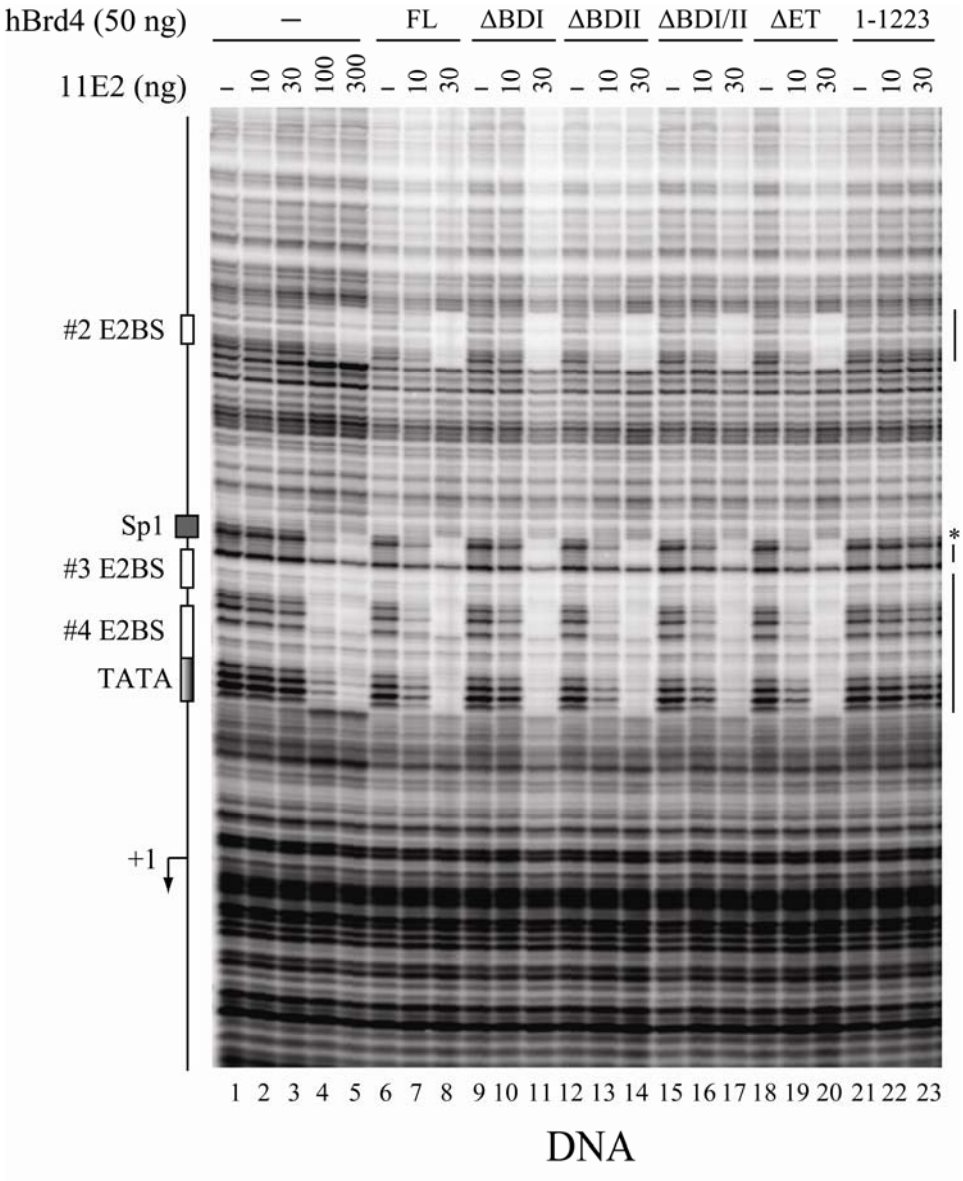

Figure 2-4. Bromodomains are dispensable for Brd4-enhanced E2 binding to HPV DNA

DNase I footprinting was performed as described in Materials and Methods. The markings are the same as outlined in the legend for Fig. 2-3.

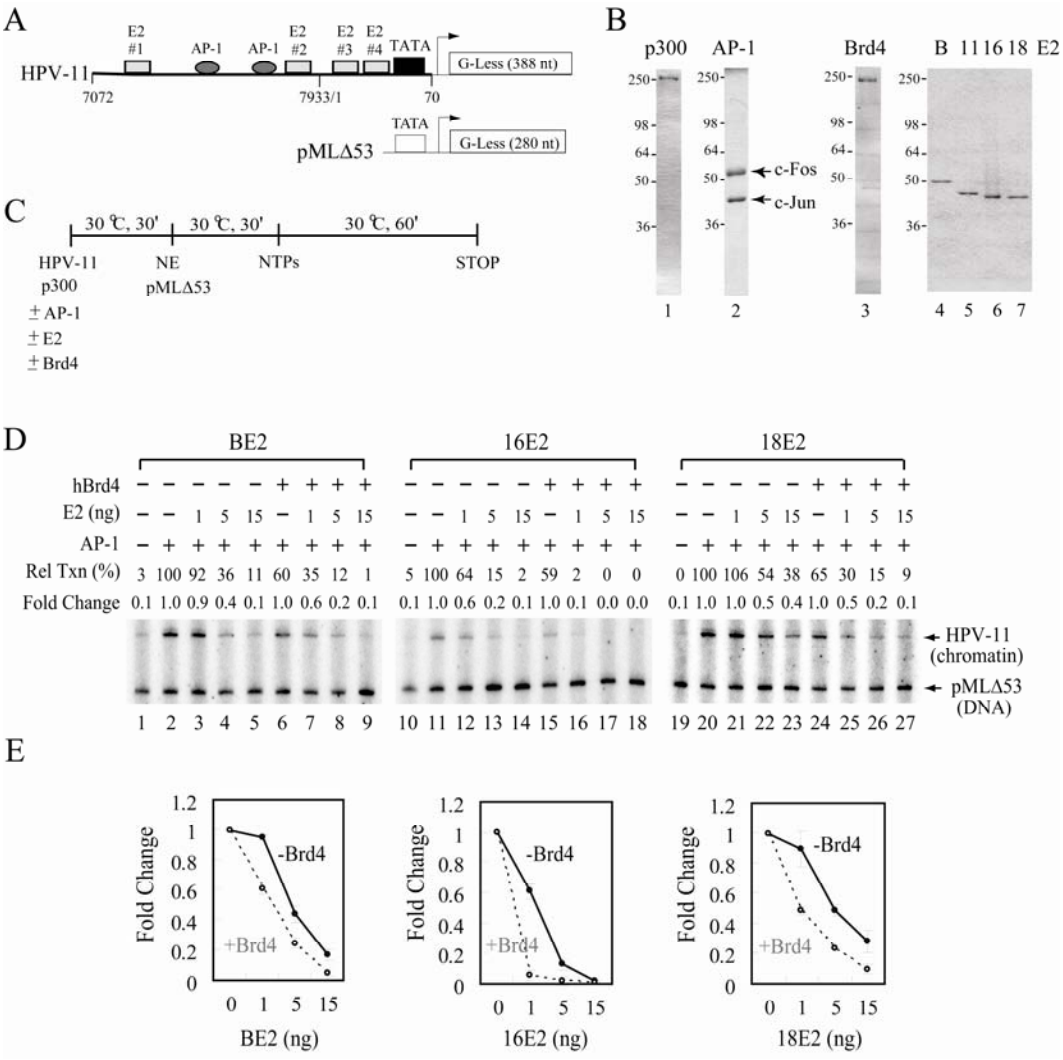
A
E2 #1
AP-1
AP-1
E2 #2
E2 #3
E2 #4
TATA
HPV-11
G-Less (388 nt)
7072
7933/1
70
TATA
pMLΔ53
G-Less (280 nt)
B
p300
AP-1
Brd4
B 11 16 18 E2
250
98
64
50
36
c-Fos
c-Jun
1 2 3 4 5 6 7
C
30 ℃, 30'
30 ℃, 30'
30 ℃, 60'
HPV-11
p300
± AP-1
± E2
± Brd4
NE
pMLΔ53
NTPs
STOP
D
BE2
16E2
18E2
hBrd4
E2 (ng)
AP-1
Rel Txn (%)
Fold Change
HPV-11 (chromatin)
pMLΔ53 (DNA)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
E
Fold Change
-Brd4
+Brd4
BE2 (ng)
16E2 (ng)
18E2 (ng)

Figure 2-5. Brd4 enhances repression of HPV chromatin transcription by different types of E2 proteins

(**A**) G-less cassette templates used for chromatin assembly and transcriptional analysis. (**B**) Coomassie blue staining of purified proteins. AP-1 (c-Jun/C-Fos) was prepared using bacterial cells and p300, FL Brd4, and B/11/16/18E2 proteins were purified from baculovirus-infected insect cells. (**C**) Outline of transcriptional analysis with HPV-11 chromatin or internal control DNA template. (**D**) Inhibition of AP-1-dependent chromatin transcription. *In vitro* transcription was performed as described under Materials and Methods with HPV chromatin and an internal control DNA template (pMLΔ53), in the absence (-) or presence (+) of AP-1, hBrd4, and different types and amounts of E2 proteins as indicated. Signals were quantified by PhosphorImager analysis. (**E**) Graph representation of transcription signals. Numbers were averaged from two independent experiments with error bars indicating the standard deviation.

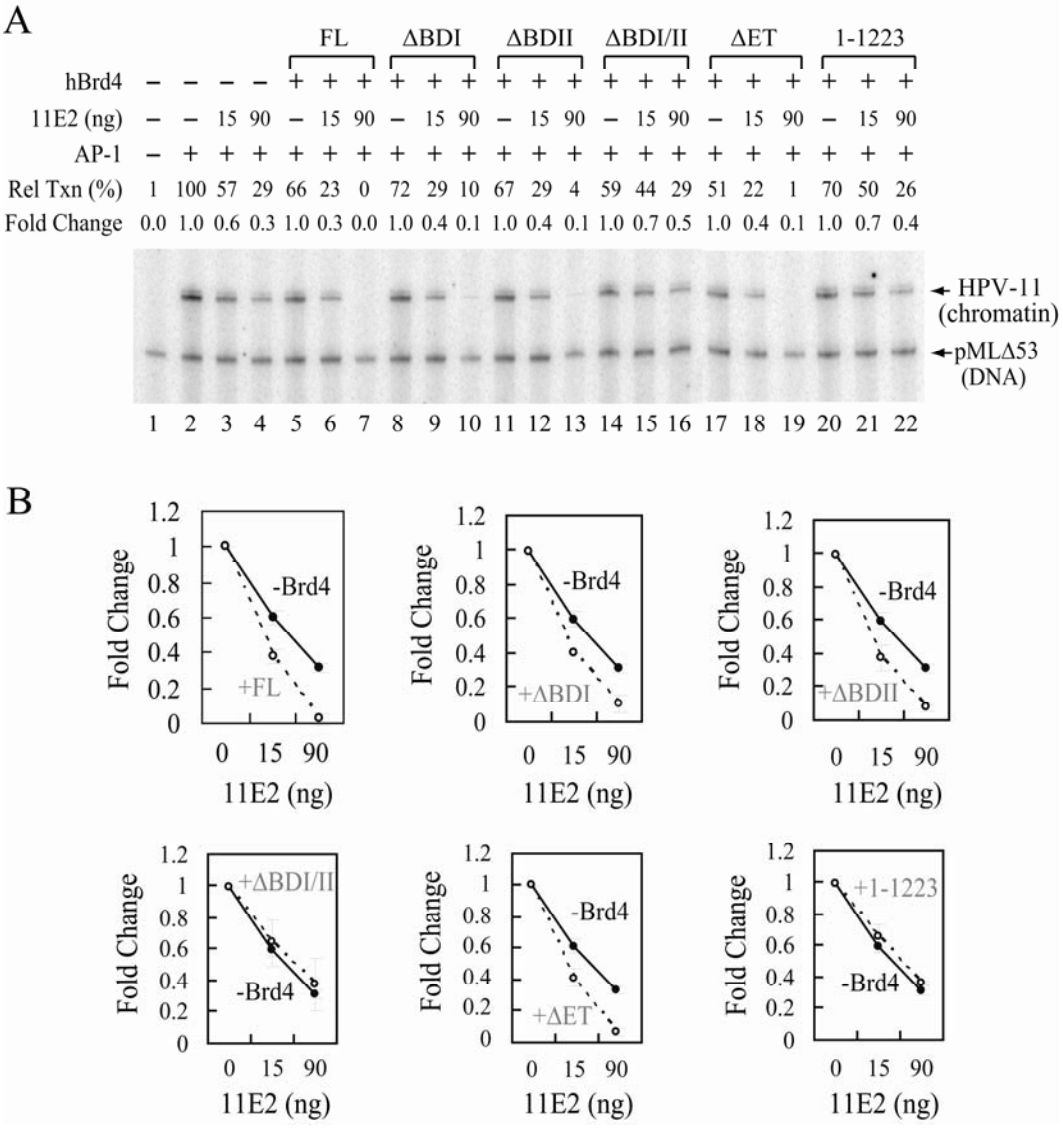

Figure 2-6. Brd4 enhances repression of HPV chromatin transcription in a bromodomain- and E2-dependent manner

(**A**) At least one bromodomain and the E2-interacting region of Brd4 are necessary for Brd4-mediated E2 repression. *In vitro* transcription was the same as described in Fig. 2-6, except that 50 ng of full-length hBrd4 (or a domain-specific deletion mutant) and 15 or 90 ng of 11E2 were used in the assay. Fold change is defined under Materials and Methods. (**B**) Graph representation of transcription signals. Numbers were averaged from two independent experiments with error bars indicating the standard deviation.

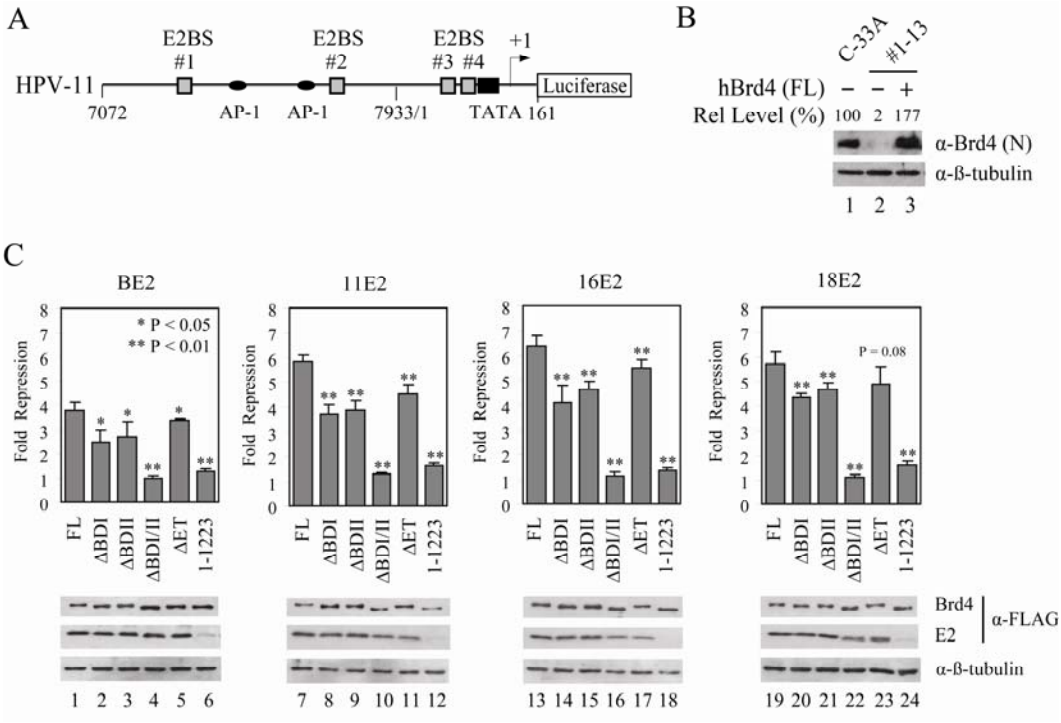

Figure 2-7. Brd4 functions as a general corepressor for different types of E2 proteins requiring a bromodomain and the CTM

(**A**) Schematic representation of the HPV-11 reporter construct. Numbers below the line indicate the positions of nucleotides in the HPV-11 genome. The transcription start site (+1) corresponds to nucleotide 93 in HPV-11. (**B**) Levels of Brd4 in C-33A and #1-13 cells with or without exogenous Brd4 expression. Transfection was performed as described in Materials and Methods. (**C**) A bromodomain and the CTM are both crucial for Brd4-enhanced E2 repression. Transfection was performed in #1-13 cells with a HPV-11 reporter, an E2 expression plasmid, and a FL or domain-deleted Brd4 expression plasmid as described under Materials and Methods. The bar graph represents two independent experiments performed in duplicate with error bars indicating the standard deviation. Student's t-test was used for statistical analysis by comparing fold repression between each mutant and the full-length (FL) protein.

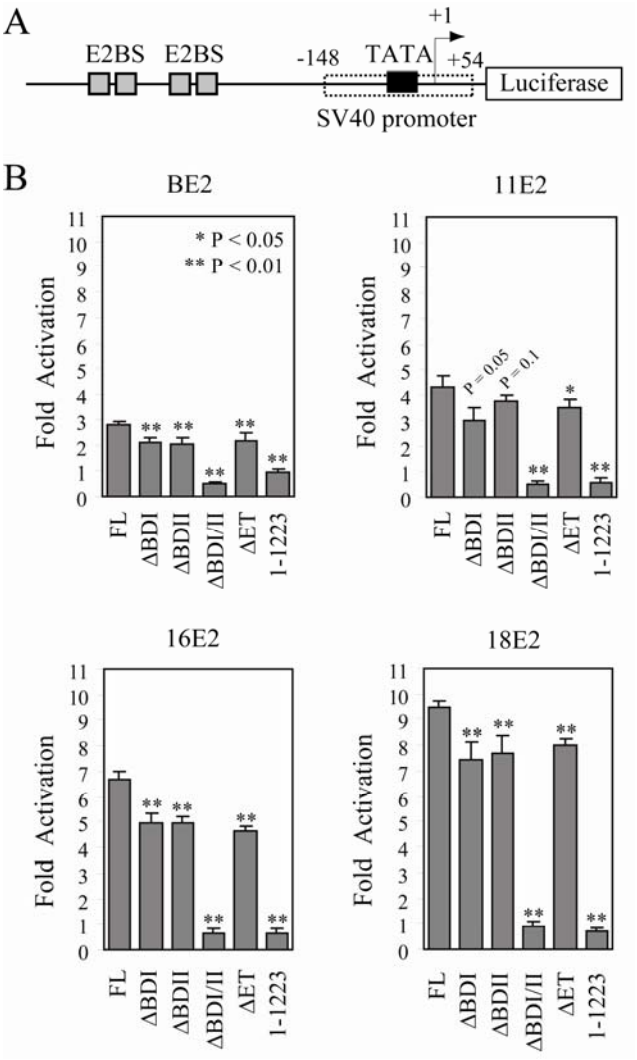

Figure 2-8. Brd4 enhances activation by different types of E2 in a bromodomain- and CTM-dependent manner

(**A**) Schematic representation of the p2x2xE2BS-luc reporter. (**B**) A bromodomain and the CTM are both necessary for Brd4-enhanced E2 transactivation. Transfection was performed in #1-13 cells with the p2x2xE2BS-luc reporter, an E2 expression plasmid, and a FL or domain-deleted Brd4 expression plasmid as described under Materials and Methods. The bar graph represents two independent experiments performed in duplicate with error bars indicating the standard deviation. Student's t-test was used for statistic analysis by comparing fold activation between each mutant and the full-length (FL) protein.

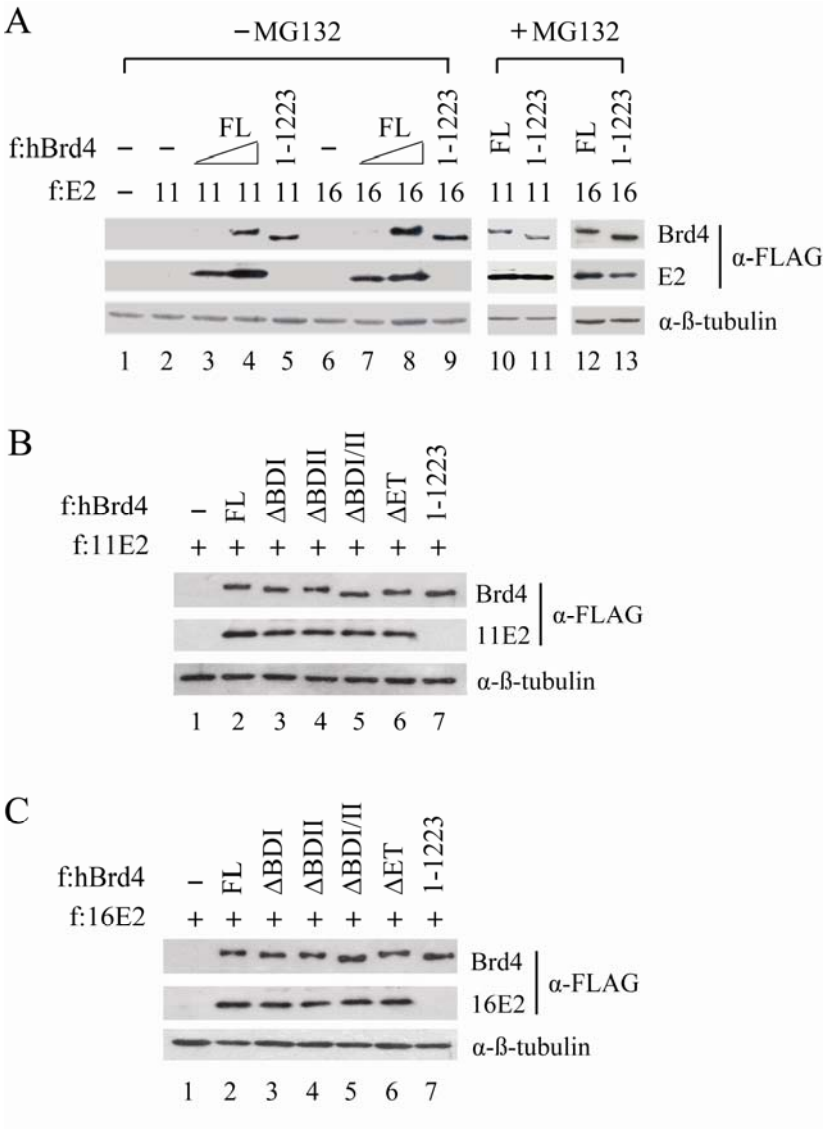

Figure 2-9. Brd4 stabilizes E2 through CTM-mediated interaction
Transfection was performed in #1-13 cells with a FLAG-tagged (f:) 11E2 or 16E2 expression plasmid, with (+) or without (-) a cotransfected FLAG-tagged FL or domain-deleted Brd4 expression plasmid. Cells were treated with the proteasome inhibitor MG132 (+) or control DMSO (-) in panel **A** or left untreated (panels **B** and **C**). Western blotting was conducted with anti-FLAG (for Brd4 and E2 detection) or β-tubulin antibodies.

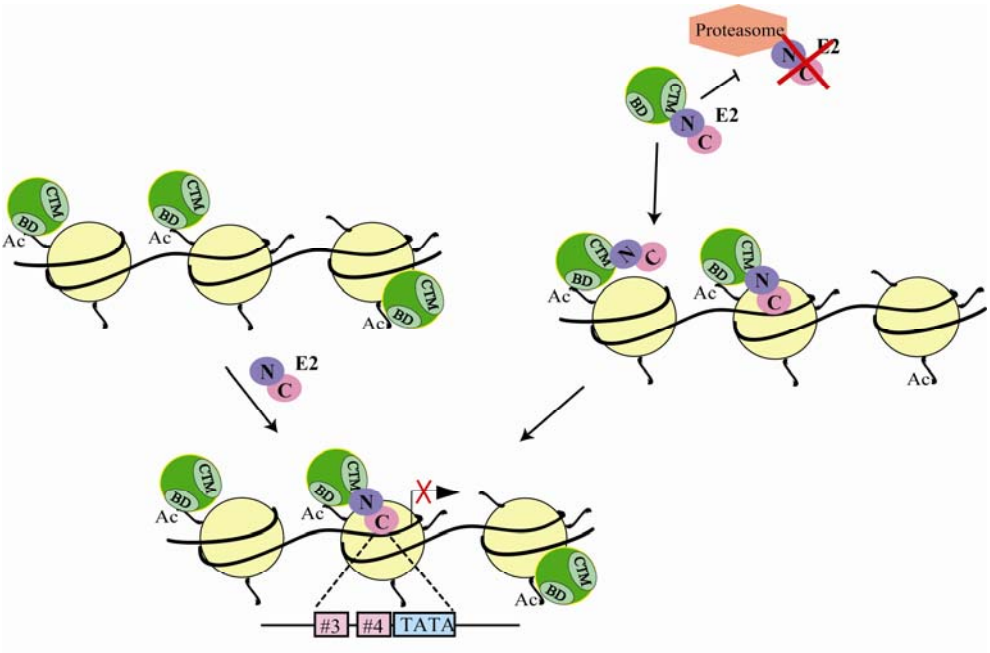

Figure 2-10. Brd4 modulates E2 transcription activity and protein stability

CHAPTER III:

FUNCTIONAL REGULATION OF BRD4 BY PHOSPHORYLATION

INTRODUCTION

Papillomaviruses (PVs) are double-stranded DNA viruses that infect epithelial cells in humans and various animals with strict host specificity. Human papillomaviruses (HPVs) can be classified as either low or high risk, depending on their propensity for malignancy. The low risk viruses such as human PV type 11 (HPV-11), HPV-6, and bovine papillomavirus type 1 (BPV-1) cause benign warts of squamous epithelia and high risk types including HPV-16, HPV-18, and HPV-31 induce the development of anogenital cancer by infection (Dell & Gaston, 2001; Lambert et al, 1988; zur Hausen, 2000).

E2 is a viral transcription/replication factor (Demeret et al, 1994; Desaintes & Demeret, 1996; Dostatni et al, 1991; Hou et al, 2002; Romanczuk et al, 1990; Stenlund, 2003; Tan et al, 1992; Thierry & Howley, 1991) encoded by PVs which recognizes the consensus binding sequence $ACCN_6GGT$ (McBride et al, 1991; Sousa et al, 1990). It consists of an N-terminal transactivation domain and a C-terminal DNA-binding domain linked by a divergent hinge region. Even

though E2's role in transcription and replication are well-conserved among different types of E2, separate properties of high risk E2 and low risk E2 have been reported. Whereas low risk E2 is exclusively localized in the nucleus, high risk E2 is shuttled between the nucleus and cytoplasm, and the cytoplasmic portion of E2 induces apoptosis. This raises the interesting possibility that the disruption of the E2 gene during viral genome integration ensures carcinogenesis by avoiding E2-mediated apoptosis (Blachon et al, 2005). Another group further reported that high risk E2 shows a stronger interaction with p53 than low risk E2, and this interaction is critical for E2-induced apoptosis in non-HPV-transformed cells, but not in HPV-transformed cells, indicating that HPV-transformed cells might require a unique pathway for E2-induced apoptosis (Parish et al, 2006b). Moreover, high risk E2-, but not low risk E2-, expressing cells induce a mitotic block and present abnormal mitotic phenotypes and genomic instability. This is possibly due to the specific binding of high risk E2 to Cdc20 and Cdh1 which are activators of APC(anaphase promoting complex), resulting in the inactivation of these proteins (Bellanger et al, 2005). Thus, high risk HPV E2 proteins have been suggested to participate in HPV's oncogenic potential through inducing genome instability, one of the contributors for cancerization.

As shown above, the biological activity of E2 is often determined by its cellular interacting factors such as Brd4. Brd4 (bromodomain-containing protein 4) belongs to the BET subgroup of the bromodomain superfamily. The BET

family proteins carry an extraterminal (ET) domain of unknown function and two bromodomains, which are acetyl-lysine binding modules often found in proteins that associate with chromatin. Many reports have implicated Brd4 in various biological processes including transcriptional regulation of viral and cellular genes (Bisgrove et al, 2007; Huang et al, 2009; Jang et al, 2005; Lee & Chiang, 2009; Lin et al, 2008a; Mochizuki et al, 2008; Ottinger et al, 2006; Ottinger et al, 2009; Schweiger et al, 2006; Wu et al, 2006; Yang et al, 2008; Yang et al, 2005; You et al, 2006; Zhou et al, 2009), protein stability (Lee & Chiang, 2009), viral genome segregation (You et al, 2004; You et al, 2005; You et al, 2006), cell cycle regulation (Dey et al, 2000; Farina et al, 2004; Haruki et al, 2005; Maruyama et al, 2002), gene arrangement in t(15;19)(q13, p13.1)-associated carcinoma (French et al, 2001; French et al, 2003; French et al, 2008), and cancer progression (Crawford et al, 2008a; Crawford et al, 2008b).

With respect to Brd4's regulation of E2 function, Brd4 is involved in BPV-1 viral genome attachment to mitotic chromosomes and segregation of its genome (Baxter et al, 2005; You et al, 2004), HPV-11 transcriptional repression (Lee & Chiang, 2009; Wu et al, 2006), activation of a heterologous promoter carrying E2 binding sites (Lee & Chiang, 2009; Schweiger et al, 2006), and modulation of E2 protein stability (Lee & Chiang, 2009). The C-terminal motif (CTM) of Brd4-mediated interaction with E2 is critical for all the functions described above.

In addition to the CTM, our group mapped an additional HPV-11 E2 (11E2)-interacting domain of Brd4 using bacterially expressed mouse Brd4 proteins: a region containing the second bromodomain (BDII). To further examine the minimum region of Brd4 required for the interaction with E2 and to determine the function of this interaction, full-length (FL) and various deletion mutants of human Brd4 (hBrd4) were generated and purified using bacterial and baculovirus expression systems. These purified Brd4 proteins were tested for interaction with E2 proteins encoded by BPV-1, low risk HPV-11, and high risk HPV-16 and HPV-18. Besides the CTM, we identified two additional E2-binding regions: E2ID (E2-interacting domain) and PDID (phosphorylation-dependent interacting domain) (Figure 3-6D). While the E2ID bound to all different types of E2 tested, PDID is recognized by only high risk E2 (HRE2) proteins (16/18E2). The interaction only occurs when PDID is phosphorlyated, and the phosphorylation is mediated by CK2 (casein kinase 2). The phosphorylated PDID also binds to E2ID, and this interaction prevents E2ID from binding to E2. The phosphorylated PDID recognizes the DNA-binding domain of HRE2, and bromodomain II is not required for this interaction. Finally, a luciferase assay shows that the overexpressed PDID functions as a dominant negative mutant specifically for HRE2s, whereas CTM induction results in an alleviation of transactivation from all different types of E2, suggesting that the PDID-HRE2 interaction is important for the HRE2-mediated transcriptional activation.

MATERIALS AND METHODS

Plasmid construction

Bacterial expression plasmids pF:hBrd4(279-579)-11d, pF:hBrd4(287-530)-11d, and pF:hBrd4(524-579)-11d were constructed to prepare Brd4 deletion mutant proteins from BL21(DE3)pLysS. The corresponding coding sequences were generated by PCR amplification with an NdeI site-containing sense primer and BamHI site-containing antisense primer using pcDNA3-F:hBrd4(FL) (Lee & Chiang, 2009) as a template. After purification and restriction enzyme treatment of the PCR product, it was cloned between NdeI and BamHI sites by swapping the insert with pF:TBP-11d (Chiang et al, 1993). To generate pGST-BE2, pGST-16E2, and pGST-18E2, E2 fragments were isolated from pF:BE2-11d, pF:16E2-11d, and pF:18E2-11d (Hou et al, 2002) using NdeI and BamHI digestion and were ligated into pGEX-2TL(+) at the same enzyme restriction sites. pGST-11HC, pGST-11C, pGST-18N, pGST-18HC, pGST-18C, pGST-E2ID(524-579), and pGST-CTM(1224-1362) were constructed similarly to the Brd4 deletion clones mentioned above by cloning the insert amplified by PCR into upstream NdeI and downstream BamHI sites of pGEX-2TL(+). The templates used for PCR amplification were as follows: pF:11E2-11d for pGST-11HC, and pGST-11C; pF:18E2-11d for pGST-18N, pGST-18HC, and pGST-18C; pcDNA3-F:hBrd4(FL) for pGST-E2ID(524-579), and pGST-CTM(1224-1362). The

plasmid pGST-11E2 (Wu & Chiang, 2001) and pGST-11E2(1-200) (Wu et al, 2006) were used to express GST-11E2 and GST-11N. To prepare M1, M2, M3, M4, M5, and M6 proteins (CK2-phosphorylation site mutants of PDID), pF:PDID(S405A)-11d, pF:PDID(S469/470A)-11d, pF:PDID(S484/488A)-11d, pF:PDID(S492/494A)-11d, pF:PDID(S498/499/T500A)-11d, and pF:PDID(S503A)-11d were used, respectively. All mutant plasmids were constructed by site-directed mutagenesis in pF:hBrd4(287-530)-11d.

For the baculovirus expression plasmids, the intermediate plasmids pF:hBrd4(1-284)-7, pF:hBrd4(287-530)-7, pF:hBrd4(598-785)-7, pF:hBrd4(721-1055)-7, pF:hBrd4(1033-1258)-7, and pF:hBrd4(1199-1362)-7 were cloned as described previously for the generation of pF:hBrd4(1-722)-7 (Lee & Chiang, 2009). Then, the XbaI-BamHI fragment containing Brd4-coding sequence was transferred between XbaI and BamHI sites of pVL1392 (Invitrogen) to generate pVL-F:hBrd4(1-284), pVL-F:hBrd4(287-530), pVL-F:hBrd4(1-722), pVL-F:hBrd4(598-785), pVL-F:hBrd4(721-1055), pVL-F:hBrd4(1033-1258), and pVL-F:hBrd4(1199-1362). The plasmids for the domain-specific deletion mutants pVL-F:hBrd4ΔBDI/CTM, pVL-F:hBrd4ΔBDII/CTM, and pVL-F:hBrd4ΔBDI/II/CTM were constructed by swapping the EcoRI-BglII fragment covering the C-terminal region of Brd4 and the vector sequence of pVL-F:hBrd4ΔBDI, pVL-F:hBrd4ΔBDII, and pVL-F:hBrd4ΔBDI/II, respectively, with the EcoRI-BamHI region including the 3' coding part of Brd4 (amino acids

645-1223) and the vector sequence of pVL-F:hBrd4(1-1223) (Lee & Chiang, 2009). For pVL-F:hBrd4ΔET/CTM, pcDNA3-F:hBrd4ΔET/CTM was generated by replacing the BstEII-XhoI region encoding Brd4 C-terminal region of pcDNA3-F:hBrd4ΔET with the same enzyme restriction fragment from pcDNA3-F:hBrd4(1-1223) (Lee & Chiang, 2009), and then the XbaI fragment covering Brd4 coding region of pcDNA3-F:hBrd4ΔET/CTM was ligated into XbaI – cleaved pVL1392 (Invitrogen) to construct pVL-F:hBrd4ΔET/CTM. For the expression of the FL and C-terminal motif deletion of Brd4, pVL-F:hBrd4FL and pVL-F:hBrd4(1-1223) were used, respectively (Lee & Chiang, 2009).

For nuclear expression of PDID and CTM in mammalian cells, pcDNA3-F:NLS:hBrd4(287-530) and pcDNA3-F:NLS:hBrd4(1224-1362) were generated in four steps of cloning. First, pF:hBrd4(1224-1362)-7 was constructed as described above for pF:hBrd4(287-530)-7 as transfer plasmids. Second, pF:NLS-11d was created for the generation of SV40 large T nuclear localization signal (NLS) peptide (PPKKKRKVA)-linked proteins. To create the plasmid containing the NLS sequence, the primers encoding NLS and several restriction enzyme sites were synthesized using the following primers: sense primer (5'-TATCCCTCCAA AAAAGAAGAGAAAGGTAGCTCATATGGTCGACGGATCCCTCGAG-3'), antisense primer (5'-CTCG AGGGATCC GTCGACCATATGAGCTACCTTTC TCTTCTTTTTTGGAGGG A-3'). Annealing primers contained NdeI-mimic sequence (to destroy exiting NdeI site after ligation with backbone vector)

upstream of the NLS sequence and additional NdeI, SalI, BamHI, and XhoI sites downstream of the NLS. To prepare the backbone plasmid which would be used for the ligation with the primers, pF:TBP-11d was digested with BamHI to cut the 3' end of the insert. The sticky ends were filled using klenow enzyme reaction to generate a blunt end. To cleave the 5' end of the insert, NdeI was treated and the insert was removed. The remaining vector was ligated with the annealed primer to create pF:NLS-11d. Third, the NdeI-BamHI fragment of pF:hBrd4(287-530)-7 and pF:hBrd4(1224-1362)-7 containing Brd4 coding sequence was introduced between the NdeI and BamHI sites of pF:NLS-11d to generate pF:NLS:hBrd4(287-530)-11d and pF:NLS:hBrd4(1224-1362)-11d, respectively. Fourth, BglII-BamHI fragment of pF:NLS:hBrd4(287-530)-11d and pF:NLS:hBrd4(1224-1362)-11d containing FLAG-tagged and NLS-linked Brd4 coding region was ligated into BamHI-cleaved pcDNA3 (Invitrogen) to construct pcDNA3-F:NLS:hBrd4(287-530) and pcDNA3-F:NLS:hBrd4(1224-1362), respectively. pcDNA3-HA:18E2 was constructed as described previously for pcDNA3-HA:11E2 (Wu et al, 2006). For the expression of FL and C-terminal truncation mutant of Brd4, pcDNA3-F:hBrd4FL and pcDNA3-F:hBrd4(1-1223) were used (Lee & Chiang, 2009).

Protein purification

Bacterially expressed FLAG-tagged human Brd4 proteins were purified following the published protocol (Chiang and Roeder, 1993). Purification of FLAG-tagged human Brd4 proteins from Sf9 insect cells was performed as described previously (Wu et al, 1999).

GST pull-down assay

To analyze protein-protein interactions, GST pull-down was performed as described previously (Wu and Chiang, 2001). Briefly, GST alone, GST-tagged E2 or Brd4 proteins were expressed in BL21(DE3)pLysS and the bacterial lysates were prepared. For the assay, 400 ng of GST proteins were immobilized onto 10 µl of glutathione-Sepharose^{TM} 4B beads (Amersham Pharmacia Biotech) at 4°C overnight. After washing, the beads were incubated with 100 ng of purified proteins at 4°C for 1 hr and washed again. 50 µl of 2X protein sample buffer was added to the protein-bound beads and the sample was analyzed using Western blotting.

For the interaction assay with phosphorylated or dephosphorylated proteins, the kinase or phosphatase reaction was performed as described below. 20 µl of each reaction was mixed with 80 µl of BC100 buffer (20 mM Tris-HCl [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol). 100 µl of reaction was bound to immobilized GST beads and processed as mentioned above.

In vitro dephosphorylation assay

200 ng of purified protein from insect cells was incubated with or without 1 μl of alkaline phosphatase (1 U/μl, Roche) at 30°C for 1 hr in a 20 μl reaction. To analyze the mobility shift by dephosphorylation reaction, the sample was separated by SDS-PAGE and visualized through Coomassie staining.

Kinase assay

200 ng of bacterially purified Brd4 protein was incubated with 200μM of ATP-containing buffer in the absence or presence of 50 U of CK2 (New England Biolabs) at 30°C for 30 min. in a 20 μl reaction. The product was analyzed through Coomassie staining. For the kinase screening assay, 400 ng of protein was incubated with a buffer containing 25 μM of cold ATP and 20 μCi of ^{32}P-γ-ATP at 30°C for 30 min. in a 10 μl reaction. All other enzymes except CK2 were from Active Motif. The reaction was processed using a Typhoon 9200 PhosphorImager.

Coimmunoprecipitation

To test *in vivo* interactions between Brd4 and E2, the stable C-33A derived Brd4 knock-down cell line #1-13 (Wu et al, 2006) was grown in 150-mm plate. 12 μg of a Brd4 expression plasmid and 4 μg of an E2 expression plasmid were transfected using Fugene 6 (Roche) as indicated in Figure 6C. 42 hr after

transfection, the cells were treated with 40 μM of MG132 for 6 hr and the lysates were harvested as described in Lee and Chiang, 2009. 10 μl of anti-FLAG M2-agarose beads (Sigma) were added to the lysates and incubated at 4°C overnight. The immunoprecipitated proteins were analyzed though Western blotting with anti-HA antibody (Covance) for the detection of E2 and anti-FLAG M2 antibody (Sigma) for Brd4.

Luciferase reporter gene assay

Human cervical cancer-derived C-33A cells or human colon cancer-derived p53 null HCT116p53$^{-/-}$ cells were used for the activation assay. Cells were grown in 24-well plates to 50% confluency and transfected with 100 ng of the p2x2xE2BS-luc reporter (Kovelman et al, 1996), 50 ng of an E2 expression plasmid (pcDNA3-HA:18E2 and pcDNA3-HA:11E2) in the absence or presence of 50 ng of pcDNA3-F:NLS:hBrd4(287-530) or pcDNA3-F:NLS:hBrd4(1224-1362) using Fugene 6 (Roche) according to the manufacturer's protocol for 24 hr. 5 mM (final concentration) of sodium butyrate was added 4 hr post-transfection. Cells were harvested in 1x reporter lysis buffer (Promega) and mixed with an assay buffer containing D-Luciferin (Pharmingen). Luciferase activity was measured with the POLARstar OPTIMA plate reader (BMG Labtechnologies). Relative activity indicates the ratio of luciferase activity measured in the presence (+) of over in the absence (-) of E2.

RESULTS

Identification of E2-interacting domain (E2ID)

To elucidate whether different types of E2 interact with the BDII-spanning region of Brd4, GST-tagged B/11/16/18E2 were individually immobilized and incubated with bacterially expressed hBrd4-deletion proteins (Figure 3-2A). Consistent with our previous work (Wu et al, 2006), Brd4 (279-579) interacts with 11E2 and all other types of E2 tested, but not with control GST (Figure 3-2A (a)). To narrow down the region required for the interaction with E2, additional deletion mutants covering the N-terminal (Brd4 (287-530)) or the C-terminal part (Brd4 (524-579)) of Brd4 (279-579) were tested. While Brd4 (287-530) totally loses E2-binding (Figure 3-2A (b)), Brd4 (524-579) retains the interaction with all types of E2 (Figure 3-2A (c)). Deletion of a few more amino acid residues significantly reduces the interaction with 11E2, indicating that Brd4 (524-579) indeed is the minimal region required for E2 interaction (Figure 3-2B). Thus, we designated Brd4 (524-579) as E2ID (E2-interacting domain).

Posttranslationally modified Brd4 is selectively recognized by high risk E2

To confirm the E2ID-mediated interaction with E2 in full-length (FL) Brd4, FL and C-terminal-truncation mutant (1-1223) of hBrd4 were purified from Sf9 insect cells and used for GST pull-down assays (Figure 3-3A and Figure 3-3B,

(1) and (2)). FL Brd4 interacts with B/11/16/ and 18 E2s with similar affinity. However, a C-terminal truncation mutant selectively binds to high risk E2 proteins (16/18E2), but not to low risk B/11E2, even when Brd4 retains its E2ID (Figure 3-3A, compare lanes 3, 4 and 5, 6). Thus, we hypothesized that E2ID may be masked by either a posttranslational modification or an intra-molecular interaction which makes it so that E2ID is not accessible for the interaction with low risk E2s in the FL context of Brd4 (See below). However, high risk E2s interacts with other Brd4 domains besides E2ID and the C-terminal motif. Therefore, a C-terminal truncation mutant only binds to high risk E2. Indeed, further mapping with other deletion mutants spanning the entire protein (Figure 3-3A, (3)-(9)) indicate that Brd4 (287-530), a region containing the BDII domain but not E2ID, is responsible for the interaction with high risk E2. Again, the C-terminal motif (Figure 3-3A, (9)) binds to all types of E2. These results are surprising because, while Brd4 (287-530) from insect cells binds specifically to high risk E2, bacterially purified Brd4 (287-530) did not interact with any of the types of E2 (compare Figure 3-2A deletion (b) and Figure 3-3A deletion (4)). We reasoned that this difference is probably due to a posttranslational modification on the protein that occurred in the insect cells and not in the bacteria. Since modifications on proteins can often alter their mobility in denaturing gels, the interaction reactions with the bacterial protein and the protein from Sf9 were analyzed in one gel side by side (Figure 3-3C). The protein from insect cells

indeed migrated slower than the bacterial protein, indicating the existence of posttranslational modification on the insect cell protein. This modification is probably critical for the interaction with high risk E2 proteins.

Collectively, two common interacting regions within Brd4 are shared by different types of E2: one is the C-terminal motif and the other is E2ID, which is possibly masked by protein modifications or intra-molecular interactions in the FL protein. High risk E2 proteins can bind to Brd4 through an additional contact point around the BDII domain and this interaction is regulated by posttranslational modification.

Brd4 contains two dominant CK2 phosphorylation sites

Yeast Bdf1 (yBdf1), another BET family member, has two separate CK2-mediated phosphorylation regions and the phosphorylation in these regions is necessary for *in vivo* yBdf1 function (Sawa et al, 2004) (Figure 3-4A, upper panel). Since Brd4 belongs to the same protein family as Bdf1, it is possible that these proteins share a similar mechanism for functional regulation. Thus, we searched for putative CK2 phosphorylation sites on Brd4 (Figure 3-4A, lower panel). Two dominant phosphorylation regions were predicted on Brd4 and are similarly positioned to those in yBdf1. The first is located in the region approximately 20 amino acids away from the C-terminal BDII (NPS; N-terminal phosphorylation sites), and the second is in the SEED domain (CPS; C-terminal

phosphorylation sites). Interestingly, the NPS is located in Brd4 (287-530), suggesting the CK2-mediated phosphorylation on the NPS may play a role in the binding with high risk E2s (Figure 3-5A). As shown in Figure 3-4B, putative CK2 phosphorylation sites are evolutionarily conserved among other human BET family proteins, suggesting a functional significance of this modification.

To see if the proteins from insect cells are indeed phosphorylated, Brd4 deletions purified from Sf9 cells were treated with alkaline phosphatase from calf intestine (CIP) (Figure 3-5A). When treated with CIP, a clear mobility shift is observed in (2) Brd4 (287-530) and (3) Brd4 (598-785), containing NPS and CPS, respectively. This data indicates that the NPS and CPS are indeed phosphorylated in Brd4 when expressed in Sf9 cells and that they are the dominant phosphorylation regions on Brd4 since the mobility of other proteins were not changed with CIP treatment.

Even though NPS was initially found to be putative CK2 phosphorylation sites, it is still possible that this region can be phosphorylated by other kinases. To explore this possibility, Brd4 (287-530), the region covering NPS which is important for the interaction with high risk E2, was incubated with various kinases (Figure 3-5B). Of about 30 different kinases, PKA and IKKε, two other Serine/Threonine kinases, phosphorylated Brd4 (287-530), but in a much lesser extent than CK2, suggesting that CK2 is a predominant kinase for the phosphorylation of Brd4 (287-530).

Phosphorylation of Brd4 (287-5530) by CK2 determines the interaction with high risk E2

Next, we asked whether phosphorylation of Brd4 (287-530) regulates the E2-Brd4 interaction. Brd4 (287-530) from insect cells was incubated with CIP buffer in the absence or presence of CIP, and was subsequently incubated with immobilized GST-E2 proteins (Figure 3-6A). When phosphate groups were removed from the protein, it lost its ability to bind to the high risk E2 proteins, indicating that the phosphorylation status of Brd4 (287-530) determines its ability to interact with high risk E2.

The next question was whether CK2 is the kinase responsible for the interaction between phosphorylated Brd4 (287-530) and high risk E2. An *In vitro* kinase assay was performed with CK2 and bacterial Brd4 (287-530) as a substrate. Phosphorylated bacterial protein gained the ability to interact with the high risk E2 (Figure 3-6B). This data indicates that phosphorylation on the Brd4 deletion (287-530) by CK2 allows for selective binding of this protein to the high risk E2. Based on its functional characteristics, we termed Brd4 (287-530) PDID (phosphorylation-dependent interacting domain) (Fig. 3D). Since Brd4 (598-785) covered another major phosphorylation region (CPS), we also tested if this deletion binds to the E2 proteins with or without CIP treatment. As shown in Figure 3-6C, Brd4 (598-785) did not interact with any types of E2 regardless of

its phosphorylation status. Even though two major phosphorylation regions (NPS and CPS) were mapped (Figure 3-4A), only phosphoryation on NPS was connected to high risk E2-binding.

Taken together, Brd4 contains three individual regions recognized by papillomavirus E2 proteins (Figure 3-6D). The C-terminal motif and E2ID of Brd4 are commonly bound by E2 proteins encoded from high risk HPVs (16E2 and 18E2), low risk HPV (11E2), and bovine papillomavirus type 1 (BE2), whereas PDID interacts only with high risk E2 proteins and only when it is phosphorylated.

Generation and characterization of phosphorylated PDID-specific peptoids

To facilitate functional characterization of phosphorylation on PDID, our collaborators (Dr. Thoams Kodadek's lab, UT Southwestern) synthesized and characterized phosphorylated PDID-specific peptoids, which are peptidomimetic oligomers composed of N-substituted glycine units. The phosphorylated PDID-specific peptoids were named Hit G and Hit I. They selectively bound to only the phosphorylated form of PDID, but not to unmodified protein in a on-bead screening assay using quantum dots (unpublished, data not shown). To explore the possibility of whether the interaction between these peptoids and phosphorylated PDID can interrupt the binding of high risk E2 to phosphorylated PDID, peptoids were incubated with PDID purified from Sf9 insect cells and

GST-tagged 16 or 18E2 proteins as illustrated in Figure 3-7A. A reaction with bacterially purified CTM also was performed as a control experiment. Indeed, the peptoids disrupted the interaction between high risk E2 and phosphorylated PDID in a dosage-dependent manner (Figure 3-7B). However, they did not affect CTM-E2 interaction, indicating that peptoids binding to the phosphorylated PDID is specific (Figure 3-7C). It is possible that the peptoids and high risk E2 bind to the same interaction surface on phosphorylated PDID so that they compete with each other for interaction with this protein. Again, these data support the notion that the phosphorylation on PDID is critical for the selective interaction with high risk E2.

Phosphorylation of PDID masks E2ID from E2-binding

To confirm that the interaction with high risk E2 is phosphorylation-dependent in the FL context of Brd4, FL and C-terminal truncation mutant (1-1223) were incubated with or without CIP and were then subsequently incubated with GST-E2 proteins (Figure 3-8A). FL Brd4 was pulled down with all types of E2 in both conditions (+/- CIP), because it contains the C-terminal motif found to be important for the interaction. The C-terminal truncation mutant bound to high risk E2s in the absence of CIP treatment as shown in Figure 3-8A. Surprisingly, removal of phosphate groups from this mutant with CIP treatment resulted in binding to all types of E2, and this result led us hypothesize that this interaction

might be mediated through E2ID on Brd4. Thus, we assumed that E2ID is masked by phosphorylation within PDID possibly through intramolecular interaction. Masked E2ID is exposed for E2-binding upon the dephosphorylation of PDID (Figure 3-8C). In other words, phosphorylation of Brd4 is required for selective binding to the high risk E2 proteins, and at the same time, prevents E2ID from binding to all types of E2. To test this hypothesis, we first performed an interaction assay using Brd4 deletions to see if Brd4 forms an internal molecular interaction through the interaction between E2ID and another part of Brd4. GST-tagged E2ID (524-579) and GST-CTM (1224-1362) were immobilized and incubated with insect cell-purified Brd4 deletions spanning the entire region of the FL protein (Figure 3-8B). Indeed, E2ID bound to PDID, but not to other regions of Brd4, suggesting that E2-interacting surface of E2ID might be masked by an E2ID-PDID interaction. On the other hand, CTM (1224-1362) does not interact with any part of Brd4, indicating that the E2ID-PDID interaction is a C-terminal region-independent event. The bacterially expressed PDID (Figure 3-8B, deletion (a)) does not associate with E2ID, suggesting that phosphorylation on PDID is required for the interaction with E2ID.

Collectively, the phosphorylation on PDID exerts two roles: 1) it provides specific interaction with high risk E2 proteins, and 2) it blocks E2ID from interaction with E2 through intra-molecular interaction with phosphorylated PDID (Figure 3-8C, left). However, dephosphorylated PDID loses its ability to interact

with both E2ID and the high-risk E2 proteins, and therefore, E2ID becomes accessible for E2-binding (Figure 3-8C, right).

BDII is dispensable for PDID function

Since PDID spans bromodomain II, which is important for chromatin targeting, and C-terminal flanking sequences covering NPS, we wondered whether BDII is required for PDID-mediated function. To address this issue, FLAG-tagged domain-specific deletion mutants were generated from Sf9 insect cells (Figure 3-9 A and B). Besides the BDII, BDI or extraterminal (ET) domain, the CTM was deleted individually as a control or together with the other deletions. The purified proteins were incubated with or without CIP, and were then incubated with GST-linked 11E2 or 16E2. As shown in Figure 3-9C, the additional deletion of BDII on C-terminal truncation mutant (ΔBDII/CTM) did not affect the PDID-mediated selective interaction with high risk E2. ΔBDII/CTM only bound to 16E2 in the absence of CIP-treatment, but dephosphorylated protein interacted with both 11E2 and 16E2. Other deletions used as a control (ΔBDI/CTM, ΔBDI/II/CTM, and ΔET/CTM) showed the same results as ΔBDII/CTM. This data suggests that BDII is not essential for the specific PDID-mediated interaction with high risk E2.

Mapping the phosphorylated amino acid residues on PDID

As summarized in Table 3-1, eleven phosphorylated residues on PDID were predicted or identified. The left most column represents the putative CK2 phosphoryaltion sites which matched with its consensus phosphorylation site (pS/TXXE/D), and these sites are illustrated above in Figure 3-4A. Previous publications have reported biological phosphorylation sites on Brd4 from HeLa cells, and among those, PDID is phosphorylated at the serine residues located at amino acid numbers 469 and 470 (S469 and S470, respectively) (Beausoleil et al, 2004; Dephoure et al, 2008). The protein chemistry technology center at UT Southwestern also identified one phosphorylated residue, T500, from insect cell-purified PDID. To understand which residues are major phosphorylation sites among those, site-directed mutagenesis was performed to generate mutant PDID proteins. Since all these sites can possibly be phosphorylated by CK2, these sereine (S) or theronine (T) residues are mutated alone or in combination with alanine (A) (Figure 3-10 A). The wild-type and mutant proteins were purified from bacterial cells (Figure 3-10B) and were incubated in the absence (-) or presence (+) of CK2 (Figure 3-10C). Compared to wild-type, these mutants did not show a significant defect in their ability to be phosphorylated, suggesting that all these sites equally contribute, or that there are additional residues which can be phosphorylated by CK2. Further mutational anaylsis will be required to resolve this issue clearly.

Phosphorylated PDID binds to the C-terminal region of high risk E2

Previously it was reported that CTM binds to the N-terminal transactivation domain of E2 (Wu et al, 2006). To understand which region of E2 is recognized by E2ID and PDID, GST-tagged11E2 and 18E2 deletion mutants covering the transactivation domain (N), hinge and DNA binding domain (HC), or DNA binding domain (C) were generated (Figure 3-11A). GST-E2 proteins were incubated with bacterially purified CTM, E2ID, PDID, or insect cell-purified PDID or FL Brd4 protein (Figure 3-11B). Consistent with previous studies, CTM recognized the N-terminal domain of both 11 and 18E2. The E2ID bound to HC and C domains from 11E2 and 18E2, indicating that the DNA binding domain of E2 is sufficient for the interaction with E2ID. Only insect cell-purified PDID, but not bacterial protein, interacted with HC and C of 18E2, indicating that phosphorylated PDID also binds to the C-terminal region of high risk E2. While FL Brd4 only interacts with the N-terminal domain of 11E2, it binds to all 18E2 fragments (N, HC, and C), suggesting FL Brd4 generated from insect cells has phosphorylation within the PDID and provides an additional interacting region besides CTM, but only for high risk E2 interaction.

High risk E2 binds to the phosphorylated PDID *in vivo*

For *in vivo* confirmation of differential Brd4 recognition by different types of E2, we performed a coimmunoprecipitation (coIP) experiment. #1-13 cells,

which are stable C-33A-derived Brd4-knockdown cells (Wu et al, 2006), were transfected with HA-tagged 11E2- or 18E2-expression plasmids with either FL or 1-1223 (C-terminal truncation mutant) Brd4 (Figure 3-11C). Since E2 is degraded by a proteasome-mediated pathway (Bellanger et al., 2001), the transfected cells were treated with forty μM MG-132 for six hours before harvest. The crude lysates were immunoprecipitated with M2 anti-FLAG antibody to pull-down Brd4 and its associated proteins. The amount of Brd4 and E2 proteins were equal among different cotransfections in the presence of MG132 treatment (Figure 3-11C, upper panel). Whereas both 11E2 and 18E2 coprecipitated with FL Brd4, only 18E2 is pulled down with 1-1223 (Figure 3-11C, bottom panel). This data indicates that Brd4 has proper phophorylation on PDID *in vivo*, and thus the C-terminal truncation mutant (1-1223) interacts and coprecipitates with only 18E2. Collectively, the CTM of Brd4 is commonly shared by different types of E2, but phosphorylated PDID specifically interacts with DNA binding domain of high risk E2 (Figure 3-11D).

PDID-mediated interaction with high risk E2 is important for its transactivation

We previously reported that Brd4 functions as a general transcription coactivator for different types of E2 proteins and that this activity depends on the bromodomains and CTM (Lee and Chiang, 2009). To explore whether the PDID-

mediated interaction with E2 is also important for the coactivator function of Brd4 on high risk E2-mediated transcription, HPV negative cervical cancer cell line C-33A was cotransfected with a E2-responsive reporter construct for an activation assay (Figure 3-12A) and different types of E2 expression plasmids (11/16/18E2) in the absence or presence of PDID or CTM expression (Figure 3-12B). Since the CTM-mediated interaction is crucial for the coactivating function of Brd4 on E2-mediated transcription, the overexpressed CTM functions as a dominant negative mutant and thus alleviates endogenous Brd4-mediated transactivation. This effect is common to different types of E2. However, the introduction of PDID only decreased 16 and 18E2-mediated transcription, indicating that the specific interaction between PDID and high risk E2 play a role in E2-mediated transacitvation.

Recently, ~50% of colorectal cancer was reported to be HPV positive and thus it is possible that there is a correlation between colorectal cancer development and HPV infection (Bodaghi et al, 2005). Moreover, we found that p53 targets PDID of Brd4 in a phosphorylation-dependent manner (Figure 4-5), and it is possible that this interaction may compete for high risk E2-PDID binding. Thus, the same experimental system used for C-33A cells was applied to p53 knock-out colon cancer cells, HCT116-p53$^{-/-}$ (Figure 3-12C). Similarly, only the overexpression of PDID specifically alleviates high risk E2-mediated transcription.

PDID and E2ID are generally important for E2-mediated transactivation

One interesting observation is that the effects of PDID on E2-mediated transcription are different depending on the level of its expression (Figure 3-13A). At higher amounts of PDID expression, the domain abolishes the transactivation by 11E2, suggesting a fundamental function of PDID in E2-mediated transactivation. This observation prompted us to test the effect of domain-specific deletion mutants of Brd4 protein on E2-mediated activation (Figure 3-13B). The C-33A-derived stable Brd4 knock-down cell line (#1-13) was cotransfected with an E2-responsive reporter plasmid (Figure 3-12A), E2 expression constructs, and wild-type or mutant Brd4 expression constructs. All Brd4 proteins were expressed at similar levels as the endogenous protein. Consistent with our previous observations, ΔBDII showed 70~80% activity compared to wild-type, and 1-1223 (ΔCTM) resulted in complete abolishment of Brd4's coactivating function. Interestingly, deletions of PDID and E2ID (ΔE2ID and ΔPDID) were as completely defective as ΔCTM, and this phenomenon was common to different types of E2, indicating a pivotal role of PDID and E2ID regions on Brd4 for its coactivating function.

DISCUSSION

We mapped two additional E2-interacting regions on Brd4 besides CTM and uncovered their regulation through posttranslational modification. We also described the functional outcome of these interactions. This study reveals several novel aspects of Brd4 and provides a new regulatory mechanism involved in its E2-mediated function.

First, Brd4 contains additional contact points with E2, besides the CTM, a previously known E2-interacting module, which are E2ID and PDID. Since the interaction between two factors was required for all the known functions Brd4 exhibits with respect to the modulation of E2 activity, the identification of new interacting domains will facilitate the understanding about the interplay between the two factors.

Second, Brd4 contains two dominant phosphorylation sites which are phosphorylated by CK2: NPS and CPS. As shown in Figure 3-4, the dominant CK2-consensus phosphorylation sites are conserved among different types of BET family proteins including yBdf1, Brd2, Brd3, Brd4, and Brdt, suggesting that this modification may carry fundamental functions for other BET proteins as it does for yBdf1 (Sawa et al, 2004). Thus, it would be interesting to test whether other BET proteins (Brd2, Brd3, and Brdt) are indeed modified by CK2.

Third, Brd4 differentially recognizes high risk E2 and low risk E2 depending on its phosphorylation status (see Figure 3-8). While E2ID interacts with all different types of E2, PDID selectively binds only to high risk E2 in a CK2 phoshorylation-dependent manner. Moreover, the phosphorylation of PDID masked the E2-interacting surface on E2ID by inducing an intramolecular interaction, thereby allowing Brd4 to recognize only high risk E2 through PDID when phosphorylated.

Fourth, both E2ID and PDID on Brd4 associate with the DNA-binding domain of the E2 protein. Since the DNA binding domain of E2 is involved in the recognition of its cognate DNA sequence and its dimerization, which is important for efficient mitotic chromosome targeting and Brd4 binding (Cardenas-Mora et al, 2008), it would be interesting to see if E2ID and PDID modulate any of these E2 functions through this protein-protein interaction.

Fifth, *in vivo*, Brd4 interacts with high risk E2 through its CTM and PDID, but it binds to low risk E2 only via CTM (see Figure 3-11D). P-TEFb also binds to Brd4 through multiple interacting points (see Figure 3-1). Deletion or overexpression of each binding domain is detrimental for their interaction and functions related to this interaction (Bisgrove et al, 2007; Jang et al, 2005). Thus, even though the disruption of E2-Brd4 interaction by overexpression or deletion of CTM completely abolishes the effect of Brd4 on E2 functions (Lee & Chiang, 2009; Schweiger et al, 2006; You et al, 2004), it is still possible that both of the

binding regions are required for a stable interaction between Brd4 and high risk E2.

Sixth, the interaction between PDID and high risk E2 is important for E2-induced transactivation, and at the same time, both PDID and E2ID are important for E2-transactivating function. The introduction of a lower amount of PDID to the cells resulted in a reduction of high risk E2-mediated activation while not affecting low risk E2 activity. However, when PDID was highly expressed, it repressed the transcription from all different types of E2. Presumably, PDID has higher affinity for high risk E2 than other cellular interacting factors (*e.q.* P-TEFb, see below), and therefore, it shows a selective effect on high risk E2-mediated transcription at a lower concentration of PDID protein. However, at higher levels of PDID, it may cause squelching of a PDID-interacting cellular protein required for E2-dependent activation, thereby resulting in general transcription repression.

Simultaneously, the experiment with domain-specific deletion mutants of Brd4 suggests two possibilities in regards to PDID and E2ID functions: 1) the deletion of either PDID or E2ID disrupts proper intramolecular interaction, presumably important for Brd4 activity, and thereby causes a defect in coactivation, and 2) since the region covering either PDID or E2ID is overlapped with one of the P-TEFb-binding regions on Brd4, deletion of each domain may cause dissociation of P-TEFb. Therefore, it results in transcriptional repression. Collectively, Brd4 differentially recognizes high risk E2 and low risk E2

depending on its phosphorylation status, and newly identified E2-intercting regions on Brd4 are important for E2-mediated transactivation.

In addition, protein kinase CK2 is an evolutionarily conserved and ubiquitous eukaryotic serine/threonine kinase that is vital for cell survival and is implicated in various cellular activities (Litchfield, 2003; Meggio & Pinna, 2003; Pinna, 2002). Although CK2 is generally considered as a constitutively active kinase, its expression is upregulated in most cancers (Homma & Homma, 2005). As of now, the link between CK2-phosphorylation on Brd4 and high risk E2-induced cancerization is elusive. However, this biochemical analysis on functional modulation of Brd4 by phosphorylation would facilitate the understanding of its versatile roles in E2 biology.

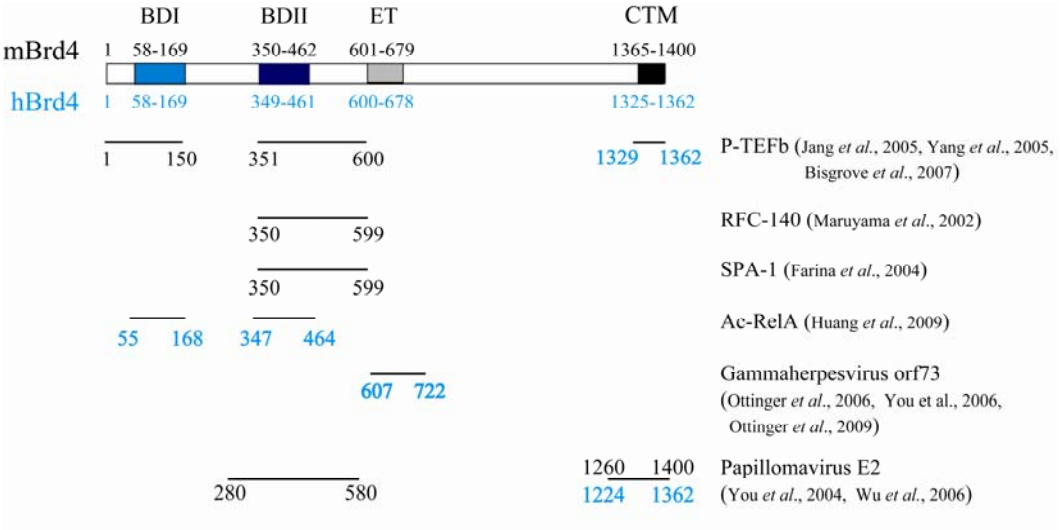

Figure 3-1. Brd4-interacting proteins
Domain structure of Brd4 and its protein-interacting regions. Numbers indicate the positions of amino acid residues of the boundaries of respective protein domains as indicated. Solid lines below the protein scheme represent the region interacting with other factors. Numbers underneath the domain scheme and the lines indicate the boundaries of amino acid residues. The amino acid number for mouse Brd4 is marked in black color and the number for human Brd4 is in blue color. Each interacting partner and its publication information are depicted on the right.

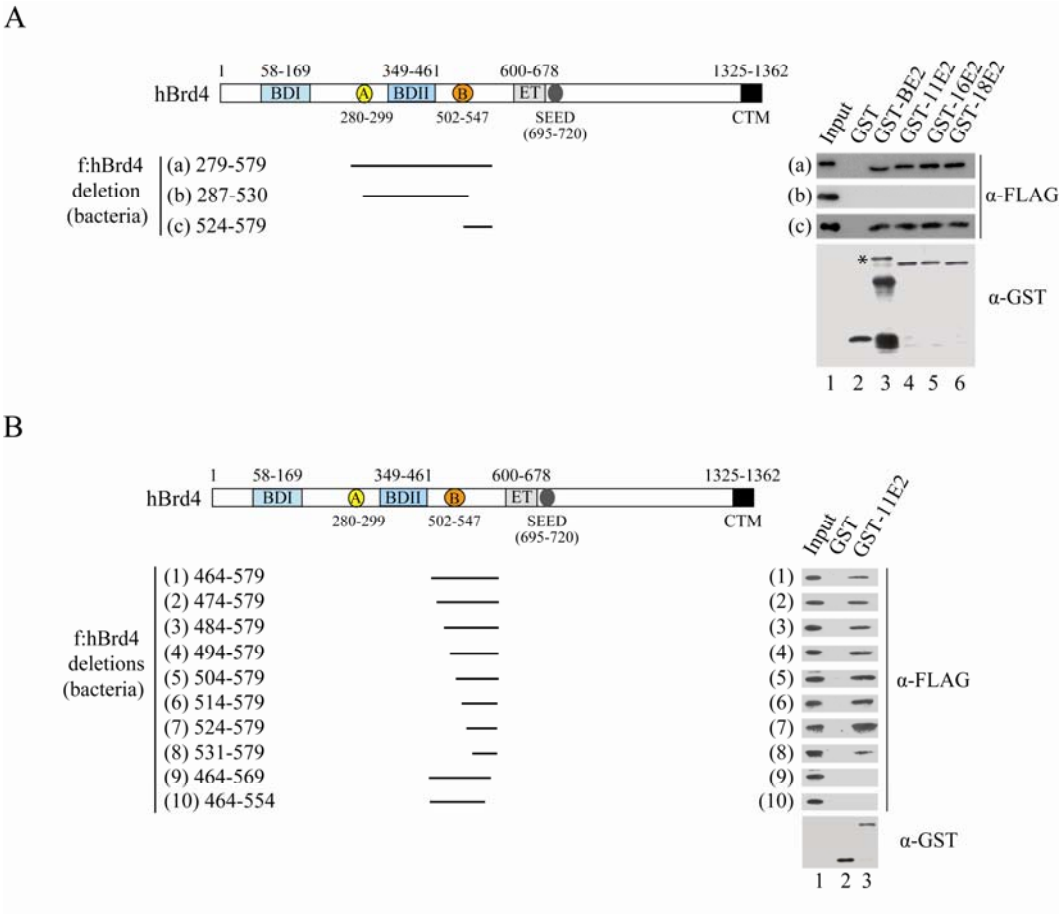

Figure 3-2. Identification of E2ID

(**A**) Identification of E2-interacting domain (E2ID). GST alone or GST-tagged E2 proteins (BE2, 11E2, 16E2 and 18E2) were immobilized with glutathione sepharose beads and incubated with bacterially purified FLAG-tagged Brd4 deletion mutants. Proteins were detected by Western blotting with anti-FLAG M2 antibody (for bound Brd4 deletions) and anti-GST antibody (for E2). Amino acid residues corresponding to each deletion are shown on the left. (**B**) The deletions spanning E2ID and its surrounding region were generated and subjected to interaction with GST-11E2.

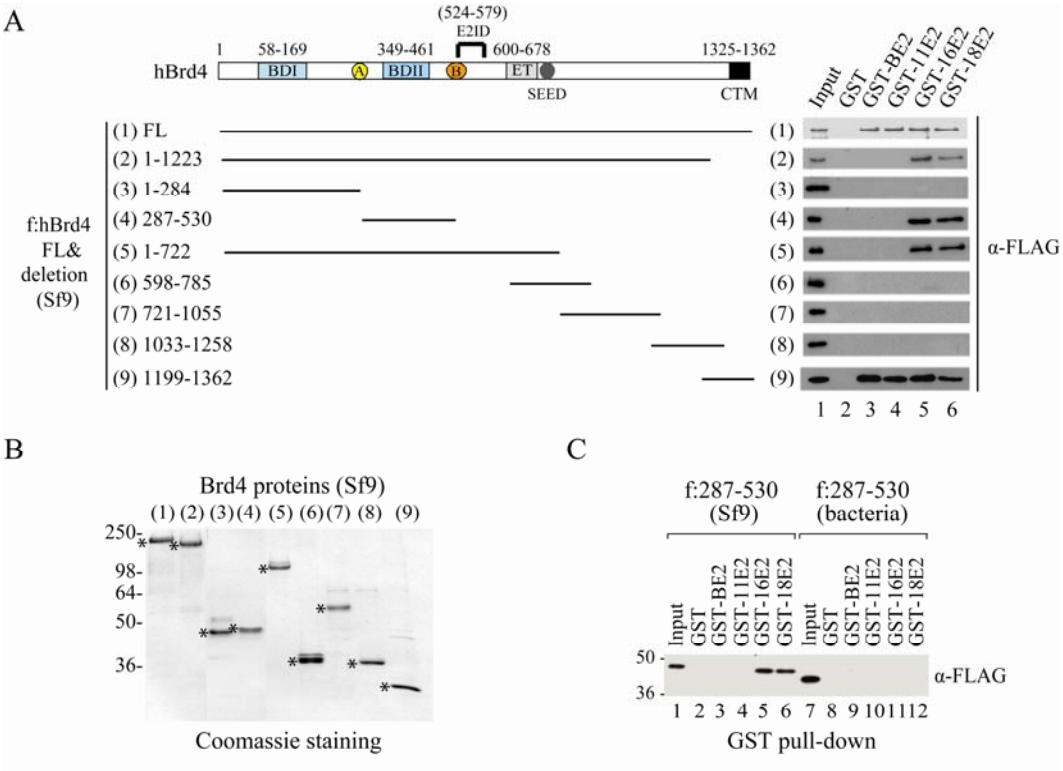

Figure 3-3. Posttranslationally modified Brd4 (287-530) is selectively recognized by high risk E2

(**A**) High risk E2 binds an additional region of Brd4 besides E2ID and CTM. GST pull-down assay was performed by incubating GST-E2 proteins with FLAG-tagged hBrd4 FL or deletion mutants. The bound f:Brd4 proteins were detected by Western blotting with anti-FLAG M2 antibody. (**B**) Coomassie-stained gel of purified Brd4 proteins. All f:Brd4 proteins were expressed and purified from Sf9 insect cells. (**C**) Posttranslationally modified Brd4 is selectively recognized by high risk E2. Brd4 deletion (287-530) from insect cells and bacterial cells were run side by side after GST pull-down and the bound Brd4 proteins were detected by Western blotting with anti-FLAG M2 antibody.

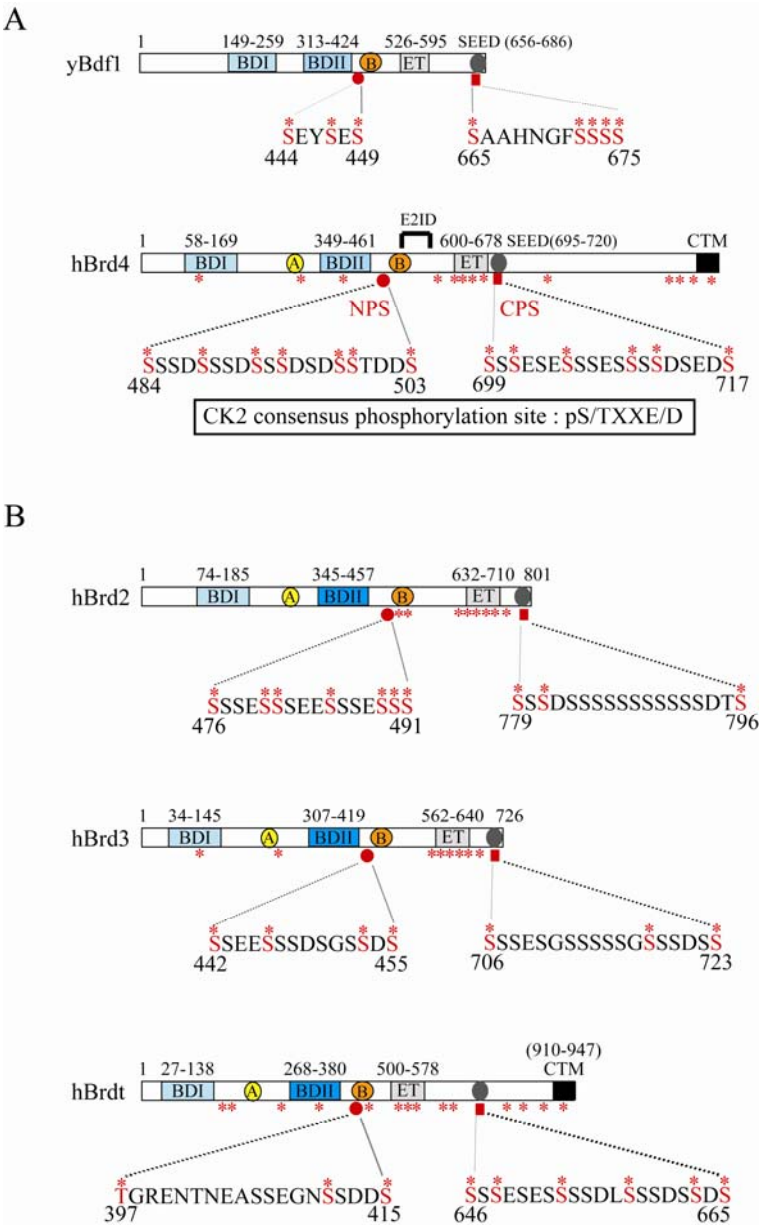
A
yBdf1
1
149-259
313-424
526-595
SEED (656-686)
BDI
BDII
B
ET
SEYSES
444
449
SAAHNGFSSSS
665
675
hBrd4
E2ID
1
58-169
349-461
600-678
SEED(695-720)
CTM
BDI
A
BDII
B
ET
NPS
CPS
SSSDSSDSSSDSDSSTDDS
484
503
SSSESESSSESSSSDSEDS
699
717
CK2 consensus phosphorylation site : pS/TXXE/D
B
hBrd2
1
74-185
345-457
632-710
801
BDI
A
BDII
B
ET
SSSESSSEESSSESSS
476
491
SSSDSSSSSSSSSSSDTS
779
796
hBrd3
1
34-145
307-419
562-640
726
BDI
A
BDII
B
ET
SSEESSSDSGSSDS
442
455
SSSESGSSSSSGSSSDSS
706
723
hBrdt
1
27-138
268-380
500-578
(910-947)
CTM
BDI
A
BDII
B
ET
TGRENTNEASSEGNSSDDS
397
415
SSSESESSSSDLSSSDSSDS
646
665

Figure 3-4. Putative CK2 phosphorylation sites on Brd4 and other BET proteins

(**A**) Putative CK2 phosphorylation sites on Brd4. Schemes represent the domain organization of yeast Bdf1 (yBdf1) and hBrd4. The amino acid residues for the domains are depicted on the top of each scheme. The asterisks and red letters indicate biological phosphorylation sites by CK2 on yBdf1 (upper) and putative phosphorylation sites by CK2 on hBrd4 (bottom). (**B**) Putative CK2 phosphorylation sites on other human BET (bromodomains and extraterminal) family proteins, hBrd2, hBrd3, and hBrdt.

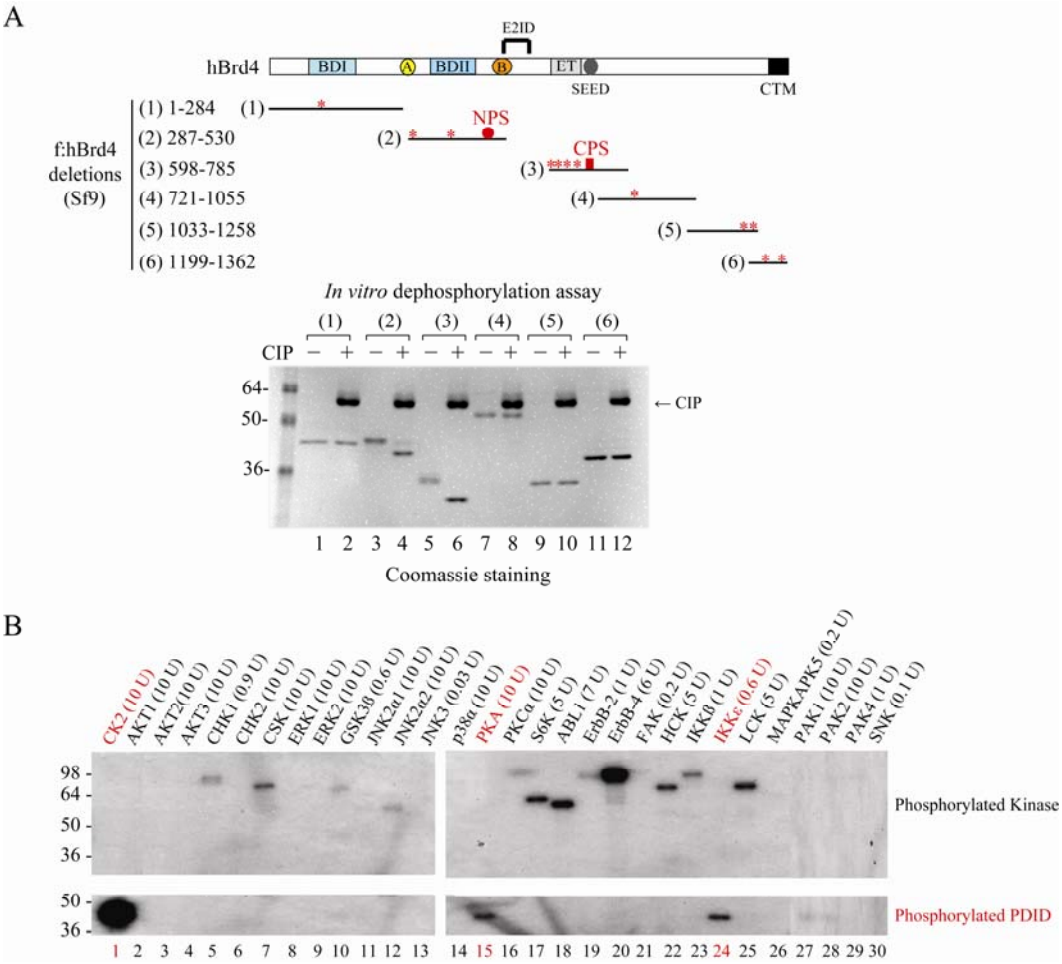

Figure 3-5. Brd4 contains two dominant CK2-mediated phosphorylation sites (**A**) The two dominant phosphorylation sites on Brd4. Upper: hBrd4 deletions used in the dephosphorylation assay. The predicted CK2 phosphorylation sites were shown as red characters on each deletion. Bottom: an *in vitro* dephosphorylation assay was performed by incubation of f:Brd4 deletions in the absence or presence of CIP. The reactions were analyzed by SDS-PAGE and Coomassie staining. (**B**) CK2 predominantly phosphorylates the PDID. Bacterially purified Brd4 (287-530) was incubated with various kinases in an optimized buffer condition. The upper gel shows autophosphorylation of kinases and the bottom gel represents phosphorylated Brd4 (287-530). The kinase phosphorylating PDID is marked in red color.

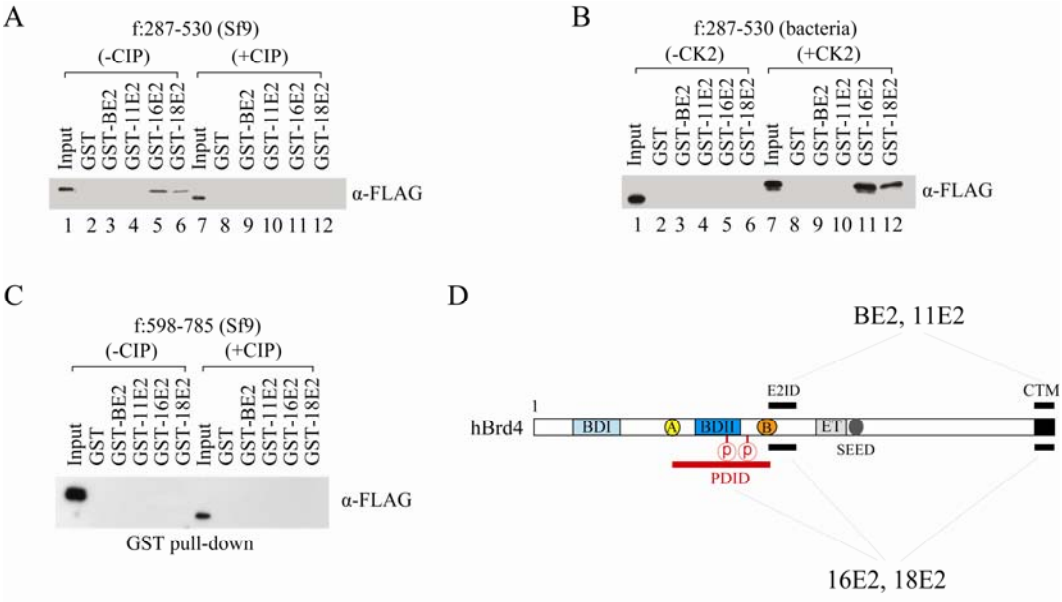

Figure 3-6. Phosphorylation of PDID by CK2 determines the interaction with high risk E2

(**A**) Phosphorylation is essential for PDID-mediated interaction with high risk E2. A GST pull-down assay was performed by incubating GST-E2 proteins with untreated or CIP-treated f:287-530 (Sf9) and bound Brd4 proteins were detected by Western blotting with anti-FLAG M2 antibody. (**B**) CK2 phosphorylation causes interaction with high risk E2. An *in vitro* CK2 kinase assay was performed by incubating Brd4 deletion f:287-530 (bacteria) with CK2. A GST pull-down assay was performed by incubating GST-E2 proteins with untreated or CK2-treated f:287-530 (bacteria) and bound Brd4 proteins were detected by Western blotting with anti-FLAG M2 antibody. (**C**) Phosphorylation of Brd4 (598-785) is not important for E2-interaction. A GST pull-down assay was performed by incubating GST-E2 proteins with untreated or CIP-treated Brd4 deletion f:598-785 (Sf9) and bound Brd4 proteins were detected by Western blotting with anti-FLAG M2 antibody. (**D**) Summary of E2-interacting regions on Brd4. Black bars indicate E2-binding regions commonly shared by B/11/16/18E2. PDID is marked as red bar and can be phosporylated as shown in circled letter "P".

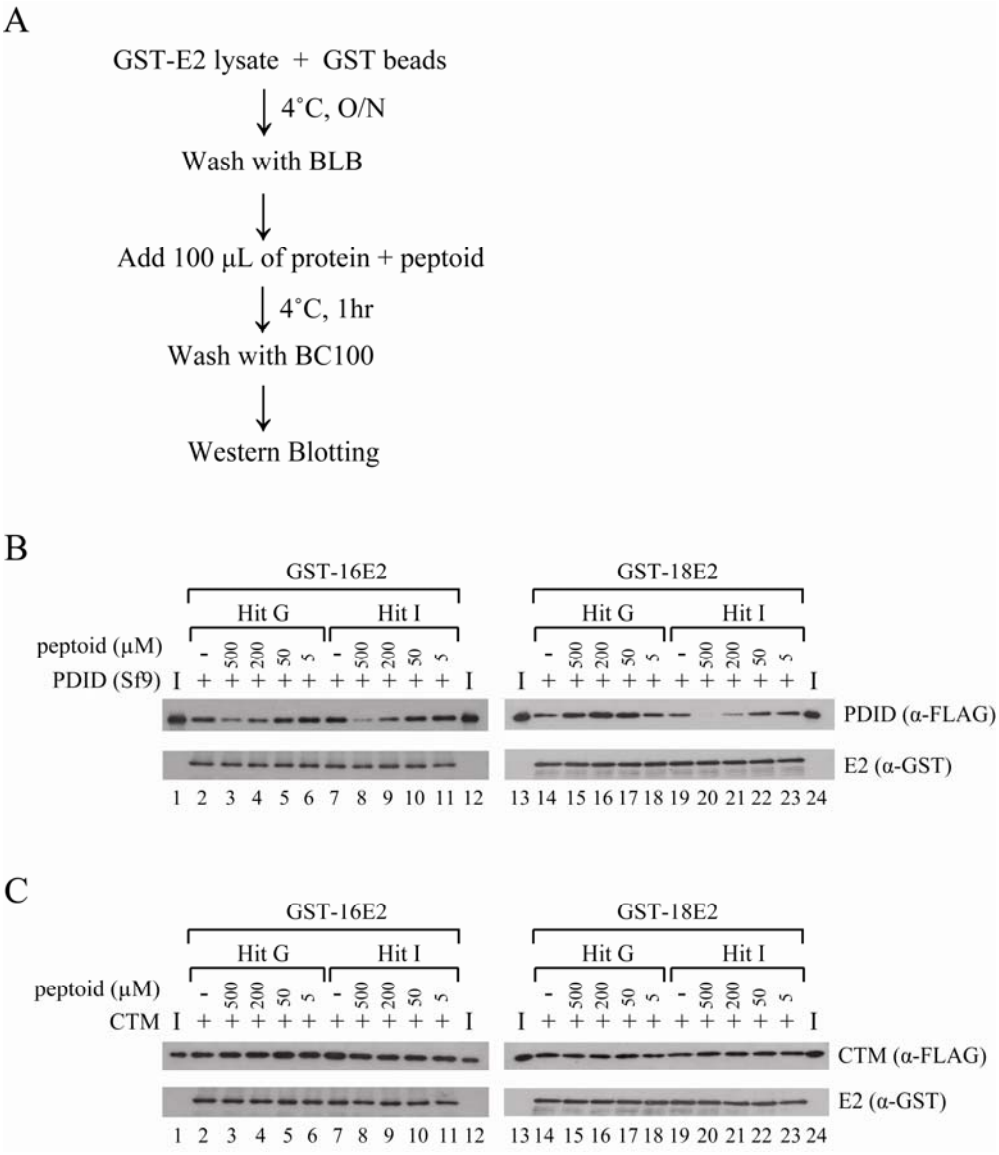
A
GST-E2 lysate + GST beads
4°C, O/N
Wash with BLB
Add 100 µL of protein + peptoid
4°C, 1hr
Wash with BC100
Western Blotting
B
GST-16E2
GST-18E2
Hit G
Hit I
peptoid (µM)
- 500 200 50 5 - 500 200 50 5
PDID (Sf9) I + + + + + + + + + + I I + + + + + + + + + + I
PDID (α-FLAG)
E2 (α-GST)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
C
GST-16E2
GST-18E2
Hit G
Hit I
peptoid (µM)
- 500 200 50 5 - 500 200 50 5
CTM I + + + + + + + + + + I I + + + + + + + + + + I
CTM (α-FLAG)
E2 (α-GST)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

Figure 3-7. Phosphorylated PDID-specific peptoids block the interaction between high risk E2 and phospho-PDID
(**A**) Experimental flow chart. The peptoid competition assay was performed as described for GST pull-down, except that peptoid was added to the interaction solution with the interacting protein and GST protein. (**B**) PDID purified from insect cells was incubated with GST-E2 proteins in the absence or presence of the indicated amount of peptoid (Hit I or G). The bound PDID and GST-E2 proteins were analyzed through Western blotting with anti-FLAG M2 and anti-GST antibodies, respectively. (**C**) Bacterially purified CTM was incubated with GST-E2 proteins in the absence or presence of peptoids (Hit I or G).

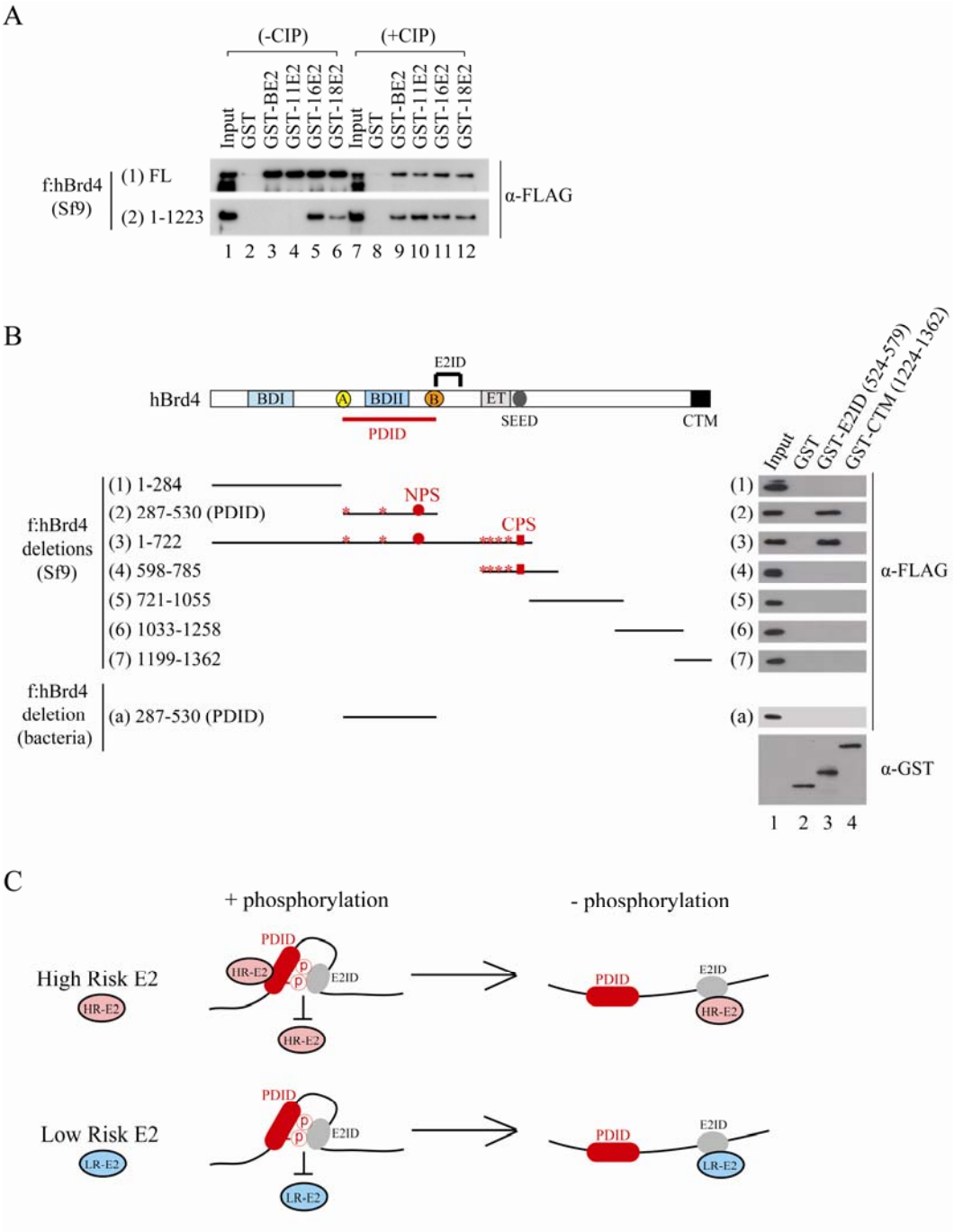
A
(-CIP)
(+CIP)
Input
GST
GST-BE2
GST-11E2
GST-16E2
GST-18E2
f:hBrd4 (Sf9)
(1) FL
(2) 1-1223
α-FLAG
1 2 3 4 5 6 7 8 9 10 11 12
B
E2ID
hBrd4
BDI
A
BDII
B
ET
SEED
CTM
PDID
Input
GST
GST-E2ID (524-579)
GST-CTM (1224-1362)
f:hBrd4 deletions (Sf9)
(1) 1-284
(2) 287-530 (PDID)
(3) 1-722
(4) 598-785
(5) 721-1055
(6) 1033-1258
(7) 1199-1362
NPS
CPS
f:hBrd4 deletion (bacteria)
(a) 287-530 (PDID)
α-FLAG
α-GST
1 2 3 4
C
+ phosphorylation
- phosphorylation
High Risk E2
HR-E2
PDID
E2ID
P
Low Risk E2
LR-E2

Figure 3-8. Phosphorylation of PDID masks E2ID from E2-binding
(**A**) A GST pull-down assay was performed by incubating GST-E2 proteins with untreated or CIP-treated FL or C-terminal truncation mutant (1-1223) of Brd4. Bound Brd4 proteins were detected by Western blotting with anti-FLAG M2 antibody. (**B**) GST-tagged E2ID or CTM (1224-1362) proteins were immobilized and incubated with FLAG-tagged Brd4 deletions purified from insect cells. Bound f:Brd4 proteins and GST-tagged proteins were detected by Western blotting with anti-FLAG M2 antibody and anti-GST antibody, respectively (right). The putative CK2 phosphorylation sites are shown in red. (**C**) Once PDID is phosphorylated, high risk E2 can recognize PDID. At the same time, phosphorylated PDID interacts with E2ID through intra-molecular interaction, thereby blocking E2ID-mediated interaction with E2.

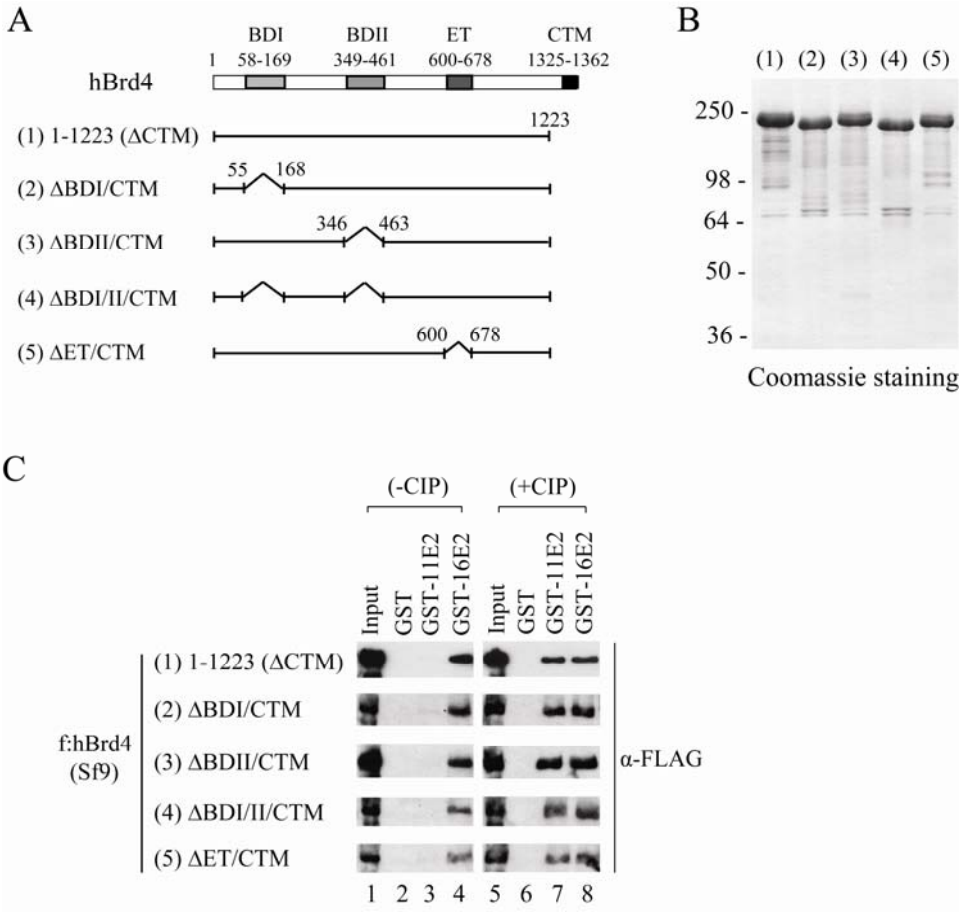

Figure 3-9. BDII is dispensable for PDID function
(**A**) Schematic representation of domain-specific deletion mutants of Brd4. The numbers represent the position of amino acid residues. (**B**) Brd4 proteins were expressed and purified from Sf9 insect cells and analyzed though SDS-PAGE and Coomassie staining. The protein size (in kDa) is marked on the left. (**C**) A GST pull-down assay was performed by incubating GST-E2 proteins with untreated or CIP-treated deletion mutant of Brd4. Bound Brd4 proteins were detected by Western blotting with anti-FLAG M2 antibody.

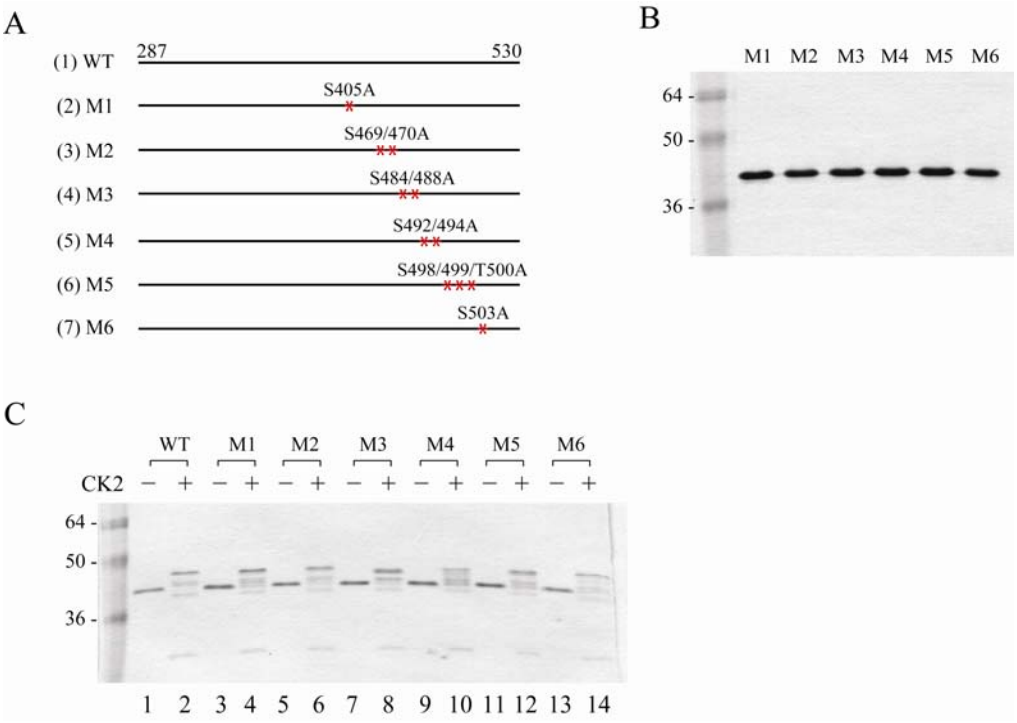

Figure 3-10. PDID point mutants

(**A**) Schematic drawing of WT and site-directed mutants of PDID. (**B**) Coomassie blue staining of purified PDID proteins. Recombinant FLAG-tagged wild-type PDID and its site-directed mutants were respectively purified from bacterial cells. Protein size markers (in kDa) are indicated on the left. (**C**) An *in vitro* kinase assay was performed by incubation of WT or mutants of PDID in the absence or presence of CK2. The reactions were analyzed by SDS-PAGE and Coomassie staining.

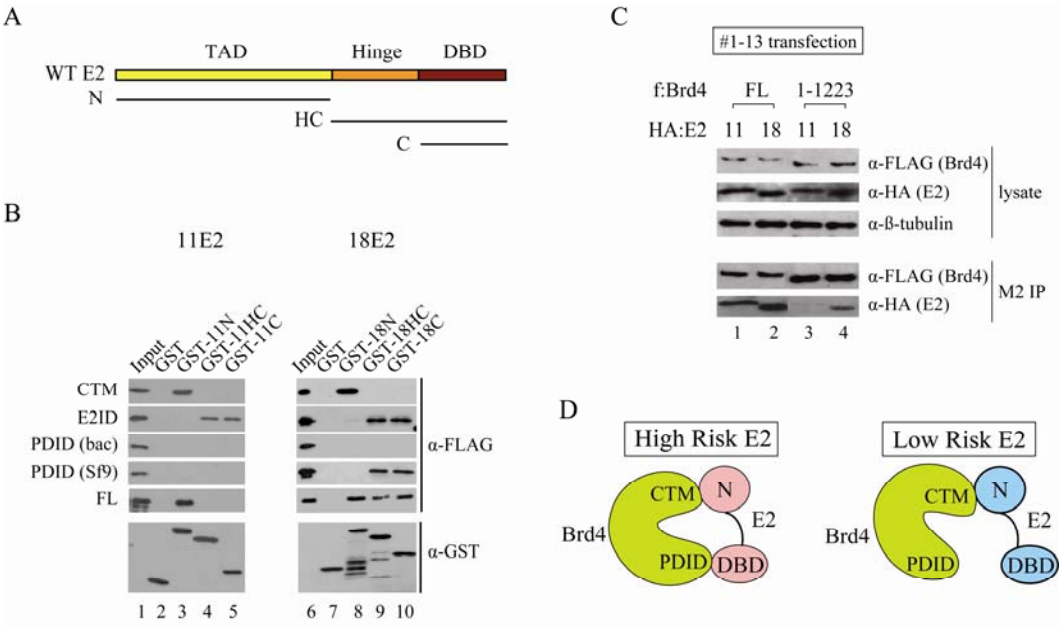

Figure 3-11. Phosphorylated PDID binds to the C-terminal region of high risk E2

(**A**) Schematic of FL and deletion mutants of E2. (**B**) GST-tagged 11E2 or 18E2 deletions were incubated with either bacterially purified CTM, E2ID, PDID or insect cell purified PDID and FL Brd4 protein. (**C**) #1-13 (Stable Brd4-knockdown cells) were transfected as indicated and treated with MG-132. The equality of amounts of lysate for immunoprecipitation (IP) among various transfections was evaluated by Western blotting with anti-β-tubulin antibody. M2 agarose beads were used to precipitate Brd4 proteins (FL and 1-1223) and their associated proteins. Brd4 and E2 proteins in lysate or in IP were detected using anti-FLAG M2 or anti-HA (Covance), respectively. (**D**) While low risk E2 binds to E2 protein through CTM of Brd4, high risk E2 recognizes CTM and PDID through its transactivation domain and DNA-binding domain, respectively.

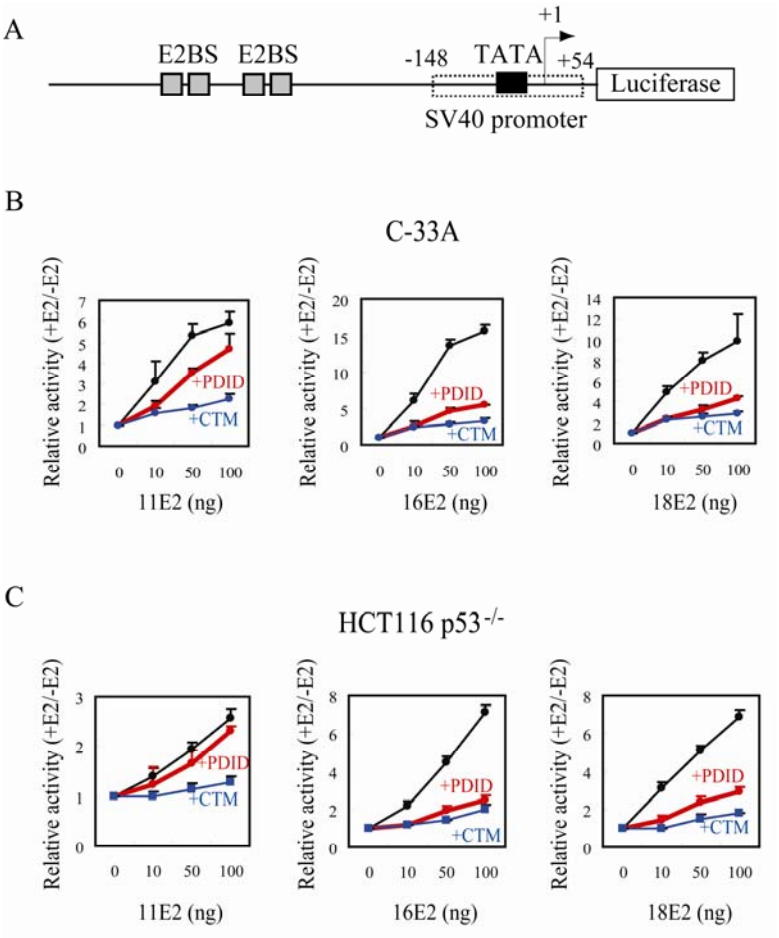

Figure 3-12. PDID-mediated interaction with high risk E2 is important for its transactivation

(**A**) Schematic representation of the p2x2xE2BS-luc reporter. The numbers indicate the positions of nucleotides relative to the transcription start site (+1) in the SV40 promoter. (**B**) Transfection was performed in C-33A cells by cotransfecting the p2x2xE2BS-luc reporter with an increasing amount of E2 expression plasmid in the absence or presence of PDID or CTM expression plasmid. The relative activity represents the ratio of luciferase activity with over without E2 expression. Error bars indicate the standard deviation calculated from two independent experiments performed in duplicate. (**C**) The same set of experiments and analysis were performed in HCT116 p53 null ($p53^{-/-}$) cells.

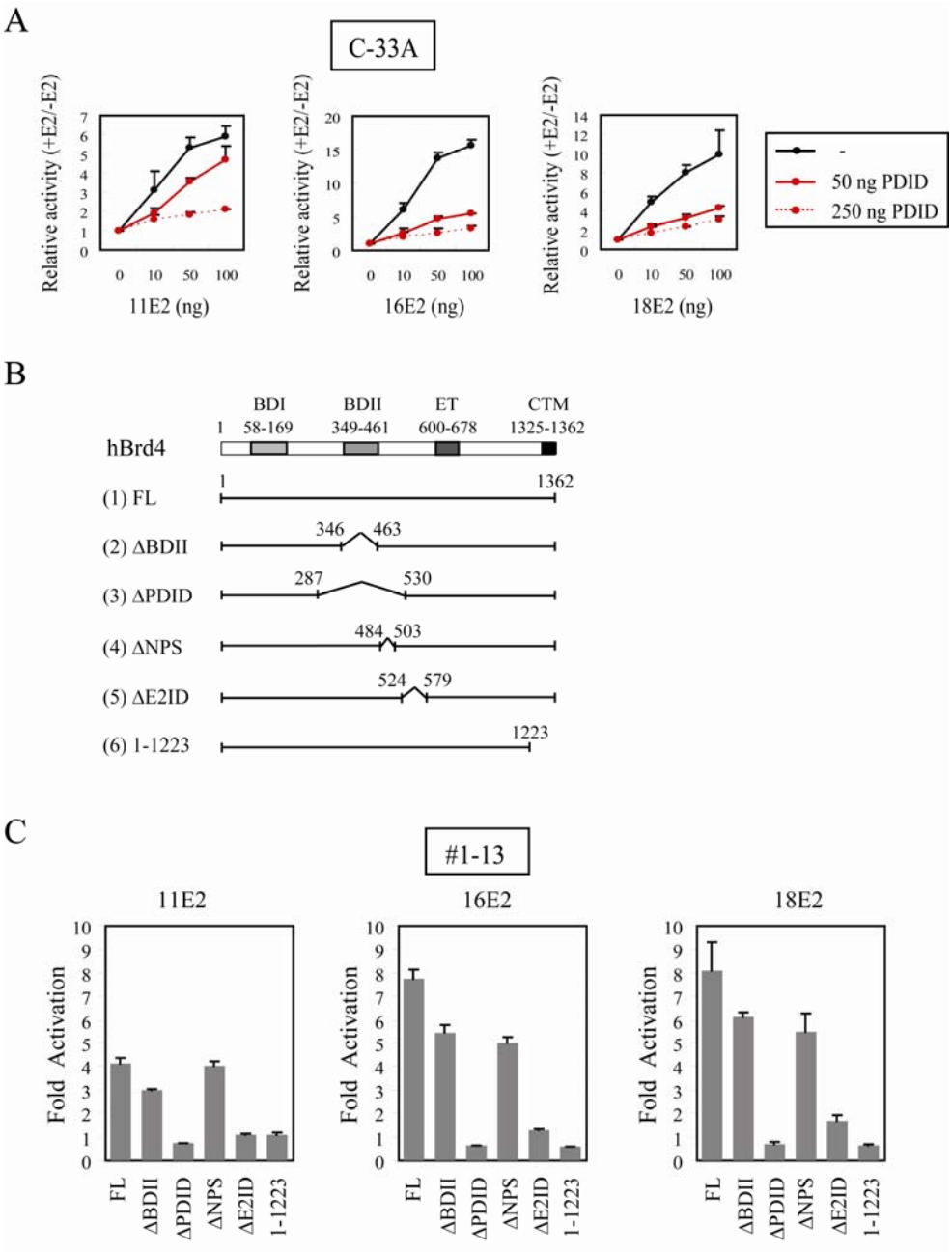
A
C-33A
Relative activity (+E2/-E2)
11E2 (ng)
16E2 (ng)
18E2 (ng)
-
50 ng PDID
250 ng PDID
B
BDI
BDII
ET
CTM
1 58-169 349-461 600-678 1325-1362
hBrd4
(1) FL
(2) ΔBDII
(3) ΔPDID
(4) ΔNPS
(5) ΔE2ID
(6) 1-1223
1
1362
346 463
287 530
484 503
524 579
1223
C
#1-13
11E2
16E2
18E2
Fold Activation
FL
ΔBDII
ΔPDID
ΔNPS
ΔE2ID
1-1223

Figure 3-13. PDID and E2ID are generally important for E2-mediated transactivation

(A) Transfection was performed in C-33A cells by cotransfecting the p2x2xE2BS-luc reporter with E2 expression plasmid in the absence or presence of increasing amounts of PDID expression construct. The relative activity represents the ratio of luciferase activity with over without E2 expression. Error bars indicate the standard deviation calculated from two independent experiments performed in duplicate. (**B**) Schematic representation of domain-specific deletion mutants of Brd4. The numbers represent the position of amino acid residues or the boundaries of protein domains deleted. (**C**) Transfection was performed in #1-13 cells with the p2x2xE2BS-luc reporter, an E2 expression plasmid, and a FL or domain-deleted Brd4 expression plasmid as described in Materials and Methods. The bar graph represents two independent experiments performed in duplicate with error bars indicating the standard deviation.

Table 3-1. Putative or identified phosphorylation residues on PDID

Putative P-sites	Biological P-sites	P-site from Sf9 cells
S405	S469	T500
S484	S470	
S488		
S492		
S494		
S498		
S499		
S503		

CHAPTER IV:

ROLE OF BRD4-INTERACTING CELLULAR FACTORS ON HPV TRANSCRIPTION

INTRODUCTION

Brd4 is a ubiquitously expressed nuclear protein with double bromodomains, which are acetyl-lysine targeting modules. It associates with chromatin via preferential recognition of acetylated lysine 14 on histone H3 and lysine 5/12 on H4 (Dey et al, 2003; Dey et al, 2000). Since Brd4 associates with the mitotic chromosome, whereas all other chromain-associating factors are dispersed, it has been suggested that it functions as a transmitter of epigenetic memory to the next generation. Brd4 does not possess DNA sequence specificity or any known enzymatic activity. Therefore, this chromatin adaptor which is involved in many biological activities does so mainly through the interaction with its many partners including: 1) P-TEFb (positive transcription elongation factor b) for transcription regulation of viral and cellular genes (Bisgrove et al, 2007; Jang et al, 2005; Mochizuki et al, 2008; Yang et al, 2008; Yang et al, 2005; Zhou et al, 2009), 2) papillomavirus E2 for viral genome segregation, transcription, and protein stability (Jang et al, 2009; Lee & Chiang, 2009; Schweiger et al, 2006;

Wu et al, 2006; You et al, 2004), 3) SPA-1 (signal-induced proliferation-associated protein 1) for cell cycle progression (Farina et al, 2004), 4) RFC-140 (replication factor 140) for DNA replication (Maruyama et al, 2002), 5) acetylated RelA (a subunit of NF-κB) for transcription regulation (Huang et al, 2009), 6) gammaherpesvirus orf73 for transcription (Ottinger et al, 2006; Ottinger et al, 2009), and 7) EBNA1 (Epstein-Barr virus nuclear antigen 1) of EBV for transcription (Lin et al, 2008a).

The eukaryotic genome is packaged into chromatin, which is a periodic nucloeprotein complex. Since chromatin dynamics influence many fundamental biological activities, many studies have recently been focused on chromatin-associating proteins, such as chromatin modifying enzymes. ACF is an ATP-utilizing chromatin assembly and remodeling factor which modulates the internucleosomal spacing of chromatin through an ATP-dependent mechanism (Ito et al, 1997). It consists of two subunits, ISWI, which has ATPase activity, and Acf1 with a bromodomain. These subunits function synergistically in the assembly of chromatin (Ito et al, 1999). Chromatin-remodeling factors participate in many DNA-related activities such as transcriptional regulation, DNA repair, homologous recombination, and chromatin assembly through their interplay with other cellular factors (Lusser & Kadonaga, 2003). Thus, the identification and functional characterization of their interacting proteins would facilitate understanding about chromatin biology.

p53 is a sequence-specific transcription factor and a tumor suppressor protein whose activity is tightly regulated by its posttranslational modification status and interacting proteins. Recently, p53 was found to be an interacting partner of the transcription factor E2 that is encoded by cancer-causing high risk HPV (Parish et al, 2006b). High risk E2, but not low risk E2, enhances p53-mediated transcription activation (Parish et al, 2006b). On the other hand, p53 represses 16E2-induced transcription activation (Brown et al, 2008). This data suggests significant interplay between two factors in their regulation of transcription, although the exact mechanism has not been revealed yet.

In this study, we identified novel Brd4-interacting proteins: Acf1, mG9a, p53, YY1, AP2, c-Jun, c-myc/max, mC/EBPα, hC/EBPβ, and Gal4-Pro. Among these, the functional effect of the interactions with Brd4- with Acf1 and p53 were examined. In particular, p53 was found to be a new HPV chromatin transcription activator which it targets the same interacting domains on Brd4 as high risk E2, suggesting its involvement in HPV biology.

MATERIAL AND METHODS

Plasmid construction

Bacterial expression plasmids pF:hBrd4(1-530)-11d, pF:hBrd4(1-722)-11d, pF:hBrd4(514-579)-11d, pF:hBrd4(598-785)-11d, pF:hBrd4(721-1055)-11d, and pF:hBrd4(1110-1362)-11d were constructed to prepare Brd4 deletion mutant proteins from BL21(DE3)pLysS. The corresponding coding sequences were generated by PCR amplification with a NdeI site-containing sense primer and a BamHI site-containing antisense primer using pcDNA3-F:hBrd4(FL) (Lee & Chiang, 2009) as a template. After purification and enzyme restriction site digestion of the PCR product, it was cloned between NdeI and BamHI sites by swapping the insert with pF:TBP-11d (Chiang et al, 1993).

In vitro protein interaction

100 ng of purified target protein was mixed with 600 ng of Brd4 protein and 100 µl of BC100. After overnight incubation at 4^{o}C, 5 µl of CW151 (anti-Brd4C antibody) was added and further reacted at 4^{o}C for 2 hr. To immobilize the antibody and associating proteins, 10 µl (bed volume) of protein A/G beads were added and incubated at 4^{o}C for an additional 1 hr. After washing, Brd4 and its associating proteins were analyzed by Western blotting with antibodies specified in Figure 4-1 legend.

ATPase assay

The assay was performed as described by another lab (Corona et al, 1999) with some modifications. The indicated amount of ACF and Brd4 was incubated in a 15 μl reaction with final concentration of 6.6 mM HEPES (pH 7.6), 0.66 mM EDTA, 1 mM DTT, 0.033% NP40, 1.1 mM $MgCl_2$, 33 μM ATP at 30°C for 30 min. 1 μl of reaction was spotted on cellulose thin-layer chromatography plate (POLYGRAM CEL 300 PEI, MACHEREY-NAGEL) and separated in 1 M formic acid and 0.5 M LiCl. ATP and free phosphate were visualized by PhosphoImager.

DNase I footprinting with chromatin and DNA templates

For figure 4-2, HPV chromatin was assembled *in vitro* using p7072-70GLess/I^+ DNA (Hou et al, 2000) with recombinant hNAP-1, *Drosophila* ACF, and HeLa core histones according to the published protocol (Wu et al, 2006). To remove ATP, the assembled chromatin was treated with the indicated amount of apyrase or was left untreated. For DNase I footprinting, a 30-μl reaction containing 2 μl of HPV chromatin or mock-assembled DNA (i.e., without HeLa core histones, hNAP-1 and ACF), with or without 50 ng of hBrd4 and a varying amount (10, 30, 100, or 300 ng) of 11E2, was incubated in transcription buffer (Wu et al., 2003) at 30°C for 30 min. The incubated chromatin or DNA (12.5 μl)

was digested with 0.25 U (chromatin) or 0.025 U (DNA) of DNase I (Invitrogen) at room temperature for 2 min. The reaction was terminated by adding 15 µl of DNase I stop solution containing 200 mM Tris-HCl (pH 7.5), 50 mM EDTA, 2% SDS, 200 µg/ml of proteinase K, and 250 µg/ml of glycogen, and then was treated with 20 µg of proteinase K at 60°C for 30 min, followed by a phenol/chloroform extraction. The soluble portion was precipitated with 200 µl of 5 M ammonium acetate, 2 µl of glycogen (10 mg/ml), and 1 ml of 100% ethanol and was incubated at -20ºC overnight. The precipitated DNA was washed with 70% ethanol and air-dried. The pellet was resuspended in 10 µl of H_2O. The resulting DNA was then amplified by asymmetric PCR for 2 cycles of the following three steps: 95ºC for 1 min, 68ºC for 2 min, and 76ºC for 2 min for in a 25-µl reaction containing 10 µl of DNA, 0.4 U of Vent DNA Polymerase (New England Biolabs), 0.1 pmole of the ^{32}P-labeled Gless(AS)-31/55 primer (Wu et al, 2006), 0.2 M dNTP, 40 mM NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM $MgSO_4$, 0.01% gelatin (Sigma), and 0.1% Triton X-100. The PCR products were mixed with 125 µl of stop solution containing 10 mM Tris-HCl (pH 7.5), 4 mM EDTA, 260 mM sodium acetate (pH 5.2), and 20 µg of tRNA, and then was precipitated with 700 µl of 100% ethanol on dry ice for 10 min. The DNA fragments were spun, dried, resuspended in 6 µl of formamide dye (90% formamide, 1x TBE, and 0.02% bromophenol blue/xylene cyanol), and were analyzed in a 6% polyacrylamide/7 M urea gel at 75 W for 2 hr. The gel was transferred onto chromatography paper

(Fisher), dried, and exposed in a PhosphorImager screen. Signals were detected by Typhoon 9200 PhosphorImager (GE Healthcare). For Figure 4-8, the p53-responsive templates described in text, were used for mock and chromatin assembly and DNase I footprinting as illustrated above, except for apyrase treatment.

In vitro transcription

HPV chromatin, assembled as described above, was used for transcription experiments according to the published scheme (Wu et al, 2006) with minor modifications. Briefly, a 30-μl reaction containing 6 μl of *in vitro*-assembled chromatin, 30 μM acetyl-CoA, and 20 ng of p300 was incubated in the absence or presence of indicated amount of proteins, in transcription buffer at 30^{o}C for 30 min. 8 μl of HeLa nuclear extract (8-10 mg/ml) and 10 ng of pMLΔ53 DNA were then added and incubated at 30^{o}C for another 30 min. Transcription was initiated by adding [α-^{32}P]CTP and NTP mix, and incubated for another 1 hr. The reactions were terminated and processed as described previously (Wu et al, 2006). Relative transcription (Rel Txn) was defined as the signal intensity quantified by PhosphorImager from the HPV chromatin relative to that performed in the presence of 60 ng of AP-1.

Coimmunoprecipitation

To test the *in vivo* interaction between Brd4 and p53, HEK293 nuclear extract (NE) was used. The extract was pre-cleared by incubation with protein A/G beads (Amersham Pharmacia Biotech) at 4°C for 1 hr to remove proteins exhibiting nonspecific binding. Then, 2 μl of preimmune serum or anti-Brd4N antibody (CW152) was added to the pre-cleared extract and was incubated at 4°C overnight. Brd4 and its associating proteins were immobilized with protein A/G beads at 4°C for additional 1 hr and analyzed through Western blotting with indicated antibodies.

GST pull-down assay

GST pull-down was performed as described previously (Wu and Chiang, 2001). Briefly, GST alone or GST-tagged p53 protein was expressed in BL21(DE3)pLysS and the bacterial lysates were prepared. For the assay, 400 ng of GST proteins were immobilized onto 10 μl of glutathione-Sepharose^{TM} 4B beads (Amersham Pharmacia Biotech) at 4°C overnight. After washing, the beads were incubated with100 ng of purified Brd4 proteins at 4°C for 1 hr and washed again. 50 μl of 2X protein sample buffer was added to the protein-bound beads and the sample was analyzed by Western blotting.

For the interaction assay with phosphorylated or dephosphorylated proteins, kinase or phosphatase reaction was performed as illustrated below. 20 μl of each reaction was mixed with 80 μl of BC100 buffer (20 mM Tris-HCl [pH

7.9], 100 mM KCl, 0.2 mM EDTA, 20% glycerol). 100 µl of reaction was interacted with immobilized GST beads and further processed as mentioned above.

In vitro dephosphorylation assay

200 ng of purified protein from insect cells was incubated with or without 1 µl of alkaline phosphatase (1 U/µl, Roche) at 30°C for 1 hr in a 20 µl reaction. To analyze the mobility shift by the dephosphorylation reaction, the sample was separated by SDS-PAGE and visualized through Coomassie staining.

Kinase assay

200 ng of bacterially purified Brd4 protein were incubated with 200 µM of ATP-containing buffer in the absence or presence of 50 U of CK2 (New England Biolabs) at 30°C for 30 min in a 20 µl reaction. The product was analyzed through Coomassie staining.

RESULTS

Brd4 interacts with various proteins

Since Brd4 exerts its functions through its protein-protein interactions, identification of novel Brd4-interacting proteins would facilitate the understanding of the diverse functions of Brd4. Thus, we aimed to screen interacting partners of Brd4 through pull-down assays with purified factors classified into different groups based on their function as follows: general transcription factors, an elongation factor, Mediator, sequence-specific transcription factors, chromatin modifiers including histone chaperone, chromatin remodeling factors, histone methyltransferases, and histone actetyltransferases, and etc. (Figure 4-1A). Each individual factor and Brd4 were incubated together and anti-Brd4C antibody was used to immunoprecipitate Brd4 and its associating factors. The proteins were further analyzed by Western blotting with appropriate antibodies as indicated in the figure legend. Among the 55 factors, 10 proteins interacted with Brd4; Acf1 (subunit of ACF remodeling factor), mouse G9a (mG9a), p53, YY1, AP2, c-Jun (subunit of AP-1), c-myc/max, mouse C/EBPα (p42 subunit), human C/EBPβ (LIP), Gal4-Pro (fusion protein of DNA binding domain of galactose 4 and the proline-rich activation domain of human CTF).

Since the factors were immunoprecipitated with anti-Brd4C antibody, if the candidate protein interacts through the C-terminal region of Brd4 recognized

by antibody, it is possible that the antibody-Brd4 interaction might compete for Brd4-factor binding, and a Brd4-interacting protein could not have been able to be pulled down with Brd4 properly. To explore this possibility, a protein interaction assay was performed with two different antibodies targeting N-terminal (hBrd4 aa 149-284) or C-terminal (hBrd4 aa 1199-1362) region of Brd4 (Figure 4-1B). Whereas mG9a and mC/EBPα (p42) were pulled down with both antibodies, C/EBPβ (LIP) showed the interaction with Brd4 only when anti-Brd4N antibody was used, indicating that C/EBPβ (LIP) might bind to C-terminal region of Brd4. Collectively, these data show that Brd4 interacts with multiple cellular factors and that this interaction could be influenced by assay conditions (See Discussion).

Brd4 neither possess ATPase activity nor does it modulates the ATPase activity of ACF, but ATP is required for Brd4-E2 binding to the chromatin template

Our preliminary data shows that the E2-Brd4 complex represses HPV transcription at a step post p300-mediated acetylation of nucleosomal core histones, but prior to the assembly of a transcriptional preinitiation complex (PIC), suggesting that chromatin remodeling may be involved in E2-Brd4-mediated transcriptional silencing (Wu & Chiang, unpublished). This notion was further supported by a restriction enzyme accessibility assay, showing that a structural change in nucleosome positioning could be detected on HPV chromatin in the

presence of E2 and Brd4 in an ATP- and E2-binding site-dependent manner (Wu & Chiang, unpublished). Since Brd4 specifically binds to ACF through the interaction with the Acf1 subunit, which is another bromodomain protein (Figure 4-2A), we hypothesized that Brd4 could be involved in a nucleosome remodeling event with following possibilities: 1) Brd4 might remodel nucleosomes using ATP hydrolysis energy, 2) Brd4 might regulate remodeling activity of ACF through its protein-protein interaction, and 3) an ATP-mediated remodeling event is required for E2-Brd4 binding, further inducing reorganization of nucleosomes. To test the first and second possibilities, an ATPase assay was performed to see whether Brd4 itself has ATPase activity, and/or whether Brd4 modulate ATPase activity of ACF via its protein-protein interaction. Since ACF has been known to hydrolyze ATP only in the presence of a chromatin template (Corona et al, 1999), bulk chromatin isolated from HeLa cell was used for assay. As shown in Figure 4-2B, Brd4 neither possesses ATPase activity nor modulates the activity of ACF. Thus, we explored the third possibility by performing DNase I footprinting with or without apyrase treatment to see if ATP depletion changes the E2-Brd4 binding on chromatin template (Figure 4-2C). ATP removal blocked the E2-Brd4 recognition of E2 binding sites on the chromatin template in a dosage-dependent manner without affecting the factor binding on naked DNA template (Figure 4-2D, compare lanes 2 and 4, and 10 and 12). Even though a more detailed mechanism

study needs to be conducted, this data suggests that ATP-dependent remodeling is required for E2-Brd4 binding, causing further nucleosome reorganization.

AP1 and p53 activate HPV chromatin transcription

Since a certain set of sequence-specific transcription factors interacts with Brd4 (see Figure 4-1A), and some of these can bind to and regulate the HPV early promoter, we performed an *in vitro* transcription assay with purified factors (Figure 4-3A) to see if any of these factors can regulate the transcription of HPV chromatin. Besides AP-1, which is well-characterized HPV transcription activator, p53 activates HPV chromatin transcription in a dosage-dependent manner (Figure 4-3B). Interestingly, sequence analysis for putative factor binding sites did not reveal any p53 binding site on the HPV early promoter (data not shown), suggesting that there could be cryptic p53 recognition sites. Since p53 is a Brd4-interacting partner, we wondered if Brd4 has any effect on p53-mediated chromatin transcription. Thus, *in vitro* transcription was performed in the absence or presence of the indicated amount of p53 and Brd4 (Figure 4-3C). p53-activated HPV chromatin transcription was suppressed by the addition of Brd4 in a dosage-dependent manner, indicating that Brd4 plays a negative role in p53-transactivation through an unknown mechanism.

p53 recognizes PDID and E2ID of Brd4

To further understand the function of the p53-Brd4 interaction, first we confirmed the interaction between the two factors *in vivo* via coimmunoprecipitation with nuclear extract (NE) of 293 cells as illustrated in Figure 4-4A. p53 is pulled down by anti-Brd4 antibody but not by pre-immune serum, indicating an *in vivo* interaction exists between p53 and Brd4 (Figure 4-4B). To map the Brd4 region recognized by p53, Brd4 deletion mutants encompassing the entire region of full-length protein were bacterially purified and used for the interaction with GST alone or GST-linked p53 (Figure 4-5A, upper panel). Deletion 1-722 and E2ID interacted with p53, suggesting that E2ID is the p53-interacting region. Interestingly, while unmodified (bacterially purified) PDID did not bind to p53, insect cell-purified PDID interacted with p53 (Figure 4-5A, compare deletion (3), upper panel and (a), bottom panel). Collectively, p53 binds to E2ID and posttranslationally modified PDID of Brd4, the same as high risk E2 does (Figure 4-5B).

Phosphorylation on PDID by CK2 determines the interaction with p53

Since PDID is phosphorylated when it is prepared from Sf9 insect cells (see Figure 3-4), and CK2-mediated phosphorylation of PDID is crucial for the interaction with high risk E2 (see Figure 3-6), we asked whether the CK2-phosphorylation on PDID is responsible for the interaction with p53. Sf9 insect cell-purified PDID and bacterially purified PDID were treated with alkaline

phosphatase from calf intestine (CIP) and CK2, respectively (Figure 4-6A). Once modified PDID is dephosphorylated, it loses its ability to interact with p53 (Figure 4-6B, lanes 1-6), indicating the importance of phosphoryaltion for the interaction. When the bacterial protein is phosphorylated by CK2, it becomes available for p53 binding (Figure 4-6B, lanes 7-12), suggesting that CK2-mediated phosphorylation is essential for p53-PDID interaction.

Brd4 enhances p53 binding to its cognate site in DNA

Our *in vitro* transcription assay and protein-protein interaction experiment showed that Brd4 affects p53-transactivation on HPV chromatin through the interaction between these factors (Figure 4-3C and Figure 4-5B). Thus, we hypothesized that Brd4 may regulate p53-responsive gene expression via the modulation of the DNA binding ability of p53, which is essential for its transactivation function. To test this idea, we performed DNase I footprinting with naked DNA or *in vitro* assembled chromatin of two different constructs including p53 binding sites which are: 1) pWWP-Luc with p21 promoter containing a p53 distal binding site (Figure 4-8A) (Wu & Chiang, 2009), and 2) pWAF-MLT containing a p53 binding site linked to the adenovirus major late core promoter preceding a 380 bp G-less cassette (Figure 4-8B) (Thomas & Chiang, 2005). p53 recognition of the DNA template is greatly enhanced in the presence of Brd4 for both constructs. However, in the chromatin template, p53

binding is altered, rather than enhanced. While the signal of hypersensitive sites is strengthened, the overall protection is reduced by the addition of Brd4. This is possibly linked to the inhibition of p53-transactivation seen in the *in vitro* transcription assay. Overall, this data suggests that Brd4 affects p53 binding to its cognate sequence from DNA and chromatin template through protein-protein interaction.

DISCUSSION

We have identified novel Brd4-interacting proteins as illustrated in Figure 4-1A. Given that Brd4 plays versatile roles based which protein(s) it is interacting with, each of these interactions possibly represent new functions of Brd4, such as modulating nucleosome remodeling through its interaction with ACF, regulating the methyltransferase activity of G9a, and functioning as a general chromatin adaptor for other sequence-specific transcription factors besides the ones already described. Even though many factors do not show the interaction with Brd4 under the assay conditions used, it is still possible that they might interact with Brd4 under optimized conditions for the following reasons: 1) the antibody used for immunoprecipitation may compete for the interaction between Brd4 and its associating protein, as proved through the experiment shown in Figure 4-1B, 2) some sequence-specific transcription factors may require the presence of DNA or chromatin template, which is omitted in this reaction, for the stable interaction with Brd4, and 3) proper posttranslational modification may be required for the protein-protein interaction. Analysis of the amino acid sequence which Brd4 binds to on Brd4-interacting proteins would be interesting, since it is possible that they share common domain recognized by Brd4. If so, it would be helpful to identify more Brd4-interacting targets.

Due to its interacting ability to bind ACF, Brd4 is speculated to modulate the nucleosome remodeling activity of ACF. However, an ATPase assay showed that Brd4 does not affect the ATPase activity of ACF. One possibility is that E2-responsive chromatin and E2 protein should be included in the ATPase assay system, since restriction enzyme accessibility assays show that Brd4-induced structural change of nucleosomes on HPV chromatin is an ATP- and an E2-binding-dependent (unpublished data). Another possibility is that the modulation of remodeling activity may not be through ATPase activity, because Brd4 binds to the Acf1 subunit, but not to the ATPase-containing subunit, ISWI. Thus, it would be interesting to test these possibilities to better understand the role of Brd4 in regulation in chromatin dynamics.

One interesting common feature of mammalian BET proteins (Brd2, Brd3, Brd4) is that they have the same preferences for the recognition of acetylated histone residues: lysine 14 on H3 and lysine 5/12 on H4 (Dey et al, 2003; LeRoy et al, 2008). Given the histone code hypothesis, their functional properties should be similar, but they are not. Conceivably, their interacting partners determine the functional readout of each family member. Thus, the identification and characterization of the function of their binding proteins will be helpful for better understanding about this chromatin-associating protein family.

It was intriguing to find that p53 is a novel HPV chromatin transcription activator and a Brd4-interacting protein. Since the involvement of Brd4 in HPV

biology absolutely occurs through its interaction with E2, it would be interesting to analyze the function of the interaction between Brd4 and p53 in HPV transcription as well as in p53-responsive gene regulation. Another fascinating aspect of this interaction is that p53 recognizes E2ID and phosphorylated PDID of Brd4 in a similar manner to high risk E2, indicating that the tumor suppressor p53 and the cancer-inducing high risk E2 may compete for Brd4-binding. Indeed, the interplay between p53 and high risk E2 has been reported recently (Brown et al, 2008; Parish et al, 2006b), and presumably, Brd4 is involved in this reciprocal action. In other words, the transcription cofactor function of Brd4 on one factor may be reversed by another factor through competitive binding to Brd4, thereby affecting each other's transcription output.

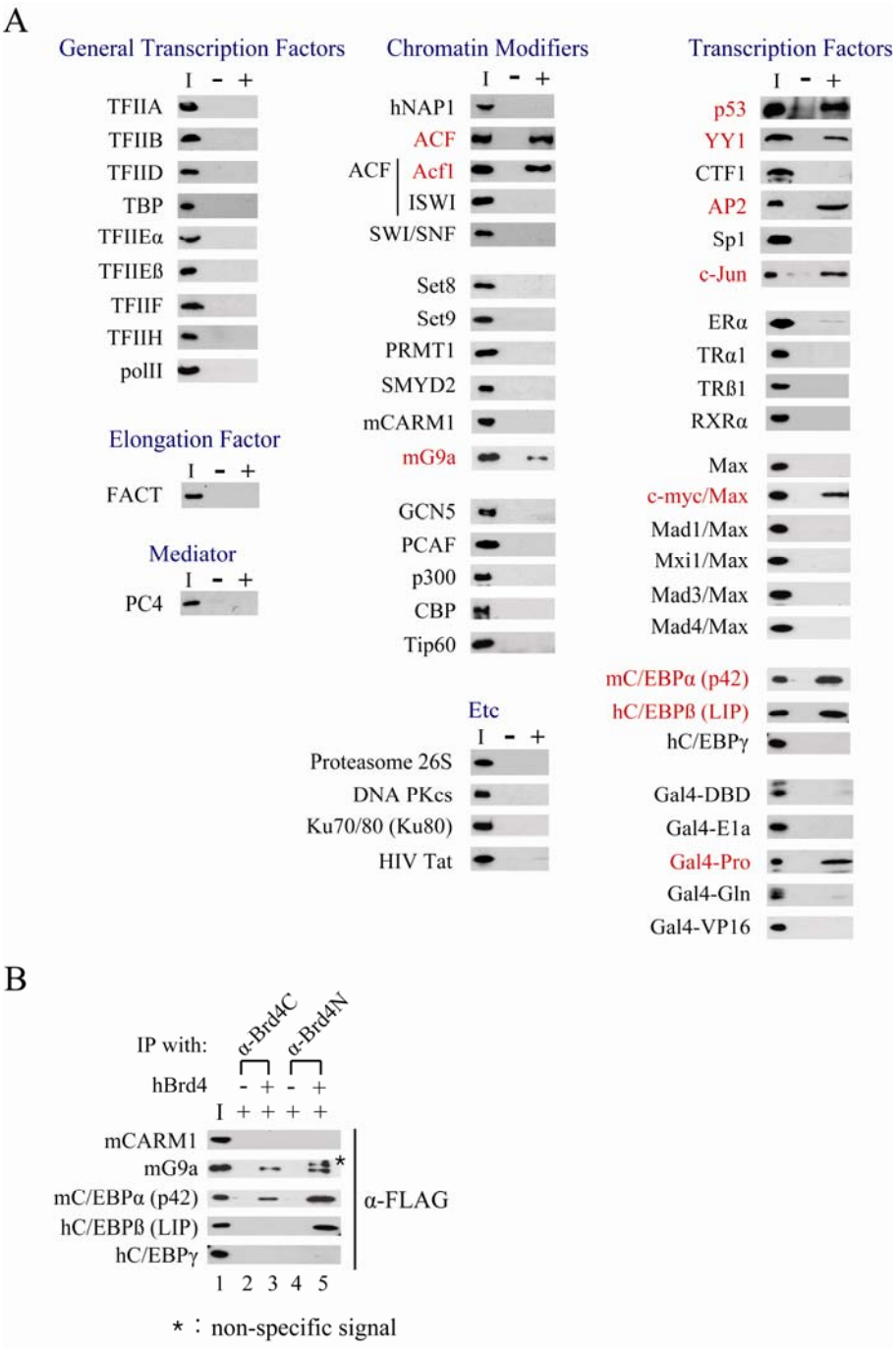
A
General Transcription Factors
I - +
TFIIA
TFIIB
TFIID
TBP
TFIIEα
TFIIEß
TFIIF
TFIIH
polII
Elongation Factor
I - +
FACT
Mediator
I - +
PC4
Chromatin Modifiers
I - +
hNAP1
ACF
ACF
Acf1
ISWI
SWI/SNF
Set8
Set9
PRMT1
SMYD2
mCARM1
mG9a
GCN5
PCAF
p300
CBP
Tip60
Etc
I - +
Proteasome 26S
DNA PKcs
Ku70/80 (Ku80)
HIV Tat
Transcription Factors
I - +
p53
YY1
CTF1
AP2
Sp1
c-Jun
ERα
TRα1
TRß1
RXRα
Max
c-myc/Max
Mad1/Max
Mxi1/Max
Mad3/Max
Mad4/Max
mC/EBPα (p42)
hC/EBPß (LIP)
hC/EBPγ
Gal4-DBD
Gal4-E1a
Gal4-Pro
Gal4-Gln
Gal4-VP16
B
IP with:
α-Brd4C
α-Brd4N
hBrd4
- + - +
I + + + +
mCARM1
mG9a
mC/EBPα (p42)
hC/EBPß (LIP)
hC/EBPγ
*
α-FLAG
1 2 3 4 5
* : non-specific signal

Figure 4-1. Screening of Brd4-interaction partners
(A) Brd4 interacts with various proteins. Bacterially or insect cell-purified proteins were incubated with Brd4 individually and precipitated with (+) or without (-) anti-Brd4C antibody (CW151). Input and pulled-down proteins were analyzed by Western blotting with anti-FLAG M2 antibody for the detection of most of proteins except for following proteins: anti-PC4 antibody for PC4, anti-Ku80 antibody for Ku70/80 (from Dr. David Chen, UT Southwestern), anti-PKcs antibody for DNA PKcs (from Dr. David Chen, UT Southwestern), anti-6His antibody (Santa Cruz) for TFIIF, p53, and TFIIA. Proteins were grouped based on their major function and the Brd4-bound proteins were marked in red. **(B)** Brd4 and target protein mix were pulled down in the absence (-) or presence (+) of two different Brd4 antibodies; anti-Brd4N antibody (CW152) and anti-Brd4C antibody (CW151). Input and pull-down proteins were analyzed by Western blotting with anti-FLAG M2 antibody. The asterisk (*) indicates a non-specific band.

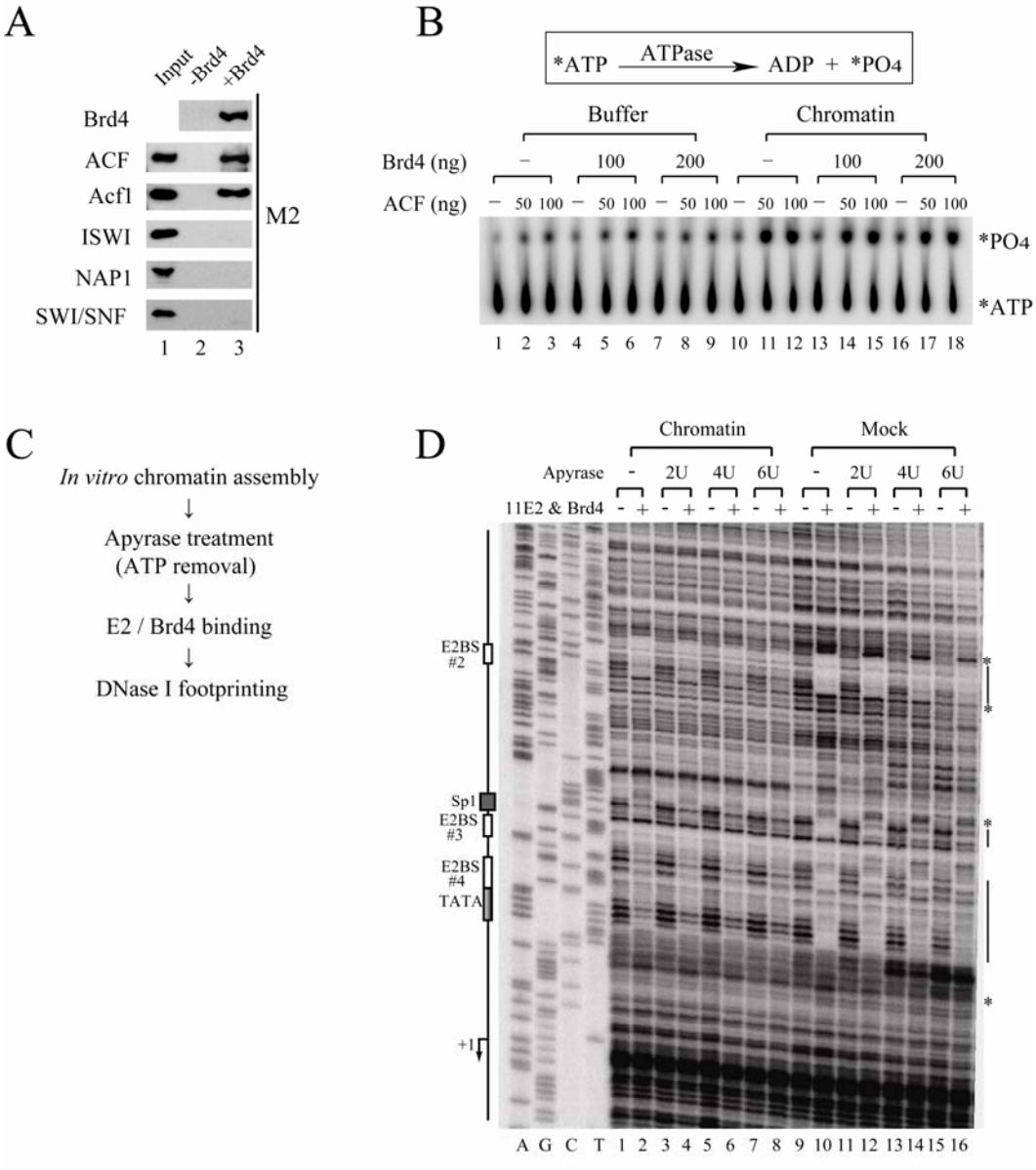
A
Input
-Brd4
+Brd4
Brd4
ACF
Acf1
ISWI
NAP1
SWI/SNF
M2
1 2 3
B
*ATP ATPase ADP + *PO4
Buffer
Chromatin
Brd4 (ng)
ACF (ng)
*PO4
*ATP
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
C
In vitro chromatin assembly
Apyrase treatment
(ATP removal)
E2 / Brd4 binding
DNase I footprinting
D
Chromatin
Mock
Apyrase
11E2 & Brd4
E2BS #2
Sp1
E2BS #3
E2BS #4
TATA
+1
A G C T 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Figure 4-2. Brd4 neither possess ATPase activity nor does it modulate ATPase activity of ACF, but ATP is required for Brd4/E2 binding to the chromatin template

(**A**) Brd4 selectively interacts with remodeling factor ACF. FLAG-tagged purified proteins were incubated in the absence (-) or presence of (+) FL Brd4. Input, Brd4, and Brd4-interacting proteins were analyzed with anti-FLAG M2 antibody. (**B**) γ-^{32}P-labeled ATP was incubated with the indicated amount of ACF and/or FL Brd4 proteins in the absence or presence of chromatin. Positions of labeled monophosphate (*PO_4) and ATP (*ATP) are indicated on the right. (**C**) Outline of chromatin footprinting. The assay was similarly performed as described for Fig. 2-3, except that the chromatin was incubated with or without apyrase before factor binding. (**D**) After apyrase treatment, *in vitro*-reconstituted HPV-11 chromatin was incubated with (+) or without (-) 30 ng of 11E2 and 50 ng of Brd4. Chromatin footprinting was conducted as described under Materials and Methods. Positions of protein factor-binding sites and the transcription start site (+1) are indicated on the left. The protected regions and hypersensitive sites (*) are marked on the right.

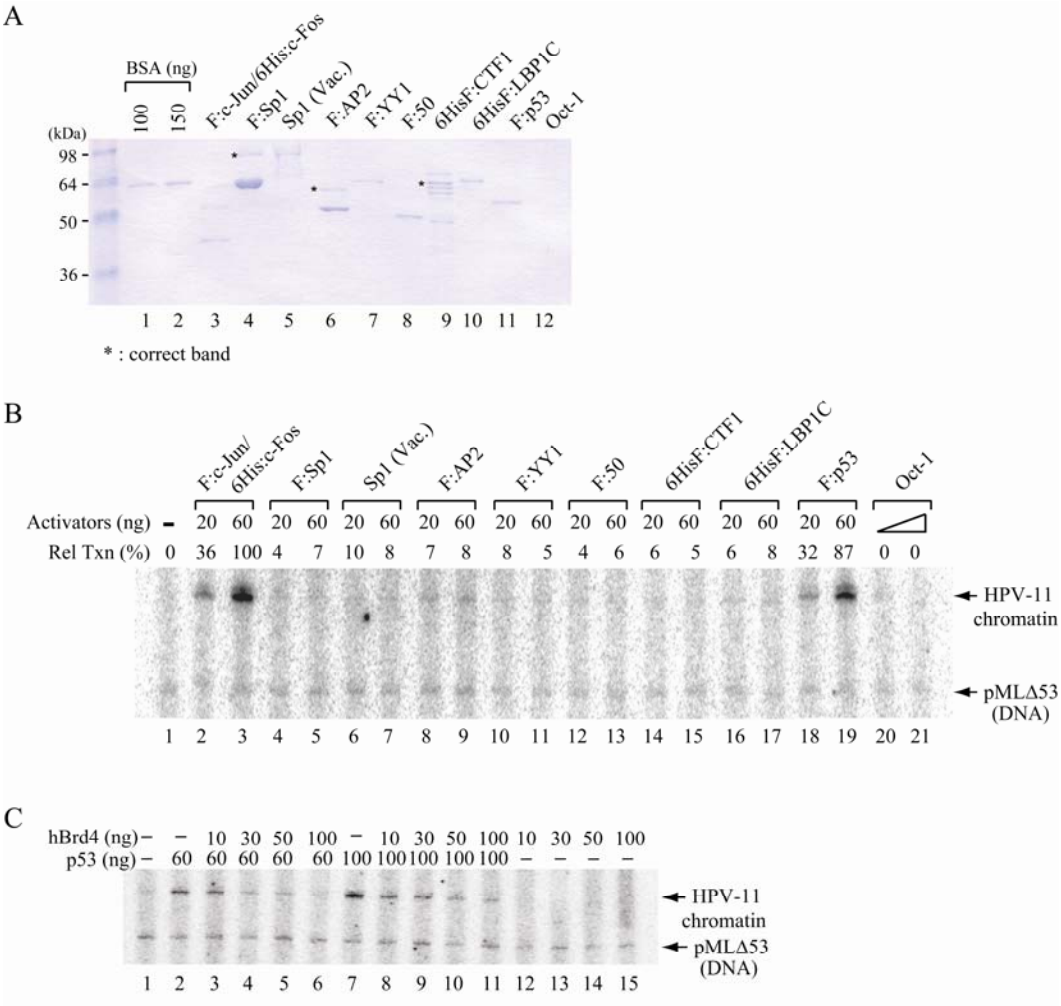

Figure 4-3. AP1 and p53 activate HPV chromatin transcription
(**A**) Coomassie blue staining of purified proteins. The asterisk (*) indicates the position of the correct protein. For quantitation, a known amount (100 or 150 ng) of BSA protein was loaded. Protein size markers (in kDa) are indicated on the left. (**B**) Activation of HPV-11 chromatin transcription by cellular factors. *In vitro* transcription was performed as described in Materials and Methods with HPV chromatin and an internal control DNA template (pMLΔ53) without (-) or with increasing amount of indicated proteins. Relative transcription (Rel Txn) was defined as the signal intensity quantified by a PhosphorImager from the HPV chromatin relative to that performed in the presence of 60 ng of AP-1 (F:c-Jun/6His:c-Fos). (**C**) Brd4 suppresses p53-mediated transactivation on HPV chromatin. *In vitro* transcription was similarly performed as described in panel (B) in the absence (-) or presence of the indicated amount of p53 or Brd4 proteins.

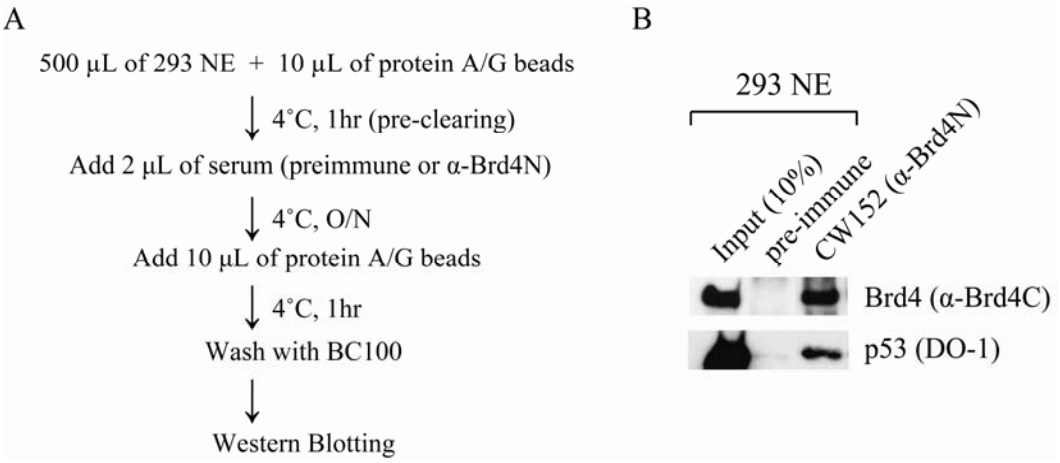

Figure 4-4. p53 binds to Brd4 *in vivo*
(**A**) Experimental flow chart. Coimmunoprecipitation was performed with nuclear extract (NE) of human embryonic kidney (HEK) 293 cells. After preclearing with protein A/G beads, Brd4 and associated proteins were immunoprecitated with either pre-immune serum or anti-Brd4N antibody (CW152) and further analyzed through Western blotting. (**B**) Brd4 and p53 were detected using anti-Brd4C antibody (CW151) and monoclonal anti-p53 antibody (DO-1, Santa Cruz).

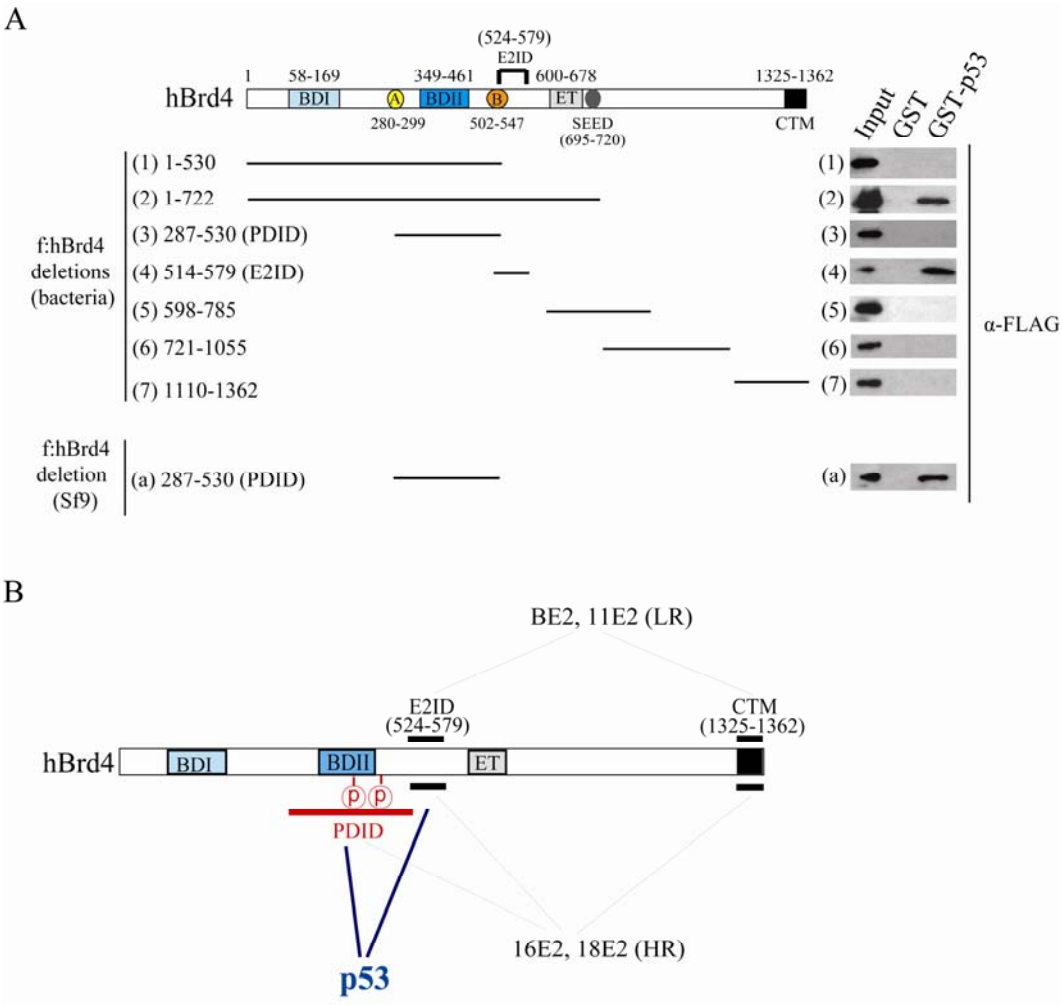

Figure 4-5. p53 recognizes PDID and E2ID of Brd4
(**A**) Mapping p53-interacting region of Brd4. GST alone or GST-tagged p53 were immobilized and incubated with bacterially purified FLAG-tagged Brd4 deletion mutants or PDID from Sf9 insect cells. Proteins were detected by Western blotting with anti-FLAG M2 antibody for bound Brd4 deletions. Amino acid residues corresponding to each deletion are shown on the left. (**B**) Summary of p53-interacting regions on Brd4. Black bars indicate E2-binding regions commonly shared by B/11/16/18E2. PDID is marked as red bar and can be phosporylated as shown in the circled letter "P".

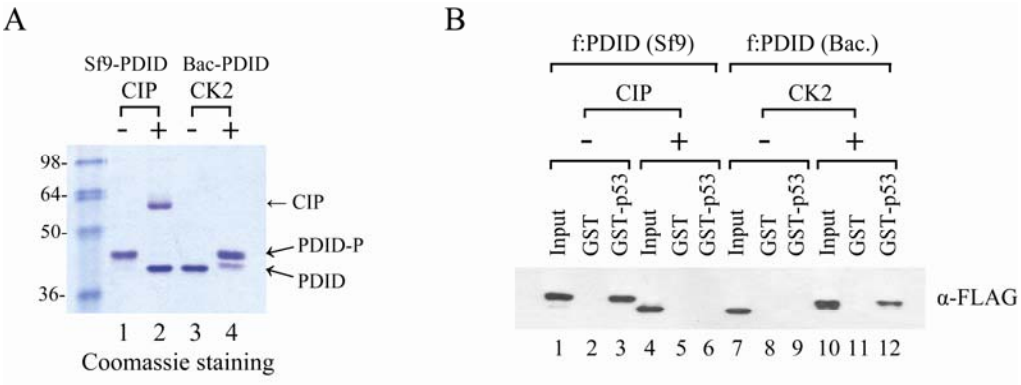

Figure 4-6. Phosphorylation on PDID by CK2 determines the interaction with p53

(A) An *in vitro* dephosphorylation assay and An *in vitro* CK2 Kinase assay were performed by incubation of insect cell-purified PDID (Sf9-PDID) with CIP and bacterially purified PDID with CK2, respectively. The reaction products were analyzed by SDS-PAGE and Coomassie staining, together with untreated PDID. (**B)** A GST pull-down assay was performed by incubation of GST alone or GST-p53 proteins with untreated or enzyme treated PDID (CIP- treated PDID (bacteria) or CK2-treated PDID (Sf9)) and bound Brd4 proteins were detected by Western blotting with anti-FLAG M2 antibody.

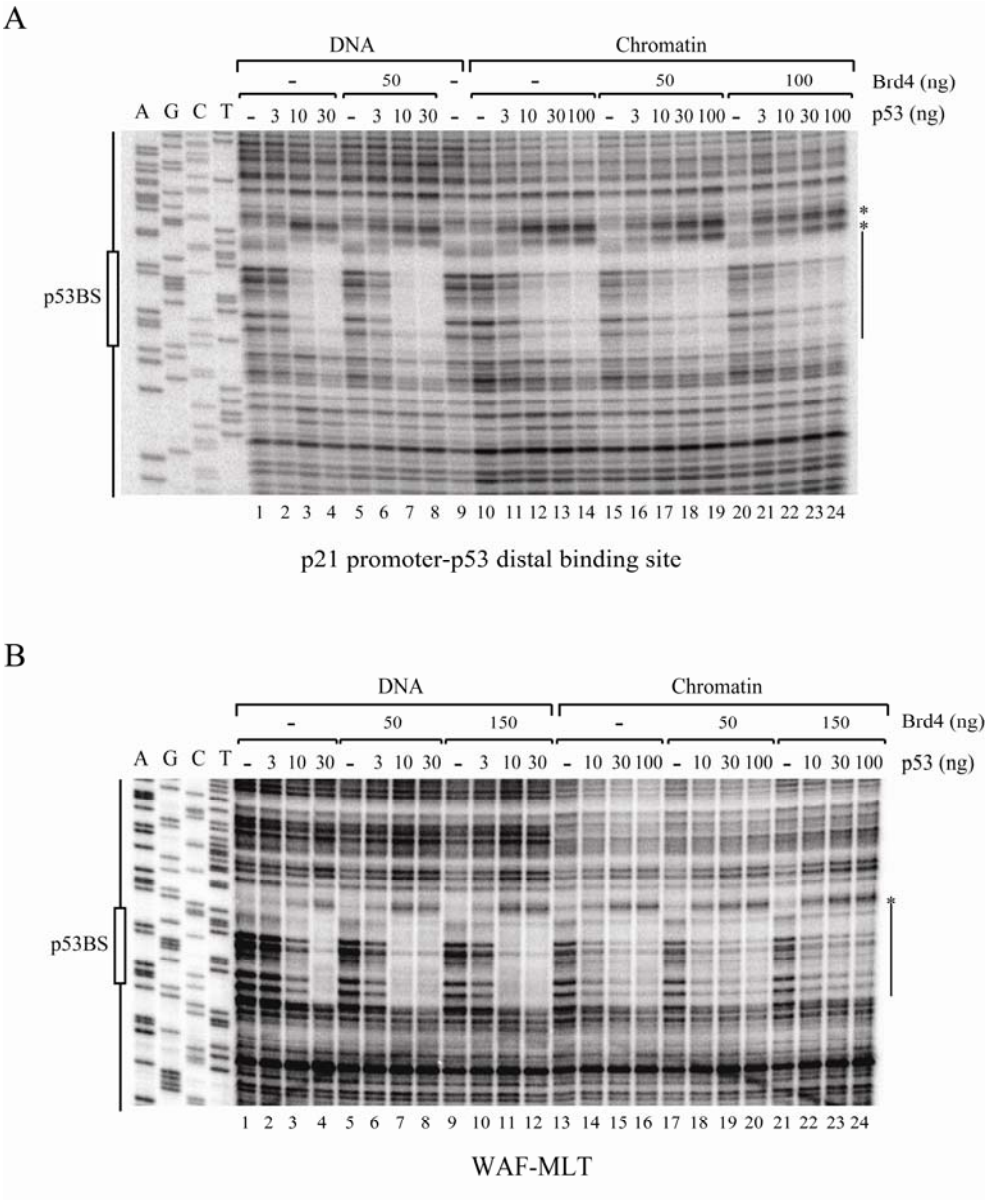
A
DNA
Chromatin
Brd4 (ng)
p53 (ng)
A G C T
p53BS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
p21 promoter-p53 distal binding site
B
DNA
Chromatin
Brd4 (ng)
p53 (ng)
A G C T
p53BS
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
WAF-MLT

Figure 4-7. Brd4 enhances p53 binding to its cognate site in DNA
(A) *In vitro*-reconstituted XXX Chromatin or naked DNA was incubated with or without (-) p53 and FL Brd4 protein as indicated. DNase I footprinting was conducted as described under Material and Methods with PCR-amplified labeled DNA fragments separated by denaturing polyacrylamide/urea gel electrophoresis and visualized following PhosphorImager analysis. Position of p53-binding site is indicated on the left. The protected region and hypersensitive sites (*) are marked on the right. **(B)** DNase I footprinting was performed the same as described for panel (A), except that XXX was used for the DNA template.

CHAPTER V:

CONCLUSIONS AND FUTURE DIRECTIONS

BRD4 MODULATES E2 TRANSCRIPTION AND STABILITY

Although Brd4 has been implicated in the transcriptional control of papillomavirus-encoded E2 protein, it was unclear how Brd4 regulates E2 function, and whether the involvement of Brd4 in transactivation and transrepression is common to different types of E2 proteins. Using DNase I footprinting performed with *in vitro*-reconstituted human papillomavirus (HPV) chromatin and nucleosome-free DNA templates, we found that Brd4 facilitates E2 binding to its cognate sequences in chromatin depending on one of its bromodomains and the E2-interacting region of Brd4 (Figure 2-3). Surprisingly, we also observed enhanced E2 footprinting in the presence of Brd4 with the DNA template in a CTM-dependent manner, possibly due to its allosteric enhancement of DNA-binding activity of E2 (Figure 2-4). The coactivator and corepressor function of Brd4 is common to different types of E2 proteins encoded by cancer-inducing high-risk HPV-16 and HPV-18, and benign wart-causing low-risk HPV-11 and bovine papillomavirus type 1 (Figures 2-5, 2-7, and 2-8). These activities require at least one intact bromodomain and were mediated by its direct association with E2 protein (Figure 2-6, 2-7, and 2-8). The general cofactor

function of Brd4 on E2-mediated transcription is, in part, mediated through enhancing the protein stability of E2 that is normally degraded via the ubiquitin-dependent proteasome pathway (Figure 2-9). However, the mechanism by which Brd4 stabilizes E2 is not yet known. Thus, further analysis is required to determine whether Brd4 affects E2 polyubiquitination, a signal for proteasome recognition, or if it blocks the accessibility of proteasome machinery.

FUNCTIONAL REGULATION OF BRD4 BY PHOSPHORYLATION

Besides the C-terminal motif, a well-known Brd4-interacting domain, we identified two novel additional E2-interacting regions of Brd4: the E2-interaction domain (E2ID) and phosphorylation-dependent interaction domain (PDID). While E2ID bound to E2 proteins encoded by bovine papillomavirus type 1, wart-causing low risk human papillomavirus type 11 (HPV-11), and cancer-inducing high risk (HR) HPV-16 and HPV-18, PDID only interacted with high risk E2 (HRE2) in a phosphorylation-dependent manner (Summarized in Figure 3-6D). In addition to HRE2-specific interaction, casein kinase 2 (CK2)-mediated phosphorylation of PDID also induced an intramolecular interaction between PDID and the E2ID, which blocked the E2-interaction through E2ID (Figure 3-8). The phosphorylated PDID recognizes the DNA-binding domain of HRE2, and

bromodomain II is dispensable for this interaction (Figures 3-9 and 3-11). Finally, a cell-based assay revealed that the PDID-HRE2 interaction is important for the HRE2-mediated transcriptional activation, and at the same time, both PDID and E2ID are important for the E2-transactivating function, suggesting a fundamental function of these domains in E2-mediated transactivation (Figures 3-12 and 3-13).

Even though PDID seems to be important in E2-mediated transcription, it is still vague as to how it contributes to the activation of E2 activity. Since PDID and E2ID interact with the DNA binding domain of E2, it would be interesting to see if they modulate E2's ability to recognize its cognate DNA sequence and also affect its dimerization through this protein-protein interaction, which can be linked to the transcription activity of E2. Also, because the region covering either PDID or E2ID is overlapped with one of the P-TEFb-binding regions on Brd4, it needs to be investigated whether the deletion of each domain would cause dissociation of P-TEFb from Brd4, resulting in inhibition of transcription.

ROLE OF BRD4-INTERACTING CELLULAR FACTORS ON HPV TRANSCRIPTION

We have identified a number of novel Brd4-interacting partners, Acf1, mG9a, p53, YY1, AP2, c-Jun, c-myc/max, mC/EBPα (p42 subunit), hC/EBPβ

(LIP), Gal4-Pro (Figure 4-1). Since Brd4 specifically binds to ACF through the interaction with the Acf1 subunit (Figure 4-2A), we tested whether Brd4 remodels chromatin using ATP hydrolysis energy and whether Brd4 regulates remodeling activity of ACF through this protein-protein interaction. An ATPase assay showed that Brd4 neither possess ATPase activity nor does it modulate the ATPase activity of ACF (Figure 4-2B). However, a DNase I footprinting assay revealed that an ATP-mediated remodeling event is required for E2-Brd4 binding, and further induces reorganization of nucleosomes (Figure 4-2C).

We also found that p53 is a novel HPV chromatin transcription activator and a Brd4-interacting protein. *In vitro* transcription showed that p53-activated HPV chromatin transcription is suppressed by the addition of Brd4 in a dosage-dependent manner, indicating a negative role of Brd4 towards p53-transactivation through an unknown mechanism (Figure 4-3C). p53 bound to E2ID and CK2-phosphorylated PDID of Brd4 the same as high risk E2 does, suggesting a possible interplay between the two Brd4-interacting partners (Figure 4-5B). DNase I footprinting performed with naked DNA or *in vitro* assembled chromatin revealed that Brd4 affects p53 binding to its cognate sequence within DNA and a chromatin template through this protein-protein interaction.

As described earlier, although many factors do not interact with Brd4, the optimization of the interaction assay conditions can further identify binding partners (See Discussion under Chapter IV). Moreover, even though an ATPase

assay showed that Brd4 does not affect the ATPase activity of ACF, it needs to be tested whether E2-responsive chromatin and E2 protein are required for the Brd4-induced changes in remodeling. Even though I suggested that Brd4 represses p53-meditated HPV chromatin transcription, the mechanism is unclear. Also, whether Brd4 functions on p53-responsive gene regulation needs to be pursued further.

APPENDICES

Appendix A. Primers for domain-specific deletion mutants of Brd4

Oligo name	Size	Sequence	Note	Description
hBrd4(3'AS-355/384)	30-mer	5' GTT AGG GTT GGA GGT CTC TGG GGG CGG GGG 3'	5' Pho.	ΔBDI
hBrd4(5'S-727/761)	35-mer	5' ACC GAG ATC ATG ATA GTC CAG GCA AAA GGA AGA GG 3'	5' Pho.	ΔBDI
hBrd4(3'AS-1228/1257)	30-mer	5' CTC TGG TCG TGG GTG CTG CTG AGA GTC GGG 3'	5' Pho.	ΔBDII
hBrd4(5'S-1612/1641)	30-mer	5' CCA GTG GTG GCC GTG TCC TCC CCG GCA GTG 3'	5' Pho.	ΔBDII
hBrd4(3'AS-1760/1791)	32-mer	5' CTC GTG CAC GGC TTT GAG CTG CTC CTG GAG CT 3'	5' Pho.	ΔE2ID
hBrd4(5'S-1960/1989)	30-mer	5' AAC AGC AAT GTG AGC AAG AAG GAG CCA GCG 3'	5' Pho.	ΔE2ID
hBrd4(3'AS-1990/2019)	30-mer	5' GGG TAC TTC TCG TTC GGG GGA GGG TGC ATA 3'	5' Pho.	ΔET
hBrd4(5'S-2257/2292)	36-mer	5' AGG AAA CCT GCC GAT GAG AAA GTT GAT GTG ATT GCC 3'	5' Pho.	ΔET
hBrd4(3'AS-1795/1824)	30-mer	5' GAA CGT CGG GAG AGA GTC GGG GTC GTC TTG 3'	5' Pho.	ΔNLS
hBrd4(5'S-1885/1921)	37-mer	5' GAG GAA GTG GAA GAG AAT AAA AAA AGC AAA GCC AAG G 3'	5' Pho.	ΔNLS
hBrd4(3'AS-1638/1671)	34-mer	5' GGG CGG GGC CAC AAC CTT GGT GGG AGG GGG CAC T 3'	5' Pho.	ΔNPS
hBrd4(5'S-1732/1763)	32-mer	5' GAG GAG GAG CGA GCC CAG CGG CTG GCT GAG CT 3'	5' Pho.	ΔNPS
hBrd4(3'AS-1050/1080)	31-mer	5' CTT CTT TGT CTT CAC AGG CTG TGG GGT GGC C 3'	5' Pho.	ΔPDID

hBrd4(5'S-1813/1845)	33-mer	5' CCC CAG CAG AAC AAA CCA AAG AAA AAG GAG AAA 3'	5' Pho.	ΔPDID

Appendix B. Primers for site-directed mutagenesis of Brd4

Oligo name	Size	Sequence	Description
PDID(5'S-S405A)	33-mer	5' ATG AGC ACA ATC AAG GCT AAA CTG GAG GCC CGT 3'	M1
PDID(3'AS-S405A)	33-mer	5' ACG GGC CTC CAG TTT AGC CTT GAT TGT GCT CAT 3'	M1
PDID(5'S-S469/470A)	36-mer	5' CCA GTG GTG GCC GTG GCC GCC CCG GCA GTG CCC CCT 3'	M2
PDID(3'AS-S469/470A)	36-mer	5' AGG GGG CAC TGC CGG GGC GGC CAC GGC CAC CAC TGG 3'	M2
PDID(3'AS-S484/488A)	39-mer	5' GCT ATC GCT GCT GGC GTC GCT GGA TGC GGG CGG GGC CAC 3'	M3
PDID(5'S-S484/488A)	39-mer	5' GTG GCC CCG CCC GCA TCC AGC GAC GCC AGC AGC GAT AGC 3'	M3
PDID(3'AS-S492/494A)	39-mer	5' CGA ACT GTC ACT GTC CGC GGA GGC ATC GCT GCT GCT GTC 3'	M4
PDID(5'S-S492/494A)	39-mer	5' GAC AGC AGC AGC GAT GCC TCC GCG GAC AGT GAC AGT TCG 3'	M4
PDID(3'AS-S498/499/500A)	36-mer	5' TCC TCA GAG TCA TCA GCC GCA GCG TCA CTG TCC GAG 3'	M5
PDID(5'S-S498/499/500A)	36-mer	5' CTC GGA CAG TGA CGC TGC GGC TGA TGA CTC TGA GGA 3'	M5
PDID(3'AS-S503A)	33-mer	5' GGC TCG CTC CTC CTC AGC GTC ATC AGT CGA ACT 3'	M6
PDID(5'S-S503A)	33-mer	5' AGT TCG ACT GAT GAC GCT GAG GAG GAG CGA GCC 3'	M6

Appendix C. Other primers

Oligo name	Size	Sequence
hBrd4(3'AS-aa579)	34-mer	5' AAC TCG AGG ATC CTC AGC TGC TAT TAT TTT TCT T 3'
hBrd4(3'AS-BamHI-a.a.1223)	34-mer	5' TTC TCG AGG ATC CTC AGC TGT CGC TGG ATG ACT T 3'
hBrd4(3'AS-BamHI-aa554)	34-mer	5' TTC TCG AGG ATC CCT ATT TTC TTT TGT GCT TTT C 3'
hBrd4(3'AS-BamHI-aa569)	34-mer	5' TTC TCG AGG ATC CTC AAG GAG GTT CCT TGG CTT T 3'
hBrd4(5'S-aa279)	31-mer	5' AAG TCG ACA TAT GCC ACA GCC TGT GAA GAC A 3'
hBrd4(5'S-aa531)	31-mer	5' AAG TCG ACA TAT GCC CCA GCA GAA CAA ACC A 3'
hBrd4(5'S-NdeI-a.a.1224)	31-mer	5' AAG TCG ACA TAT GTT CGA GCA GTT CCG CCG C 3'
hBrd4(5'S-NdeI-aa464)	31-mer	5' AAG TCG ACA TAT GCC AGT GGT GGC CGT GTC C 3'
hBrd4(5'S-NdeI-aa474)	31-mer	5' TTG TCG ACA TAT GCC CCC TCC CAC CAA GGT T 3'
hBrd4(5'S-NdeI-aa484)	31-mer	5' TTG TCG ACA TAT GTC ATC CAG CGA CAG CAG C 3'
hBrd4(5'S-NdeI-aa494)	31-mer	5' AAG TCG ACA TAT GTC GGA CAG TGA CAG TTC G 3'
hBrd4(5'S-NdeI-aa504)	31-mer	5' AAG TCG ACA TAT GGA GGA GGA GCG AGC CCA G 3'
hBrd4(5'S-NdeI-aa514)	31-mer	5' AAG TCG ACA TAT GCT CCA GGA GCA GCT CAA A 3'
hBrd4(5'S-NdeI-aa524)	31-mer	5' TTG TCG ACA TAT GCA GCT TGC AGC CCT CTC T 3'
5'S-NLS(NdeI/BamHI)	55-mer	5' TAT CCC TCC AAA AAA GAA GAG AAA GGT AGC TCA TAT GGT CGA CGG ATC CCT CGA G 3'
3'AS-NLS(NdeI/BamHI)	53-mer	5' CTC GAG GGA TCC GTC GAC CAT ATG AGC TAC CTT TCT CTT CTT TTT TGG AGG GA 3'

Appendix D. Primers for E2

Oligo name	Size	Sequence
11E2(3'AS-aa290)	34-mer	5' AAC TCG AGG ATC CTC ATT GCA GTT GCA CTA TAG G 3'
11E2(3'AS-aa30)	34-mer	5' TTC TCG AGG ATC CCT AAA TGT GTT TGT GTA TAT C 3'
11E2(3'AS-aa310)	34-mer	5' AAC TCG AGG ATC CTC ACA AAT GTT TAT ATT TGT C 3'
11E2(3'AS-aa330)	34-mer	5' AAC TCG AGG ATC CTC ATG CAT TTT TAT GTG GTG C 3'
11E2(3'AS-aa348)	34-mer	5' AAC TCG AGG ATC CTC AAC TGT TTA AAA ATT GCT G 3'
11E2(5'S-aa251)	29-mer	5' AAG TCG ACA TAT GTG TGT GGC CAA CAT CA 3'
BPV-E2(3'AS-BamHI-aa209)	34-mer	5' AAC TCG AGG ATC CTC ATG GGC GAT CTC TAA AAT C 3'
BPV-E2(3'AS-BamHI-aa323)	34-mer	5' AAC TCG AGG ATC CTC ATG CCT TTA ACA GGT GGA A 3'
BPV-E2(5'S-NdeI-aa210)	33-mer	5' AAG TCG ACA TAT GAC GGA GTC TGG GTC GCA TCC 3'
BPV-E2(5'S-NdeI-aa210)	34-mer	5' AAG TCG ACA TAT GGA CGG AGT CTG GGT CGC ATC C 3'
BPV-E2(5'S-NdeI-aa324)	34-mer	5' TTG TCG ACA TAT GGG AGG GTC ATG CTT TGC TCT A 3'
11E2(3'AS-I73A)	33-mer	5' ATG CAT TTG CAT TTC AGC AGC ATT ATG TCC TTT 3'
11E2(3'AS-R37A)	33-mer	5' TAA TAC ACT TTC CAA TGC TAT GCA TTT CCA ATG 3'
11E2(5'S-I73A)	33-mer	5' AAA GGA CAT AAT GCT GCT GAA ATG CAA ATG CAT 3'
11E2(5'S-R37A)	33-mer	5' CAT TGG AAA TGC ATA GCA TTG GAA AGT GTA TTA 3'

Appendix E. GST-tagged bacterial expression plasmids

Plasmid name	Insert	Vector	Cloning sites
pGEX-5E2	NdeI - BamHI digestion of pF:5E2-11d	pGEX-2TL(+)	NdeI - BamHI
pGEX-8E2	NdeI - BamHI digestion of pF:8E2-11d	pGEX-2TL(+)	NdeI - BamHI
pGEX-BE2	NdeI - BamHI digestion of pF:BE2-11d	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(1-160)	PCR with 11E2(5'S-NdeI) and 11E2(3'AS-aa160)	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(1-30)	PCR with 11E2(5'S-NdeI) and 11E2(3'AS-aa30)	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(165-200)	PCR with 11E2(5'S-N165) and 11E2(3'AS-aa200)	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(251-290)	PCR with 11E2(5'S-aa251) and 11E2(3'AS-aa290)	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(251-330)	PCR with 11E2(5'S-aa251) and 11E2(3'AS-aa330)	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(251-367)	PCR with 11E2(5'S-aa251) and E2-Ter(Bx-3'AS)	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(I73A)	point mutation with 11E2(3'AS-I73A) and 11E2(5'S-I73A)	pGEX-E2(FL)	NdeI - BamHI
pGEX-E2(N165)	PCR with 11E2(5'S-N165) and E2-Ter(Bx-3'AS)	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(N284), 11C	PCR with 11E2(5'S-N284) and E2-Ter(Bx-3'AS)	pGEX-2TL(+)	NdeI - BamHI
pGEX-E2(N310)	PCR with 11E2(5'S-aa301) and E2-Ter(Bx-3'AS)	pGEX-2TL(+)	NdeI – BamHI
pGEX-E2(R37A)	point mutation with 11E2(3'AS-R37A) and 11E2(5'S-R37A)	pGEX-E2(FL)	
pGEX-E2(R37K/I73A)	point mutation with 11E2(3'AS-R37A) and 11E2(5'S-R37A)	pGEX-E2(R37K)	
pGEX-hBrd4(1033-1258)	PCR with hBrd4(5'S-NdeI-aa1033) and hBrd4(3'AS-BamHI-aa1258)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(1-1043)	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa1043)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(1110-1362)	PCR with hBrd4(5'S-NdeI-aa1110) and hBrd4(3'AS-BamHI)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(1199-1362)	PCR with hBrd4(5'S-NdeI-aa1199) and hBrd4(3'AS-BamHI)	pGEX-2TL(+)	NdeI - BamHI

pGEX-hBrd4(1224-1362), CTM	PCR with hBrd4(5'S-NdeI-aa1224) and hBrd4(3'AS-BamHI)	pGEX-2TL(+)	NdeI – BamHI
pGEX-hBrd4(1-284)	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa284)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(149-284)	PCR with hBrd4(5'S-NdeI-aa149) and hBrd4(3'AS-BamHI-aa284)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(1-530)	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa530)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(1-719)	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa719)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(287-1362)	PCR with hBrd4(5'S-NdeI-aa287) and hBrd4(3'AS-BamHI)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(287-530), PDID	PCR with hBrd4(5'S-NdeI-aa287) and hBrd4(3'AS-BamHI-aa530)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(524-579), E2ID	PCR with hBrd4(5'S-NdeI-aa524) and hBrd4(3'AS-BamHI-aa579)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(598-1362)	PCR with hBrd4(5'S-NdeI-aa598) and hBrd4(3'AS-BamHI)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(598-785)	PCR with hBrd4(5'S-NdeI-aa598) and hBrd4(3'AS-BamHI-aa785)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(721-1043)	PCR with hBrd4(5'S-NdeI-aa721) and hBrd4(3'AS-BamHI-aa1043)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(721-1055)	PCR with hBrd4(5'S-NdeI-aa721) and hBrd4(3'AS-BamHI-aa1055)	pGEX-2TL(+)	NdeI - BamHI
pGEX-hBrd4(short)	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa722)	pGEX-2TL(+)	NdeI - BamHI

Appendix F. pGEM-7-based plasmids

Plasmid name	Insert	Vector	Cloning sites
pF:hBrd4(1033-1258)-7	PCR with hBrd4(5'S-NdeI-aa1033) and hBrd4(3'AS-BamHI-aa1258)	pFLAG(S)-7	NdeI - BamHI
pF(S)-7			
pF:hBrd4(1110-1362)-7	PCR with hBrd4(5'S-NdeI-aa1110) and hBrd4(3'AS-BamHI)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(1199-1362)-7	PCR with hBrd4(5'S-NdeI-aa1199) and hBrd4(3'AS-BamHI)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(1224-1362)-7	PCR with hBrd4(5'S-NdeI-aa1224) and hBrd4(3'AS-BamHI)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(1-284)-7	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa284)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(1-530)-7	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa530)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(1-719)-7	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa719)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(1-722)ΔBDI-7	inverse PCR with hBrd4(5'S-727/761) and hBrd4(3'AS-355/384)	pF:hBrd4(1-722)-7, short	
pF:hBrd4(1-722)ΔBDII/NLS-7	inverse PCR with hBrd4(5'S-1885/1921) and hBrd4(3'AS-1795/1824)	pF:hBrd4(1-722)ΔBDII-7	
pF:hBrd4(1-722)ΔBDII-7	inverse PCR with hBrd4(5'S-1612/1641) and hBrd4(3'AS-1228/1257)	pF:hBrd4(1-722)-7, short	
pF:hBrd4(1-722)ΔE2ID-7	inverse PCR with hBrd4(5'S-1960/1989) and hBrd4(3'AS-1760/1791)	pF:hBrd4(1-722)-7, short	
pF:hBrd4(1-722)ΔET-7	inverse PCR with hBrd4(5'S-2257/2292) and hBrd4(3'AS-1990/2019)	pF:hBrd4(1-722)-7, short	
pF:hBrd4(1-722)ΔNLS-7	inverse PCR with hBrd4(5'S-1885/1921) and hBrd4(3'AS-1795/1824)	pF:hBrd4(1-722)-7, short	
pF:hBrd4(1-722)ΔNPS-7	inverse PCR with hBrd4(5'S-1732/1763) and hBrd4(3'AS-1638/1671)	pF:hBrd4(1-722)-7, short	
pF:hBrd4(1-722)ΔPDID/E2ID-7	inverse PCR with hBrd4(5'S-1960/1989) and hBrd4(3'AS-1050/1080)	pF:hBrd4(1-722)-7, short	

pF:hBrd4(1-722)ΔPDID-7	inverse PCR with hBrd4(5'S-1813/1845) and hBrd4(3'AS-1050/1080)	pF:hBrd4(1-722)-7, short	
pF:hBrd4(1-722)-7, short	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa722)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(279-579)-7	PCR with hBrd4(5'S-NdeI-aa279) and hBrd4(3'AS-BamHI-aa579)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(287-1362)-7	PCR with hBrd4(5'S-NdeI-aa287) and hBrd4(3'AS-BamHI)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(287-530)-7	PCR with hBrd4(5'S-NdeI-aa287) and hBrd4(3'AS-BamHI-aa530)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(531-579)-7	PCR with hBrd4(5'S-NdeI-aa531) and hBrd4(3'AS-BamHI-aa579)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(598-1362)-7	PCR with hBrd4(5'S-NdeI-aa598) and hBrd4(3'AS-BamHI)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(598-785)-7	PCR with hBrd4(5'S-NdeI-aa598) and hBrd4(3'AS-BamHI-aa785)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(721-1055)-7	PCR with hBrd4(5'S-NdeI-aa721) and hBrd4(3'AS-BamHI-aa1055)	pFLAG(S)-7	NdeI - BamHI
pF:hBrd4(721-1362)-7	PCR with hBrd4(5'S-NdeI-aa721) and hBrd4(3'AS-BamHI)	pFLAG(S)-7	NdeI - BamHI
pHA:BE2-7	NdeI - BamHI digestion of pF:BE2-11d	pGEM-HA:E2	NdeI - BamHI
pHA:16E2-7	NdeI - BamHI digestion of pF:16E2-11d	pGEM-HA:E2	NdeI - BamHI
pHA:18E2-7	NdeI - BamHI digestion of pF:18E2-11d	pGEM-HA:E3	NdeI - BamHI

Appendix G. FLAG-tagged bacterial expression plasmids

Plasmid name	Insert	Vector	Cloning sites
pF:hBrd4(1-1043)-11d	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa1043)	pET-11d	NdeI - BamHI
pF:hBrd4(1-1043)ΔET-11d	insert swapping with SacI fragment from pF:hBrd4(1-722)ΔET-7	pF:hBrd4(1-1043)-11d	SacI
pF:hBrd4(1110-1362)-11d	PCR with hBrd4(5'S-NdeI-aa1110) and hBrd4(3'AS-BamHI)	pET-11d	NdeI - BamHI
pF:hBrd4(1199-1362)-11d	PCR with hBrd4(5'S-NdeI-aa1199) and hBrd4(3'AS-BamHI)	pET-11d	NdeI - BamHI
pF:hBrd4(149-284)-11d	PCR with hBrd4(5'S-NdeI-aa149) and hBrd4(3'AS-BamHI-aa284)	pET-11d	NdeI - BamHI
pF:hBrd4(1-530)-11d	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa530)	pET-11d	NdeI - BamHI
pF:hBrd4(1-719)-11d	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa719)	pET-11d	NdeI - BamHI
pF:hBrd4(279-579)-11d	PCR with hBrd4(5'S-NdeI-aa279) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(287-1362)-11d	PCR with hBrd4(5'S-NdeI-aa287) and hBrd4(3'AS-BamHI)	pET-11d	NdeI - BamHI
pF:hBrd4(287-530)-11d	PCR with hBrd4(5'S-NdeI-aa287) and hBrd4(3'AS-BamHI-aa530)	pET-11d	NdeI - BamHI
pF:hBrd4(464-554)-11d	PCR with hBrd4(5'S-NdeI-aa464) and hBrd4(3'AS-BamHI-aa554)	pET-11d	NdeI - BamHI
pF:hBrd4(464-569)-11d	PCR with hBrd4(5'S-NdeI-aa464) and hBrd4(3'AS-BamHI-aa569)	pET-11d	NdeI - BamHI
pF:hBrd4(464-579)-11d	PCR with hBrd4(5'S-NdeI-aa464) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(474-579)-11d	PCR with hBrd4(5'S-NdeI-aa474) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(484-579)-11d	PCR with hBrd4(5'S-NdeI-aa484) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(494-579)-11d	PCR with hBrd4(5'S-NdeI-aa494) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(504-579)-11d	PCR with hBrd4(5'S-NdeI-aa504) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(514-579)-11d	PCR with hBrd4(5'S-NdeI-aa514) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(524-579)-11d	PCR with hBrd4(5'S-NdeI-aa524) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(531-579)-11d	PCR with hBrd4(5'S-NdeI-aa531) and hBrd4(3'AS-BamHI-aa579)	pET-11d	NdeI - BamHI
pF:hBrd4(598-1362)-11d	PCR with hBrd4(5'S-NdeI-aa598) and hBrd4(3'AS-BamHI)	pET-11d	NdeI - BamHI

pF:hBrd4(598-785)-11d	PCR with hBrd4(5'S-NdeI-aa598) and hBrd4(3'AS-BamHI-aa785)	pET-11d	NdeI - BamHI
pF:hBrd4(721-1055)-11d	PCR with hBrd4(5'S-NdeI-aa721) and hBrd4(3'AS-BamHI-aa1055)	pET-11d	NdeI - BamHI
pF:hBrd4(721-1362)-11d	PCR with hBrd4(5'S-NdeI-aa721) and hBrd4(3'AS-BamHI)	pET-11d	NdeI - BamHI
pF:hBrd4(short)-11d	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa722)	pET-11d	NdeI - BamHI
pF:NLS:hBrd4(1224-1362)-11d	NdeI - BamHI digestion of pF:hBrd4(1224-1362)-11d	pF:NLS-11d	NdeI - BamHI
pF:NLS:hBrd4(287-530)-11d	NdeI - BamHI digestion of pF:hBrd4(287-530)-11d	pF:NLS-11d	NdeI - BamHI
pF:NLS:hBrd4(524-579)-11d	NdeI - BamHI digestion of pF:hBrd4(524-579)-11d	pF:NLS-11d	NdeI - BamHI
pF:NLS:hBrd4(598-1362)-11d	NdeI - BamHI digestion of pF:hBrd4(598-1362)-11d	pF:NLS-11d	NdeI - BamHI
pF:NLS:hBrd4(ΔBDII/NLS)-11d	NdeI - BamHI digestion of pF:hBrd4(ΔBDII/NLS)-11d	pF:NLS-11d	NdeI - BamHI
pF:NLS:hBrd4(ΔNLS)-11d	NdeI - BamHI digestion of pF:hBrd4(ΔNLS)-11d	pF:NLS-11d	NdeI - BamHI
pF:NLS-11d	annealed primers: 5'S-NLS(NdeI/BamHI) and 3'AS-NLS(NdeI/BamHI)	pET-11d	NdeI - BamHI
pF:PDID(S405A)-11d	point mutation with PDID(5'S-S405A) and PDID(3'AS-S405A)	pF:hBrd4(287-530)-11d	
pF:PDID(S469/470A)-11d	point mutation with PDID(5'S-S469/470A) and PDID(3'AS-S469/470A)	pF:hBrd4(287-530)-11d	
pF:PDID(S484/488A)-11d	point mutation with PDID(5'S-S484/488A) and PDID(3'AS-S484/488A)	pF:hBrd4(287-530)-11d	
pF:PDID(S492/494A)-11d	point mutation with PDID(5'S-S492/494A) and PDID(3'AS-S492/494A)	pF:hBrd4(287-530)-11d	
pF:PDID(S498/499/T500A)-11d	point mutation with PDID(5'S-S498/499/500A) and PDID(3'AS-S498/499/500A)	pF:hBrd4(287-530)-11d	
pF:PDID(S503A)-11d	point mutation with PDID(5'S-S503A) and PDID(3'AS-S503A)	pF:hBrd4(287-530)-11d	
pF:hBrd4(1033-1258)-11d	PCR with hBrd4(5'S-NdeI-aa1033) and hBrd4(3'AS-BamHI-aa1258)		NdeI - BamHI

pF:hBrd4(721-1043)-11d	PCR with hBrd4(5'S-NdeI-aa721) and hBrd4(3'AS-BamHI-aa1043)		NdeI - BamHI
pF:hBrd4(1-284)-11d	PCR with hBrd4(5'S-NdeI) and hBrd4(3'AS-BamHI-aa284)		NdeI - BamHI

Appendix H. His-tagged bacterial expression plasmids

Plasmid name	Insert	Vector	Cloning sites
p6His:11E2-Hinge-11d	PCR with 11E2(5'S-N201) and 11E2(3'AS-aa283)	p6His:TBP-11d	NdeI - BamHI
p6His:11E2-NTD-11d	NdeI - BamHI digestion of pGEX-E2(1-200)	p6His:TBP-11d	NdeI - BamHI
p6His:BE2-CTD-11d	PCR with BE2(5'S-aa324) and BE2(3'AS-4450-BamHI)	p6His:TBP-11d	NdeI - BamHI
p6His:BE2-Hinge-11d	PCR with BE2(5'S-aa210) and BE2(3'AS-aa323)	p6His:TBP-11d	NdeI - BamHI
p6His:BE2-NTD-11d	PCR with BE2(5'S-NdeI) and BE2(3'AS-aa209)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(1199-1362)-11d	PCR with hBrd4(5'S-NdeI-aa1199) and hBrd4(3'AS-BamHI)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(1224-1362)-11d	PCR with hBrd4(5'S-NdeI-aa1224) and hBrd4(3'AS-BamHI)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(149-284)-11d	PCR with hBrd4(5'S-NdeI-aa149) and hBrd4(3'AS-BamHI-aa284)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(464-554)-11d	PCR with hBrd4(5'S-NdeI-aa464) and hBrd4(3'AS-BamHI-aa554)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(464-569)-11d	PCR with hBrd4(5'S-NdeI-aa464) and hBrd4(3'AS-BamHI-aa569)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(464-579)-11d	PCR with hBrd4(5'S-NdeI-aa464) and hBrd4(3'AS-BamHI-aa579)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(474-579)-11d	PCR with hBrd4(5'S-NdeI-aa474) and hBrd4(3'AS-BamHI-aa579)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(484-579)-11d	PCR with hBrd4(5'S-NdeI-aa484) and hBrd4(3'AS-BamHI-aa579)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(494-579)-11d	PCR with hBrd4(5'S-NdeI-aa494) and hBrd4(3'AS-BamHI-aa579)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(504-579)-11d	PCR with hBrd4(5'S-NdeI-aa504) and hBrd4(3'AS-BamHI-aa579)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(514-579)-11d	PCR with hBrd4(5'S-NdeI-aa514) and hBrd4(3'AS-BamHI-aa579)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(524-579)-11d	PCR with hBrd4(5'S-NdeI-aa524) and hBrd4(3'AS-BamHI-aa579)	p6His:TBP-11d	NdeI - BamHI
p6His:hBrd4(531-579)-11d	PCR with hBrd4(5'S-NdeI-aa531) and hBrd4(3'AS-BamHI-aa579)	p6His:TBP-11d	NdeI - BamHI

Appendix I. Mammalian expression plasmids

Plasmid name	Insert	Vector	Cloning sites
pcDNA3-F:hBrd4(1-1043)	BglII-EcoRV digested pF:hBrd4(1-1043)-11d	pcDNA3	BamHI-EcoRV
pcDNA3-F:hBrd4(1-1223)	insert swapping with BstEII-XhoI fragment of PCR product (aa1033-1223)	pcDNA3-F:hBrd4(FL)	BstEII-XhoI
pcDNA3-F:hBrd4(1224-1362)	BglII-BamHI digestion of pF:hBrd4(1224-1362)-7	pcDNA3	BamHI
pcDNA3-F:hBrd4(287-1362)	BglII-EcoRV digested pF:hBrd4(287-1362)-11d	pcDNA3	BamHI-EcoRV
pcDNA3-F:hBrd4(287-530), PDID	KpnI-BamHI digestion of pF:hBrd4(287-530)-7	pcDNA3	KpnI-BamHI
pcDNA3-F:hBrd4(524-579), E2ID	BglII-EcoRI digestion of pF:hBrd4(524-579)-11d	pcDNA3	BamHI-EcoRI
pcDNA3-F:hBrd4(598-1362)	BglII-EcoRV digested pF:hBrd4(598-1362)-11d	pcDNA3	BamHI-EcoRV
pcDNA3-F:hBrd4(EcoRI-NotI)	EcoRI-NotI digestion of pcDNA4C-F:hBrd4(FL)	pcDNA3	EcoRI-NotI
pcDNA3-F:hBrd4(FL)	NotI digestion of pVL-F:hBrd4(FL)	pcDNA3	NotI
pcDNA3-F:hBrd4(short)	BglII-BamHI digestion of pVL-F:hBrd4(1-722), short	pcDNA3	BamHI
pcDNA3-F:hBrd4-NUT	EcoRI-XhoI digestion of PCR product from pGFP:Brd4-NUT	pcDNA3-F:hBrd4(1-1043)	EcoRI-XhoI
pcDNA3-F:hBrd4ΔBDI	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDI-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:hBrd4ΔBDI/CTM	BstEII-XhoI digestion of pcDNA3-F:hBrd4(1-1223)	pcDNA3-F:hBrd4ΔBDI	BstEII-XhoI
pcDNA3-F:hBrd4ΔBDI/II	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDI/II-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:hBrd4ΔBDI/II/CTM	BstEII-XhoI digestion of pcDNA3-F:hBrd4(1-1223)	pcDNA3-F:hBrd4ΔBDI/II	BstEII-XhoI

pcDNA3-F:hBrd4ΔBDII	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDII-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:hBrd4ΔBDII/CTM	BstEII-XhoI digestion of pcDNA3-F:hBrd4(1-1223)	pcDNA3-F:hBrd4ΔBDII	BstEII-XhoI
pcDNA3-F:hBrd4ΔBDII/NLS	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDII/NLS-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:hBrd4ΔBDII/NLS/CTM	BstEII-XhoI digestion of pcDNA3-F:hBrd4(1-1223)	pcDNA3-F:hBrd4ΔBDII/NLS	BstEII-XhoI
pcDNA3-F:hBrd4ΔE2ID	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔE2ID-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:hBrd4ΔET	EcoRI-NotI digestion of pVL-F:hBrd4ΔET	pcDNA3	EcoRI-NotI
pcDNA3-F:hBrd4ΔET/CTM	BstEII-XhoI digestion of pcDNA3-F:hBrd4(1-1223)	pcDNA3-F:hBrd4ΔET	BstEII-XhoI
pcDNA3-F:hBrd4ΔNLS	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔNLS-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:hBrd4ΔNLS/CTM	BstEII-XhoI digestion of pcDNA3-F:hBrd4(1-1223)	pcDNA3-F:hBrd4ΔNLS	BstEII-XhoI
pcDNA3-F:hBrd4ΔNPS	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔNPS-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:hBrd4ΔPDID	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔPDID-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:hBrd4ΔPDID/E2ID	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔPDID/E2ID-7	pcDNA3-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pcDNA3-F:NLS:hBrd4(1224-1362)	BglII-BamHI digestion of pF:NLS:hBrd4(1224-1362)-11d	pcDNA3	BamHI
pcDNA3-F:NLS:hBrd4(287-530)	BglII-BamHI digestion of pF:NLS:hBrd4(287-530)-11d	pcDNA3	BamHI
pcDNA3-F:NLS:hBrd4(524-579)	BglII-BamHI digestion of pF:NLS:hBrd4(524-579)-11d	pcDNA3	BamHI
pcDNA3-F:NLS:hBrd4(598-1362)	BglII-BamHI digestion of pF:NLS:hBrd4(598-1362)-11d	pcDNA3	BamHI
pcDNA3-F:16E2	BglII-EcoRI digestion of pF:16E2-11d	pcDNA3	BamHI-EcoRI

pcDNA3-F:18E2	BglII-EcoRI digestion of pF:18E2-11d	pcDNA3	BamHI-EcoRI
pcDNA3-F:BE2	BglII-EcoRI digestion of pF:BE2-11d	pcDNA3	BamHI-EcoRI
pcDNA3-HA:11E2	BglII-BamHI digestion of pHA:11E2-7	pcDNA3	BamHI
pcDNA3-HA:16E2	BglII-BamHI digestion of pHA:16E2-7	pcDNA3	BamHI
pcDNA3-HA:18E2	BglII-BamHI digestion of pHA:18E2-7	pcDNA3	BamHI
pcDNA3-HA:BE2	BglII-BamHI digestion of pHA:BE2-7	pcDNA3	BamHI
pF:CMV2-11E2	PCR from pF:11E2-11d	pFLAG-CMV-2	Not I-BamHI
pF:CMV2-16E2	PCR from pF:16E2-11d	pFLAG-CMV-2	Not I-BamHI
pF:CMV2-18E2	PCR from pF:18E2-11d	pFLAG-CMV-2	Not I-BamHI

Appendix J. Baculovirus expression plasmids

Plasmid name	Insert	Vector	Cloning sites
pVL-F:hBrd4(1033-1258)	XbaI-BamHI digestion of pF:hBrd4(1033-1258)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(1-1043)	XbaI-BamHI digestion of pF:hBrd4(1-1043)-11d	pVL1392	XbaI-BamHI
pVL-F:hBrd4(1110-1362)	XbaI-BamHI digestion of pF:hBrd4(1110-1362)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(1-1223)	BglII-XbaI digestion of pcDNA3-F:hBrd4(1-1223)	pVL1392	BglII-XbaI
pVL-F:hBrd4(1199-1362)	XbaI-BamHI digestion of pF:hBrd4(1199-1362)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(1224-1362)	XbaI-BamHI digestion of pF:hBrd4(1224-1362)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(1-284)	XbaI-BamHI digestion of pF:hBrd4(1-284)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(1-530)	XbaI-BamHI digestion of pF:hBrd4(1-530)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(1-719)	XbaI-BamHI digestion of pF:hBrd4(1-719)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(1-722), short	XbaI-BamHI digestion of pF:hBrd4(1-722)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(279-579)	XbaI-BamHI digestion of pF:hBrd4(279-579)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(287-1362)	XbaI-BamHI digestion of pF:hBrd4(287-1362)-11d	pVL1392	XbaI-BamHI
pVL-F:hBrd4(287-530)	XbaI-BamHI digestion of pF:hBrd4(287-530)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(524-579)	XbaI-BamHI digestion of pF:hBrd4(524-579)-11d	pVL1392	XbaI-BamHI
pVL-F:hBrd4(531-579)	XbaI-BamHI digestion of pF:hBrd4(531-579)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(598-1362)	XbaI-BamHI digestion of pF:hBrd4(598-1362)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(598-785)	XbaI-BamHI digestion of pF:hBrd4(598-785)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(721-1043)	XbaI-BamHI digestion of pF:hBrd4(721-1043)-11d	pVL1392	XbaI-BamHI
pVL-F:hBrd4(721-1055)	XbaI-BamHI digestion of pF:hBrd4(721-1055)-7	pVL1392	XbaI-BamHI
pVL-F:hBrd4(721-1362)	XbaI-BamHI digestion of pF:hBrd4(721-1362)-11d	pVL1392	XbaI-BamHI
pVL-F:hBrd4(EcoRI-NotI)	EcoRI-NotI digestion of pcDNA4C-F:hBrd4(FL)	pVL1393	EcoRI-NotI

pVL-F:hBrd4(FL)	XhoI-SmaI digestion of pVL-F:hBrd4(1-1043)	pVL-F:hBrd4(EcoRI-NotI)	XhoI-SmaI
pVL-F:hBrd4ΔBDI	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDI-7	pVL-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pVL-F:hBrd4ΔBDI/II	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDI/II-7	pVL-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pVL-F:hBrd4ΔBDII	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDII-7	pVL-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pVL-F:hBrd4-NUT	XbaI digestion of pcDNA3-F:hBrd4-NUT	pVL	XbaI
pVL-F:hBrd4ΔBDI/CTM	EcoRI-BamHI digestion of pVL-F:hBrd4(1-1223)	pVL-F:hBrd4ΔBDI	EcoRI-BglII
pVL-F:hBrd4ΔBDI/II/CTM	EcoRI-BamHI digestion of pVL-F:hBrd4(1-1223)	pVL-F:hBrd4ΔBDI/II	EcoRI-BglII
pVL-F:hBrd4ΔBDI/II/NLS	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDI/II/NLS-7	pVL-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pVL-F:hBrd4ΔBDI/II/NLS/CTM	EcoRI-BamHI digestion of pVL-F:hBrd4(1-1223)	pVL-F:hBrd4ΔBDI/II/NLS	EcoRI-BglII
pVL-F:hBrd4ΔBDII/CTM	EcoRI-BamHI digestion of pVL-F:hBrd4(1-1223)	pVL-F:hBrd4ΔBDII	EcoRI-BglII
pVL-F:hBrd4ΔBDII/NLS	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔBDII/NLS-7	pVL-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pVL-F:hBrd4ΔBDII/NLS/CTM	EcoRI-BamHI digestion of pVL-F:hBrd4(1-1223)	pVL-F:hBrd4ΔBDII/NLS	EcoRI-BglII
pVL-F:hBrd4ΔET	XbaI-SmaI digestion of pF:hBrd4(1-1043)ΔET-11d	pVL-F:hBrd4(FL)	XbaI-SmaI
pVL-F:hBrd4ΔET/CTM	XbaI digestion of pcDNA3-F:hBrd4ΔET/CTM	pVL1392	XbaI
pVL-F:hBrd4ΔNLS	BglII-EcoRI digestion of pF:hBrd4(1-722)ΔNLS-7	pVL-F:hBrd4(EcoRI-NotI)	BamHI-EcoRI
pVL-F:hBrd4ΔNLS/CTM	EcoRI-BamHI digestion of pVL-F:hBrd4(1-1223)	pVL-F:hBrd4ΔNLS	EcoRI-BglII

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