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Multiple Roles of Brd4 in the Human Papillomavirus Life Cycle

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Abstract
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PAPILLOMAVIRUS LIFE CYCLE

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Jianxin You

While human papillomavirus (HPV) vaccines protect against acquiring new infections, there is currently no antiviral treatment for eradicating persistent HPV infections. In this study, I demonstrated that the cellular chromatin binding protein, Brd4, in association with HPV E2 protein, is important for multiple HPV functions including replication, maintenance of viral genomes, and regulation of viral gene transcription. These studies suggest that the E2–Brd4 complex could be an effective target to disrupt the HPV life cycle. Using bimolecular fluorescence complementation, we demonstrate that E2 from high–risk HPV16 interacts with Brd4 on cellular chromosomes throughout mitosis while the BET bromodomain inhibitor, JQ1(+), dissociates Brd4–E2 complexes from mitotic chromosomes. These results suggest that Brd4 is important for tethering HPV16 E2 to mitotic chromosomes for stable viral genome maintenance and that abrogating Brd4's chromatin association might disrupt stable HPV genome maintenance. I also found that JQ1(+) treatment of cells stably maintaining papillomavirus genomes reduces viral mRNA levels, demonstrating that HPV association with cellular chromatin through Brd4 is essential for HPV transcription and further supporting the importance of Brd4 for the HPV life cycle. My work also identified a novel role of Brd4 in HPV16 DNA replication. Immunofluorescence analyses show Brd4 is recruited to nuclear foci actively replicating HPV16 genomes. Replication assays further confirm that Brd4 is essential for HPV16 genome replication. Interestingly, JQ1(+) treatment stimulates viral genome replication. Since HPV genome amplification is normally limited to upper epithelial layers, we predict premature stimulation of viral DNA amplification induced by JQ1(+) in basal epithelial cells might activate host immune responses to clear HPV infection. Finally, this work identified a specific function of Brd4 in papillomavirus transcription activation. Using ChIP analysis and an E2–responsive luciferase assay, we show Brd4 actively recruits P–TEFb to papillomavirus genomes to support E2 transactivation function. Together, this study uncovers two novel functions of Brd4 in the HPV life cycle, improving our understanding of this complex virus–host relationship. Furthermore, we identify the E2–Brd4 complex as a promising antiviral target for eliminating HPV persistent infection.

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DEDICATION

This thesis is dedicated to my family and to my husband, Geoff.

To my family, thank you for loving me and for always believing in me. Making this journey so far away from you was difficult but just knowing that you were all supporting me and cheering me on from afar gave me the strength, confidence, and motivation to face any challenges and complete this journey.

To Geoff, thank you for all the sacrifices you made so that we could be together these last couple years. Your love and support got me through the tough times and your unwavering confidence in me truly helped elevate my self-esteem and for this, I am eternally grateful.

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While human papillomavirus (HPV) vaccines protect against acquiring new infections, there is currently no antiviral treatment for eradicating persistent HPV infections. In this study, I demonstrated that the cellular chromatin binding protein, Brd4, in association with HPV E2 protein, is important for multiple HPV functions including replication, maintenance of viral genomes, and regulation of viral gene transcription. These studies suggest that the E2-Brd4 complex could be an effective target to disrupt the HPV life cycle. Using bimolecular fluorescence complementation, we demonstrate that E2 from high-risk HPV16 interacts with Brd4 on cellular chromosomes throughout mitosis while the BET bromodomain inhibitor, JQ1(+), dissociates Brd4-E2 complexes from mitotic chromosomes. These results suggest that Brd4 is important for tethering HPV16 E2 to mitotic chromosomes for stable viral genome maintenance and that abrogating Brd4's chromatin association might disrupt stable HPV genome maintenance. I also found that JQ1(+) treatment of cells stably maintaining papillomavirus genomes reduces viral mRNA levels, demonstrating that HPV association with cellular chromatin through Brd4 is essential for HPV transcription and further supporting the importance of Brd4 for the HPV life cycle. My work also

identified a novel role of Brd4 in HPV16 DNA replication. Immunofluorescence analyses show Brd4 is recruited to nuclear foci actively replicating HPV16 genomes. Replication assays further confirm that Brd4 is essential for HPV16 genome replication. Interestingly, JQ1(+) treatment stimulates viral genome replication. Since HPV genome amplification is normally limited to upper epithelial layers, we predict premature stimulation of viral DNA amplification induced by JQ1(+) in basal epithelial cells might activate host immune responses to clear HPV infection. Finally, this work identified a specific function of Brd4 in papillomavirus transcription activation. Using ChIP analysis and an E2-responsive luciferase assay, we show Brd4 actively recruits P-TEFb to papillomavirus genomes to support E2 transactivation function. Together, this study uncovers two novel functions of Brd4 in the HPV life cycle, improving our understanding of this complex virus-host relationship. Furthermore, we identify the E2-Brd4 complex as a promising antiviral target for eliminating HPV persistent infection.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: Introduction

1.1: The Human Papillomavirus Life Cycle and HPV-Associated Cancers

1.1.1: The Papillomavirus Life Cycle

Papillomaviruses are small, non-enveloped, DNA viruses that infect dividing basal cells of stratified squamous and cutaneous epithelia. HPV is the most common sexually transmitted virus infection and over 150 different types of HPVs have been identified to date (99). The most common HPV subtypes associated with cancer include HPV type 16, 18, and 31, which are termed "high-risk" HPVs (HR-HPV) while the majority of HPV types are considered "low-risk" (LR-HPV) since they are not usually associated with human cancers (42, 43, 142, 185, 205). LR-HPV types are commonly associated with benign anogenital warts and warts of the hands and feet (43).

Viral entry into cells occurs when the virus gains access to the basal epithelial layer through microwounds (42). The viral capsids initially bind to the basement membrane and infect basal keratinocytes present in the wound (Fig. 1.1, (97, 236)). After entry and uncoating, the virus establishes early infection in the nucleus and replicates its genomes to about 100 genome copies per cell (42).

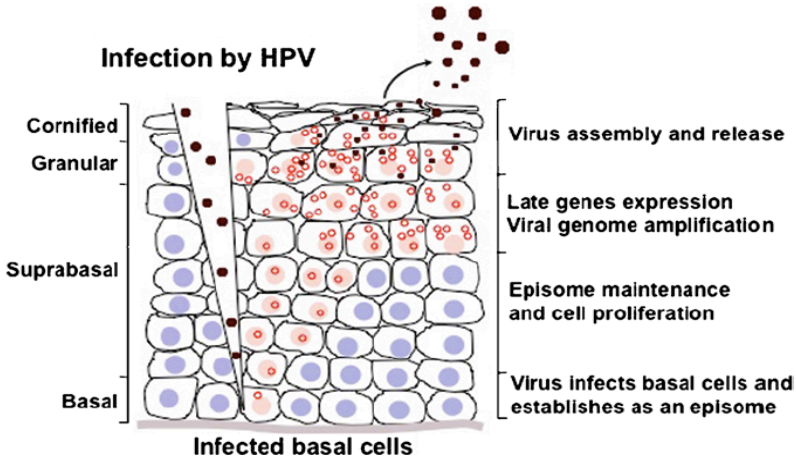

Figure. 1.1. Model of the productive HPV life cycle (adapted from Springer, *Cancer Associated Viruses*, 2012, 19/Human Papillomaviruses and Cancer, You J. and Wells S., Fig. 19.2, DOI 10.1007/978-1-4614-0016-5_19, © Springer Science and Business Media (228)).

Papillomaviruses have a circular double-stranded DNA genome, which is termed an episome. The viral genome is about 8000 base pairs in length and can be divided into three regions (Fig. 1.2). The long control region (LCR) consists of the origin of replication, transcriptional enhancers, and promoters. The early region encodes viral proteins expressed early during infection from early promoters (PE) including the oncoproteins, E6 and E7, the replication proteins, E1and E2, and the accessory proteins, E4 and E5. The late region encodes the capsid proteins, L1 and L2, which are exclusively expressed from the late promoter (PL) during late infection (92). Expression of the viral proteins is tightly regulated and is linked to the differentiation program of the infected epithelial cell (136). During early infection, E1 and E2 are expressed at low

level to replicate the viral genome once per cell cycle. This is the maintenance phase of the HPV life cycle in which the virus establishes a stable, persistent infection in replicating basal cells. Since both viral protein expression and viral genome replication are maintained at minimum level, the virus is able to evade the host immune system so that infection can persist in basal epithelial cells for many months, or in rare cases, years or decades (21).

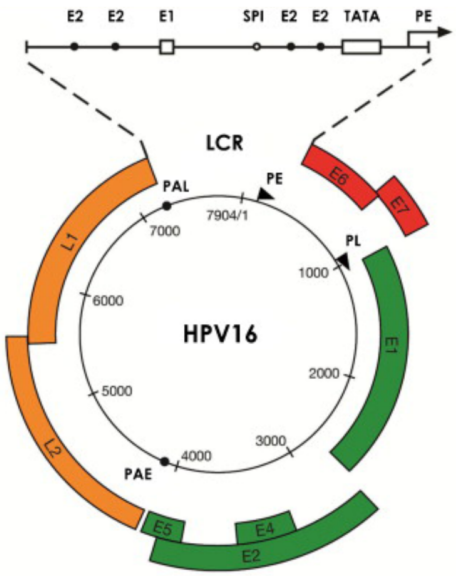

Figure 1.2. Organization of the HPV16 genome (adapted from Doorbar *et al*., 2012 (43)).

Normal human squamous epithelia grow as stratified layers where only cells in the basal layer are able to divide (210). After basal cell division, one daughter cell becomes a new basal cell while the other migrates up the epithelial layer and undergoes differentiation. These differentiated cells normally withdraw from the cell cycle and begin keratin synthesis and nuclear breakdown (210). However, during HPV infection,

the viral oncoproteins, E6 and E7, prevent the normal cell cycle exit of the differentiated cells to set up a cellular environment conducive for viral genome amplification and virion production (52, 62). E7 functions to drive infected cells into S phase by binding to phosphorylated retinoblastoma (pRb) proteins and inhibiting their interaction with E2F transcription factors (141). E7 also associates with other proteins involved in cell proliferation including cyclin-dependent kinase inhibitors and histone deacetylases (117, 141). E6, on the other hand, primarily functions to inactivate p53 to block growth arrest and apoptosis in response to the cell cycle changes mediated by E7 (79). Furthermore, E6 has several other oncogenic functions including activating telomerase, altering transcription, reducing immune recognition, and disrupting cell adhesion and differentiation (79).

Differentiation of infected epithelial cells triggers activation of the viral late promoter, which leads to high expression of E1 and E2 (43). These elevated levels of the viral replication proteins initiate the genome amplification phase of the papillomavirus life cycle in which viral genomes amplify to thousands of copies per cell (Fig. 1.1). Late after the onset of genome amplification, the viral capsid proteins are expressed and virions are assembled. Virions are shed from the top layer of the epithelia with desquamated cells in the absence of cell lysis or necrosis, thus avoiding inflammation while contributing to virus persistence (73).

1.1.2: HPV-Associated Cancers

Infection by HR-HPVs is associated with cervical cancer, which is the second most prevalent cancer in women worldwide and kills roughly 288,000 women annually (142, 185, 205). In addition, HR-HPV is the causative agent in many anal cancer cases (236). In recent years, it has become increasingly apparent that HPV is also an important cause of some oropharyngeal cancers (236).

Persistent HR-HPV infection over long periods is the single most important risk factor for the development of HPV-associated cancer (189, 214). Furthermore, integration of the HPV genome into the host chromosome is found in most cervical cancer cases, suggesting that this is a critical event in cancer development (29, 89). It is thought that extended periods of manipulating cell proliferation and cell cycle checkpoints by the HPV oncoproteins gradually induces genomic instabilities, which allow the viral genome to become integrated into the host chromosome (47). Intriguingly, several studies have indicated a preference for HPV integration at common fragile sites in the host genome and have suggested that changes in the expression of cellular genes near the integration site might participate in cancer development (31, 123, 195, 196). When these integrated viral genomes were analyzed, it was discovered that most integrations disrupt the E2 gene so that it is no longer expressed (166). This is important because E2 negatively regulates E6 and E7 expression and derepression of these viral oncogenes is required for malignant progression (45, 72, 90). However, cells immortalized with HR-HPV are non-tumorigenic at low passage numbers. They require extended growth or expression of additional oncoproteins such as *ras* or *fos* to become

tumorigenic, suggesting that HPV infection is necessary but not sufficient for cancer development (8, 48, 156). Indeed, it was established that accumulation of secondary mutations in the cellular genome over several years of HPV persistent infection is required for cell transformation to cancer (235).

HPV-associated cancers are composed of cells that have lost the ability to differentiate and can no longer support the complete viral life cycle. In addition, the viral genomes in these cancer cells are usually integrated so they are unable to replicate. Together, this means that cancer cells do not produce HPV virions and are therefore a dead end for the virus. Since this does not benefit the virus, an interesting question to ponder is why HR-HPVs cause cancer. One possibility is that development to cancer could simply be a mistaken side effect of inactivating pRb and p53 surveillance pathways, which are necessary for HPV replication.

1.1.3: Current Treatments for HPV Infection

Infection with HPV is very common and is normally identified and cleared by the host immune system before cancer can develop (76, 88). Still, about 450,000 new cases of cervical cancer are diagnosed each year (155). The wide utilization of the Papanicolaou (Pap) smear in the developed world has enabled early detection of cellular abnormalities associated with HPV infection and has resulted in an 80% reduction in the number of cervical cancer cases in the US (182). Furthermore, there are now two commercially available prophylactic HPV virus-like particle vaccines that protect against HPV types 16 and 18 (Cervarix) and HPV types 6, 11, 16, and 18 (Gardasil). While these

vaccines will likely further reduce the incidence of HPV-associated diseases in the developed world, these diseases will likely remain prevalent in developing countries where the Pap smear and HPV vaccines are not as widely available. In addition, the HPV vaccines do not protect against every high-risk HPV type and are ineffective at treating established infections so there is still a strong need for antiviral drugs to eliminate ongoing HPV infections (9, 198).

1.2: E2 Functions in the HPV Life Cycle

1.2.1: E2 Domain Structure

E2 is the major regulatory protein encoded by all papillomaviruses. The E2 protein consists of an N-terminal transactivation domain linked to a DNA binding/dimerization domain. The two domains are connected by a flexible "hinge" linker sequence. All papillomaviruses have the potential to also express alternatively spliced short E2 variants that lack most of the transactivation domain. These short forms usually function as inhibitors of full-length E2 by competing for E2 binding sequences (114, 135).

Through a conserved DNA binding domain, E2 proteins bind specific consensus motifs located in the viral LCR (Fig. 1.1, (6, 71, 127, 139)). In the alpha subgroup of papillomaviruses, which includes the high-risk HPV subtypes, there are four E2 binding sites in the LCR important for regulating viral transcription, replication, and episome maintenance. Interestingly, despite no sequence similarity, the E2 DNA binding domain has significant structural similarity with that of the EBV EBNA1 protein (20).

The amino-terminal 200 amino acids of E2 form the conserved transactivation domain. This region is essential for both transcription activation and repression of viral genes as well as for viral replication (126, 133). The crystal structure of the E2 transactivation domain for several PVs including HPV16 and HPV18 has been solved (7, 70). In all PVs analyzed, the transactivation domain forms a cashew shaped structure with the amino acids important for transcription located on the convex face while the amino acids important for replication are located on the concave face of the domain.

The E2 DNA binding domain also contains a dimerization region. E2 proteins form stable dimers upon translation (126, 134, 140). These E2 dimers can be homodimers consisting of two full-length or short E2 isoforms or they can be a heterodimer of a full-length E2 and a short E2. The heterodimers have been shown to support viral replication and transcription but are unable to tether the viral genome to host chromosomes for episome partitioning (102-104).

The E2 hinge region is highly variable between PV subtypes in both sequence and length and is thought to be an unstructured, flexible linker between the transactivation and DNA binding domains (63, 67, 126). The hinge is not required for E2's replication and transcription functions but serves as a necessary spacer to avoid steric hindrance between the two domains (213). Notably, the hinge region contains several phosphorylation sites that are important for E2 auxiliary functions such as protein stability and subcellular localization (125, 158, 177, 178, 233).

1.2.2: E2's Role in Viral Genome Maintenance

During the persistent phase of early papillomavirus infection, viral episomal genomes replicate once per cell cycle and are maintained in the nucleus at about 100 copies per cell (65). To ensure stable maintenance of these episomes between mitotic divisions, the virus must have a way to faithfully partition them to the nuclei of daughter cells. Since papillomaviruses do not encode a centromere protein, they require another mechanism to ensure that the viral genomes remain within the nuclear envelope following nuclear membrane reassembly. Otherwise, the viral genomes could be excluded from the nucleus after mitosis and lost through degradation or dilution.

It is well established that E2 is the key viral factor required for stable maintenance of viral episomes in dividing cells through tethering the viral genomes to mitotic chromosomes (15, 159, 183). The E2 DNA binding domain interacts with E2 binding sites in the viral LCR while the transactivation domain associates with a cellular factor bound to the mitotic chromosomes (3). In the case of bovine papillomavirus, the cellular factor important for E2 association with mitotic chromosomes is bromodomain-containing protein 4 (Brd4) (17, 224). However, it is still unclear whether Brd4 also performs this function for other papillomavirus species.

One study showed by immunofluorescence staining that E2 proteins from various HPV subtypes bind to different regions of mitotic chromosomes, suggesting that each PV subtype might target a different cellular factor on the chromosomes for episome tethering (147). Another protein implicated in E2 tethering to mitotic chromosomes is the topoisomerase binding protein, TopBP1. TopBP1 was shown to interact with HPV16 E2

and regulate the affinity of E2 for host chromatin (23, 40). In addition, E2 and TopBP1 colocalize on mitotic chromosomes during late stages of mitosis, supporting a role for this protein in E2-mediated episome tethering (40). The DNA helicase, ChlR1, was also shown to interact with several PV E2s including HPV16 E2 (153). Furthermore, using disruption of this interaction or knock-down of ChlR1, the E2-ChlR1 interaction was demonstrated to be necessary for E2 binding to mitotic chromosomes and for maintenance of episomes in dividing cells (153). However, ChlR1 is thought to load E2 and the associated viral genomes onto chromosomes only during S phase since E2 and ChlR1 were only found colocalized on chromosomes during early mitosis. It is therefore possible that E2 proteins tether the viral genome to chromosomes through a complex process involving more than one cellular factor.

1.2.3: E2's Role in Viral Genome Replication

The PV origin of replication contains an E1 binding site, an A/T rich sequence, and one or more E2 binding sites (201). E2 primarily functions in the initiation of viral genome replication by recruiting and loading the viral helicase, E1, onto the replication origin (171). Upon binding the origin, E1 assembles into double-hexamers and recruits the cellular replication machinery, while E2 is displaced from the origin for DNA replication to proceed (124, 175).

Viral DNA replication takes place in nuclear foci and E2 is required for formation of these replication foci (193, 208). Besides the viral and cellular replication factors, other cellular factors found within HPV replication foci include members of the ATM and ATR DNA damage pathway (55, 164, 170). The cellular DNA damage response is

believed to facilitate HPV genome replication by recruiting cellular repair proteins to synthesize viral DNA (66, 164, 170). E1 is the primary activator of the DNA damage response in cells because its helicase domain is able to bind non-specifically to cellular DNA and initiate inappropriate DNA unwinding, which causes damage throughout the cellular genome and a robust DNA damage response (55, 170). E2 functions to direct E1 binding specifically to the viral origin and with it, the DDR proteins are also localized to the viral replication foci, thereby reducing damage to the cellular genome caused by E1 while facilitating HPV DNA replication (170).

1.2.4: E2's Dual Functions in Viral Gene Transcription

E2 is the principle transcriptional regulator of papillomaviruses and acts through binding specifically to E2 binding sites in the viral upstream regulatory region (URR) and recruiting cellular factors (187). E2 can function as either an activator or repressor of viral transcription depending on the context of the E2 binding sites and the type of cellular factors that E2 recruits. Cellular proteins recruited to the early promoter by E2 include the transcription activators: CBP, p/CAF, BRCA1, Brm, and Brd4 as well as chromatin modifying proteins: SMCX, EP400, and TIP60 (96, 101, 109, 110, 184). A number of studies suggest that E2 regulation of the early promoter is dose-dependent, activating transcription at low concentrations and repressing transcription at higher concentrations (60, 192, 194). Steger *et al*. postulated that, at low concentrations, E2 binds its high-affinity, distal sites in the LCR and recruits transcriptional activators to the early promoter. As E2 levels increase, all four binding sites become occupied, which blocks the binding of transcriptional activators (192). How this dose-dependent

transcription regulation functions during the HPV lifecycle is still unclear. E2 levels are kept low during the maintenance phase of infection and increase to high levels during genome amplification in differentiated cells. It is therefore possible that E2's dual role in transcription regulation functions as a negative feedback loop in late infection where high E2 levels repress the viral oncogene expression to allow the cells to terminally differentiate, releasing virions into the environment with the sloughed off epithelia.

1.3: Brd4 Cellular Functions

1.3.1: Brd4 Structure and General Function

Brd4 is a member of the bromodomain and extra terminal domain (BET) family, which in mammals includes Brd2, Brd3, Brd4, and BrdT. These proteins all contain two tandem bromodomains (BDI and BDII) and an extra terminal (ET) domain but Brd4 is unique in that it also carries a long C-terminal domain (CTD) (Fig. 1.3, (50, 204)). The BET family proteins are ubiquitously expressed in cells with the exception of BrdT, which is only found in the ovaries and testes (151, 180). The double bromodomains of the BET family proteins are highly conserved and recognize acetylation marks at lysine 14 on histone H3 and lysines 5, 12, and 16 on histone H4 (36, 204). Until recently, only two isoforms of Brd4 were known to exist in cells. The long isoform is predominant and is responsible for most of the functions attributed to Brd4 (215). The Brd4 short isoform lacks the long C-terminal region after the ET domain and its function is not well understood, although it has been suggested to function as an inhibitor of full-length Brd4 (215). Notably, a recent publication identified a second short Brd4 isoform termed

"isoform B" (54). Like the original short isoform, isoform B lacks the C-terminal region after the ET domain but in its place contains a divergent 75 amino acid segment (54). This novel isoform was shown to modulate chromatin structure to insulate DNA from DNA damage signaling (54).

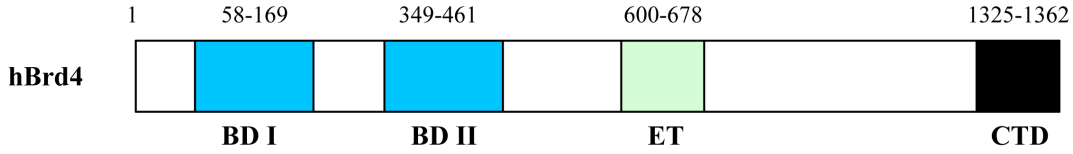

Figure. 1.3. Organization of the domains of human Brd4. BD: Bromodomain, ET: Extra-terminal domain, CTD: C-terminal domain. The amino acid numbers are indicated above the image.

1.3.2: Brd4's Functions in Cellular Transcription

Brd4 associated with chromatin is a key factor for transcription of a number of cellular genes (38, 222, 225). Probably the most important and well-studied function of Brd4 during transcription is recruitment of positive transcription elongation factor B (P-TEFb) to the transcription initiation complex (86, 223). P-TEFb is composed of the kinase Cdk9 and Cyclin T1 subunits and functions to activate transcription elongation by phosphorylating RNA polymerase II (RNAP II) and the transcription inhibitory factors, NELF and DSIF (161). The Brd4 C-terminal domain mediates the interaction with the Cdk9 subunit of P-TEFb (19). When P-TEFb is not associated with Brd4, it is bound in

an inhibitory complex with 7SK snRNP and hexamethylene bis-acetamide inducible 1 (HEXIM1). Brd4 binding releases P-TEFb from the inhibitory complex and activates Cdk9 kinase activity (13, 84).

Brd4 was recently identified as an atypical kinase that, like P-TEFb, can phosphorylate the RNAP II CTD tail at serine 2 (35). This particular phosphorylation activates transcription elongation (14). It was suggested that Brd4 is the major kinase that phosphorylates RNAP II CTD serine 2 during transcription initiation and elongation while P-TEFb is mostly important during transcription elongation (35). It will be interesting to discover whether other cellular proteins are phosphorylated by Brd4.

Brd4 also functions as a protein scaffold, interacting with a number of transcription factors and chromatin modifiers (32, 46, 86, 163). Rahman *et al*. demonstrated that several proteins bind the Brd4 ET domain including GLTSCR1, NSD3, and JMJD6 and contribute to Brd4's transcription activation function independently of P-TEFb (163). Brd4 also interacts with the transcription coactivator, Mediator, which is a protein complex that interacts with several general transcription factors and serves as a bridge between transcription factors and RNAP II (217).

1.3.3: Brd4 as a Mitotic Bookmark

The BET proteins are unique in that they remain associated with chromatin during mitosis unlike other bromodomain-containing proteins, which are usually displaced from mitotic chromosomes (36, 37, 94, 120, 160). Brd4 functions as a mitotic bookmark, binding chromatin near particular set of genes to preserve epigenetic memory between cell divisions (38, 229). Genes important for embryonic development are often

bookmarked during mitosis (93). Therefore, it is not entirely surprising that Brd4 is essential for proper embryonic development (78). Indeed, mice heterozygous for the Brd4 gene have several developmental abnormalities and Brd4-null mouse embryos die early in development (78).

A number of the genes bookmarked by Brd4 are important for cell cycle progression and Brd4 binding ensures that they are transcribed soon after mitosis (38). As a result, altering the level of Brd4 in cells either by siRNA knockdown or ectopic expression affects Brd4's function as a mitotic bookmark and influences cell cycle progression (37, 121, 132). For example, Brd4 siRNA knockdown or depletion with antibodies causes a reduction in expression of G1 genes, cell cycle arrest at G2/M, and reduced cellular proliferation (37, 132). In addition, ectopic over-expression of Brd4 induces G1/S arrest, which is thought to be related to Brd4's interaction with replication factor C1 (RFC1) (121). Furthermore it was found that either Brd4 knock-down or over-expression causes aberrant chromosome segregation and failures in cytokinesis, which is partially due to Brd4's regulation of Aurora B kinase expression (225). Interestingly, during mitotic stress induced by nocodozole treatment, JNK activation triggers Brd4 release from chromatin to prevent genomic instability and allow complete progression through mitosis (145).

1.3.4: Brd4 Regulates Chromatin Structure

Post-translational modifications of histone tails are important for regulating higher-order chromatin structure and the epigenetic reader proteins that recognize these

modifications often mediate the chromatin structure organization (64). Not surprisingly, Brd4 has been shown to regulate cellular chromatin compaction (229). One study that linked Brd4 to chromatin reorganization focused on post-mitotic transcription re-activation and demonstrated that Brd4 facilitates transcription re-activation after mitosis by decompacting chromatin around specific promoters to promote rapid transcription activation (229). Another study from our lab showed that Brd4 is important for maintenance of the global chromatin architecture. In this work, we demonstrated that Brd4 knockdown induces global chromatin decondensation while inhibition of Brd4 binding to chromatin via ectopic expression of the Brd4 bromodomains triggers a severely fragmented chromatin morphology (206). Furthermore, this study demonstrated that Brd4 proteins interact intermolecularly on chromatin, suggesting that Brd4 forms a molecular scaffold to maintain proper chromatin structure (206). Most recently it was demonstrated that a newly identified Brd4 isoform B interacts with and recruits members of the condensin II chromatin remodeling complex to insulate chromatin from DNA damage signaling (54). It will be interesting to determine how Brd4 regulates global chromatin structure and whether Brd4 interacts with other chromatin remodeling factors.

1.3.5: Brd4 in Human Cancers

Brd4 is a ubiquitously expressed protein associated with diverse cellular processes through its transcriptional regulation of a multitude of cellular genes. Incidentally, some Brd4-associated cellular processes link Brd4 to human cancers. One of the first studies to implicate Brd4 in cancer observed that ectopic expression of Brd4 reduced cell mobility

and invasiveness in a breast cancer model and demonstrated that Brd4 controls the expression of extra-cellular matrix factors that are linked to cancer cell metastasis (30).

Brd4 was also identified as a target of a chromosome translocation that results in the rare but highly lethal squamous cell cancer, NUT midline carcinoma (NMC) (58). This cancer arises from the t(15;19) translocation that fuses the testes-specific gene, NUT, to the N-terminal half of Brd4, which includes the bromodomains. Interestingly, some NMC cases were found with fusions of NUT to the Brd3 bromodomains (59). NUT fusion to the double bromodomains allows NUT to sequester histone acetyl-transferases to distinct chromatin foci in order to block global transcription and inhibit cellular differentiation (165, 219). The Brd4-NUT protein also functions to drive c-MYC expression to block cellular differentiation (69). Furthermore, Brd4-NUT increases SOX2 protein levels to drive cellular transformation (207).

NMC tumors are resistant to conventional chemotherapy and there is currently no effective cure for this fatal disease (16, 57). Importantly, the recently developed BET bromodomain inhibitor, JQ1, shows great promise as a potential treatment for NMC (51). JQ1 and the structurally similar I-BET are cell-permeable small molecules that bind within the acetyl-lysine binding pocket of BET protein bromodomains to inhibit the BET protein association with acetylated histones (51, 144). These molecules are highly selective for BET family bromodomains with very minimal binding to other bromodomain-containing proteins. Notably, JQ1 induces cellular differentiation and growth arrest in NMC cell lines and in a mouse xenograph model of NMC. In addition, JQ1 treatment also causes marked tumor regression and prolonged animal survival (51).

Brd4 has been identified as a therapeutic target in several different human cancers including acute myeloid leukemia, multiple myeloma, Burkitt's lymphoma, neuroblastoma, and prostate cancer (34, 130, 162, 218, 234). In cell culture studies of these diseases, JQ1 treatment induced potent growth arrest (34, 130, 162, 218, 234). Furthermore, JQ1 treatment in an animal model of prostate cancer resulted in greatly reduced tumor burden (218). This anti-tumor effect of Brd4 inhibition was initially attributed to Brd4's important role in regulating MYC, a transcription factor that is often mutated or upregulated in a variety of cancers (34, 130). Indeed, the cancers most greatly affected by Brd4 dysregulation tend to be driven by elevated MYC expression (12). In addition, Brd4 knock-down or bromodomain inhibition causes MYC suppression and down-regulation of its target genes (34, 130). More recently, Brd4 was found to also control expression of other cancer-related genes including the anti-apoptotic oncoprotein, Bcl2, and cell cycle driver, Cdk6, suggesting that cancers controlled by abnormal expression of these genes might also be susceptible to Brd4 inhibition (32).

1.4: Brd4 Functions in the HPV Life Cycle

1.4.1: The E2-Brd4 Interaction

Brd4 was first identified as an E2-associated factor in a proteomic screen of proteins that bind bovine papillomavirus 1 (BPV1) E2TA and this was later confirmed by yeast two-hybrid screening (146, 224). E2 interacts through its transactivation domain with the extreme C-terminus of Brd4 (Fig. 1.4, (2)). The other members of the BET family proteins lack this C-terminal region found in Brd4 and they therefore do not

interact with E2. However, it was found that the E2 proteins from all types of papillomaviruses interact with Brd4 although with a wide range of binding affinities (128).

After the discovery of E2's association with Brd4, mutagenesis studies were used to identify the E2 regions crucial for Brd4 interaction. Two conserved amino acids in the E2 transactivation domain, arginine 37 and isoleucine 73, were found to mediate E2 binding to Brd4. When these sites are mutated to alanines, E2 interaction with Brd4 is almost completely abolished (174). It was also found that while the C-terminal DNA binding domain of E2 is not necessary for Brd4 binding, the dimerization function of this domain greatly increases E2-Brd4 binding (26).

Brd4 normally associates with chromatin with a rapid on-off dynamic but when bound to E2, Brd4's association with chromatin is greatly stabilized (36, 129). Dimerized E2 further increases Brd4's affinity for chromatin likely by promoting assembly of E2-Brd4 complexes on the chromatin (26). Similarly, binding of Brd4 to E2 or ectopically expressed Brd4 CTD stabilizes E2's association with the E2 binding sites in the viral genome (108). Several studies also demonstrated that Brd4 binding stabilizes E2 at the protein level (61, 108). E2 proteins are relatively short-lived and their levels are controlled by ubiquitination and proteosomal degradation (18, 157, 158). Binding to the Brd4 CTD blocks the interaction between E2 and the Cullin-3 ubiquitin ligase, contributing to E2 stabilization (232). It was also shown that E2 is degraded in the cytoplasm but Brd4 binding retains E2 in the nucleus and inhibits E2 nuclear export (113).

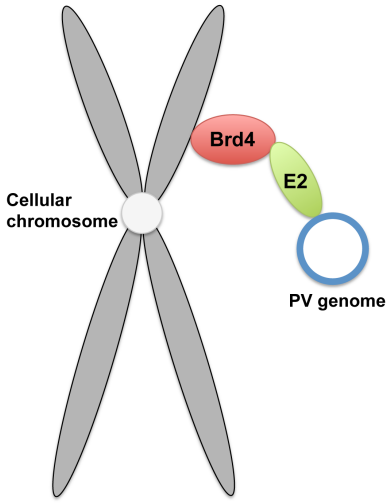

Figure 1.4. Model of Brd4 tethering of E2 and the viral episome to cellular chromosomes.

1.4.2: Brd4's Function in Papillomavirus Genome Maintenance

As described in section 1.2.2, E2 functions to tether viral episomes to host mitotic chromosomes to ensure viral genomes are maintained between cell divisions. For BPV1, Brd4 mediates E2 tethering to host mitotic chromosomes (Fig. 1.4, (224)). Brd4 normally diffusely coats mitotic chromosomes but in the presence of BPV1 E2, both proteins colocalize in a punctate pattern on mitotic chromosomes (129). When the E2-Brd4 interaction is abolished by mutating E2 at arginine 37 and/or isoleucine 73, E2 no longer associates with mitotic chromosomes (17). Furthermore, ectopic expression of the Brd4 CTD competitively inhibits the E2-Brd4 interaction causing E2's dissociation from mitotic chromosomes (224, 226). Moreover, expression of the Brd4 CTD causes BPV1 transformed cells to lose the viral episomes and revert to an untransformed phenotype (226).

It was unclear whether Brd4 also functions as the tethering protein during HPV infections. One study examined E2 proteins from several PV types and their association pattern with mitotic chromosomes by immunofluorescence (147). They found that the different HPV E2 proteins associate with mitotic chromosomes in distinctive patterns. HPV E2's from the same genera as BPV1 such as HPV1 E2 colocalize with Brd4 in punctate dots along the chromosome arms. However, the E2 from other HPV subtypes were found associated with pericentromeric regions of chromosomes and the E2 from high-risk HPV types such as HPV16 and 18 could not be detected on mitotic chromosomes by immunofluorescence unless the cells were pre-extracted before fixation. After this treatment, HPV16 and 18 E2 were found bound to pericentromeric regions of host chromosomes but there is still question about whether pre-extraction alters E2 nuclear localization.

Another group sought to determine whether inhibiting the Brd4 interaction with HR-HPV E2 protein affects viral episome tethering to mitotic chromosomes (2). They used a Brd4 CTD peptide fused to HIV Tat to deliver the peptide into cells that maintain either HPV16 or HPV31 genomes to abrogate the E2-Brd4 interaction. By analyzing metaphase spreads, they found that the Brd4 CTD fusion peptide abolished the association of HPV16 or 31 episomes with mitotic chromosomes, while a Brd4 CTD mutant peptide that does not bind E2 did not affect episome tethering to chromosomes. These results suggest that the Brd4 interaction with HR-HPV E2 proteins is necessary for viral genome tethering to mitotic chromosomes.

1.4.3: Brd4's Functions in Papillomavirus Transcription and Replication

E2 is the major transcription regulatory factor that functions to either activate or repress viral gene expression (see section 1.2.4). Mutational analyses identified amino acids in the E2 transactivation domain important for E2-mediated transcription activation. Two highly conserved residues, arginine 37 and isoleucine 73, were found to be crucial for E2 transactivation function since alanine substitutions at these sites rendered the E2 protein transcriptionally inactive (168, 213). Interestingly, it was later found that these two residues are also necessary for binding to Brd4. Subsequent studies discovered that Brd4 is essential for E2 transcription activation function (17, 128, 174). Indeed, both Brd4 knock-down or abrogation of the E2-Brd4 interaction by ectopic expression of Brd4 CTD inhibited transcription activation by E2 from several PV subtypes (128, 174, 179).

The R37A and I73A E2 point mutants were also found to abolish E2-dependent transcription repression, indicating a role for Brd4 in E2 transcriptional repression (216). Subsequent studies found that siRNA knock-down of Brd4 or disruption of the E2-Brd4 interaction using Brd4 CTD both inhibited E2's transcription repression function, confirming Brd4 as an important player in repression of PV gene expression (173, 184, 216).

In cervical cancer cells where the HPV genome is integrated into the host DNA, the E2 gene is invariably missing or disrupted so that E2 is no longer expressed. This is thought to be crucial for cervical cancer development since loss of E2 triggers derepression of the viral oncogenes (see section 1.1.2). This dysregulated expression of E6 and E7 was found to require Brd4 recruitment of P-TEFb to the viral oncogene

promoter integrated in the host genome (220). Importantly, this study discovered that E2 inhibits Brd4's interaction with P-TEFb and ectopic expression of E2 in HeLa cells prevents Brd4 recruitment of P-TEFb to the integrated viral promoter, providing a mechanism for E2-mediated repression in cervical cancer cells.

Although, Brd4 functions to recruit P-TEFb to activate the integrated viral early promoter in cervical cancer cells, it is still not known how Brd4 functions in transcription activation of episomal genomes during natural HPV infection. The papillomavirus episomes associate with cellular histones to form nucleosomes so it is possible that Brd4 interacts with these nucleosomes on the viral genomes to recruit transcription factors or chromatin modulator proteins (49, 167). Additionally, two studies demonstrated that E2 associates with transcriptionally active cellular chromatin through its interaction with Brd4, suggesting that E2 may bind to Brd4 to ensure that the viral episomes are retained in transcriptionally active regions of the nucleus to avoid transcriptional silencing (85, 87). Interestingly, these studies also showed that Brd4 associates with cellular chromatin near common fragile sites, which are prone to DNA damage, providing evidence that Brd4 recruits papillomavirus genomes to these sites to facilitate viral genome replication (87).

1.4.4: Brd4's Association with Other Viruses

Brd4 and some of the other BET proteins are targeted by other viruses besides the papillomaviruses. The herpesviruses, Kaposi's sarcoma-associated herpes virus (KSHV), and Epstein-Barr virus (EBV), encode viral proteins that not only have similar functions to the papillomavirus E2 protein but also interact with Brd4 (115, 149, 202, 227). The

KSHV latency-associated nuclear antigen (LANA1) protein regulates KSHV transcription and also tethers the viral genome to host mitotic chromosomes. Brd4 was found to interact with LANA1 on mitotic chromosomes, suggesting that it might be important for KSHV viral genome tethering to cellular chromosomes (227). Similarly, EBV encodes the Epstein-Barr nuclear antigen 1 protein (EBNA1), which functions in viral replication, viral genome tethering to cellular mitotic chromosomes, and viral transcription regulation (115). Brd4 binding to EBNA1 was shown to be important for EBNA1's transactivation function (115). Brd4 also binds the family of repeats (FR) enhancer regions within the EBV genome to possibly regulate viral transcription (115).

During human immunodeficiency virus (HIV) infection, the viral TAT protein activates expression of the viral genes by recruiting P-TEFb to the integrated HIV genome. Brd4 was shown to compete with TAT for P-TEFb binding and effectively inhibit transcription of HIV genes (19). More recently, there is evidence that blocking Brd4 function with the BET inhibitors such as JQ1 could be a useful tool to reactivate latent HIV (11, 22). Brd2 was also identified as an important suppressor of HIV transcription in latently infected cells and knock-down of Brd2 or treatment with the BET inhibitors also contributed to HIV latency reactivation (22).

Merkel cell polyomavirus (MCV) is a relatively newly discovered virus that is associated with about 80% of Merkel cell carcinoma cases (MCC) (77). Like other polyomaviruses, MCV encodes a large tumor (LT) antigen as well as a few alternatively spliced truncations of LT (105). The LT protein is important for regulating viral genome replication and viral transcription (105). Our group discovered that the MCV LT protein

binds Brd4, which recruits RFC1 protein to the viral replication complex to support MCV genome replication (209).

Most recently, Brd4 was identified as an important interaction partner for the murine leukemia virus (MLV) integrase protein (33, 181). As described in previous sections, Brd4 binds acetylated histones and is mostly associated with transcriptionally active regions of cellular chromatin (36, 204). In the above studies, Brd4 was shown to bind the MLV integrase protein to direct viral integration near cellular transcriptional start sites, which ensures the viral genes are expressed and not silenced (33, 181).

CHAPTER 2: Analysis of the HPV16 E2 and Brd4 Interaction Using BiFC

Research presented in this chapter was accomplished in collaboration with a postdoctoral fellow in our laboratory, Ranran Wang, with equal contribution. I have contributed to the experimental design and execution, and manuscript writing for this publication. Together with Ranran, I have ensured that the BiFC signal was specific for the E2-Brd4 complex (Figure 2.1, 2.2), examined the E2-Brd4 BiFC in interphase and mitotic cells (Figure 2.3), and analyzed the E2-Brd4 BiFC signal in the presence of the Brd4 C-terminal domain (Figure. 2.4). I conducted the experiments testing the effect of both the HPV genome and JQ1(+) on E2-Brd4 BiFC signal intensity and chromatin association (Figure 2.5, 2.6).

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2.1: Chapter Summary

The human papillomavirus (HPV) vaccines effectively protect against new infections of up to four HPV subtypes. However, these vaccines are not protective against many other clinically relevant HPV subtypes and are ineffective at treating established HPV infections. There is therefore a significant need for antiviral treatments for persistent HPV infections. We hypothesize that the complex between the HPV E2 protein and Brd4 would be an effective anti-HPV drug target since this pair mediates several viral processes including genome maintenance, replication, and transcription of HPV proteins. Using bimolecular fluorescence complementation (BiFC) technology, we observe the HPV16 E2 and Brd4 interaction on cellular chromatin in interphase and mitotic cells. The E2-Brd4 BiFC is significantly diminished by mutating the Brd4 binding sites in E2 or by a dominant negative inhibitor of the E2-Brd4 interaction, demonstrating that BiFC may be useful for identifying inhibitors of protein-protein interactions. Importantly, when Brd4 is released from chromatin using the bromodomain inhibitor JQ1(+), the E2-Brd4 complex relocates into foci that no longer associate with mitotic chromosomes, pointing to JQ1(+) as a promising antiviral inhibitor of HPV genome maintenance during HPV persistent infection.

2.2: Introduction

Infection by the high-risk human papillomavirus subtypes is associated with several types of cancer including various anogenital cancers as well as head and neck cancers (236). Papillomaviruses infect undifferentiated basal epithelial cells where the virus establishes a persistent infection. In the majority of cases, infection is eventually cleared by the host immune system but in the rare cases when the infection is allowed to persist for several years, mutations can arise that develop into cancer (41).

There are now two commercially available prophylactic HPV virus-like particle vaccines that protect against HPV types 16 and 18 (Cervarix) and HPV types 6, 11, 16, and 18 (Gardasil). Although the vaccines effectively prevent initial infections, they are ineffective at curing persistent infections (10, 198). Alternative approaches are therefore needed for treating ongoing infections.

Currently, there are no virus-specific antiviral therapies to cure HPV infection. The E1 helicase protein is the only enzyme encoded by papillomaviruses making it a promising target for drug design. However, small molecule inhibitors against E1 ATPase activity were unfortunately found to be inactive in cellular assays (190, 212). The highly conserved papillomavirus E2 protein interacts with E1 and is required for several viral functions making this protein another promising candidate for drug targeting. Indeed, White *et al*. identified small molecule inhibitors of the E2-E1 interaction for HPV 6 and 11, demonstrating the feasibility of small molecule inhibitors of virus-mediated protein-protein interactions (211).

The papillomavirus E2 protein is an attractive target for drug design because it mediates several viral processes during PV infection. E2 is crucial for viral genome replication since it recruits the E1 helicase protein to the viral origin (171). Additionally E2 both positively and negatively regulates transcription from the viral promoter (60, 192, 194). E2 also functions in viral genome maintenance during persistent infection by tethering PV genomes to mitotic chromosomes to ensure their faithful partitioning into daughter cells during cell divisions (15, 82, 112, 159, 183).

An important E2 interacting partner is Brd4 (224). The Brd4 C-terminal domain binds to the E2 transactivation domain and helps E2 perform many of its viral functions (2, 128). Our laboratory has shown that Brd4 is an essential E2 cofactor during HPV genome replication (208) (see Chapter 3). Brd4 is also recruited by E2 to help regulate viral transcription (128, 174, 184, 216). In addition, Brd4 and BPV1 E2 interact on mitotic chromosomes to mediate viral genome maintenance (Fig. 1.4, (17, 24, 129, 224)).

Since E2 in complex with Brd4 controls multiple important HPV functions, it has been proposed that antiviral inhibitors targeting this interaction would likely abrogate the HPV life cycle and clear the infection (190). In support of this hypothesis, past studies have demonstrated that blocking E2-Brd4 with either Brd4 binding-deficient E2 mutants or expression of Brd4 CTD impairs viral transcription activation and HPV genome replication (128, 174, 208). Furthermore, abrogation of the E2-Brd4 interaction abolishes tethering of both HPV16 and HPV31 viral episomes to mitotic chromosomes, which could eventually result in cell clearance of the virus genomes as cells divide (2).

In this work, we utilized bimolecular fluorescence complementation (BiFC) technology to study the Brd4 interaction with HPV16 E2 in live and fixed cells. This technique allowed us to visualize physiological levels of E2 and Brd4 interacting in live cells at all cell cycle stages and demonstrated Brd4's involvement in E2 tethering to mitotic chromosomes. Furthermore, the utility of BiFC for drug screening was demonstrated using Brd4 CTD as an E2-Brd4 inhibitor that effectively abolishes E2-Brd4 BiFC. Finally, we show that the bromodomain inhibitor, JQ1(+), releases E2-Brd4 BiFC from mitotic chromosomes, indicating this drug as a potential agent to interrupt and clear HPV persistent infection.

2.3: Results

2.3.1: BiFC signal is specific for the Brd4-E2 interaction

Brd4 is the molecular tether for BPV1, linking E2 and the viral genome to host mitotic chromosomes to ensure stable maintenance of the viral genomes between cell divisions (17, 24, 129, 224). However, despite the fact that Brd4 interacts with the E2 proteins of the human papillomaviruses, it is still unclear whether Brd4 also plays a role in episome tethering for the HPV subtypes (2, 129, 224). We employed bimolecular fluorescence complementation (BiFC) technology to study the interaction between HPV type 16 E2 and Brd4 and determine the importance of Brd4 for tethering HPV16 E2 to mitotic chromosomes. BiFC is a useful method to examine protein-protein interactions in live and fixed cells. The technique involves fusing the proteins of interest individually to

complementary fragments of a fluorescent protein and expressing them together in cells. Interaction between the two fusion proteins brings the fluorescent protein fragments within close proximity, allowing the fluorescent protein to reform and emit fluorescent signal (95). With this method, specific protein interactions can be identified and located within live or fixed cells through visualization of fluorescent signal.

For these studies, Brd4 was fused to the N-terminal portion (VN) of the yellow fluorescent protein, Venus, while either HPV16 E2 or BPV1 E2TA was fused to the C-terminal portion of Venus (VC). The resulting constructs both contain Flag and hemagglutinin (HA) epitope tags to monitor the proteins in cells. We first confirmed that the BiFC signal in our experiments was specific for the E2-Brd4 interaction. The HPV-negative cervical cancer cell line, C33A, was used in these studies since it provides a similar cellular environment that human papillomaviruses encounter during natural infection. We and other investigators have previously observed by immuofluorescence staining of C33A cells that Brd4 localizes in punctate speckles throughout the nucleus and when E2 is present, it colocalizes with Brd4 in these nuclear speckles (129, 224). Interestingly, in live C33A cells cotransfected with VN-Brd4 and either VC-E2TA or VC-16E2, we could detect the E2-Brd4 BiFC signal in nuclear speckles, demonstrating a real-time interaction of BPV1 E2TA and HPV16 E2 with Brd4. Similar results were seen in fixed cells, which allowed us to examine the cells under high magnification (Fig. 2.1 A). In contrast to the above results, cells cotransfected with the empty VN construct and either VC-E2TA or VC-16E2 did not produce any BiFC signal despite the high expression of these constructs measured by anti-Flag immunostaining and

immunoblotting (Fig. 2.1 A, B). In addition, co-expressing VN-Brd4 and the empty VC construct also did not generate BiFC signal (data not shown). These observations confirmed that the BiFC signal was generated through specific interaction between Brd4 and E2.

To further test the specificity of E2-Brd4 BiFC, we examined E2 mutants defective in Brd4 binding. E2TR is a truncated protein expressed from the BPV1 E2 ORF, which lacks the transactivation domain and functions as a repressor of E2TA activity during the viral life cycle (114, 135). This protein also lacks the Brd4 binding domain and, therefore, does not interact with Brd4 (224). For HPV16 E2, the R37A/I73A double point mutant has been shown to bind Brd4 with much lower efficiency than wild type E2 (128). These E2 proteins were compared with E2TA and wild type 16E2 for interaction with Brd4 using BiFC. C33A cells were cotransfected with VN-Brd4 and one of the VC-E2 proteins (E2TA, E2TR, 16E2, or 16E2 R37A/I73A). Western blot analysis showed that expression levels were comparable between the E2 proteins except E2TR, which consistently had lower expression than the other E2 proteins (Fig. 2.2 C). Importantly, the VN-Brd4 fusion protein expressed at a much lower level than endogenous Brd4, suggesting that it is not likely to induce an over-expression artifact (Fig. 2.2 D). Co-expression of Brd4 with the wild type E2 proteins generated a strong BiFC signal in nuclear speckles (Fig. 2.2 A). In contrast, co-expression of Brd4 with the E2 mutants only showed dim BiFC nuclear foci (Fig. 2.2 A). The cells were also immunostained with anti-Flag antibody to visualize expression of the Flag-tagged Venus fusion proteins and the percentage of Flag-positive cells with BiFC

signal was quantified. While virtually all E2TA- or 16E2 WT-transfected cells had robust BiFC signal, the majority of cells transfected with E2TR or 16E2 R37A/I73A were either negative for BiFC signal or had very dim BiFC nuclear speckles, which were counted as positive BiFC in the quantification (Fig. 2.2 B). Notably, the dim BiFC signal of 16E2 R37A/I73A-Brd4 or E2TR-Brd4 could only be detected in cells with very high expression of Venus fusion proteins (as indicated by the strong Flag signal). The high quantities of Brd4 and E2 mutant Venus fusion proteins present together in cells could contribute to the high BiFC background, which was also observed in previously published studies (98, 143). The Brd4 and 16E2 R37A/I73A BiFC also suggests that, while the affinity of this E2 mutant for Brd4 is greatly reduced, it still retains some ability to bind Brd4. This is supported by co-immunoprecipitation showing that the HPV16 E2 R37A/I73A mutant could pull-down a detectible amount of Brd4 protein (Fig. 2.2 E). These data demonstrate that breaking the E2-Brd4 interaction by mutagenesis could greatly reduce the E2-Brd4 BiFC signal, confirming that the BiFC signal was specifically generated through the E2-Brd4 interaction.

2.3.2: E2 and Brd4 interact during interphase and mitosis

Previous immunofluorescence analyses have shown that Brd4 interacts with E2 from various papillomavirus types during interphase and mitosis (128). However, the cell cycle phases at which HPV16 E2 and Brd4 interact are less clear. By immunofluorescence, we detected 16E2 colocalized in small speckles on chromatin with endogenous Brd4 in C33A interphase cells (Fig. 2.3 A). Similarly, in C33A cells cotransfected with VN-Brd4 and VC-16E2 WT plasmids, E2-Brd4 BiFC fluorescence

was detected in small speckles on interphase chromatin (Fig. 2.3 B). The immunofluorescence technique was not sensitive enough to detect HPV16 colocalized in speckles with endogenous Brd4 on mitotic chromosomes (data not shown). However, using BiFC technology, we observed HPV16 E2-Brd4 BiFC fluorescence in small dots on mitotic chromosomes from all phases of mitosis (Fig. 2.3 B). This result was also observed with VN-Brd4 and VC-E2TA (data not shown). In cells transfected with VC-16E2 and VN empty vector, neither BiFC signal nor Flag-16E2 immunofluorescence was detected on mitotic chromosomes (Fig. 2.3 C). This experiment demonstrates that BiFC is a useful tool to study the E2-Brd4 interaction in live cells throughout the cell cycle. In addition, this study shows that HPV16 E2 and Brd4 interact on chromosomes during mitosis and suggests a role for Brd4 in HPV16 E2-mediated episome tethering and maintenance in host cells.

2.3.3: Brd4 CTD disrupts E2-Brd4 BiFC

Papillomavirus E2 proteins interact with the Brd4 CTD and previous studies demonstrated that the Brd4 CTD could competitively inhibit the E2 interaction with full-length Brd4 and disrupt multiple viral processes (2, 174, 216, 224). We sought to test whether the CTD could abrogate E2-Brd4 BiFC signal with the thought that this technique could be used to screen small molecule inhibitors of this important interaction. C33A cells were cotransfected with the VN-Brd4 plasmid, one of the VC-E2 plasmids, and a construct that expressed either Xpress-tagged Brd4 CTD or LacI, which served as a negative control. Both the Brd4 CTD and LacI proteins contained nuclear localization sequences to focus the proteins in the nucleus. In live cells, there was an obvious

reduction in the number of BiFC positive cells in the CTD transfections compared to cells transfected with LacI (data not shown). We further examined the BiFC signal in fixed cells co-stained with HA to detect the E2 and Brd4 fusion proteins and Xpress to detect CTD or LacI (Fig. 2.4 A and B). As seen in live cells, expression of LacI did not affect E2-Brd4 BiFC signal. However, despite the lower expression of CTD measured by immunoblotting (Fig. 2.4 D), there was a marked decrease in cells with BiFC fluorescence in CTD-positive cells (Fig. 2.4 A and B). The percentage of HA- and Xpress-positive cells with BiFC signal was quantified, revealing a significant reduction in BiFC-positive cells when CTD was expressed as compared to LacI (Fig. 2.4 C). This study shows that a dominant negative inhibitor of the E2-Brd4 interaction could disrupt E2-Brd4 BiFC, further confirming that the BiFC signal is specifically generated through the E2-Brd4 interaction. Additionally, this experiment demonstrates the potential for BiFC as a technique to identify inhibitors of this important interaction.

2.3.4: The HPV genome enhances E2-Brd4 BiFC

During natural papillomavirus infection, E2 forms a homodimer and binds E2 binding sites in the long control region of the viral genome (44). We were curious whether the presence of the HPV genome would affect E2-Brd4 BiFC since it was previously shown that Brd4 interacts more efficiently with dimerized E2 proteins (26). In this experiment, we cotransfected C33A cells with VC-16E2, VN-Brd4, and either empty vector or the HPV16 genome at a 1:2 ratio (BiFC constructs:HPV16 genome/vector) to increase the probability that cells with BiFC signal also contain HPV16 genome or empty vector. Intriguingly, cells transfected with the HPV16 genome had a more than two-fold

increase in E2-Brd4 BiFC signal intensity compared to cells transfected with empty vector. (Fig. 2.5 A, B) From this result, we suspect that the HPV genome promotes E2 dimerization, which enhances E2-Brd4 interaction. However, it is also possible that other viral mRNA or proteins expressed from the HPV genome might somehow promote the E2-Brd4 interaction. Further experiments are needed to better understand this result.

2.3.5: JQ1(+) treatment releases E2-Brd4 BiFC proteins from mitotic chromosomes

Brd4 associates with acetylated histones through its double bromodomains and the chemical compound JQ1(+) effectively inhibits this association, releasing Brd4 from chromatin (51). Since Brd4 is thought to tether E2 to mitotic chromosomes for viral genome maintenance, we hypothesized that releasing Brd4 from chromosomes by JQ1(+) would disrupt the association of the E2-Brd4 complex with mitotic chromosomes. To test this, we used the BiFC system to monitor the effect of JQ1(+) treatment on the E2-Brd4 interaction with mitotic chromosomes and interphase chromatin. C33A cells were cotransfected with the VN-Brd4 and VC-16E2 plasmids and 24 hours later treated with 500nM JQ1(+) or the inactive stereoisomer JQ1(-) for various lengths of time. Interestingly, within 30 minutes of JQ1(+) treatment, the E2-Brd4 BiFC signal was no longer in the chromatin-associated speckles but instead formed punctate foci that grew larger with increased incubation in JQ1(+) (Fig. 2.6 A). In contrast, E2-Brd4 BiFC signal remained in the chromatin-associated speckles in cells treated with JQ1(-) (Fig. 2.6 A).

It was important to rule out the possibility that these punctate foci were non-specific aggregates of the BiFC proteins. We therefore repeated the above experiment but transfected cells with the VN-Brd4 plasmid and VC control construct followed by JQ1(+) treatment starting at 24 hours post transfection. As was seen in Fig 2.1, no BiFC fluorescence was formed with these two proteins (data not shown). Furthermore, the Flag immunofluorescence staining of the BiFC constructs was diffuse in the nucleus in contrast to the enrichment of Flag proteins in large foci seen for the VN-Brd4/VC-16E2 transfected cells treated with JQ1(+) (Fig. 2.6 A). This result suggests that these punctate foci are not likely to be non-specific protein aggregates but specifically form between E2 and Brd4 upon JQ1(+) treatment.

As another way to confirm the specificity of these foci, we determined whether the E2-Brd4 BiFC proteins in the large foci would return to chromatin-associated speckles when JQ1(+) was washed away from the cells. Cells transfected with the E2 and Brd4 BiFC plasmids were incubated with JQ1(+) for 24 hours and then thoroughly washed and cultured in media lacking JQ1(+) for up to three hours. Surprisingly, the E2-Brd4 BiFC began returning to the small speckles on chromatin within five minutes of removing JQ1(+) and, in most cells, was completely restored to its usual speckled pattern after one hour (Fig. 2.6 A). From this result we concluded that these large foci are not likely to be non-specific protein aggregates but rather large, reversible E2-Brd4 complexes formed specifically when Brd4 is released from chromatin.

We next examined the effect of JQ1(+) on the 16E2-Brd4 association with mitotic chromosomes. C33A cells were transfected with the 16E2 and Brd4 BiFC constructs and, 48 hours later, treated with 500 nM JQ1(+) or JQ1(-) for only 1 hour to minimize the drugs' effect on cell cycle and BiFC protein levels. Similar to mitotic cells without drug treatment in Fig. 2.3, mitotic cells treated with JQ1(-) had 16E2-Brd4 BiFC in small dots associated with the chromosomes in 36 out of 51 (70.6%) of transfected mitotic cells (Fig. 2.6 B). Conversely, the 16E2-Brd4 BiFC in mitotic cells treated with JQ1(+) was in large foci clearly dissociated from the chromosomes and only 6 out of 50 (16.0%) transfected mitotic cells had E2-Brd4 BiFC colocalized with chromosomes (Fig. 2.6 B). These foci were likely dissociated from the chromosomes but their signals superimposed on the chromosomes under the microscope. Since it was not possible to determine for sure that these foci were dissociated from chromosomes, we counted these cells as having E2-Brd4 BiFC still associated with chromatin making the above quantification a conservative estimate. Interestingly, we observed much fewer E2-Brd4 BiFC foci in the JQ1(+) treated cells compared to the JQ1(-) treated cells, suggesting that when dissociated from chromosomes during mitosis, the E2-Brd4 interaction might become less stable. It is also important to note that the Flag staining was completely excluded from the mitotic chromosomes in JQ1(+) treated cells, suggesting that 16E2 tethering to mitotic chromosomes is dependent on Brd4's associated with chromatin. We observed similar JQ1(+) effects on the E2TA-Brd4 association with mitotic chromosomes (data not shown). These results reveal the potential of JQ1(+) as a possible antiviral for disruption

of HPV episome maintenance during persistent infection and suggest an important role of Brd4 for tethering HPV16 E2 to mitotic chromosomes.

2.4: Discussion

Here, we used BiFC technology to demonstrate that HPV16 E2 and Brd4 interact on interphase chromatin and mitotic chromosomes. This association with mitotic chromosomes is abolished when Brd4 tethering to chromatin is inhibited by JQ1(+), demonstrating the potential of JQ1(+) as an antiviral agent to treat HPV persistent infections by abrogating episome maintenance. We also confirmed through these findings that Brd4 is an important factor for HPV16 E2 tethering to mitotic chromosomes and is therefore likely important for HPV16 episome maintenance between cell divisions.

Brd4 is known as the molecular tether for BPV1 episome maintenance but it appears that the tethering process for the HPV subtypes is more complex and involves multiple cellular factors. Besides Brd4, other proteins such as TopB1 and ChlR1 have been implicated in HPV episome maintenance (23, 40, 153). One question that remains is determining the specific function of Brd4 and these other proteins during episome tethering. It will also be important to determine if these proteins have redundant roles in episome maintenance or if they are each essential for the tethering process. From our JQ1(+) study, since no E2 immunofluorescent signal was detected on mitotic chromosomes in the presence of JQ1(+), we predict that Brd4 plays an essential role in

E2 tethering to chromosomes. Further investigation is required to determine the functional importance of the other factors involved in HPV episome maintenance.

We and other investigators have observed that HPV16 E2 interacts very weakly with Brd4 compared to other papillomavirus E2s such as BPV1 E2 (128). However, we saw a dramatic increase in E2-Brd4 BiFC signal intensity in the presence of the HPV genome. The simplest explanation for this is supported by work from others showing that E2 dimerizes when binding the HPV genome and as a dimer, E2 binds Brd4 more efficiently (26, 44). However, it is also worth noting that the HPV genome is known to associate with histones and form a structure similar to cellular chromatin that Brd4 likely interacts with through its double bromodomains (49, 167). The functional consequences of Brd4 binding HPV chromatin have not been investigated but it is possible that Brd4 binding viral chromatin could stabilize its interaction with E2. Additionally, one or more of the viral proteins expressed from the viral genome might have an unknown function in regulating the E2-Brd4 interaction but further analysis is needed to confirm this explanation. Nevertheless, our finding suggests that during infection when the viral episomes are present, the HPV16 E2 interaction with Brd4 is likely a much stronger interaction than was previously appreciated.

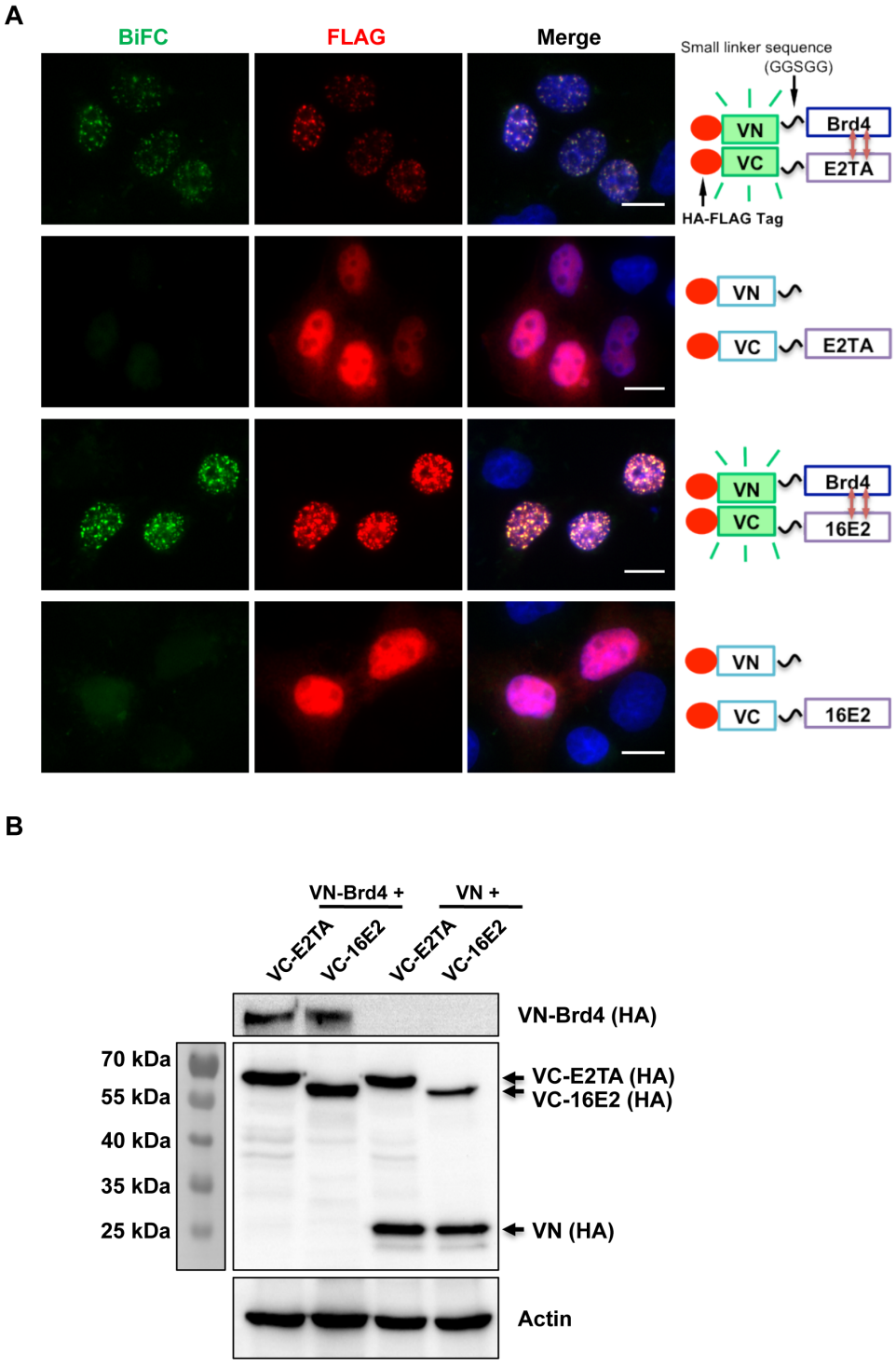
In C33A cells, Brd4 bound to cellular chromatin appears as nuclear speckles by immunofluorescence and E2 colocalizes with Brd4 in these speckles. Remarkably, upon treatment with JQ1(+), the E2 and Brd4 BiFC partners relocate into large, punctate spheres that are reversible when JQ1(+) is removed. Interestingly, VN-Brd4 and VC-

Brd4 BiFC partners also form nuclear spheres after treatment with JQ1(+) (data not shown) and, furthermore, we have observed that these punctate spheres formed by the Brd4-Brd4 and E2-Brd4 BiFC partners also contain PML protein (data not shown). This result suggests that when blocked from binding chromatin, Brd4 and any associated proteins localize to PML nuclear bodies where the proteins might be post-translationally modified or maybe simply stored in the nuclear bodies. Additional studies are necessary to better understand this result.

BET inhibitors like JQ1(+) are currently being tested in clinical trials as a potential treatment for various cancers such as AML and NUT midline carcinoma (231). In our experiments, we found that E2 was no longer tethered to mitotic chromosomes upon treatment with JQ1(+). This is important because during a persistent HPV infection, the virus relies on the tethering of E2 to chromosomes to ensure that its genomes are maintained and not lost during the latest stage of mitosis when the nuclear envelope reforms. It is therefore possible that JQ1(+) treatment of cells with persistent HPV infection could abolish tethering of E2 and the viral genome to host chromosomes resulting in a gradual loss of HPV genomes in each cell and eventual clearance of the infection. However, as will be discussed in later chapters, further studies in naturally infected cells are necessary to determine the overall impact of JQ1(+) on the HPV life cycle especially since Brd4 is involved in HPV genome replication and viral transcription regulation.

2.5: Figures

Figure 2.1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FLAG
Merge
Small linker sequence
(GGSGG)
VN
VC
Brd4
E2TA
HA-FLAG Tag
VN
VC
E2TA
VN
VC
Brd4
16E2
VN
VC
16E2
B
VN-Brd4 +
VN +
VC-E2TA
VC-16E2
VC-E2TA
VC-16E2
VN-Brd4 (HA)
70 kDa
55 kDa
40 kDa
35 kDa
25 kDa
VC-E2TA (HA)
VC-16E2 (HA)
VN (HA)
Actin

Figure 2.1. The BiFC signal is specifically generated through the E2-Brd4 interaction. (A) C33A cells were cotransfected with pairs of Venus N constructs (VN or VN-Brd4) and Venus C constructs (VC-E2TA or VC-16E2) as indicated on the right panel. Forty-eight hours post transfection, the cells were fixed and stained with anti-Flag antibody (red) and DAPI. Bar, 10 μm. (B) C33A cells were transfected as described in (A) and protein lysates were immunoblotted using anti-HA or anti-Actin antibodies. Arrows mark the VC-E2s or VN constructs expressed in cells.

Figure 2.2

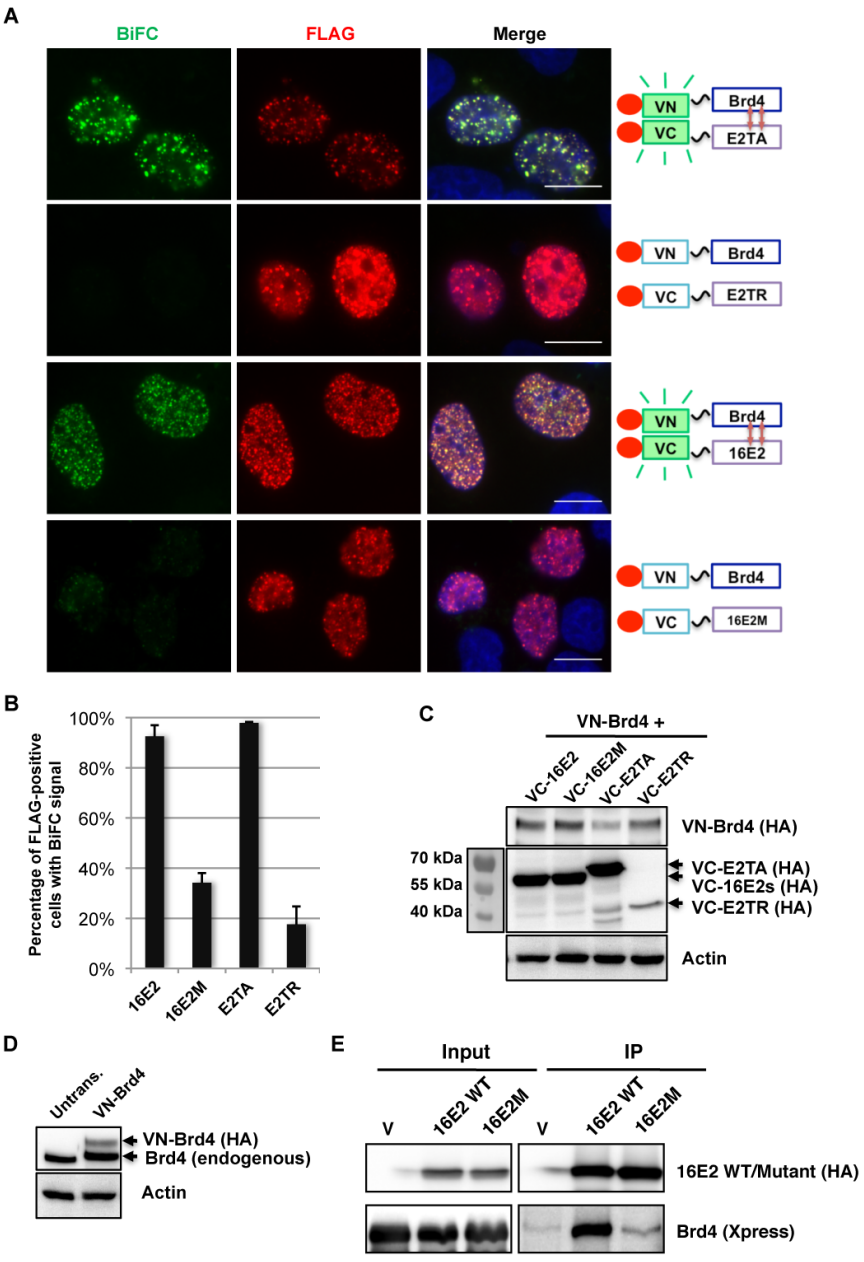

A
BiFC
FLAG
Merge
VN
VC
Brd4
E2TA
E2TR
16E2
16E2M
B
Percentage of FLAG-positive cells with BiFC signal
100%
80%
60%
40%
20%
0%
16E2
16E2M
E2TA
E2TR
C
VN-Brd4 +
VC-16E2
VC-16E2M
VC-E2TA
VC-E2TR
VN-Brd4 (HA)
70 kDa
55 kDa
40 kDa
VC-E2TA (HA)
VC-16E2s (HA)
VC-E2TR (HA)
Actin
D
Untrans.
VN-Brd4
VN-Brd4 (HA)
Brd4 (endogenous)
Actin
E
Input
IP
V
16E2 WT
16E2M
16E2 WT/Mutant (HA)
Brd4 (Xpress)

Figure 2.2. The E2-Brd4 BiFC signal is inhibited by mutating the Brd4 binding sites in E2. (A) C33A cells were cotransfected with VN-Brd4 and Venus C BiFC constructs (VC-E2TA, VC-E2TR, VC-16E2, or VC-16E2 R37A/I73A (16E2M)) as indicated on the right panel. Forty-eight hours post transfection, the cells were fixed and stained with anti-Flag antibody (red) and DAPI. Bar,10 μm. (B) For each transfection in (A), the percentage of cells showing BiFC signal was quantified from approximately 200 positively transfected cells. Average and standard deviation were calculated from three independent experiments. (C) C33A cells were either untreated or transfected as described in (A) and protein lysates were immunoblotted using anti-HA or anti-Actin antibodies. Arrows mark the VC-E2 constructs expressed in cells. (D) Protein lysates from untreated C33A cells or cells transfected with VN-Brd4 and VC-16E2 were immunoblotted using anti-Brd4 or anti-Actin antibodies. (E) C33A cells were cotransfected with Flag-HA-tagged 16E2 WT, R37A/I73A (16E2M), or empty vector as well as Xpress-tagged Brd4. At 48 hour post transfection, nuclear extracts were collected and used in Flag (M2) immunoprecipitations. The input and IP samples were analyzed by Western blotting using anti-HA and anti-Xpress antibodies.

Figure 2.3

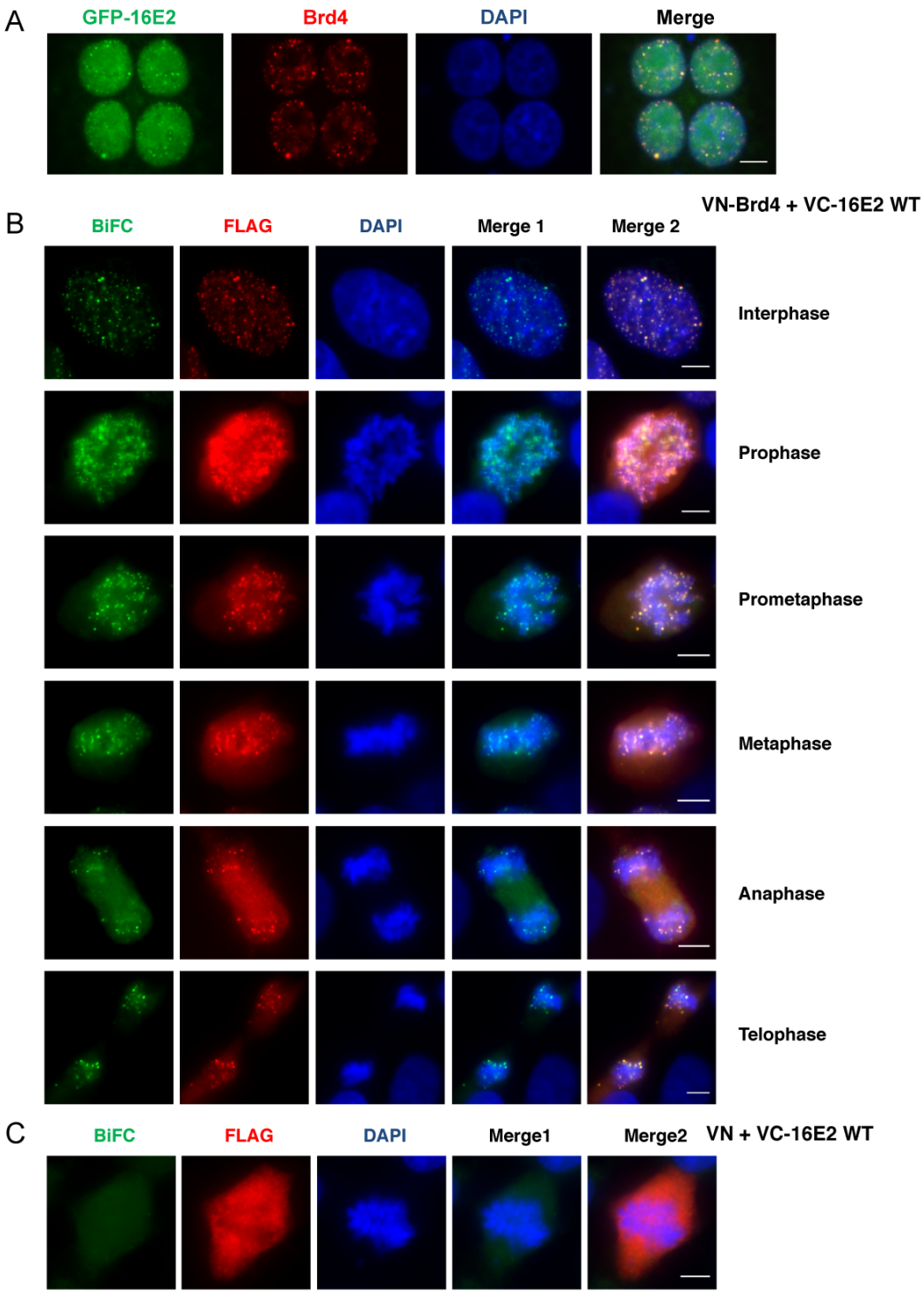

A
GFP-16E2
Brd4
DAPI
Merge
B
VN-Brd4 + VC-16E2 WT
BiFC
FLAG
DAPI
Merge 1
Merge 2
Interphase
Prophase
Prometaphase
Metaphase
Anaphase
Telophase
C
BiFC
FLAG
DAPI
Merge1
Merge2
VN + VC-16E2 WT

Figure 2.3. HPV16 E2 and Brd4 interact on chromatin in interphase and mitotic cells. (A) C33A cells were transfected with GFP-16E2 and 48 hours post transfection were fixed and stained with anti-Brd4C antibody (red) and DAPI. (B) C33A cells were cotransfected with VN-Brd4 and VC-16E2 BiFC constructs. Forty-eight hours post transfection, cells were fixed and stained with anti-Flag antibody (red) and DAPI. Merge 1 is a merge of the BIFC and DAPI panels and Merge 2 is a merge of BIFC, Flag and DAPI panels. (C) C33A cells were cotransfected with Venus N and VC-16E2 BiFC constructs. Forty-eight hours post transfection, the cells were stained with anti-Flag antibody (red) and DAPI. Bar, 5 μm.

Figure 2.4

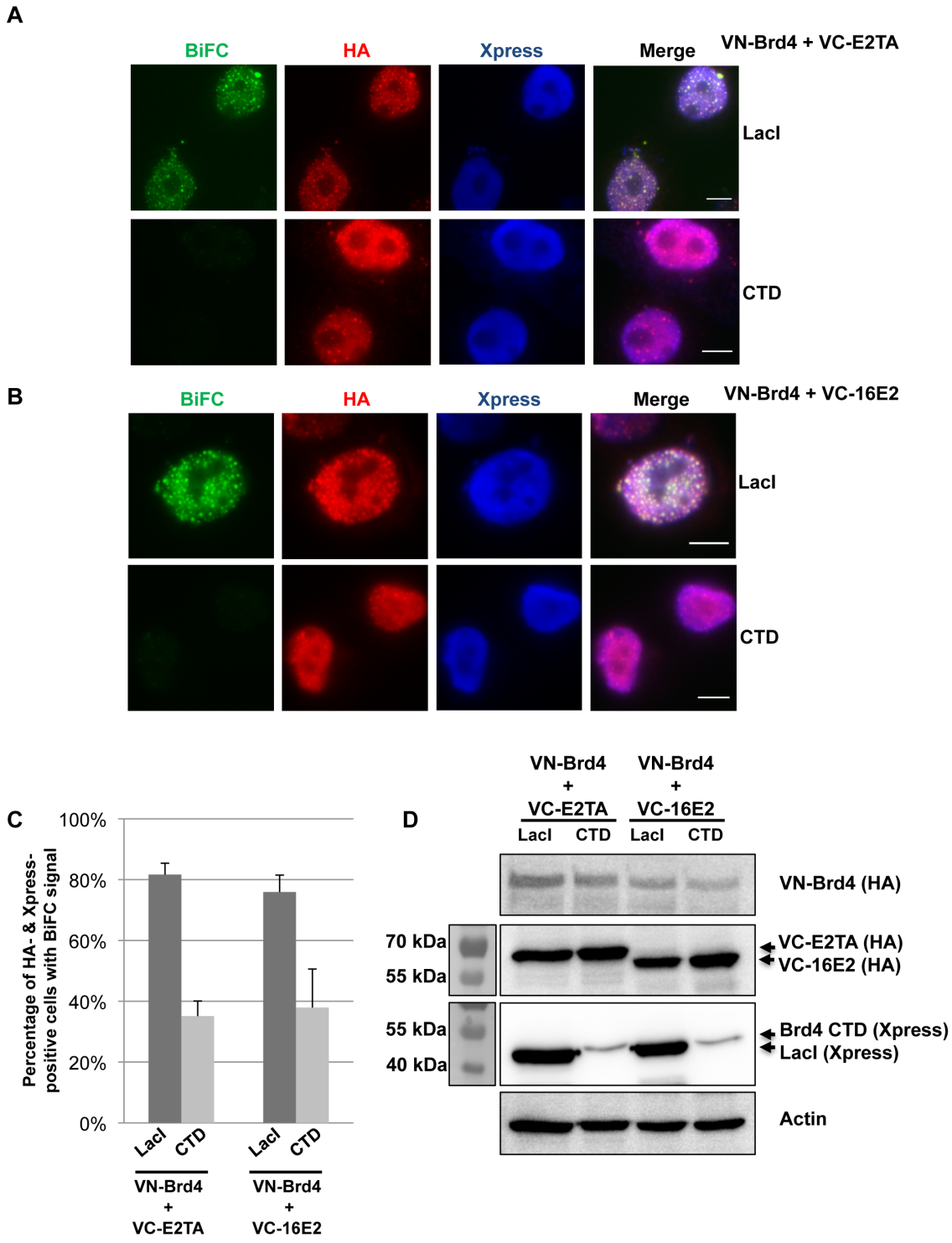

A
BiFC
HA
Xpress
Merge
VN-Brd4 + VC-E2TA
LacI
CTD
B
BiFC
HA
Xpress
Merge
VN-Brd4 + VC-16E2
LacI
CTD
C
Percentage of HA- & Xpress-positive cells with BiFC signal
100%
80%
60%
40%
20%
0%
LacI
CTD
LacI
CTD
VN-Brd4 + VC-E2TA
VN-Brd4 + VC-16E2
D
VN-Brd4 + VC-E2TA
VN-Brd4 + VC-16E2
LacI
CTD
LacI
CTD
VN-Brd4 (HA)
70 kDa
55 kDa
VC-E2TA (HA)
VC-16E2 (HA)
55 kDa
40 kDa
Brd4 CTD (Xpress)
LacI (Xpress)
Actin

Figure 2.4. Brd4 CTD effectively disrupts the E2-Brd4 interaction measured by BiFC. (A, B) C33A cells were cotransfected with VN-Brd4, VC-E2TA or VC-16E2, and either Xpress-LacI or Xpress-Brd4 CTD constructs as indicated on the right panel. Forty-eight hours post transfection, the cells were fixed and stained with anti-HA antibody (red) and anti-Xpress antibody (blue). (C) For each transfection in (A) and (B), the percentage of cells showing BiFC signal was quantified from approximately 100 Xpress- and HA-positive cells. Average and standard deviation were calculated from three independent experiments. Bar, 5 μm. (D) C33A cells were transfected as described in (A) and (B). Protein lysates were immunoblotted using anti-HA, anti-Xpress, or anti-Actin antibodies. Arrows mark the VC-E2s, Xpress-LacI, or Xpress-CTD constructs expressed in cells.

Figure 2.5

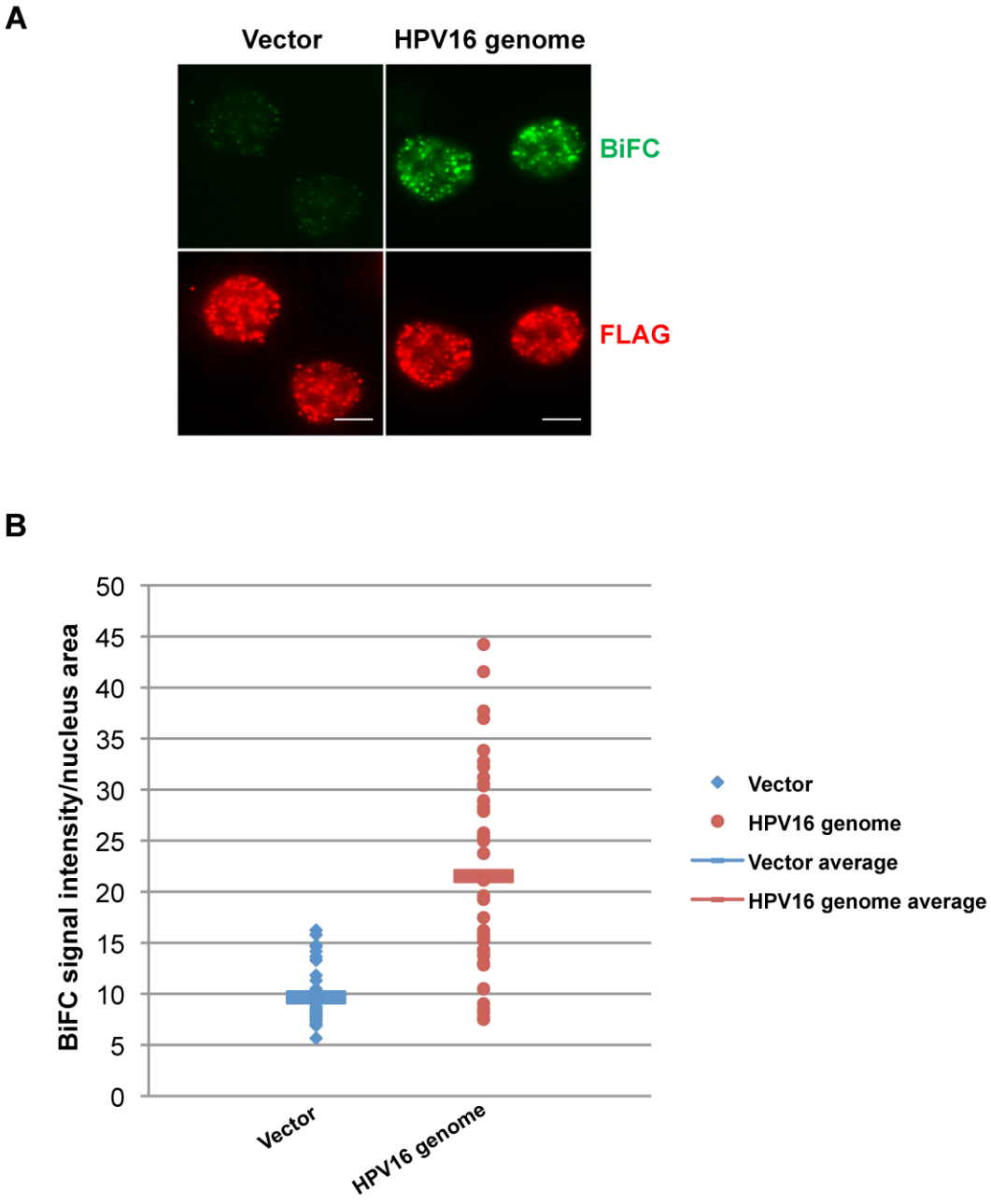

A
Vector
HPV16 genome
BiFC
FLAG
B
BiFC signal intensity/nucleus area
50
45
40
35
30
25
20
15
10
5
0
Vector
HPV16 genome
Vector
HPV16 genome
Vector average
HPV16 genome average

Figure 2.5. The E2-Brd4 BiFC signal is enhanced by the presence of HPV16 genome. (A) C33A cells were cotransfected with VN-Brd4, VC-16E2, and either pUC19 or pEFHPV-16W12E at a 1:2 ratio. Forty-eight hours post transfection, cells were fixed and stained with anti-Flag antibody (red) and DAPI. Bar, 5 μm. In the vector control, the BiFC signal is dimmer than in previous figures because there is much less E2/Brd4 BiFC DNA transfected. (B) Scatter plot of the average BiFC signal intensity divided by nucleus area in cells transfected as in (A). Data were collected from 50 vector transfected cells and 50 HPV16 genome transfected cells using ImageJ. This experiment was repeated twice with similar results. Bars indicate the mean of all cells examined.

Figure 2.6

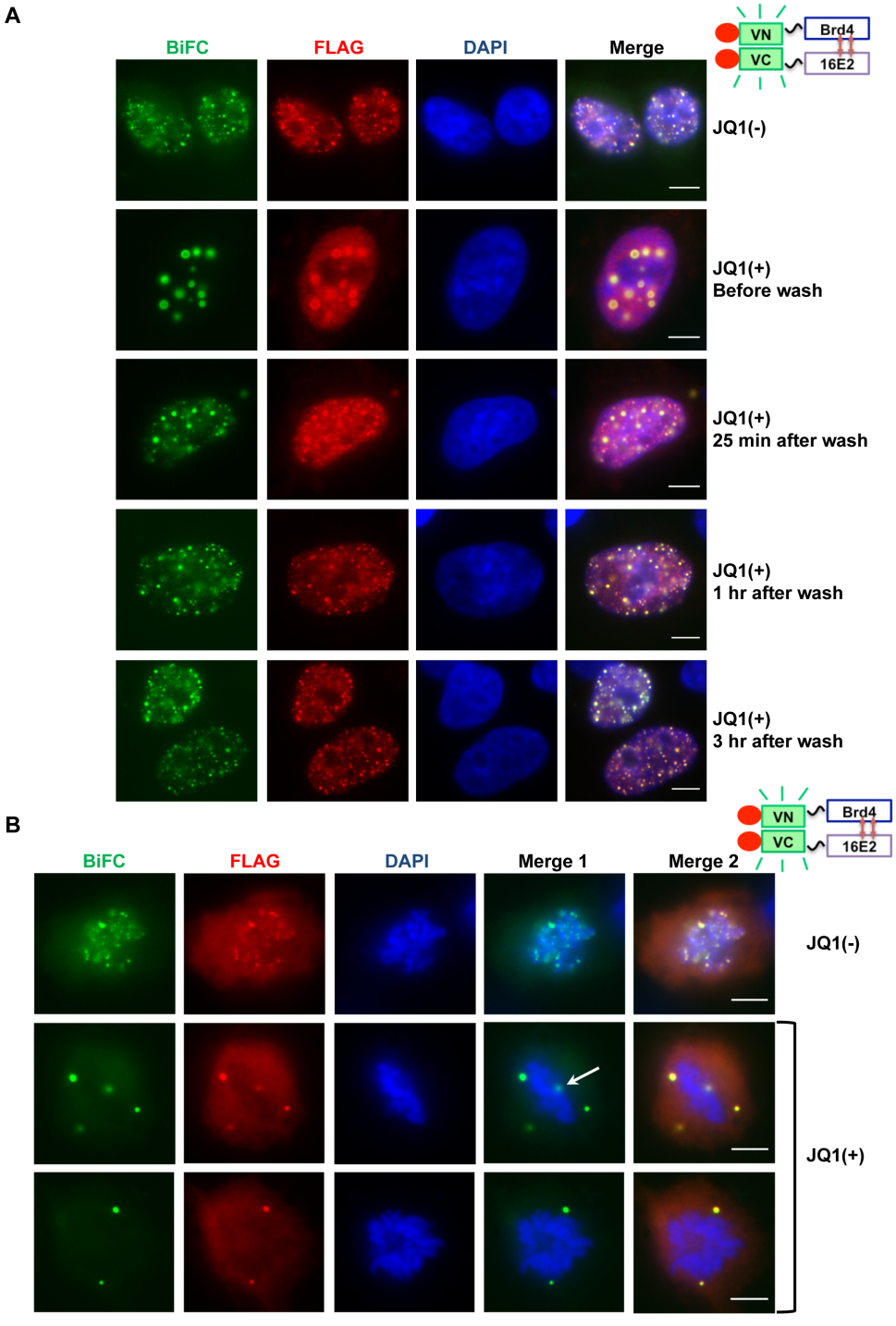

A
BiFC
FLAG
DAPI
Merge
VN
VC
Brd4
16E2
JQ1(-)
JQ1(+)
Before wash
JQ1(+)
25 min after wash
JQ1(+)
1 hr after wash
JQ1(+)
3 hr after wash
B
BiFC
FLAG
DAPI
Merge 1
Merge 2
JQ1(-)
JQ1(+)

Figure 2.6. Releasing Brd4 from chromatin by JQ1(+) abolishes the E2-Brd4 interaction on mitotic chromosomes. (A) C33A cells were cotransfected with VN-Brd4 and VC-16E2 and treated with 500 nM JQ1(-) or JQ1(+) at 24 hours post transfection. Forty-eight hours post transfection, cells were either fixed immediately (before wash) or washed several times and cultured for the times indicated on the right panel. All cells were fixed and stained with anti-Flag antibody (red) and DAPI. (B) C33A cells were cotransfected with VN-Brd4 and VC-16E2. Forty-eight hours post transfection, cells were treated with 500 nM JQ1(-) or JQ1(+) for 1 hour. The cells were then fixed and stained with anti-Flag antibody (red) and DAPI. Merge 1 is a merge of the BIFC and DAPI panels and Merge 2 is a merge of BIFC, FLAG and DAPI panels. Bar, 5 μm. The white arrow marks a BiFC focus superimposed on a mitotic chromosome.

CHAPTER 3: Brd4 is Essential for HPV16 DNA Replication

Research presented in this chapter was accomplished in collaboration with a postdoctoral fellow in our laboratory, Xin Wang, with equal contribution. I have contributed to the experimental design, execution, and manuscript writing for the publication. Together with Xin, I have examined the recruitment of Brd4 to the HPV replication foci by immunofluorescence (Figures 3.1-3.5) and tested Brd4 stimulation of HPV replication *in vitro* (Figure 3.6). I have examined the association of the E2, E1, and Brd4 proteins by co-immunoprecipitation (Figures 3.2 and 3.5). Xin performed the time course experiments (Figures 3.2 and 3.7), analyzed the viral genomes by immuno-FISH (Figure 3.3), and performed the *in vivo* replication experiments (Figures 3.5 and 3.7). Neha Pancholi offered feedback on the manuscript and provided technical assistance. James Bradner offered feedback on the manuscript and provided the JQ1(-) and JQ1(+) compounds.

This research was originally published in Journal of Virology. Xin Wang*, Christine M. Helfer*, Neha Pancholi, James E. Bradner, and Jianxin You. Recruitment of Brd4 to the Human Papillomavirus Type 16 DNA Replication Complex is Essential for Replication of Viral DNA. J. Virol. 2013 vol. 87(7) pp. 3871-3884. DOI: 10.1128/JVI.03068-12. *equal contribution

3.1: Chapter Summary

Replication of the HPV DNA genome relies on viral factors, E1 and E2, and the cellular replication machinery. Brd4 interacts with E2 to mediate papillomavirus genome maintenance and viral transcription. In this study, we examine the E2-Brd4 interaction in the presence of the HPV16 E1 protein and viral genome. We show that Brd4 is recruited to actively replicating HPV16 origin foci together with HPV16 E1, E2, and a number of the cellular replication factors: RPA70, RFC1, and DNA polymerase δ. Mutagenesis disrupting the E2-Brd4 interaction abolishes the formation of the HPV16 replication complex and impairs HPV16 DNA replication in cells. Brd4 is further demonstrated to be necessary for HPV16 viral DNA replication using a cell-free replication system in which Brd4 depletion by siRNA silencing leads to impaired HPV16 DNA replication whereas recombinant Brd4 protein is able to rescue viral DNA replication. In addition, releasing endogenous Brd4 from cellular chromatin using the bromodomain inhibitor JQ1(+) enhances HPV16 DNA replication, demonstrating that the role of Brd4 in HPV DNA replication can be uncoupled from its function in chromatin-associated transcriptional regulation and cell cycle control. This study reveals a new role for Brd4 in HPV genome replication, providing novel insights into understanding the life cycle of this oncogenic DNA virus.

3.2: Introduction

Papillomavirus genome replication occurs during two different stages of the viral life cycle. In the infected basal epithelial cells, the viral genome replicates an average of

once per cell cycle during S phase, in synchrony with the host DNA replication (65). This allows the viral genome to be maintained as stable episomes at 50 to 100 copies per cell. This stage of DNA replication ensures a persistent infection in the basal layers of the epidermis. Terminal differentiation of infected cells triggers vegetative viral DNA replication, producing viral genomes, which can then be assembled into virions and released from the surface of differentiated epithelium (74).

The viral E1 and E2 proteins in combination with various components of the cellular DNA replication machinery carry out replication of the PV genome (74). E2 binds to several consensus E2 binding sites near the HPV origin of replication (Ori) and recruits E1 to the viral Ori (133, 221). The cooperative binding of E1 and E2 proteins to the viral Ori form an E1-E2-Ori complex, in which E1 builds a hexameric ring around the viral DNA and functions as the helicase to unwind the HPV Ori for initiation of viral DNA replication (176). For successful completion of the viral DNA replication, many components of the cellular replication machinery are recruited by E1 and E2 to the viral origin of replication. For example, E1 has been shown to recruit the cellular DNA polymerase alpha/primase subunits to the viral replication origin (28, 122, 154). E1 interaction with the chaperone protein hsp40 and the single-stranded DNA-binding protein replication protein A (RPA) has also been shown to enhance E1 binding to the Ori and to facilitate processing of the replication fork, respectively (116, 118). Furthermore, interaction of E1 and hSNF5 proteins has been shown to stimulate HPV DNA replication (111).

Brd4 is a critical host interacting partner for the PV E2 protein (224). As presented in Chapter 2, Brd4 interacts with HPV16 E2 during interphase and mitosis and we and others have shown that Brd4 is an important factor in tethering the E2/viral genome complexes to host mitotic chromosomes to ensure faithful partitioning of replicated viral episomes to the nuclei of both daughter cells (Fig. 1.4, (17, 24, 75, 129, 224)). Brd4 also interacts with E2 to regulate viral transcription from the viral early promoter (see Chapter 4) (128, 174, 184, 216).

Brd4 is primarily known for its role in cellular transcription where it recruits P-TEFb to the pre-initiation complex (86, 223). However, Brd4 has been shown to interact with the PCNA sliding clamp unloader, ATAD5, and replication factor C (RFC), suggesting that Brd4 plays a role in cellular DNA replication (83, 121, 163). In accordance with Brd4's role in DNA replication, a study from our laboratory discovered that Brd4 functions in Merkel cell polyomavirus (MCV) genome replication through interaction with MCV large T antigen (209). HPV and MCV share similar properties in many aspects of their viral life cycles. For example, the viral genomes of both HPV and MCV are maintained as circular double-stranded DNA episomes during latent infection. These viral genomes replicate poorly in monolayer cell cultures and both types of virions are commonly shed from healthy human skin surfaces. In addition, MCV large T antigen and the HPV E1/E2 viral replication proteins are functionally conserved in binding the viral replication origin and interacting with Brd4. Together, these observations suggest that Brd4 might also be important for HPV DNA replication. In addition, Ilves *et al.* demonstrated that abrogation of the E2-Brd4 interaction reduces BPV1 genome

replication in some cell lines although the underlying mechanism was not fully investigated (82). These observations prompted us to investigate the role of Brd4 in HPV replication.

Here, we show that Brd4 is recruited to actively replicating HPV16 Ori foci together with HPV16 E1, E2, and a number of the cellular replication factors, including RPA70, RFC1, and DNA polymerase δ. The Brd4 function in HPV16 DNA replication was further demonstrated using several different approaches, including E2 mutagenesis, Brd4 silencing, and rescue of HPV16 replication in a cell-free replication system with recombinant Brd4 protein. We also reveal that dissociating Brd4 from chromatin with the bromodomain inhibitor JQ1(+), stimulates HPV Ori replication. Together, our findings uncover a new role of Brd4 in HPV DNA replication that is distinct from its chromatin-associated transcriptional regulation function, providing novel insights into understanding the life cycle of this oncogenic DNA virus.

3.3: Results

3.3.1: Brd4 colocalizes with E2 in large nuclear foci in the presence of HPV16 genomes

Previous studies that examined the E2 and Brd4 interaction did so in the absence of other viral components such as the HPV genome and viral proteins. In this study, we set out to investigate the interaction of Brd4 and HPV16 E2 in cells carrying the HPV16 genome and E1 protein. Interestingly, when C33A cells are transfected with HA-16E1, GFP-16E2 and the HPV16 genome, Brd4 is found colocalized in large, punctate dots with E2 (Fig. 3.1). In contrast, in the absence of the HPV16 genome, exogenously

expressed GFP-16E2 is diffuse as reported previously (170, 193), while Brd4 staining gives a characteristic speckled pattern in the nucleus (Fig. 3.1). This suggests that the HPV16 genome can enrich the interaction and colocalization of Brd4 and HPV16 E2 into subnuclear domains and/or promote the recruitment of the HPV16 E2/Brd4 complex to the HPV16 replication centers. This intriguing result prompted us to further investigate the potential function of the E2-Brd4 interaction in HPV16 replication.

3.3.2: HPV16 E1, E2, and Brd4 are recruited to the viral Ori replication foci.

The HPV16 genome contains the viral upstream regulatory region (URR), which includes transcription regulatory elements and the viral DNA replication origin. HPV E2 initiates viral gene transcription by recruiting many transcription factors, including Brd4, to the URR (86, 96, 101, 108-110, 223). The data shown in Fig. 3.1 suggests that Brd4 might be recruited by E2 to the viral URR to contribute to E2 viral transcription and/or replication function. Additionally, Brd4 might simply be enriched at the HPV16 genome through the binding of acetylated histones on HPV minichromosomes (138). To set up a system that would allow us to specifically examine the function of Brd4 in HPV16 replication instead of viral transcription and minichromosome association, we constructed an artificial episome that contains the 145 base pair minimum HPV replication Ori (Fig. 3.2 A), which we termed pHPVOri. This episome excludes most of the URR transcription regulatory elements, such as the binding sites for C/EBP, NF1, OCT-1, and the TATA box of the viral early promoter, retaining only three E2 binding sites (E2BS), one E1 binding site (E1BS), a GT-1 binding site, and an SP1 binding site (Fig. 3.2 A). According to mutagenesis studies performed in HPV11 URR, neither GT-1 site nor SP1

site deletion dramatically affects viral early promoter activities, suggesting that these sites might not contribute significantly to the early promoter transcription (230). Therefore, this plasmid with the minimum HPV16 Ori allows us to separate the HPV16 E2 transcription and replication functions and to specifically examine the recruitment of Brd4 and its impact on HPV16 replication.

Using the pHPVOri plasmid, we observed a similar phenomenon as seen in Fig. 3.1 with E2 and Brd4 colocalized in large foci only in the cells cotransfected with pHPVOri, E1 and, E2 constructs (data not shown). To visualize cells transfected with the HPV16 Ori construct and to also study the foci formation in the presence of HPV16 E1 protein, we inserted an HA-16E1 expression cassette, which includes HA-tagged 16E1 under the control of a CMV promoter, in pHPVOri to generate the pHPVOri/E1 plasmid (Fig. 3.2 A). With this plasmid, HPV16 Ori-positive cells can be identified as those stained positive for HA-16E1.

During HPV genome replication, E2 binds to its binding sites in HPV16 Ori and recruits E1 and cellular replication factors to the viral DNA replication complex (27, 199-201). To monitor the localization pattern of Brd4, E1, and E2 in cells carrying HPV16 Ori, cells transfected with GFP-16E2 and pHPVOri/E1 were stained by IF in three colors to show Brd4 in red, E1 in blue, and E2 displaying GFP green fluorescence. In these cells, we detected a clear colocalization pattern of Brd4, E2, and E1 assembled into large foci in 55.1% ± 3.4% of E1 and E2 double-positive cells (Fig. 3.2 B, experiment $n \geq 3$). The formation of Brd4-E1-E2 foci requires the HPV16 Ori because in cells transfected with GFP-16E2 and HA-16E1, which does not carry the HPV16 Ori, both E1 and E2

staining are diffuse in the nucleus of most cells and in only 19.8% ± 1.5% of E1 and E2 double positive cells, the viral proteins colocalize as tiny dots (Fig. 3.2 B, experiment n ≥ 3). This phenomenon was also reported in a previous study (170). Interestingly, in these cells, Brd4 staining is markedly altered from its normal punctate pattern to a more diffuse pattern that only partially colocalizes with the tiny E1/E2 dots (Fig. 3.2 B). In cells transfected with GFP-16E2 and HPVOri that does not carry the E1 expression cassette, E2 staining is diffuse despite the presence of HPVOri, while Brd4 maintains its usual speckled pattern (Fig. 3.2 B). This result is surprising because we expected E2 to bind the E2BSs within the Ori plasmid and to recruit Brd4. However, Brd4 was recruited to the large foci only when E1, E2, and HPV16 Ori were all present (Fig. 3.2 B). This observation suggests that E1 plays an important role in building these foci and recruiting Brd4. Together, these results indicate that recruitment of Brd4 into the large nuclear foci is dependent on the formation of the intact E1-E2-Ori replication complex. We also performed a time course experiment to look at the kinetics of viral replication focus formation over time (Fig. 3.2 C). Smaller Brd4, E1, and E2 replication foci were observed at earlier times post transfection. The size of the replication foci as detected by E1 staining continued to increase after 12 hours post transfection and reached a plateau at 36 hours post transfection.

The Brd4/E2/E1 complex was further investigated by Brd4 immunoprecipitation using cells transfected with CMV-Flag16E2 and either empty vector or HA-16E1. Consistent with previous findings, Brd4 is able to interact with and pull-down E2 (Fig.

3.2 D). Interestingly, E1 was also pulled-down with E2 and Brd4 providing biochemical evidence that these proteins assemble into a complex.

3.3.3: Brd4 colocalizes with the HPV16 DNA replication complex

Previous studies show that the E1 and E2 proteins co-expressed in cells localize to defined nuclear foci and induce a cellular DNA damage response (137, 170). To rule out the possibility that the E1, E2, and Brd4 foci observed in this study are sites of host chromosomal DNA replication and to demonstrate that these foci contain the HPV Ori, immuno-FISH was performed. C33A cells cotransfected with E2 and pHPVOri/E1 were examined by immunofluorescence to detect the viral proteins and by FISH to detect the HPV16 Ori. FISH using HPV16 Ori-specific probes detected large foci of HPV16 Ori similar to those detected by IF as shown in Fig. 3.2 B, while no signal was detectable when non-specific probes targeting the MCV genome were used (Fig. 3.3 A, B). In addition, both E1 and E2 colocalized with the HPV16 Ori in large nuclear foci in nearly all cells carrying viral replication foci (Fig. 3.3 A, B). Brd4 was also detected in these foci containing the HPV16 Ori (data not shown). These results further demonstrate that the large nuclear foci containing E1, E2, and Brd4 are centered on the HPV16 Ori.

Since the HPV16 foci we observed contain the viral replication proteins, E1 and E2, and the HPV16 Ori, we next determined if these foci are centers of viral replication. To test this, C33A cells cotransfected with GFP-16E2 and various vectors carrying 16E1 and/or Ori were pulse labeled with BrdU to detect its incorporation into newly synthesized DNA. As shown in Fig. 3.3 C, in cells transfected with GFP-16E2 and HPV16 Ori, GFP-16E2 and BrdU staining was diffuse in the nucleus and Brd4 was in its

normal nuclear speckled pattern. Cells transfected with pHPVOri/E1 and the empty GFP vector, pEGFPC1, also displayed a background BrdU staining and the normal speckled Brd4 pattern, while most of the GFP protein was removed by the acetone fixation used in the BrdU staining (Fig. 3.3 C). The diffuse BrdU staining in these cells cotransfected with HPV16 Ori and either E1 or E2 alone indicates that they do not have active HPV16 Ori replication (Fig. 3.3 C). Since these cells also have unaltered Brd4 localization pattern, the result suggests that both E1 and E2 are needed to support viral replication and Brd4 recruitment. In accordance with this notion, cells cotransfected with GFP-16E2 and pHPVOri/E1 displayed clear colocalization of GFP-16E2 and BrdU in large HPV16 Ori foci in 82% ± 8.8% of transfected cells (Fig. 3.3 C, experiment n = 3). Interestingly, Brd4 and E1 were also clearly present in the BrdU-labeled foci with E2 in these cells (Fig. 3.3 C and data not shown). It was clear that formation of these large foci is dependent on HPV16 Ori, since 54% ± 11.1% of cells transfected with GFP-16E2 and HA-16E1 that do not carry HPV16 Ori had small nuclear dots with GFP-16E2, Brd4 and BrdU colocalized while the rest of the cells had diffuse GFP-16E2 staining in the nucleus (Fig. 3.3 C, experiment n = 3). This result is consistent with previous reports showing that E1 and E2 can form small foci that colocalize with BrdU and cellular replication factors on host chromatin (170, 193). The small foci formed in the absence of HPV16 Ori are likely sites of E1- and E2-mediated non-specific unwinding/replication on the cellular genome. Our results demonstrate that Brd4 and the HPV replication proteins are present together at the HPV Ori in active viral replication compartments and suggest that Brd4 might be involved in HPV replication.

HPV genome replication requires the cellular replication machinery and it has previously been shown that host replication factors are recruited to HPV replication compartments (170, 193). To further demonstrate that the large Brd4-E2-E1 foci are viral replication centers, we next determined if host replication proteins are recruited to these foci. For this, C33A cells cotransfected with Flag-16E2 or GFP-16E2 and various vectors carrying 16E1 and/or Ori were immunostained for the RPA component, RPA70, replication factor C1 (RFC1), or DNA polymerase δ. As shown in Fig. 3.4, cells transfected with both E2 and pHPVOri/E1 had large E2-positive nuclear foci, which colocalized with all three replication proteins tested. RPA70 was observed colocalized with large foci in 67% ± 7.8% of cells while RFC1 and DNA polymerase δ colocalized with large foci in 51% ± 5.8% and 57.5% ± 2.7% of transfected cells respectively. In the absence of HPV16 Ori, E2 and E1 formed small foci that only partially colocalized with RPA70 and RFC1 in 61% ± 0.8% and 32% ± 4.9% of transfected cells, respectively (Fig. 3.4 A and B, experiment n = 3). DNA polymerase δ did not localize to the small foci and was diffuse in the nucleus without HPV Ori (Fig. 3.4 C, experiment n = 3). Without E1, E2 cotransfected with the HPV16 Ori construct did not form nuclear foci, and all three replication factors remained diffuse (Fig. 3.4). These results offer additional support that the large foci formed in cells transfected with both E2 and pHPVOri/E1 are sites of HPV DNA replication. The fact that Brd4 colocalizes with these replication factors in the HPV16 Ori foci provides further support for its role in HPV16 replication.

3.3.4: Brd4 is important for transient HPV16 replication in cells

Since Brd4 is clearly recruited to the HPV replication Ori foci that are actively replicating, we further investigated the role of the Brd4-E2 interaction in HPV16 DNA replication using HPV16 E2 mutant R37A/I73A, which is unable to interact with Brd4. As observed before, in cells transfected with wild type E2 and pHPVOri/E1, large foci were found in 56.9% ± 5.1% of E2-positive cells (Fig. 3.5 A, experiment n = 3). Conversely, when the E2-Brd4 interaction was abolished with E2 R37A/I73A mutations, large foci or tiny dots were found in <10% of cells and most cells had diffuse staining of E2, E1, and Brd4 (Fig. 3.5 A and data not shown, experiment n = 3). In the few cells with E1/E2 foci, Brd4 staining was more diffuse and only partially colocalized with the foci. Because large HPV replication foci cannot be efficiently formed in the E2 R37A/I73A cells, this set of results suggested that the E2-Brd4 interaction is essential for recruitment of Brd4 and formation of the HPV16 Ori replication foci. We also studied the E2 E39A mutant, which does not interact with E1 and, therefore, does not support HPV replication. As was predicted, cells transfected with both E2 E39A mutant and pHPVOri/E1 were not able to support large HPV replication foci formation as in the cells containing wild type E2 (Fig. 3.5 A). This result confirms that the E1 and E2 interaction is important for the formation of HPV replication foci. Interestingly, this E2 E39A mutant did not completely eliminate E1/E2 colocalization since, in 26.3% ± 3.5% of the transfected cells, E1, E2, and Brd4 were observed to colocalize in tiny dots (Fig. 3.5 A, experiment n = 3). However, these foci are much smaller than those observed in wild type E2 transfected cells. It is possible that, while the E2 E39A mutant failed to recruit E1 to

form the large HPV Ori replication complexes, E1 can still bind to the HPV Ori in an E2-independent manner to form the small foci as has been previously described (39). Brd4 is recruited in these small foci presumably through binding to the E2 E39A mutant. We also examined the E2 R302K and R304K mutants, which do not interact with DNA but retain Brd4 binding (26), for their ability to form replication foci. Similar to the E39A mutant, in cells transfected with pHPVOri/E1 and E2 R302K or R304K, 35.2% had tiny dots while the rest had diffuse staining of E2, E1, and Brd4 (Fig. 3.5 A and data not shown). Large replication foci were not found with these DNA binding-deficient mutants. From these results it seems that both the E2-Brd4 and E2-E1 interactions and E2-DNA binding are necessary for the formation of large HPV replication foci, suggesting that these interactions are important for viral replication.

To further analyze the impact of the E2-Brd4 and E1-E2 interactions on HPV16 Ori DNA replication, we performed an *in vivo* replication assay comparing various E2 mutants with wild type E2. C33A cells were transfected with pHPVOri/E1 and the E2 constructs indicated in Fig. 3.5 B. Episomal DNA was collected at 6 and 48 hours post transfection for Southern blot analysis. The 6-hour DNA samples, which served as transfection controls, showed that transfection efficiencies were similar, and an equal amount of the initial viral DNA template was loaded for each lane (Fig. 3.5 B). Western blot analysis showed that E1 and the various E2 constructs were also expressed in cells at comparable levels (Fig. 3.5 C). For analysis of newly synthesized HPV16 Ori, the DNA samples collected at 48 hours post transfection were digested with XhoI to linearize the plasmid and with DpnI to remove input plasmid. As shown in Fig. 3.5 B, cells

transfected with wild type E2 supported efficient replication of HPVOri. This HPVOri replication was dependent on E2, as cells transfected with empty vector did not replicate the HPV16 Ori plasmid. As expected, the E2 E39A mutant with abrogated E1-E2 interaction was nearly completely impaired in HPV16 Ori replication. More importantly, the E2 R37A/I73A double mutations, which disrupt the E2-Brd4 interaction, also significantly reduced HPV16 Ori replication (Fig. 3.5 B, $P<0.05$, $n=3$). Co-immunoprecipitation analysis showed that the E2 R37A/I73A double mutant interacts with 16E1 similarly to wild type 16E2 (Fig. 3.5 D) suggesting that its reduced DNA replication activity is not caused by a defect in E1 binding. We previously showed that the 16E2 R37A/I73A double mutant retains low-level binding to Brd4 (Fig. 2.2), which could explain why this double mutant still maintains some replication activation activity. Notably, the E2 single mutants, R37A and I73A, could replicate HPV16 Ori slightly better than the R37A/I73A double mutant (Fig. 3.5 B). The partial inhibition of the HPV16 replication by these two single mutants, especially R37A, has been reported in a previous study using detailed structure-function analysis to separate the transcriptional activation and replication activities of HPV16 E2 (168). We suspect the single mutants can support a little more HPVOri replication than the E2 R37A/I73A mutant because they likely retain even more interactions with Brd4 than the E2 R37A/I73A double mutant. Together, these results suggest that the E2-Brd4 interaction is necessary for HPV16 Ori replication.

3.3.5: Brd4 is important for HPV DNA replication in vitro

Brd4 plays an important role in regulating host gene transcription and cellular growth (222). Brd4 knockdown could therefore induce cell cycle arrest, which would indirectly inhibit HPV DNA replication. This prevented us from using Brd4 silencing as a fair approach to test the functional role of Brd4 in HPV DNA replication in cells. To overcome this problem, we set up a cell-free replication system as an alternative strategy. In the *in vitro* replication assay, protein lysates to be used as a source of replication factors were isolated from cells transfected with HPV16 E1 and E2 proteins and either non-targeting control siRNA or Brd4 specific siRNA (Fig. 3.6). These lysates were mixed with the HPV16 genome template and *in vitro* replication components to set up the reaction (see details in Materials and Methods). As shown in Fig. 3.6 A, lysates from cells expressing both E1 and E2 boosted HPV16 viral DNA synthesis by more than 12-fold compared to the cell lysates without E1 and E2 (compare CO lanes to No E1/E2 lane), although a low level of E1/E2-independent viral DNA synthesis was detected in the No E1/E2 lysate. Compared with reactions using control siRNA-transfected C33A cells, Brd4 knockdown cell lysates showed a more than 3-fold reduction in HPV16 DNA replication *in vitro* (Fig. 3.6 A, n=3). This is not likely due to a change in the viral protein expression because E1 and E2 expression was not affected by Brd4 knockdown (Fig. 3.6 D). This result suggests that Brd4 might directly contribute to HPV16 DNA replication.

To demonstrate that this reduction of HPV16 DNA replication in Brd4 knockdown cell lysates was not due to cell cycle arrest caused by Brd4 knockdown,

recombinant Brd4 expressed and purified from insect cells was added to replication reactions to restore Brd4 levels (Fig. 3.6 E). Compared to the control, addition of recombinant Brd4 increased the HPV16 genome replication by about 2.8-fold (Fig. 3.6 A, compare rBrd4 lanes with Control lanes, n=3). In contrast, rBrd4 did not increase HPV16 replication when cellular Brd4 was not depleted (Fig. 3.6 A). The recombinant Brd4 also rescued *in vitro* HPV16 genome replication in Brd4 knockdown cell lysates in a dose-dependent manner, causing about a 7-fold increase in HPV16 DNA replication (Fig. 3.6 B, compare 2 μg lanes to KD lanes, n=3). Moreover, recombinant Brd4 purified from *E. coli* could also restore HPV16 replication in Brd4 knockdown lysates (data not shown).

Since E1 protein of HPV1a is sufficient to initiate viral DNA replication (68) and the Brd4 CTD, which can block the E2-Brd4 interaction, does not significantly inhibit HPV16 replication in C33A cells (82, 174), we investigated if the Brd4 activity in HPV16 replication is solely dependent on its interaction with E2. In the *in vitro* replication assay using cellular extracts containing 16E1 but not 16E2, HPV16 replication could be detected in the control siRNA-treated samples and was also inhibited upon Brd4 knockdown (Fig. 3.6 C, F). rBrd4 could partially restore the 16E1-mediated HPV16 replication (Fig. 3.6 C). However, rBrd4 was able to more efficiently stimulate the viral replication in the reaction mixtures containing both E1 and E2 (compare the quantification data in Fig. 3.6 B and C), suggesting that the E2-Brd4 interaction is important for the viral DNA replication *in vitro*. Together, the results shown in Fig. 3.6

demonstrate that Brd4 stimulates HPV16 DNA replication *in vitro* and is directly associated with HPV16 DNA replication.

3.3.6: Release of endogenous Brd4 from chromatin stimulates HPV16 DNA replication

Most of Brd4's cellular functions are associated with its chromatin localization through a dynamic "on and off" interaction with acetylated histones (36). Since Brd4 can relocate from cellular chromatin to the HPV16 Ori replication complex, we postulated that releasing Brd4 from chromatin might facilitate HPV16 DNA replication. The chemical compound, JQ1(+), provides an excellent tool to test this possibility because it can prevent the binding of Brd4 to tetra-acetylated histone H4 peptides (51). We decided to test whether release of Brd4 from chromatin using JQ1(+) affects HPV16 Ori DNA replication. Brd4 binds acetylated chromatin and is normally observed in a nuclear speckled pattern in C33A cells (75). Treating C33A cells with 300 nM JQ1(+) could efficiently alter Brd4 localization from punctate foci on chromatin to a dimmer and more diffuse pattern throughout the nucleus, indicating the release of endogenous Brd4 from chromatin (Fig. 3.7 A). Lower concentrations of JQ1(+) only slightly released Brd4 from chromatin while the inactive stereoisomer, JQ1(-), or DMSO vehicle control did not noticeably affect Brd4 staining in C33A cells (Fig. 3.7 A). To determine if JQ1(+) could affect HPV16 DNA replication, pHPVOri/E1 and E2 cotransfected cells were split into five dishes and each treated with 100, 200, and 300 nM JQ1(+), 300 nM JQ1(-), or an equal volume of DMSO. The extracted episomal DNA samples were digested with both DpnI and XhoI and used in Southern blotting to analyze the HPV16 Ori replication product, whereas the samples without DpnI digestion were used as loading control. As

shown in Fig. 3.7 B, 300 nM JQ1(+) treatment, which efficiently releases Brd4 from chromatin, increased HPV16 Ori replication while the other drug concentrations had little effect. Treatment with 300 nM JQ1(+) did not affect E2 expression but slightly increased E1 expression likely due to stimulation of pHPVOri/E1 DNA replication in these cells (Fig. 3.7 B). JQ1(+) also stimulated HPV replication in cells carrying Ori and E1 encoded by two separate plasmids, HPVOri and HA-16E1, cotransfected with CMV4-Flag-16E2 (data not shown), further supporting a direct role of JQ1(+) in stimulating the viral DNA replication. Using flow cytometry analysis, we confirmed that 300 nM JQ1(+) treatment does not cause cell cycle arrest (Fig. 3.7 C, experiment n = 3), suggesting that the induced HPV16 Ori DNA replication was not caused by a cell cycle effect associated with JQ1(+) treatment. We also examined how JQ1(+) affects the kinetics of HPV16 replication focus formation by monitoring BrdU incorporation over time. Compared to DMSO-treated cells, JQ1(+) treatment leads to increased BrdU focus size during the early time points and the percentage of large BrdU replication foci throughout the experiment (Fig. 3.7 D). These results suggest that the release of Brd4 from cellular chromatin stimulates HPV16 DNA replication, likely by relocating Brd4 to the HPV16 Ori replication complex. The data also demonstrate that the role of Brd4 in HPV DNA replication could be uncoupled from its cell cycle and transcriptional regulation function.

3.4: Discussion

In this study we discovered a novel role for Brd4 in HPV genome replication. Brd4 was found colocalized with the HPV replication factors, E1 and E2, and the viral genome in actively replicating viral foci. It was further demonstrated that the E2-Brd4 interaction is necessary for formation of these replication foci and for efficient HPV genome replication *in vivo*. We also showed that cellular lysates depleted of Brd4 do not support HPV *in vitro* replication while purified Brd4 protein can restore replication in these lysates. Finally, we provide evidence that release of Brd4 from cellular chromatin enhances HPV DNA replication.

While our data demonstrate an important role of Brd4 in HPV DNA replication, Brd4's specific function during viral replication is still unknown. Brd4 is known to interact with cellular replication factors including RFC1 and ATAD5 (83, 121, 163) and our laboratory previously showed that Brd4 functions in Merkel cell polyomavirus replication by recruiting RFC1 to MCV replication foci (209). We can detect both RFC1 and ATAD5 in HPV replication centers (data not shown) so it is possible that, similar to MCV, Brd4 functions in HPV replication to recruit specific cellular replication proteins to the viral foci. Our finding that release of Brd4 from chromatin promotes HPV DNA replication might support this hypothesis since Brd4 removal from chromatin could make Brd4 more free and accessible to bind and recruit replication proteins to viral replication centers. Further studies are needed to address this possible role of Brd4 in replication factor recruitment.

Sakakibara *et al*. also examined Brd4 function during HPV genome replication and were able to detect Brd4 in small, newly formed viral replication foci but showed Brd4 was displaced from larger, mature foci (169). This finding differs from our results most likely because of the different cell types used in the two studies; primary human keratinocytes were used in their study while our experiments used a cervical cancer cell line. Nevertheless, this finding supports the theory that Brd4 is required for the early formation of replication centers. This study also demonstrated that Brd4 is associated with early replication foci but is displaced once the replication foci mature. In support of our observation that Brd4 release from chromatin stimulates HPV DNA replication, this result suggests that for efficient viral genome amplification, the replication centers need to detatch from cellular chromatin along with Brd4, which might explain why they observed both Brd4 and acetylated host chromatin displaced from mature replication foci. Since our data was from experiments using a transformed cell line and the experiments presented in Sakakibara *et al*. were mostly qualitative immunofluorescent analyses of Brd4 colocalization in replication foci, future experiments should focus on utilizing functional replication assays to determine whether Brd4 is important for HPV replication in naturally HPV infected cells.

HPV is often found integrated into common fragile sites in HPV-associated cancers (31, 123, 186, 195, 196). Common fragile sites are stretches of the genome that are highly prone to DNA damage and replication stress (56). Since these regions are frequently sites of DNA damage repair and papillomaviruses have been shown to co-opt cellular DNA damage response (DDR) proteins for viral genome amplification (137,

170), we and others speculate that HPV replication centers might localize near common fragile sites to access the DDR proteins (169). Intriguingly, a recent report demonstrated that Brd4 binds chromatin near common fragile sites and recruits HPV replication complexes to these sites to facilitate viral replication (87). In light of this finding, it is unclear why Brd4 seems to be eventually displaced from HPV replication foci and why JQ1(+) treatment stimulates viral replication. Obviously further studies are needed to address these questions in order to fully understand Brd4's function in papillomavirus genome replication.

The interesting results obtained from the JQ1(+) studies presented here and in chapters 2 and 4 suggest that this chemical compound could be a valuable tool to eliminate HPV infection as it can release Brd4 from chromatin and abrogate viral episome maintenance and viral gene transcription. More importantly, since HPV genome amplification normally occurs exclusively in terminally differentiated cells to avoid detection by host immune surveillance, an impromptu viral genome amplification induced by JQ1(+) in the infected basal cells could trigger activation of the immune response to clear the viral latent infection before cancer development. This small molecule therefore offers a promising lead for development of antiviral inhibitors to treat HPV latent infection.

3.5: Figures

Figure 3.1

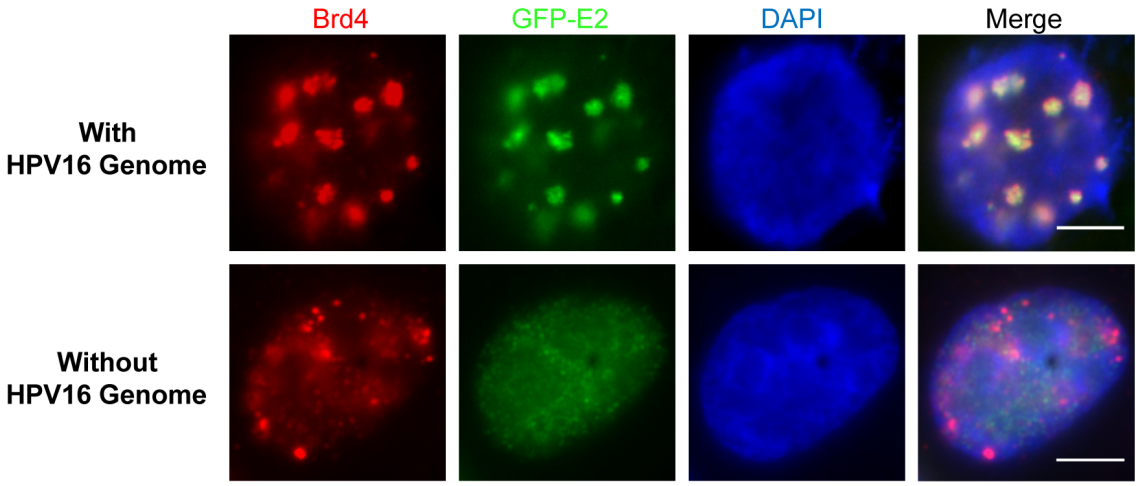
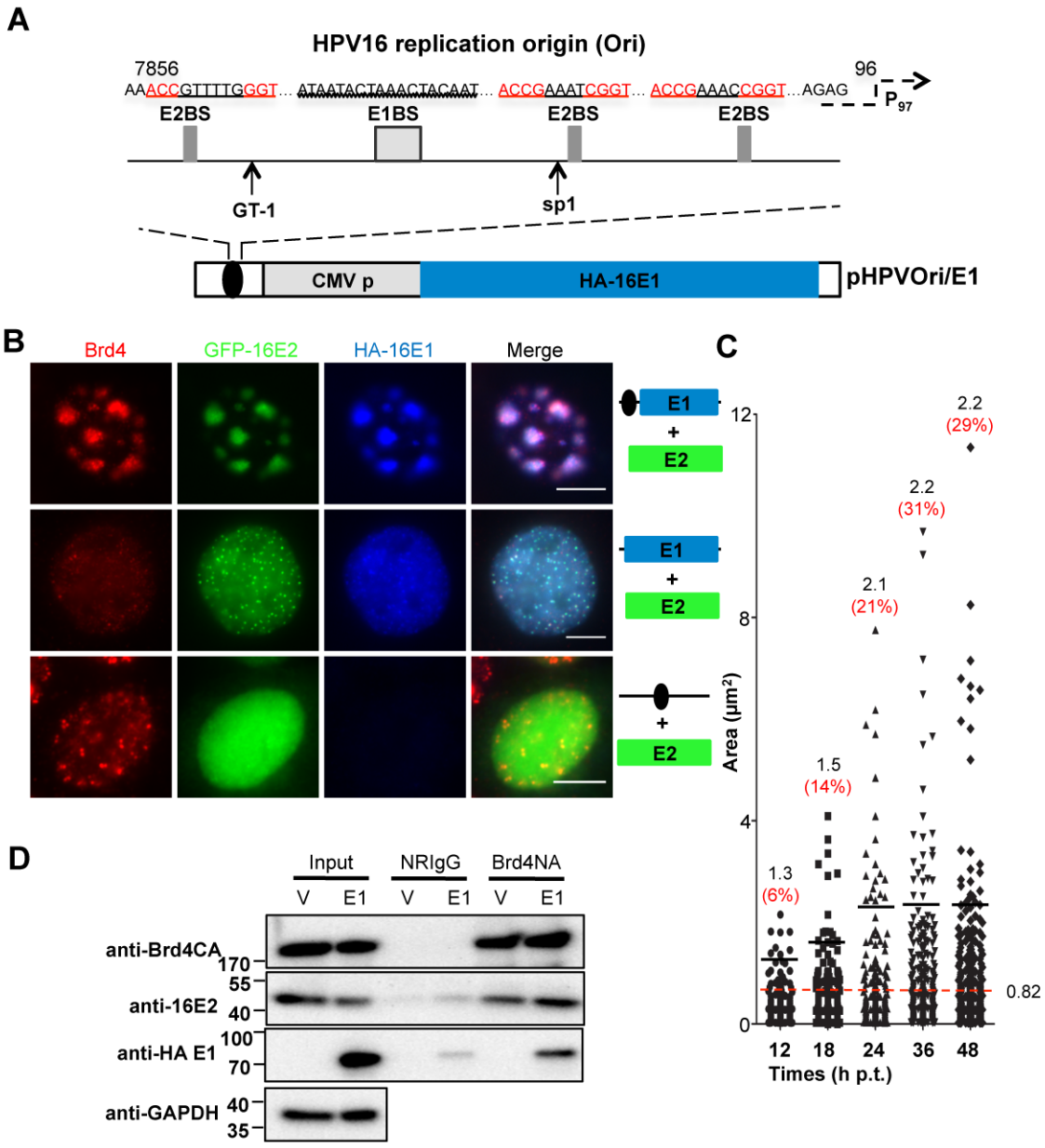


Figure 3.1. Brd4 and HPV16 E2 colocalize in punctate nuclear foci only in the presence of HPV16 genome. C33A cells were cotransfected with pUC19-HPV16, GFP-16E2, and HA-16E1. The pUC19-HPV16 plasmid was replaced by pUC19 in the "without HPV16 genome" transfection control. At 48 hours post transfection, cells were fixed and stained using anti-Brd4 antibody and counterstained with DAPI. Bar, 5 μm.

Figure 3.2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E2BS
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E2BS
E2BS
P97
GT-1
sp1
CMV p
HA-16E1
pHPVOri/E1
B
Brd4
GFP-16E2
HA-16E1
Merge
E1
+
E2
C
Area (μm²)
12
8
4
0
2.2
(29%)
2.2
(31%)
2.1
(21%)
1.5
(14%)
1.3
(6%)
0.82
12
18
24
36
48
Times (h p.t.)
D
Input
NRIgG
Brd4NA
V
E1
anti-Brd4CA
170
anti-16E2
55
40
anti-HA E1
100
70
anti-GAPDH
40
35

Figure 3.2. Brd4 and HPV16 E2 colocalization in nuclear foci is dependent on both HPV E1 and viral origin. (A) Schematic diagram of the artificial pHPVOri/E1 episome. E1BS, E1 binding site. E2BS, E2 binding site. P97, TATA box of viral early promoter. GT-1, transcription factor GT-1. Sp1, specificity protein 1. (B) C33A cells were cotransfected with GFP-16E2 and either pHPVOri/E1, HA-16E1, or HPVOri. At 60 hours post transfection, cells were fixed and co-stained using anti-Brd4 (red) and anti-HA (blue) antibodies. Bar, 5 μm. (C) C33A cells were cotransfected with GFP-16E2 and HPVOri/E1 and co-stained as in panel B at 12, 18, 24, 36, and 48 hours post transfection. Foci with an area larger than this mean value were considered big foci. The mean area of these big foci (numbers in black) and the percentage of big foci in total foci (numbers in red) were calculated for each time point and are shown at the top of each column. (D) C33A cells were cotransfected with CMV4-Flag16E2 and either empty vector (V) or HA-16E1 (E1). At 48 hours post transfection, nuclear proteins were harvested and immunoprecipitated with the indicated antibodies. The precipitates were immunoblotted with specific antibodies as indicated.

Figure 3.3

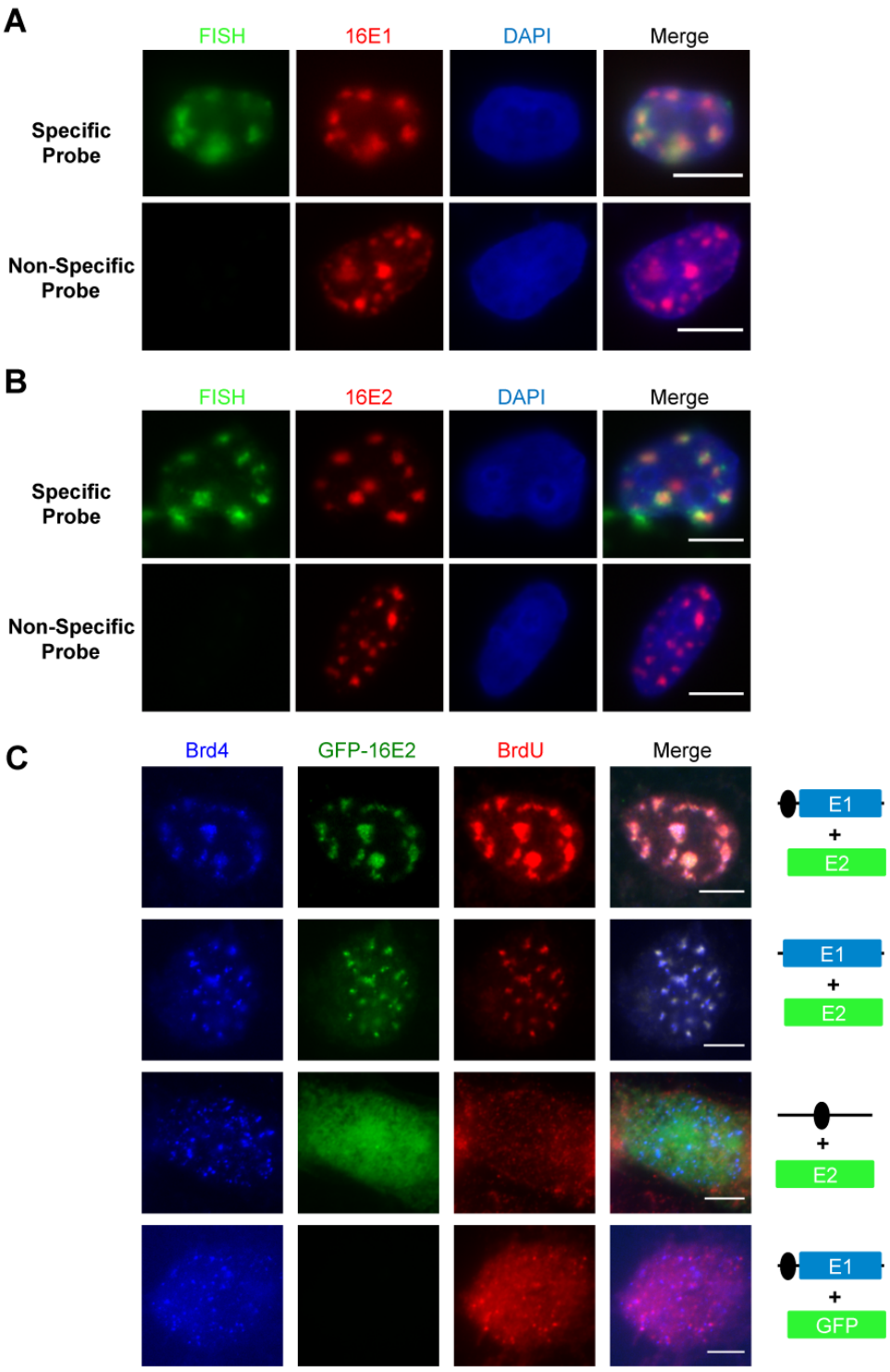
A
FISH
16E1
DAPI
Merge
Specific Probe
Non-Specific Probe
B
FISH
16E2
DAPI
Merge
Specific Probe
Non-Specific Probe
C
Brd4
GFP-16E2
BrdU
Merge
E1
+
E2
E1
+
E2
+
E2
E1
+
GFP

Figure 3.3. Brd4 colocalizes with HPV16 E1 and E2 proteins in foci harboring actively replicating HPV episomes. (A and B) C33A cells were cotransfected with pHPVOri/E1 and CMV4-Flag16E2. At 48 hours post transfection, biotin-labeled probes targeting pHPVOri/E1 (Specific Probe) were used to detect the replicating DNA while probes targeting Merkel cell polyomavirus genome (Non-Specific Probe) were used as a non-specific control. HPV16 E1 (A) and E2 (B) were stained using anti-HA and anti-Flag antibody, respectively. The cells were also counterstained with DAPI. Bar, 5 μm. (C) C33A cells were cotransfected with GFP-16E2 or pEGFPC1 and either pHPVOri/E1, HA-16E1, or HPVOri. At 42 hours post transfection, the cells were labeled with BrdU for twenty minutes and grown for an additional hour prior to acetone fixation. Cells were immunostained for Brd4 (blue) and BrdU (red). Bar, 5 μm.

Figure 3.4

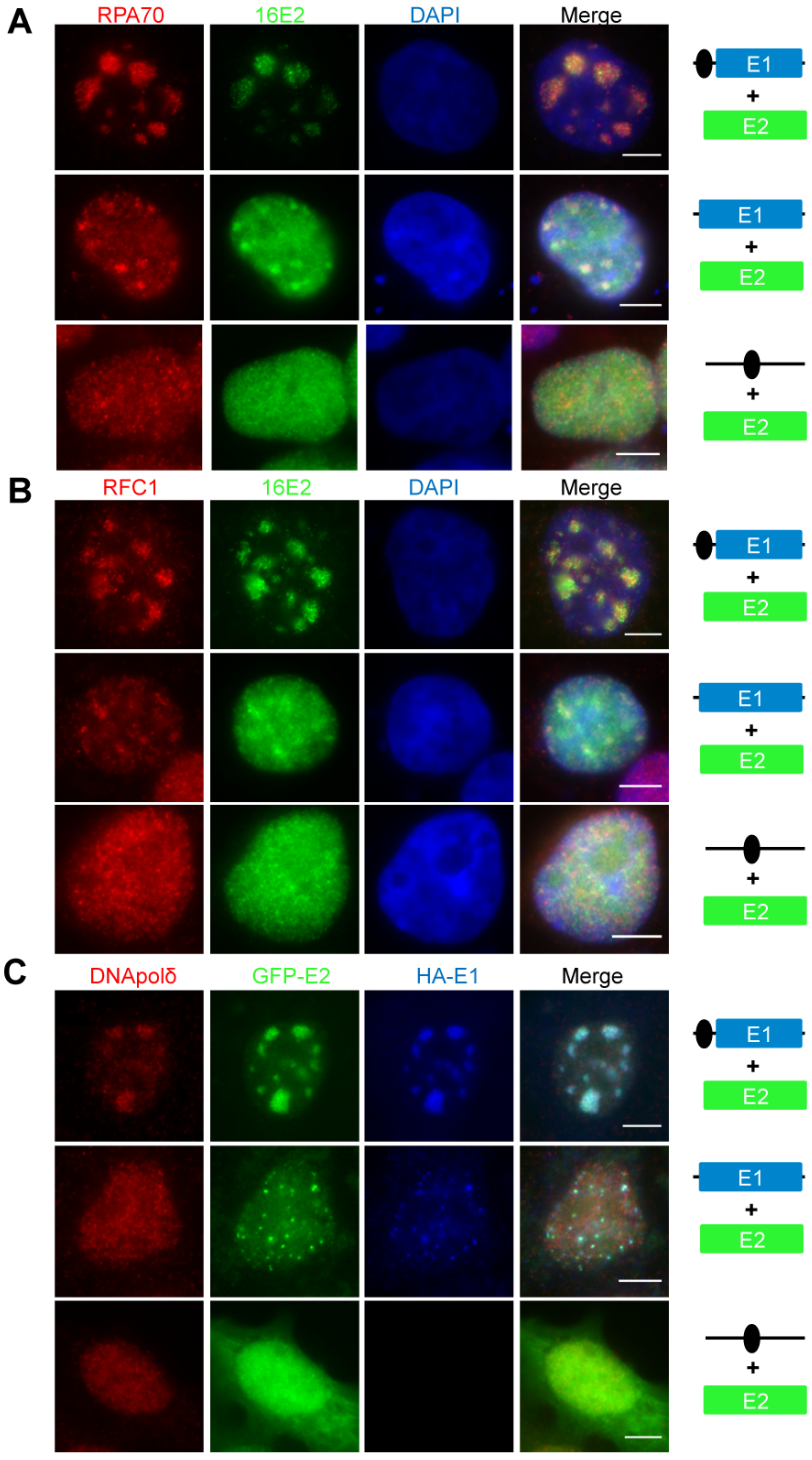
A
RPA70
16E2
DAPI
Merge
E1
+
E2
E1
+
E2
+
E2
B
RFC1
16E2
DAPI
Merge
E1
+
E2
E1
+
E2
+
E2
C
DNApolδ
GFP-E2
HA-E1
Merge
E1
+
E2
E1
+
E2
+
E2

Figure 3.4. Host replication factors are recruited to the HPV16 E1/E2 foci in a HPV origin-dependent manner. (A and B) C33A cells were cotransfected with Flag-16E2 expression vector and either pHPVOri/E1, HA-16E1, or HPVOri. At 48 hours post transfection, the cells were fixed and immunostained for E2 (α-Flag; green) and RPA70 (red) (A) or RFC1 (red) (B). The cells were also counterstained with DAPI. (C) C33A cells were cotransfected with GFP-16E2 and either pHPVOri/E1, HA-16E1, or pHPVOri. At 64 hours post transfection, cells were fixed and co-stained with anti-DNA polymerase δ (red) and anti-HA (blue) antibodies. Bar, 5 μm.

Figure 3.5

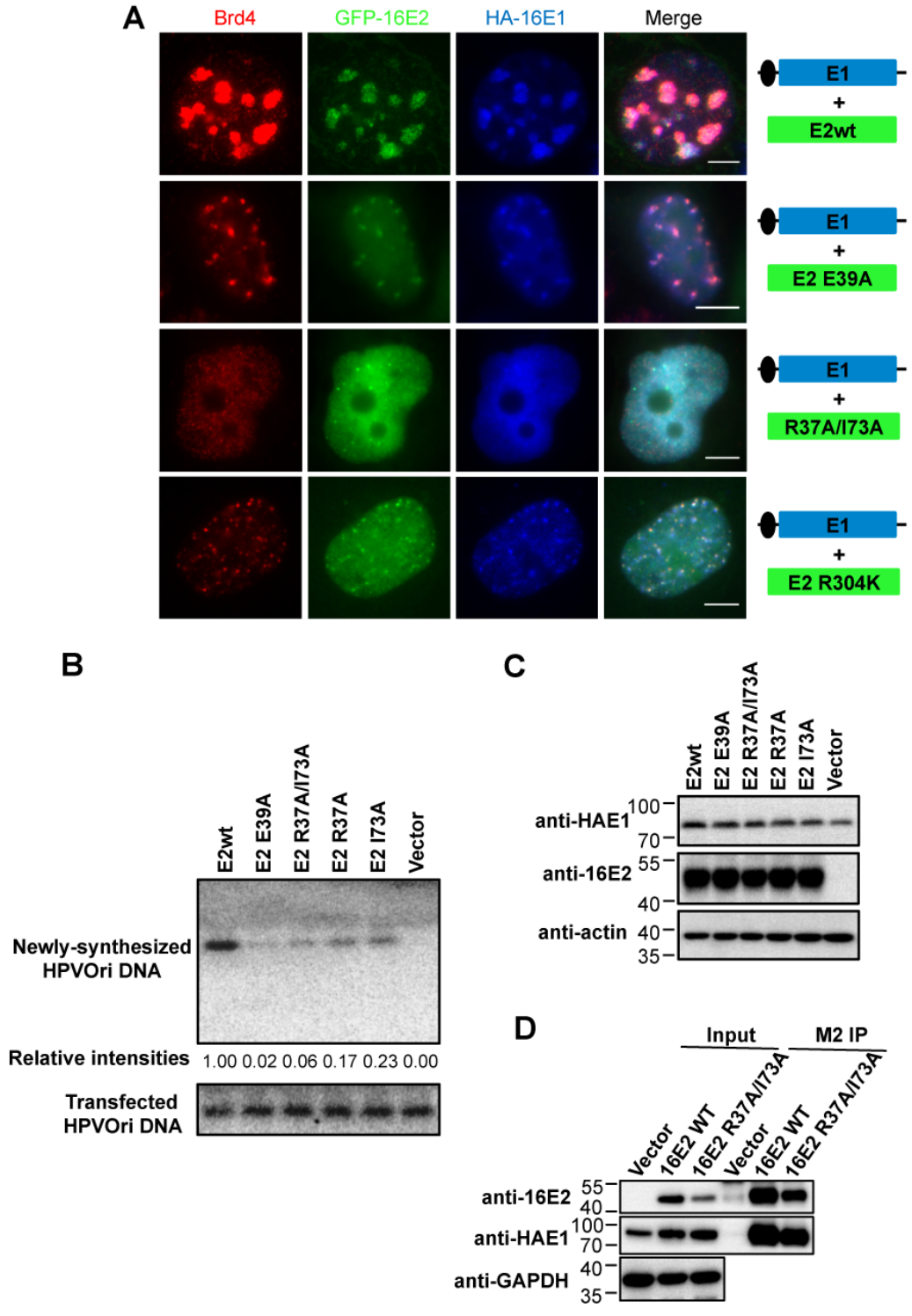
A
Brd4
GFP-16E2
HA-16E1
Merge
E1
+
E2wt
E1
+
E2 E39A
E1
+
R37A/I73A
E1
+
E2 R304K
B
E2wt
E2 E39A
E2 R37A/I73A
E2 R37A
E2 I73A
Vector
Newly-synthesized HPVOri DNA
Relative intensities 1.00 0.02 0.06 0.17 0.23 0.00
Transfected HPVOri DNA
C
E2wt
E2 E39A
E2 R37A/I73A
E2 R37A
E2 I73A
Vector
anti-HAE1
100
70
anti-16E2
55
40
anti-actin
40
35
D
Input
M2 IP
Vector
16E2 WT
16E2 R37A/I73A
Vector
16E2 WT
16E2 R37A/I73A
anti-16E2
55
40
anti-HAE1
100
70
anti-GAPDH
40
35

Figure 3.5. The Brd4-E2 interaction is important for HPV16 replication. (A) C33A cells were cotransfected with pHPVOri/E1 and the GFP-16E2 construct indicated in the right panel. At 48 hours post transfection, cells were fixed and co-stained with anti-Brd4 (red) and anti-HA (blue) antibodies. (B) C33A cells were cotransfected with pHPVOri/E1 and either CMV4 (Vector) or the CMV-Flag-16E2 construct indicated in (B). Episomal DNA was extracted at 6 hours post transfection for transfection controls and 48 hours post transfection for newly synthesized DNA. These were digested with XhoI or XhoI/DpnI respectively, and analyzed by Southern blotting. The intensity of each replicated DNA band was quantified by ImageJ and normalized to E2wt. (C) Cells were transfected as in (B). At 48 hours post transfection, extracted protein was analyzed by Western blotting using anti-16E2 and anti-HA antibodies. (D) C33A cells were cotransfected with HA-16E1 and either CMV4 (Vector), CMV4-Flag-16E2 wild type, or CMV4-Flag-16E2 R37A/I73A. Cell lysates were immunoprecipitated with anti-Flag antibody. The precipitates were immunoblotted with specific antibodies as indicated.

Figure 3.6

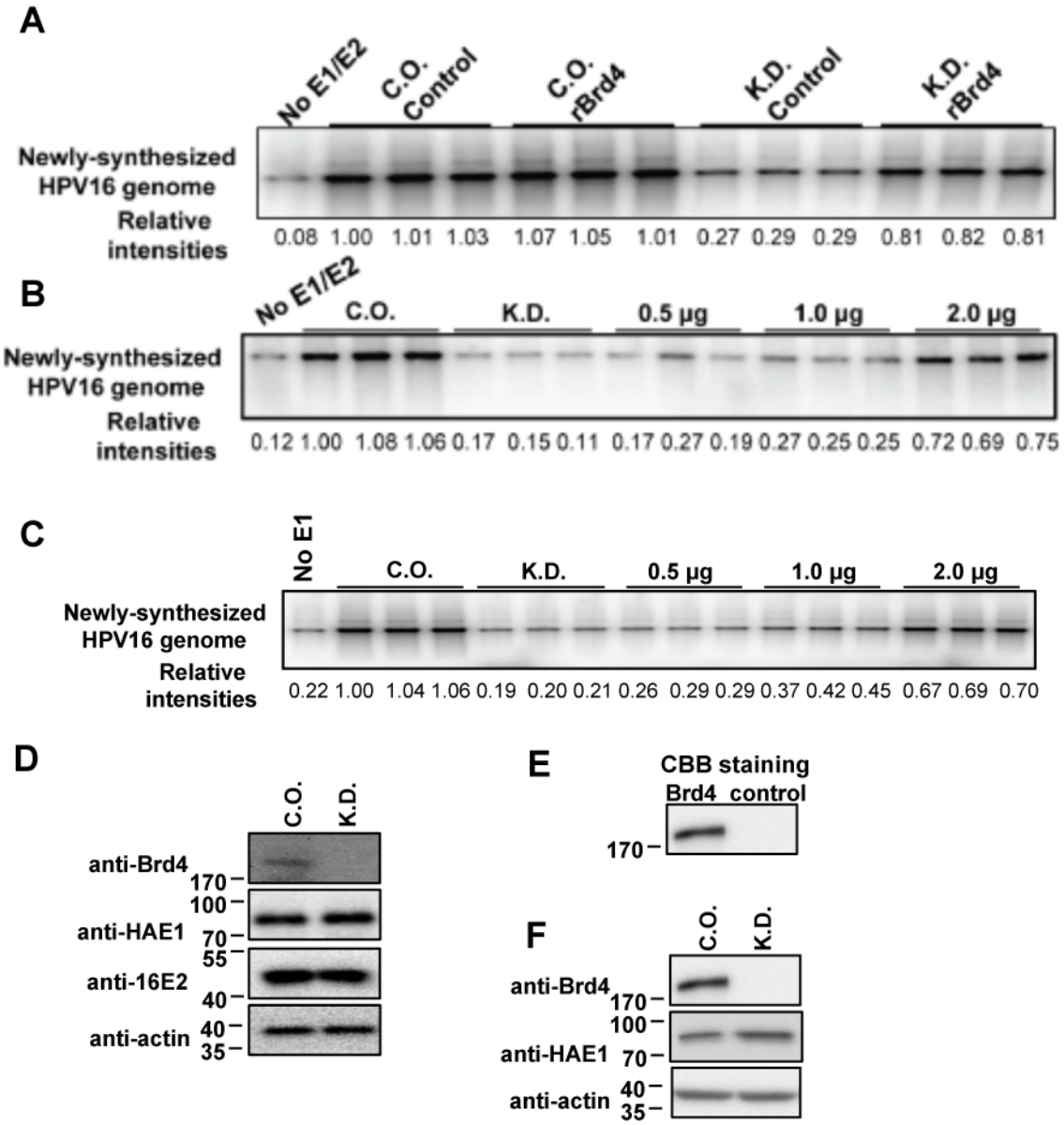
A
No E1/E2
C.O. Control
C.O. rBrd4
K.D. Control
K.D. rBrd4
Newly-synthesized HPV16 genome
Relative intensities
0.08 1.00 1.01 1.03 1.07 1.05 1.01 0.27 0.29 0.29 0.81 0.82 0.81
B
No E1/E2
C.O.
K.D.
0.5 μg
1.0 μg
2.0 μg
Newly-synthesized HPV16 genome
Relative intensities
0.12 1.00 1.08 1.06 0.17 0.15 0.11 0.17 0.27 0.19 0.27 0.25 0.25 0.72 0.69 0.75
C
No E1
C.O.
K.D.
0.5 μg
1.0 μg
2.0 μg
Newly-synthesized HPV16 genome
Relative intensities
0.22 1.00 1.04 1.06 0.19 0.20 0.21 0.26 0.29 0.29 0.37 0.42 0.45 0.67 0.69 0.70
D
C.O.
K.D.
anti-Brd4
170
100
anti-HAE1
70
55
anti-16E2
40
anti-actin
40
35
E
CBB staining
Brd4 control
170
F
C.O.
K.D.
anti-Brd4
170
100
anti-HAE1
70
40
anti-actin
35

Figure 3.6. Brd4 directly stimulates HPV16 replication *in vitro*. (A) C33A cells were transfected with siRNA targeting Brd4 (KD) or nontargeting control siRNA (CO). At 36 hours post transfection, cells were transfected with pOZN-16E2 and HA-16E1. Cell extracts were prepared at 82 hours post transfection for the *in vitro* replication assay using religated HPV16 genomes as the template. Cells transfected with the nontargeting siRNA but not the E1/E2 constructs were used as a negative control (no E1/E2). In the "rBrd4" reaction, 2 μg rBrd4 expressed and purified from Sf9 insect cells was added to the replication assays with Brd4 knockdown extract to restore HPV16 replication. In the "Control" condition, an equal amount of non-specific proteins isolated from the wild type baculovirus-infected Sf9 cells were used. The intensity of each replicated DNA band was quantified by ImageJ and normalized to one of the control replicates. (B) Cell extracts and rBrd4 were prepared as in (A). Increasing amounts of rBrd4 was added to the replication assays using Brd4 knockdown extracts. The intensity of each replicated DNA band was quantified by ImageJ and normalized to one of the control replicates. (C) Cell extracts were prepared as in (A) except at 36 hours post transfection, cells were transfected with only HA-16E1. Cells transfected with the nontargeting siRNA but not the E1 construct were used as a negative control (no E1). Increasing amounts of rBrd4 purified from insect cells were added to the replication assays using Brd4 knockdown extracts. The intensity of each replicated DNA band was quantified by ImageJ and normalized to one of the control replicates. (D) Western blot analysis of the HPV16 E1, E2, and Brd4 proteins in cellular extracts used for *in vitro* replication. (E) Coomassie

brilliant blue staining of rBrd4 purified from insect cells. (F) Western blot analysis of the HPV16 E1 and Brd4 proteins in cellular extracts used in panel (C).

Figure 3.7

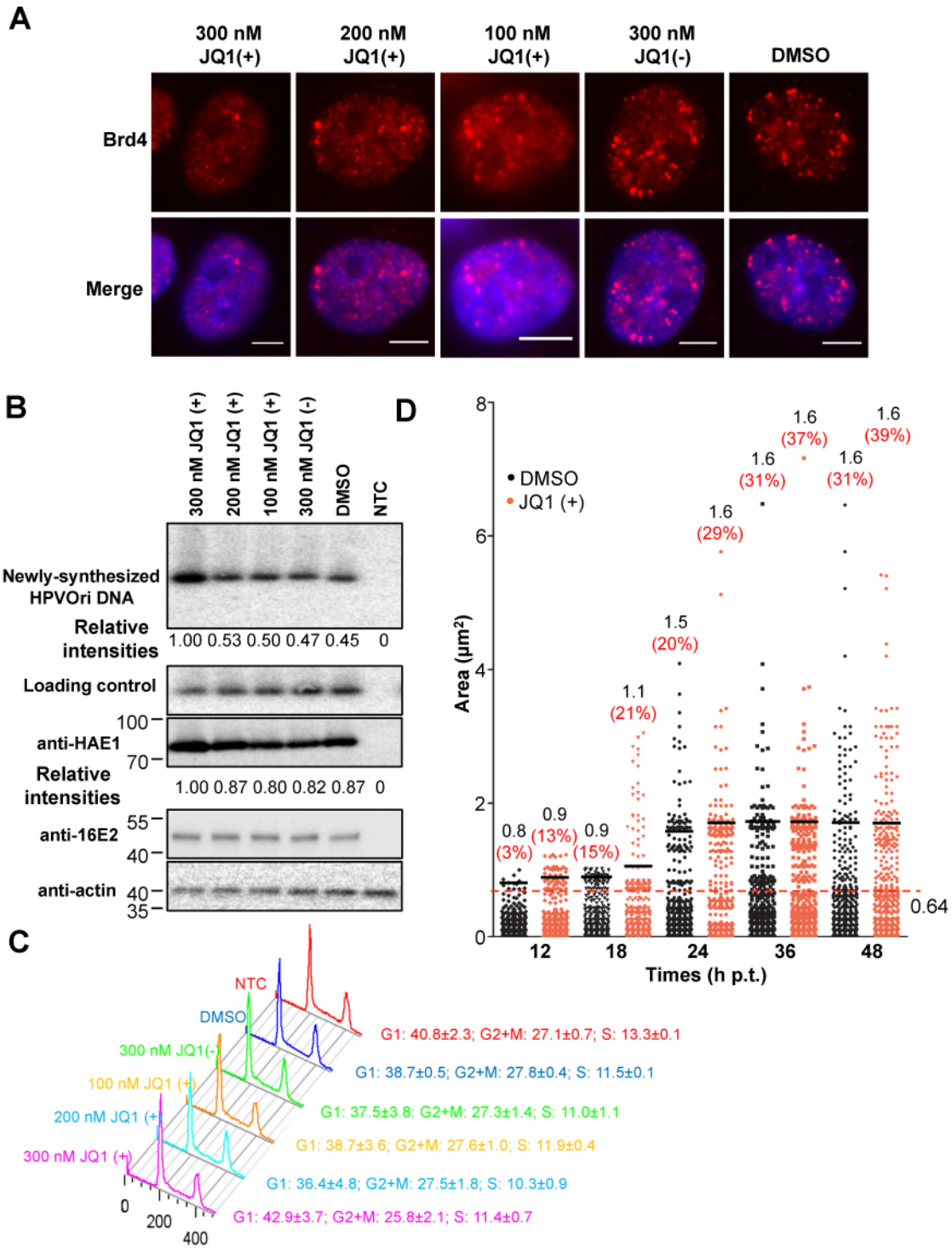
A
300 nM JQ1(+)
200 nM JQ1(+)
100 nM JQ1(+)
300 nM JQ1(-)
DMSO
Brd4
Merge
B
300 nM JQ1 (+)
200 nM JQ1 (+)
100 nM JQ1 (+)
300 nM JQ1 (-)
DMSO
NTC
Newly-synthesized HPVOri DNA
Relative intensities
1.00 0.53 0.50 0.47 0.45 0
Loading control
100
anti-HAE1
70
Relative intensities
1.00 0.87 0.80 0.82 0.87 0
anti-16E2
55
40
anti-actin
40
35
C
NTC
DMSO
300 nM JQ1(-)
100 nM JQ1 (+)
200 nM JQ1 (+)
300 nM JQ1 (+)
G1: 40.8±2.3; G2+M: 27.1±0.7; S: 13.3±0.1
G1: 38.7±0.5; G2+M: 27.8±0.4; S: 11.5±0.1
G1: 37.5±3.8: G2+M: 27.3±1.4; S: 11.0±1.1
G1: 38.7±3.6; G2+M: 27.6±1.0; S: 11.9±0.4
G1: 36.4±4.8; G2+M: 27.5±1.8; S: 10.3±0.9
G1: 42.9±3.7; G2+M: 25.8±2.1; S: 11.4±0.7
0
200
400
D
DMSO
JQ1 (+)
Area (μm²)
0
2
4
6
8
0.8 (3%)
0.9 (13%)
0.9 (15%)
1.1 (21%)
1.5 (20%)
1.6 (29%)
1.6 (31%)
1.6 (37%)
1.6 (31%)
1.6 (39%)
0.64
12
18
24
36
48
Times (h p.t.)

Figure 3.7. Releasing Brd4 from chromatin by JQ1(+) increases HPV16 replication. (A) C33A cells were treated with JQ1(+), JQ1(-), or DMSO at the indicated concentrations for 24 hours. Cells were then fixed and immunostained with anti-Brd4 antibody and counterstained with DAPI. Bar, 5 μm. (B) C33A cells transfected with pHPVOri/E1 and CMV4-Flag16E2 were split equally into 6 dishes. Cells were treated with 100, 200, 300 nM JQ1(+), 300 nM JQ1(-), or an equal volume of DMSO. At 48 hours post transfection, episomal DNA was extracted, digested with DpnI and XhoI, and 600 ng was used to analyze the replication product while 2.5 ng XhoI treated DNA was used as loading control in a Southern blot. Cellular extracts were analyzed by Western blotting using anti-16E2, anti-HA, and anti-actin antibodies. NTC, non-transfection control. (C) C33A cells were treated as described in (A). Flow cytometry analysis was performed to evaluate the impact of JQ1(+) treatments on the cell cycle. (D) C33A cells were transfected as in (B) and treated with DMSO or 300 nM JQ1(+). Cells were labeled using BrdU as in Figure 3.3 C and co-stained with anti-HA E1 and anti-BrdU antibodies at 12, 18, 24, 36, and 48 hours post transfection. The area of BrdU foci was quantified for about 30 cells from each sample using ImageJ. The red dashed line represents the mean area of BrdU foci in the DMSO-treated sample at 48 hours post transfection. Foci with an area larger than the mean value were considered big foci. The mean area of these big foci (numbers in black) and the percentage of big foci in total foci (numbers in red) were calculated for each time point and are shown at the top of each column.

CHAPTER 4: Brd4 has Multiple Functions in E2-Mediated Papillomavirus Transcription Activation

I was the principal researcher that designed and performed the experiments in this chapter. A former postdoctoral fellow in our laboratory, Junpeng Yan, performed some preliminary experiments that initially got us interested in this project. Junpeng also cloned some of the DNA constructs used in this work.

The manuscript presenting this research has been submitted to *Viruses*. Christine M. Helfer, Junpeng Yan, and Jianxin You. The Cellular Bromodomain Protein Brd4 has Multiple Functions in E2-Mediated Papillomavirus Transcription Activation. Submitted May 26, 2014.

4.1: Chapter Summary

The cellular bromodomain protein Brd4 functions in multiple processes of the papillomavirus life cycle including viral replication, genome maintenance, and gene transcription through its interaction with the viral protein, E2. However, the mechanisms by which E2 and Brd4 activate viral transcription are still not completely understood. In this study, we show that recruitment of P-TEFb, a functional interaction partner of Brd4 in transcription activation, is important for E2's transcription activation function. Furthermore, chromatin immunoprecipitation (ChIP) analyses demonstrate that P-TEFb is recruited to the native papillomavirus episomes. We also show that E2's interaction with cellular chromatin through Brd4 correlates with its papillomavirus transcription activation function since JQ1(+), a bromodomain inhibitor that efficiently dissociates E2-Brd4 complexes from chromatin, potently represses papillomavirus transcription. Our study identifies a specific function of Brd4 in papillomavirus gene transcription and highlights the potential use of bromodomain inhibitors as a method to disrupt the HPV life cycle.

4.2: Introduction

Papillomaviruses are small, double-stranded DNA viruses with an approximately 8 kb episomal genome that can be divided into three regions: the LCR, the early gene region, and the late gene region (Fig. 1.2, (80)). The LCR contains the origin of replication and a transcriptional enhancer region containing multiple binding sites for the viral E2 protein and cellular transcription factors (41). There are six early viral genes: E1, E2, E4, E5, E6, and E7. These early genes are expressed from one or more early promoters as polycistronic mRNAs (80). The prototypical papillomavirus, BPV1, has six early promoters that each encode a different polycistronic transcript while the high-risk HPVs, such as HPV types 16 and 18, only contain one early promoter (42, 91). The polycistronic transcripts encoded from the early promoters utilize the same poly-adenylation site and are processed by cellular splicing factors (92).

Papillomavirus gene expression is strictly regulated by the differentiation status of the infected cell (81, 188). During initial infection in basal epithelial cells, small amounts of E1 and E2 are expressed from the early promoter to support low-level maintenance replication of the viral genome. The viral oncoproteins E6 and E7 promote proliferation of infected cells and establish a cellular environment conducive for the genome amplification stage of the PV life cycle (21). Upon differentiation of the infected cells, the differentiation-dependent late promoter located within the E7 gene activates robust E1 and E2 expression to support viral genome amplification as well as expression of the L1 and L2 capsid proteins for assembly of new virions (81, 188).

E2 serves as the master regulatory protein of viral early gene transcription by binding to several sites upstream of the early promoter to tightly regulate viral gene expression (187). This tight regulation ensures that just enough E6 and E7 are expressed to drive differentiated cells into S phase while avoiding the development of neoplasias and carcinomas which can be induced by E6/E7 over-expression (21). E2 can both activate and repress transcription from the early promoter and this is thought to be partially regulated by E2 levels where low levels of E2 activates viral transcription while elevated E2 levels repress the viral early promoter (60, 192, 194). E2 also recruits cellular transcription factors and chromatin modulatory proteins to the early promoter to mediate viral transcription activation and repression (96, 101, 109, 110, 174, 184).

Brd4 is an important factor for cellular transcription as well as transcriptional regulation of a variety of different viruses including papillomaviruses, Epstein-Barr virus, and HIV (19, 86, 115, 223). In cells, Brd4 interacts with the Cdk9 and Cyclin T1 subunits of P-TEFb, displacing the negative regulators, HEXIM1 and 7SKsnRNA, from the P-TEFb complex to transform P-TEFb into its transcriptionally active form (19, 86, 215). Brd4 then recruits active P-TEFb to the transcription pre-initiation complex of many cellular genes where it stimulates transcription elongation by phosphorylating serine 2 in the C-terminal domain (CTD) of RNA polymerase II (86, 223).

Interestingly, for papillomaviruses, Brd4 binding is important for E2's dual transcription regulatory functions. (174, 184, 216). In E2-dependent luciferase reporter assays where E2 expression stimulates luciferase gene transcription, Brd4 knock-down or abrogation of the E2-Brd4 interaction inhibits E2 transactivation of the luciferase reporter

(128, 174, 179). Similarly, in luciferase reporter assays where E2 functions to repress luciferase gene expression, both Brd4 knock-down and disruption of the E2-Brd4 interaction reduce E2's ability to repress expression of the reporter gene (184, 216). Furthermore, in cervical cancer cells where the HPV genome is integrated into the cellular DNA and the E2 gene is disrupted, Brd4 activates viral oncogene transcription independently of E2 by recruiting P-TEFb to the HPV early promoter. Upon reintroduction of E2 into these cells, E2 functions to repress viral oncogene expression by interacting with Brd4 and competitively inhibiting the Brd4-P-TEFb interaction (220). Wu *et al*. also showed that the transcription repression function of E2 and Brd4 is at least partially mediated by preventing the assembly of the pre-initiation complex near the viral promoter (216). In contrast, the exact mechanism(s) whereby Brd4 contributes to viral transcription activation during papillomavirus infection is still unknown.

In this study, we sought to uncover the mechanism underlying Brd4's role(s) in E2-dependent transcription activation. Using an E2-responsive reporter assay, we demonstrate that Brd4 recruitment of P-TEFb is important for E2-dependent transactivation. We also found that P-TEFb is recruited to the native papillomavirus genome. Furthermore, we provide evidence that Brd4 tethering of E2 to the cellular chromatin is necessary for the transactivation of the E2-responsive reporter. Lastly, in cells carrying the papillomavirus genomes, we demonstrate that inhibiting Brd4's association with cellular chromatin using the bromodomain inhibitor, JQ1(+), effectively reduces transcription of the viral early genes E1, E2, E6, and E7. Together, these findings suggest that Brd4 interaction with E2 is not only necessary for recruiting P-

TEFb to the papillomavirus early promoter, but might also be important for tethering E2 and the viral genome complexes to particular regions of the cellular chromatin to support viral gene transcription.

4.3: Results

4.3.1: P-TEFb is Important for Papillomavirus E2-Mediated Transcription Activation

Brd4 has previously been shown to assist E2 in transactivating viral genes but Brd4's precise role in this process has remained elusive (128, 174). Since Brd4 functions in cellular transcription activation mainly by recruiting P-TEFb to cellular gene promoters, we decided to investigate whether Brd4 activates papillomavirus transcription by recruiting P-TEFb to the viral promoters. We first utilized an E2-responsive luciferase reporter assay to study this E2 transactivation function (100, 174). The p2x2xE2BS-luciferase reporter construct contains two pairs of E2 binding sites upstream of a minimal SV40 promoter and the luciferase gene (Fig. 4.1 A). When either BPV1 E2TA or HPV16 E2 is cotransfected with the reporter plasmid, it binds the E2 binding sites and activates the expression of luciferase (Fig. 4.1 B). As previously reported, however, the E2 mutants, BPV1 E2TR and HPV16 E2 R37A/I73A (16E2 RI), which do not bind Brd4, are unable to transactivate the luciferase reporter (Fig. 4.1 B) (128, 174). We next determined if the defect in transcription activation observed in the Brd4 binding-deficient E2 mutants is due to their inability to recruit P-TEFb through Brd4 association. To test this, we fused the Cdk9 subunit of P-TEFb to the E2 mutants, E2TR or 16E2 RI, and tested them in the luciferase reporter assay. As shown in Fig. 4.1 B, Cdk9 fusion with

either E2TR or 16E2 RI mutant markedly restored the transactivation activities well above that seen for wild type E2TA and 16E2. This enhanced transactivation activity was also observed when Cdk9 was fused to 16E2 WT or E2TA (data not shown). The Cdk9 fusion to E2TR expressed at similar level as E2TR protein (Fig. 4.1 C) but the HPV16 E2 proteins express below the level detectable by Western blot, so it was not possible to compare their protein levels. However, because both E2TR and 16E2 RI have been shown by us and others to be completely inactive in the E2 transactivation reaction (128, 224), this highly stimulated transactivation activity observed with the Cdk9-E2TR and Cdk9-16E2 RI constructs was not likely caused simply by a higher expression of these fusion proteins relative to E2TR or 16E2 RI. We further proved this by comparing the transactivation activity of low levels (1X) of Cdk9-E2TR or Cdk9-16E2 RI with 1X, 2X, and 4X more of E2TR or 16E2 RI in the luciferase reporter assay. As shown in Fig. 4.1 D, while E2TR and 16E2 RI could not transactivate the luciferase reporter even when 4 times more expression construct was transfected, very low levels of the Cdk9-E2 mutant fusion proteins still robustly transactivated the luciferase reporter, confirming that the restored transactivation by the Cdk9-E2 mutant fusion proteins is not likely due to increased protein levels. It rather suggested a gain of function introduced by the Cdk9 moiety of the fusion proteins. These results demonstrate that P-TEFb is important for E2 transactivation function, indicating that Brd4 likely functions to recruit P-TEFb to the E2-responsive promoter to support viral transcription.

We next tested whether fusing the E2 mutants to a kinase-dead Cdk9 D167N mutant (Cdk9m) abolished the transactivation activity of the fusion proteins (119). The

D167N mutation did not affect the Cdk9-E2 fusion protein levels (Fig. 4.1 C). These mutant Cdk9-E2 fusion constructs transactivated the luciferase reporter expression at a consistently lower level than the E2 mutants fused to wild type Cdk9, although their activity was still well above that seen for WT E2 proteins (Fig. 4.1 B). This is likely because the Cdk9 D167N mutant retains some low-level kinase activity. Alternatively, because this Cdk9 D167N mutant can still bind Brd4 and we have previously shown that Brd4 can form Brd4-Brd4 homodimers (206, 223), we suspect that Brd4 dimers bound to Cdk9 D167N-E2 can recruit endogenous P-TEFb to the luciferase reporter to partially activate transcription elongation of the luciferase gene.

4.3.2: P-TEFb is Recruited to the Papillomavirus Genome

To confirm the role of P-TEFb in viral genome transcription, we tested if P-TEFb is recruited to the native papillomavirus genome. For this set of experiments we used the H2 and W12 (clone 20863) cells that maintain either BPV1 or HPV16 episomal genomes, respectively (107, 191). Both of these cell types stably maintain the viral episomes and support papillomavirus early promoter transcription (4, 5, 172, 226). ChIP assays were performed with a Cdk9 antibody to examine the occupancy of P-TEFb on the papillomavirus genome. As a positive control, an affinity purified Brd4 N-terminal antibody was also used in the ChIP analysis since Brd4 is known to associate with the papillomavirus genome through its interaction with E2 (224). Primers recognizing either the BPV1 or HPV16 genome were used in ChIP qPCR to detect the binding of P-TEFb and Brd4 to the viral episome as we have described previously (226). Both Cdk9 and Brd4 antibodies immunoprecipitated the BPV1 and HPV16 genomes at significantly

higher levels than the negative control ChIP using normal rabbit IgG (NRIgG) (Fig. 4.2 A and B). Importantly, no BPV1 or HPV16 signal was detected by qPCR from ChIP experiments using uninfected C127 cells, confirming that these primers specifically amplify a segment of the viral genome (data not shown). These results demonstrate that P-TEFb is recruited to the papillomavirus episome likely through interaction with Brd4.

4.3.3: Brd4 CTD Disrupts Papillomavirus Transcription Activation by the Cdk9-E2 Fusion Proteins

E2 and P-TEFb both interact with the extreme C-terminus of Brd4 and expression of the Brd4 CTD effectively inhibits Brd4's interaction with these two proteins (86, 220, 223, 224). In addition, Brd4 CTD abrogates E2 transactivation of the p2x2xE2BS-luciferase reporter (174). Our findings suggest that this is likely because Brd4 CTD prevents the recruitment of Brd4 as well as P-TEFb to the E2-responsive promoter. We hypothesized that if Brd4's only function in E2 transactivation is to recruit P-TEFb, then inhibiting Brd4's interaction with E2 and P-TEFb using Brd4 CTD will likely have no effect on transactivation function of the E2 proteins that are already fused to P-TEFb. Surprisingly, when Cdk9-E2 fusion constructs were coexpressed with the Brd4 CTD expression construct, Brd4 CTD significantly inhibited the transactivation function of all Cdk9-E2 fusions (Fig. 4.3 A). The weaker inhibition of Cdk9-E2TA by Brd4 CTD is likely because E2TA has a stronger binding affinity to Brd4 than HPV16 E2 so more Brd4 CTD is needed to efficiently break the E2TA-Brd4 interaction (128). Indeed, we found that higher concentration of Brd4 CTD dramatically inhibited transactivation by Cdk9-E2TA (Fig. 4.3 B). For an unknown reason, Brd4 CTD consistently increased β-

galactosidase expression from the CMV promoter, which prevented us from normalizing the luciferase activity to β-galactosidase activity. We instead examined E2 protein levels. Western blot analysis showed that Brd4 CTD expression does not decrease the levels of Cdk9-E2TA or Cdk9-E2TR. Instead, as previously reported, Brd4 CTD even caused moderate stabilization of Cdk9-16E2 WT and Cdk9-16E2 RI proteins (Fig. 4.3 C) (108). Since the P-TEFb complex is already tethered to E2 by direct fusion, these results suggested that, other than recruiting P-TEFb, Brd4 likely has additional roles in E2-mediated transactivation, which is tested in the experiments described below. Alternatively, it is possible that Brd4 CTD binds Cdk9 in the E2 fusions and induces a conformational change that abrogates P-TEFb function.

4.3.4: Releasing Brd4 From Chromatin by JQ1(+) Reduces E2-mediated Transcription Activation

Previous studies published by Jang *et al*. showed that Brd4 directs E2 to transcriptionally active regions of cellular chromatin (85, 87). We hypothesized that this recruitment of E2 by Brd4 to active regions of the nucleus would give the virus easy access to the cellular transcription machinery and thereby assist in E2-mediated transcription activation. In this case, inhibiting Brd4 chromatin binding would likely impair E2-mediated transactivation. To examine the importance of Brd4's association with chromatin for E2 transactivation function, we abrogated Brd4's interaction with acetylated histones on chromatin using a small molecule inhibitor, JQ1(+), as we have described previously (75, 208). C33A cells stably expressing BPV1 E2TA were transfected with the p2x2xE2BS-Luciferase construct or an empty vector and then treated

with 500 nM of JQ1(+) or its inactive stereoisomer, JQ1(-), for 15 hours prior to the luciferase assay. As shown in Fig. 4.4 A, E2TA transactivation of the luciferase reporter was drastically reduced in cells treated with JQ1(+) while cells transfected with empty vector had very low background luciferase activity. A similar result was observed when C33A cells transfected with the HPV16 E2 and p2x2xE2BS-Luciferase constructs were treated for 15 hours with 500 nM of JQ1(+) or JQ1(-) prior to the luciferase assay (Fig. 4.4 B). Similar to Brd4 CTD expression, JQ1(+) consistently increased β-galactosidase levels precluding us from normalizing the luciferase reporter to this protein. The E2 protein levels, which were unaffected by JQ1(+) treatment, were used as transfection controls instead (Figs. 4.4 C and D). Moreover, low levels of HPV16 E2 could still robustly activate luciferase expression in the presence of JQ1(-) whereas a high level of E2 protein was transcriptionally inactive with JQ1(+) treatment (Fig. 4.4 E and F). This reduction in luciferase expression was also observed with as low as 100 nM JQ1(+) and as short as 8 hours of treatment (data not shown). These results suggested that Brd4 binding to chromatin is essential for E2 transactivation activity. Together with the findings by Jang *et al*., we hypothesized that chromatin-bound Brd4 functions to direct E2 to transcriptionally active regions of the nucleus to facilitate E2 transcription activation (85, 87).

To confirm that JQ1(+) abolishes E2's association with cellular chromatin through interaction with Brd4, the strength of E2's chromatin association in the presence of JQ1(-) or JQ1(+) was examined as described by McPhillips *et al*. (129). When HPV16 E2 and Brd4 are ectopically expressed in C33A cells, the E2-Brd4 complexes bound to cellular

chromatin appear as nuclear speckles in immunofluorescently-stained cells (Fig. 4.5 and (75)). Treatment with JQ1(-) did not affect the localization of E2 and Brd4 in these speckles (Fig. 4.5, JQ1(-) unextracted). However, after only 2 hours of JQ1(+) treatment, E2 and Brd4 no longer localized to small speckles but instead formed larger, punctate nuclear spheres or became diffuse in the nucleus (Fig. 4.5, JQ1(+) unextracted). These large spheres formed by E2 and Brd4 in the presence of JQ1(+) has been described previously and were shown to be E2-Brd4 complexes that form off the cellular chromatin (see Chapter 2, (75)). The strength of E2's binding to cellular chromatin was next tested by pre-extracting proteins using solutions with increasing salt concentrations prior to cell fixation. In this experiment, the cells were incubated in buffer containing NP-40 (to permeablize the nucleus) and different salt concentrations (to extract proteins unbound or weakly bound to cellular chromatin). All proteins besides those tightly bound to chromatin were washed away and then the cells were fixed for immunofluorescence analysis. Upon JQ1(-) treatment, Flag-16E2 and Brd4 remained associated with cellular chromatin in nuclear speckles after pre-extraction with buffer containing up to 250 mM NaCl (Fig. 4.5). Conversely, with JQ1(+) treatment and pre-extraction with 150 mM NaCl, only diffuse Flag-16E2 and Brd4 staining was detected in the nucleus but pre-extraction with higher salt concentrations removed nearly all of the Flag-16E2 and Brd4 signals (Fig. 4.5). Similar results were observed with BPV1 E2TA (data not shown). This experiment demonstrates that Brd4 release from chromatin by JQ1(+) treatment weakens E2's association with chromatin, confirming that Brd4 mediates E2 binding to cellular chromatin.

4.3.5: JQ1(+) Treatment Reduces Papillomavirus Gene Expression

In the next experiment, we examined whether JQ1(+)-induced dissociation of Brd4 from chromatin could also repress E2-mediated transactivation of gene transcription from the native papillomavirus genome. For this study, H2 and W12 (clone 20863) cells were treated with JQ1(-) or JQ1(+) for 15 hours prior to mRNA isolation. Viral gene expression was measured with qRT-PCR using primers specific for either the BPV1 or HPV16 early genes: E1, E2, E6, and E7. As shown in Fig. 4.6, JQ1(+) treatment caused efficient repression of all the viral early genes compared to JQ1(-) treatments (Fig. 4.6 A and B). This reduction in viral transcripts was not due to any effect of JQ1(+) on papillomavirus episome levels (Fig. 4.6 C and D). Notably, GAPDH mRNA levels were not significantly affected by the JQ1(+) treatment, indicating that JQ1(+) does not induce a global shutdown of cellular gene transcription. It is also important to note that both H2 and W12 cells looked healthy and grew normally after 15 hours of JQ1(+) treatment, suggesting that the reduced viral mRNA levels did not result from any impact of JQ1(+) on cell proliferation. These results, together with the findings in Figs. 4.4 and 4.5, suggest that JQ1(+) releases E2 and Brd4 from cellular chromatin and this dissociation correlates with dramatically reduced transcription of the papillomavirus early genes. However, the papillomavirus genome also associates with cellular histones and assembles into minichromosomes so it is possible that the Brd4 bromodomains directly interact with viral minichromosomes and abrogating this interaction might also inhibit viral transcription (49, 167).

4.4: Discussion

Brd4 has long been known to function in papillomavirus transcription activation but its precise role in this process remained elusive. Here, P-TEFb was found recruited to the papillomavirus genome along with Brd4 and was also shown to restore transactivation function to Brd4 binding-deficient E2 mutants, suggesting an important role of Brd4/P-TEFb recruitment for papillomavirus transcription activation. Furthermore, our work is the first to identify JQ1(+) as a potent inhibitor of papillomavirus gene expression, suggesting an important role of Brd4's chromatin association for viral transcription regulation. These findings support a model of Brd4 function in E2-dependent viral transcription activation (Fig. 4.7).

E2 and Cdk9 both interact with the extreme C-terminal region of Brd4 (86, 220, 223, 224). This raises the question as to how Brd4 associates with E2 at the viral promoter while also recruiting P-TEFb. Our earlier report demonstrated that Brd4 proteins can interact intermolecularly to form homodimers (206). We therefore predict that Brd4 dimerizes at the papillomavirus transcription complex (Fig. 4.7). It has been well established that low levels of E2 activate viral transcription whereas elevated E2 levels repress the viral early promoter (60, 192, 194). It is possible that, in the presence of low-level E2 expression, one Brd4 protein in the homodimers associates with E2 on the viral promoter while the other recruits P-TEFb. These molecular interactions therefore allow E2 to recruit the Brd4/P-TEFb complexes to the viral promoter to stimulate viral transcription activation (Fig. 4.7). On the other hand, high levels of E2 protein can saturate all of the Brd4 C-terminal binding sites, thus replacing the Brd4-P-

TEFb interaction and preventing the recruitment of P-TEFb to the viral promoter region for transcription activation. This could eventually lead to repression of viral transcription as has been observed when E2 is ectopically expressed in cells with integrated HPV genomes (220). Future studies will test this hypothesis and determine whether Brd4 dimerization is essential for PV transcription regulation.

JQ1(+) treatment potently inhibited transactivation of the p2x2xE2BS-Luciferse reporter and the papillomavirus early promoters. This transcription inhibitory effect by JQ1(+) is specific for papillomaviruses since GAPDH mRNA levels were not significantly affected by JQ1(+) and expression of beta-galactosidase from a constitutive CMV promoter was actually elevated with JQ1(+) treatment. This observation also suggests that JQ1(+)-induced inhibition of E2 transcription activation is not a consequence of global gene expression shutdown. In fact, it was recently found that JQ1(+) effectively reactivates HIV transcription in latently infected cells (13, 22). The JQ1(+)-induced inhibition of E2 transcription activation was also observed with only 8 hours of treatment, suggesting that this inhibition effect is not likely due to an impact on the cell cycle. Furthermore, we did not detect a significant change in papillomavirus genome levels in H2 and W12 cells after 15 hours of JQ1(+) treatment. In a previous study we showed that JQ1(+) activates HPV16 genome replication in a transient transfection experiment, which would likely result in increased viral genome copies (Chapter 3, (208)). However, with the drastically reduced viral transcript levels in combination with abrogated viral genome maintenance in dividing cells infected with

native viruses (Chapter 2, (75)), we predict that prolonged JQ1(+) treatment would eventually induce a pronounced loss of the papillomavirus episomes.

Other than Brd4, JQ1(+) also affects the chromatin association of all BET family members as well as other bromodomain-containing proteins (51). We therefore sought to specifically inhibit Brd4 chromatin binding to determine if the reduced papillomavirus transcription is exclusively from Brd4 inhibition. We previously utilized ectopic expression of the Brd4 double-bromodomains (BDI/II) to dissociate Brd4 from chromatin and showed that Brd4 BDI/II does not affect Brd2 chromatin binding, indicating that this is a more specific method to abrogate Brd4 chromatin interactions (206). Unfortunately, although Brd4 BDI/II efficiently inhibited the E2 transactivation activities, it also drastically destabilized the E2 protein, preventing us from fairly assessing the effect of BDI/II on E2-mediated transcription activation (data not shown). However, because Brd4 is the only bromodomain-containing protein that carries the C-terminal domain for E2 binding, the observed JQ1(+) effects on E2 chromatin association and transcription activation are not likely resulted from the inhibition of other JQ1(+) targets besides Brd4.

Our analysis of E2 and Brd4 chromatin association upon JQ1(+) treatment demonstrates that E2's chromatin association matches that of Brd4. Given that the other BET family members do not interact with E2, we believe this experiment suggests that Brd4 tethering of E2 to cellular chromatin might play an important role in E2 transcription activation of viral genes. Our observations are in line with the study published by Schweiger *et al*., which showed that Brd4 binding is required for E2

transcriptional activation (174). The authors found that inhibiting E2-Brd4 with Brd4 CTD or knocking-down Brd4 with siRNA significantly reduced E2-dependent transcription activation, demonstrating a role for Brd4 in the transcriptional activation function of E2. In studies from the McBride group, it was shown that Brd4 directs E2 to actively transcribed regions of cellular chromatin (85, 87). We predict the virus might rely on Brd4 to take its genome to areas of the nucleus where it can efficiently co-opt the cellular transcriptional machinery. However, this is not the only possible explanation to account for our findings so further studies are needed to confirm this hypothesis. For example, Brd4 is involved in transcription activation of a number of cellular genes and JQ1(+) has been shown to inhibit transcription of some of these genes (34, 148, 207, 234). It is therefore possible that at least part of the reduction in papillomavirus transcription from JQ1(+) treatment is due to a loss of cellular transcription factors important for papillomavirus transcription. Furthermore, Brd4 might contribute to other functions necessary for viral transcription. For instance, the papillomavirus episomal genome assembles into viral minichromosomes with cellular histones and it is possible that Brd4 binds acetylated histones on the viral genome (49, 167). This binding might serve to recruit chromatin modulating factors or other transcription factors to the viral promoters. Additionally, we have observed that Brd4 can phosphorylate E2 *in vitro* (data not shown). It will be interesting to determine if this Brd4 function can be detected in cells once a suitable antibody is generated and if this could be a regulatory switch by which Brd4 controls E2's transcription functions. Uncovering the mechanisms underlying

E2 and Brd4 function in viral transcription regulation will provide a better understanding of how HPV gene expression is regulated throughout the viral life cycle.

We previously showed that JQ1(+) has the potential to be a valuable tool to eliminate HPV infection since it can not only release E2-Brd4 complexes from mitotic chromosomes, which could disrupt viral episome maintenance, but also stimulate HPV16 DNA replication, which could trigger an early and inopportune antiviral immune response (Chapter 2 and 3, (75, 208)). In this study, we further discovered that JQ1(+) also abrogates papillomavirus gene expression. Future studies will address the important question of whether the combined effects of JQ1(+) on the HPV life cycle result in clearance of HPV persistent infection.

4.5: Figures

Figure 4.1

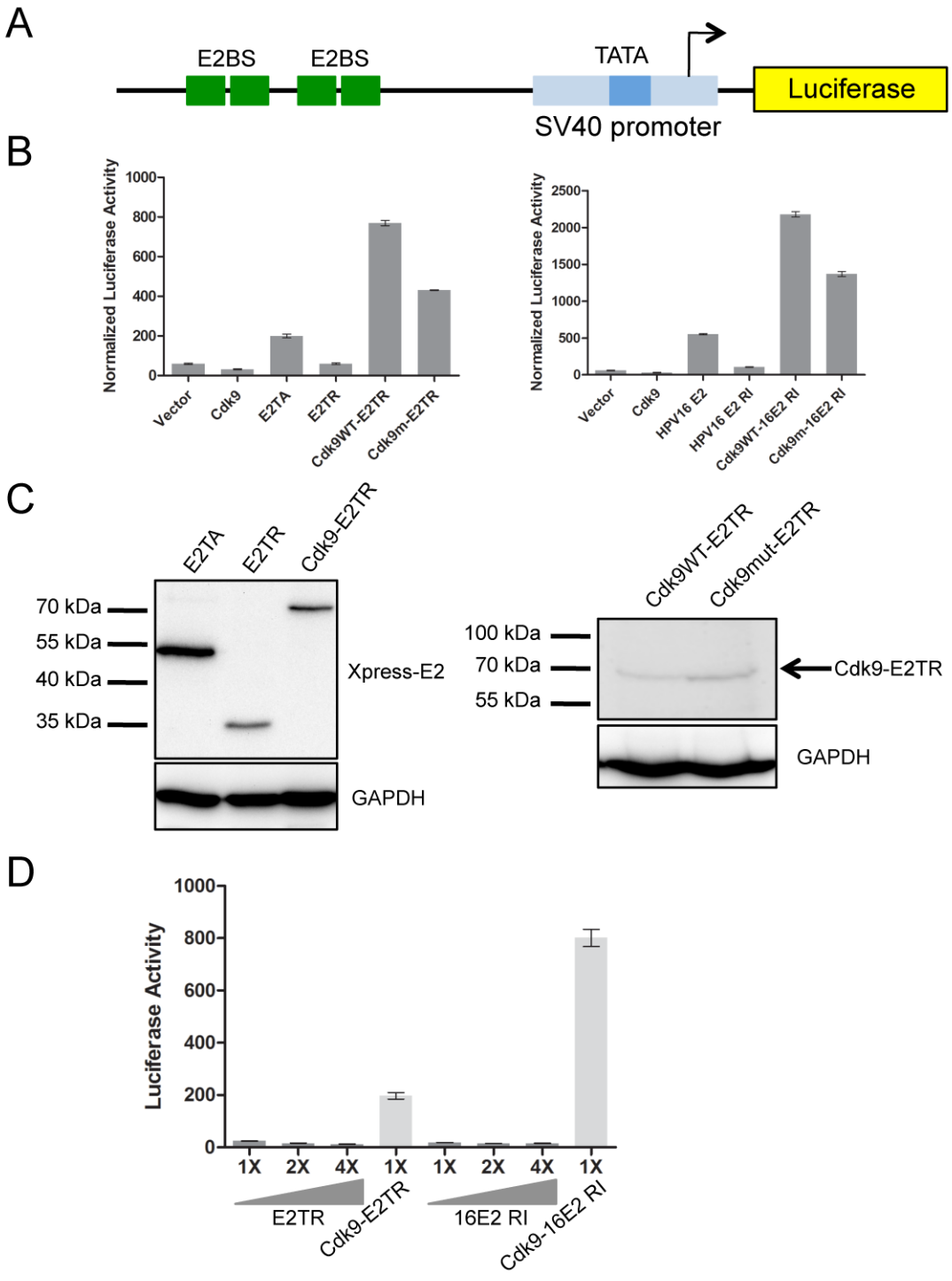
A
E2BS
E2BS
TATA
Luciferase
SV40 promoter
B
Normalized Luciferase Activity
1000
800
600
400
200
0
Vector
Cdk9
E2TA
E2TR
Cdk9WT-E2TR
Cdk9m-E2TR
Normalized Luciferase Activity
2500
2000
1500
1000
500
0
Vector
Cdk9
HPV16 E2
HPV16 E2 RI
Cdk9WT-16E2 RI
Cdk9m-16E2 RI
C
E2TA
E2TR
Cdk9-E2TR
70 kDa
55 kDa
40 kDa
35 kDa
Xpress-E2
GAPDH
Cdk9WT-E2TR
Cdk9mut-E2TR
100 kDa
70 kDa
55 kDa
Cdk9-E2TR
GAPDH
D
Luciferase Activity
1000
800
600
400
200
0
1X
2X
4X
1X
1X
2X
4X
1X
E2TR
Cdk9-E2TR
16E2 RI
Cdk9-16E2 RI

Figure 4.1. P-TEFb is important for E2-mediated papillomavirus transcription activation. (A) Schematic diagram of the 2x2xE2BS-Luciferase reporter. Two pairs of E2 binding sites (E2BS) are upstream of the minimal SV-40 promoter and firefly luciferase gene. (B) C33A cells were cotransfected with p2x2xE2BS-Luciferse, CMV-β-gal, and either an empty vector (Vector), a Cdk9 expression plasmid (Cdk9), or the indicated E2 expression plasmids. Forty-eight hours post transfection, the cells were processed for luciferase and beta-galactosidase measurements. The luciferase values were normalized to beta-galactosidase expressed from a constitutive CMV promoter. Average and standard deviation were calculated from three experiments. (C) Nuclear proteins from cells transfected as in (B) were extracted and immunoblotted using anti-Xpress and anti-GAPDH antibodies. (D) Cells were cotransfected as in (B) but with 1X of the Cdk9-E2 fusion constructs or increasing amounts of E2TR/16E2 RI (1X, 2X, or 4X). Empty vector was used to make the amount of DNA used for each transfection equal. Forty-eight hours post transfection, the cells were processed for luciferase activity and beta-galactosidase activity measurements. Average and standard deviation were calculated from three experiments.

Figure 4.2

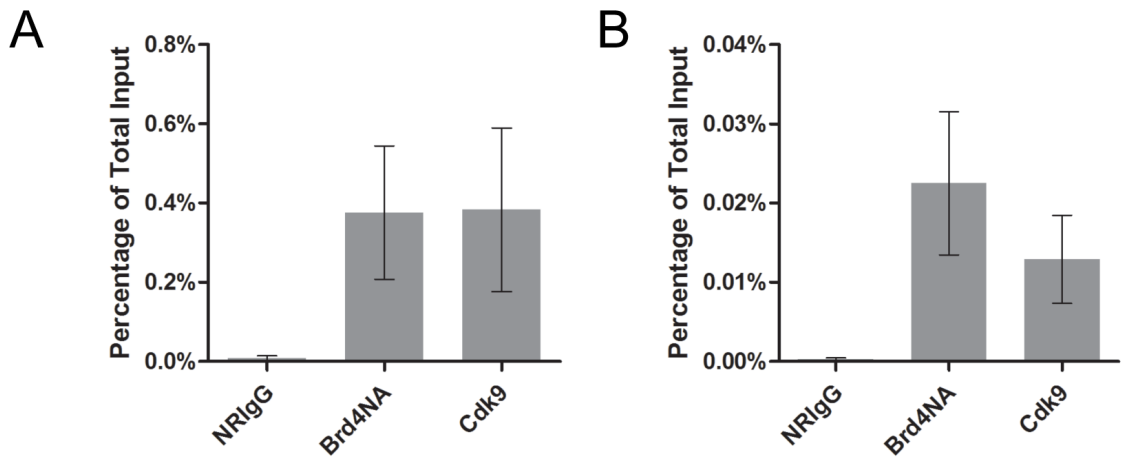

Figure 4.2. P-TEFb is recruited to the papillomavirus genome. (A) H2 cells were subjected to ChIP assay using normal rabbit IgG (NRIgG), Brd4NA antibody, or anti-Cdk9 antibody. ChIP samples were analyzed by qPCR using primers targeting the BPV1 genome. (B) W12 (clone 20863) cells were subjected to ChIP assay as in (A) and ChIP samples were analyzed by qPCR using primers targeting the HPV16 genome. For both (A) and (B), the values represent the average and standard deviation of three independent experiments.

Figure 4.3

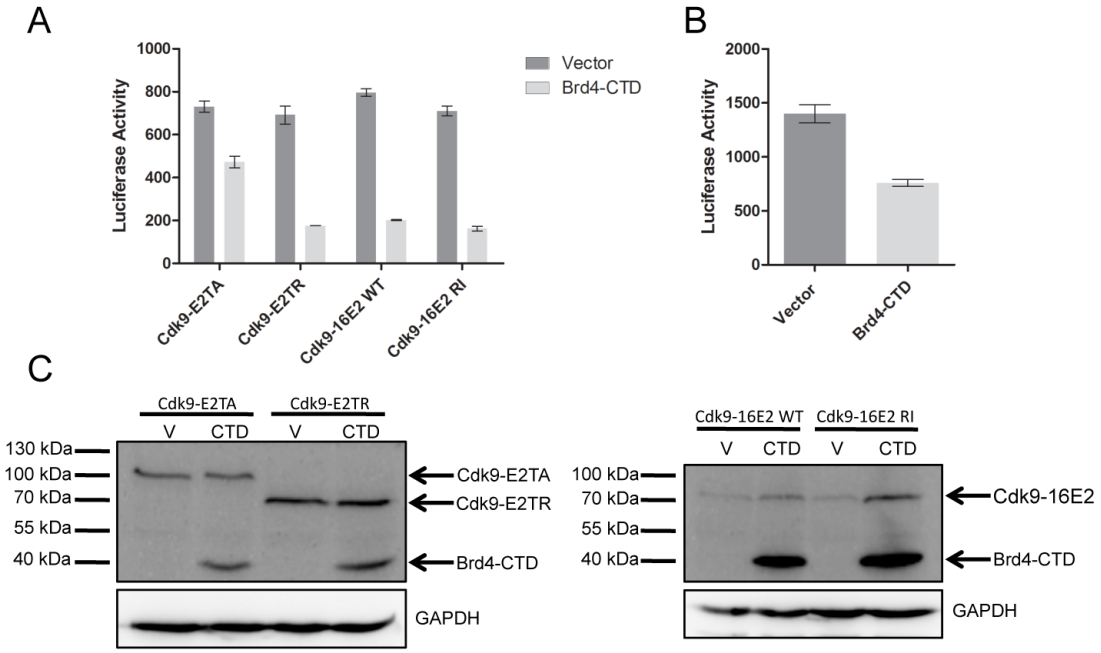

Figure 4.3. Brd4 CTD inhibits transcription activation by the Cdk9-E2 fusion proteins. (A) Along with the p2x2xE2BS-Luciferase construct, C33A cells were cotransfected with the indicated Cdk9-E2 fusion constructs and either an empty vector (Vector) or Brd4 CTD expression construct at a 1:6 ratio (Cdk9-E2:V/CTD). Forty-eight hours post transfection, the cells were processed for luciferase activity measurement. (B) Cells were transfected as in (A) except a 1:8 ratio of Cdk9-E2TA:V/CTD was used. Forty-eight hours post transfection, the cells were processed for luciferase activity measurement. In (A) and (B), average and standard deviation were calculated from three experiments. (C) Cells were transfected as in (A) and nuclear lysates were immunoblotted with anti-Xpress and anti-GAPDH antibodies.

Figure 4.4

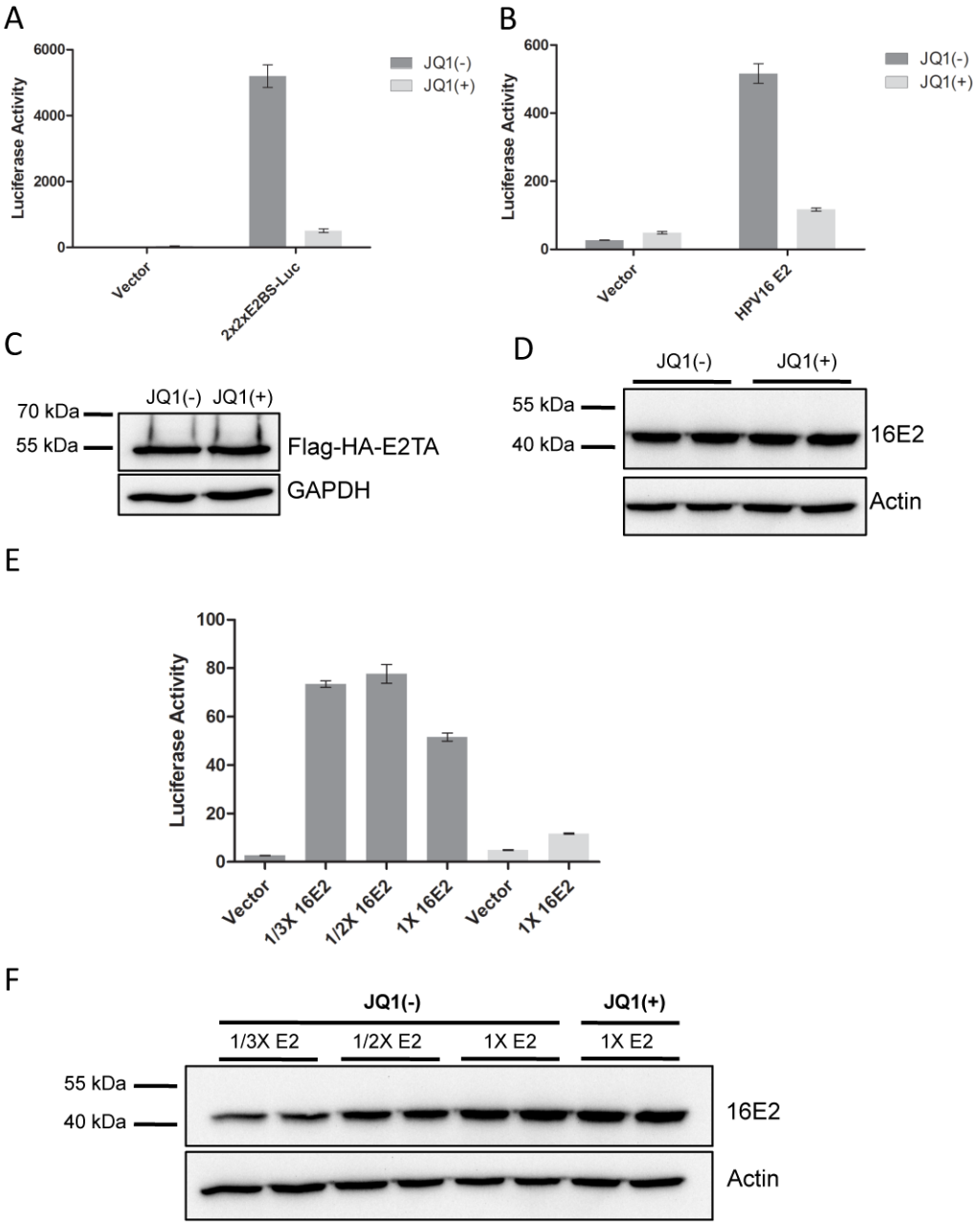
A
Luciferase Activity
6000
4000
2000
0
JQ1(-)
JQ1(+)
Vector
2x2xE2BS-Luc
B
Luciferase Activity
600
400
200
0
JQ1(-)
JQ1(+)
Vector
HPV16 E2
C
JQ1(-) JQ1(+)
70 kDa
55 kDa
Flag-HA-E2TA
GAPDH
D
JQ1(-)
JQ1(+)
55 kDa
40 kDa
16E2
Actin
E
Luciferase Activity
100
80
60
40
20
0
Vector
1/3X 16E2
1/2X 16E2
1X 16E2
Vector
1X 16E2
F
JQ1(-)
JQ1(+)
1/3X E2
1/2X E2
1X E2
1X E2
55 kDa
40 kDa
16E2
Actin

Figure 4.4. JQ1(+) inhibits E2 transcription activation. (A) C33A cells stably expressing Flag-HA-tagged BPV1 E2TA were transfected with empty vector (Vector) or the p2x2xE2BS-Luciferase construct. At thirty-three hours post transfection, the cells were treated with 500 nM JQ1(-) or JQ1(+) until collection at forty-eight hours post transfection. The cells were processed for luciferase activity measurement. (B) C33A cells were cotransfected with the p2x2xE2BS-Luciferase construct and an empty vector (Vector) or the Flag-16E2 construct. As in (A), the cells were treated with 500 nM JQ1(-) or JQ1(+) for 15 hours then collected at forty-eight hours post transfected for luciferase activity measurement. Average and standard deviation were calculated from three experiments for (A) and (B). (C) Cells stably expressing Flag-HA-tagged BPV1 E2TA were transfected and treated with JQ1 as in (A). Nuclear proteins were harvested and immunoprecipitated with anti-Flag (M2) beads. The precipitates were immunoblotted with anti-HA-HRP antibody. GAPDH levels were analyzed from the input with anti-GAPDH antibody. (D) C33A cells were transfected and treated with JQ1 as in (B). Protein extracts were immunoblotted with anti-HPV16 E2 and anti-actin antibodies. (E) C33A cells were cotransfected as in (B) except with increasing amounts of 16E2 (1/3X, 1/2X, 1X). Empty vector was used to make the amount of DNA used for each transfection equal. The cells were treated with JQ1 and collected for luciferase activity measurement as in (B). The average and standard deviation were calculated from three experiments. (F) Cells were transfected and treated with JQ1 as in (E). Protein extracts were immunoblotted with anti-HPV16 E2 and anti-actin antibodies.

Figure 4.5

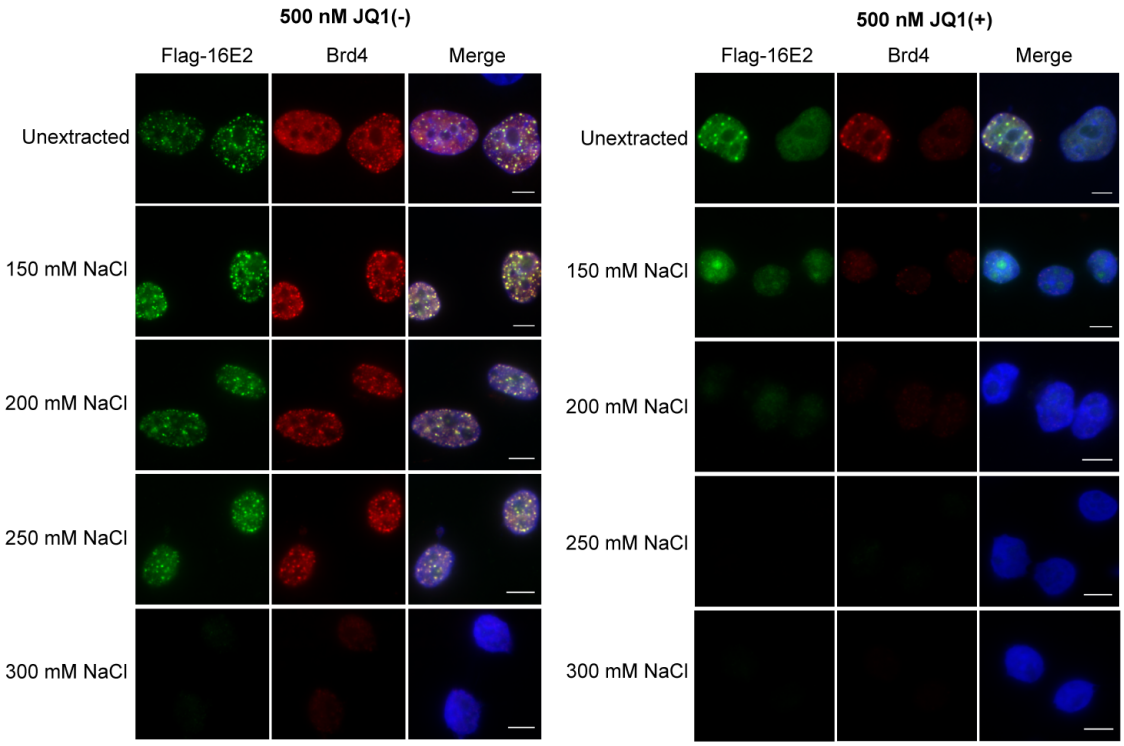

Figure 4.5. JQ1(+) releases E2 from chromatin. C33A cells were cotransfected with Flag-16E2 and Brd4 expression constructs at a 1:1 ratio. Two hours prior to collection, cells were treated with 500 nM JQ1(-) or JQ1(+). Forty-eight hours post transfection, coverslips were collected and either fixed immediately (unextracted) or pre-extracted in buffer containing the indicated concentration of NaCl prior to fixation. The coverslips were then stained with anti-Flag (green) and anti-Brd4CA (red) antibodies and counterstained with DAPI. This experiment was repeated a total of three times and the most representative images are presented. Bar, 5 μm.

Figure 4.6

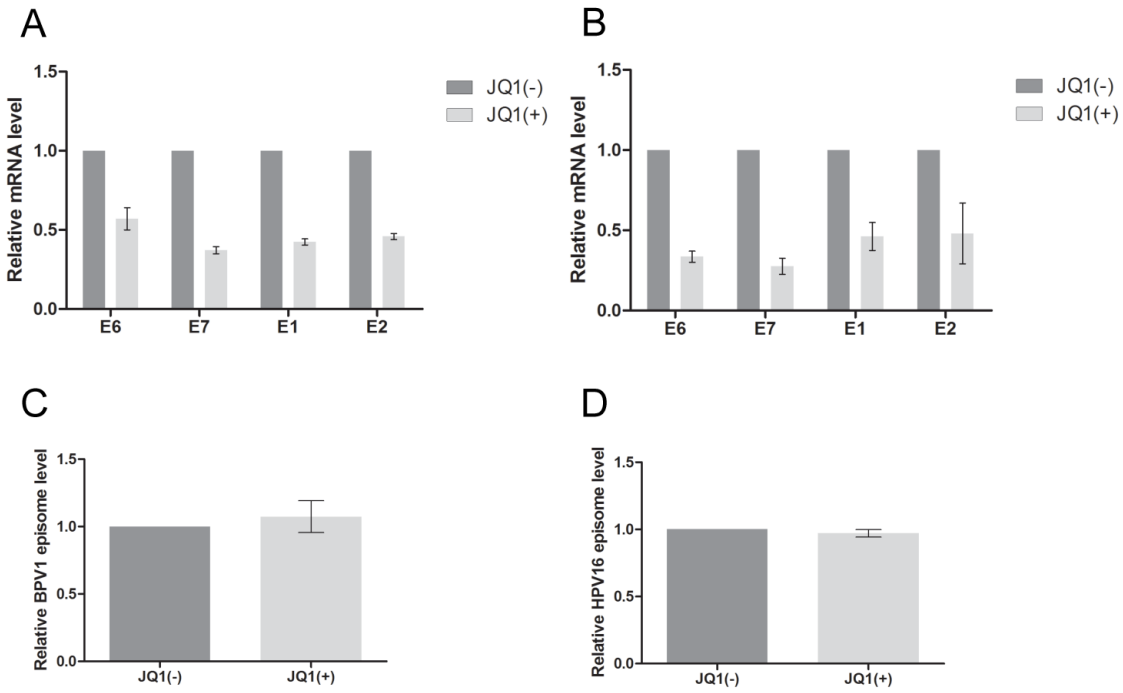
A
Relative mRNA level
1.5
1.0
0.5
0.0
E6
E7
E1
E2
JQ1(-)
JQ1(+)
B
Relative mRNA level
1.5
1.0
0.5
0.0
E6
E7
E1
E2
JQ1(-)
JQ1(+)
C
Relative BPV1 episome level
1.5
1.0
0.5
0.0
JQ1(-)
JQ1(+)
D
Relative HPV16 episome level
1.5
1.0
0.5
0.0
JQ1(-)
JQ1(+)

Figure 4.6. JQ1(+) treatment inhibits papillomavirus early gene expression. (A) H2 cells were treated with 1 μM JQ1(-) or JQ1(+) for 15 hours. RNA from these cells was reverse transcribed and the levels of the indicated BPV1 early gene mRNAs were measured by qRT-PCR. The mRNA level for each viral gene was normalized to GAPDH mRNA level. The JQ1(+)-treated sample values are presented relative to the JQ1(-) sample values. (B) W12 (clone 20863) cells were treated with 100 nM JQ1(-) or JQ1(+) for 15 hours. As in (A), RNA was collected and reverse transcribed and the cDNA was used in qRT-PCR analyses to measure HPV16 early gene mRNAs. The mRNA level for each viral gene was normalized to GAPDH mRNA level. The JQ1(+)-treated sample values are presented relative to the JQ1(-) sample values. (C) H2 cells were treated with 1μM JQ1(-) or JQ1(+) for 15 hours. Whole genomic DNA was extracted and the BPV1 episome level in each sample was measured using qPCR. The BPV1 episome level was normalized to the GAPDH DNA level in the samples. (D) W12 (clone 20863) cells were treated with 100 nM JQ1(-) or JQ1(+) for 15 hours and whole genomic DNA was extracted. The HPV16 episome level in each sample was measured using qPCR. The HPV16 episome level was normalized to the GAPDH DNA level in the samples. For all these experiments, the average and standard deviation were calculated from three independent experiments.

Figure 4.7

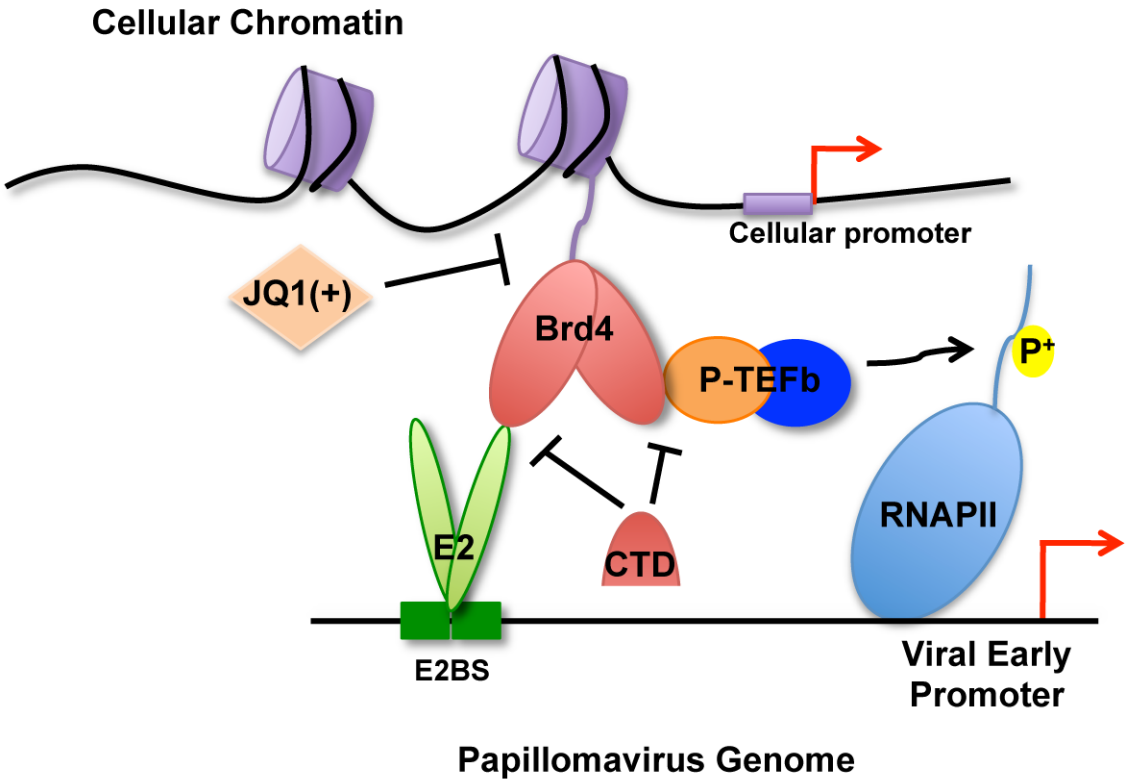
Cellular Chromatin
Cellular promoter
JQ1(+)
Brd4
P-TEFb
P+
E2
CTD
RNAPII
E2BS
Viral Early
Promoter
Papillomavirus Genome

Figure 4.7. A model of Brd4 functions in E2-mediated viral transcription activation. Papillomavirus E2 protein binds to the E2 binding sites (E2BS) upstream of the early promoter as a dimer. E2 interacts with Brd4 at the C-terminus while the N-terminal Brd4 bromodomains direct E2 and the viral genome to transcriptionally active regions of the cellular genome. Brd4 can form homodimers and also recruit P-TEFb to the viral promoter to phosphorylate the RNA pol II CTD to activate transcription elongation. However, JQ1(+) blocks Brd4 association with cellular chromatin, impeding the recruitment of the viral genomes to transcriptionally active areas of the cellular genome and thereby inhibiting E2-mediated transcription activation of the papillomavirus promoters. The Brd4-CTD can abrogate the E2-Brd4 interaction as well as the Brd4-Cdk9 interaction, thus blocking P-TEFb recruitment to the viral promoter and preventing the activation of transcription elongation by P-TEFb.

Table 4.1. The qPCR primer sequences used in Figures 4.2 and 4.6.

Primer Name	Primer Sequence
BPV1 Genome Forward	5’ GAAGAGGATGGAGACAGCATGC 3’
BPV1 Genome Reverse	5’ TCTGTGCGCATGTACAAATTGC 3’
HPV16 Genome Forward	5’ CAATGCGACACAAACGTTCTGC 3’
HPV16 Genome Reverse	5’ CTATAGAAGGATCGGAAGGG 3’
BPV1 E6 Forward	5’ TCCATTCTCAGGGTTGGATTG 3’
BPV1 E6 Reverse	5’ CACAGTAGCAGCATCTTATGC 3’
BPV1 E7 Forward	5’ CGTTGCTGATTTTAAGTCCATGTG 3’
BPV1 E7 Reverse	5’ GTCTTCACAGCAAAAGTCAGCT 3’
BPV1 E1 Forward	5’ CTGACTGAGGCAGAATGTGAAAG 3’
BPV1 E1 Reverse	5’ TGGAGTTTCAGATGCTTCGG 3’
BPV1 E2 Forward	5’ GCTGTTAGAACTGAGAACACACTG 3’
BPV1 E2 Reverse	5’ GCCTTTCTTAAAGCACCGTTTAGG 3’
Mouse GAPDH Forward	5’ CCAGCCTCGTCCCGTAGA 3’
Mouse GAPDH Reverse	5’ CGCCCAATACGGCCAAA 3’
HPV16 E6 Forward	5’ TACCACAGTTATGCACAGAGC 3’
HPV16 E6 Reverse	5’ GCTTTTCTTCAGGACACAGTG 3’
HPV16 E7 Forward	5’ GCATGGAGATACACCTACATTGC 3’
HPV16 E7 Reverse	5’ CGAATGTCTACGTGTGTGCTTTG 3’
HPV16 E1 Forward	5’ GAAGAGGGTACGGGATGTAATG 3’
HPV16 E1 Reverse	5’ CATGTGCTGTCTCTGTTTCTGC 3’
HPV16 E2 Forward	5’ CAGACCTACGTGACCATATAGACT 3’
HPV16 E2 Reverse	5’ CTGCACTTCCACTGTATATCCATG 3’
Human GAPDH Forward	5’ GTGAAGGTCGGAGTCAACGGA 3’
Human GAPDH Reverse	5’ CCATGGGTGGAATCATATTGGAAC 3’

CHAPTER 5: Conclusions and Future Directions

5.1: Brd4 Inhibition as a Method to Clear Viral Infection

5.1.1: Brd4 Inhibition to Disrupt the HPV Life Cycle

In this thesis, I have studied the interaction between HPV16 E2 and Brd4 as well as the importance of this interaction for different viral functions. More importantly, I have identified the BET inhibitor, JQ1, as a promising tool to abrogate critical events of the HPV life cycle. My studies demonstrated that Brd4 association with chromatin is necessary for HPV16 E2-Brd4 complexes to bind mitotic chromosomes (Chapter 2), suggesting that Brd4 is important for HPV16 E2-mediated episome maintenance during the early stage of HPV infection. This result also suggests that JQ1 treatment could be an effective method to abrogate this E2 function, which could result in loss of viral genomes with each cell division.

I also found that Brd4 is essential for HPV genome replication and that releasing Brd4 from chromatin after JQ1 treatment actually enhances viral replication. From the perspective of trying to treat a persistent HPV infection, elevated viral replication might initially seem undesirable. However, during the persistent phase of the HPV life cycle, the virus maintains its genomes at a very low copy number to avoid activation of the host immune response. Therefore, premature induction of viral genome amplification during persistent infection might trigger an immune response to clear the viral infection. This hypothesis can be tested using a mouse model of HPV infection since mice have been shown to tolerate JQ1 with minimal adverse effects (25, 51).

Lastly, we have discovered that JQ1 treatment dramatically reduces papillomavirus transcription suggesting that Brd4-mediated HPV association with cellular chromatin is important for papillomavirus gene transcription. It is possible that Brd4 functions to direct E2 and the viral genomes to active regions of the nucleus so that the virus can easily access the cellular transcription machinery (85, 87). Without this targeting effect, the viral transcription is very inefficient.

The next obvious question from these findings is what effect Brd4 bromodomain inhibition would have during an actual HPV infection when episome maintenance, viral replication, and viral transcription are all occurring at once. I predict that the combined loss of viral genome maintenance and greatly reduced HPV gene transcription would be enough to disrupt the viral life cycle during an early HPV infection. This obviously needs to be tested so future work will analyze cells that maintain a persistent HPV infection and determine if prolonged treatment with a non-toxic dose of JQ1 clears these cells of HPV infection. Additionally, we can examine the effect of JQ1 on organotypic raft cultures of HPV31 infected cells (106). Other have demonstrated that HPV31 positive cells grown in organotypic raft cultures can produce and release virions from the upper cell layer (131). It would therefore be interesting to determine if JQ1 treatment of these cultures disrupts the viral life cycle so that virions are no longer generated from the culture.

5.1.2: Brd4 Inhibition as a Tool to Clear Other Viral Infections and Treat Virus-Related Diseases

As discussed in Section 1.4.4, a number of other viruses besides papillomaviruses utilize Brd4 for important viral functions. It is therefore possible that inhibition of Brd4 could be an effective method to disrupt the life cycles of these viruses as well. Interestingly, it was found that Brd4 is important for P-TEFb recruitment to the EBV C promoter for activation of EBNA gene transcription elongation (152). Similar to our findings with papillomaviruses, this group demonstrated that Brd4 inhibition by JQ1 significantly reduces transcription from the C promoter (152). It will be interesting to determine if this repression of the EBNA latency genes has an impact on EBV latent infection.

During HIV infection, Brd4 competes with the HIV TAT protein for P-TEFb binding and thereby inhibits P-TEFb recruitment to the integrated viral genome and represses HIV gene transcription (19). In addition, Brd2 acts as an important enforcer of HIV latency (22). Since Brd4 and Brd2 both function to inhibit HIV lytic infection, it was not surprising that the BET inhibitors were found to relieve this repression of HIV transcription and effectively reactivate latent HIV (11, 22). This finding identified JQ1 and other BET inhibitors as promising drugs to reactivate latent pools of HIV so the infection can be treated and hopefully eliminated completely.

Besides viral infections, the BET inhibitors have shown great promise as a drug therapy for several types of cancers (see Section 1.3.5). Recently, it was found that a type of non-Hodgkin's lymphoma associated with KSHV infection is sensitive to BET

inhibition by JQ1 (197). This group demonstrated that treatment of these cells with JQ1 down-regulates Myc levels and causes cellular senescence, cell cycle arrest, and apoptosis. This finding indicates that the BET inhibitors can also be useful tools to treat additional virus-associated diseases.

5.2: The Dynamic E2-Brd4 Interaction During the Viral Life Cycle

5.2.1: The Interaction Between E2 and Brd4

Using the BiFC technique, our studies demonstrated that HPV16 E2 interacts with Brd4 during all stages of mitosis, suggesting that this interaction is important for tethering E2 and viral episomes to mitotic chromosomes to ensure that the viral genomes are maintained between cell divisions. Besides this important discovery, this study also demonstrated the usefulness of BiFC for analyzing protein-protein interactions in live or fixed cells throughout the cell cycle. In the future, BiFC can be used to examine the interaction between other papillomavirus E2s and Brd4 at different cell cycle stages and throughout the HPV life cycle in order to better understand how this interaction is regulated and when during infection this interaction is most important.

5.2.2: E2 Transition Between E1 and Brd4 Binding

E1 and Brd4 interact with E2 on opposite faces of the E2 transactivation domain and therefore can potentially bind E2 simultaneously (1, 2). However, we have observed that increasing E1 levels competitively inhibits Brd4's interaction with E2 (Fig. 5.1). It is therefore possible that E2's transition between its roles in episome tethering, viral genome replication, and viral transcription is regulated by the relative abundance of E2,

E1, and Brd4 in cells. E1 and E2 are expressed at a similar level in undifferentiated cells but upon cellular differentiation, expression of both proteins greatly increases with E1 protein reaching a higher level than E2 (150). Furthermore, in human skin, we found that Brd4 levels also increase upon cellular differentiation (data not shown). Because the relative protein levels of E2, E1, and Brd4 change during the HPV life cycle, it will be interesting to test if these changes regulate the various E2 functions in the viral life cycle.

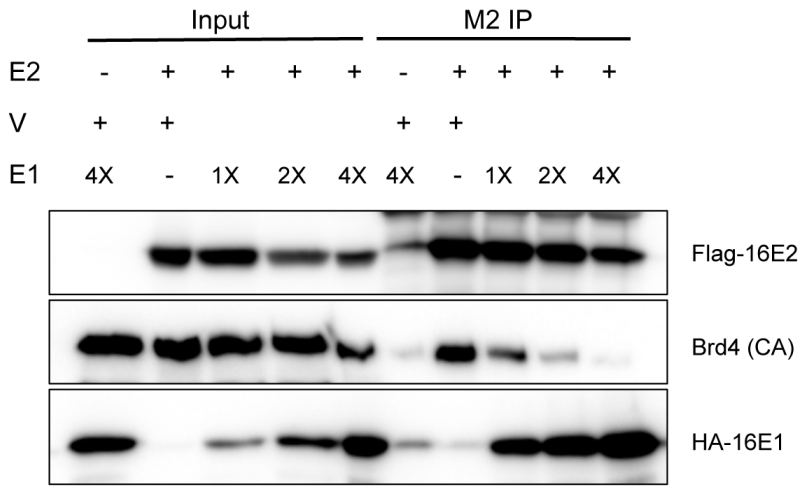

Figure 5.1. E1 inhibits Brd4's association with E2 in a dose-dependent manner. C33A cells were cotransfected with Flag-16E2 and differing amounts of HA-16E1 or empty vector (V) as indicated. The empty vector was used as a negative control to make the final DNA concentration equal for all the transfections. Forty-eight hours post transfection, nuclear extracts were immunoprecipitated with anti-Flag (M2) antibody. Both input samples and precipitates were immunoblotted with specific antibodies as indicated.

One report suggested that E2 phosphorylation status might regulate its binding to E1 (203). This group showed that BPV1 E2 mutated at four serines in its hinge domain abolishes phosphorylation at these sites and increases E2 binding to E1 while reducing E2 association with mitotic chromatin. Interestingly, we have found that Brd4 purified from insect cells can phosphorylate E2 *in vitro* (data not shown). Future studies will determine if Brd4 actually phosphorylates E2 *in vivo* and assess whether E2 phosphorylation affects E2's binding to mitotic chromosomes and E1. If these studies are successful, we can further examine E2 phosphorylation status throughout the HPV life cycle to gain a better understanding of how E2 function is regulated.

5.3: Brd4's Roles During HPV Replication

5.3.1: Brd4's Role During HPV Genome Replication

Our study demonstrated that Brd4 association with E2 is essential for the formation of viral replication factories and for efficient replication of the HPV genomes (Chapter 3). However, Brd4's exact function during viral replication is still unclear. Similar to our hypothesis for Brd4's role in viral transcription activation, we speculate that Brd4 might direct the viral replication complex to particular regions of the nucleus where the virus can access cellular replication proteins and DNA damage response factors. This is supported by recent findings that Brd4 tethers the HPV replication complex to cellular chromatin near common fragile sites, which are especially prone to DNA damage (87). It is also possible that Brd4 functions to directly recruit cellular or DNA damage response factors to the HPV replication foci. Brd4 is known to interact

with RFC1 as well as the RFC homolog, ATAD5, which are both found in HPV replication foci (data not shown and (83, 121, 163, 208)). Future efforts will focus on better understanding Brd4's role(s) in HPV replication and, more specifically, determining if Brd4 functions to recruit cellular replication factors to HPV replication centers.

A recent study from the McBride group showed that Brd4 and cellular chromatin colocalize with early HPV replication foci but are eventually displaced from the viral foci as these foci mature into full-blown viral genome amplification factories (169). This group speculated that displacement of Brd4 and cellular chromatin from viral replication foci might be necessary for HPV genome amplification to proceed (169). In this model, Brd4 would function early during replication to direct the replication foci to specific nuclear domains in order to facilitate virus access to cellular replication and DDR factors. However, once these replication foci were established, Brd4 and the cellular chromatin would be displaced from viral foci to allow viral genome amplification to proceed. Intriguingly, in our study, we found that releasing Brd4 from cellular chromatin by JQ1 stimulates HPV genome replication (Chapter 3). It is possible that for replication foci established before JQ1 treatment, the release of Brd4 from cellular chromatin after JQ1 treatment promotes the displacement of Brd4 and cellular chromatin from the viral replication foci and stimulates full-blown HPV genome amplification. Future studies should further investigate Brd4 displacement from replication foci and determine whether this displacement is essential for viral genome replication. Furthermore, it will be important to determine if viral replication factories need to eventually dissociate from

cellular chromatin to amplify the viral genomes and if JQ1 treatment promotes this detachment from cellular chromatin by releasing Brd4.

5.4: Brd4's Roles in HPV Transcription Regulation

5.4.1: Brd4's Role in Papillomavirus Transcription Activation

Part of my thesis work focused on better understanding the role of Brd4 in papillomavirus transcription activation (Chapter 4). I showed that Brd4 recruitment of P-TEFb to the viral promoter is important for E2 transactivation function (Chapter 4). However, one question that remains is how Brd4 itself is recruited to the viral promoter. E2 recruitment is the simplest explanation but we have shown previously that E2 and P-TEFb bind the same region on Brd4 and interfere with each other's association with Brd4 (220). Therefore, the mechanism by which Brd4 and P-TEFb are recruited to the HPV transcription start site must be more complex. We have previously shown that Brd4 can form homodimers so it is possible that one Brd4 molecule binds E2 at the viral promoter while the other Brd4 molecule in the homodimer associates with P-TEFb (see Fig. 4.7) (206). To test this idea, we can mutate the dimerization domain within the Brd4 bromodomains to abolish Brd4 dimerization and determine if Brd4 can still recruit P-TEFb to the HPV promoters. Alternatively, it is also possible that some Brd4 molecules interact independently of E2 with acetylated histones associated with the viral chromatin and thereby recruit P-TEFb to the papillomavirus promoter (49, 138, 167). In this model, some Brd4 molecules would interact with E2 to enhance E2 stability and E2's association with the promoter (108) while other Brd4 molecules would interact with viral chromatin

near the papillomavirus transcription start site independent of E2 in order to recruit P-TEFb. E2-independent localization of Brd4 to the HPV promoters can be assessed by determining if a Brd4-specific antibody can ChIP HPV genomes mutated to abolish E2 expression. In addition, we can assess whether JQ1(+) treatment affects Brd4's ability to immunoprecipitate the papillomavirus episomes from infected cells.

5.4.2: Brd4's Role in Papillomavirus Transcription Repression

Brd4 has also been shown to be important for E2's transcription repression function (184, 216). One study demonstrated that E2 and Brd4 act to repress papillomavirus transcription by preventing the assembly of the pre-initiation complex near the viral promoter (216). However, it is also possible that Brd4 represses viral transcription by other mechanisms such as recruiting chromatin-remodeling factors or other transcription repression factors to the viral genome (184). In future studies, we will focus on uncovering Brd4's role(s) in papillomavirus transcription repression. In addition, it will be interesting and important to determine how E2 and Brd4 transition between their transcription activation and repression functions during the viral life cycle.

Concluding Remarks

This work studied a key virus-host interaction during the papillomavirus life cycle. It demonstrates the remarkable ability of viruses to co-opt a single cellular factor to mediate multiple viral processes. It also shows that specifically targeting this essential

virus-host complex can be an effective strategy to disrupt important stages of the viral life cycle.

CHAPTER 6: Materials and Methods

Cell culture

Cells of the human papillomavirus-negative cervical cancer cell line, C33A, were purchased from ATCC and maintained as monolayers in high glucose Dulbecco's modified Eagle's medium (DMEM, GIBCO) containing 10% fetal bovine serum (FBS, Hyclone) and 1% penicillin-streptomycin (Invitrogen). The mouse fibroblast cell line maintaining BPV1 episomes, H2, were maintained as monolayers in DMEM containing 10% FBS and 1% penicillin-streptomycin. Generation of the C33A cells stably expressing Flag-HA-tagged E2TA was described previously (224). The HPV16-positive cervical cancer epithelial cells, W12 (clone 20863), were provided by Dr. Paul Lambert and were maintained at subconfluence on mitomycin-c treated 3T3M feeder cells in F medium composed of 1 part DMEM and 3 parts F-12 medium (GIBCO) supplemented with 5% FBS, cholera toxin (8.4 ng/ml, Sigma), adenine (24 μg/ml, Sigma), epidermal growth factor (10 ng/ml, R&D), hydrocortisone (0.4 μg/ml, Millipore), and insulin (5 μg/ml, Sigma) (53). The *Spodoptera frugiperda* (Sf9) cell line was maintained as a monolayer in Sf-900 III Serum free medium (SFM, GIBCO).

Transient transfections

For immunofluorescence staining, immunofluorescence-fluorescence *in situ* hybridization (immuno-FISH) analysis, Southern blotting, and Western blotting, cells were transfected with FuGENE 6 (Promega) or Lipofectamine 2000 (Invitrogen)

transfection reagents following the manufacturers' instructions. For the *in vitro* replication assay, immunoprecipitation (IP) experiments, luciferase reporter assays, and Western blot analysis, cells were transfected using the calcium phosphate method. For calcium phosphate transfection, 25 μg DNA was mixed with 163 μl 2 M $CaCl_2$ in a 1.3 ml final volume. The DNA/$CaCl_2$ mixture was slowly dropped into 1.3 ml 2×HBS (55 mM HEPES, 0.4 M NaCl and 1.5 mM Na_2HPO_4, pH 7.0) while vortexing. The DNA mixture was then overlayed onto the cells with culture medium. Small interfering RNA (siRNA) transfection was performed using the calcium phosphate method described above or DharmaFECT II siRNA transfection reagent (Thermo Scientific Dharmacon) following the manufacturer's instructions. For bromodeoxyuridine (BrdU) labeling, cells were transfected with FuGENE HD (Promega) following the manufacturer's instructions. At 42 hours post transfection, the cells were treated with 10 μM BrdU for 20 minutes, washed 2 times with medium, and cultured an additional hour before being fixed with acetone.

Reagents

The compound, 4',6-diamidino-2-phenylindole (DAPI), was dissolved in water as a 500X stock (Invitrogen). siGENOME siRNAs targeting human *BRD4* and control non-targeting siRNA 1 were purchased from Thermo Scientific Dharmacon. The Brd4 bromodomain-specific inhibitor, JQ1(+), and its isomer compound, JQ1(-), were from Dr. James Bradner and were dissolved in dimethyl sulfoxide (DMSO) as 1000X stocks. BrdU (Sigma) was dissolved in DMSO to 10 mM.

Recombinant plasmid construction

The pUC19-HPV16 plasmid containing HPV16 genome inserted into the BamHI site of pUC19 and the pEFHPV-16W12E plasmid were gifts from Dr. Paul F. Lambert. HA-16E1 was provided by Dr. Mart Ustav. CMV4-Flag16E2 has been described previously (224). The cDNA for full-length human Brd4 was PCR amplified and subcloned into the BamHI and NotI sites of pcDNA4/HisMax C vector (pcDNA4C, Invitrogen) to generate pcDNA4C-hBrd4, in which human Brd4 was fused with an Xpress tag on the N-terminus. The Brd4 gene was also inserted into the BamHI/XhoI sites of pGEX-6P-1 (GE Healthcare) to obtain pGEX-6P-1-hBrd4 producing a GST-tagged hBrd4 protein. pOZN-HPV16 E2 encodes a codon optimized E2 gene fused to HA and Flag tags on its N-terminus and was generated by subcloning the HPV16 E2 coding sequence into the pOZN vector using XhoI and NotI sites. HPV16 E2 was PCR amplified and inserted into BamHI/XhoI sites of pEGFPC1 (Clontech) to obtain GFP-16E2. HPV16 E2 mutant plasmids, GFP-16E2 E39A, GFP-16E2 R37A/I73A, GFP-16E2 R304K, CMV4-Flag16E2 E39A, CMV4-Flag16E2 R37A/I73A, CMV4-Flag16E2 R37A, CMV4-Flag16E2 I73A, and pOZN-VC-16E2 R37A/I73A were constructed using QuikChange site-Directed Mutagenesis Kit (Stratagene) following the manufacturer's protocol. To obtain pHPVOri, the HPV16 replication Ori (7855 nt - 96 nt in HPV16 genome) was PCR amplified using primers $^{5'}$GCGGATCCCAAACCGTTTTGGGTT$^{3'}$ and $^{5'}$GCGGATCCCTCTTTTGGTGCATAAAATG$^{3'}$, and inserted into the BamHI site

of pcDNA4/HisMax C vector. The HA-16E1 open reading frame including its upstream promoter and downstream polyadenylation signals was digested with EcoRI/XhoI restriction enzymes and inserted into the EcoRI/XhoI sites of pHPVOri and pcDNA4/HisMax C to obtain pHPVOri/E1 and HA-16E1, respectively. The HPV16 genome was isolated from pUC19-HPV16 by BamHI digestion and re-ligation was performed under a very diluted condition (3-4 μg/ml). Un-ligated DNA was removed using Plasmid-Safety ATP-dependent DNase (Epicentre) following the manufacturer's protocol. To construct the plasmid encoding Xpress-tagged Brd4-CTD (pcDNA4C-NLS-Brd4-CTD), the SV40 nuclear localization signal (NLS) was inserted in frame in front of the Brd4-CTD (Brd4aa 1047-1362) coding region by PCR and the product was subcloned into pcDNA4C using BamHI and NotI sites. The Venus N (Venus aa 1-155) and Venus C (Venus aa 156-238) coding sequences were amplified from pCS2-Venus provided by Dr. Atsushi Miyawaki. To construct Brd4 or E2 fusions with VN or VC, a short linker sequence (GGSGG) was introduced in the C-terminal end of VN/VC fragments by PCR, and the amplified DNA fragments were cloned into the pOZN vector at the XhoI site. Brd4, E2TA, E2TR or 16E2 DNA fragments excised from their pOZN constructs using XhoI and NotI digestion were ligated into pOZN-VN-short linker and pOZN-VC-short linker to generate in-frame fusions of these molecules with either Venus N or Venus C. The pcDNA4C-NLS-LacI plasmid was generated by cloning an NLS into the pcDNA4C vector using a BamHI site and the PCR-amplified LacI cDNA fragment into the pcDNA4C vector using BamHI and EcoRI sites. For the pcDNA4C-Cdk9 construct, Cdk9 cDNA was PCR amplified and subcloned into the EcoRI and NotI sites of

pcDNA4C. The E2 expression constructs pcDNA4C-E2TA, pcDNA4C-E2TR, pcDNA4C-16E2, and pcDNA4C-16E2 R37A/I73A were generated by PCR amplifying the indicated E2 gene and subcloning into the EcoRI and NotI sites of pcDNA4C. For pcDNA4C-Cdk9-E2TA, pcDNA4C-Cdk9-E2TR, pcDNA4C-Cdk9-16E2, and pcDNA4C-16E2 R37A/I73A, the Cdk9 gene was fused to the indicated E2 gene using PCR. This fusion product was then subcloned into the EcoRI and NotI sites of pcDNA4C. The constructs containing Cdk9 D167N were generated by mutating the indicated site using the QuikChange site-directed mutagenesis kit (Stratagene). pCMV-LacZ (β-galactosidase) was purchased from Clontech. The 2x2xE2BS-Luciferase reporter was described previously (100, 187). All plasmid constructs were verified by DNA sequencing.

IF staining and immuno-FISH

Cells were cultured on coverslips and fixed with 3% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 20 minutes. The coverslips were then incubated in blocking/permeabilization buffer (3% bovine serum albumin and 0.5% Triton X-100 in PBS) and then incubated with primary antibodies for 1 hour at room temperature. After incubation, cells were washed 3 times using blocking/permeabilization buffer and incubated with fluorescently labeled secondary antibodies for 1 hour. In some cases, cells were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI).

For experiments with pre-extraction prior to cell fixation (Fig. 4.5), cells grown on coverslips were pre-extracted on ice for 10 minutes in CSK buffer (10 mM PIPES (pH

6.8), 30 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100, from 150 to 300 mM NaCl, and supplemented with protease inhibitors) before being fixed with 4% PFA in PBS for 20 minutes. The coverslips were then incubated in blocking/permeabilization buffer and then incubated with primary antibodies for 1 hour at room temperature. After incubation, cells were washed 3 times using blocking/permeabilization buffer and incubated with secondary antibodies for 1 hour. The cells were counterstained with DAPI.

For immuno-FISH staining, cells were fixed with 1% PFA for 10 minutes at room temperature and washed with cold PBS twice on ice. Proteins were stained following the IF protocol described above and RNA was digested with 25 μg/ml RNase A for 1 hour at 37°C. Cells were then fixed with 4% PFA for 10 minutes at room temperature, washed with cold PBS twice, washed with 2X SSC (300 mM NaCl, 30 mM trisodium citrate, pH 7.0) twice, and once each with 70%, 80%, and 100% ethanol. Probes were labeled with biotin-dUTP (AppliChem) using a nick translation assay and incubated with cells for 5 minutes at 95°C. Cells were then incubated in a moist chamber at 37°C overnight. The *in situ* hybridization signal was developed using the Trypticase soy agar (TSA)-biotin system (PerkinElmer) following the manufacturer's instructions.

Primary antibodies used for IF and immuno-FISH include: anti-Xpress (Invitrogen), anti-HA (Santa Cruz), anti-Flag M2 (Sigma), anti-RFC1 (H-300) (Santa Cruz), anti-RPA70 (Cell Signaling), anti-BrdU (Invitrogen), anti-Brd4C (recognizing Brd4 aa1313-1362), and anti-DNA polymerase δ (Santa Cruz). Secondary antibodies used include: Alexa Fluor 594 goat anti-rabbit IgG (Invitrogen), Alexa Fluor 594 goat

anti-mouse IgG (Invitrogen), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen), and Alexa Fluor 350 goat anti-mouse IgG (Invitrogen).

Microscopy and image analysis

All IF and immuno-FISH images were collected using an inverted fluorescence microscope (Olympus, IX81) equipped with an UPlanSApo 40×/0.95 NA lens (Olympus), an UPlanSApo 100×/1.4 oil immersion lens (Olympus) and a high-resolution charge-coupled device camera (QImaging, FAST1394) at room temperature. Photos were taken using either a 40× or 100× lens with immersion oil type-F (Olympus). Image data were analyzed and presented using SlideBook 5.0 software (Intelligent Imaging Innovations, Inc.). Images were cropped and scale bars were added using ImageJ software. Relative intensities were analyzed using ImageJ software.

The percentage of cells showing different foci or different localization pattern were quantified from approximately 50 to 200 positively transfected cells. Means and standard deviations (SD) were calculated from at least three independent experiments.

For Fig. 2.5, immunofluorescent images were analyzed using ImageJ software. The "Adjust Threshold" function of the ImageJ software was used to identify DAPI-stained nuclei. The average BiFC signal intensity divided by nucleus area was measured using the "Analyze Particles" function of the software. The BiFC signal intensity of 50 cells transfected with vector and 50 cells transfected with HPV16 genome was measured

and divided by the nucleus area to get the values plotted. This experiment was repeated 2 times with similar results.

Reverse Transcription (RT)-q-PCR

Total RNA was isolated using a NucleoSpin RNA II Kit (Macherey-Nagel) following the manufacturer's instructions. Reverse transcription was performed using a 20 μl reaction mixture containing 350 ng of total RNA, oligo(dT) primer (Invitrogen), dNTP (Invitrogen), and M-MLV reverse transcriptase (Invitrogen), following the manufacturer's instructions. Real time PCR was performed using a CFX96 real time PCR detection system (Bio-Rad) with IQ SYBR Green supermix (Bio-Rad). The mRNA level of each gene was normalized to GAPDH mRNA level. Primer sequences are shown in Table 4.1.

Immunoblotting

Cells were collected at 48 hours post transfection and washed once with PBS. The cell pellets were lysed in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol (DTT), supplemented with protease inhibitors (Roche)). The cells were then incubated on ice for 10 minutes, and Nonidet P-40 (NP-40) was added to a final concentration of 0.6%. After vortexing and centrifugation at 5,000 rpm for 5 minutes, the nuclear pellet was resuspended in ice-cold buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT, supplemented with protease inhibitors) and either incubated on ice for 15

minutes with vortexing or passed through a 20-gauge needle 10 times and mixed at 4°C for 1 hour. The nuclear proteins were isolated by centrifugation at 14,000 rpm for 5-15 minutes. The samples were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto PVDF membrane, and immunoblotted with specific antibodies. Antibodies employed in the Western blot analysis include: anti-Brd4C (recognizing Brd4 aa1313-1362), anti-HPV16 E2 (Millipore), anti-Xpress (Invitrogen), anti-Actin (Chemicon), anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) (United States Biological), and anti-HA-HRP (Roche). Western blots were developed using ECL solution (PerkinElmer) and images were captured using a Fuji imaging system.

Protein expression and purification

To express rBrd4 in insect cells, either wild type or Ac-IIT-hBrd4 baculovirus was used to infect Sf9 cells. Cells were lysed in IPP400 (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, and protease inhibitors) by passing through a 20-gauge needle 15 times. Supernatant was isolated by centrifugation at 14,000 rpm for 30 minutes at 4°C, and the salt concentration was adjusted to 0.15 M. Recombinant IIT-Brd4 was purified using IgG-Sepharose 6 Fast Flow (GE Healthcare) following the manufacturer's instructions. The protein-bound Sepharose was washed with IPP150 (20 mM HEPES, 0.15 M NaCl, 1 mM EDTA, 0.1% NP-40, and protease inhibitors) four times and IPP400 supplemented with 0.1% NP-40 twice. rBrd4 was released from the Sepharose using TEV protease

(Sigma) following the manufacturer's instructions. Expressed proteins were analyzed using SDS-PAGE, Coomassie brilliant blue staining, and/or immunoblotting.

Immunoprecipitation

C33A cells were pelleted at 48 hours post transfection and resuspended in buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, and 1 mM dithiothreitol (DTT) supplemented with protease inhibitors (Roche Applied Science)). The resuspended cells were incubated on ice for 10 minutes, and NP-40 was added to a final concentration of 0.6%. After vortexing and centrifugation at 5,000 rpm for 5 minutes, the nuclear pellet was resuspended in ice-cold buffer B (20 mM HEPES (pH 7.9), 0.4 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT, supplemented with protease inhibitors). To extract nuclear proteins, nuclei were passed through a 20-gauge needle ten times and extracted at 4°C for 1 hour. Nuclear proteins were isolated by centrifugation at 14,000 rpm for 15 minutes, diluted in buffer A, and pre-cleared with 3 μg normal rabbit IgG (NRIgG) and 10 μl protein A sepharose 4 fast flow beads (GE Healthcore) at 4°C for 1 hour. Pre-cleared lysates were then divided equally and immunoprecipitated with 1.8 μg of either NRIgG or Brd4NA antibody and 10 μl protein A beads (pre-blocked with 1% BSA in PBS at 4°C for 2 hour) at 4°C for 7 hours. The beads were then washed 3 times with 60 mM KCl base buffer (20 mM Tris (pH 8.0), 10% glycerol, 5 mM $MgCl_2$, 60 mM KCl, and protease inhibitors (Roche Applied Science)) and eluted with sample buffer. Input and IP samples were resolved by SDS-PAGE and proteins were immunoblotted as described above.

In Fig. 4.4, C33A cells stably expressing Flag-HA-tagged E2TA were treated with 500 nM JQ1(-) or JQ1(+) for 15 hours. The cells were then pelleted and a nuclear extraction was performed as described above. About 20 μg of nuclear protein were used as input for analysis of GAPDH protein level. The remaining lysate was diluted in buffer A and incubated with 10 μl of pre-blocked (1% BSA in PBS for 1 hour at 4°C) anti-Flag M2 affinity gel beads (Sigma) overnight at 4°C. The beads were then washed 3 times with 150 mM KCl base buffer (20 mM Tris (pH 8.0), 10% glycerol, 5 mM $MgCl_2$, 0.1% Tween-20, 150 mM KCl, and protease inhibitors (Roche Applied Science)) and eluted with sample buffer. Input and IP samples were resolved on a SDS-PAGE gel and proteins were immunoblotted as described above.

Chromatin Immunoprecipitation (ChIP)

For ChIP analysis, formaldehyde was added directly to cell culture media to a final concentration of 1%. Fixation was completed after incubation for 10 minutes at room temperature and stopped by adding glycine to a final concentration of 0.125 M. Cells were scraped, collected, centrifuged and swelled in cell lysis buffer (5 mM PIPES (pH 8.0), 85 mM KCl, 1% NP-40, 0.1 mM PMSF, and 1 μg/ml leupeptin, aprotinin and Pepstatin A). After 1 hour incubation on ice, nuclei were collected by centrifugation at 4,000 rpm for 10 minutes at 4°C, resuspended in nuclei lysis buffer (50 mM Tris-HCl (pH 8.0), 1% SDS, 10 mM EDTA, 0.1 mM PMSF, and 1 μg/ml leupeptin, aprotinin and Pepstatin A) and incubated on ice for 10 minutes. Samples were sonicated on ice to an average DNA length of 500 bp and centrifuged at 14,000 rpm. The chromatin solution

was pre-cleared with Staph A cells (pre-blocked with 1 mg/ml sheared herring sperm DNA and 1 mg/ml BSA overnight at 4°C) for 15 minutes at 4°C. Chromatin from about 10^7 cells was incubated with 5 μg of normal rabbit IgG (NRIgG, Millipore), an affinity-purified rabbit polyclonal antibody Brd4NA (recognizes BRD4 aa 156-284) (207), or a Cdk9 antibody (Santa Cruz, sc-484). After rotating at 4°C overnight, chromatin and antibody complexes were immunoprecipitated by mixing with pre-blocked Staph A cells at 4°C for 15 minutes. Immunoprecipitates were centrifuged at 14,000 rpm for 3 minutes. The supernatant from the NRIgG antibody sample was used as total chromatin input. Staph A immuno-complexes were washed twice with dialysis buffer (50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.2% Sarkosyl, 0.1 mM PMSF) and four times with ChIP wash buffer (100 mM Tris, pH 9.0, 500 mM LiCl, 1% NP-40, 1% deoxycholic acid, 0.1 mM PMSF). Immuno-complexes were eluted from the Staph A cells using elution buffer (50 mM $NaHCO_3$, 1% SDS). Crosslinking was reversed by adding NaCl to the eluted supernatants to a final concentration of 300 mM and incubating at 67°C overnight. RNA was removed by incubation with RNase A at 37°C for 30-60 minutes. DNA samples were purified using a PCR purification kit (Qiagen) and eluted in 50 μl elution buffer. Real-time PCR was performed as described above using primers listed in Table 4.1. Two micro liters of immunoprecipitated DNA or total input chromatin diluted 1:50 were used as templates for the PCR reactions.

Whole Genomic Extraction

Cells were treated with JQ1(-) or JQ1(+) for 15 hours prior to harvest. Trypsinized cells were washed with PBS and pelleted by centrifuging at 3000 rpm for 5 minutes at 4°C. The pellets were resuspended in lysis buffer (400 mM NaCl, 10 mM TrisCl (pH 7.4), 10 mM EDTA (pH 7.0), 0.2% SDS) and 45 μg of RNaseA (Roche) was added. The lysates were then passed through a 22-gauge needle ten times and incubated at 37°C for 30 minutes. Protein was then digested with 100 μg proteinase K (Roche) overnight at 37°C. A phenol/chloroform extraction was then performed by adding 500 μl phenol/chloroform/isoamyl alcohol (25:24:1) (Sigma) to the samples then mixing for 5 minutes at room temperature and microcentrifuging at 14,000 rpm for 5 minutes. The aqueous supernatant was recovered and mixed with chloroform/isoamyl alcohol (24:1) (Sigma) for 5 minutes at room temperature. After microcentrifugation at 14,000 rpm for 5 minutes, the DNA in the aqueous supernatant was isolated and precipitated by adding 2.5 volumes of isopropanol and 1/10 volume of 3M sodium acetate and incubating overnight at -20°C. The DNA was pelleted by microcentrifuging at 14,000 rpm for 20 minutes at 4°C. The pellet was washed with 70% ethanol, microcentrifuged at 14,000 rpm at room temperature, dried, and resuspended in water. Viral episome levels were measured by qRT-PCR as described above using primers listed in Table 4.1. The episomal DNA level was normalized to the GAPDH gene.

Luciferase transactivation assay

C33A cells were transfected using the calcium phosphate method and lysed in Reporter Lysis Buffer (Promega) at 48 hours post transfection. Luciferase activities were

measured according to the manufacturer's instructions (Luciferase Assay System; Promega). In Figure 4.1, the luciferase activity was normalized to β-galactosidase activity measured using β-galactosidase Enzyme Assay System (Promega). For Figures 4.3 and 4.4, luciferase activities calculated from three independent experiments were presented and Western blotting analyses were performed to ensure that these luciferase activities resulted from similar amount of E2 proteins.

Southern blot analysis and *in vitro* replication assay

Episomal DNA for Southern blot analysis was isolated by the Hirt extraction method. Less than 1 million cells were re-suspended in 250 μl Buffer I (50 mM Tris and 10 mM EDTA, supplemented with 50 μg/ml RNase A, pH 7.5), and frozen at -80°C for more than 20 minutes. Cells were mixed with 250 μl 1.2% SDS and incubated for 5 minutes at room temperature. After incubation in 350 μl Buffer III (3 M CsCl, 1 M KAc, and 0.67 M HAc) for 10 minutes at room temperature, the samples were centrifuged at 16,000 × g for 10 minutes. Supernatant was loaded onto a miniprep column (Qiagen) and washed twice with 10 mM Tris, 50 μM EDTA, 80 mM KAc, and 60% ethanol, pH 7.5. DNA was eluted with water or elution buffer (Qiagen). To linearize DNA and remove transfected DNA, 600-800 ng DNA from Hirt extraction was digested with XhoI/DpnI for 2 hours at 37°C. To detect transfected DNA, episomal DNA was extracted at 6 hours post transfection and digested with XhoI. Probes targeting the HPV16 E1 gene were labeled with [α-^{32}P] dCTP (3000 Ci/mmol) using Prime-It II random primer labeling kit

(Agilent Technologies) following the manufacturer's instructions. The hybridization was performed at 65°C overnight.

For the *in vitro* replication assay, C33A cells were transfected with siRNA targeting Brd4 (siBrd4) or non-target siRNA. At 36 hours post transfection, cells were re-transfected with HA-16E1 and pOZN-16E2 plasmids. After 48 hours, cells were swollen in 20 mM HEPES, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF, pH8.0, and lysed with a Dounce homogenizer on ice. Supernatant was frozen at -80°C overnight. The *in vitro* replication reaction mixture contained: 40 mM creatine phosphate (pH 7.7, di-Tris salt), 7 mM $MgCl_2$, 100 μg/ml creatine kinase, 0.5 mM DTT, 3.3 μM [α-^{32}P] dCTP (3000 Ci/mmol), 200 ng re-ligated HPV16 genome, and 150-200 μg cellular extracts, supplemented with 4 mM ATP, 200 μM each of CTP, UTP, GTP, and 80 μM each of dATP, dTTP, dGTP in a 50 μl volume. Mixtures were incubated for 2 hours at 37°C. DNA was extracted with phenol, precipitated with ethanol, and linearized with BamHI. Purified recombinant Brd4 was added into the Brd4 rescue reactions. The band intensities of radiographs were analyzed using a Phosphorimager (Typhoon 9400; GE Healthcare).

Flow cytometry

Cells were detached from plates with 0.05% Trypsin-EDTA (GIBCO), re-suspended/fixed with 70% ethanol, and stained with 25 μg/ml propidium iodide. RNA was digested with 100 μg/ml RNase A. Flow cytometry results were analyzed using FlowJo software.

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