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A multimeric complex of adenoviral small e1a and host cell proteins condenses chromatin

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular Biology

By

Sarah Anne Johnson

2014

ABSTRACT OF THE DISSERTATION

A multimeric complex of adenoviral small e1a and host cell proteins condenses chromatin

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Sarah Anne Johnson

Doctor of Philosophy in Molecular Biology

University of California, Los Angeles 2014

Professor Arnold J. Berk, Chair

Classically, transcriptional regulation is discussed as the recruitment and assembly of RNA Polymerase II (Pol II) and its pre-initiation complex (PIC) on promoter regions of DNA. However, upstream of this event, transcriptional regulation also requires the regulation of chromatin structure between repressive, condensed forms of chromatin and active, decondensed chromatin to allow transcription of cellular genes. Chromatin structure is regulated via modifications on the histone tails by histone modifiers, such as the lysine acetyltransferase p300/CBP(P300). The actions of repressive and activating transcriptional regulators binding to chromatin result in the highly regulated expression of genes in cells.

Adenovirus infects the terminally differentiated cells of the human respiratory epithelium. In order to create a conducive environment for the viral life cycle, the first viral protein expressed, e1a interacts with RB and p300 proteins to reprogram the host cell's epigenetic landscape. This dissertation shows that in order to repress some host cell genes, small e1a rearranges host cell chromatin into higher order condensed chromatin. Furthermore, the condensation of chromatin by small e1a requires the RB and P300 interaction, the maintenance

of RB in its repressive state and e1a interactions with host cell chromatin modifiers. This work also hopes to refine the e1a-P300 interaction in e1a-induced chromatin condensation, and through those studies illuminate understanding of P300 and its protein domains.

Overall, further studies are required, but this work furthers understanding of the precise impact of adenoviral small e1a interactions with host cell proteins. More broadly, these studies further knowledge not only in how adenovirus oncogenic transformations, but overall cellular functions, that when mis-regulated, lead to cancer.

The dissertation of Sarah Anne Johnson is approved.

Michael F. Carey

Samson A. Chow

Arnold J. Berk, Committee Chair

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2014

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To my younger brother, Daniel. Our friendly sibling rivalry will never cease to motivate.

To my advisor, Arnie. His love of science, and the excitement he experiences in the pursuit of scientific knowledge is inspirational. Without his enthusiasm for research, failed experiments would have been a much bigger pill to swallow.

To my labmates throughout the years. Late nights and weekends in lab were always more fun with you all around.

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"Adenoviral Small e1a/RB/p300 interaction condenses chromatin," **Johnson, S.***, and Berk, A.; *Cold Spring Harbor: Mechanisms of Eukaryotic Transcription*, Cold Spring Harbor, NJ; September, 2011; poster presentation

"Using fluorescence microscopy to analyze Mediator and Pol II interactions," **Johnson, S***, Pennella, M., and Berk, A.; *MBI Departmental Retreat*, UCLA Conference Center, Lake Arrowhead, CA, October 2010; poster presentation

"Using fluorescence microscopy to analyze Mediator and Pol II interactions," **Johnson, S***, Pennella, M., and Berk, A.; *MBI Student Seminar Series*, UCLA, February 2010; oral presentation

 "Microarray studies of salivary gland morphogenesis." **Johnson, S*.**, Musselmann, K., and Yamada, K.; *Annual Post baccalaureate Poster Day,* National Institutes of Health, Bethesda, MD, May 2008.poster presentation

CHAPTER 1

Introduction

Adenovirus (Ad) is one of the most abundant viruses to cause illness in humans. In humans, there are ~57 adenovirus types divided between seven different species, A-D (Fields et al, 2007). The most widely studied Ad serotypes are 2 and 5 found within the C-species of adenoviruses (Walsh et al 2011). Named after the tissue source of its original discovery, adenoid tissue, the Ad family of viruses encompasses non-enveloped, double-stranded DNA tumor viruses that cause mild infections in the upper or lower respiratory tract, gastrointestinal (GI) tract, or conjunctiva in humans.

Decades of study of Ad has greatly enhanced our knowledge of gene regulatory mechanisms, facilitated the discovery of RNA splicing and general protein machinery in mammalian cells. Adenoviruses transform rodent cells in culture (Pope and Rowe, 1964; Freeman et al., 1967; McAllister et al., 1969a,b), form tumors in immunocompromised animals (Trentin et al., 1962), and are able to drive human primary cells into S-phase through misregulation of the cell cycle. The oncogenic nature of this DNA tumor virus' mechanisms during the course of infection serves as an excellent model for the progression of healthy cells into cancerous cells by misregulation in cell division (cell cycle) genes, and drastic changes to gene expression patterns.

Adenovirus structure, organization, and function

A single, adenovirus particle is a non-enveloped icosahedron-shaped protein shell measuring 70-100nm in diameter (Shenk et al, 1996; Russell, 2009) called the capsid, with the 36 KB double-stranded DNA viral genome packaged inside (FIG 1.1A). The adenoviral genome is divided into two categories of functional transcription units that contain all of the protein coding information the virus needs to propagate itself. The expression of these two functional

units is divided between the early and late-phases of infection during the adenovirus life cycle (Shenk et al, 1996, Zhao et al, 2012), (FIG 1.1B)

In the early phase of viral reproductive cycle, the early expressing units (E1A, E1B, E2A, E2B, E3, and E4) are transcribed into proteins most well known to regulate viral transcription, replicate viral DNA, and suppress the host response to infection (Nevins, 1981; Shenk et al, 1996). The late expressing units (L1-L5) are transcribed in the late-phase of the viral reproductive cycle, and transcribe into mRNAs for the proteins of the viral capsid and proteins involved in the assembly of the viral capsid as the virus comes to the end of its reproductive cycle. Four smaller transcripts, pIX, IVa, and VA RNA's I and II, are also produced. The 103 BP inverted terminal repeats (ITRs) are located at the termini of the genome and are involved in viral DNA replication, and the packaging signal (ψ) located from nucleotides 190 to 380 at the left end is involved in packaging of the genome into virion capsids (Shenk et al, 1996)(FIG 1.1B).

Adenoviral early region 1A protein

Adenoviruses generally infect differentiated, quiescent cells of the human respiratory tract. For a successful viral replication, the virus requires the infected cell to produce the proteins required for nucleic acid and polypeptide synthesis. In short, these infected quiescent cells require growth stimulation. After initial infection, and the localization of the Ad genome to the infected cell's nucleus, the very first protein to be expressed by the virus is the early region 1A (E1A). The virus uses the E1A gene product to optimize the cellular environment in which to express the remainder of the viral genes (Berk et al, 1979; Jones and Shenk, 1979).

Early in adenovirus studies, the E1A protein was found to be the minimum adenoviral protein required to immortalize rodent cells (Houweling et al., 1980), as well as induce limited

cell-cycle progression. However, unlike the normal epithelial-mesenchymal transition that occurs in carcinoma progression, E1A induces a mesenchymal-epithelial transition in the adenovirus infected human cells (Frisch, 1994), suggesting that the functional implications of the E1A interactions with targeted host cell proteins are highly cell context specific with the overall function to reprogram each unique cell phenotype in the virus' favor.

The adenovirus *E1A* gene encodes for two major protein isoforms of 289 and 243 residues (FIG 1.1 C). The two major isoforms are translated from two different splice variants of the same transcriptional unit (Harlow et al, 1985). Throughout different adenovirus serotypes, the amino acid sequence of E1A consists of three conserved regions (CR), CR1, CR2, and CR3. (Moran and Mathews, 1987; van Ormondt et al, 1980). CR1 and CR2 are encoded from Exon 1 of the viral transcript, while CR3 is found only in the splice variant that translates into the larger 243 amino acid isoform of E1A. Also found within some adenovirus serotypes is a fourth region, encoded from Exon 2, the CR4 region.

The 243 amino acid protein isoform of E1A is commonly referred to as small e1a (hereafter referred to as "e1a"), and contains the highly conserved CR1 and CR2 regions of the protein, as well as the less conserved CR4 (FIG 1.1C). The larger 289 amino acid protein, E1A is composed of all four major regions CR1-CR4 (FIG 1.1 C). CR3 of the large E1A is responsible for the transcriptional activation of the other viral genes (Lee et al, 1991), and is known to interact with MED 23 of the Mediator of Transcription complex (Boyer et al., 1999).

E1A is not a DNA binding protein, and does not bind DNA directly. (Chatterjee et al, 1988; Ferguson et al 1985). Therefore, the functions of both e1a and E1A in manipulation of the cellular environment depend entirely on interactions of these conserved regions with host cell proteins (Ferguson et al, 1985) (FIG 1.1 C, FIG 1.2 A). Each conserved region is a distinct

functional module with its own independent connection to networks of specific cellular functions. Interestingly, a vast majority of e1a-targeted cellular proteins are located in the nucleus, but half of the E1A molecules are also present in the cytoplasm of the cell(Rowe et al, 1983), and very little is known about the interactions of E1A in the cell cytoplasm except for a few components of the proteasomal degradation complex (Turnell et al 2000). It is through these specific interactions through its conserved regions that E1A is a viral molecular hub through which the virus reprograms the infected host cell (Harlow et al, 1986; Yee et al, 1985), and further understanding of these interactions enhances overall understanding of oncogenic transformation of healthy cells.

e1a regulation of the cell cycle through CR1 and CR2 interactions

In a normal cell, the E2F transcription factors regulate genes required to enter S-phase (Dick and Rubin, 2013). In G_1, G_0 end-differentiated (Chong et al., 2009) and senescent cells (Chicas et al., 2012), the retinoblastoma (RB) family of proteins bind to E2F activation domains (ADs) (Lee et al., 2002; Xiao et al., 2003). The RB-E2F interaction blocks the E2F ADs and recruits repressive chromatin remodelers (such as histone deacetylases) to repress E2F-regulated genes, (FIG 1.2 B), repressing histone methylases and demethylases (Chicas et al., 2012; Dick and Rubin, 2013). In proliferating cells during S-phase, RB dissociates from E2F's after phosphorylation by cyclin D-CDK4/6 and cyclin E/A-CDK2. Hyperphosphorylation of the RB protein induced conformational changes that result in the disruption of the RB-E2F interactions (FIG 1.2 B) (Rubin et al., 2005). The removal of the hyper-phosophorylated RB protein also causes the dissociation of repressing chromatin modifying complexes from RB (Dick and Rubin, 2013). The de-repression of E2F activation function by RB-phosphorylation results in the expression of hundreds of genes required to enter S-phase (FIG 1.2 B).

Early in infection, e1a binds the RB family of cell-cycle repressor proteins. Specifically, the CR1 and CR2 of e1a bind the RB family of cell-cycle repressor proteins. CR1 and CR2 of e1a are ~30 amino acids apart and work together synergistically to quickly displace the E2Fs from RB. The interaction of the LxCxE motif of CR2 with the B-pocket domain of RB (FIG 1.2 A) brings CR1 in close proximity to the E2F binding site in between the two pocket domains of RB allowing e1a to out compete and disrupt the E2F-RB interaction discussed earlier (FIG 1.2 A, FIG 1.2 B). An e1a mutant with three point mutations in CR1 and deletions of e1a's CR2 (hereafter referred to as RBb-) prevents an interaction with all members of the RB-family of proteins (Ferrari et al, 2014).

It is well known that these early interactions are required to drive the infected cells past the G1/S cell cycle checkpoint and into the S-phase of the cell cycle (Ghosh and Harter, 2003; Howe and Bayley, 1992). The e1a CR1 and CR2 interactions with unphosphorylated RB displace RB from E2Fs, thus activating E2F-regulated cell cycle genes. (Bagchi et al., 1990; Fattaey et al., 1993; Ikeda and Nevins, 1993;)Dyson et al., 1992) (FIG 1.2). Once e1a displaces RB from E2Fs, however, it seems the e1a maintains RB in a repressive state and utilized in repression of host cell genes. The mechanism of e1a-RB repressive complex is discussed further in Chapter 2

e1a CR1 and N-terminal interactions

During an adenovirus infection, e1a's N-terminus and CR1 bind to numerous host cell proteins characterized as lysine acetyltransferases (KATs) or otherwise involved in the regulation of host cell chromatin structure. Specifically, e1a interacts with CBP/P300, PCAF, GCN5, P400, and TRAAP (Lundblad et al, 1995; Yang et al, 1996; Arany et al., 1995; Fuchs et

al., 2001; and Lang et al., 2003). e1a's interactions with PCAF, GCN5, and TRAAP are well characterized, but the functional relevance of these interactions is unclear

P400 is a core component of the SWI2/SNF2 complex. e1a mutants defective in binding P400 are not able to oncogenically transform mammalian cells (Fuchs et al 2001). In addition to the requirement for oncogenic transformation, as with PCAF, GCN5, and TRAAP, there is not much known of the functional relevance of the e1a-P400 interaction. However, the interaction of e1a with CBP/P300 is slightly better understood.

CREB binding protein (CBP) and P300 are well-characterized coactivators. P300 was originally isolated as a 300 kilodalton sized protein through its interaction with e1a's CR1 and N-terminus (FIG 1.1C), (Eckner et al., 1994) and later discovered to be highly homologous and almost interchangeable in function with CBP (Chrivia et al., 1993; Kwok et al. 1994). P300 is well known to function as histone acetyltransferases (HATs) (Bannister et al. 1996), a class of proteins that add acetyl groups to the amino acid lysine residues of the histone tails in order to reduce the tight interaction between histones and DNA in the nucleosome (Hodawawedekar and Marmostein, 2007). Specifically, P300 acetylates lysine 18 of histone 3 (H3K18ac) and lysine 27 of histone 3 (H3K27ac) in the histone octamer (Jin et al, 2011). P300 also acetylates the lysines of non-histone proteins such as RB, and E2Fs (Chan et al, 2001; Martinez-Balbas et al, 2000), indicating that P300's catalytic activity is perhaps indispensable for adenovirus mediated cellular transformation.

e1a binds P300 through its N-terminal region (residues 1–25) and residues within the CR1(Whyte et al., 1989; Stein et al., 1990; Wang et al., 1993; Barbeau et al., 1994). Primarily, e1a binds to the transcriptional adaptor zinc finger-2 (TAZ2) (or CH3) domain (FIG 1.2C) (Arany. Et al., 1995; Eckner, et al., 1994) although interactions with other domains have been

reported (Ferreon et al., 2009). The regions of CR1 that bind P300 and RB do not overlap (Wang et al., 1993) which is probably why simultaneous interaction of both P300 and RB is necessary for maximal biological activity of e1a (Wang et al., 1995). A single point mutant in the second arginine of P300, and five point mutations within CR1 of e1a to alanine prevent an interaction of e1a in co-immunoprecipitation experiments (Ferrari et al., 2014). This mutant, hereafter referred to as P300b-, still interacts with the RB pocket family of proteins, and is unable to induce S-phase (Ferrari et al., 2014). Furthermore, many cancers are found to have mutated P300. Further characterization and understanding of the e1a-P300 interaction will lead to further understanding of the importance of P300's catalytic activity in general cellular functions as well as oncogenic transformation.

e1a CR4 and c-terminal interactions

While many of e1a's N-terminal, CR1, and CR2 interactions are well known to induce cell proliferation and oncogenic transformation, little is actually known about the function of the C-terminal interactions of e1a with the host cell proteins CtBP, FoxK1/2, and Dyrk1A/1B (Boyd et al, 1993; Schaeper et al., 1995; Komorek et al, 2010). In fact, early studies of e1a C-terminal mutants suggested a paradoxical suppression of e1a-induced transformation by e1a's C-terminal interactions (Yousef et al, 2012).

Perhaps, the best-characterized interaction of e1a's C-terminus is with C-terminal binding protein (CtBP)., CtBP was originally discovered and named because of its association with e1a (Boyd, et al. 1993). Most CtBP proteins are involved in the regulation of transcription, and largely function as transcriptional co-repressors in the regulation of genes involved in cellular growth, oncogenic transformation, and apoptosis (Komorek et al., 2010). Mammalian CtBP proteins are known to form complexes with human polycomb protein (Sewalt et al, 1999), or

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through histone deacetylase interactions (HDAC) (Subramanian and Chinnadurai, 2003). CtBP proteins interact with a PXDLS amino acid motif in their target proteins,. In fact, mutations of the PXDLS motif in the C-terminus of e1a (FIG 1.1 C) (Zhao et al., 2007), disrupt CtBP binding to e1a (Pelka et al, 2008).

. In the context of the adenoviral infection, it is known that acetylation of lysines 239 of small e1a prevents an interaction with CtBP (Zhang et al., 2000). Additionally, an e1a-CtBP interaction promotes the cytoplasmic localization of CtBP over a nuclear one (Madison et al., 2002). However, questions still remain about the dynamics of e1a's interaction with CtBP and P300, and the functional consequences these interactions have in the viral reprogramming of the infected host cell.

Epigenetic reprogramming by e1a

Epigenetics describes the changes in gene expression in eukaryotic organisms that is not due to alterations in the DNA sequences, but to other aspects of the eukaryotic genome. Epigenetic abnormalities exist in virtually all human cancers, and are one of the drivers in tumor development (Tsai and Baylin, 2011).

In the eukaryotic cell, DNA is organized and packaged into chromatin within the nucleus of the cell. The basic unit of chromatin is a nucleosome composed of DNA and histone proteins. Histone proteins are highly conserved in the eukaryotic kingdom and are vital to the organization and structure of the genomic information encoded in the DNA (Van Holde, 1988; Wolffe, 1999). One nucleosome is composed of 147 base pairs of DNA wrapped 1.7 times around an octamer of histone proteins (two copies of the H2A, H2B, H3, and H4 histone proteins) in a left-handed super helix (Thomas & Kornberg, 1975), (FIG 1.3A). The nucleosomes are then thought to fold

into highly ordered 30nm chromatin fibers that compact meters of DNA into the nucleus of each individual cell (Olins & Olins, 1974, 2003; Woodcock, 2005).

The N-terminal tails of the histones are exposed and external to the core of the nucleosome (FIG 1.3A). In the early 1960's it was hypothesized that specific chemical modifications on individual amino acid residues on the histone tails affect chromatin structure and function (Allfrey and Mirsky, 1964). We know now that not only are histone tails chemically modified, but that specific classes of enzymes acetylate, phosphorylate, methylate, or ubiquitinate these specific residues of the N-termini of the histone tails (Fischle et al, 2005; Smith and Peterson, 2005), (FIG 1.3 B). The modifications of histone tails are able to regulate the higher order structure of chromatin (Bednar et al., 1998). The more prominent enzymes responsible for these modifications are known as histone acetyltransferases (HATs), histone methyltransferases (HMTs), and histone deacetylases (HDACs).

Acetylation and deacetylation of lysine residues in the N-terminal tails play fundamental roles in chromatin structure and function. During adenovirus infection, the e1a isoform causes a global decrease in H3K18ac and H3K27ac levels in the infected cells, but does not affect other amino acid modifications of H3 (Horwitz et al., 2008, Ferrari et al 2012). This global hypoacetylation by the virus is dependent on the e1a CR1 and N-terminal interactions with CBP/P300 (Horwitz et al., 2008, Ferrari et al 2012). Mutants of e1a that are unable to bind CBP/P300 are also defective in cell transformation (Deng et al., 2005), indicating again that e1a's interactions with P300 are vital to its oncogenic ability.

Conclusion

Adenovirus has taught us much about transcriptional regulation and cell cycle control. The adenoviral e1a protein is a molecular hub to coordinate the actions of the cell's own

molecular networks through specific interactions with host cell proteins through its conserved regions.

Earlier work purports that e1a binding P300 inhibits its HAT activity (Hamamori et al, 1999). At the simplest level, a loss of P300 HAT activity may explain the global hypoacetylation induced by e1a in infected cells (Ferrari et al, 2012; Horwitz et al., 2008). Furthermore, as mentioned earlier, once e1a removes RB from E2F-regulated promoters, little is understand about the downstream effects of the e1a-RB interaction. However, it is also well known that e1a must interact simultaneously with both P300 and RB to transform cells (Wang et al., 1995), and that this interaction promotes acetylation of RB by P300, and preventing a phosphorylated RB maintaining RB in a repressive state (Chan et al, 2001) suggesting that a P300-e1a-RB repressive interaction exists downstream of the removal of RB from E2F-regulated genes.

Do the P300-e1a-RB interactions influence gene expression in infected cells? In the Berk laboratory, RNA-seq studies of cells expressing e1a defective for binding of either P300 or RB revealed some host cell genes whose activation or repression requires simultaneous interactions between e1a and P300 and RB (Ferrari et al, 2014). Chromatin-immunoprecipitation (ChIP) experiments show increased association of P300 and RB throughout the gene body of repressed genes, and this association requires interactions with P300 and RB (Ferrari et al, 2008; 2014). The ChIP analyses also show increased H3 cross-linking at repressed promoters that are highly associated with P300 and RB (Ferrari et al, 2008). Therefore, after initial interactions with RB and P300, we modeled that e1a forms a repressive complex to repress host cell genes 24 hours after adenoviral infection (FIG 1.4).

However, there are many questions that still remain about this model of P300-e1a-RB induced repression, specifically about the chromatin dynamics of the infected cell. Does

adenoviral small e1a condense chromatin? If so, are interactions with RB, P300, or other host cell proteins required for this interaction? To answer these questions, I used a direct to determine chromatin condensation is induced by e1a. Furthermore, I show specifically the e1a-P300, P300-e1a-RB, and other host cell protein interactions are required for e1a-induced chromatin condensation.

The results of this dissertation refine our understanding of the oncogenic transformation of e1a through its interactions with the host cell proteins RB and P300.
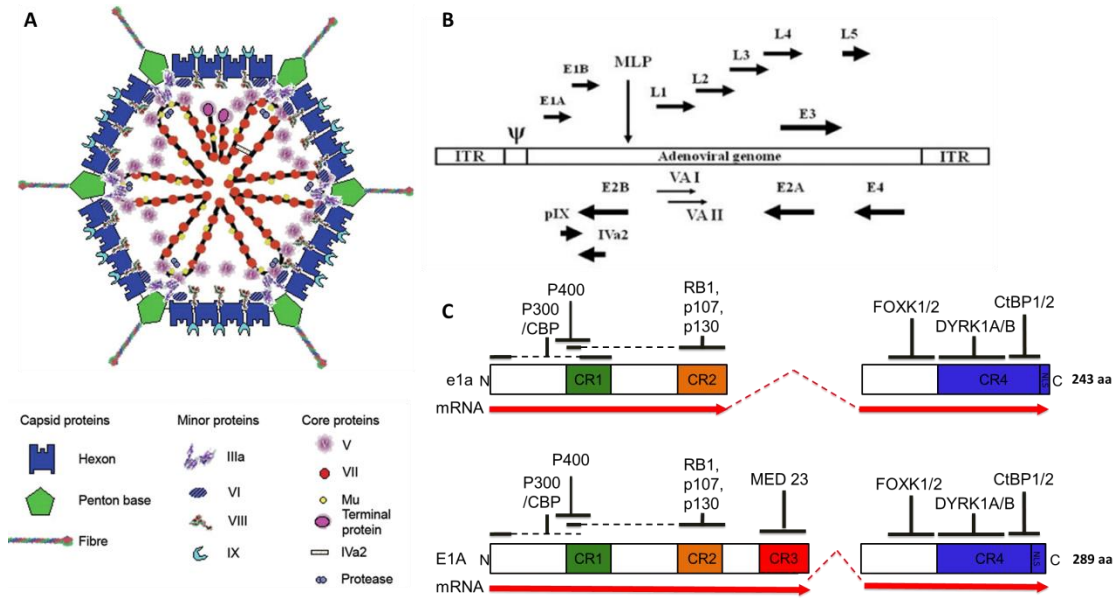
Figure 1.1 Adenovirus structure, genome, and proteins

    A. Structure of adenovirus. A schematic depiction of the structure based on cryo-electron microscopy and crystallography. The locations of the capsid and minor components are reasonably well defined and are not to scale. The disposition of the core proteins and the virus DNA is largely conjectural. Adapted from Russell, 2009.

    B. Transcription map of human adenovirus serotype 5. "➔" indicate direction of transcription, Ψ indicates the viral packaging signal. The viral genome is flanked by inverted terminal repeats (ITR).

    C. Major E1A conserved region interactions with host cell proteins. "e1a" indicates the 243 amino acid isoform, while "E1A" indicates the larger 289 amino acid isoform. Domains are not to scale.
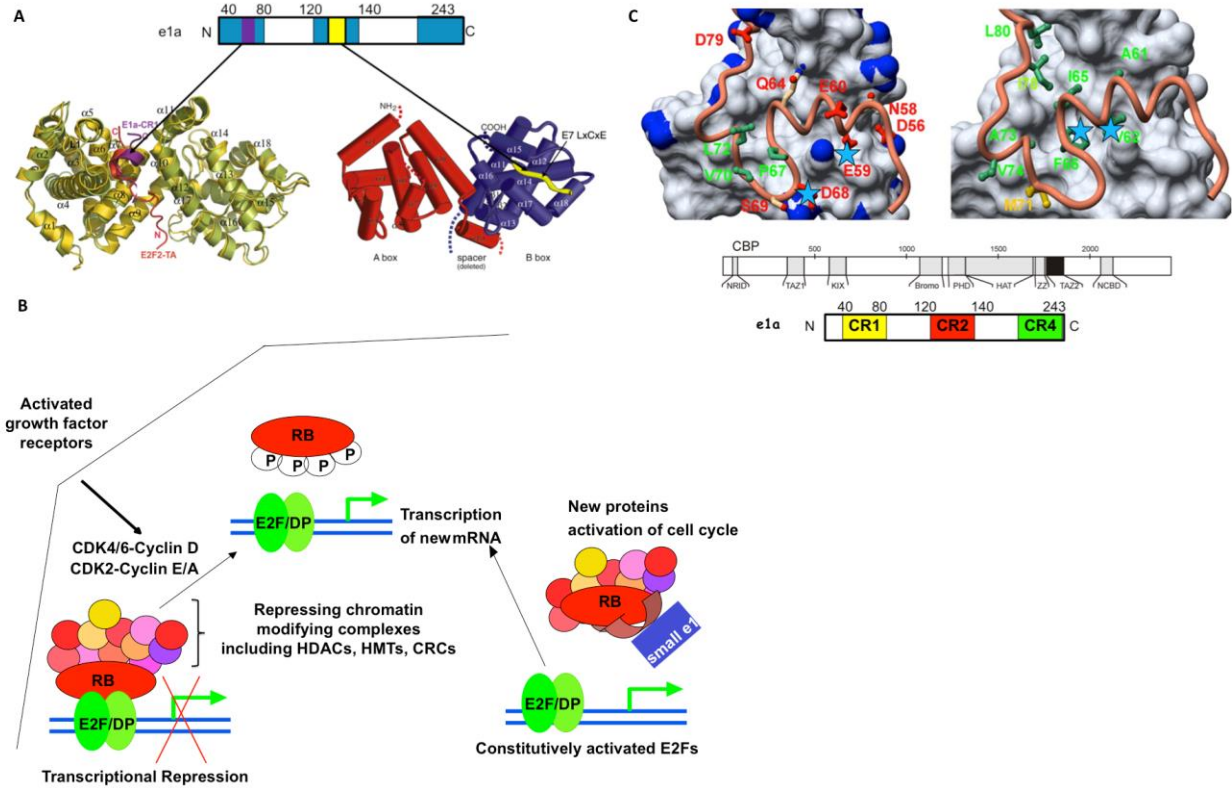
Figure 1.2 e1a interactions with RB and P300

A. *Left*: e1a CR1(residues 37-49) in purple co-crystallized with the pocket domains of RB(380-387) in gold. Superimposed in red is the alpha helix of E2F2. E2F2 cannot interact with RB when e1a interacts with the RB pocket domains. *Right*: in yellow is the LxCxE motif of HPV E7, a motif conserved in CR2 of e1a. Point mutations in the three residues in CR1 of e1a, in addition to a deletion of CR2 of e1a eliminate RB interaction (Structures adapted from Liu and Marmostein, 2007; Lee et al, 1998).

B. In normal cell-cycle progression, phosphorylation of RB (red sphere) by CDK2-cyclinE/D releases chromatin repressive chromatin remodeling complexes (colored spheres in lower left) from E2Fs (green spheres in upper middle). During adenovirus infection, e1a displaces hypophosphorylated RB from E2Fs by e1a (lower right), maintains the association of repressive complex associations with RB so that when the RB is transferred to other genes, it continues to be associated with the repressive complexes a repressed these genes.

C. *Left:* structure of the electrostatic interactions between P300's TAZ2domain and e1a (Blue star is the nitrogen atoms of TAZ2). *Right:* structure of e1a's hydrophobic side chains interacting with TAZ2, Residues 53-91 of CR1 of e1a in coral. (Structures adapted from Ferreon et al, 2008)
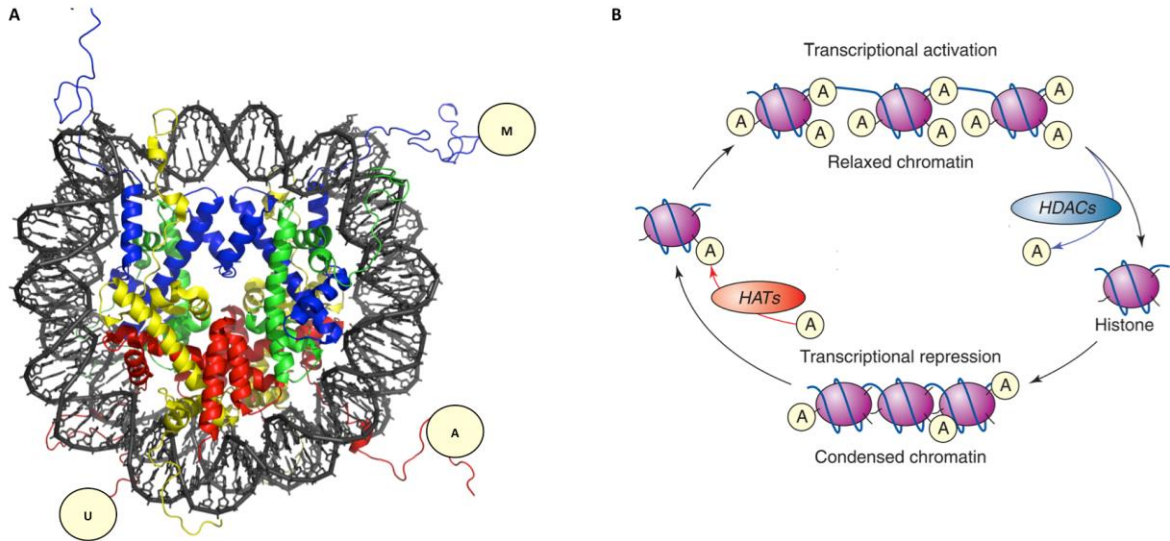
Figure 1.3 Histone tail modification within the nucleosome

A. The 7-Å-crystal structure of nucleosome adapted from Richmond et al, 1984. Each nucleosome comprises an octamer of histone molecules, which consists of an H3–H4 tetramer and two H2A–H2B dimers(Red, Blue, Green, and Yellow). DNA(black) wraps around the histone core. The amino (N) termini of histones project out of the nucleosome core and interact with DNA. These histone tails can be epigenetically modified, and function as signal integration platforms.

B. Histone acetylation of lysine residues on the histone tails are regulated by acetylation and deacetylation mechanisms catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. On the other hand, chromatin with a tight conformation is transcriptionally inactive. Adapted from Chuang et al, 2009
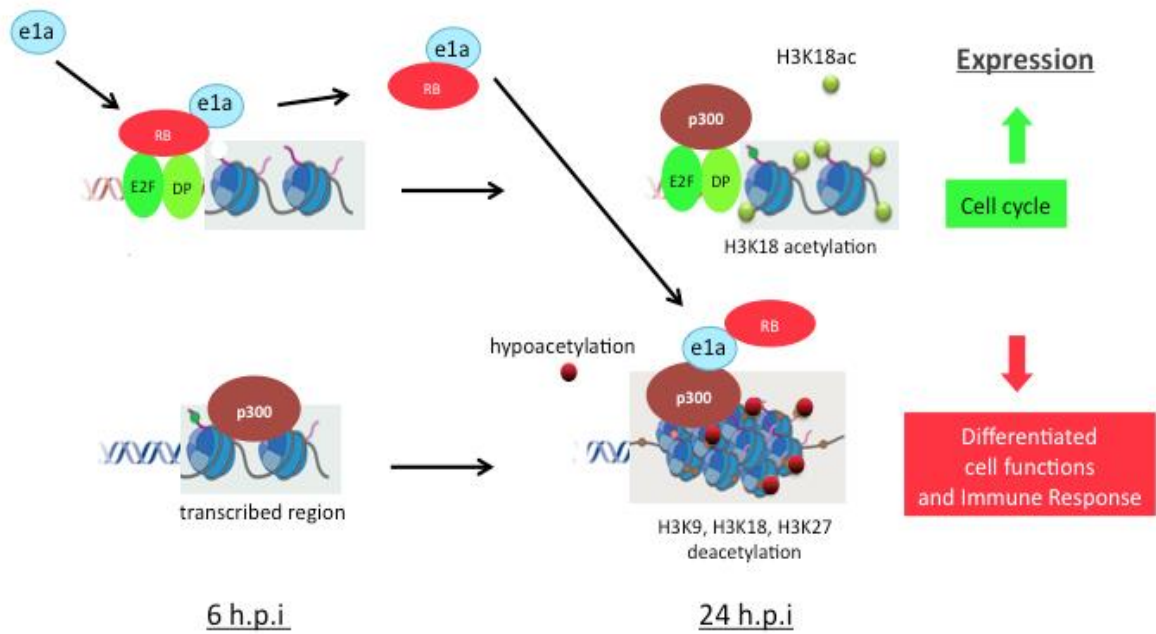
Figure 1.4 Repression of host cell genes by P300-e1a-RB e1a first binds and dissociates RB-family proteins from the E2Fs on the promoters of cell cycle genes. e1a then delivers RB to genes highly associated with P300 such as immune response and differentiation specific genes.

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CHAPTER 2

Adenoviral small e1a condenses chromatin

INTRODUCTION

As discussed earlier, recent work in the Berk laboratory discovered an unexpected mechanism targeting RB and P300 to the gene bodies of host cell genes repressed during infection. In cells infected with an adenovirus expressing only the small e1a isoform, we see a large class of genes whose repression requires an e1a-P300 interaction. These same repressed genes have a higher average P300 association within their "gene body," when compared to genes unchanged by e1a infection, and whose initial expression was equivalent to this P300-associated repressed class of genes. These P300-associated repressed genes also show e1a induced RB-association, H3 hypoacetylation, and reduced pol2-association within these same gene bodies (Ferrari et al, 2014).

A model of the P300-e1a-RB interaction, as seen in the Ferreon et al 2009 structure, brings RB in close proximity to P300's acetyltransferase (AT) domain (FIG 2.1A). It is also know that e1a induces P300 acetylation of RB at K873 and K874 near the RB C-terminus by targeting P300AT activity to these lysines in a P300-e1a-RB complex (FIG 2.1B), (Chan et al., 2001). This acetylation is hypothesized to prevent RB phosphorylation by cyclin-CDKs and maintain its repressive conformation (Chan et al, 2001). In fact, 24 hours post-infection (h.p.i.), despite S-phase entry and high CDK2 or cyclin E activity in infected cells (Ferrari et al, 2014) known RB cyclin-CDK target sites are not phosphorylated (FIG 2.4 F phospho S807/811) (Ferrari et al, 2014). Additionally, earlier ChIP-chip experiments in the Berk laboratory observed that cross-linking of total histone H3 increased in response to e1a at promoter regions of repressed genes with increased P300 and RB association (Ferrari et al., 2008).

The epigenetic landscape of a cell strongly indicates the physical arrangement of chromatin structure. High levels of histone acetylation indicate a higher decondensed chromatin

state while low levels of histone acetylation indicate a higher condensed chromatin state(Allfrey and Mirsky, 1964). Based on the increased enrichment of H3, the reprogramming of the host cell's epigenetic landscape, and the presence of RB and P300 at host cell repressed genes, the work in Chapter 2 asked: 1) Does e1a cause chromatin condensation, 2) What host cell interactions (specifically RB and P300) are important for e1a-induced chromatin condensation, and 3) Is RB acetylation important for e1a-induced chromatin condensation?

MATERIALS AND METHODS

Cell Culture

BJ-Tert fibroblasts were grown to confluency at $37°$ C and 5% CO_2 in 80% Dulbecco's modified Eagles' Medium (DMEM) + 20% 199 Media supplemented with 100U/mL penicillin, 100ug/mL streptomycin, and 10% heat-inactivated fetal calf serum(FCS)(Tissue Culture Biologicals). Cells were then incubated two days and were either mock infected or infected with the indicated Ad5-based vectors.

RRE.B1 cells were cultured in F12 Ham Media supplemented with 100U/mL penicillin, 100ug/mL streptomycin, 10mM methotrexate (to ensure constitutive chromosomal insertion of the DHFR_LacO cDNA construct), and 10% heat-inactivated FCS.

Ad and NLM vectors

Vectors were similar to the Ψ5 vector that uses in vivo Cre-mediated site-specific recombination to substitute the E1A and most of the E1B region, leaving the IX gene intact (Hardy et al., 1997). However, the vectors used (called Ψ5*) have the CMV IE promoter/enhancer deleted. The sequence is wt Ad5 from 1 to 555, including the wt Ad5 E1A enhancer and TSS, followed by CGAAGCT, followed by dl1500 sequence (Montell et al., 1984) from 540 to 1574 in the Ad2 sequence containing the Ad2 E1A AUG at 559, the 12S mRNA 5'

splice site at 973, a 9 BP deletion encompassing the 13S 5' splice site at 1111, the 3' splice site for E1A mRNAs at 1225, and the E1A UAA termination codon at 1540. Ad2 1574 is followed by the SV40 late poly A region (SV40 nt 2643-2557), followed by a *loxP* site, followed by dl309 sequence (Jones and Shenk, 1979) from the Bgl II site at 3327 to the right end of the dl309 genome. The dl312 vector deletes out e1a based upon the same mutations made in Jones and Shenk, 1979.

All e1a constructs were fused to LacI-mCherry after the SV40 NLS sequence, so that the open reading frame was SV40 NLS-e1a-LacI-mCherry. In the e1a-P300b- mutant, the e1a amino acid R2 was mutated to glycine, and e1a amino acids E25, V62, F66, and E68 were mutated to alanine based on the Ferreon et al, 2009 structure. In the e1a RBb- mutant, CR2 deletion from e1a included amino acids 111-127 and e1a amino acids L43, L46, and Y46 were mutated to alanine based on the Liu and Marmostein 2007 structure. In YFP-P300 AT2, the P300 amino acids H1415, E1423, Y1424, L1428, Y1430, and H1434 were mutated to alanines modeled after the Kraus et al, 1999 mutant. In the YFP-RB RR and YFP-QQ, the RB amino acids K873 and K874 were mutated to arginine and glutamine respectively based on the Chan et al, 2001 work. The "P400b-" mutant fused to NLM has the following e1a mutations E25A, E26A, V27A, L28A, D30A, L32A, P35A, and S36A. The "CtBPb-" mutant fused to NLM has e1a mutations P233A, D235A, L236A, and S237A. All deletion and point mutagenesis was performed using the Agilent QuikChange XL kit.

Infections

For western blots of extracts of infected BJ-Tert cells, the multiplicity of infection (moi) was 200 for wt e1a, P300b- vector, RBb- vector, and dl312. These multiplicities of infection

yielded approximately equal amounts of wt and mutant e1a proteins as judged by western

blotting with mAb M73 (FIG 2.3 F).

Chromatin condensation assays

    RRE.B1 cells were transiently transfected with 2ug of each indicated expression vector

following standard protocols with Invitrogen Lipofectamine 2000 transfection reagent. After 48

hours, cells were fixed and DAPI stained as described in Balamotis et al, 2009. Confocal

microscopy of transfected RRE.B1 cells (Verschure et al., 2005) was also performed as

described earlier for A03 cells (Balamotis et al., 2009). Quantification of array areas was

analyzed using ImageJ particle analysis as outlined in FIG A.2 B. P-values for differences

between the distributions of data shown in boxplots was calculated using Kaleidograph® to

perform one-way ANOVA and a Tukey's HSD post-hoc comparison.

HDAC Inhibition assay

    For the western blots, RRE.B1 cells were treated with F12 Ham Media supplemented with

100U/mL penicillin, 100ug/mL streptomycin, 10mM methotrexate media with 10mM

methotrexate made 5% DMSO or 10uM TSA in 5% DMSO for 4 hours at 37°C. Histones were

extracted and assayed via western blotting as in Horwitz et al, 2008.

    RRE.B1 cells were transiently transfected as above. 44 hours after transfection, the media

was removed from RRE.B1 cells and replaced with F12 Ham Media supplemented with

100U/mL penicillin, 100ug/mL streptomycin, 10mM methotrexate made 5% DMSO or 10uM

TSA in 5% DMSO for 4 hours at 37°C. Cells were then fixed and stained as above.

Antibodies

H3K18ac (814) prepared and validated as described (Ferrari et al., 2012); H3K27ac, a gift from Michael Grunstein; RB1 (4H1 mAb, Cell Signaling 9309L); anti-E1A mAb M73 (Harlow et al., 1985); Histone H3 antibody (mAbcam 10799); Phospho-Rb (Ser807/811) (Cell Signaling Antibody #9308)

RESULTS

A complex of P300-e1a-RB and other host cell proteins condenses chromatin

To assay chromatin condensation, I used a microscopic method with specialized CHO cells (RRE.B1) that allows direct observation of chromatin condensation (FIG A.3 A, B). Previous work by Versuchure et al in 2005 utilized the same cells to visually observe chromatin condensation induced by heterochromatin protein 1 alpha (HP1) (FIG A.2 A).

When LacI-mCherry with an N-terminal SV40 nuclear localization signal (NLM) was expressed in RRE.B1 cells, the expressed NLM bound to the ~10,000 lac operators in the *lacO* array, allowing the *lacO* array to be visualized spread through ~5-10% of the nuclear volume (FIG 2.2 A). However, when e1a was fused to the N-terminus of NLS-LacI-mCherry (e1a-NLM), the *lacO* array condensed into a much smaller area (FIG 2.2 A). Overall, the array areas measured across more than six experiments had a mean value in cells expressing e1a-NLM ~1/2 that of the area in cells expressing NLM (FIG 2.2 C). To ensure that condensation by e1a was not simply due to high-level e1a expression, and that the condensation required binding of e1a-NLM to the *lacO* array, expression vectors for NLM and a YFP-e1a fusion were co-expressed into RRE.B1 cells. NLM did not condense the *lacO* array in the presence of e1a-YFP (FIG 2.2 B). Therefore, it is not simply a high-level expression of e1a that induces condensation of the *lacO* array. Furthermore, a large E1A fusion to NLM did not condense the *lacO* array (FIG 2.2

B), suggesting that chromatin condensation is a small e1a specific function, and does not require the CR3 of large E1A (FIG 1.1 C). Chromatin condensation is therefore a small e1a function.

We hypothesized based on the earlier mentioned RNA-seq results, a P300-e1a-RB complex must be tethered to the array to the lac repressor DNA-binding domain for e1a to induce condensation of the *lacO* array.. To elucidate if the RB or P300 interactions with e1a are required for chromatin condensation, various mutants of e1a fused to NLM were expressed in RRE.B1 cells. In multiple replicate experiments, fusion of an e1a RB binding mutant (RBb-) or an e1a P300 binding mutant (P300b-) showed no difference in *lacO* array area compared to the NLM *lacO array area* (FIG 2.2 B, 2.4 A). These results indicate that e1a must simultaneously bind both RB and P300 molecules in order to condense chromatin. Furthermore, co-expression of e1aRBb- and e1aP300b- fusions to NLM did not rescue e1a-induced condensation (FIG 2.3 A).

e1a requires interactions with specific chromatin remodelers to condense chromatin

e1a also interacts with other proteins known to be involved in transcriptional regulation, either at the chromatin remodeling level like P400, or as a co-repressor protein like CtBP. While little is know of the e1a interactions of P400 and CtBP on host cell gene expression, their functions in gene expression implicate they may also be required for e1a-induced chromatin condensation, and therefore we hypothesized that e1a requires an interaction with either P400 or CtBP to induce changes in chromatin structure.

When a P400 binding mutant of e1a based on mutations by Fuchs et al, 2001(P400b-) was expressed in RRE.B1 cells, there was no statistical difference in the *lacO* array area of P400b- when compared to NLM *lacO* array area. (FIG 2.2 B). This indicates that e1a must also interact with the chromatin remodeler P400 in order to condense chromatin. In stark contrast, an

e1a CtBP binding mutant (CtBPb-) based on work by Pelka et al 2008 produced *lacO* array areas statistically similar to the e1a-NLM *lacO* array areas. Therefore, CtBP is not required for e1a- did not affect e1a's ability to condense the LacO array (FIG 2.2 B).

Histone deacetylation is required for chromatin condensation by e1a

As discussed earlier in Chapter 1, chromatin dynamics are tightly controlled by the actions of HATs like P300 and histone deacetylatses (HDACs). There are no known e1a-HDAC interactions, but there are a number of known RB-HDAC interactions that are potentially kept intact in the e1a-RB complex. To assess the requirement for HDACs in e1a-induced chromatin condensation, a general chemical inhibition of HDAC activity was done to determine if e1a requires HDAC activity to condense chromatin. Tricostatin A (TSA), by competing for the HDAC active site, is a well-characterized inhibitor of all but the Sirtuin class of HDACs (Yoshida, et al, 1995). Specifically, TSA inhibits the Class I and II histone deacatylases that interact with RB (Luo et al 1998; Yosida et al, 1995). To assess the effect on overall RRE.B1 H3 acetylation levels, untransfected RRE.B1 cells were treated with TSA for four hours at 37 C. TSA increased overall H3K18 and H3K27 acetylation compared to DMSO treatment alone (FIG 2.3 A).

After confirming effective HDAC inhibition by TSA in RRE.B1 cells, NLM or e1a-NLM was expressed in RRE.B1 cells. Forty-four hours after transfection, the cells were treated with DMSO alone or TSA. The e1a-NLM *lacO* array areas of TSA treated cells were statistically larger than the e1a-NLM *lacO* array areas of DMSO treated cells (FIG 2.3 B). No difference was seen between the DMSO treated and the TSA treated NLM *lacO* array areas. Therefore, e1a requires an HDAC to condense chromatin.

RB acetylation by P300 required for chromatin condensation

As suggested by the results in FIG 2.4 A, e1a requires simultaneous interactions with RB and P300 to condense chromatin. As discussed earlier in the chapter, e1a promotes RB acetylation by P300 at K873 and K874 (FIG 2.1 B, C) (Chan et al, 2001). To assess the importance of RB's acetylation for e1a-induced chromatin condensation, YFP- fused RB mutants with arginine (R) or glutamine (Q) substitutions at K873/874 were co-expressed in RRE.B1 cells with NLM or e1a-NLM. Neither the YFP-RB RR nor the YFP-RB QQ mutant interfered greatly with the e1a-RB interaction, as both localized to the e1a-NLM LacO array statistically similar to YFP-RB WT (FIG 2.4 B). However, the RB RR mutant significantly inhibited e1a-NLM chromatin condensation of the *lacO* array when compared to e1a-NLM co-expressed with YFP-RB WT (FIG 2.4 C). In contrast, there was no significant difference between the RB QQ mutant when compared to e1a-NLM co-expression with YFP-RB WT (FIG 2.4 C). Since Q is a chemical mimic of acetylated lysine, these results argue that e1a-induced chromatin condensation requires e1a-promoted acetylation of RB K873/874 by P300.

Based on the Chan et al, 2001 model, it is P300 that acetylates RB at K873/874, and this acetylation is enhanced by the simultaneous e1a interactions with RB and P300 (FIG 2.1 B,C). To determine if P300 KAT activity is required for chromatin condensation, wild-type P300 (P300 WT) or a kinase inactive P300 mutant (P300 AT) (Kraus et al., 1999) fused to YFP was co-expressed in RRE.B1 cells with NLM or e1a-NLM. Interestingly, YFP-P300 AT co-localized less to e1a-NLM than did YFP-P300 WT (FIG 2.4 D). Despite this decreased recruitment, the over expression of the P300 AT mutant prevented condensation by e1a-NLM, but had no effect on NLM's larger array (FIG 2.4 E). Therefore, e1a requires the acetyltransferase activity of P300 to condense chromatin.

DISCUSSION

When e1a was bound to a large, extended array of lacO sites in RRE.B1 CHO cells (Verschure et al., 2005) by expression of an e1a-NLS-LacI-mCherry fusion (e1a-NLM), the volume occupied by the lacO array condensed to ~1/4 the volume visualized with NLM alone (FIG 2.2 A). It is important to note that large E1A did not condense chromatin (FIG 2.2 B), despite the fact that the entirety of the small e1a isoform is present within the larger isoform (FIG 1.1 C). As CR3, unique to large E1A, (FIG 1.1 C) is well characterized as a transcriptional activator, this result was somewhat expected (Ablack et al., 2012; Pelka et al., 2009a; Pelka et al., 2009b). However, the unique chromatin condensation by small e1a potentially provides further explanation for why the virus utilizes, and requires two distinct isoforms of this early adenoviral protein to oncogenically transform cells (Montell et al, 1984). Moreover, this ability to condense chromatin required interaction of both P300 and RB with the same e1a molecule (FIG 2.4 A), HDAC activity (FIG 2.3 B), the KAT activity of P300 (FIG 2.4 E), and 873/874 acetylated lysines in RB (FIG 2.4 C).

RB remained hypophoshorylated in wild-type e1a and e1a P300b- infected cells, but become phosphorylated when cells were infected with an e1a RBb- adenovirus (FIG 2.4 F). Perhaps the lack of phosphorylation is due to an intact, but compromised interaction with P300 (FIG 2.2 C). As stated earlier, it is presumed that this hypophosphorylation is required for RB to maintain a repressive conformation that interacts with the HDACs of the repressing chromatin-modifying enzymes (Dick and Rubin, 2013). Therefore, these repressing chromatin-modifying enzymes cause chromatin associated with P300-e1a-RB to become hypoacetylated and condensed, repressing transcription. The loss of chromatin condensation in the HDAC inhibition assays indicates that e1a does require chromatin-modifying enzymes to condense chromatin.

Though this interaction may not be a direct e1a-HDAC interaction, it is plausible that it is invariably due to an e1a-RB interaction.

Furthermore, P400 is a SWI/SNF chromatin remodeler. SWI/SNF complexes also function in other examples of repression (Martens and Winston, 2003). The requirement of the e1a P400 binding region (aa 25-36) (Fuchs et al., 2001) for direct visualization of e1a-mediated chromatin condensation (FIG 2.2 B) is consistent with the model that remodelers can "close" chromatin as well as "open" it. Additionally, the e1a P400b- mutant was still able to localize YFP-RB or YFP-P300 to the LacO array (FIG 2.2 C) despite the loss of chromatin condensation. Therefore, the P300-e1a-RB complex may in actuality also require an interaction with P400 to finalize chromatin condensation in repressed genes of infected cells and form a P300/P400-e1a-RB complex in infected cells. Further studies elucidating the finite interactions between these four proteins are required to fully elucidate how these interactions induce chromatin condensation.

CONCLUSION

In adenovirus biology, it was unclear why adenoviral small e1a must interact with P300 in order to transform mammalian cells. As discussed earlier, recent P300 ChIP-sequencing studies revealed an increased enrichment of P300 throughout the gene body following infection with an adenovirus expressing only small e1a, and paradoxically, even though P300 is a known transcriptional activator, these genes were repressed. Specifically, this association of e1a with P300 in the transcribed regions of repressed genes is with e1a molecules that are already co-bound to hypophosphorylated repressive RBs (and all bound repressive chromatin modifying complexes) removed by e1a from E2F transcription factors. In Chapter 2's studies, it was concluded that the P300-e1a-RB repressive complex is not only repressing genes, but is able to

physically rearrange chromatin structure into a condensed state. Furthermore, this repressive complex also requires histone deactylase activity and the P400 chromatin remodeler further adding to our understanding of why e1a requires such interactions with RB and P300 to oncogenically transform cells.

However, in the course of these studies, a few surprising results indicated that the e1a-P300 interaction is not completely understood. Specifically, the continued recruitment of YFP-P300 WT to the LacO array by the e1a-P300b- mutant that does not co-IP with P300 (Ferrari et al, 2014), and the reduced interaction of e1a-NLM with YFP-P300 AT2. These unexpected observations led to further questions that are explored in Chapter 3 of this thesis.
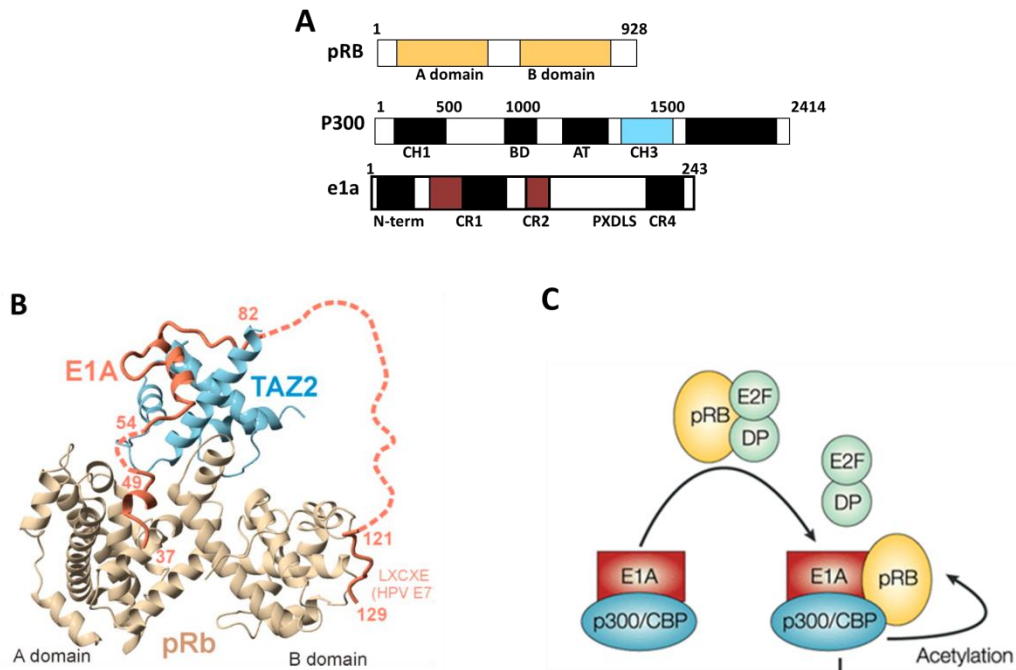
Fig 2.1 P300-e1a-RB repressive complex promotes acetylation of RB

  A. Diagrams of e1a, RB, and P300. Colored domains match the structure seen in (B).
  B. Crystal structure of P300-e1a-RB ternary complex (based on Wang et al, 1995 and Ferreon et al, 2009 of e1a (Coral), the TAZ2 domain of P300 (Blue)/and RB (Tan) interaction with a predicted flexible linker connecting CR1 and CR2 of small e1a.
  C. Model of e1a-induced stimulation of acetylation of RB by P300 adapted from Frisch and Mymrk, 2002. The acetylation of RB at those sites prevents phosphorylation of RB by CDKs, placing RB in a hyper-repressive state.
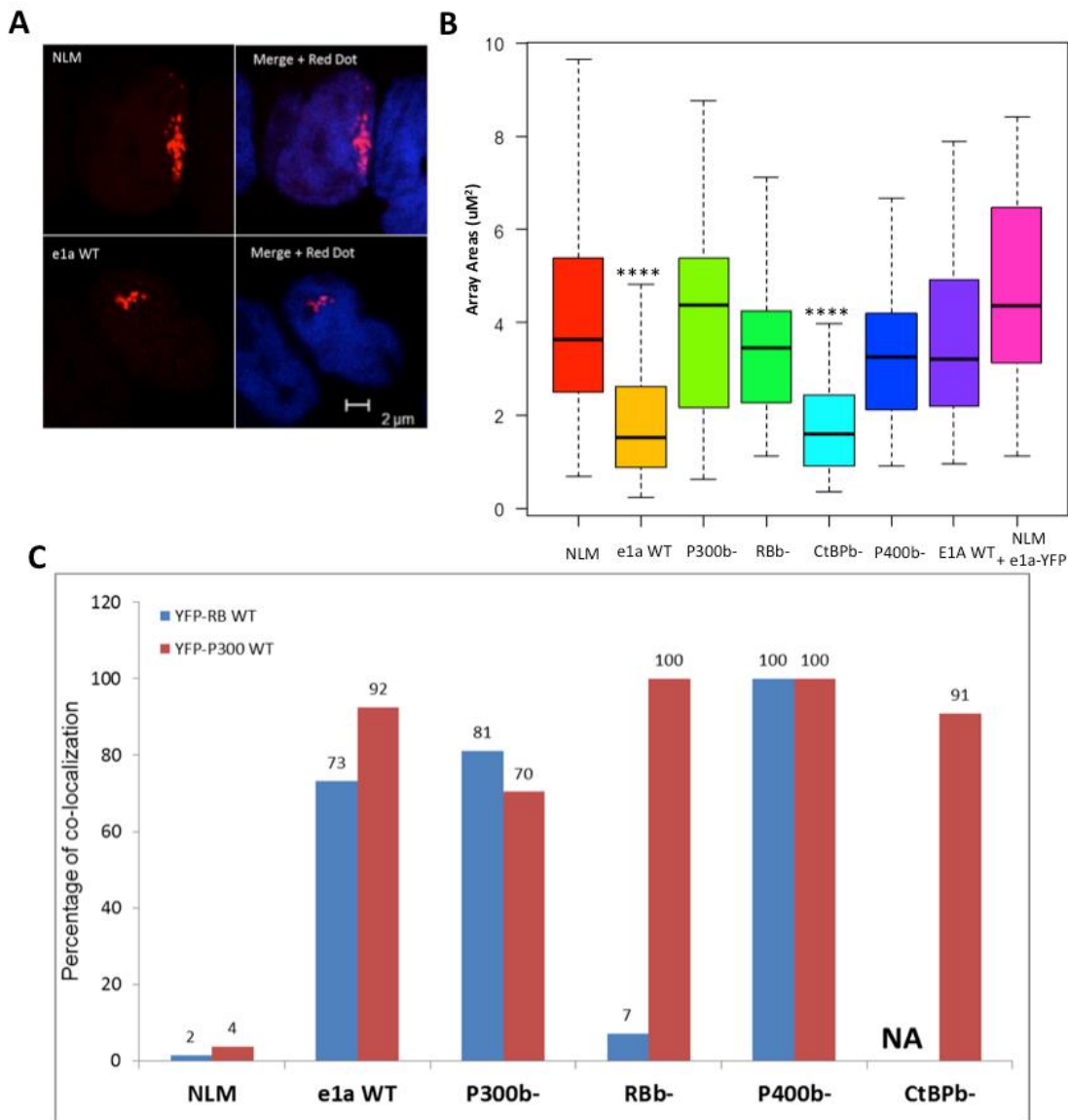
Fig 2.2 e1a-induces chromatin condensation

D. Confocal micrographs of mCherry fluorescence in RRE.B1 cells transfected with vectors for NLS-LacI-mCherry (NLM) and e1a-NLS-LacI-mCherry (e1a-NLM).

E. Boxplots of mCherry-fluorescent areas in the confocal slice with the largest area. "****" indicate a p-value <0.0001 between the distribution of areas from NLM and the indicated e1a wt (e1a) or mutant fused to NLM.

F. Average percentage of "N" cells with YFP-P300(Red) or YFP-RB(Blue) localized at the LacO array with NLM, e1a WT, or various.e1a mutants. No data shown for CtBPb- with YFP-RB
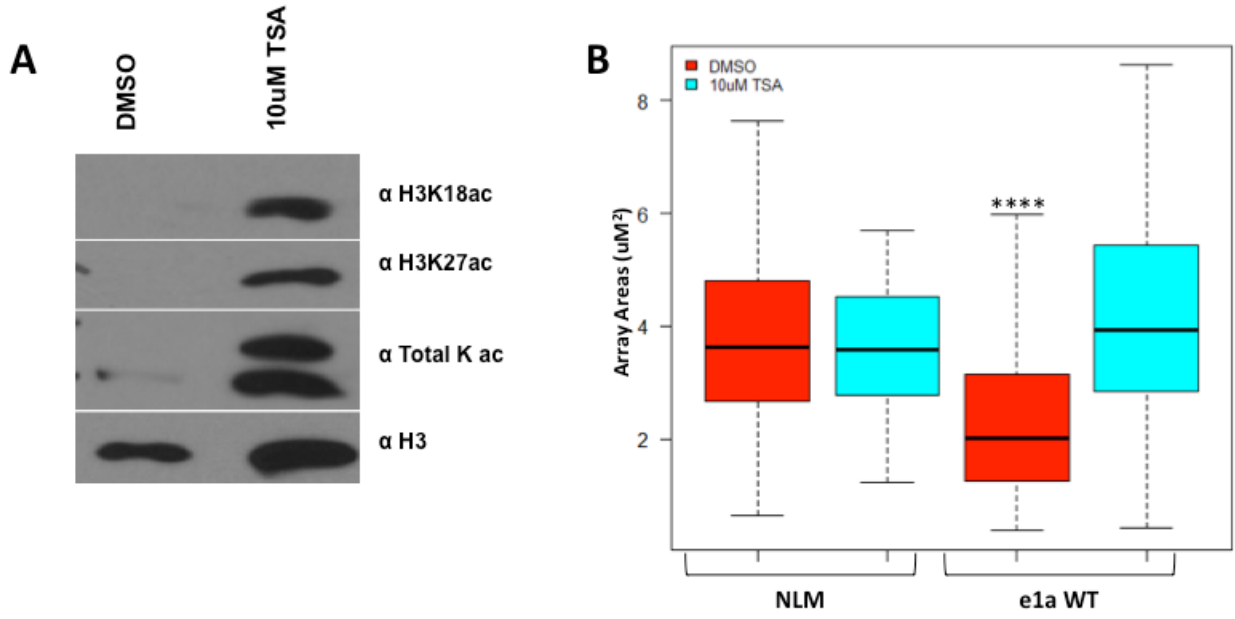
Fig 2.3 e1a requires histone deacetylase activity to condense chromatin
  A. Western blots of acid-extracted histones from RRE.B1 cells treated for 4 hours @ 37 C with DMSO or 10uM TSA.
  B. Boxplots as in (A) of RRE.B1 cells treated as in (C).
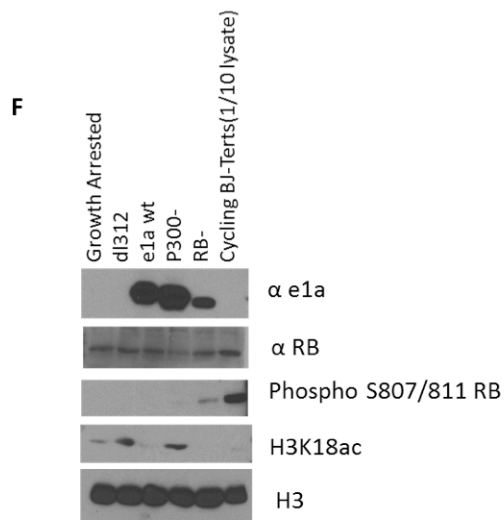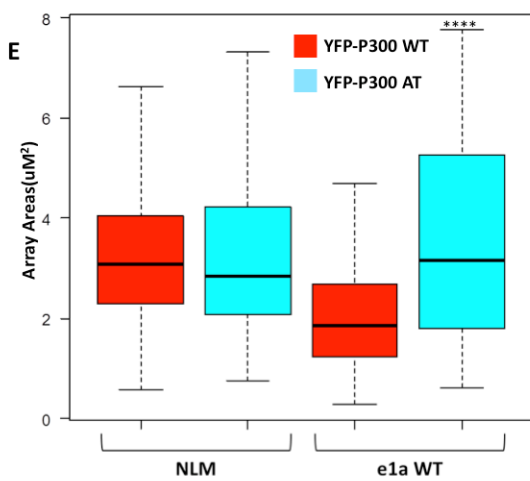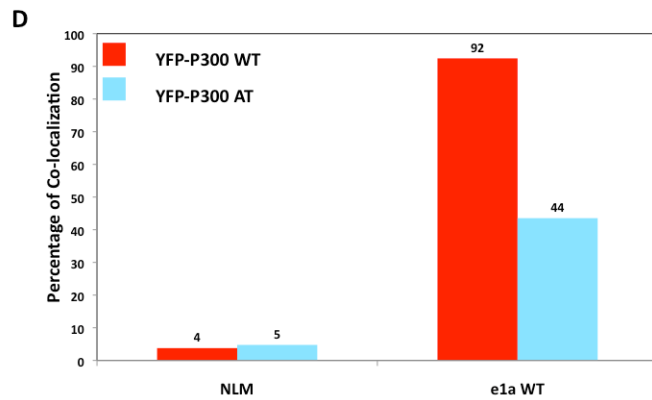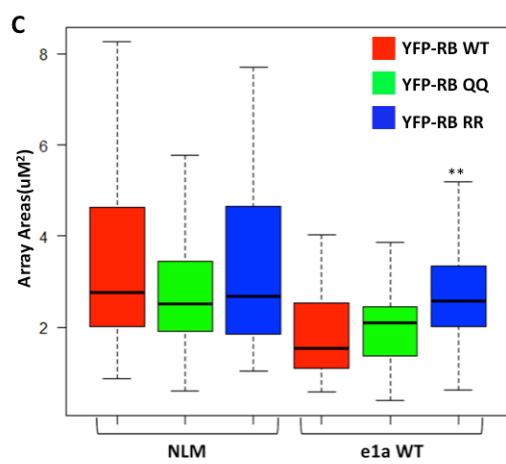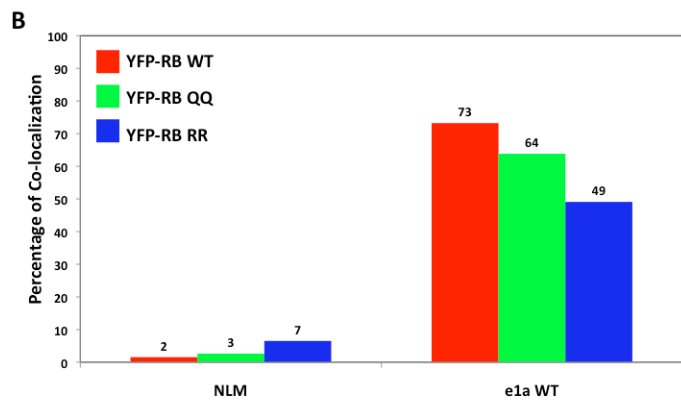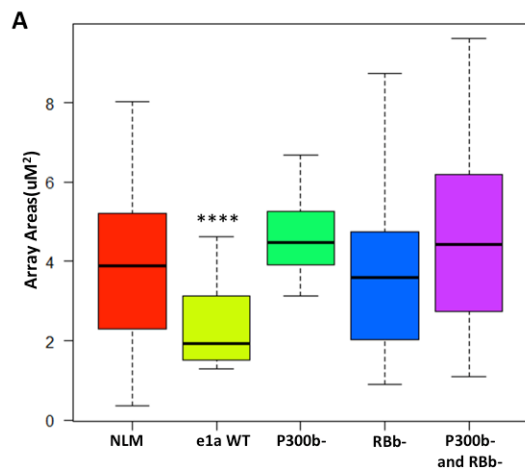
Fig 2.4 e1a requires RB acetylation by P300 to condense chromatin

A. Boxplots of mCherry-fluorescent areas in the confocal slice with the largest area. "****" indicates a p-value <0.0001 between the distribution of areas from NLM and the indicated e1a wt or mutant fused to NLM

B. Average percentage of "N" cells with YFP-RB WT (Red), YFP-RB QQ(Green) or YFP-RB RR (Blue) localized at the LacO array with NLM or e1a WT.

C. Boxplots of mCherry-fluorescent areas in the confocal slice with the largest area. "**" indicates a p-value=0.01 between e1a wt co-transfected with RB WT and a co-transfection with the RB mutants

D. Average percentage of "N" cells with YFP-P300 WT (Red), YFP-P300 AT (Teal) localized at the LacO array with NLM or e1a WT.

E. Boxplots of mCherry-fluorescent areas in the confocal slice with the largest area. "****" indicates a p-value<0.0001 between e1a wt co-transfected with P300WT and a co-transfection with the mutants.

F. Western blots of BJ-Terts 24 hours post-infection or after splitting. First three panels are RIPA-collected lysates, and bottom two panels are blots of acid-extracted histones.

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CHAPTER 3

Refining e1a-P300 interactions

INTRODUCTION

As discussed earlier, P300 is a histone acetyltransferase (Bannister et al. 1996), acetylating H3K18 and H3K27 to reduce the tight interaction between histones and DNA in the nucleosome into a decondensed state. (Hodawawedekar and Marmostein, 2007; Jin et al, 2011). P300 also acetylates non-histone proteins to participate in many important cellular processes including proliferation, differentiation, and apoptosis. However, other studies propose that P300 also promotes gene transcription by acting as a protein bridge, or scaffold that connects different transcription factors to the basic transcriptional apparatus (Goodman et al., 2000; Shikama et al, 1999). This allows for the assembly of multi-component transcription coactivator complexes via P300's bromo domain (BD) (Blobel et al, 2000;Chan et al, 2001A; Manning et al, 2001). Through these domains, P300 interacts with numerous transcription factors and other coactivators.

The catalytic core of P300 is composed of the BD, CH2 region and AT domain (FIG 2.1 A) (Manning et al, 2001). The AT domain acetylates histones and other proteins, and the BD binds acetyl lysines (Dhalluin, et al, 1999; Kraus et al, 1999;). The CH2 region has been predicted to contain a domain that typically binds trimethylated histone H3 Lys4 (H3K4me3), unmodified histone-H3 tails or other modified chromatin (Ragvin et al., 2004). The P300 BD and CH2 region are both required for chromatin binding, but how these domains cooperate to read out chromatin modifications is unknown (Manning et al, 2001; Sanchez et al, 2011).

As discussed earlier, e1a binds to the transactivation zinc-finger (TAZ) domains of P300 (Ferreon et al, 2009). The two TAZ domains, called TAZ1 and TAZ2, contain two cysteine/histidine-rich regions, called CH1 and CH3, respectively (FIG 2.1A). The sequences of the CH1 and CH3 domains are structurally homologous but bind different proteins (De Guzman

et al., 2005; Freedman et al., 2002) suggesting different functions based on unique protein interactions. In *in vitro* binding assays, it was also shown that e1a can bind the CH1 domain of P300 and that this interaction can promote an RB interaction (Fera et al., 2012), but it is unknown if this interaction promotes acetylation of RB. Additionally, one effect of an e1a-P300 interaction is the acetylation of e1a's K239, but the functional consequences of e1a's acetylation at this position is not well understood nor is it know whether the CH1 or CH3 domain of P300 is required for this K239 acetylation (Zhang et al., 2000; Madison et al., 2002).

As e1a interacts with or requires almost all of P300's functional domains, an exact understanding of the impact of e1a's interactions with P300 will lead to a more precise understanding of the function of P300 in transcriptional regulation, specifically chromatin structure. In this chapter, I attempt to elucidate which domains of P300 are required for 1) e1a interaction *in vivo* and 2) e1a-induced chromatin condensation.

MATERIALS AND METHODS

Cell Culture

RRE.B1 cells were cultured in F12 Ham Media supplemented with 100U/mL penicillin, 100ug/mL streptomycin, 10mM methotrexate(to ensure constitutive chromosomal insertion of the DHFR_LacO cDNA), and 10% heat-inactivated FCS.

NLM vectors

All e1a constructs were fused to LacI-mCherry after the SV40 NLS sequence, so that the open reading frame was SV40 NLS-e1a-LacI-mCherry. The K239R and K239Q mutants had K239 mutated in the e1a sequence to arginine and glutamine respectively. In the e1a-P300b-mutant, the e1a amino acid R2 was mutated to glycine, and e1a amino acids E25, V62, F66, and E68 were mutated to alanine based on the Ferreon et al, 2009 structure. In the e1a RBb- mutant,

CR2 deletion from e1a included amino acids 111-127 and e1a amino acids L43, L46, and Y46 were mutated to alanine based on the Liu and Marmostein 2007 structure.. In YFP-P300 AT2, the P300 amino acids H1415, E1423, Y1424, L1428, Y1430, and H1434 were mutated to alanines modeled after the Kraus et al, 1999 mutant. The CH1 domain deletion in YFP-P300 CH1 included P300 amino acids 323-424. The bromo domain deletion in YFP-P300 BD included P300 amino acids 1071—1241. All deletion and point mutagenesis was performed using Agilent QuikChange XL kit.

Chromatin condensation assays

RRE.B1 cells were transiently transfected with 2ug of each construct following standard protocols of Invitrogen Lipofectamine 2000 transfection reagent. After 48 hours, cells were fixed in 4% formaldehyde at RT for 10 min, and then stained with DAPI as described in Balamotis et al, 2009. Confocal microscopy of transfected RRE.B1 cells (Verschure et al., 2005) was performed as described earlier for A03 cells (Balamotis et al., 2009). P-values for differences between the distributions of data shown in boxplots was calculated using Kaleidograph® to perform one way ANOVA and a Tukey's HSD post-hoc comparison.

RESULTS

e1a does not require the CHI for chromatin condensation

In the P300b- mutant, the five point mutations in the N-terminus and CR1 of e1a were designed to disrupt the e1a-P300 TAZ2 (CH3) interaction within the P300-e1a-RB repressive ternary complex (Ferreon et al. 2009; Ferrari et al, 2014). In the course of Chapter 2's studies, it was discovered that the e1a-P300b-mutant still recruits YFP-P300 WT to the *lacO* array despite its inability to co-immunoprecipitate P300 from extracts of IMR90 fibroblasts and HeLa cells (Ferrari et al., 2014). As expected, e1a-P300b- also recruits YFP-RB to the LacO array (FIG 2.2

E). Despite continued RB and P300 localization with the *lacO* array, the e1a-P300b- mutant does not induce chromatin condensation (FIG 2.2 B). This suggests that the e1a-TAZ2 interaction is functionally important for chromatin dynamics. This suggests that despite continued interaction with P300, the e1aP300b- interaction with P300 compromises other potential e1a-P300 interactions important for e1a-induced chromatin condensation.

As discussed earlier, the functional implications of e1a's interaction with CH1 is not well characterized. To elucidate a CH1 domain requirement for e1a-induced chromatin condensation, a CH1 domain deleted YFP-P300 (YFP-P300 CH1) construct was created to potentially eliminate an *in vivo* e1a-P300 CH1 interaction in RRE.B1 cells. YFP-P300 CH1 was co-expressed with the NLM, e1aWT-NLM, e1aP300b-NLM, and e1aRBb-NLM constructs in RRE.B1 cells. While there was reduced co-localization of YFP-P300 CH1with e1aP300b- compared to WT e1a, there was no reduction in co-localization of WT e1a with YFP-P300 CH1 compared to YFP-P300 WT (FIG 3.2 A). There was also no reduced ability to interact with the RBb- mutant (FIG 3.2 A). Furthermore, the loss of P300's CH1 domain did not impact e1a-induced chromatin condensation (FIG 3.2 B, C) nor did it significantly rescue the loss of chromatin condensation by the e1aP300b- or e1aRBb- mutant (FIG 3.2 C). Therefore, the e1a-CH1 P300 interaction is not required for chromatin condensation. Further examination of the requirement of e1a for other P300 domains in chromatin condensation was explored.

<center>**e1a requires the P300 bromo domain for chromatin condensation**</center>

As stated earlier in the introduction, bromo domains interact with the acetylated lysines of histone tails. Therefore, to assess the requirement of the P300 BD in e1a induced chromatin condensation, a P300 bromo domain deleted (YFP-P300 BD) fusion was transiently co-expressed in the RRE.B1 cell line with NLM or e1a-NLM.   Somewhat surprisingly, the YFP-

<center>47</center>

P300 BD is highly localized to the e1a WT *lacO array*, as well as to e1a P300b-(FIG 3.3A). Despite this continued interaction, however, there is loss in e1a-NLM *lacO array* when YFP-P300 BD is co-expressed (FIG 3.3 B). Therefore, e1a requires the P300 bromo domain for chromatin condensation, but not necessarily for a e1a-P300 interaction.

e1a K239 acetylation by P300

It was shown that e1a requires AT interaction for e1a-induced chromatin condensation (FIG 2.4 E. FIG 3.3 B), as well as a reduced interaction with e1a-NLM (FIG 2.4 D). Earlier it was concluded that e1a required P300 acetyltransferase activity for RB acetylation. However, P300 is known to acetylate e1a at K239, and this acetylation is known to prevent a CtBP interaction (Madison et al., 2002; Zhang et al., 2000). The CtBP-e1a interaction is not required for e1a-induced chromatin condensation (FIG 2.2B). Is K239 acetylation by P300 important also important for e1a-induced chromatin condensation, as well as to promote an e1a-P300 interaction?

To answer these questions, a K239R or a K239Q-NLM construct was expressed in the RRE.B1 cells. The K239R fusion led to a drastic increase in *LacO* array area in replicated experiments (FIG 3.4 A, B), but the K239Q acetylated lysine mimic did condense the *lacO* array to the same extent as wild-type e1a (FIG 3.4 A, B). Therefore, e1a acetylation at K239 is important for e1a-induced chromatin condensation. The next question, therefore, is whether e1a K239 is important for the e1a-P300 interaction?

YFP-P300 WT was co-expressed with NLM, e1a, or the K239 mutants, and assayed for co-localization. Surprisingly, unlike the e1aP300b- mutant, the e1a K239R mutant was no longer able to recruit YFP-P300 WT to the *LacO* Array (FIG 3.5 A, B). YFP-P300 AT or YFP-P300 BD did not rescue this effect as neither was recruited to the K239R LacO array (FIG 3.5

B). However, the K239Q chemical mimic of an acetylated e1a restored YFP-P300 WT interaction suggesting that an acetylated K239 is required for an e1a-P300 interaction (FIG 3.5 A, B). To further elucidate the importance of P300's acetyltransferase activity in the e1a-P300 interaction, K239R or K239Q-NLM were co-expressed in the RRE.B1 cells with YFP-P300 WT. Also, as seen with wild-type e1a, YFP-P300 AT was recruited less than 50% of the time to the K239Q *lacO* arrays (FIG 3.5 B). However, YFP-P300 BD still co-localizes to the K239Q *lacO* array, as well as to wild-type e1a LacO array (FIG 3.5 B).

Despite the lack of co-localization between K239R and P300, the over expression of YFP-P300 WT with the e1aK239R mutant did lead to a partial rescue of e1a induced chromatin condensation (FIG 3.5 C). Neither the K239R nor the K239Q mutant induced chromatin condensation when YFP-P300 AT2 or YFP-P300 BD was co-expressed (FIG 3.5 C).

DISCUSSION

While exploring the e1a chromatin condensation mechanism, it was discovered that there was an in vivo interaction between the e1aP300b- mutant and P300 in fluorescent co-localization assays (FIG 3.1 A, B). Despite RB and P300's presence at the *lacO* array (FIG 3.1B), the P300b- mutant did not induce chromatin condensation (FIG 2.3B) nor was the ability to condense chromatin rescued when P300 was expressed with the YFP-P300 WT construct (FIG 3.2 D; FIG 3.3 B). Furthermore, the fact that there was still co-localization of e1aP300b-NLM and P300 CH1 in ~40% of cells (FIG 3.2 A, B) suggests strongly that P300's CH1 or TAZ2 interactions with e1a are not the primary e1a-P300 interactions.

The reduced interaction of wild-type e1a with YFP-P300 AT, in addition to a complete loss of interaction in the e1a P300b-, (FIG 3.3 A) suggests that the acetyl transferase domain of P300 is the most important for an e1a-P300 interaction over the previously characterized e1a-

P300 TAZ 2 interaction. Additionally, the complete loss of any wild-type or mutant P300 interaction with the e1a K239R mutant (FIG 3.5 B) despite an potentially intact interaction between the N-terminus of e1a with the P300 Taz2 domain further suggests that it is the e1a K239ac that is the strongest driver of an e1a-P300 interaction.

In fact, the finding that the P300 bromo domain is required for e1a-mediated chromatin condensation (FIG 3.2 B), but not for an interaction with wild-type e1a, e1a K239Q, or e1a P300b-, is interesting in light of the required P300 acetylation of e1a and RB (FIG 3.2B). The continued interaction of the P300 BD with e1a WT, e1a K239Q, and the e1a P300b- mutant, however, suggests that P300's bromo domain may be more important for interactions with acetylated lysines in chromatin, than in other protein interactions in a scaffold-like interaction as seen in FIG 3.6. In transfection assays of YFP-P300 BD, endogenous P300 with an intact bromo domain is theoretically still available for interaction. Therefore, it may be that the order of interaction is that e1a interacts with TAZ 2 of the YFP-P300 BD, and then an intact AT of that YFP-P300 BD is capable of acetylating K239. Then endogenous P300, with an intact BD, is able to interact with K239ac and properly assemble a scaffold-like interaction as seen in FIG 3.6. Furthermore, the AT domain lies between Taz 2 and the BD of P300 (FIG 2.1 B). Therefore, it may be that e1a interacting with the BD and Taz2 of P300 are important for stabilizing the acetyl transferase activity of the P300 AT domain in the e1a-induced chromatin condensation.

CONCLUSIONS

Chapter 3 further refined the functional implications of the e1a-P300 interaction in e1a induced chromatin condensation. Specifically the requirement of e1 for an intact interaction with the Taz2 domain of P300 for oncogenic transformation may be due to a stabilization of the acetyltransferase activity of P300. Furthermore, this intact acetyltransferase activity and

interaction with e1a K239ac may be of utmost importance for the repression of host cell genes because of the requirement for e1a induced chromatin condensation. Further experiments elucidating the impact of the acetyltransferase activity on H3 hypoacetylation and host cell gene repression in adenovirus infected cells need to be performed. Further study is also required to elucidate the minimum e1a interactions with various P300 domains to induce not only chromatin condensation, but also e1a induced oncogenic transformation.
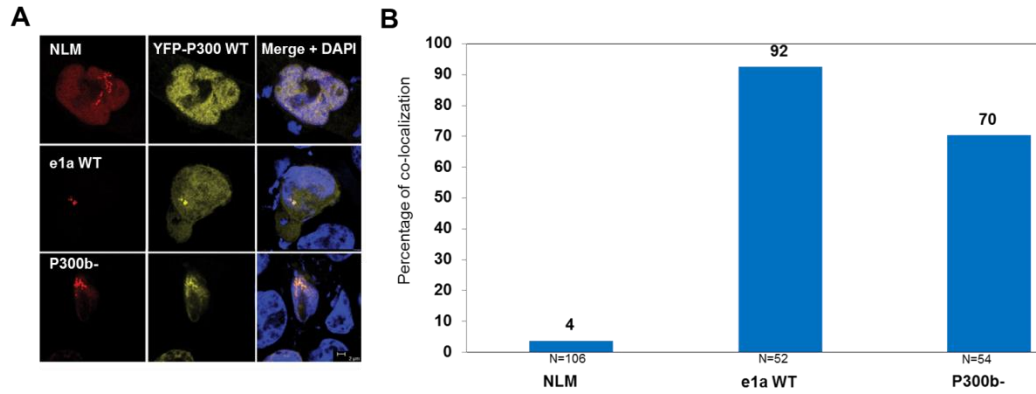
Figure 3.1: P300b- mutant still recruits YFP-P300 WT

A. Confocal micrographs of RRE.B1 cells co-transfected with vectors for all NLM constructs with either YFP-P300 WT

B. Average percentage of "N" cells with YFP-P300 localized at the LacO array with NLM, e1a WT, or the P300b- mutant e1a.
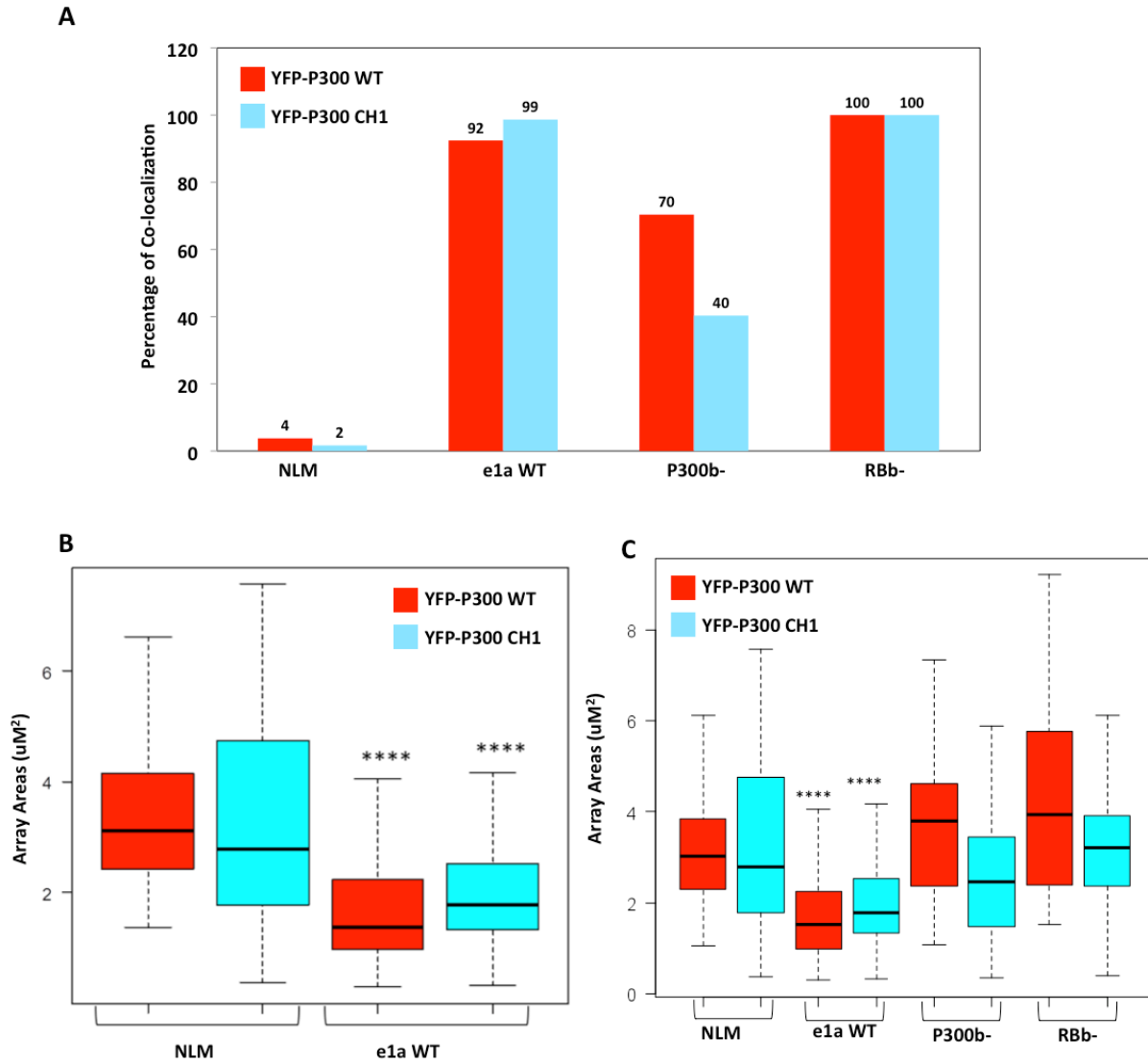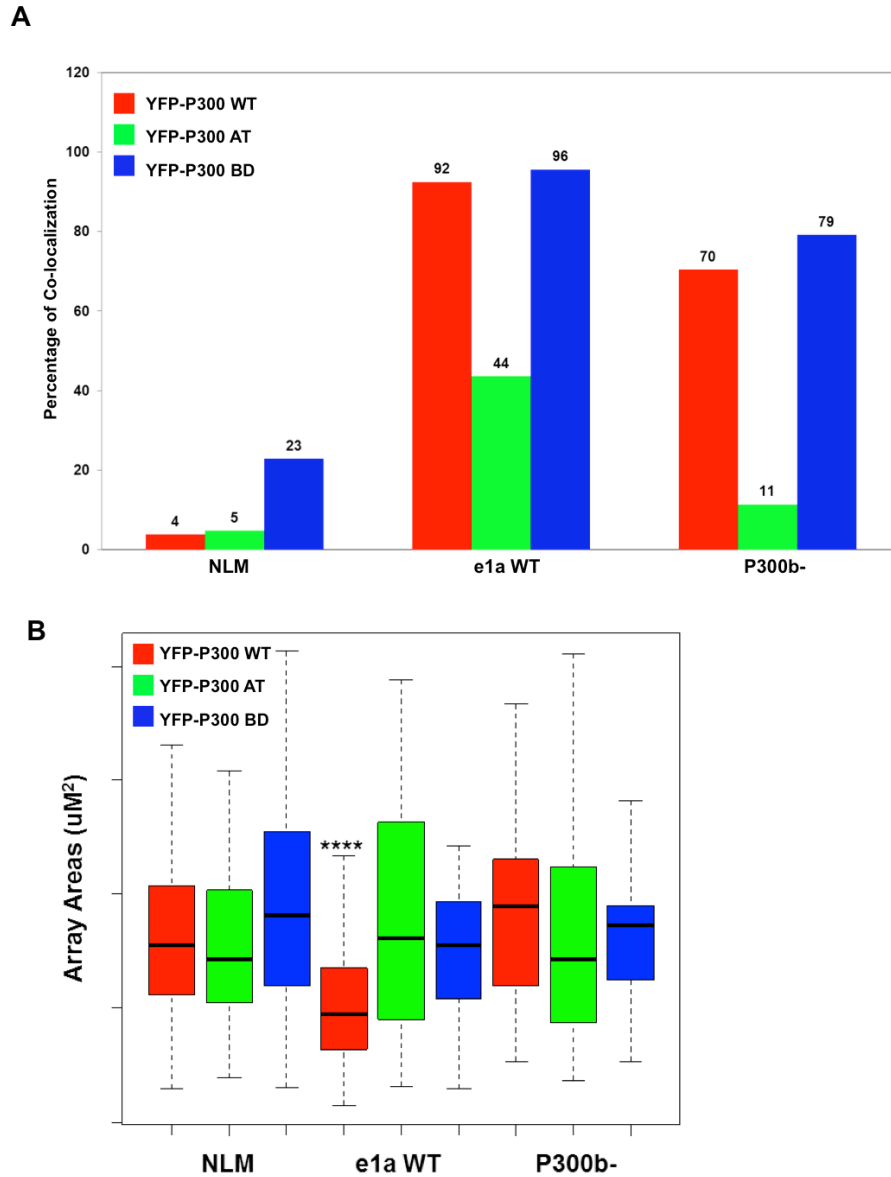
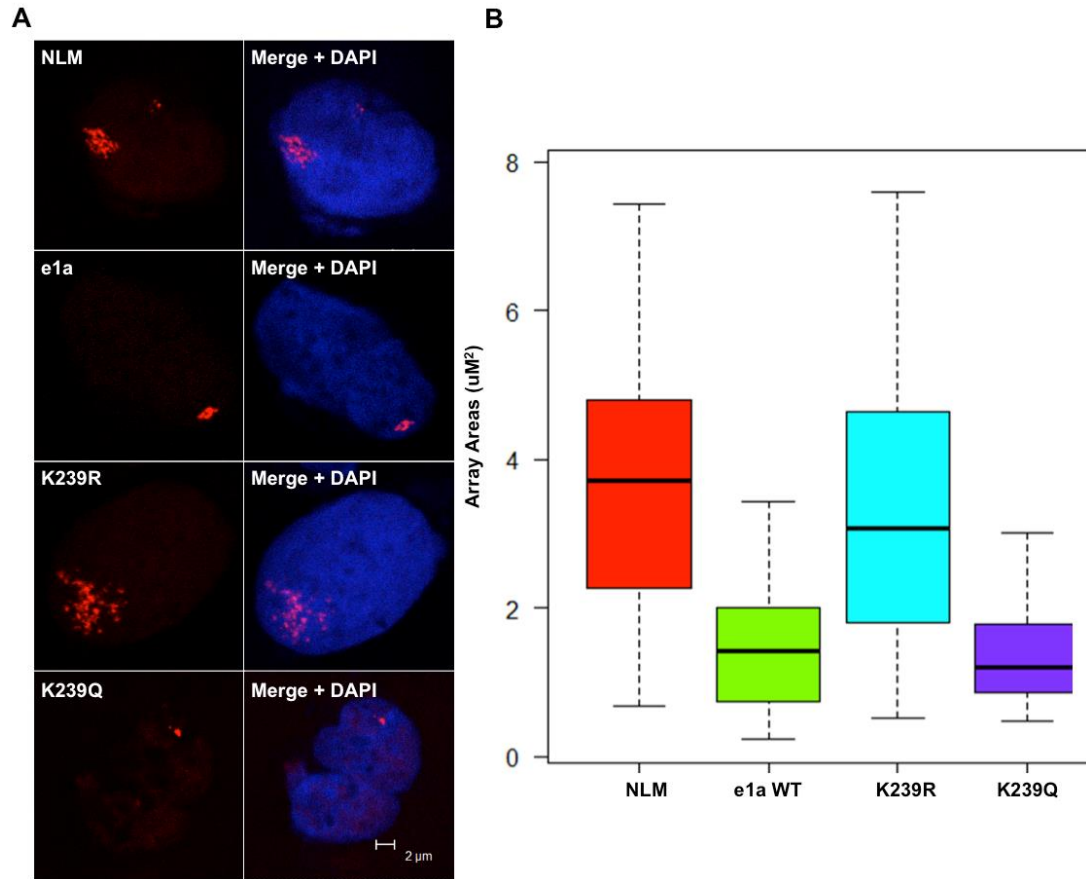**Figure 3.2: e1a does not require the P300 CH1 domain to condense chromatin**

    A. Average percentage of "N" cells with YFP-P300 WT (yellow) or CH1 (blue) localized at the LacO array with the specific NLM constructs. Same cells used were to quantify LacO Array area in C- D.

    B-C. Boxplots of mCherry-fluorescent areas in the confocal slice with the largest area. A "****" indicates a p-value of <0.0001 between the NLM construct co-transfected with the YFP-P300 construct and the equivalent e1a-NLM construct.
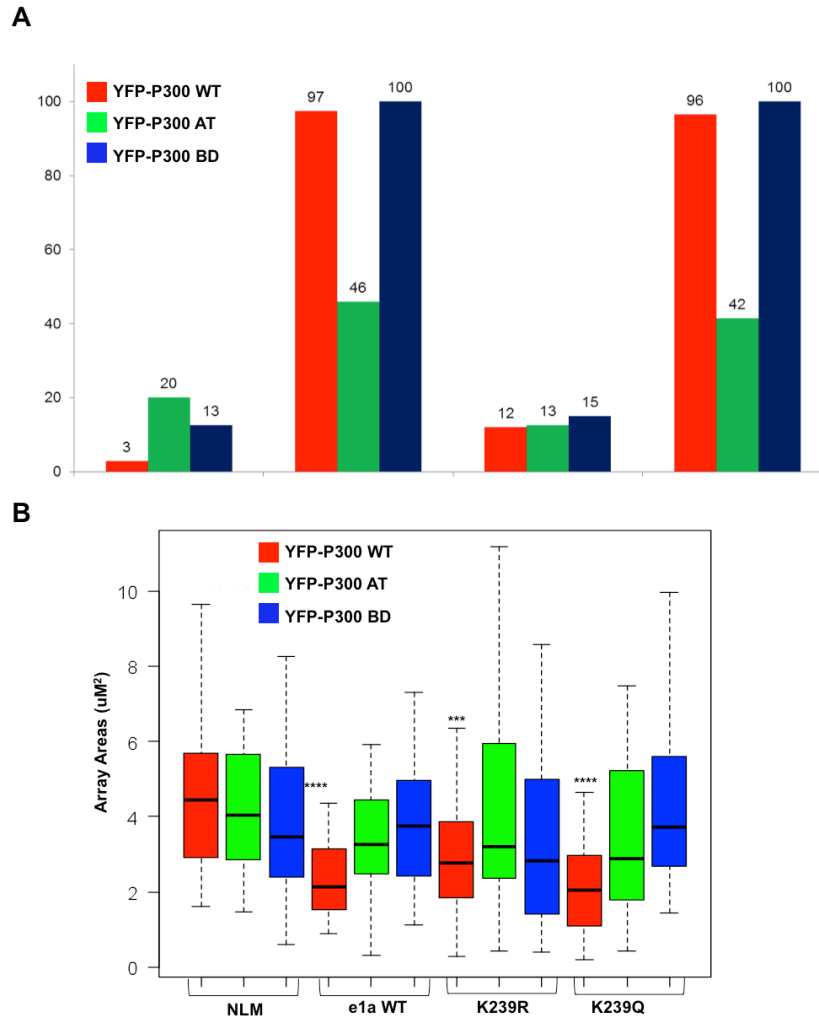
Figure 3.3: e1a requires P300's acetyltransferase domain for interaction
  A. Average percentage of "N" cells with YFP-P300 WT(red), AT2(green) or BD(blue) localized at the LacO array with the specific NLM constructs. YFP-P300 WT numbers are a repeat of FIG 3.1 A.
  B. Boxplots of mCherry-fluorescent areas in the confocal slice with the largest area. A "****" indicates a p-value of <0.0001 between the NLM construct co-transfected with the YFP-P300 construct and the equivalent e1a-NLM construct. Data was quantified from the same cells as A.

Figure 3.4: e1a requires an acetylated lysine to condense chromatin
  A. Confocal micrographs of RRE.B1 cells co-transfected with vectors for K239 mutants and YFP-P300 WT
  B. . Boxplots of mCherry-fluorescent areas in the confocal slice with the largest area. A "****" indicates a p-value of <0.0001 between the NLM construct and the equivalent e1a-NLM construct.

Figure 3.5: e1a requires an acetylated K239 to interact with P300
  A. Confocal micrographs of RRE.B1 cells co-transfected with vectors for K239 mutants and YFP-P300 WT
  B. Average percentage of "N" cells with YFP-P300 WT(red), YFP-P300 AT2(green), or YFP-P300 BD(blue) localized at the LacO array with the specific NLM constructs. Same cells used were to quantify LacO Array area in C.
  C. Boxplots of mCherry-fluorescent areas in the confocal slice with the largest area. A "****" indicates a p-value of <0.0001, and "***" indicates a p-value of .001 between the NLM construct co-transfected with the YFP-P300 construct and the equivalent e1a-NLM construct.
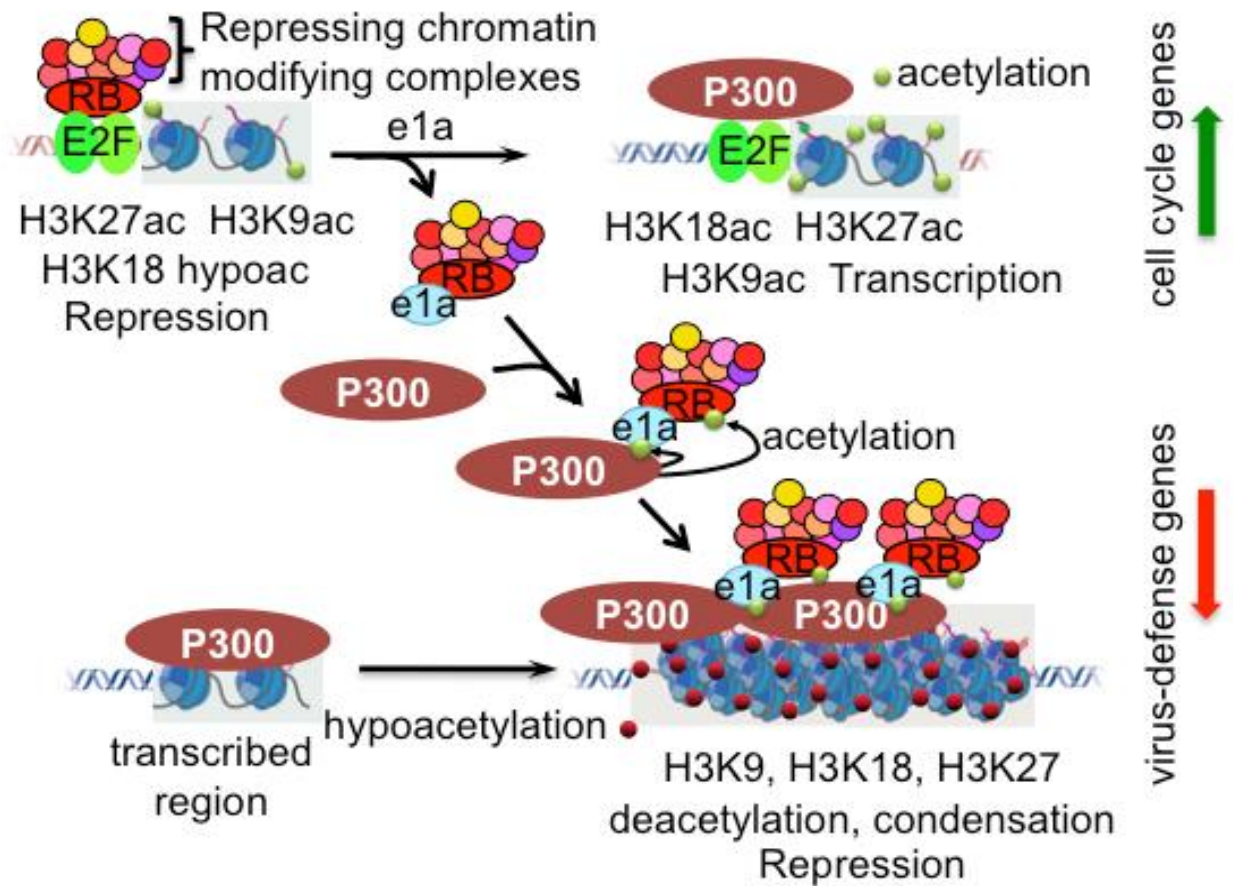
Figure 3.6: Model for e1a regulation of host cell gene activation and repression through interactions with RBs and P300 (adapted from Ferrari et al., 2014).

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APPENDIX
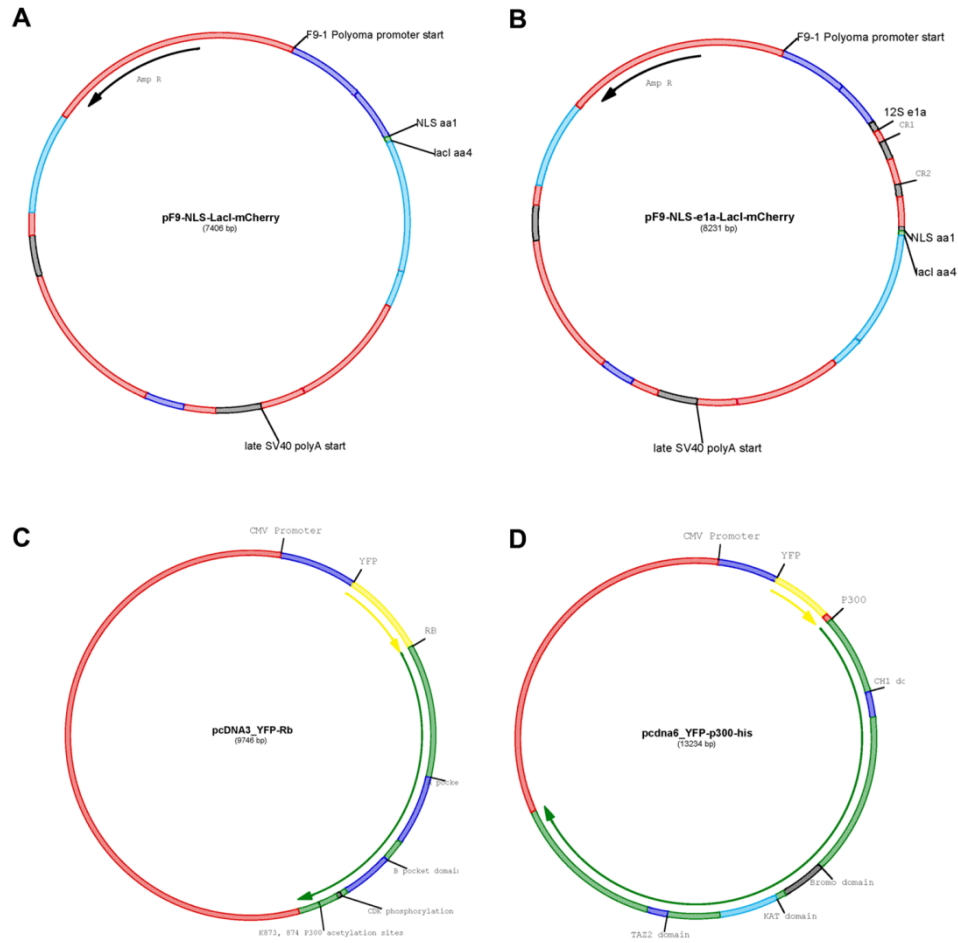
Figure A.1: Vector Maps of expression vectors

A. F9 promoter-NLS-LacI-mCherry plasmids for expression of fusion proteins used in chromatin condensation assays.

B. The small e1a cDNA sequence was inserted via *SalI* restriction digest before the NLS-LacI-mCherry cDNA sequences. 30-45 bp linker sequences exist between fusions for linker amino acid spacers between proteins.

C. pcDNA 3-YFP-RB expression vector. The cytomegalovirus promoter sequence is followed by YFP-RB sequences. The RB cDNA was cloned into a pcDNA3 YFP plasmid via the BamHI sites of the multiple cloning site. Major protein domains and amino acids discussed in this thesis are marked.

D. pcDNA6.1 YFP-P300 expression vector. P300 followed by six histidine sites cDNA from the original Kraus et al., 1999 plasmid was cloned after the YFP sequences using the XhoI sites of the multiple cloning site. Major protein domains and amino acids discussed in this thesis are marked.
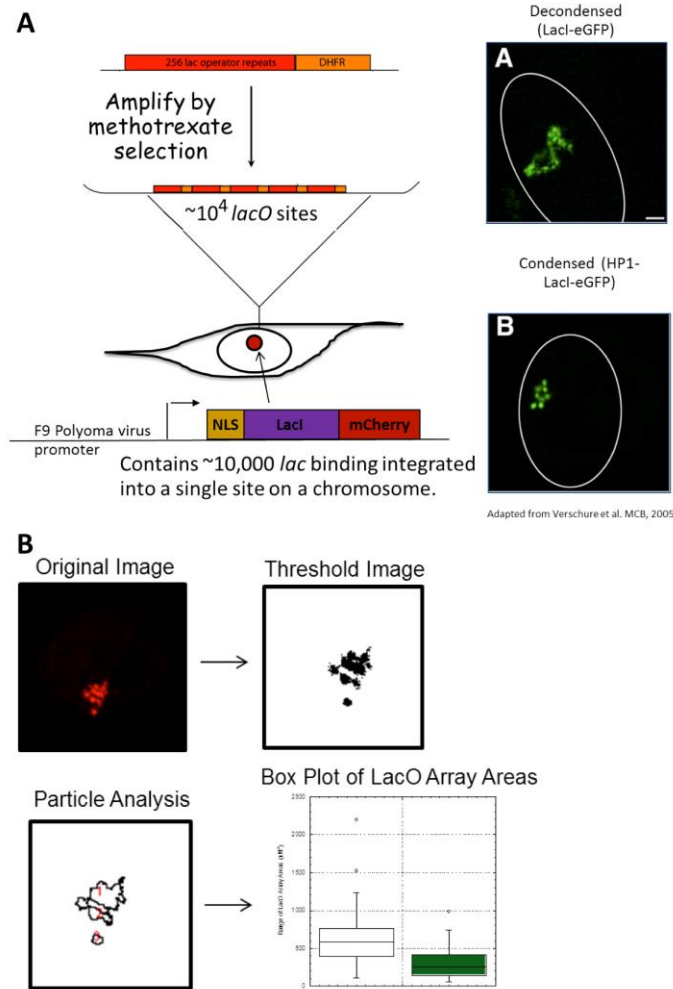
Figure A.2: The RRE.B1 Cell Line

A. The RRE.B1 cell line is a specialized Chinese hamster ovary cell line containing amplified repeats of the *lac* operator within one chromosome to create a ~80 Mb region with ~10,000 high affinity *lac* operators. A to LacI repressor results in a highly localized visual LacO array whose chromatin structure is manipulated by fusing proteins associated with chromatin condensation and decondensation to the LacI-fluorophore fusion protein. Transfection of an expression vector for a Lac I-GFP fusion results in GFP fluorescence at the LacO array in the nuclei of RRE cells creating a highly localized area of fluorescence. Fusions of LacI-GFP or HP1-LacI-GFP allow visualization of a decondensed(A) or condensed(B) LacO array(Adapted from Verscure et al, 2005)

B. .In ImageJ software, the original micrograph file all background fluorescence is removed via thresholding. Then, the ImageJ particle analysis plugin is run, and the total area of fluorescence measured in microns^2 . The distribution and mean of each experimental data set are graphed into box plot using Kaleidagraph ® data analysis software