Project 1

Characterisation of the Functional Interaction between HPV16 E2 and Rint-1

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Project Abstract:

Human papillomavirus (HPV) infection causes wide range of infections in human beings resulting in 100% of cervical cancers and other anogential and oropharyngeal cancers. HPV protein E2 plays a vital role in subsequent productive and persistent viral infections; HPV E2 protein being a multi functional protein binds to several cellular proteins involved in viral entry pathways and seems to have a role in modulating intracellular trafficking. Previous work in the Parish lab has isolated an interaction between HPV16 E2 and Rint-1 and hypothesized that the association of HPV E2 with membrane associated protein Rint-1 is a part of a novel mechanism in which HPV modulates sub-cellular trafficking during HPV infection. The aim of this project is to further investigate the interaction between Rint-1 and HPV E2 and we successfully characterised the functional interaction between Rint-1 and HPV16 E2 *in vivo* and from the results obtained, we confirmed that Rint-1 interacts with N-terminus of E2. We also tried to analyse why Rint-1 is interacting with E2 *in vivo* but not *in vitro* with phosphatase treated IP. We also determined the localisation of HPV16 E2 protein and Rint-1 protein in sub-cellular elements by sub-cellular fractionation and confirmed that Rint-1 and E2 together localise in cytoskeletal fraction. We also tried to analyse the localisation of HPV16 E2 following siRNA mediated depletion of Rint-1 by Knockdown experiment. From this work, we can speculate that associations of E2 with Rint-1will have a role in early stages of HPV infection.

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I. Introduction:

Human papillomavirus (HPVs) are small double stranded DNA viruses which are non-enveloped with icasohedral symmetry and belong to family *papilloma viridae*. It was in the 1970's that human papillomaviruses were identified as the causative agents of warts (benign skin lesions) in humans and mammals (Bernard, 2005). They are categorized as a diverse group of viruses which infect different amniotes (mammalian species, reptiles, turtles and also birds) (de Villiers et al., 2004). The advancement in molecular biology techniques in 1980's made it possible to detect numerous human papillomavirus types in malignant and benign mucosal cells such as cervical cancer cells and many other laryngeal cells and genital warts (Mammas et al., 2014) and (de Villiers et al., 2004) . The International Committee on Taxonomy of Viruses (ICTV) data shows more than 200 types of HPV are currently classified, of which about 150 HPV types have been isolated and sequenced (de Villiers et al., 2004, Mammas et al., 2014, Doorbar et al., 2012). The phylogenetic analysis and advancement of technical approaches has changed the description of HPVs several times. Currently, to define an HPV type as a new type, there should be a minimum of 10% dissimilarity of the L1 gene sequence with other known HPV types (Bernard, 2005).

History:

In the late 1950s, the discovery of electron microscopy made it possible to

observe lots of viruses and as a result of those observations and basic nucleic acid sequence study, both papillomaviruses and polyomaviruses were discovered and categorized as similar type of viruses and kept in a common family called *papovaviridae* (Bernard, 2005). As it is a known fact that the taxonomic classification must be based on natural existence, the ICTV, concluded that these two viruses belong to two separate families and since then papillomaviruses have been placed in the family *papillomaviridae* and was officially updated in ICTV classification (Bernard, 2005, de Villiers et al., 2004).

HPV Classification:

Based upon the specific characteristics of genome organization, phylogenic similarities and biological variance, HPVs are classified in to 5 generas like Alpha, Beta, Gamma, Mu and Nu-papilloma viruses (de Villiers et al., 2004). All of the genitally transmitted viruses are in the Alpha genus; the major sexually transmitted viruses from this group are HPV-6, HPV-11, HPV-16 and HPV-18 (Doorbar et al., 2012, Bernard, 2005). Approximately 1% of the population who are sexually active are infected with HPV-6 and HPV-11 through oral and sexual routes causing benign lesions (Mammas et al., 2014). The major threat of cancers are from the virus types HPV-16 and HPV-18 from genus alpha, which cause some mucosal lesions that may convert in to high risk cancers such as cervical cancers (Mammas et al., 2014, Bernard, 2005). The Beta papillomaviruses like HPV5 cause skin cancers in immuno suppressed patients or people with genetic defects where as the Gamma

papillomaviruses e.g. HPV4 causes small skin warts, the clinical results of this sometimes mislead with the results of alpha papilloma virus HPV2 (de Villiers et al., 2004, Doorbar et al., 2012). The rest of the human papillomaviruses are placed in genera's Mu and Nu, causing general warts in the human population (Doorbar et al., 2012, Mammas et al., 2014). The five genera of HPVs genomic diversity and its host cell interactions leading to different clinical manifestations are shown in the figure 1.

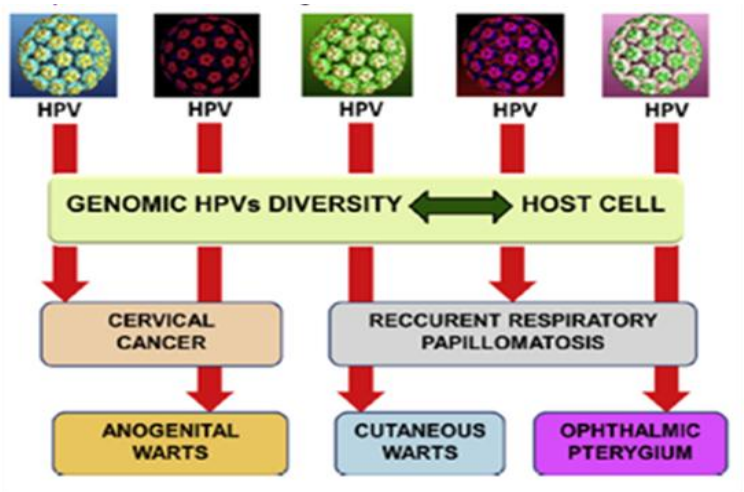


Figure no.1: Different HPV genera and their clinical manifestations. source: "Genomic diversity of human papillomaviruses and clinical manifestations: An overview in adulthood and childhood" figure taken from (Mammas et al., 2014).

Of the alpha HPVs, HPV16 and HPV18 are studied the most due to their medical and biological importance as they account for more than 70% of the cervical, vaginal and anal cancers (Doorbar et al., 2012).

HPV16 mode of transmission:

The major mode of HPV transmission is sexual transmission and skin to skin contact (Baussano et al., 2010). And there could be chances of vertical transmission of oncogenic HPV from infected mother to foetus through placenta which is yet to be explored (Porterfield, 2011). The latency period is highly variable; from younger women to older women of different age groups and infections can sometimes last for decades (Baussano et al., 2010).

HPV Genome:

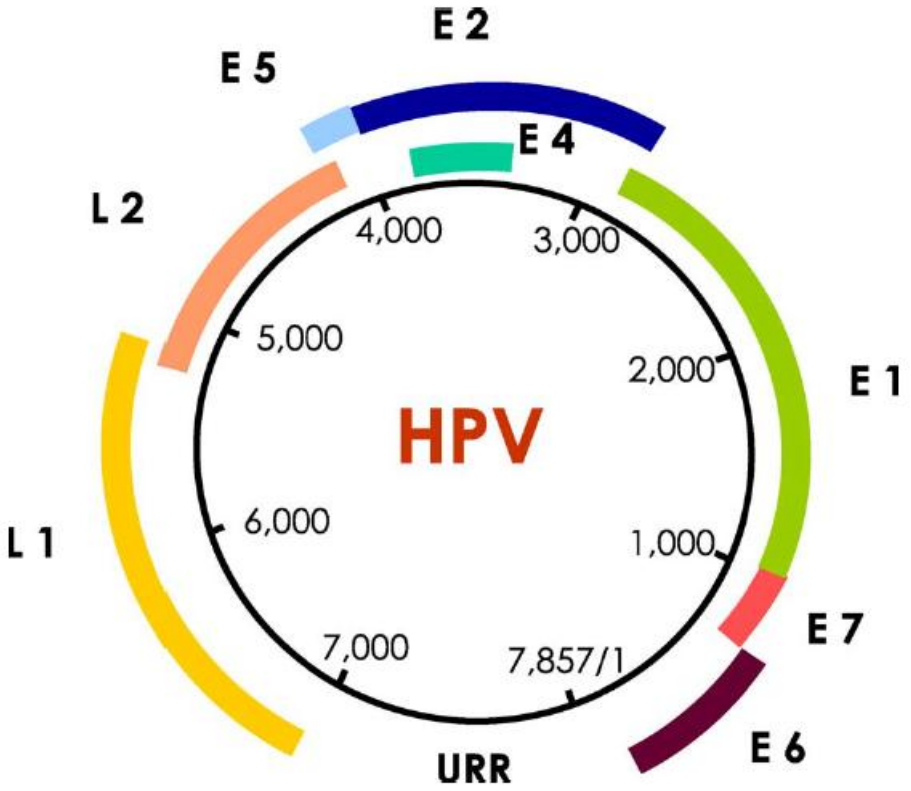


Figure no.2: showing the arrangement of early (E) genes, capsid (L1 and L2) genes and upstream regulatory region (URR) schematically. Figure source: “HPV in the etiology of human cancer” figure taken from (Munoz et al., 2006).

As shown in figure no.2, the HPV genome is an approximately 8000 base pairs (bp) circular DNA molecule that is packaged inside the capsid proteins L1 and L2. The genome consists of six early proteins E1, E2, E4, E5, E6 and E7 which facilitate in the replication and assembly of the viral genome inside the infected cell. The upstream regulatory region (URR) of about 1000bp is involved in viral gene regulation, genome replication and finally assembling the virions (Munoz et al., 2006).

HPV16 Virus Life Cycle:

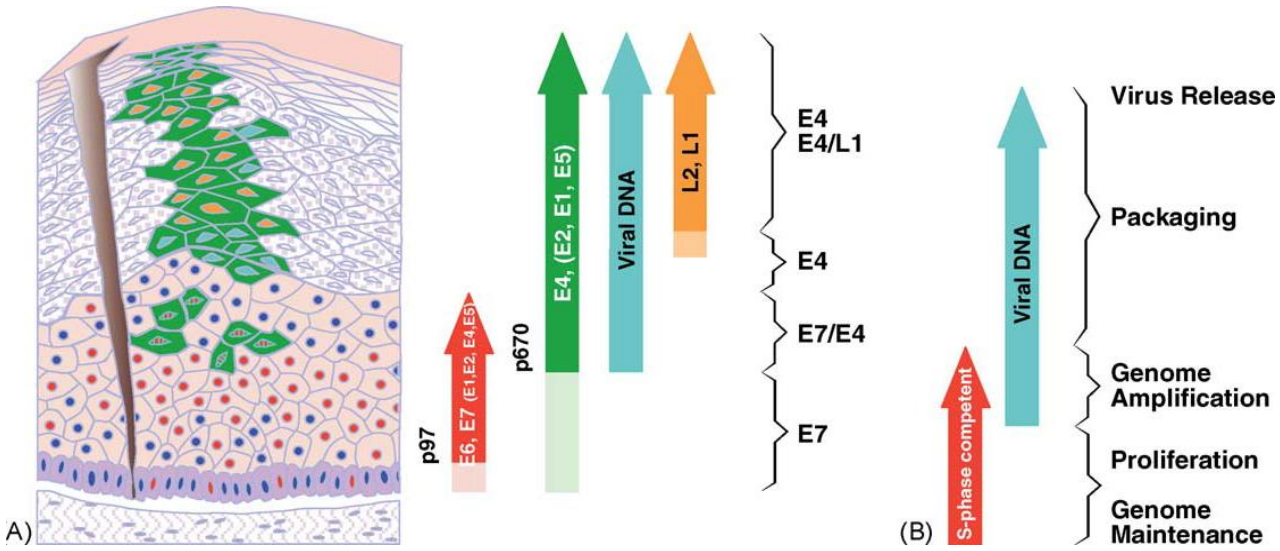


Figure no.3 A): Representation of skin, the patterns of gene expression of HPV16 and how the infected cell migrates to epithelial surface. The p97 promoter directs E6 and E7 gene expression required for S-phase entry and in the upper layers of epithelium, due to up-regulation of the p670 promoter, E4, E2, E1 and E5 expression increases, which will amplify the viral genome, and due to modifications in mRNA splicing, the E4 will remain in the upper layers where the capsid proteins L1 and L2 are present. (B) Shows different stages of the HPV life cycle from genome maintenance to mature viral release. Figure source: “The papillomavirus life cycle, Journal of Clinical Virology”. Figure taken from (Doorbar, 2005)

In the life cycle of virus, early stages of infection occur by the infectious particle attack on basal layer cells as shown in figure 3. Normally the virus enters the stratified epithelium through a break, which may be caused by abrasion or certain exposure to trauma. The virus reaches the basal epithelial cells and infection is initiated (Doorbar, 2005). Virus attachment to the cells takes place through the cell surface receptors (Li M, 1998). The uncoating of the virus seems to occur through the disruption of disulphide bonds in the viral

capsid, mediating the viral genome entry into the nucleus of the cell (Li M, 1998). Inside the nucleus of the basal cells, the genome is initially maintained at a low copy number for which E1 and E2 protein expression is required (Doorbar, 2005, Doorbar et al., 2012).

After infection, the proliferative phase occurs where the basal cells with viral genomes increase in number. The viral genome is maintained from 10 to 200 copies per cell in the basal layer and to maintain the viral episomes, the early proteins (E1, E2, E6 and E7), are expressed at low levels (Doorbar, 2005). During the proliferative phase the expressed E6 and E7 proteins work together to allow the infected cell to enter S-phase of the cell cycle where the viral genome copy number is gradually increased facilitated by the E1 and E2 proteins (Doorbar, 2005). After viral genome amplification, the HPV life cycle completes by the expression of two functional proteins in the more superfacular differentiated cells. The two functional proteins are minor coat protein L2 and major capsid protein L1 (Doorbar et al., 1997). After the exit of cell from cell cycle, the L1 facilitates the self assembly of virus like particles with the help of L2 in packaging (Roden et al., 2001). The HPV genome is encapsided in a capsid containing 360 L1 protein copies and around 12 L2 protein copies which are arranged in an icasohedral shell with around 72 pentameric capsomeres (Modis, 2002). Maturation of the virus takes place on the surface of dying skin, for successful completion of this process; the virus leaves the infected cell before re-infection. The E4 protein presence which is present in bulk amount can enhance the virion release, which can then infect the upper epithelial layer (Doorbar et al., 1997).

E2 protein:

The human papillomavirus E2 proteins are highly conserved in different human papillomavirus types and are involved in viral gene regulation and viral genome replication (Hegde and Androphy, 1998). HPV E2 is approximately 50 kDa in size and consists of a carboxy-terminal DNA dimerization or DNA binding domain (DBD) of 100 amino acids linked to amino-terminal transactivation domain (TAD) of 200 amino acids. These two domains are linked by a hinge domain (Yaniv, 1988, McBride AA, 1988, McBride et al., 1988). X-ray crystallography shows that the TAD is made up of 3 α-helices which form a twisted plane (Antson et al., 2000) and the carboxy terminal domain forms an anti-parallel β-barrel structure (Dell et al., 2003). The DNA binding domain binds to palindromic E2 binding sites on URR of the viral genome (12 bp motifs) and this TAD is involved in regulation of transcription by complex formation with E1 (Pang and Thierry, 2013). E2 binds to the viral helicase E1 on the replication origin and together facilitate viral DNA replication (Mohr et al., 1990).

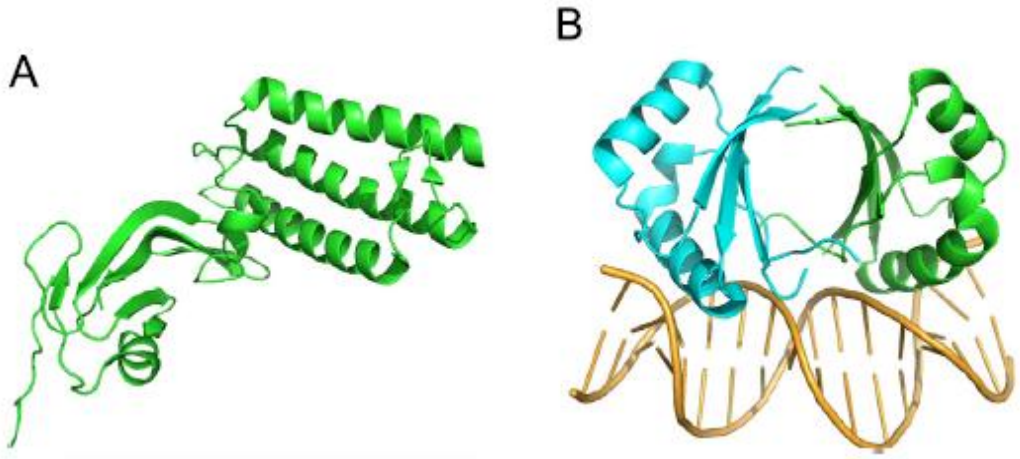


Figure no 4: (A) represents the transactivation domain of HPV16 E2 protein and figure (B) represents the dimeric DNA binding domain of HPV18 E2 bound to DNA. Figure source: “The papillomavirus E2 proteins”. Figure taken from (McBride, 2013).

E2 Transactivation domain:

The 200 amino acid TAD folds to form a conserved structural domain with a cashew shaped structure (figure 4 A) (Antson et al., 2000). TAD has some residues inside it, which are responsible for transcription activation and repression and replication. Some residues on the outer convex surface (R37 and R173) facilitate transcription regulation and some on its concave surface (E39) facilitates interaction with E1 proteins and replication (Antson et al., 2000, McBride, 2013). For maintaining the viral DNA, TAD associates E2 with mitotic chromosomes in the dividing cells (Xue et al., 2010, Mohr et al., 1990). Mutations in this domain can result in the disruption of viral genome and mitotic chromosomes tethering (Mohr et al., 1990). Deletions of this TAD prevents association of E2 with mitotic chromatin (McBride, 1998). TAD is very sensitive; single point mutations or deletion of few amino acids in this domain can prevent the attachment of protein to chromatin and also inhibit

replication and transcription. Attachment to chromatin is essential for viral genome partitioning (Abroi et al., 2004).

DNA binding and Dimerization domain:

The 85 to 100 amino acid length DBD binds to specific motifs within the viral genome. E2 DBD forms a very stable dimer, which is required for association with DNA (figure 4 B) (McBride et al., 1988). The consensus binding motif of E2 is defined as ACC(N_6)CGGT or ACCG(N_4)CGGT and is located in upstream regulatory region (URR) (Androphy et al., 1987). This E2 DNA binding site bends in a way around the domain facilitating the major grooves to interact with the recognition helices (Hegde et al., 1992). The charge on the interaction surface of the DNA also facilitates E2 in selecting flexible or non-flexible targets (Kim et al., 2000).

E2 Hinge region:

The hinge domain of E2 has no defined structure, but forms a flexible link between TAD and DBD. The length of the hinge varies between papillomavirus genera but is usually similar within different variants of the same genus (Gauthier et al., 1991). The HPV E2 hinge region contains mostly proline, threonine, arginine, serine and glycine residues (Winokur and McBride, 1992). Though the hinge shows no role in replication or transcription functions, it mediates spacing in between the TAD and DBD which helps in avoiding steric hindrance in between these two domains (Winokur and McBride, 1992). The phosphorylation of the hinge region aids auxillary functions such as chromatin binding, intracellular localization and also

maintaining protein stability (Garcia-Alai et al., 2006). Also one of the major roles of the hinge region is in interacting with other cellular proteins such as TNPO3, SMN1, SRSF7, SRSF2 etc., (Lai et al., 1999).

E2 expression and binding to the HPV URR:

E2 binds to consensus sites within the upstream regulatory region (URR) of viral DNA and regulates viral DNA transcription and replication. By associating with E1, E2 binds to the origin of replication and enhances viral genome replication and suppresses the expression of E6 and E7 (Goodwin EC, 1998). The number of E2 binding motifs varies in different papillomavirus genera, for example the alpha papillomaviruses have four E2 binding motifs with highly conserved spacing (McBride, 2013). At the 3' end of the URR, the origin of replication is present which is made up of an E1 binding sites adjacent to an E2 binding site (Mohr et al., 1990).

E2 Intracellular localization:

E2 proteins predominantly localize in the nucleus, but can also mediate in nuclear and cytoplasmic shuttling (Blachon et al., 2005). The nuclear localization signals of certain papillomaviruses are known; one is situated within the DBD and other was pointed on TAD (McBride, 1998). These signals are transplantable to other proteins (McBride, 1998). The hinge region helps binding of E2 to nuclear matrix (Zou et al., 2000). E2 forms nuclear foci in the presence of E1 which are thought to be the regions of viral genome replication (Fradet-Turcotte et al., 2011). The E2 protein plays an important role in viral infection through its association with the minor capsid protein L2, and thereby

attaching to ND10 domain of nucleus. This domain is also present on the adjacent sites of replication foci shows the nuclear bodies have different roles in viral life cycle (Day et al., 2004).

E2 associated cellular proteins:

HPV E2 is a multifunctional protein and functions in many diverse aspects of the virus life cycle by recruiting cellular proteins (McBride, 2013). These cellular proteins have been broadly categorized depending on their role into functional groups such as RNA processing, cell cycle regulation, protein degradation, nuclear import and apoptosis (McBride, 2013, Radaelli et al., 2012). Examples for some of the E2 host proteins are TopBP1, ChlR1 and Brd4 (Donaldson et al., 2007, Parish et al., 2006, Jang et al., 2009). The functional interaction of HPV16 E2 with TopBP1 cellular protein is required for the viral DNA replication and genome segregation (Donaldson et al., 2007). In the absence of TopBP1, HPV16 E2 is thought to associate with an alternative complex of cellular proteins and binds to host chromatin (Donaldson et al., 2007). E2 binds to ChlR1, a DNA dependent helicase protein which catalyses the unwinding of single and double stranded DNA by utilizing the energy produced during ATP hydrolysis (Parish et al., 2006). E2 binding to ChlR1 is essential for E2 association with mitotic chromosomes and viral genome segregation (Parish et al., 2006). In addition to the pathways listed above, intracellular trafficking has been suggested to be mediated by interaction of E2 with cellular proteins which play a vital role in vesicle mediated transport but many of these novel interactions have yet to be characterized (Muller and Demeret, 2012). Recent studies in the Parish laboratory have demonstrated an interaction between HPV16 E2 and a membrane-associated protein Rint-1

(Rad50-interacting protein 1) which functions in retrograde transport between donor acceptor compartments, Golgi and endoplasmic reticulum. E2 and Rint-1 co-localize in cytoplasmic compartment (Parish, unpublished).

Membrane-associated protein Rint-1:

Rint-1 (Rad50-interacting protein 1) (figure 5) is an 87 kDa protein which consists of 792 amino acids (Xiao et al., 2001, Lin et al., 2007). Rad50 is a member of structural maintenance of chromosomes (SMC) family of proteins and was first identified in yeast and given the name based on its role in recombinational DNA repair activity (Xiao et al., 2001). Rad50 is involved in wide range of cellular activities like telomere maintenance (Kong et al., 2006), DNA break repair, meiosis, and cell cycle checkpoint (Xiao et al., 2001) by interacting with numerous other cellular proteins. Experimental work conducted by Xiao *et al*., has identified a novel interaction of a 87 KDa protein with the carboxy terminal of Rad50 and named it as Rint-1 (Xiao et al., 2001) and the interaction of Rint-1 with Rad50 was specifically executed during M and S phases of cell cycle and participate in cellular processes like cell cycle check point activation control (Xiao et al., 2001). Rint-1 also serves as a connecting link between Rb family protein p130 and Rad50 by interacting with p130 protein at amino acids 358 – 440, which is required for telomere maintenance by blocking telomerase independent telomere elongation, therefore deletion of either Rint-1 or p130 results in elongated telomeres (Kong et al., 2006). Rint-1 through its N-terminal region also associates with ZW10 (dynamitin interacting protein) (Aoki et al., 2009). ZW10 is located inside Golgi and ER apparatus (Arasaki et al., 2006). Together Rint-1 and ZW10 forms complex with syntaxin

18 complex which is an ER localized N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) and target membrane associated receptor (SNAP) complex which mediates an important role in vesicle trafficking between ER and Golgi apparatus (Hirose et al., 2004, Aoki et al., 2009). Deletion of either Rint-1 or ZW10 results in dispersion of Golgi structure which suggests that both of these proteins are required for the structural maintenance of Golgi (Hirose et al., 2004, Aoki et al., 2009). All these data show that Rint-1 is involved in multiple functions such as cell cycle progression control, telomere maintenance and sub-cellular trafficking by interacting with other proteins (Xiao et al., 2001, Kong et al., 2006, Aoki et al., 2009, Hirose et al., 2004). Some reports also suggest that over expression of Rint-1 in primary non-transformed cells of murine astrocytes has the ability to confer tumorigenicity, hence it is also validated as novel glioblastoma (GBM) oncogene (Quayle et al., 2012).

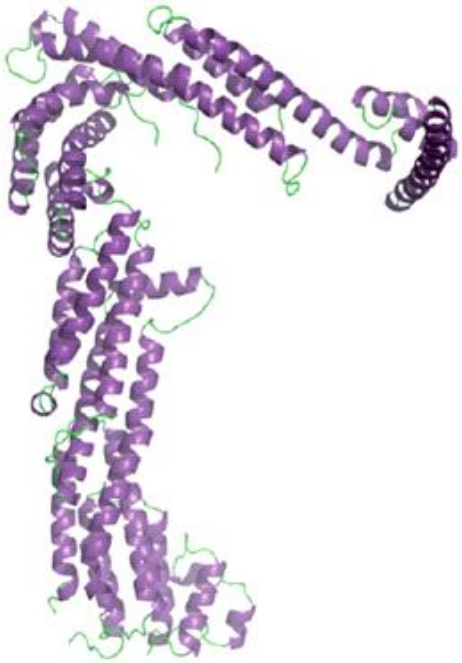


Figure no.5: Structural model of Rint-1 protein. Figure source: “Structural analysis of the RZZ complex reveals common ancestry with multisubunit vesicle tethering machinery”. Figure taken from (Civril et al., 2010).

Role of Rint-1 in Golgi dynamics and cell cycle progression:

The Golgi apparatus and endoplasmic reticulum (ER) are required for co-ordinating protein synthesis through post translational modifications in the ER and sorting in the Golgi apparatus. Experimental work conducted by some scientists' shows that deficiency of Rint-1 in cells has a severe effect on the Golgi apparatus which impairs the normal function of Golgi apparatus and protein sorting (Lin et al., 2007). Rint-1 plays a vital role in anterograde transport from ER to Golgi by interacting with other cellular proteins that likewise have a role in retrograde transport from Golgi to ER (Hirose et al., 2004). Rint-1 also serves as scaffold component essential for Golgi structural integrity maintenance. Also, in enzymatic reactions of disassembly and reassembly of Golgi apparatus, Rint-1 was shown to be an important effector of upstream signalling in controlling structural conversion steps (Lin et al., 2007, Xiao et al., 2001).

Intracellular trafficking:

All cells are covered with membrane which acts as a barrier between the inside of the cell and its outer environment. Cellular processes such as respiration and DNA replication takes place within intracellular membrane bound compartments. Unlike prokaryotic cells, which have only one plasma membrane, eukaryotic cells have additional intracellular membrane bound compartments which regulate specific functions such as mitochondria for respiration and nucleus for DNA replication. The compartment is widely

expanded (approximately 1000 to 10,000 greater in volume) resulting in the formation of larger cells and all the cell functions are widely separated (Tokarev, 2009). The membrane bound compartments in eukaryotic cells interact with the cellular environment by vesicle mediated intracellular trafficking which move proteins between intracellular compartments. The proteins and lipids are transported between these intracellular compartments in cargo-loaded vesicles originating from donor compartments which are formed with the help of specific adaptor and coat proteins such as COPI, COPII and clathrin. These vesicles are then targeted to acceptor compartments, where the vesicles attach with the help of tethers and membrane fusion takes place with the help of different SNARE proteins (reviewed by (Tokarev, 2009). Two major pathways are involved in shuttling materials inward and outward termed exocytic and endocytic pathways, respectively. There are many proteins involved in vesicle recognition, formation and fusion; these include protein components from the vesicle coat and SNAREs. The compartment specific v-SNARE in the transport vesicle pairs with complimentary t-SNARE on its target compartment, the stable SNARE complex mediates membrane fusion (Tokarev, 2009, Vassilieva and Nusrat, 2008). Three different SNARE proteins, syntaxin 18, BNIP1 and p31 together with the membrane proteins ZW10, Sly1 and Rint-1 form the syntaxin 18 complex which facilitates vesicle transport. Rint-1 in association with ZW10 in syntaxin 18 complex plays an important role in vesicle trafficking in between ER and Golgi (Aoki et al., 2009, Hirose et al., 2004).

Role of HPV16 E2 in association with Rint-1 in sub-cellular trafficking:

Previous work conducted in the Parish laboratory has shown an interaction between HPV16 E2 and Rint-1 *in vivo* (Wijendra *et al.,* unpublished). HPV16 E2 binds to Rint-1 between amino acids 220 to 264. HPV16 E2 and Rint-1 co-localise in the cytoplasmic compartments of transfected cells, which are thought to be ER-like structures. E2 is predominantly a nuclear protein with very little cytoplasmic localisation (Blachon et al., 2005) whereas Rint-1 localises to ER-like structures (Parish *et al.,* unpublished). Interestingly, co-expression of these two proteins resulted in a significant accumulation of both proteins in ER-like structures. In addition, it has been also suggested that, HPV16 E2 regulates sub-cellular trafficking during HPV infection by associating with Rint-1. This was demonstrated using a vesicle trafficking experiment in which cells were transfected with GFP-tagged temperature sensitive vesicular stomatitis virus (VSVG). When cells are incubated at 40˚C for 48 hours, GFP-VSVG is retained in the ER. However, incubation at 32˚C results in movement of GFP-VSVG to the Golgi and then to the plasma membrane. Expression of HPV16 E2 resulted in a significant delay of vesicle movement to the Golgi and also to the plasma membrane, suggesting that E2 has a role in modulating sub-cellular trafficking during HPV infection.

II. Project Aims:

1. Hypothesis:

- We hypothesize that HPV16 E2 interaction with cellular binding protein Rint-1, is important for sub-cellular trafficking during HPV infection.

2. Overall aim:

- To investigate the functional interaction between HPV16 E2 and Rint-1.

3. Sub-aims:

- Determine the Rint-1 binding site on HPV16 E2 using *in vitro* binding assays.
- Analyze the interaction of Rint-1 with truncated E2 proteins *in vivo*.
- Analyze the localization of HPV16 E2 protein and Rint-1 protein in sub-cellular elements by sub-cellular fractionation.
- To determine the localization of HPV16 E2 following siRNA-mediated depletion of Rint-1.

III. Materials and Methods:

Cell Culture:

C33a derived from cervical carcinoma cell line and U20S derived from human osteosarcoma cell lines were cultured and maintained in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum. The medium was changed every 2/3rd day and the cells were incubated at 37°C and 5% CO_2 at constant humidity. Once the cells became 90% confluent, they were trypsinized with 1ml of trypsin per 10cm dish and once detached, 9ml of fresh DMEM was added and the cells were mixed well. 10µl of cell suspension was pipetted into a haemocytometer and cells were counted and seeded according to the required number in to a new 10cm dish.

Plasmid Maxi-prep:

100ml of Luria Broth (LB) supplemented with Ampicillin (100 µg/ml) was inoculated with a bacterial colony and incubated at 37°C overnight with vigorous shaking at approximately 210rpm. Next day the culture was transferred into a centrifuge bottle and the bacterial cells were harvested by centrifugation at 6000 xg for 15 minutes at 4°C. The supernatant was discarded. Following the Qiagen Maxi prep protocol, the pellet was resuspended in 10ml P1 buffer (Qiagen kit) containing RNase A. 10ml of P2 buffer (Qiagen kit) was then added and mixed by inverting the tubes for 5 to 6 times. The samples were then incubated at room temperature for 5 minutes. 10ml of chilled buffer P3 (Qiagen kit) was then added and samples were

mixed by inverting the tubes 5 to 6 times. Samples were incubated on ice for 15 minutes and then centrifuged at 20,000 xg for 30 minutes at 4°C. The supernatant containing plasmid DNA was carefully removed and transferred to new tubes and centrifuged again at 20,000 xg for 15 minutes at 4°C. The supernatant was collected. A Qiagen-tip 500 column was then equilibrated by applying 10ml of equilibrium buffer QBT (Qiagen kit) and the column was emptied by gravity flow. The supernatant with plasmid DNA was then added and allowed to enter the resin by gravity flow. After the supernatant had passed through the column, it was washed twice with 30 ml of buffer QC (Qiagen kit). After washing, the tip was placed on to a centrifuge tube and the DNA eluted with 5ml buffer QF (Qiagen kit). After the elution, the DNA was precipitated by adding 10.5 ml of isopropanol, mixed gently and centrifuged at 15,000 xg for 30 minutes at 4°C. After centrifugation, the supernatant was carefully removed and the pellet was washed with 5ml ethanol and centrifuged at 15,000 xg for 10 minutes at 4°C. The pellet was air-dried for 10 minutes and DNA was dissolved in TE buffer (pH 8.0) and the final plasmid DNA concentration was determined using a Nanodrop.

In Vitro **Binding Assay:**

*Protein expression and GST pull down***:**

5 ml of LB containing Ampicillin (100 µg/ml) was inoculated from a glycerol stock of *E.coli* transformed with plasmids that express either GST, GST-E2 NH (containing the N-terminus and hinge region of HPV 16 E2) and GST-E2

HC (containing the hinge region and C-terminus of HPV 16 E2) and incubated overnight at 37°C and 210 rpm. The following day, 45 ml of LB was inoculated with the starter culture which was incubated at 37°C and 210 rpm until the optical density at 600nm (OD_{600}) of the culture was 0.6. Protein expression was then induced with 1 mM IPTG and the culture was then incubated for a further 4 hours 37°C and 210 rpm. The cultures were then transferred to 50ml Falcon tubes and centrifuged at 6000 xg at 4°C for 30 minutes. The bacterial pellets were the stored at -20°C.

Purification:

The frozen pellet was thawed on ice and resuspended in 2 ml of lysis buffer (25 mM Tris-HCl pH 8.0, 250 mM NaCl, 1% lysozyme, 1x protease inhibitors and 5mM DTT) and incubated on ice for 30 minutes. The suspension was then sonicated at 35% amplitude for 3x 10 seconds with 10 seconds rest on ice between each sonication before centrifugation at 20,000 xg, 4°C for 10 minutes. The lysates were transferred to new tubes and pellet was discarded. 500 µl of the lysate was added to Eppendorf tubes with 20µl of glutathione sepharose resin (resin was washed three times with the lysis buffer without DTT, inhibitors or Lysozyme) and nutated in the cold room overnight. The next day, the beads were centrifuged at 2000 xg for 2 minutes. The supernatant was removed and discarded without disturbing the beads and the beads were washed three times in lysis buffer without inhibitors and DTT, after the final wash, the supernatant was removed and an equal volume of lysis buffer was added to the beads and mixed thoroughly. 20µl suspension was taken in to a new tube for separation by SDS-PAGE; 10 µl of 2 x protein dye was added

and samples were boiled at 95°C for 10 minutes and used to run on 10% SDS gel which was then stained with coomassie.

Coomassie Staining:

After running the samples on SDS-PAGE, the gel was carefully removed from the cassette placed in a plastic box and washed with distilled water 5 times. The gel was covered with Coomassie stain and placed on a gentle rocker for 1 hour. After an hour the gel was washed with distilled water and placed in distilled water on a gentle rocker for another 1 hour after an hour the water was replaced with fresh one and rocked for overnight. Next day the gel was imaged and the relative amount of each protein was quantified using a Fusion device (Peqlab).

C33a cell transfection:

C33a cells were seeded at 3 X 10^6 cells per dish and allowed to adhere. 5µg of Rint-1 DNA was placed in Eppendorf tubes and to that 500µl of serum free DMEM was added and (1:2) ratio X-treme gene was added carefully without touching the walls of Eppendorf tubes, mixed gently and incubated at room temperature for 15 minutes. After 15 minutes the complex prepared in the Eppendorf tubes was added drop wise to the C33a cell dishes. The medium was mixed gently by swirling and placed in the incubator at 37°C. Next day the old media was replaced with 10 ml of fresh DMEM. After 48 hours of transfection the cells were harvested, the old media was removed, washed

with PBS and the cells were scraped in 500 µl of chilled PBS, collected in to tubes and centrifuged at 1000 xg for 5 minutes, The supernatant was removed and pellet was stored at -80°C. The cell pellet was resuspended in 300µl of IP lysis buffer [Tris (50 mM) pH 7.4, NaCl (100mM), NaF (200mM), Triton X-100 (1 %), glycerol (10 %) and distilled water] with inhibitors (1%) and DTT (0.1 mM), incubated on ice for 30 minutes, sonicated at 35% amplitude, 10 seconds on and 10 seconds off for 30 seconds. The samples were centrifuged at 10,000 xg at 4°C for 15 minutes and Rint-1 lysate was carefully collected and saved on ice.

GST-Pull down Assay continuation:

The purified GST, E2-NH and E2-CH beads and Rint-1 lysates were thawed on ice and to approximately 20µl of glutathione resin with bound GST protein, 100µl of Rint-1 C33a cell lysate and 100µl of IP binding buffer with inhibitors and DTT (100 mM) was added. Samples were nutated in the cold room for 2 hours and centrifuged at 2000 xg, 4°C for 2 minutes. The supernatant was discarded and beads were washed three times with wash buffer [Tris (100 mM) pH 7.4, NaCl (100 mM), NP 40 (1%), KCl (200 mM)]. After the final wash, approximately to 20 µl of the beads added 20 µl of wash buffer and 10 µl of 6x sample buffer. For the inputs, to 25 µl of untransfected lysate and Rint-1 lysate, added 5 µl of 6x sample buffer, boiled the samples for 10 minutes and saved the samples to separate by SDS-PAGE.

SDS-polyacrylamide gel electrophoresis:

The resolving gel mix and stacking gel mix was prepared following the standard protocol, the glass plates were arranged and using a glass pipette approximately 7 ml of resolving gel mix was added to the level marked, isopropanol was added on top to level it up and once the resolving gel was set, the isopropanol was removed, rinsed with distilled water and stacking gel mix was carefully added. The combs were inserted and allowed to set for 30 minutes. The gel was placed in the running equipment with 1 X running buffer (standard protocol) and the combs were removed and proteins were loaded in the wells and initially ran at a voltage 100 V until the samples left the stacking gel and the voltage was increased to 150 V until the blue layer near the base gets disappeared.

Western blotting:

The PVDF membrane was soaked for a few seconds in methanol and washed with distilled water and equilibrated in 1 X transfer buffer for 5 minutes. The proteins on the gel were transferred on to PVDF membrane using a standard protocol, in 1 X transfer buffer at 100 V for 1 hour. Once the proteins are transferred on to the membrane, they were blocked with 5 % milk in 1 X TBS-T [Tris buffered saline with Tween 20 (0.1 %)] for 1 hour at room temperature with gentle rocking. After blocking, the membrane was incubated in 5% milk in TBS-T with primary antibody (Goat Rint-1 (Santa Cruz) 1:1000) for 1 hour at room temperature with gentle rocking. The membrane was washed in 1 X TBS-T for 5 times with 5 minutes interval and membrane was incubated in 5

% milk in TBS-T with secondary antibody (Anti sheep/goat HRP; 1:10,000) for 1 hour at room temperature and again membrane was washed in 1 X TBS-T for 5 times with 5 minutes interval and membrane was placed on a cling film and covered with ECL reagent, incubated for 3 minutes and proteins were detected by chemiluminescence using Fusion digital imaging system.

Co-immunoprecipitation:

Transfection:

C33a cells were seeded (3 x 10^6) cells per 10 cm dish to carry out the transfection. In Eppendorf tubes 5 µg of Rint-1 DNA was taken and to it added 5 µg of DNA for flag tagged full length E2, E2-NH and E2-CH respectively, 300 µl of serum free DMEM was added, mixed gently and added X-treme gene (2:1) ratio and incubated at room temperature for 20 minutes, after 20 minutes the complex prepared in the Eppendorf tubes were added drop wise to the C33a dishes and incubated at 37°C, 5% CO_2 for 48 hours.

Harvesting:

The cells were washed with PBS and 500 µl of cold PBS was added to the dishes and the cells were scraped and collected in tubes and centrifuged at 3000 xg for 5 minutes, supernatant was carefully removed without disturbing the cell pellet and pellet was placed on ice.

Lysing:

The cell pellet was resuspended in 300 µl of IP lysis buffer [Tris (50 mm) pH 7.4, NaCl (100mM), NaF (200mM), Triton X (1 %), glycerol (10 %) and distilled water.] with inhibitors (1%) and DTT (0.1 mM) and incubated for 30 minutes at 4°C. The cells were sonicated twice at 30% amplitude for 10 seconds and centrifuged at 10,000 xg at 4°C for 10 minutes to remove the cell debris. The lysate was carefully collected and saved at 4°C.

Immunoprecipitation:

250 µl of IP binding buffer (1:1) ratio was added to 250 µl of lysate [binding buffer: Tris (50 mM) pH 7.4, KCl (100mM), EDTA (0.1 M), NP-40 (0.20%), Bovine serum albumin (BSA) (0.10%), glycerol (2.50%), DTT (2 mM), inhibitors (1 %) and distilled water], to it 2 µl of Flag antibody was added and for mock sample mouse IgG was added. 10 µl of Protein A conjugated sepharose beads was added and whole complex was nutated in the cold room at 4 °C for 2 hours. The beads were washed 3 times with 1 ml IP wash buffer [Tris (100 mM) pH 7.4, NaCl (100mM), NP- 40 (0.40 %), DTT (2 mM), inhibitors (1%) and distilled water]. After the final wash, 25 µl of IP wash buffer was added to approximately 25 µl of beads, and 10 µl of 6 x sample buffer. For the input samples, 10 µl of 6 x sample buffer was added to 50 µl of untransfected lysate, Rint-1 plus E2 full length, E2-CH and E2-NH lysates. The samples were boiled at 95 °C for 10 minutes and separated on an 8 % SDS-gel. The proteins on the gel were then transferred on to PVDF membrane and blocked in 5 % milk in TBS-T for 1 hour and the milk was

replaced with 5 % milk in TBS-T with primary antibody goat Rint-1 (santa Cruz) 1:500 for 1 hour at room temperature, washed 5 times in 1 x TBS-T followed by incubating in 5 % milk in TBS-T with secondary antibody (sheep/goat HRP) 1:10000 for 1 hour and washed the membrane again in 1 x TBS-T for 5 times and finally the membrane was covered in ECL reagent for 3 minutes and detected using a Fusion digital imaging system.

Sub-cellular fractionation experiment:

2.5 µg of E2 full length DNA, 2.5 µg of Rint-1 DNA was used and the amount of DNA was made up to 5 µg with 2.5 µg of Salmon sperm DNA. In three different tubes added E2 DNA and Salmon sperm DNA, Rint-1 DNA and salmon sperm DNA and E2 DNA and Rint-1 DNA were added respectively. 300 µl of serum free DMEM and 10 µl (1:2) of X-treme gene was then added and the mixtures incubated at room temperature for 20 minutes. After incubation samples were added drop wise to the C33a cell dishes and placed in an incubator at 37°C, 5% CO_2. The next day the medium was replaced and after 48 hours of transfection, the cells were harvested by seeding them at 5 x 10^6 cells per set and collected in to 10 ml tubes and centrifuged at 500 g for 5 minutes, the supernatant was discarded and pellet was resuspended in 5 ml of PBS and centrifuged at 500 xg for 5 minutes, supernatant was discarded and pellet was resuspended in 500 µl of PBS and transferred to Eppendorf tubes and centrifuged in micro centrifuge at 1000 xg for 5 minutes. The supernatant was discarded and pellet was saved on ice at 4°C for fractionation experiment.

Fractionation:

Fractionation experiment was carried out following a standard Thermo-scientific protocol. To approximately 20 µl of cell pellet 200 µl of ice cold CEB (cytoplasmic extraction buffer) with 2 µl of protease inhibitor was added and incubated on ice at 4°C for 10 minutes with gentle mixing with hand. The samples were centrifuged at 500 xg for 5 minutes and the cytoplasmic extract transferred in to pre chilled tube on ice at 4°C. 200 µl of ice cold MEB (membrane extraction buffer) with 2 µl of protease inhibitors was added to the cell pellet, the tubes were vortexed for 5 seconds and incubated at 4°C with gentle mixing for 10 minutes. The samples were then centrifuged at 3000 xg for 5 minutes, and the membrane extract transferred to a pre chilled tube on ice at 4°C. 100 µl of ice cold NEB (nuclear extraction buffer) with 1 µl of protease inhibitor was added to the cell pellet and vortexed for 15 seconds, samples were incubated at 4°C for 30 minutes with gentle mixing, centrifuged at 5000 xg for 5 minutes and the supernatant was transferred to a pre chilled tube on ice at 4°C. 100 µl of chromatin bound extraction buffer was added to the cell pellet (Chromatin bound extraction buffer was prepared with 100 µl of room temperature NEB, 5 µl of $CaCl_2$ (100 mM), 3 µl of micrococcal nuclease and 1 µl of protease inhibitor), and the samples were vortexed for 15 seconds and incubated for 15 minutes RT, vortexed again for 15 seconds and centrifuged at 16 000 xg for 5 minutes. The chromatin bound nuclear extract was transferred to pre chilled tube and stored on ice at 4°C. 100 µl of room temperature PEB (pellet extraction buffer) with 1 µl of protease inhibitor was added to the cell pellet which was vortexed for 15 seconds and incubated at room temperature for 10 minutes, centrifuged at 16,000 xg for 5 minutes and the cytoskeletal extract was transferred to pre chilled tube on ice. To 15 µl of

the fractions, added 15 µl of 6x sample buffer boiled the samples for 10 minutes and the fractions are separated on 10% and 12% SDS-PAGE.

siRNA Knockdown:

Oligofectamine was used for the transfection. In 2 tubes 1 ml of serum free DMEM was taken and 20 µl of oligofectamine was added. 20 µl of control siRNA or Rint-1 siRNA was added to separate tubes and incubated at room temperature (RT) for 30 minutes. The transfection mixes were added to 6 cm C33a cell dishes drop wise (before adding the dishes were washed with PBS and added 1 ml of Serum free DMEM) and the dishes were placed in the incubator at 37˚C, 5% CO_2 overnight. Next day the serum free DMEM was replaced with 4 ml of DMEM with 10% fetal bovine serum and placed again in the incubator at 37˚C for 24 hours. The samples were harvested in cold PBS and the pellet was placed on ice at 4°C. The cells were lysed in 300 µl of IP lysis buffer with DTT (0.1 mM) and inhibitors (1%) and incubated on ice for 30 minutes. Samples were sonicated twice at 30% amplitude for 10 seconds and centrifuged at 16,000 xg for 5 minutes at 4˚C. The lysate was collected and saved for downstream analysis to check the knockdown by western blotting.

Phosphatase Assay:

Transfection was carried out as mentioned in the co-immunoprecipitation methods using 5 µg of E2 DNA and Rint-1 DNA and X-treme gene (1:2) in C33a dishes. The cells were harvested by using 500 µl of cold PBS, 48 hours

following transfection. Cell pellet was stored on ice, lysis buffer was prepared without phosphatase inhibitors. (NaF and β-glycerophosphate and with added protease inhibitors and DTT) and 300 µl added to each cell pellet and incubated on ice for 30 minutes, sonicated twice at 30% amplitude for 10 seconds and centrifuged at 16 000 xg for 5 minutes. The lysate was collected and distributed equally in to 4 new tubes, the lysate in one tube was incubated on ice and rest of the tubes are Mock phosphatase treated or phosphatase treated by adding 2 µl of phosphoserine [P0114 (Sigma)] and incubated at 37 ˚C for 1 hour. After one hour, 30 µl of lysate was used as input and rest of the lysate was used to co-immunoprecipitate with HPV 16 sheep E2 antibody (experiment was performed as mentioned in Co-IP method).

IV. Results

1) Characterise Rint-1 expression constructs and antibodies:

To work with Rint-1 protein, mainly to check the functional interactions of Rint-1 with E2 protein, transfections needed to be performed, for this purpose purified plasmid DNA was required. The HA tagged Rint-1 plasmid DNA was purified by maxi prep (Qiagen).

a) Plasmid DNA purification:

The Rint-1 plasmid DNA was purified for further downstream work. The concentration of DNA was determined using a Nano drop to measure the absorbance of DNA at 260/280 nm. The DNA concentration was 1227.4 ng/µl and the 260/280 ratio was 1.92, which indicates there is probably some RNA contamination or protein contamination.

The purified DNA was used to transfect both U2OS cells and C33a cells to optimise the transfection conditions and check the efficiency of transfection. The transfections in U2OS cells did not work well but it worked efficiently in C33a cells. So, this Rint-1 lysate was used to characterise the efficiency of different Rint-1 antibodies.

b) Testing different Rint-1 antibodies

Western blot with four different primary antibodies; Rabbit 2073 (1:500), Rabbit 2074 (1:500) [these two are gifts from Androphy lab

(USA)], HA mouse (1:500) (Covenance) and goat Rint-1(1:500) (Santa Cruz Biotechnologies) and respective secondary antibody (1:10,000); anti-Rabbit HRP, anti-mouse HRP and anti-goat HRP (as mentioned in materials and methods). The results are presented in figure 1. After comparison of all the Rint-1 antibodies used, in figure 1, a, b, c and d, it was concluded that Goat Rint-1 antibody was highly specific compared with other three.

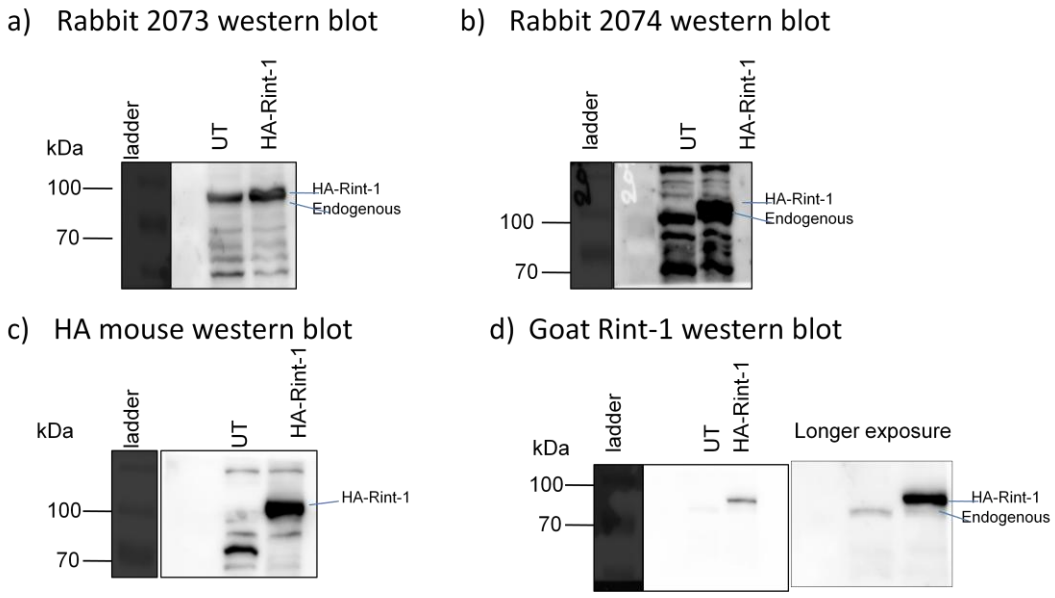


Figure.1: Characterization of different Rint-1 antibodies. Figure 1 a, b, c and d shows Rabbit 2073, Rabbit 2074, HA mouse and Goat RInt-1 western blots respectively and Rint-1 lane shows the presence of Rint-1 protein at approximately 80 kDa.

2) Determine the RInt-1 binding site within HPV16 E2 *in vitro*.

As per my aim to further characterise the interactions between E2 and Rint-1, I tried to determine the Rint-1 binding site on HPV16 E2 using GST-pull down an *in vitro* binding assay. Before proceeding to the pull down step, the presence of purified GST proteins was checked by SDS-PAGE and coomassie staining.

a) Coomassie staining

The amount of protein required for pull down assay was quantified by coomassie staining and the results were shown in figure 2.a. The intensity of the bands in GST lane, E2NH and E2CH lane shows there is an approximately equal amount of each protein in all the lanes.

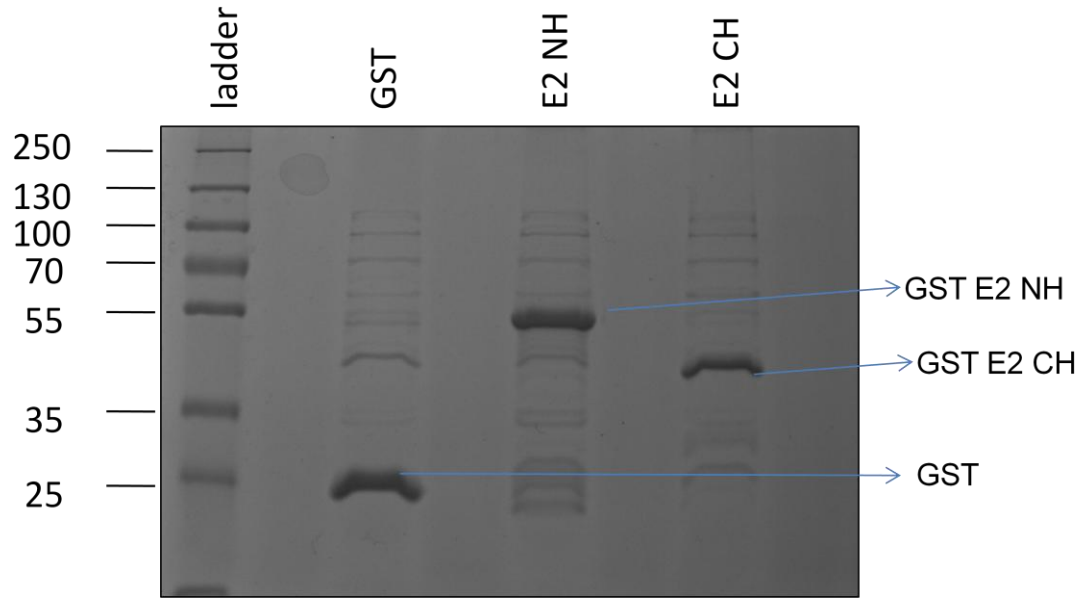


Figure 2.a): Purification of GST E2 proteins: Coomassie staining results. The presence of bands in GST lane, E2NH and E2CH lanes was visualised and the amount of protein resin mixture required for GST-pull down assay was quantified as 20µl for 100 µl of Rint-1 lysate.

From the above results, I made sure that there is approximately equal amount of each protein and estimated required amount as 20µl.

b) GST- pull down *in vitro* binding assay

Following the above results, 20 µl of protein-bound resin was incubated with 100 µl of Rint-1 lysate (method was explained in materials and methods). The samples were washed and separated by SDS–PAGE and western blotted with goat Rint-1 primary antibody and anti-goat HRP secondary. The results in figure 2.b show a representative result of three experimental repeats. From figure 2.b, the absence of Rint-1 binding to E2 NH, E2 HC and GST lanes in presence of Rint-1 indicates that Rint-1 was not binding to any of the E2 domains *in vitro*. This result was the same in three experimental repeats.

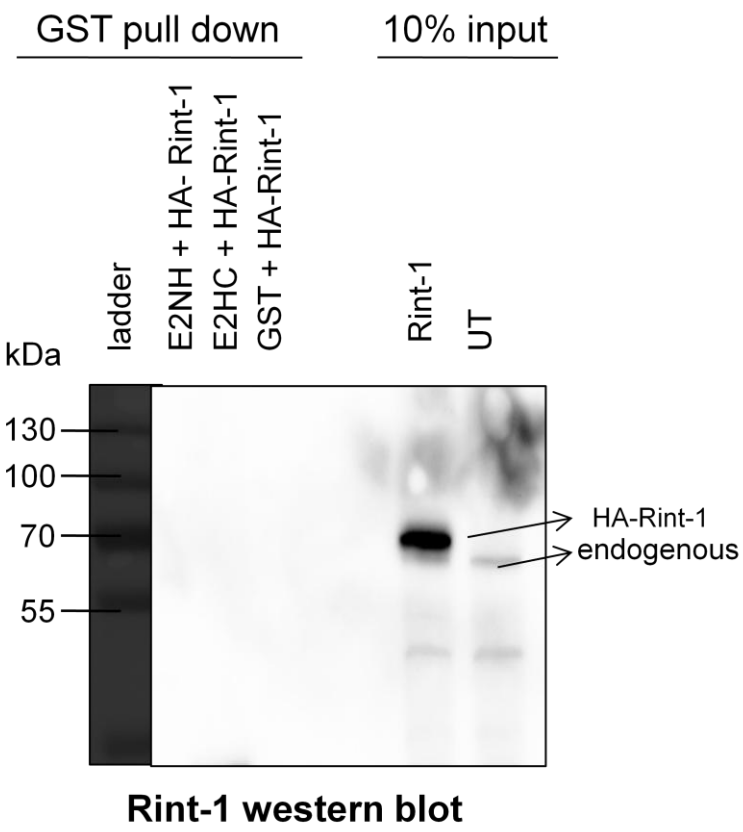


Figure 2.b): GST-pull down *in vitro* binding assay results. The Rint-1 western blot results shows at approximately 80 kDa, the presence of Rint-1 protein was observed in the Rint-1 input samples, but there was no protein bands in E2 NH (HPV16 E2 N-terminus), E2 HC (HPV16 E2 C-terminus) and GST lanes in presence of Rint-1 protein.

3) Analysis of the interaction of Rint-1 with truncated E2 proteins *in vivo.*

Because Rint-1 protein was not binding to HPV16 E2 proteins *in vitro*, the interaction of Rint-1 protein with HPV16 E2 was assessed *in vivo* by co-immunoprecipitation.

a) Co-immunoprecipitation

Five µg of truncated E2 DNAs with 5 µg of Rint-1 DNA and (1:2) X-treme gene was used for transfection in C33a cells. Antibodies raised against Rint-1 and HPV E2 were used for immunoprecipitation (materials and methods) and a representative result of three experimental repeats was shown in figure 3.1.a. The appearance of specific bands at approximately 80kDa indicates Rint-1 binding to Flag tagged E2 full length and E2 NH and not to E2 HC. However, due to the presence of less protein in E2 HC lane in Rint-1 western blot and more protein in E2 HC lane in E2 western blots compared with E2 NH lanes in both the western blots, it cannot be concluded that the Rint-1 protein was binding to N-terminus but not C-terminus. The graphs in figure.3.1.b and c, shows the IP proteins and input proteins in X-axis and its percentage of protein in Y-axis of densitometric scanning.

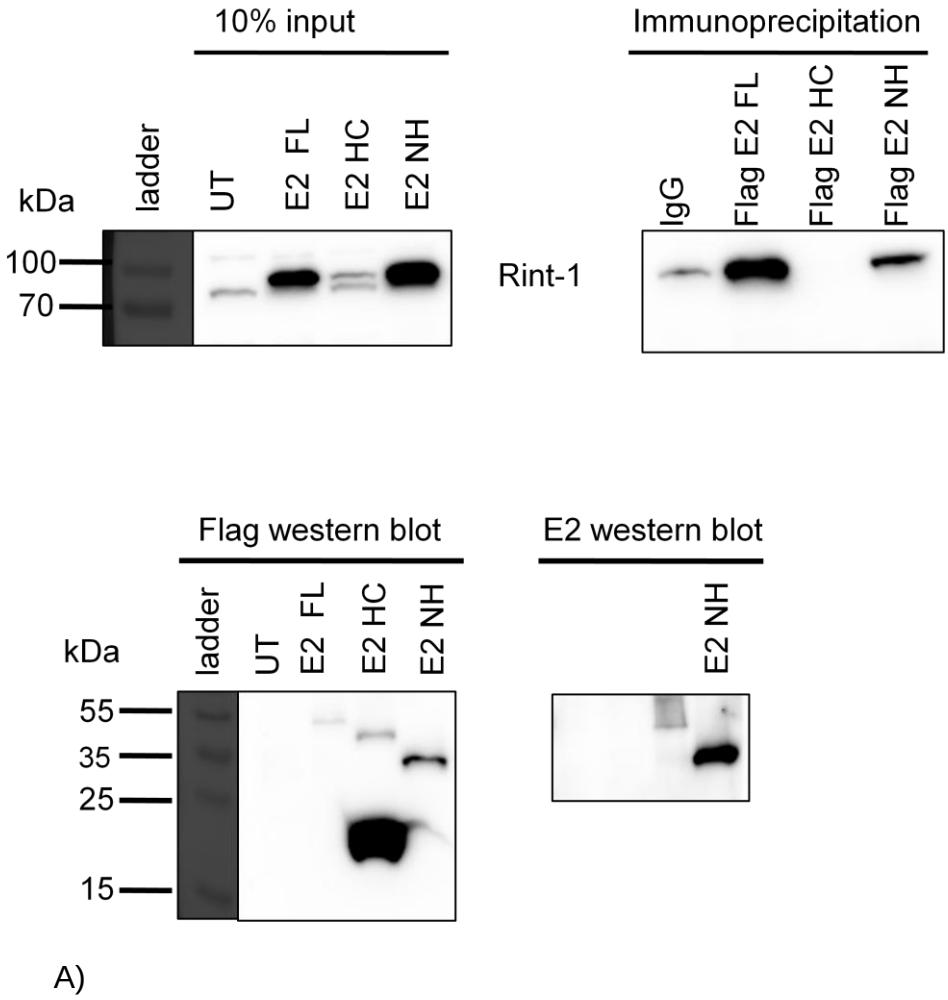
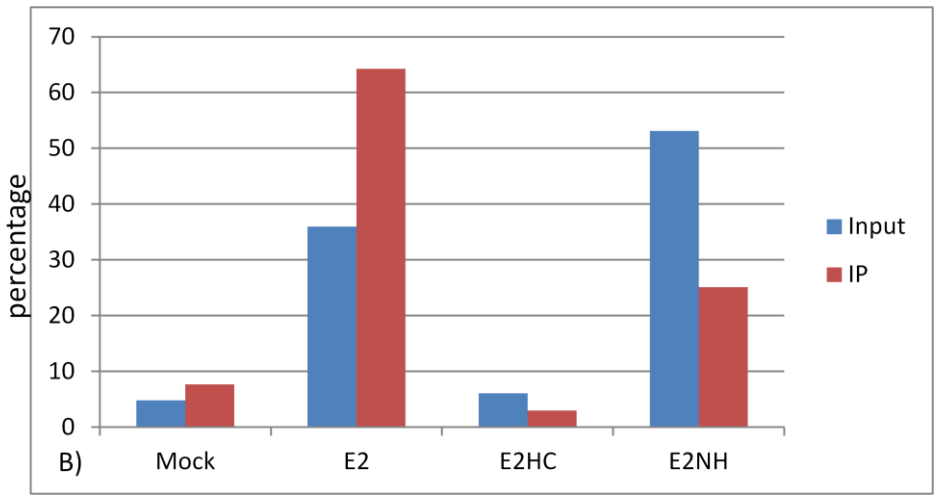
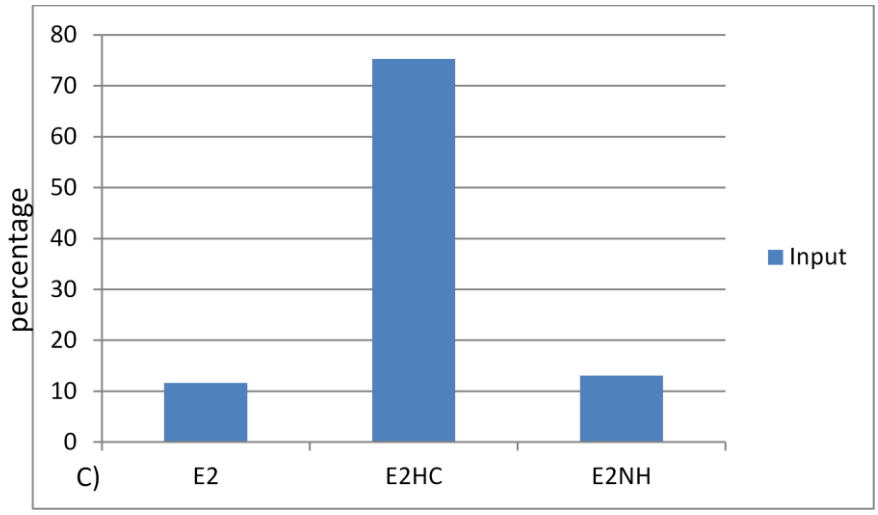


Figure 3.1.a): Co-immunoprecipitation. The Rint-1 and E2 was co-immunoprecipitated from C33a cell lysate and the immunocomplexes and 10% inputs were resolved on SDS-PAGE and western blotted by using goat Rint-1 primary antibody (1:500) and anti-sheep/goat HRP secondary antibody (1:10,000) and for inputs, Flag primary antibody (1:1000) and HPV 16 E2 primary antibody (1:500) and anti-mouse HRP secondary and anti-sheep HRP secondary antibody (1:10,000) respectively. In the Rint-1 western blot, Rint-1 protein was seen at approximately 80 kDa. In the E2 western blot, E2 FL was seen at approximately 55 kDa and E2 NH and E2 CH were seen at approximately 35 kDa and 25 kDa respectively.



Rint-1



E2

Figure 3.1.b) and c): Graphical representation of three Co-Ip experimental repeats. In figure 4 B) X-axis shows inputs for E2 FL, E2 CH and E2 NH and immunoprecipitants of Rint-1 and E2 FL, Rint-1 and E2 CH and Rint-1 and E2 NH in Rint-1 and Y-axis shows the percentage of the protein in Rint-1 western blot. Figure 4 C) shows inputs, E2 FL, E2 CH and E2 NH in X-axis and its percentage on Y-axis in E2 western blot.

From the above results, it is difficult to conclude that Rint-1 was binding to one domain of E2 more than the other, therefore further optimisation of the

conditions by reducing the amount of E2 HC DNA used for transfection. 1 µg of E2 HC DNA was used instead of 5 µg and by following the same steps of transfection as for the above experiment (method was explained in materials and methods section), the experiment was repeated and results are presented in figure 3.2.a. The result in 3.2.b and c shows that Rint-1 associates with the N-terminus but not C-terminus of E2. Yet it cannot be concluded from these results that Rint-1 binds to N-terminus but not C-terminus because there is still less protein in E2 HC lane in Rint-1 western blot and more protein in E2 western blots compared with E2 NH lanes in both western blots.

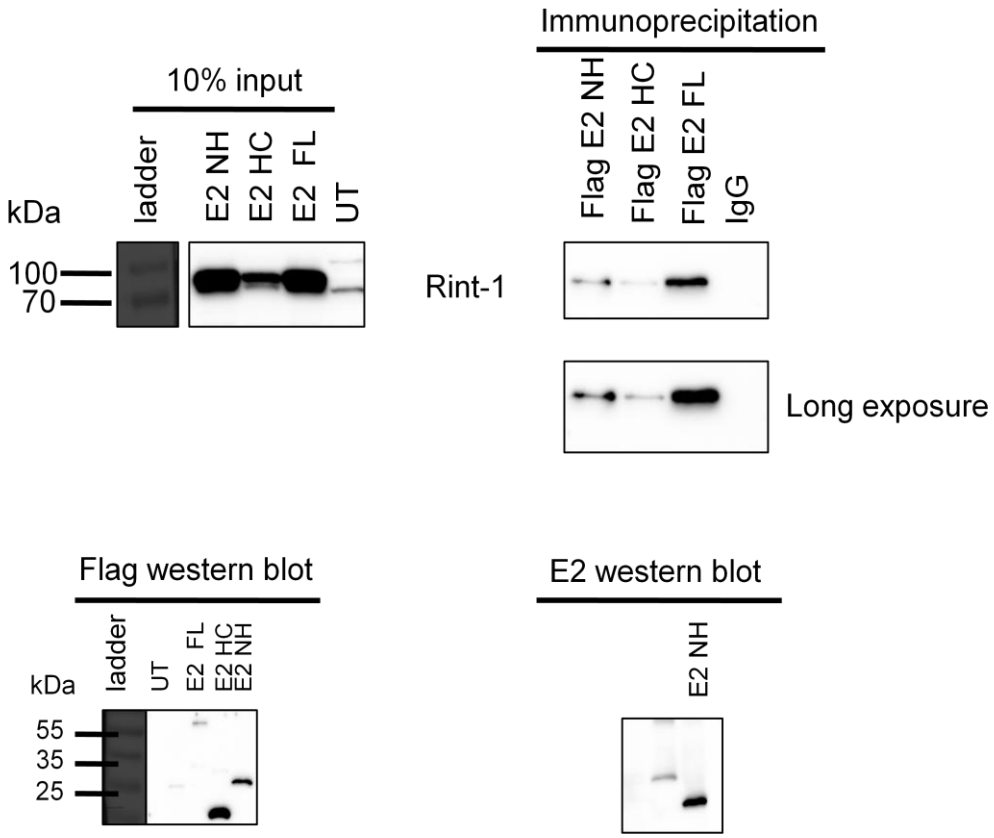
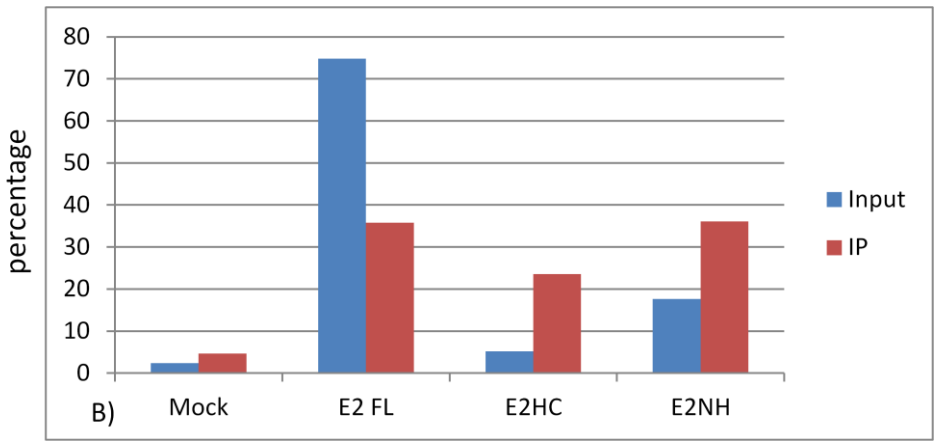
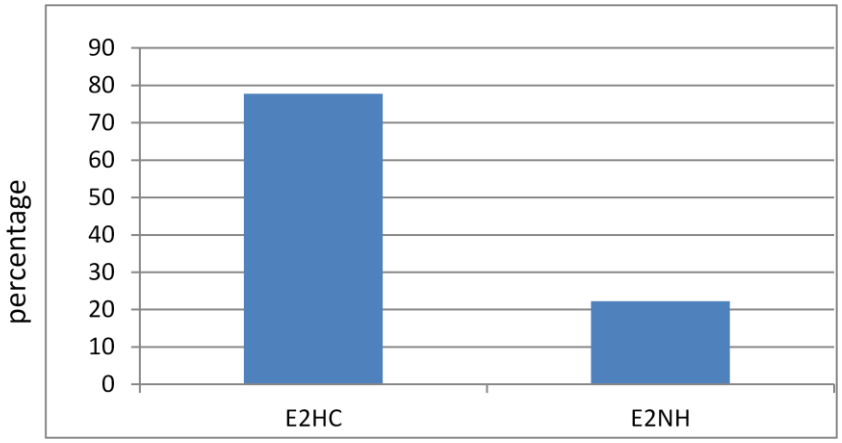


Figure 3.2.a): Co-immunoprecipitation. The Rint-1 and E2 was co-immunoprecipitated using 5 µg of Rint-1 DNA, 5 µg of E2 FL and E2 NH DNA and 1 µg of E2 CH DNA from C33a cell lysate and the immunoprecipitated complexes and 10% inputs were resolved on SDS-PAGE and western blotted. In the Rint-1 western blot, Rint-1 protein was seen at approximately 80 kDa. In the E2 western blot, E2 FL was seen at approximately 55 kDa and E2 NH and E2 CH were seen at approximately 35 kDa and 25 kDa respectively.



Rint-1



E2

Figure 3.2.b) and c): Graphical representation of co-ip. In figure 3.2.b) X-axis shows inputs for E2 FL, E2 CH and E2 NH and immunoprecipitants of Rint-1 and E2 FL, Rint-1 and E2 CH and Rint-1 and E2 NH and Y-axis shows the percentage of the protein in Rint-1 western blot. Figure 3.2.c) shows inputs E2 CH and E2 NH in X-axis and its percentage on Y-axis in E2 western blot.

The conditions of this experiment were further optimised by transfecting the C33a cells with 200ng and 500ng of E2 HC DNA with 5 µg of Rint-1 DNA and for comparison 5 µg of E2 NH DNA with 5 µg of Rint-1 DNA was transfected (as mentioned in Materials and Methods section).

b) E2 HC western blots

The samples were separated by SDS-PAGE and western blotted for Rint-1 and E2 by using (1:500) goat Rint-1 (Santa Cruz Biotechnologies) and (1:500) HPV16 sheep E2 primary antibodies and anti-sheep/goat HRP secondary antibodies (mentioned in materials and methods) and the results were shown in figure 3.3. From the figure, In the Rint-1 western blot, at approximately, 80 kDa Rint-1 protein was seen in E2 HC lanes (200ng and 500ng DNA) and E2 NH lane (5 µg DNA). At approximately 15 kDa and 35 kDa, E2 HC and E2 NH proteins were seen in Flag western blots. The band intensity in E2 HC 500ng lane was similar to E2 NH lane. The β-actin western blot served as a loading control to check the amount of protein loaded in the lanes are all equal.

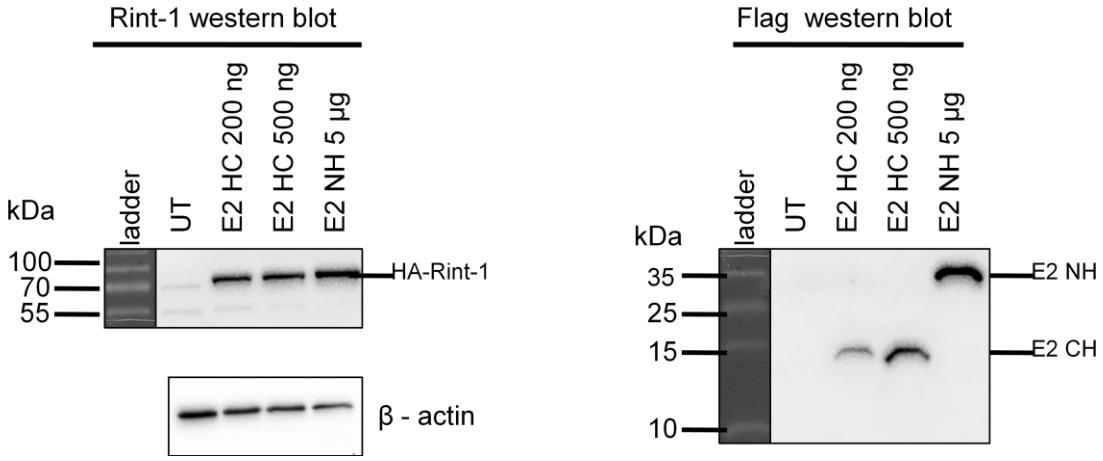
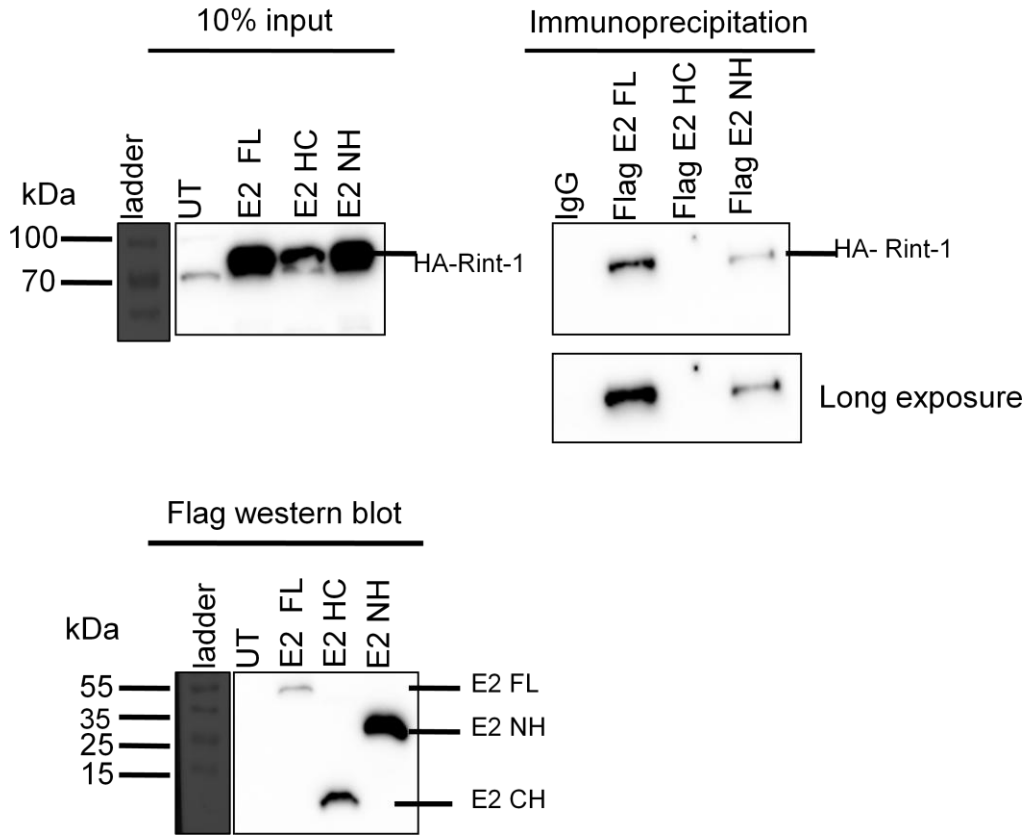


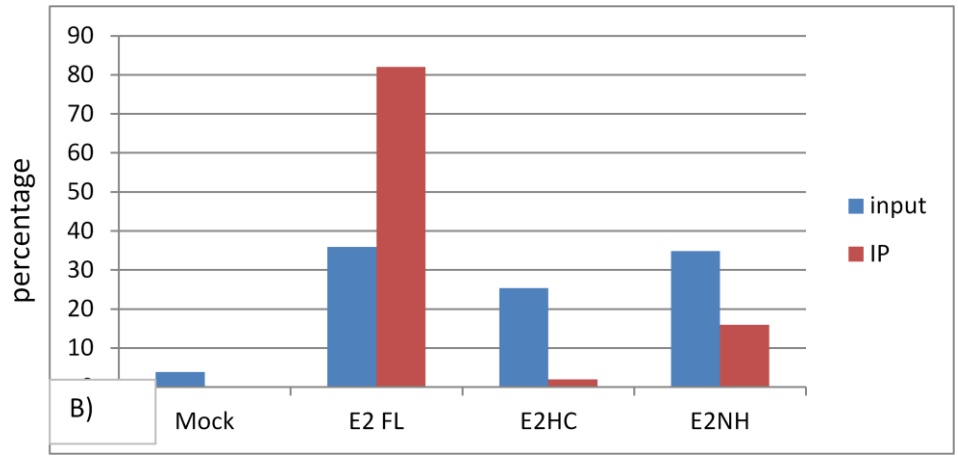
Figure 3.3): E2 western blot. Figure shows Rint-1 and Flag western blot results. Lane 1 was untransfected (UT), lane 2 and 3 are E2 HC 200ng and 500ng DNA with 5 µg of Rint-1 DNA and lane 4 was 5 µg of E2 NH DNA with 5 µg of Rint-1 DNA. The presence of Rint-1 protein was confirmed at 80 kDa in Rint-1 western blot and at approximately 15 kDa and 35 kDa in flag western blot, the presence of E2 HC and E2 NH proteins were confirmed. β -actin western blot was internal loading control.

From the above results, the amount of E2 HC DNA required for co-immunoprecipitation was optimised as 500ng. The co-immunopecipitation experiment was repeated with 500ng of E2 HC DNA instead of 5 µg and rest are all the same as above experiment (method explained in materials and methods). The results are shown in figure 3.4.a. The absence of immunoprecipitated Rint-1 in the E2 HC lane and presence of Rint-1 in the E2 NH lane confirms that Rint-1 interacts to the N-terminus of E2 but not the C-terminus. This was further confirmed by the presence of equal intensity of bands in E2 CH lanes compared to E2 NH lanes in both Flag and E2 western blots.



A)

Figure 3.4. a): Co-Immunoprecipitation. The Rint-1 and E2 was co-immunoprecipitated using 5 µg of Rint-1 DNA, 5 µg of E2 FL and E2 NH DNA and 500ng of E2 CH DNA from C33a cell lysate and the immunoprecipitated complexes and 10% inputs were resolved on SDS-PAGE and western blotted. In the Rint-1 western blot, Rint-1 protein was seen at approximately 80 kDa. In the E2 western blot, E2 FL was seen at approximately 55 kDa and E2 NH and E2 CH were seen at approximately 35 kDa and 25 kDa respectively.



B)

Rint-1

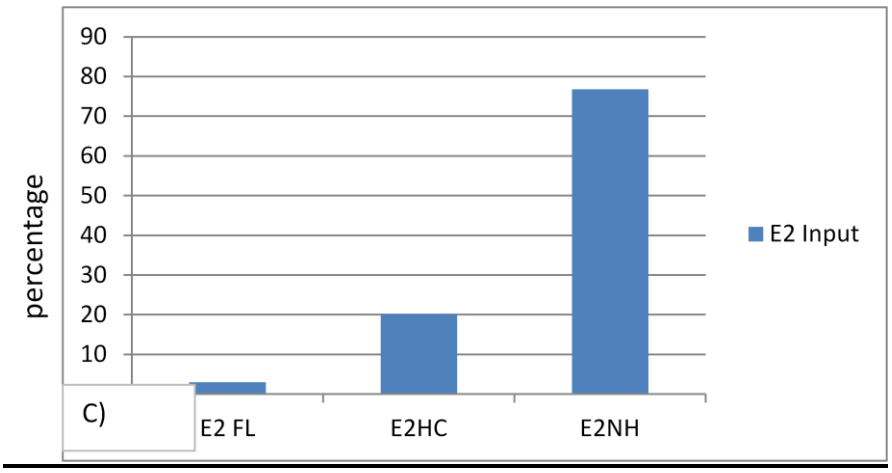


Figure 3.4.b) and c): Graphical representation of co-ip. In figure 4 B) X-axis shows inputs for E2 FL, E2 CH and E2 NH and immunoprecipitants of Rint-1 and E2 FL, Rint-1 and E2 CH and Rint-1 and E2 NH and Y-axis shows the percentage of the protein present in Rint-1 western blot. Figure 4 C) shows inputs E2 FL, E2 CH and E2 NH in X-axis and its percentage on Y-axis in E2 western blot.

From the above results, it was confirmed that Rint-1 protein interacts with N-terminus of HPV16 E2 protein *in vivo*.

4) Phosphatase Assay:

The data presented so far suggest that Rint-1 was interacting with E2 *in vivo* but not *in vitro.* So furthermore, I wanted to investigate whether any post-translational modification for example phosphorylation is required for the reason behind the interaction. Therefore, I tried analysing by phosphatase treated IP. Five µg of E2 full length DNA with 5 µg of Rint-1 DNA (1:2) X-treme gene was used to transfect C33a cells (explained in materials and methods section). E2 and Rint-1 lysate was divided in to 3 sets and the 1st set was kept

at 4˚C, the 2nd set was mock phosphatase treated and the 3rd set was phosphatase treated with lambda phosphatase (as explained in methods) and samples were resolved by SDS-PAGE and western blotted (as mentioned in materials and methods) the results are presented in figure 4. As shown in figure 4, though the experiment seems to have worked, as there is a reduction in band intensity in phosphatase treated lane in the inputs, probed with phosphothreonine primary antibody there was more Rint-1 co-immunoprecipitated in the phosphatase treated lane. This phosphatase treated IP results suggests that, phosphorylation is not the reason for Rint-1 protein not binding to E2 *in vitro*.

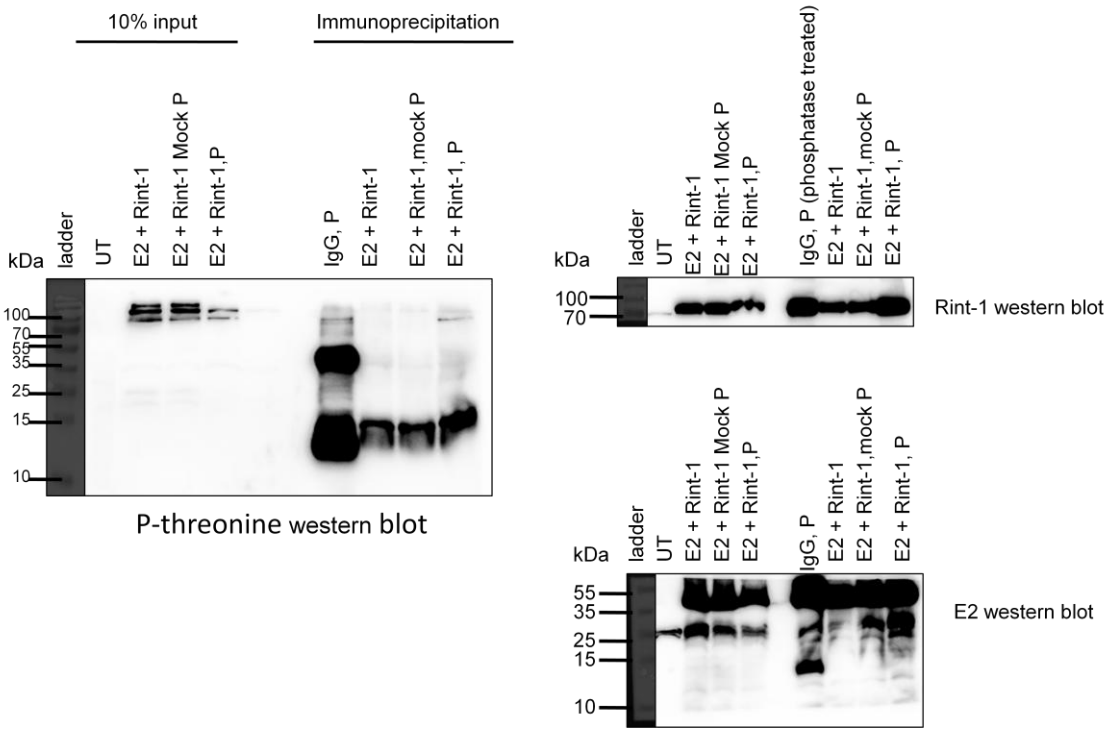


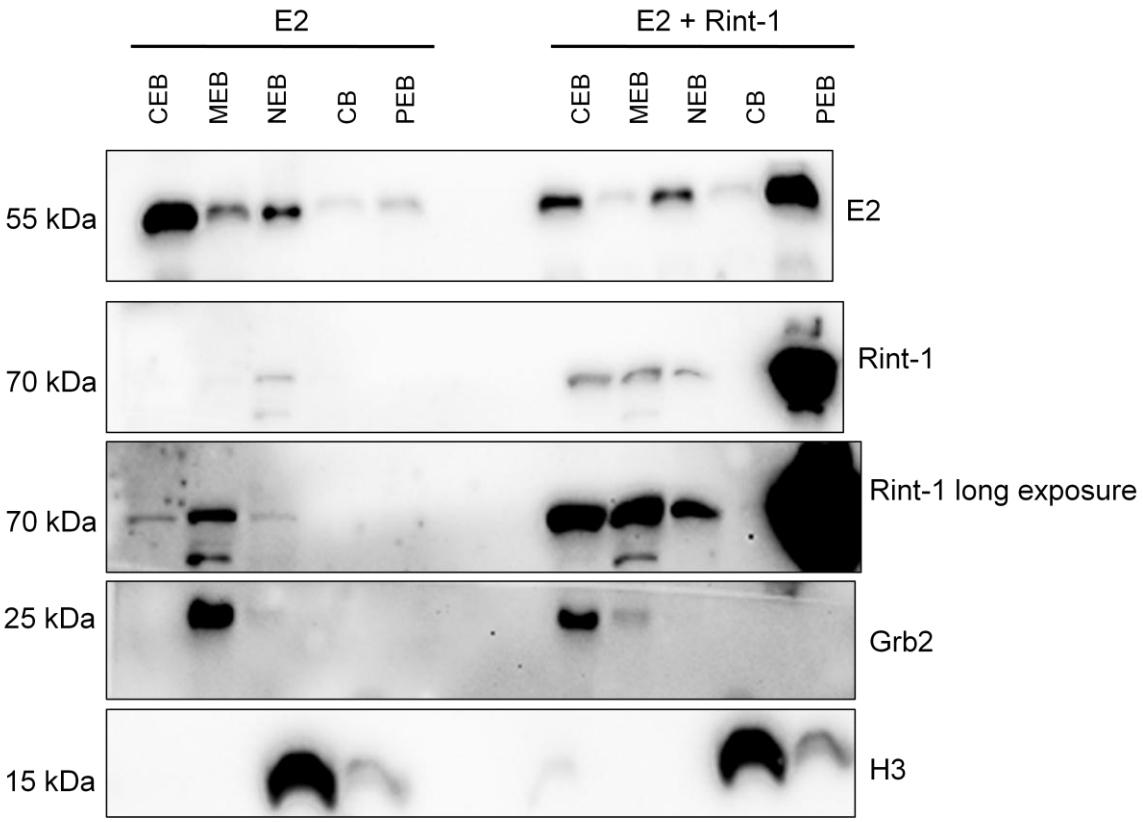
Figure 4: Phosphatase treated IP. Figure shows phosphatase IP, Rint-1 and E2 western blot results. The Rint-1 and E2 was co-immunoprecipitated using 5 µg of Rint-1 DNA, 5 µg of E2 FL and E2 NH DNA and 500ng of E2 CH DNA from C33a cell lysate and the immunoprecipitated complexes and inputs were either phosphatase treated or not phosphatase treated and the samples were resolved on SDS-PAGE and western blotted with (1:1000) phospho-threonine primary antibody (Cell Signalling antibody #9386) and (1:10,000) anti-mouse secondary. The second set was western blotted for Rint-1 using (1:500) goat-Rint-1 primary, (1:10,000) anti-goat secondary and for E2 using (1:500) TVG E2 primary and (1:10,000) anti-mouse HRP secondary antibody. The results shows E2 has been masked by IgG.

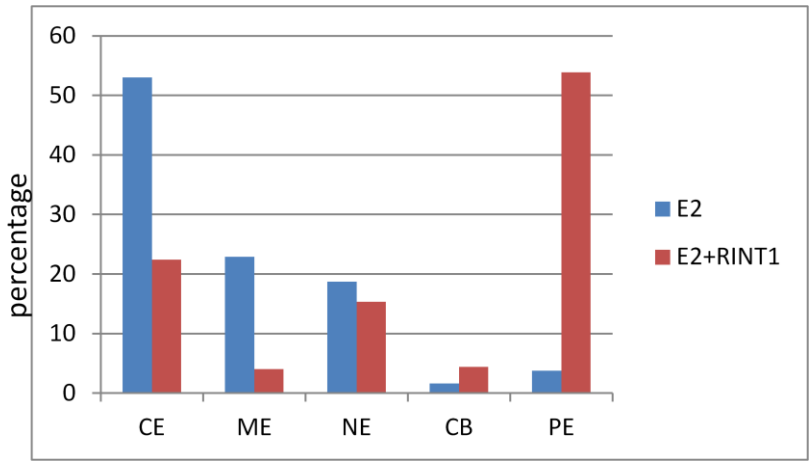
From, the above results it was confirmed that phosphorylation was not the reason for Rint-1 not binding to E2 *in vitro*. There could be other post translational modifications involved in this such as sumoylation, ubiquitination etc. As Rint-1 protein interaction with HPV16 E2 proteins was confirmed *in vivo*.

5) Sub cellular Fractionation:

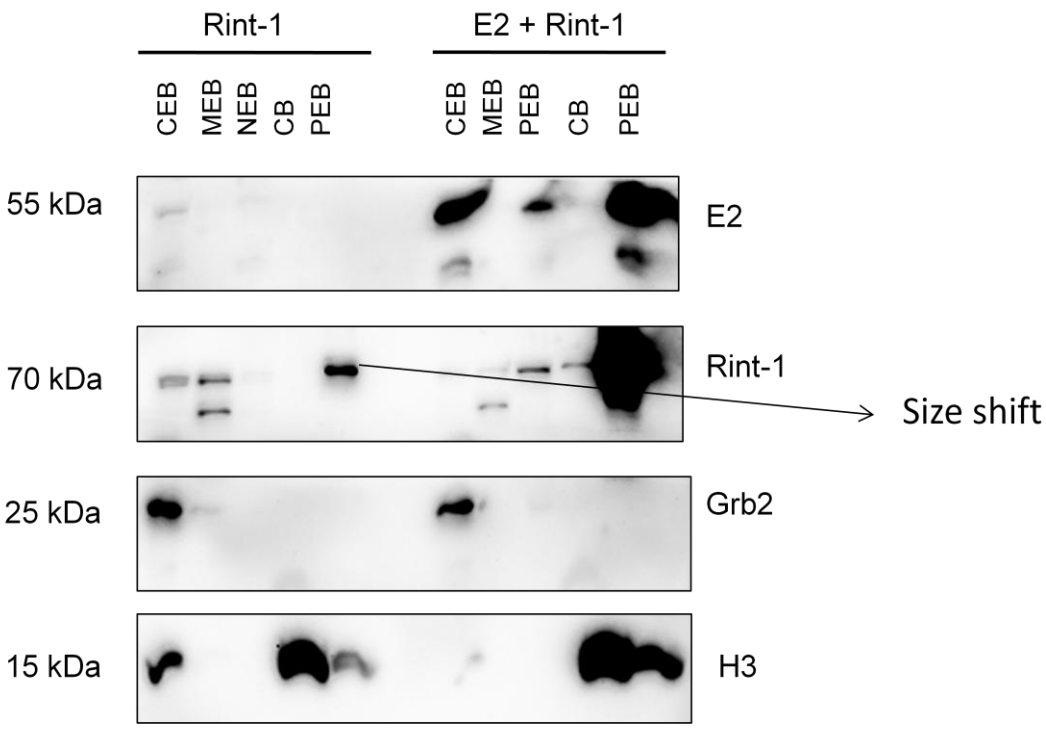
To confirm that Rint-1 and E2 relocalise to cytoplasmic compartments, I analyzed the localization of HPV16 E2 proteins and Rint-1 proteins in sub cellular localisation by sub cellular fractionation.

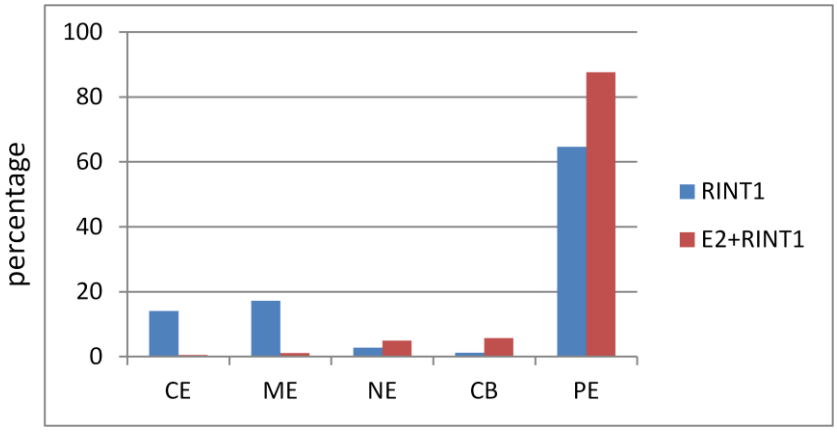
2.5 µg of E2 (full length DNA), Rint-1 DNA and E2 and Rint-1 DNA was transfected into C33a cells and fractionated. The 5 fractions [cytoplasm extract (CE), membrane extract (ME), nuclear extract (NE), chromatin bound extract (CB) and the pellet extract (PE)] of cells with Rint-1, E2 and E2 and Rint-1 co-expressed, were compared and analysed with each other through Rint-1 and E2 western blots. For fraction controls, samples were western blotted for Grb2 (cytoplasm), Orc2 (nuclear soluble), H3 (chromatin) and vimentin (cytoskeletal/pellet) and results were shown in figure 5.a and b. In the E2 alone samples, E2 was mainly localised in the nucleus and also in cytoplasam, Rint-1 protein was expressed alone predominantly localised in the membrane extract and there is a size shift in the pellet fraction, which might indicate post-translational modification. However, Rint-1 co-transfected with E2 resulted in a dense accumulation of protein in cytoskeletal/pellet fraction in all the three repeats. To prove the amount of protein loaded in the wells was not the reason for the dense accumulation of E2 and Rint-1 in the pellet fraction, the UT fractions were compared with E2 alone and Rint-1 alone samples. These samples had very little E2 or Rint-1 in the pellet fraction as shown in figure 5.c. This proves E2 and Rint-1 together localise in pellet fraction. The standard deviation of three fractionation repeats of E2 and E2 + Rint-1, Rint-1 and E2 + Rint-1 was shown in Figure 5.d.





E2 protein localisation





Rint-1 protein localisation

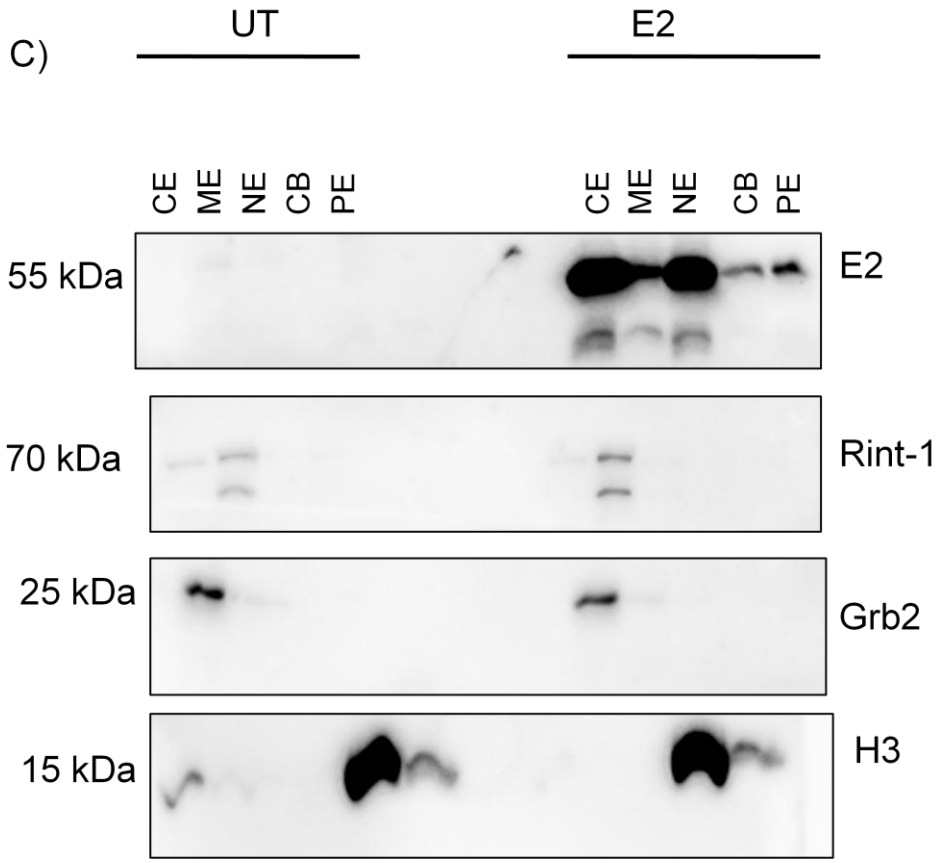
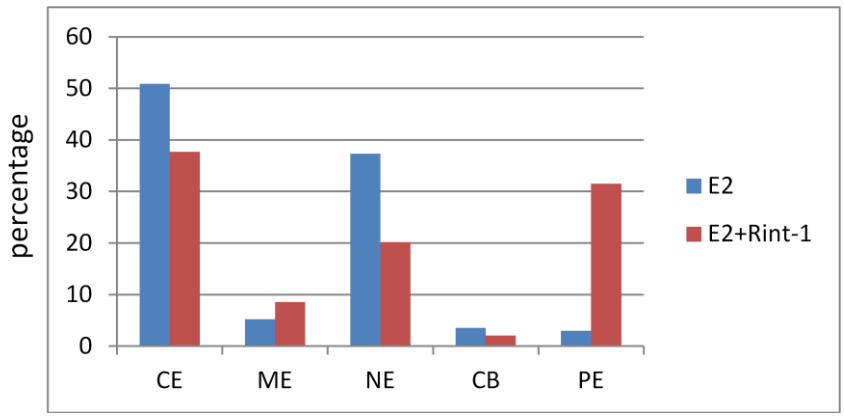
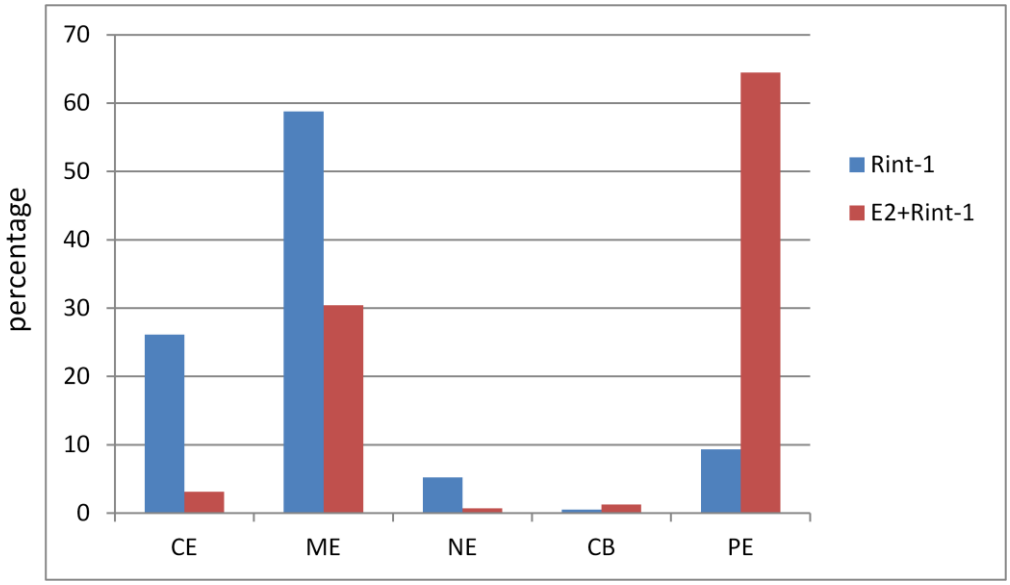


Figure 5. a, b, c): 5 fractions from cytoplasm (CE), membrane (ME), nuclear (NE), chromatin (CB) and cytoskeletal/pellet (PE) of E2, Rint, E2+Rint-1 and untransfected (UT) are shown with Rint-1, E2 and control western blots. There is a size shift in the Rint-1 pellet fraction in the Rint-1 western blot. Western blots were quantified by taking the the 5 fractions of E2, Rint-1 and E2 and Rint-1 on X-axis and its percentage on Y-axis.



E2 protein +/- Rint-1



Rint-1 protein +/- E2

Figure 5.d) : Standard deviations of three fractionation repeats of E2, E2+Rint-1 and Rint-1, E2+Rint-1.

6) Rint-1 siRNA Knockdown

Although it has been shown previously that E2 and Rint-1 localises in the cytoskeletal fraction, we furthermore, wanted to address this by determining the localisation of HPV16 E2 following siRNA-mediated depletion of Rint-1.

For this, to optimise the amount of Rint-1 siRNA required for the depletion of Rint-1, 50nM, 100nM and 200nM of Rint-1 siRNA and 200nM of control siRNA were transfected in C33a cells using Oligofectamine reagent and depletion was analised by Rint-1 western blot. β-actin western blot was also carried out for a loading control and results were shown in figure 6. From the results, the gradual depletion of Rint-1 from 50nM lane to 200nM lane was observed. This indicates that siRNA mediated knockdown of Rint-1 was successful and that 200nM siRNA was optimal and optimised the amount of Rint-1 siRNA required for depletion of Rint-1 as 200nM.

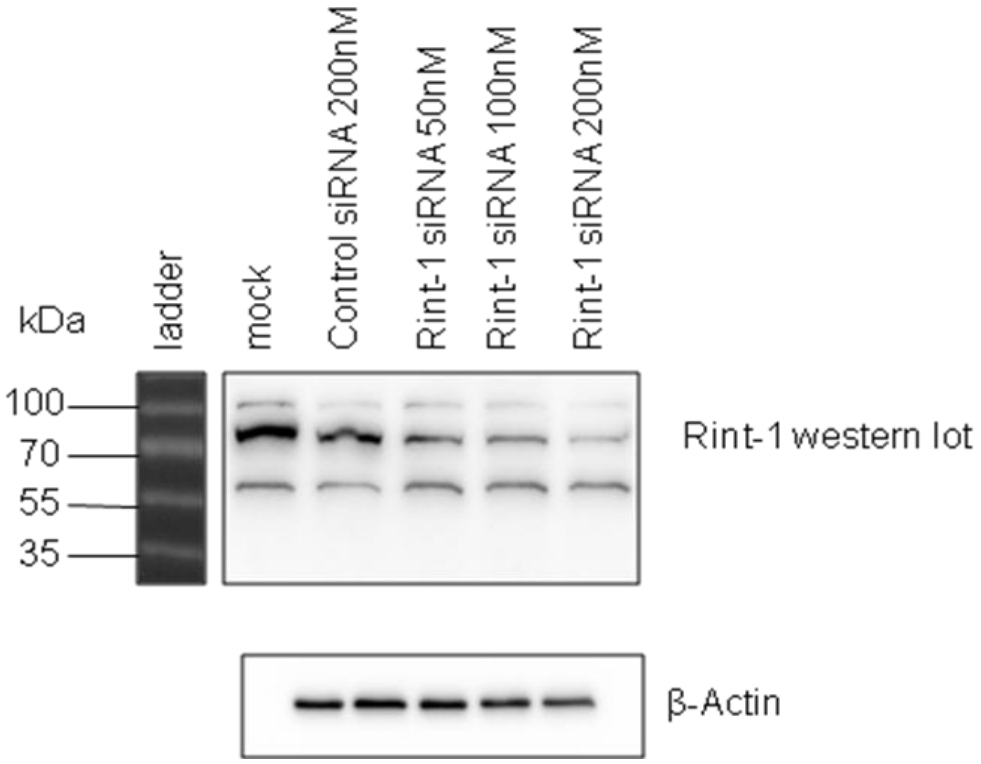


Figure 6: Rint-1 siRNA knockdown: C33a cells were transfetced with control siRNA 200nM, Rint-1 siRNA 50nM, 100nM and 200nM respectively and were analysed by Rint-1 western blotting to evaluate the knockdown. Loading control, β-actin western blot results shows the amount of protein loaded in all the wells are same.

As the knockdown was successful in C33a cells, to check the localisation of HPV16 E2, the siRNA knockdown was repeated in U2OS cells (as they are engineered to stably express E2) and E2 localisation determined by

immunoflorescence. Unfortunately, the knockdown in U2OS cells was not successful, and the immunoflorescence showed negative results in two repeats because the U2OS cells were not adequately expressing E2. If the experiment worked then it might have made it easier to check the localisation of HPV E2 and Rint-1 by immunoflorescence and sub-cellular fractionation. However, due to lack of time I could not proceed further with optimising the experimental conditions and trying in new clones of U2OS cells.

v. Discussion

Human papillomavirus (HPV) causes a wide range of infections in human beings resulting in a high percentage of cervical cancers and other anogential and oropharyngeal cancers. HPV E2 protein plays a vital role in subsequent productive and persistant viral infections (Muller et al., 2012). Most of the infections tend to persist with or without immune activation (Doorbar, 2005). HPV E2 being a multi functional protein, can interact with numerous other cellular proteins involved in different functions, by interacting with some proteins essential for viral transport pathways, HPV E2 might play a vital role during early stages of HPV infection. Unexpectedly, HPV E2 protein seems to have a role in modulating intracellular trafficking by interacting with cellular proteins involved in vesicle-mediated transport, which tends to alter the intracellular membrane organelle dynamics and likewise affecting its maintenance (Muller et al., 2012). Mostly these associated cellular protein targets are concentrated on Golgi apparatus, So Golgi in association with type II MHC molecules can act as centre for translocating processed viral antigens and there by altering the infected keratinocyte's antigen presentation (Muller et al., 2012). All these reasons show HPV E2 might have the potential to target host cell intracellular trafficking pathways by interacting with several cellular proteins which function in vesicle mediated transport.

The membrane bound compartments in eukaryotic cells interact with cellular environment by vesicle mediated intra-cellular trafficking which aid in the transportation of proteins and lipids in between intracellular compartments by cargo loaded vesicles originated from donor compartments formed of specific

adaptor or coat proteins (COPI, COPII and clathrin), then targeted to adaptor compartments where vesicle attachment takes place through tethers and with the involvement of different N-ethylmaleimide sensitive factor attachment protein receptor (SNARE) proteins, membrane fusion occurs (Tokarev, 2009) and by exocytic pathways and endocytic pathways, shuttling of materials takes place. Three different SNARE proteins, BNIP 1, p31 and syntaxin 18 together with some membrane proteins, ZW10, Rint-1 and Sly1 form the syntaxin 18 complex which mediates vesicle transport. Together Rint-1 and ZW10 form a complex within the syntaxin 18 complex, it targets membrane associated receptor (SNAP) complex by an ER localised SNARE complex, which facilitates a vital role in vesicle trafficking between ER and Golgi apparatus (Hirose et al., 2004, Aoki et al., 2009).

Rint-1 is a membrane-associated protein that helps to mediate anterograde transport from ER to Golgi (Xiao et al., 2001) and regulates retrograde transport from the golgi back to the ER by interacting with cellular proteins (Arasaki et al., 2006). Deletions of Rint-1 from cells have adverse effects on Golgi functions such as protein sorting.

Previously, the Parish group has isolated an interaction between HPV16 E2 and Rint-1 *in vivo* and concluded that HPV16 E2 and Rint-1 co-localise in the cytoplasmic compartments of transfected cells which resembled ER like structures and they hypothesized that the association of HPV E2 with membrane associated protein Rint-1 is a part of a novel mechanism in which HPV modulates sub-cellular trafficking during HPV infection.

In my project, we further characterised the functional interaction between Rint-1 and HPV16 E2. In the first instance, we purified the Rint-1 plasmid DNA for transfections. U2OS cells (derived from osteosarcoma cell lines) were chosen for transfections because they are engineered to express E2. Rint-1 DNA was transfected using Lipofectamine reagent, but the transfection didn't work in two repeats, as lipofectamine was killing the cells. Therefore, we tried to optimise the transfection conditions, and Rint-1 DNA was transfected again in U2OS cells using calcium phosphate precipitation method, though the transfection worked this time, the cells were no longer expressing E2. So, we were unable to proceed further to check the interaction between Rint-1 and E2 using U2OS cells. To further examine the interaction, we transfected the Rint-1 DNA in C33a cells (derived from cervical carcinoma cell line) using X-treme gene and the transfection was successful. To proceed further, the lysate was used to test the efficiency of four different Rint-1 antibodies; Rabbit 2074 and Rabbit 2073, goat Rint-1, and HA which detects the HA epitope on exogenously expressed Rint-1, and found that optimised goat Rint-1 antibody was the most specific. Furthermore, to determine the Rint-1 binding site on HPV16 E2, an *in vitro* GST pull down binding assay was performed followed by western blot for Rint-1. Unfortunately the *in vitro* assay showed negative results in all the three repeats and it was concluded that Rint-1 does not bind to HPV16 E2 *in vitro*. To further examine the interaction, we tried to analyse the interaction of Rint-1 with truncated E2 proteins *in vivo* by co-immunoprecipitation (Co-IP), for this I transfected Rint-1 DNA and flag tagged truncated E2 DNAs in C33a cells and IP with flag antibody and western blotted to determine whether E2 truncations associated with Rint-1. Results from three repeats confirmed that Rint-1 was interacting with N-terminus of

E2. As the HPV E2 TAD, consists of intracellular localisation signals, which are transplantable to associated cellular proteins, the association of Rint-1 with N-terminus of HPV E2 could be the reason behind the involvement of HPV in sub-cellular trafficking during HPV infection because Rint-1 proteins was known to regulate retrograde transport from Golgi back to ER.

From the immunoprecipitation results, it was confirmed that the Rint-1 protein was interacting with the N-terminus of E2 protein. At the same time it raised a question, why was Rint-1 was interacting with E2 *in vivo* but not *in vitro*? There could be several reasons for this, such as post-translational modifications of E2, for example phosphorylation. Therefore, we tried to analyse this by phosphatase treating the lysate used in the IP, for this experiment Rint-1 and E2 were co-transfected and the lysates treated with phosphatase before Co-IP. Though the experimental results were not clear, the phosphatase treatment appeared to work as there was a reduction in phospho-proteins in the phosphatase treated inputs compared with the other samples in phospho-threonine western blot. However there was more co-immunoprecipitated Rint-1 in the phosphatase treated lanes, which suggests phosphorylation is not the reason for why Rint-1 protein was not interacting with E2 *in vitro*. There could be other post translational modifications involved in this, for example methylation, acetylation, ubiquitination, sumoylation, which needed to be explored in future research. E2 proteins are already known to have post translational modifications through ubiquitination and sumoylation because sumoylation is a post translational modification predominantly seen in transcriptional factors and E2 proteins are well known to play a key role in transcriptional control (Wu et al., 2009).

Furthermore, we tried to analyze the localisation of HPV16 E2 protein and Rint-1 protein in sub-cellular elements by sub-cellular fractionation, the 5 fractions [cytoplasm extract (CE), membrane extract (ME), nuclear extract (NE), chromatin bound extract (CB) and the pellet extract (PE)] of Rint-1, E2 and Rint-1 in association with E2 were compared and analysed with each other through Rint-1 and E2 western blots and fractions control western blots. In the E2 alone fractions, E2 was mainly localised in the nucleus and also in cytoplasm. In Rint-1 alone fractions, Rint-1 protein was predominantly localised in the membrane element. Rint-1 co-transfected with E2 however resulted in a dense accumulation of protein in cytoskeletal/pellet fraction in all the three repeats. Previous experimental work conducted in the Parish laboratory confirmed HPV E2 and Rint-1 co-localise in cytoplasmic compartment of transfected cells and have a role in sub-cellular trafficking during HPV infection. Now my experimental work showed together Rint-1 and E2 are localised in cytoskeletal element. In my opinion, this might be the reason for higher levels of viral Ag transport between nuclear periphery and the host cell which can regulate nuclear localisation of cellular proteins. In future, experimental work should be done to conclude what role this could play in HPV infection.

Later on, we tried to determine the localisation of HPV16 E2 following siRNA mediated depletion of Rint-1. We optimised the amount of siRNA required for Rint-1 depletion as 200nM and the knockdown was successful in C33a cells. As the knockdown was optimised in C33a cells, to determine the localisation of HPV16 E2 following siRNA mediated depletion of Rint-1, transfection of

Rint-1 siRNA in U2OS cells was required (as U2OS cells express E2) but my previous transfections in U2OS cells was not successful, so we tried transfecting Rint-1 siRNA in new clone of U2OS cells (clone F) to check the knockdown and to proceed with immunoflouresence to see the localisation of E2 in Rint-1 depleted cells, Unfortunately the siRNA knockdown was not successful in U2OS cells and these cells were also not expressing E2 as a result of which localisation of E2 and Rint-1 was not visualised in immunoflouresence experiment. Due to lack of time repeating this knockdown by optimising the conditions and trying of new clones of U2OS cells was not possible but in future research someone needs to optimise these conditions and repeat this experiment to determine the localisation of HPV16 E2 following siRNA-mediated depletion of Rint-1. Other experiments can also be done with Rint-1 siRNA like RT-qPCR and can detect the knock down of mRNA.

Inside the cell, proteins are transported by vesicles in between donor and acceptor compartments, and through retrograde transport some of the vesicles shuttle back to donor compartments. Rint-1 proteins are known to regulate retrograde transport and E2 proteins can interact with certain host cellular proteins and target the intracellular trafficking. The association of Rint-1 with E2 could affect the dynamics of intracellular organelles and can be involved in viral replication during early stages of viral infection by maintaining the viral DNA in between the cells and translocating the viral antigens through retrograde transport. From this, we can speculate that association of E2 with Rint-1 might have a role during early stages of viral infection by targeting host cell intracellular trafficking pathways during HPV life cycle.

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Project 2

Role of Adenovirus E1A in viral gene regulation

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Word count: 13,300

Project Abstract:

Adenoviruses are used as a model system to study the transcription of viral and cellular genes during viral infection, and dissect the molecular basis of cellular transformation. The Adenovirus (Adv) early gene product, 13S E1A serves to transactivate viral early genes; the CR3 domain of E1A plays a vital role in this process. The aim of this project was to investigate further the role of E1A interaction with the cellular histone-directed H3 K4 methyltransferases, Set1A and Set1B, in transactivation. We successfully confirmed by reciprocal co-immunoprecipitation in HEK 293 cells, the *in vivo* association of Set1A and Set1B with Adv E1A. We also determined by chromatin immunoprecipitation that Set1A and Set1B associate with viral early gene promoters in Adv5-infected cells, suggesting that Set1A and Set1B might be important for transactivation function. We also demonstrated that Adv E1A associates with functional histone H3- and H4- directed methyltransferase activities in HEK 293 cells. We also identified a number of, potentially new, E1A-interacting proteins by mass spectrometry, of which E1A association with MCM proteins was validated in HEK 293 cells. This work has established potential new roles for Set1A and Set1B in E1A transactivation, and, identified a number of novel E1A-interacting proteins.

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I. Introduction

1. Adenonviruses

Adenoviruses are small, non-enveloped, linear double-stranded DNA viruses with an icosahedral nucleocapsid symmetry belonging to the family *Adenoviridae* (Rowe et al., 1953). Adenoviridae are classed into five distinct genera: Mastadenoviridae, Atadenoviridae, Aviadenoviridae, Siadenoviridae and Ichtadenoviridae based on their host range. Adenoviruses infect many animals including mammals, birds, reptiles, frogs and fish. They generally tend to cause upper respiratory tract infections in humans, but they can also cause conjunctivitis, cystitis, gastroenteritis and some rashes etc. Mostly the infections persist for long periods, especially in immunocompromised patients. Apart from understanding the infectious process, adenoviruses have long been used as a model system to study the molecular and cellular biology of human cells because they are well characterised, low risk and easy to work with. Adenoviruses can also be classified as tumour causing DNA viruses, as they induce tumours in experimental animals. Hence, they can serve as good models to study and understand the molecular basis of cancer (Turnell, 2008).

1.1. History:

In 1953, Wallace Rowe and his colleagues first discovered Adenovirus from the adenoid and tonsil tissue of infected children after tonsillectomy, hence the name (Rowe et al., 1953). But the role of human Adenovirus and its role in cancer came to light after the experimental work by John Trenitin and colleagues who investigated the tumourigenic properties of adenovirus as part of “The quest for human cancer viruses”. They discovered that human Adenovirus (hAdv) type 12 causes cancers, typically sarcomas, when injected

into baby hamsters (Trentin et al., 1962). Further studies revealed that tumourigenesis was dependent upon virus dose and serotype, as well upon host age, host genome and host immunity (reviewed by Gallimore and Turnell, 2001).

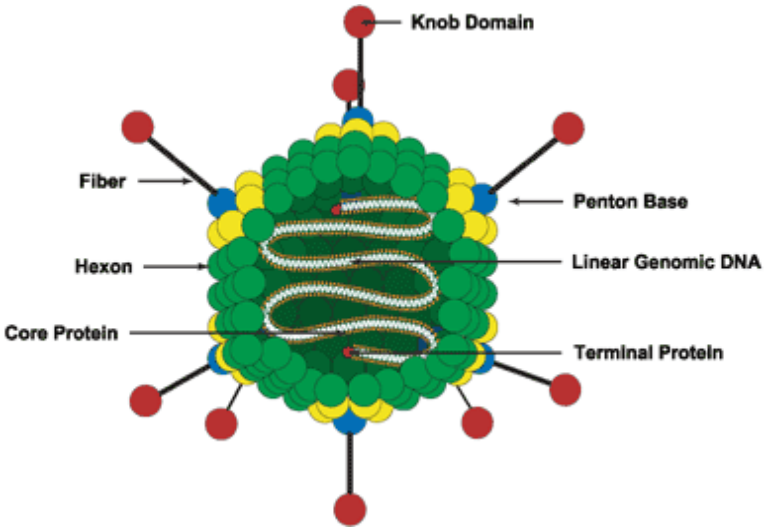
1.2. Classification:

According to the Baltimore virus classification system, Adenoviruses are classified as group I (ds DNA) viruses. The international committee on taxonomy of viruses (ICTV) provided a systematic classification of Adenoviruses and kept them in the family *Adenoviridae* and divided in to 5 sub-families or genera: Mastadenovirus, Siadenovirus, Atadenovirus, Aviadenovirus and Ichtadenovirus (Norrby et al., 1976). The Human Adenoviruses A, B, C, D, E and F types are categorised principally due to their ability to agglutinate red blood cells, and come under the genus Mastadenovirus (masta-, mammal) which consists of at least 51 different serotypes. The different adenovirus types have different cellular tropisms and can cause different infections, like respiratory disease caused by hAdv-B and C serotypes, conjunctivitis caused by hAdV-B and D serotypes, and gastroenteritis by hAdv-F serotype. Oncogenicity in rodents is caused by serotypes of hAdv-A and B sub-groups where sub-group - A shows higher level of oncogenicity and sub-group- B shows moderate level (Norrby et al., 1976).

1.3. Genome:

The Adenovirus genome is a linear, non-segmented, ds DNA virus of approximately 35,000 base-pairs in size and lies within a capsid containing 12 pentons and 240 hexons and additional structural proteins (Cusack, 2005).

The terminal ends of the genome have inverted repetition (ITR) sequences (100 – 140bp). The origin of replication (ORI) lies in between 36 to 46bp of ITRs, and a terminal protein (TP) is attached to each end of the genome. The adenovirus genome-encoding genes are divided in to two sets of early gene transcripts (E1 – E4) and late gene transcripts (L1 – L5) depending upon their expression before, or after, viral DNA replication. E1A and E1B gene products are essential for viral replication, E2 proteins are involved in providing machinery for transcription of late genes and viral DNA replication. The E3 proteins are required for modulating the host immune response, whereas the E4 proteins are involved in promoting functions like viral DNA replication and host protein synthesis shut off, as well as being involved in viral mRNA metabolism. The virion consists of 13% of DNA mass and 87% of protein (Green and Pina, 1963).



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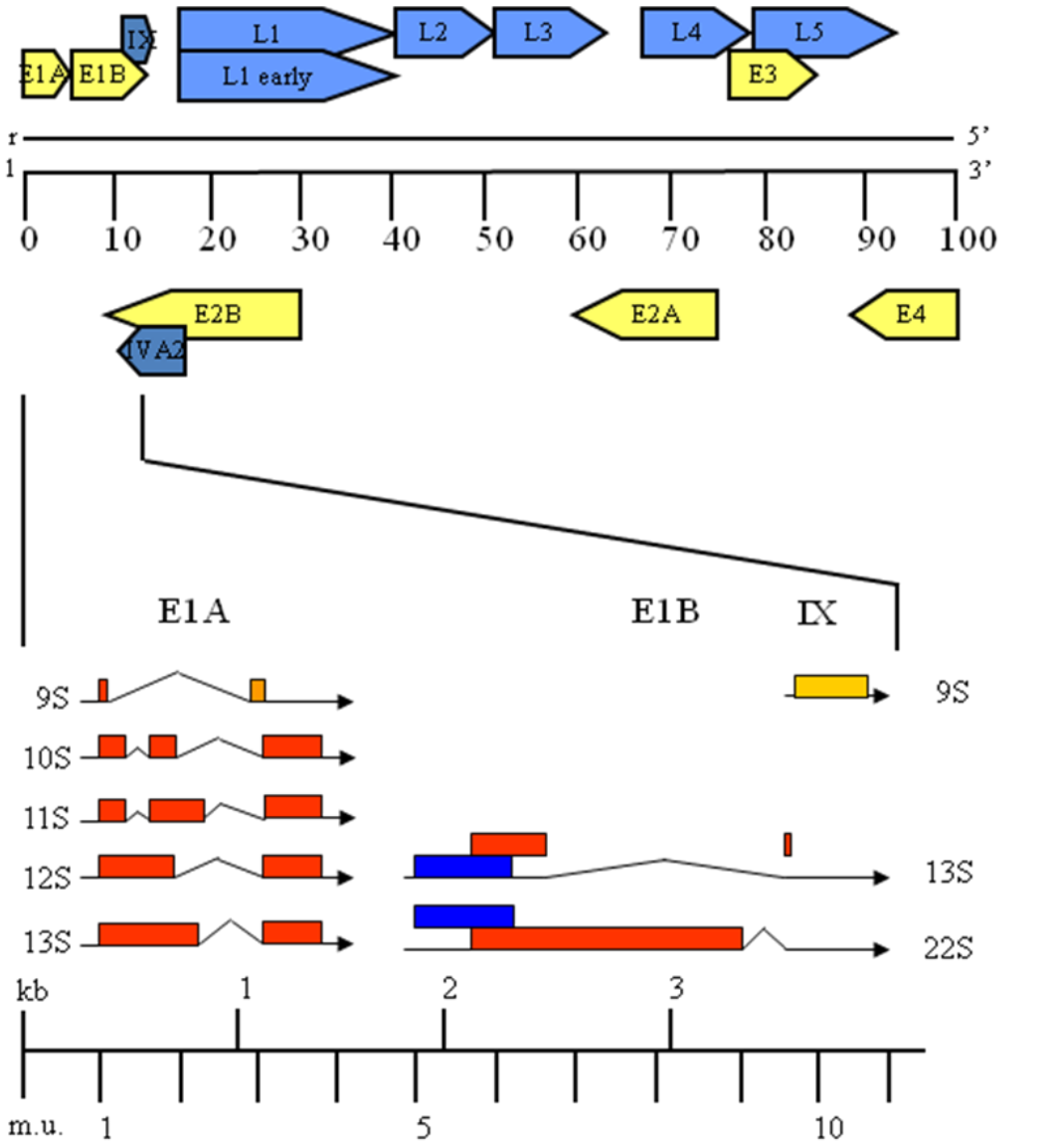

Figure adopted from Shenk 1996

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Figure 1. A) Structure of the Adenovirus Virion: Model of the adenovirus virion showing the knob domain, fibre and penton base, as well as hexon, core proteins terminal protein and linear genomic DNA.
B) Schematic representation of the genome of Ad2/5: The upper panel shows the genes expressed during viral infection. The lower panel shows the multiple mRNAs of the E1A and E1B transcription units. E represents early region proteins, L represents late proteins and IX and VIA2 as intermediate proteins.

1.4. Life cycle:

Adenovirus entry into the host cell is coordinated by receptor-mediated endocytosis. The viral fibre proteins associate with known cell surface receptors, CD46 for hAdv group-B viruses and CAR (coxsackievirus and adenovirus receptor) for the other serotypes; αv integrins also facilitate adenovirus entry. The virus fibre proteins bind to the cell receptor which promotes endocytosis and viral entry. After the successful entry of the virus inside the host cell, the capsid components get dissociated and virions are released in the cytoplasm. Through cellular microtubules the virus gets entry in to nuclear core complex, where the viral DNA is released inside the nucleus with the help of several cell signalling factors and activation of protein A kinase and also p38 /MAPK cascade (Meier and Greber, 2004). Inside the nucleus the viral DNA can dissociate from the core proteins and become associated with histone molecules and new virions are produced ultimately through a coordinated programme of viral gene expression, viral protein synthesis, viral DNA replication and virion assembly (Dery et al., 1987).

1.5. Replication:

Adenovirus expresses three proteins that function in viral DNA replication: preTerminal Protein (pTP), which acts as a primer for the initiation of DNA synthesis; E2 DBP which binds viral genome DNA, and E2 Ad DNA polymerase (AdPol) which catalyses nascent viral DNA synthesis. There are also a number of cellular proteins that facilitate viral DNA replication. Transcription factors NFI and NFIII bind to Ad origins of replication and help recruit the pTP-AdPol complex, whilst topoisomerases I and IIA help relax supercoiled DNA. These cellular proteins cooperate with viral proteins to

promote viral DNA synthesis, where AdPol catalyses the initial covalent association of dCMP to a pTP serine residue which then serves as a primer for Adpol-mediated viral DNA synthesis via strand displacement (reviewed by (Ramachandra M., 1999).

1.6. E1A:

The adenovirus E1A gene is the first gene to be expressed following virus infection (Yousef et al., 2009). E1A is required for viral replication and cellular transformation and plays a vital role in transcription activation. There are five E1A mRNAs produced: 9S, 10S, 11S, 12S and 13S, formed by means of differential splicing. The 12S and 13S mRNAs are transcribed during the early stages of viral infection whereas the 9S, 10S and 11S mRNAs are all transcribed during the late stage of viral infection, though their function remains uncertain -reviewed by (Boulanger and Blair, 1991, Lillie et al., 1987). In Adv2 and Adv5, 13S and 12S E1A splice variants encode E1A proteins of 289 amino acids and 243 amino acids, respectively. 12S and 13S gene products both share extensive sequence identity across the entirety of the E1A molecule except for the presence of an additional 46 amino acid sequence in the 289 R (residue) proteins. This 46 amino acid sequence in addition to its adjacent sequences are highly conserved amongst different adenovirus serotypes and species and is known as conserved region 3 (CR3). CR1, CR2 and CR3, were also identified as regions of high conservation after sequencing and characterizing the E1A mRNAs in both infected and transformed cells; CR4 was identified much later by comparison of the C-terminal regions of E1A from different serotypes (Avvakumov et al., 2002). Indeed, comparison of the E1A coding region from 15 different adenovirus

serotypes that infect human and simian species, identified CR4 in the C-terminal region of E1A between residues 255 to 288 (Avvakumov et al., 2004). E1A is heavily post-translationally modified, principally by phosphorylation during infection. The molecular weight of E1A upon SDS-PAGE therefore has a large variance in size, between 28 and 58 kDa. Both the 289R and 243R E1A proteins play a role in transcription, the larger 289R protein has a role in transcriptional activation of cellular and viral genes-CR3 is essential for transactivation by E1A. Later experimental work concluded an Auxiliary region 1 (AR1) acidic region adjacent to CR3 from residues 189–200 is important for early viral promoter transactivation by E1A (Anne-Christine Strom, 1998).

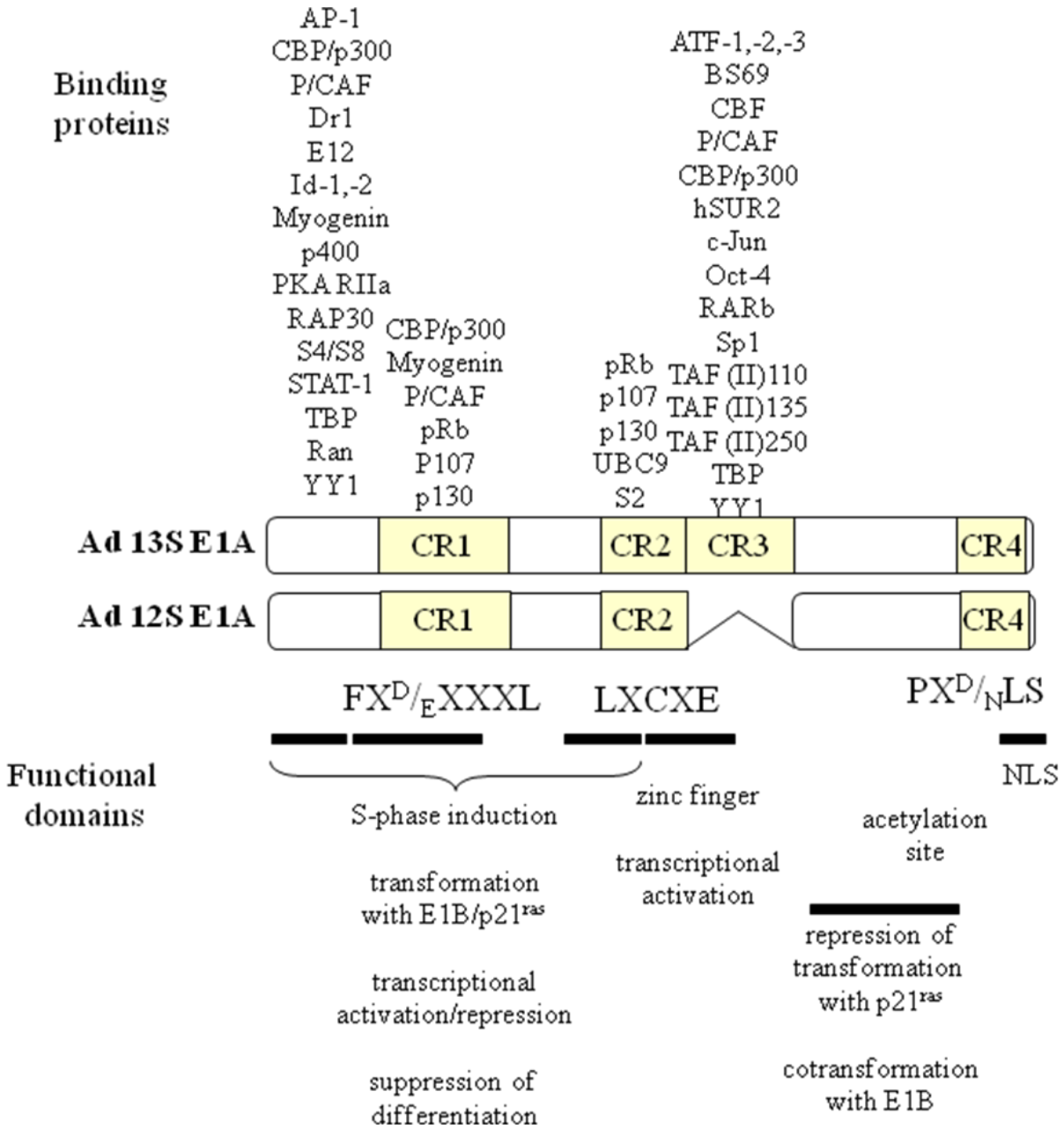


Figure adopted from Gallimore and Turnell, 2001.

Figure 2. Diagrammatic representation of AdvE1A conserved regions and its associated functional domains and binding proteins: This shows the AdvE1A conserved regions and its associated cellular proteins that bind to N-terminus region, CR1, CR2, CR3 and CR4 domains and their functions are also shown.

1.6.1. Functions of E1A:

E1A proteins are involved in large number of biological activities including activation and repression of transcription, DNA synthesis, apoptosis, mitosis and cellular transformation and immortalization in association with cooperating oncogenes such as E1B or $p21^{ras}$. It is also known to function as an anti-oncogene during tumourigenesis and is involved in suppressing transformation and metastasis. As mentioned earlier, E1A proteins are known to function in cellular and viral transcription programmes and as such associate with numerous cellular proteins that regulate transcription.

1.6.2. E1A role in transactivation of cellular and viral genes:

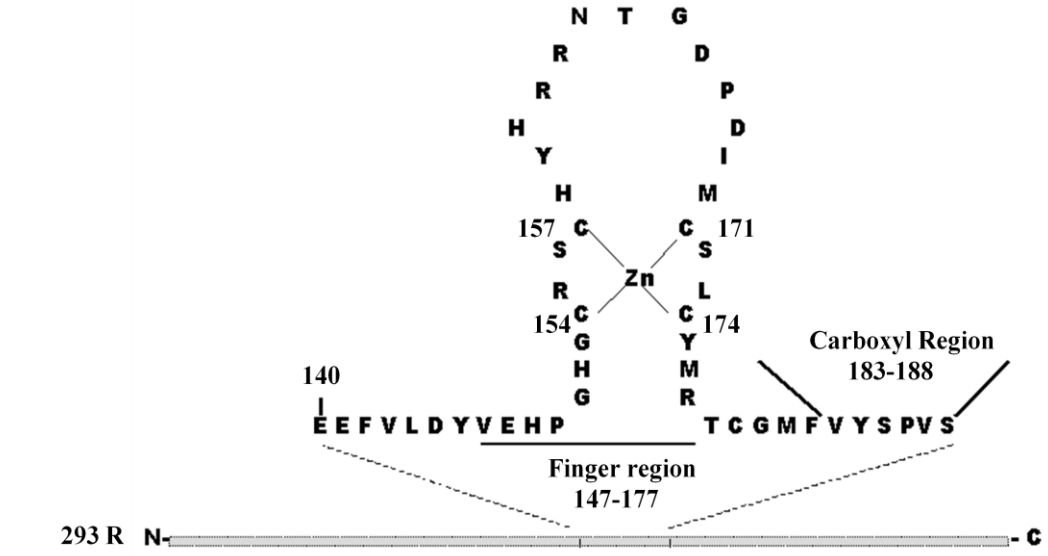
The adenovirus genome is packaged in the virion with the help of viral core proteins V and VII. During the early stages of viral infection, viral DNA can dissociate from the core proteins and the 'naked' viral DNA, with the help of host cell histone proteins, gets rapidly chromatinized (Dery et al., 1985). Transcription is assumed to take place both from core template and cellular histone template and E1A is known to activate transcription from different cellular and viral genes. This temporal regulation of transcription of adenoviral genes by E1A is required for replication and for reprogramming the cell transcripts to induce the S-phase entry (Dery et al., 1985). Due to lack of sequence specificity, E1A can't bind DNA directly, for this E1A transactivates gene promoters by associating with factors that regulate transcription, like ATF-2 and YY1, co-activators like CBP/p300, repressors like pRB, p107 and p130, co-repressors like CtBP, the general transcriptional machinery, such as TBP and TAFs and nucleosomal remodelling factors like SW1/SNF

(Gallimore, 2001, Turnell, 2008).

1.6.3. CR3-mediated transcription:

Amongst all the transcription pathways of E1A, transactivation mediated by CR3 is very important, as it ultimately promotes viral replication. The CR3 region of the 13S subunit of E1A can activate transcription of viral genes and cellular genes by binding to number of transcription factors and proteins that function as components of the transcriptional machinery (Jones, 1995).

The CR3 region of E1A is involved in interacting with different transcriptional factors to enhance their activity; it transactivates different cellular and viral promoters by means of protein – protein interactions. Experimental studies such as mutational analysis of CR3 showed that between residues 180-188 of CR3 in the carboxyl terminal region there is a promoter targeting region which interacts with numerous transcription factors and TAFs (TBP associated factors). TAFs interact with transcription factor activation surface and can serve as multi subunit protein complex components (Pelka et al., 2008, Yousef et al., 2009). These interactions ultimately result in localization of E1A to target promoters in the viral infected cells (Pelka et al., 2008, Yousef et al., 2009). The CR3 domain also consists of a zinc finger region from residues 147 to 177 that is also essential for transactivation. The zinc finger domain is important for binding the general transcription machinery like TBP for transactivation (Webster, 1991). In addition to TBP, some other cellular components like TAFs, and mediator component, Sur2 are also required for the CR3-mediated transcription (Boyer, 1999); Sur2 is essential for CR3 transactivation function.



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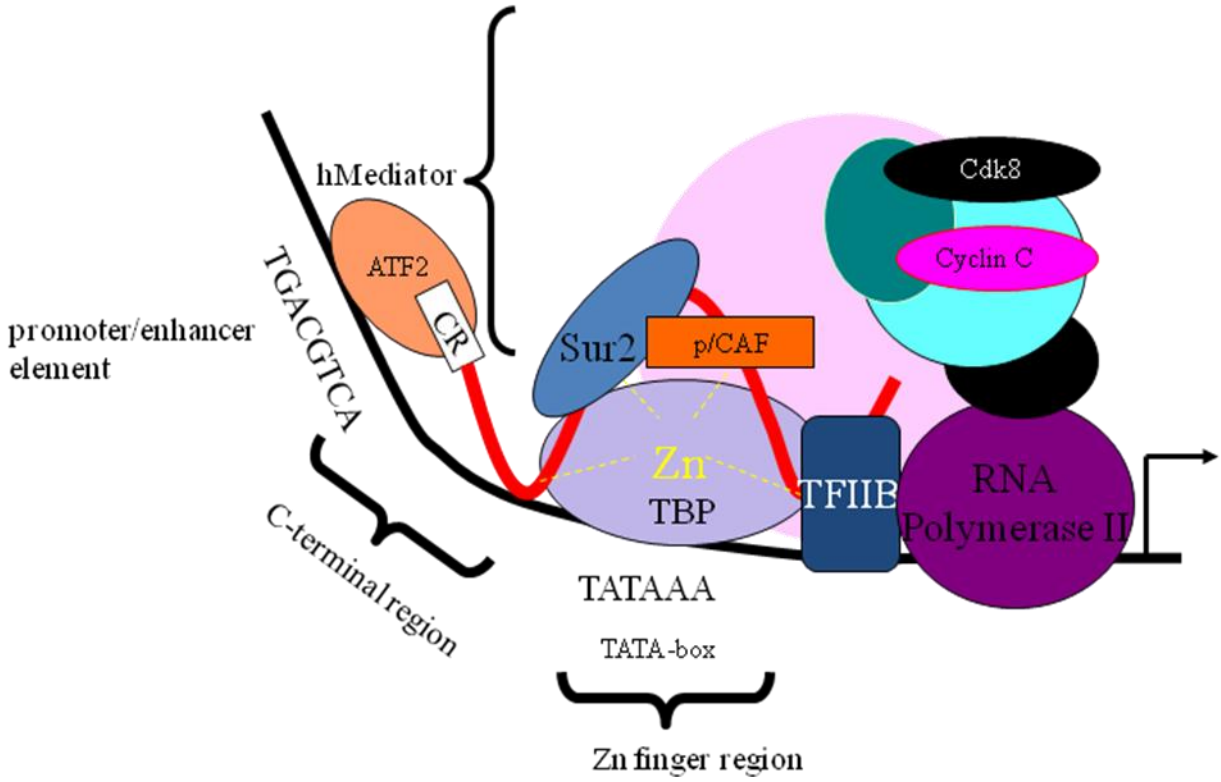


B) Figure adopted from Geisberg *et al*., 1994.

Figure 3. A) Shows the N-terminal region, Zinc finger and C-terminal region of CR3 domain of Adv 13S E1A. **B)** Shows the model for CR3 binding to cellular proteins in order to stimulate transcription.

1.6.4. Role of the 12S E1A gene product in transcription:

Whereas the large, 13S E1A gene product transactivates viral and cellular promoters directly through interaction with promoter-bound transcription factors, the smaller, 12S E1A gene product can both activate and repress

transcription through interaction with numerous transcriptional regulators (Gallimore and Turnell, 2001). For instance, the 243R E1A species interacts specifically with the pRB family (pRB, p130 and p107) of transcriptional repressors and tumour suppressor proteins through interactions with CR1 and CR2 (Figure 2; (Gallimore and Turnell, 2001). E1A association with pRB mimics the CDK-dependent phosphorylation of pRB and leads to the disruption of pRB-E2F repression complexes on E2F-dependent promoters, such that E1A associates with pRB and removes pRB from E2F-regulated promoters (reviewed by (Frisch and Mymryk, 2002). This allows for the activation of S-Phase genes by the E2F family.

The N-terminal region and CR1 of E1A interacts specifically with CBP, p300 and P/CAF histone-directed acetyltransferases through residues in the N-terminal region and CR1 (Figure 2; Gallimore and Turnell, 2001). Indeed, it has been determined that the 12S E1A gene product promotes global histone H3 lysine 18 hypoacetylation through interaction with cellular acetyltransferases (Horwitz et al., 2008). Interestingly, however, microarray studies suggest that E1A causes the global relocalization of CBP/p300 during infection to genes that promote cell cycle, inhibit differentiation and inhibit antiviral responses and, as such, histone H3 lysine 18 becomes hyperacetylated at these sites (Ferrari et al., 2008). Conversely, E1A removes pRB from these same promoters to de-repress E2F-dependent transcription from these loci.

Mutational analyses have revealed that the ability of E1A to promote quiescent cells to enter S-phase resides in its capacity to interact with CBP/p300 or the pRB family of proteins (Jelsma, 1989), whereas the ability of E1A to induce mitosis resides in its ability to bind both CBP/p300 and the pRB

family of proteins (Jelsma, 1989). As such, the ability of E1A to promote cellular transformation is also due to its ability to bind CBP/p300 and the pRB family of proteins (Gallimore and Turnell, 2001).

A role for the C-terminal region of E1A in transcriptional control has also been determined. A mutant virus which expresses only exon-2 of E1A activates transcription in both human cells and in experimental animals (Mymryk and Bayley, 1993). One cellular protein known to interact with the exon-2 gene product of E1A is the C-terminal binding protein (CtBP) which has been found to function as a transcriptional repressor (Gallimore and Turnell, 2001). E1A interaction with CtBP also promotes E1A/E1B transformation, though recent evidence suggests that CtBP does not suppress E1A/ras-mediated transformation (Cohen et al., 2013). CtBP interaction with E1A is modulated by acetylation (Zhang et al., 2000). CBP/p300 acetylates E1A, adjacent to the known CtBP binding site at K-239, and inhibits CtBP association, and consequently, gene repression (Zhang et al., 2000).

1.6.5. Role of the Proteasome in CR3-mediated transcription

Proteasomes are the protein complexes located inside the nucleus and cytoplasm of the cell that function to degrade the damaged or unwanted proteins by means of proteolysis. The 26S proteasome is 2.5 – MDa in size and consists of at least 31 different subunits which degrade both poly-ubiquitylated and non-ubiquitylated subunits. This proteasome mediated degradation of protein complexes is known to have a role in signal transduction, transcription, cell cycle control and apoptosis by modulating the regulatory proteins involved in these processes (Ciechanover, 1998). The degradation of protein is achieved mainly in two step process, first the target protein gets ubiquitylated and then degradation occurs through the 26S

proteasome (Ciechanover, 1998). This 26S proteasome is an ATP-dependent protease complex which consists of a 20S catalytic core protein and 19S regulatory protein complexes. The 19S regulatory complex is a 700 KDa protein complex with 20 subunits mainly divided in to base and lid sub-complexes. The base complex in mammalian cells consists of 6 ATPases (S7, S4, S10, S6, S6' and S8) and 3 non-ATPases (S1, S2 and S5a) and the lid complex consists of 8 non – ATPases. These base complex 6 ATPases are assembled to form hexameric ring structure which will form a connecting link between 19S complex and the 20S proteasome and they together can degrade the non-ubiquitylated proteins by ATP-dependent manner. The lid complex pair with base 20S proteasome complex for the degradation of ubiquitylated target proteins in ATP dependent manner (Voges, 1999).

It has previously been determined that the N-terminal region of E1A is found associated with S8 and S4 ATPases of the 19S regulatory complex subunits (Turnell et al., 2000). In the absence of oncogenes, E1A is known to regulate the p53 expression and ultimately lead to apoptosis. Mutational studies have revealed that the N-terminal region of E1A by interacting with proteasome can inhibit the degradation of p53 (Turnell et al., 2000).

Other work conducted in the Turnell laboratory has found that CR3 of E1A binds to proteasomes, and that these interactions are essential for E1A transactivation function. Indeed, CR3 associates with 19S ATPase proteins independent of 20S (APIS), and also binds 20S proteasomes and 26S proteasomes, independently (Rasti et al., 2006). Crucial to the purported role of 19S ATPases in CR3 function it was also determined that S8 knockdown, reduced adenovirus-mediated early region gene expression, considerably. The proteasomal-mediated turnover of E1A was also found to be required to

promote CR3-dependent transcriptional activation, such that the E1A degron and sequences required for transactivation overlapped. It was also determined that E1A and 19S and 20S proteasomes are found associated with both viral early promoter, and gene sequences, implicating proteasomes in both viral gene initiation and elongation programmes.

S8-CR3 interactions promote the ability of E1A to induce transactivation of viral early promoters in mammalian cells during Adv infection *in vivo*. Chromatin immunoprecipitation experiments conducted in the Turnell laboratory have shown that proteasomes associate with Adv early region gene promoters and gene sequences. Indeed, these experiments revealed that CR3 association with 20S, 26S and APIS are essential for E1A-dependent transactivation of early region genes (Rasti et al., 2006).

1.7. Histone-directed Methyltransferase activity in gene activation and repression:

The massive genomic information encoded within DNA in the form of linear sequences is protected by a shield of histone proteins surrounding the DNA in the form of nucleosome, which is a unit of chromatin. The core histones H2A, H2B, H3 and H4 are components of the nucleosome. The N-terminus region of this histones can extend outside the packed DNA area and the amino acids present on the tail of histone will undergo post translational modifications like acetylation, phosphorylation, methylation, ubiquitylation etc, and these modifications will serve as a platform for transcription factors, DNA binding proteins and chromatin remodelers to control and regulate gene expression (Gallimore and Turnell 2001). Histone modifications in histone tails are residue-specific. Some modifications promote transcription, others repress transcription.

1.7.1. SET1:

SET derived its name from *Drosophila* suppressor and enhancer genes **S**U (Var), **E** (Var) and ***t****rx*. The Set domain consists of Histone-directed (H) methyltransferase (HMTase) or lysine-directed methyltransferase (KMTase) activity. There are eight classes of KMTase activity identified, KMT-1 to KMT-8. SET1 is the first member of the KMT2 class isolated from yeast, and is a part of the methyltransferase complex, COMPASS, which functions as a histone-directed H3 lysine 4 (H3K4) methylase. The size of this SET1 complex is approximately 450 KDa. In humans, mixed lineage leukaemia (MLL) gene is found to have homology to the *Drosophila* trx gene and the SET1/COMPASS proteins in humans that are homologous to yeast Set1 are Set1A, Set1B, MLL1, MLL2, MLL3 and MLL4. Set1A is the first H3K4 methyltransferase to be identified which is responsible to promote H3K4 mono-, di- and tri-methylation, and facilitate gene transcription (Shilatifard, 2012). All these methyltransferases exist as multi protein complexes and have some shared subunits like RbBp5, Wdr5, Ash2 and Dpy30 which are common between yeast COMPASS and the human MLL/Set1 complex. These complexes are broadly involved in the activation of gene expression and gene recombination (Lee and Skalnik, 2005); (Shilatifard, 2012).

Sequencing analysis shows Set1A protein share 39% similarity with an unknown Set domain protein, K1AA1076 known as Set1B. Set1B associates with all the subunits of Set1A like Rbbp5, Ash2, CFp1, Wdr5 and Wdr82 with in the 450 KDa complexes. Experimental analysis has demonstrated that the Set1B complex also trimethylates histone H3 at the lysine, K4, residue. Both these Set1A and Set1B together will bind to certain set of target genes and

play a important role in chromatin structural control and gene expression control (Lee et al., 2007).

I. Project Aims

1. Hypothesis:

- We hypothesize that adenovirus E1A interaction with cellular binding proteins, such as the Set1A and Set1B histone-directed methyltransferases, is important for adenovirus transactivation function.

2. Overall aim:

- To investigate the role of Adenovirus E1A, and cellular Adenovirus E1A-binding proteins in viral gene regulation.

3. Sub-aims:

- To investigate the roles of 12S and 13S E1A gene products during infection.
- To characterise E1A interactions with cellular proteins *in vivo*.
- To investigate E1A association with histone H3- and H4-methyltransferases *in vivo*.
- To investigate Set1A and Set1B association with Adv early region promoters in Adv5-infected cells.
- To investigate the role of Set1A and Set1B in viral early gene expression.
- To identify novel E1A binding proteins from HEK293 cells.

II. Materials and Methods

1. Cell Biology:

1.1. Dulbecco Modified Eagles Medium (DMEM):

Commercially available HEPES-buffered DMEM purchased from Sigma, was supplemented with 8 % (v/v) foetal bovine serum (FBS) and 2mM L-glutamine (Sigma) and stored at 4˚C before use.

1.2. Phosphate Buffered Saline (PBS):

Commercially available Dulcecco A (PBS) Tablets (Oxides) were dissolved in sterile distilled water (1 tablet/100ml), autoclaved and stored at room temperature. PBS tablet consists of 0.8g/L sodium chloride, 0.02g/L potassium chloride, 0.115g/L disodium hydrogen phosphate and 0.02g/L potassium dihydrogen phosphate (pH 7.4).

1.3. Saline:

One Saline tablet (Oxides) was dissolved in 0.5 litres of sterile distilled water and sterilized by autoclaving and stored at -4˚C.

1.4. Trypsin:

Commercially available trypsin (1X) was purchased from Sigma, aliquoted in to 5ml tubes and stored at -20˚C.

1.5. Maintenance of cell lines:

Ad5 E1-transformed, human embryo kidney (HEK) 293 cells, Ad12 E1-transformed human embryo retinoblasts (HER2), and human A549 small cell lung carcinoma cells were cultured and maintained in HEPES-buffered DMEM supplemented with 8% (v/v) FBS and 2mM L-glutamine and the cells were incubated at 37°C and 5% CO_2 at constant humidity. Once the cells became 90% confluent, they were washed once with 10ml PBS and treated with 1ml of trypsin (1X) per 10cm dish. Once detached, 9ml of fresh DMEM was added and the cells were mixed well. Ten µl of cell suspension was pipetted into a haemocytometer and cells were counted and seeded accordingly, onto new 10cm dishes.

1.6. Adenoviral infection:

A549 cells at approximately 90% confluence were selected for infection. The dishes were washed twice with 5ml serum-free DMEM. Adenovirus was diluted in serum-free DMEM at a multiplicity of infection (m.o.i.) of 10 particle forming units (p.f.u)/cell, unless otherwise stated, and added drop wise to the dishes and incubated at 37˚C for 2 hours in a humidified incubator with 5% (v/v) CO_2. Dishes were rocked gently at 15 minutes intervals to ensure the even dispersal of virus. After 2 hours the excess virus was removed carefully and the dishes supplemented with 10 ml fresh DMEM containing serum and incubated at 37˚C until harvesting.

2. Protein Biochemistry:

2.1. Bradford assay:

To determine protein concentration, 4 µl of the protein sample was diluted with 1 ml of Bradford reagent (BioRad). Bovine serum albumin (BSA) 0-30 µg was used to generate a calibration curve from which the unknown protein concentrations were determined. The absorbance was measured using a spectrophotometer at 595 nm against the blank (distilled water).

2.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE):

Typically, 50 µg of protein lysate, or immunoprecipitated samples, were separated by SDS-PAGE (10 % (w/v) Acrylamide/Bis-acrylamide [37.5:1- Severn Biotech Ltd], 0.1M Tris-Bicine pH 8.3, 0.1% (v/v) SDS, 0.3% (v/v) TEMED (N, N, N', N'-tetramethyl-ethylenediamine). APS (ammonium persulphate) was added to a final concentration of 0.06% (w/v) to initiate acrylamide polymerization. Prior to polymerization the gel mixture was poured into a gel cassette (Hoefer), and a comb was inserted to create wells for loading the samples. The combs were removed after gel polymerized and the wells were rinsed with dH_2O and placed the cassette in running buffer (Tris-Bicine (0.1 M), pH 8.3, SDS (0.1% (w/v)). Samples were loaded in the wells alongside suitable pre-stained protein standards and run at 10-20mA overnight depending on the proteins being investigated.

2.3. Urea-PAGE:

For immunodetection of adenovirus E1A, immunoprecipitated samples were separated by urea-PAGE in the absence of SDS [urea (8 M), Tris (15.2 mM),

glycine (96 mM) pH 8.5, 0.3% (v/v) TEMED and 0.06% (w/v) APS]. The gel mixture was poured into a urea gel cassette apparatus and levelled with a water-saturated Butan-2-ol, which was washed off with distilled water when the gel was polymerised. Before running the sample, the gel was pre-run at 25 mA in urea gel running buffer [Tris (15.2 mM), glycine (96 mM) pH 8.5] for 2 hours. The samples along with 25 µg of protein lysate input, in equal volumes of urea gel sample buffer, were loaded and ran at 15 mA for 4 hours (twice).

2.4. Coomassie staining:

After running the samples on SDS-PAGE, the gel was carefully removed from the cassette and placed in a plastic box and washed with distilled water. The gel was covered with 0.1% (w/v) Coomassie brilliant blue R-250 stain (Sigma) in methanol/glacial acetic acid/water (4:1:5 v/v) and placed on a gentle rocker for 20 minutes and destained again in methanol/glacial acetic acid/water (4:1:5 v/v).

2.5. Histone-directed Methyltransferase assay:

Confluent A549 cell dishes were lysed in Hi Lo buffer, protein concentration determined, and lysates incubated with anti-E1A antibodies (20 µg) by rotation at 4˚C overnight. The next day 20 µl of packed Protein-G Sephasrose beads were added to the mixtures and incubated, by rotation, for an additional 2 hours at 4˚C. The beads were then washed 4X with Hi Lo buffer. To the beads, 2µl radioactive-adenosyl-L-[Methyl ^{3}H]-methionine, 2µl of 10 mg/ml of core histones (H2A, H2B, H3 and H4) and 16 µl of methyl transferase buffer (50mM Tris pH 8, 30 mM KCl, 10 mM $MgCl_2$ and 10 mM β-mercaptoethanol) were added and samples incubated for 1.5 hours at 30˚C. After this time

sample buffer was added and samples were separated by SDS-PAGE. The gel was then stained with coomassie, dried and exposed to X-ray film at -20ºC, and developed as appropriate.

2.6. Mass Spectrometry:

Adv E1A immunoprecipitates from A549 cells were washed twice with detergent free lysis buffer. Proteins were then denatured in 0.5ml 9M Urea/50mM ABC (50mM Ammonium Bicarbonate (ABC), and incubated at RT for 30 minutes to 1 hour, vortexing occassionally. The beads were then pelleted and the supernatant containing the protein was collected. To the supernatant, 50 mM DTT (final concentration) was added and incubated at 56˚C for 30 minutes. 100 mM iodoacetamide (final concentration) was then added to the supernatant and incubated in the dark for 30 minutes at room temperature. 300 µl of the supernatant was added to the FASP filters (Amicon Ultra 30K) and centrifuged at maximum speed for 10 to 15 minutes (until the lysate was reduced to approximately 50 µl). The flow through was discarded and the remaining lysate was added to the filters and subject to further centrifugation. The filters were then washed 4X with 50 mM ABC, whereupon the FASP filters were moved to new tubes and resuspended in sequencing grade trypsin (1 µg of trypsin per IP), diluted with 50 mM ABC to give a final volume of 300 µl. Samples were incubated at 37ºC overnight. The next day samples were centrifuged at high speed for 10 to 15 minutes to obtain tryptic peptides. Filters were washed once with 300 µl of 50 mM ABC and eluates combined, dried and stored at -20ºC.

3. Immunochemistry:

3.1. Harvesting cells for Immunoprecipitation:

Cells were washed twice with ice-cold isotonic saline solution and lysed with 1 ml/dish of HiLo salt solution [50mM Tris (pH 7.5), 1% (v/v) NP-40 and 0.825M NaCl] at 4˚C for 10 minutes. The cells were harvested and sonicated at 30% amplitude, 10 sec on and 10 sec off and then centrifuged at 4˚C for 20 minutes at 13,000 ×g. The lysate was collected and saved at 4˚C and to it was added a primary antibody to precipitate the appropriate immunocomplex overnight at 4˚C. The next day 30 µl of protein G agarose beads (Sigma) were added to the immunocomplexes and rotated in the cold room for 2 hours. Immunocomplexes bound to the beads were then washed 4 times with 1 ml HiLo buffer. The beads were suspended with either 20 µl of urea sample buffer [Tris (18.6 mM), Glycine (115 mM) and bromophenol blue (w/v 0.1%)] for running the samples on urea-PAGE, or resuspended in 20 µl of SDS sample buffer for running the samples on SDS-PAGE.

3.2. Western blotting:

Following SDS-PAGE and urea gel-PAGE, the proteins on the gel were transferred on to nitrocellulose membrane using a standard protocol. The cassette was set up by placing a 3 MM Whatman filter that had been pre-soaked in transfer buffer (0.05 M Tris, 0.1 M glycine and 20% (v/v) methanol) upon a blotting sponge. A nitrocellulose membrane that had also been pre-wetted in transfer buffer was then placed upon the Whatman paper, after which the gel was placed on top of the nitrocellulose membrane, and was again overlaid by another sheet of pre-soaked 3 MM Whatman filter paper and a blotting sponge in transfer buffer. The cassette was then transferred to the

transfer apparatus (Hoefer) filled with transfer buffer and run at 250 mAmps for 6 hours. After protein transfer, the membrane was stained with 0.1 (w/v) Ponceau S (Sigma) in 3% (w/v) trichloroacetic acid, to visualize proteins. Membranes were then washed with 1 X TBS-T [Tris buffered saline with Tween 20 (0.1%)] and subsequently blocked in 5 % (w/v) milk in 1 X TBS-T for 30 minutes. After blocking the membrane was placed in a plastic bag containiing 5 % (w/v) milk in 1 X TBS-T with primary antibody at the appropriate dilution, and were heat sealed and incubated overnight at 4˚C on a gentle rocker. Next day the membrane was washed 3 times with 1 X TBS-T and incubated in horseradish peroxide anti-species secondary antibody (Dako) for 2 hours on a gentle rocker at room temperature. The membrane was then washed 4 times with 1 X TBS-T (at 15 minutes intervals). The membrane was then incubated with enhanced chemiluminescence reagent (ECL; Millipore) for 1 minute. The membrane was placed on a cling film (Saran wrap sheet) exposed to X-ray film sheets (Kodak) for the desired period of time depending on the protein (few seconds to a few minutes) and the film was then developed in a developer (X-OMAT).

3.3. Chromatin immunoprecipitation (ChIP) assay:

A549 cells were infected with wild type Adv5 at an m.o.i. of 1 pfu/cell. 16 hours post infection the cells were treated with Formaldehyde (final concentration 1% (v/v)) to cross-link histones to DNA and incubated for 10 minutes at 37˚C. Later, the cells were washed twice with 5 ml of ice-cold PBS and harvested in 1 ml of PBS and centrifuged. The pellet was re-suspended in 0.550ml of SDS lysis buffer (50 mM Tris-HCl pH 8.1, 1% (v/v) SDS and 10mM EDTA) and kept on ice for 10 minutes. The lysate was then sonicated twice at 30% amplitude,

10 seconds, to shear the DNA. 50 µl of the sonicated lysate was taken from the supernatant and diluted to make up to 200 µl by lysis buffer and kept as input. The remainder of the supernatant was diluted 10 fold with ChIP dilution buffer (20 mM Tris-HCl pH 8.1, 1.1% (v/v) Triton X-100, 0.01% (w/v) SDS, 1.2 mM EDTA and 150 mM NaCl) and pre-cleared with 50 µl of Protein A agarose slurry (containing 1mg/ml BSA and 400µg/ml Salmon Sperm DNA) for 2 hour at 4˚C by gentle agitation. Equal volumes of cleared lysates were incubated with the appropriate antibody and mixed by rotation in the cold room at 4˚C overnight. To collect the histone/antibody complex, 50 µl of a Protein A agarose slurry (containing 1mg/ml BSA and 400µg/ml Salmon Sperm DNA) was added to the samples and incubated for 2 hours at 4˚C. The beads were then washed 3 to 4 times, by gently spinning on a rotating platform, in 1 ml of the following buffers; 1 X low salt immune complex wash buffer (20 mM Tris-HCl pH 8.1, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 2mM EDTA and 150 mM NaCl); 1 X high salt immune complex wash buffer (20 mM Tris-HCl, pH 8.1, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 2 mM EDTA and 150 mM NaCl); 1 X LiCl immune complex buffer (10 mM Tris-HCl pH 8.1, 1% (v/v) NP40, 0.25 M LiCl, 1 mM EDTA and 1% (w/v) sodium deoxycholate) and 2X TE (10 mM Tris-HCl pH 8.0, and 1 mM EDTA). Protein-DNA complexes were then eluted from the Protein A beads using 0.5 ml of freshly prepared elution buffer (0.1 M $NaHCO_3$ in 1% (w/v) SDS) by incubation at room temperature for 30 minutes with gentle rotation. Eluates were combined and transferred to fresh tubes whereupon histone-DNA crosslinks were reversed by the addition of 20µl 5M NaCl and heating at 65˚C for 4 hours. Input histone-DNA crosslinks were reversed by the addition of 8µl of 5M NaCl and heating at 65˚C for 4 hours. After reversal of cross-links 10 µl of 0.5 M EDTA, 20 µl of 1M Tris-HCl pH 6.5,

and 2 µl of 10 mg/ml Proteinase K was added to the eluates and incubated at 45˚C for 1 hour. Following phenol/chloroform extraction, the DNA was recovered and the DNA pellet was visualised by ethanol precipitation and by adding 20 µg of glycogen. Later the DNA pellet was washed twice with 70% ethanol and air dried, resuspended in 50µl sterile water and frozen for PCR.

4. Molecular Biology techniques:

4.1. Polymerase chain reaction (PCR):

PCR was performed to amplify DNA following ChIP using 2U Fast-start Taq polymerase (Roche) following the manufacturer's guidelines. A GC-Rich solution buffer was used in order to alter the melting nature of DNA and was used for templates with high GC-content such as the promoter regions amplified here. PCR was performed using a thermal cycler for 10 minutes at 94˚C for DNA denaturation and 30 cycles DNA amplification (60 sec at 94˚C, 30 sec at 50˚C and 120 sec at 72˚C). The amplified DNA was separated by agarose gel electrophoresis. The oligonucleotides used for amplification were as follows:

E1A promoter: tgacgtagtagtgtggcggaagtgt (Forward-F), tataaatacactacacgtcagctga (Reverse- R). E1B promoter: gtgtctagagaatgcaatagtagtacggat (F), taaccaagattagcccacggcgcattatat (R). E2e promoter: tcatgtagtttattcgggttgagtagtctt (F), atactgcgcgctgactcttaaggactagtt (R). E3 promoter: tggtgtaccaggaaagtcccgctcccacca (F), caagcgaggagctcaccgactcgtcgttga (R). E4 promoter: gcagagcgagtatatataggactaaaaaat (F), taccttattttggattgaagccaatatgat (R).

4.2. Agarose Gel electrophoresis:

DNA fragments were separated upon 0.8% (w/v) agarose (Sigma) in 1X TBE (Tris-Borate-EDTA) containing (0.5 µg/ml) ethidium bromide. Samples were diluted with 6X gel buffer (30% (v/v) Glycerol (BDH), 0.25% (w/v) bromophenol blue (Sigma) and 0.25% (w/v) xylene cyanol FF in sterile distilled water) and loaded in the wells. Electrophoresis was carried out at 60 V in 1X TBE running buffer for 45 minutes and the gel was observed under a UV transilluminator to visualise DNA fragments.

III. Results

1. Investigating the roles of 12S and 13S E1A gene products during infection:

Adenovirus E1A proteins play an important role in transactivation function and cell cycle regulation during infection. Adv-regulated transactivation of Adv early region gene promoters is dependent on the expression of the 13S E1A species, whilst the smaller 12S E1A gene product promotes cell cycle entry through binding pRB and CBP/p300. Initial experiments were therefore designed to study the differences between 12S and 13S in promoting Adv early region gene expression. To do this I infected A549 cells with Adv5 expressing either 12S-only or 13S-only at 10 pfu/cell and harvested cells at 4h, 8h, 18h and 24h post infection in UTB buffer, separated proteins on 10% SDS-PAGE and Western blotted for E1A, p53, E4Orf6, E4Orf3, E2A and E1B-55K, as well as loading control, β-actin (Figure 1A). From the results it was clear that the expression of, E4Orf6 and E4Orf3, which are required for efficient viral DNA replication was wholly dependent upon 13S E1A expression, as these proteins were not identified following infection with the 12S-only virus (Figure 1A). E1B-55K and E2A expression were also largely dependent upon 13S E1A expression, but 12S E1A induced their expression at considerably reduced levels (Figure 1A). p53 tumour suppressor protein levels were stable over this time-course following 13S E1A expression, and was increased following 12S E1A expression, presumably as E4Orf6 is not expressed (Figure 1A). From the results obtained we confirm earlier studies indicating that 13S subunit plays a key role in transactivation, and 12S E1A does not play a major role in the transactivation of early region gene

promoters. We next optimised the infection conditions for different doses of wild type Adv 5 (0.5, 1, 5 and 10 pfu/ cell) for infection of A549 cells and harvested infected cells at 24h and 48h post infection in UTB buffer; Western blot results are presented in Figure 1B. It can be seen that E1A and E1B-55K were detected at all viral doses. There was comparatively more E1A protein at 24h relative to 48h, consistent with its role in the early phase of infection, whereas E1B-55K protein levels were very similar at different moi's (Figure 1B). p53 was degraded at all doses of virus, but was more rapidly degraded in those cells infected with higher doses of virus (Figure 1B). β-actin western blot is shown as loading control. From the results we decided that the optimal dose required for infection was 1 pfu/cell.

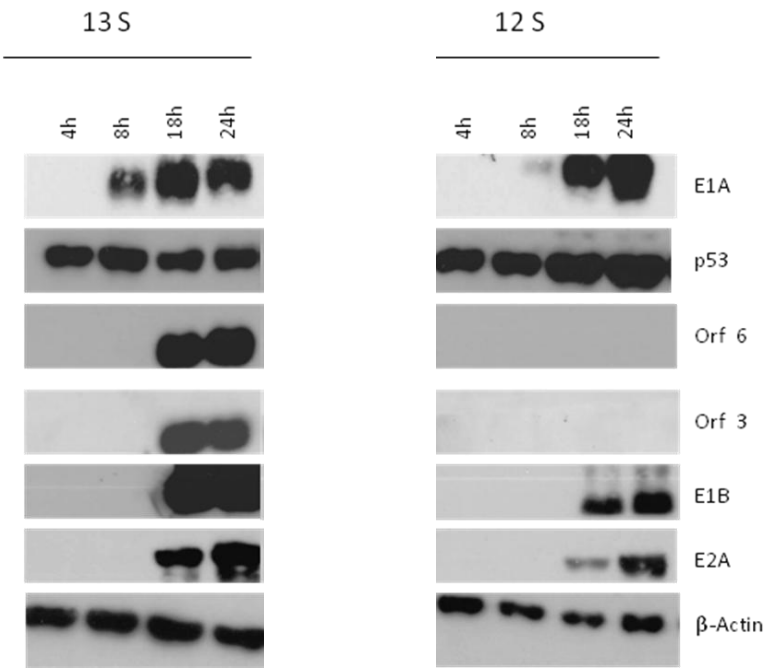


Figure 1A. Adv5 12S E1A-only and 13S E1A-only infection of A549 cells. A549 cells were infected with Adv5 12S-only or Adv5 13S-only viruses and 50 μg of protein from 4hours, 8hours, 18hours and 24hours post-infection was separated on 10% SDS-PAGE and Western blotted for E1A, E1B, E2A, p53, E4ORF3, E4ORF6 using E1A (primary, M58 (1:2000) and anti-mouse HRP secondary (1:2000), E1B-55K (primary, 2A6 (1:2000) and anti-mouse HRP secondary (1:2000), E2A (primary, B6-8 (1:1000) and anti-mouse HRP secondary (1:2000), p53 (primary DO-1 (1:20) and anti-mouse HRP secondary (1:2000), E4Orf3 (primary 6A11, (1:50) and anti-rat HRP secondary (1:2000) and E4Orf6 (primary RSA3, (1:20) and anti-mouse HRP secondary (1:2000). Proteins ran at approximately 40 kDa, 55 kDa, 11 kDa, 34 kDa and 70 kDa for E1A, E1B-55K, E4ORF3, E4ORF6 and E2A, respectively.

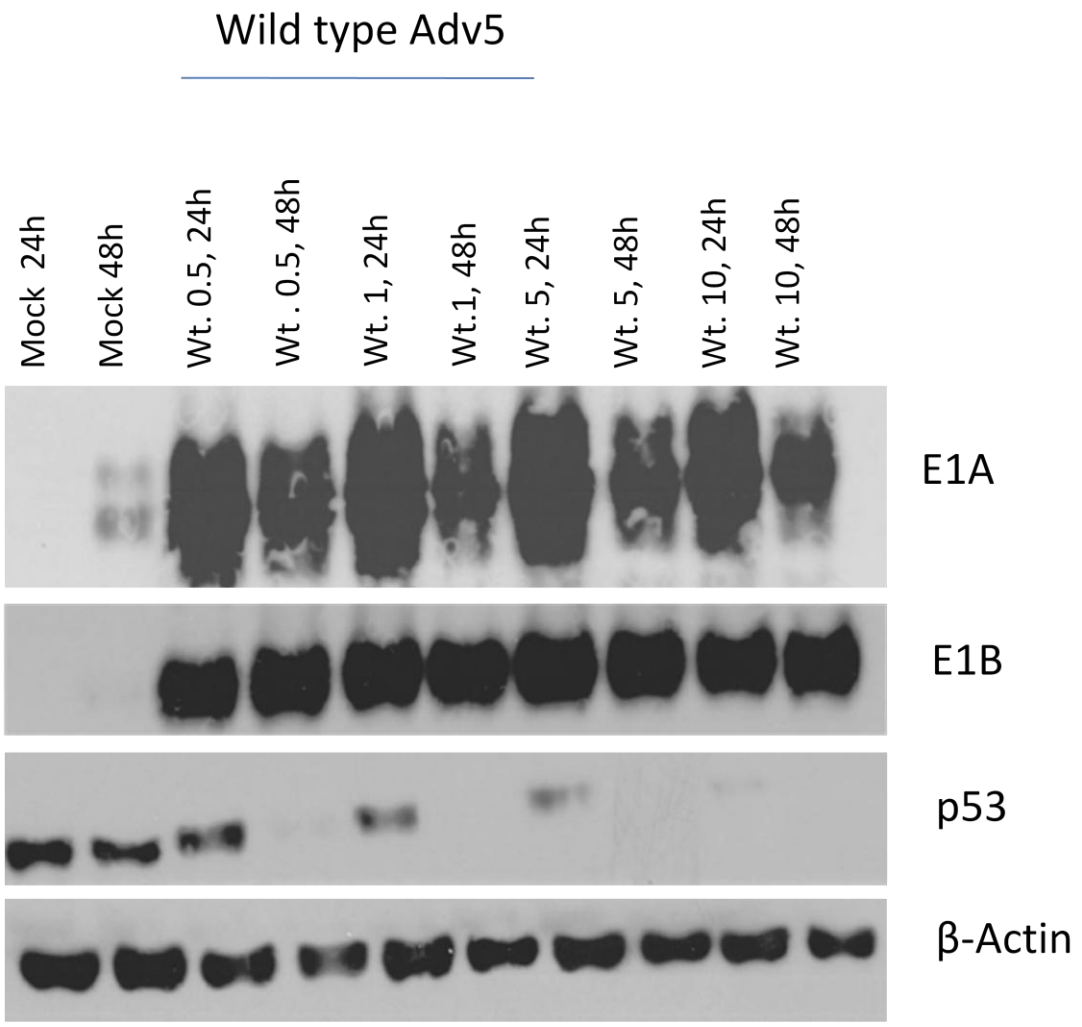


Figure 1B. Adv5 wild-type infection of A549 cells.
A549 cells was infected with Adv wild type at 0.5, 1, 5 and 10 pfu/cell and mock without infection and harvested in UTB buffer 24h and 48h post-infection and the samples were separated on 10% SDS-PAGE and Western blotted for E1A, E1B, p53 and actin using primary and secondary antibodies as described in Figure 1A.

2. Characterisation of E1A interactions with cellular proteins.

A. Co-immunoprecipitation of E1A with known binding partners

To study the cellular functions of E1A, especially to understand the basis of E1A dependent transactivation, it is important to characterise the functional interactions between E1A and cellular binding-proteins. Therefore, I harvested HEK293 lysates in HiLo buffer and performed immunoprecipitation with antibodies raised against known E1A-binding partners: CBP an N-terminal and CR1 binding protein; TBP, which binds to the N-terminal region and CR3; pRb family of proteins (pRb, p107 and p130) which bind to CR1 and CR2; and CtBP a C-terminal binding protein (Figure 2A). From the results obtained we can conclude that antibodies raised against pRb family proteins pRb, p107 and p130 co-immunoprecipitate E1A most efficiently, when compared to C-terminal and N-terminal binding proteins CtBP, CBP and TBP (Figure 2A). The association of E1A with pRb family proteins is important for E1A induction of cell cycle and cellular transformation, and repression of cellular differentiation. It is also evident that CBP bound more efficiently to the 12S E1A species (lower band), whilst the particular anti-TBP and anti-CtBP used in this study were not good for co-immunoprecipitation of E1A (Figure 2A).

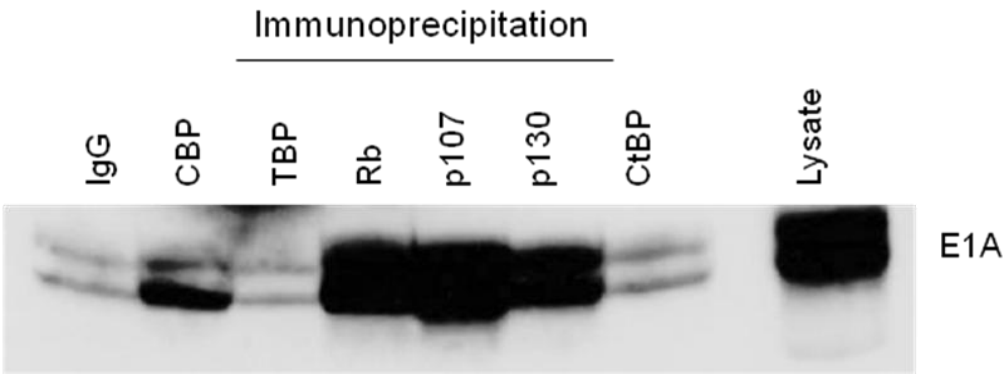


Figure 2A. Co-precipitation of E1A with known binding partners
Cellular binding proteins were immunoprecipitated from HEK293 cell lysate and normal IgG was used for the negative control. Immunocomplexes were isolated on Protein G Agarose beads, and separated along with 10% input (positive control) by urea-PAGE (in the absence of SDS) and E1A association was analysed using an anti-E1A (M58) primary antibody (1:2000) and anti-mouse HRP secondary (1:2000).

B. E1A immunoprecipitation of known binding partners in vivo.

After analysing the E1A binding cellular proteins through immunoprecipitation of known binding proteins followed by an E1A western blot, I proceeded to characterise further the functional interactions of E1A with cellular binding proteins through immunoprecipitation of E1A from HEK293 lysates followed by Western blotting with known E1A-binding partners (Figure 2B). The results confirmed the strong interaction of Rb family proteins pRB, p107 and p130 with E1A, as E1A co-immunoprecipitated all of these proteins (Figure 2B), E1A also co-immunoprecipitated CtBP efficiently (Figure 2B).

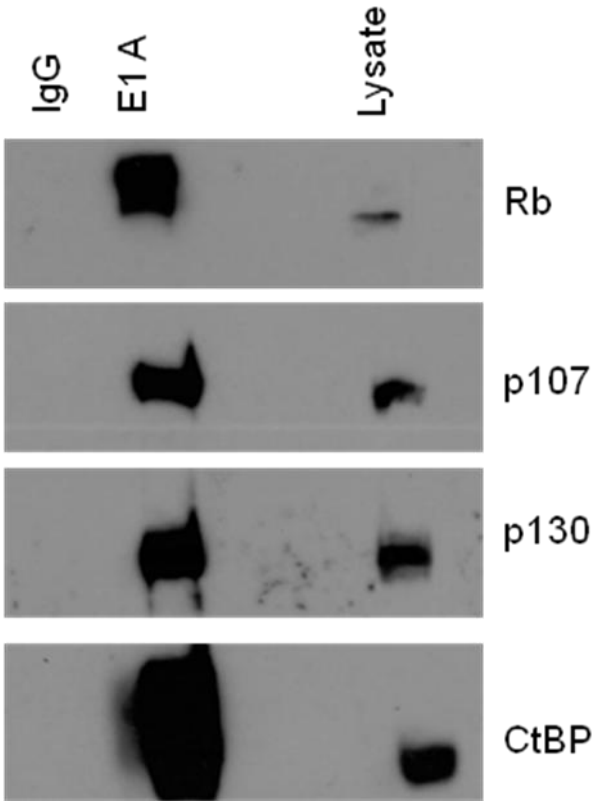


Figure 2B. E1A co-immunoprecipitation of pRb proteins and CtBP.
E1A protein was immunoprecipitated from HEK293 cell lysate using E1A monoclonal antibody M73. IgG was used as negative control. After isolation upon Protein G Agarose beads, immunocomplexes along with 10% input lysate were resolved on 10% SDS-PAGE and western blotted using primary antibodies, pRb (1:10), CtBP (1:10), p107 (1:500) and p130 (1:500), and appropriate horseradish peroxidase anti-species secondary antibody (Dako) mouse (1:2000) for Rb, CtBP and p130 and Rabbit (1:3000) for p107.

C. *In Vivo* binding of Set1A and Set1B histone-directed metyltransferases to E1A.

Previous work conducted in Dr. Turnell's laboratory has indicated that there exists an interaction between H3K4 methyltransferases Set1A and Set1B and

Adv5 E1A, in both Adv5 E1-transformed cells and Adv5-infected cells (Munir and Turnell, unpublished observations). To confirm this I immunoprecipated Set1A and Set1B from Adv5 HEK293 and Adv12 HER2 cell lysates using rabbit polyclonal antibodies raised against Set1A and Set1B (Figure 2C). From the experimental results it was clear that both Set 1A and 1B interacted with E1A *in vivo*. In HER2 cells the interaction appeared to be stronger than in HEK293 cells especially using the Set1A 1420-1 and Set1B K1OB-1 antibodies (Figure 2C). These data confirm previous observations that E1A associates with Set1A and Set1B *in vivo*.

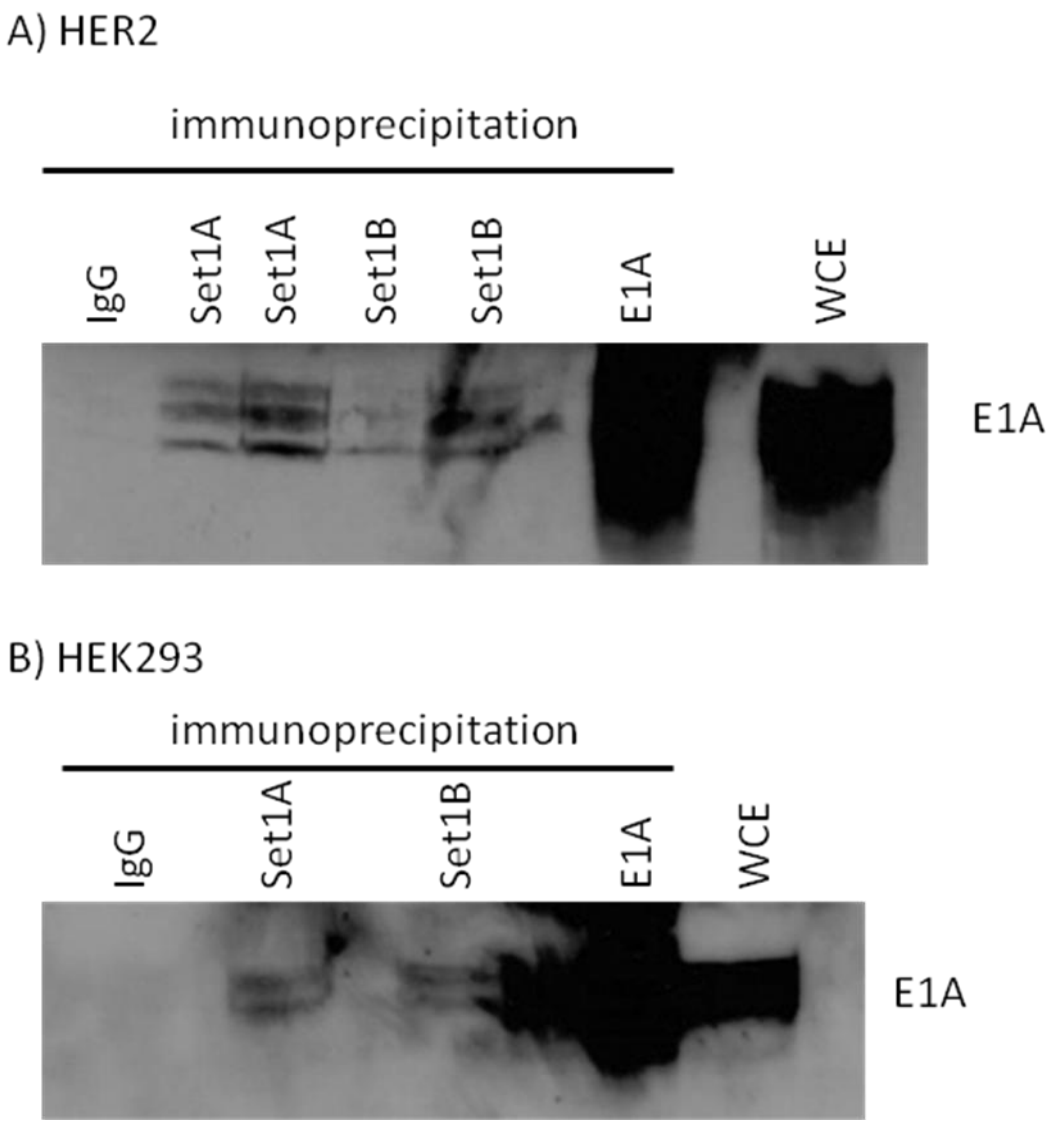


Figure 2C. Set1A and Set1B associate with E1A *in vivo.*
Anti-Set1A, (741-1 and 1420-1) and Set 1B (K76A-2 and K1OB-1), antibodies were used for immunoprecipitation from HER2 cells, whilst Set 1A (741-1) and Set1B (K1OB-1) antibodies were used for immunoprecipitation from HEK293 cell lysates. IgG was used as negative control. Imunocomplexes along with 10% whole cell extract (WCE), as a positive control, were resolved on urea-PAGE and western blotted for E1A using primary antibody M58 (1:2000) and horseradish peroxidase anti-mouse secondary antibody (Dako) (1:2000).

D. E1A co-immunoprecipitates Set1A and Set1B from HEK293 cells.

Given that I was able to show that Set1A co-precipitated E1A from HEK293 and HER2 cells, I next proceeded to determine if anti-E1A antibodies would co-immunoprecipitate Set1A and Set1B from these cells (Figure 2D). I therefore immunoprecipitated E1A from 293 cell lysates in HiLo buffer, using the M37 monoclonal antibody, and immunoprecipitated E1A from HER2 cells using a mix of the 163 and 170 monoclonal antibodies (Figure 2D). Consistent with co-precipitation of E1A with Set proteins (Figure 2C), I was able to co-immunoprecipitate Set1A and Set1B from HEK 293 cells (Figure 2D). Both Set1A and Set1B were identified as two distinct bands upon SDS-PAGE (Figure 2D). Whether these bands correspond to different isoforms or reflects post-translational modifications awaits clarification. I was unable, however, to co-immunoprecipitate Set1A and Set1B from HER2 cells. Whether the inability of Adv12 E1A antibodies to co-immunoprecipitate Set proteins reflects the relative ability of these E1A antibodies to immunoprecipitate E1A, or that the Set binding site overlaps with the antibody epitope, requires further investigation. From the results presented in Figures 2C and 2D however, we can conclude that E1A interact with both Set1A and Set1B methyltransferases

in vivo.

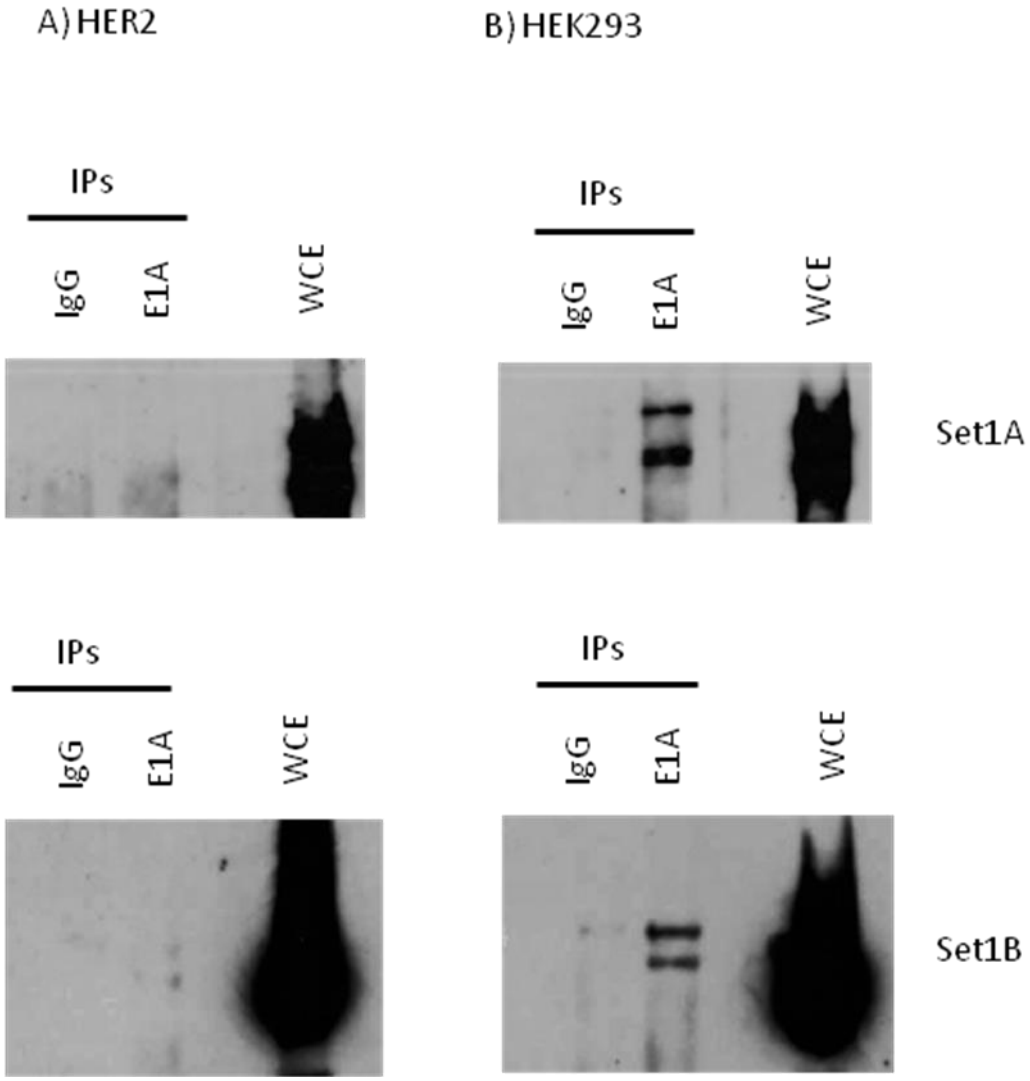


Figure 2D. E1A associates with Set1A and Set1B *in vivo.*

E1A was immunoprecipitated from HER2 cell lysates (using antibody 163 and 170) and HEK293 cell lysates (using M37 antibody). IgG was used as negative control. After isolation upon Protein G agarose beads, both the immunocomplexes along with 10% WCE (whole cell extract), were resolved on 10% SDS-PAGE and western blotted for Set 1A and Set 1B using Set1A primary antibody, 741-1(1:2000) and Set1B primary antibody, K10B-1 (1:2000). Horseradish peroxidase anti-rabbit secondary antibody was also used (Dako) (1:3000).

3. E1A associates with histone H3- and H4- directed methyltransferase activities *in vivo*.

Previous experimental work in the Turnell laboratory has shown that GST-Adv E1A 12S and 13S can immonoprecipiate histone H3- and H4- directed methyltransferase activities from A549 cells (Munir and Turnell, unpublished observations). To determine whether E1A proteins from HEK293 cells also associate with histone H3- and H4- directed methyltransferases, I performed a methyltransferase assay upon anti-E1A immunoprecipitates. Specifically, HEK293 cells were lysed in HiLo buffer and subsequently incubated with a number of different anti-E1A antibodies and antibodies raised against the S8 component of the 19S proteasome (as a positive control). Following E1A immunoprecipitation upon Protein G agarose, IPs were incubated with radioactive adenosyl-L-[Methyl ^{3}H]-methionine and core histones (H2A, H2B, H3 and H4) for 1.5 hours at 30˚C and the samples were resolved by SDS-PAGE. The gel was then stained with coomassie, dried and exposed to X-ray film at -20ºC, and developed to analyse the methyltransferase activity associated with E1A (Figure 3). Results shows E1A associate with both Histone H3 and H4 directed methyltransferase activities and that the α-E1A antibody M37 was highly efficient, compared to the other α-E1A antibodies used in this study (Figure 3). S8 antibodies also co-immunoprecipitated both Histone H3 and H4 directed methyltransferase activities (Figure 3). From the results it can be concluded that E1A associates with functional histone-directed methyltransferase activities *in vivo*, which might have implications for the ability of E1A to regulate transcription.

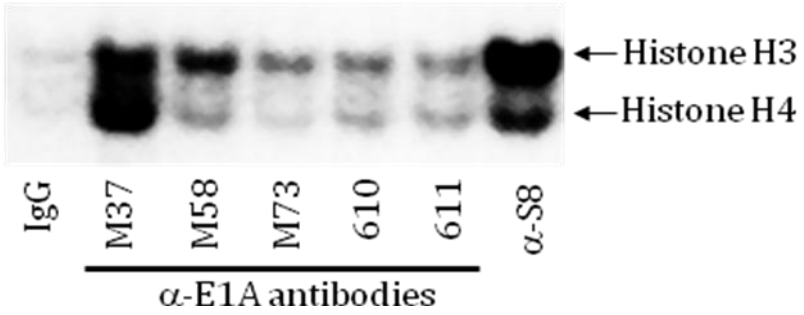


Figure 3. E1A associates with functional Histone-directed Methyltransferases. α-E1A antibodies M37, M58, M73, 610 and 611 (20 μg) were used to immunoprecipitate E1A from HEK293 cells. An antibody against the known E1A binding protein, S8, was used as positive control, and IgG was used as a negative control. Immunoprecipitates were isolated upon Protein G Sepharose beads for 2 hours at 4˚C, incubated with radioactive adenosyl-L-[Methyl ^{3}H]-methionine and core histones for 1.5 hours at 30˚C and separated on 10% SDS-PAGE, stained with coomassie, dried and exposed to X-ray film.

4. Set1A and Set1B associate with AdV early region promoters in AdV5-infected cells

From the observations that Set methyltransferases can associate with Adv E1A *in vivo*, I next wanted to determine whether Set1A and Set1B can be found associated with E1A on early region gene promoters during *w.t.* Ad5 infection. For this purpose I performed a Chromatin immunoprecipitation (ChIP) assay with anti- SET1A, Set1B and E1A antibodies from A549 cells which had been infected with *w.t.* Ad5 (1 p.f.u. /cell). 12 hours post-infection cells were treated with formaldehyde for 10 minutes and sonicated to shear the DNA; chromatin-DNA complexes were subsequently immunoprecipitated using antibodies against E1A (M58 and M73), Set1A and Set1B (Set 1A, 741-1 and Set1B, K1OB-1) and IgG as a negative control. Immunoprecipitated viral DNA was recovered by ethanol precipitation followed by phenol/chloroform extraction and was analysed by performing PCR using

oligonucleotide primer pairs specific to E1A, E1B, E2e, E3 and E4 (Figure 4). From the results obtained it was interesting to see that E1A, Set1A and Set1B were all found associated with E1A, E1B, E2e, E3 and E4 promoters (Figure 4). The E1A M73 antibody immunoprecipitated all of the early region promoters highly efficiently, whilst the M58 antibody co-immunoprecipitated E1B and E4 promoter elements, more efficiently than it did E1A, E2e and E3 promoter elements (Figure 4). Set1A and Set1B was found to associate with E1A, E1B, E2e, E3 and E4 promoters, albeit at modest levels when compared to E1A (Figure 4). From the results presented however, it seems that Set1A and Set1B do regulate Adv early gene expression at the promoter level. It is also likely, that Set1A and Set1B recruitment to Adv5 early region promoter elements is dependent on its ability to interact with E1A, though this awaits clarification.

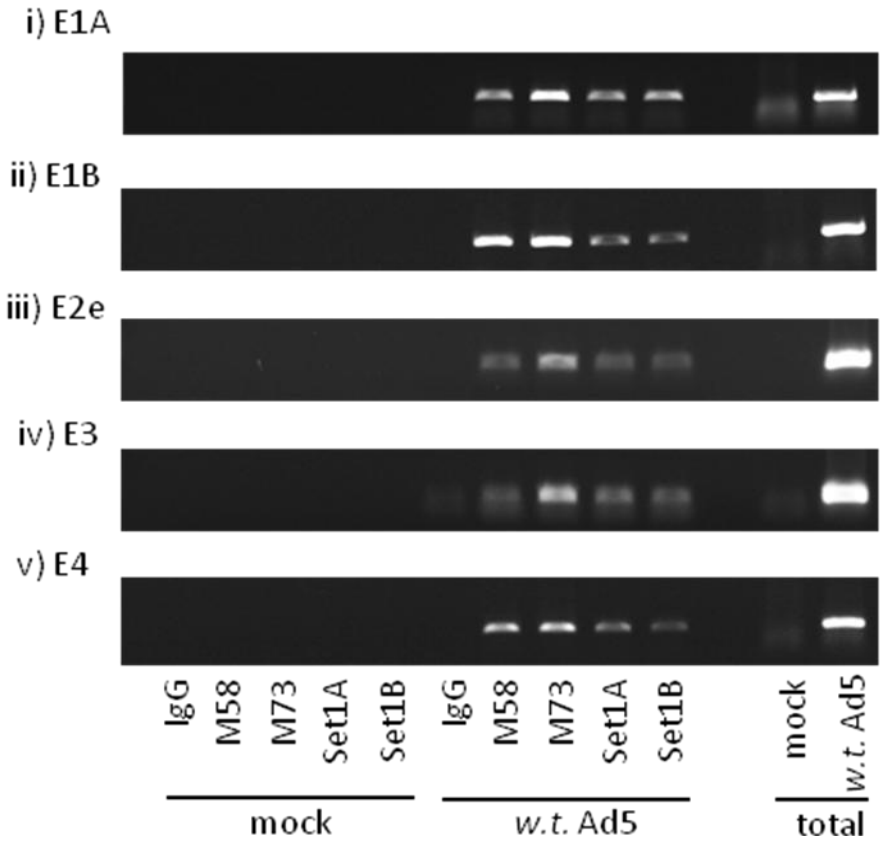
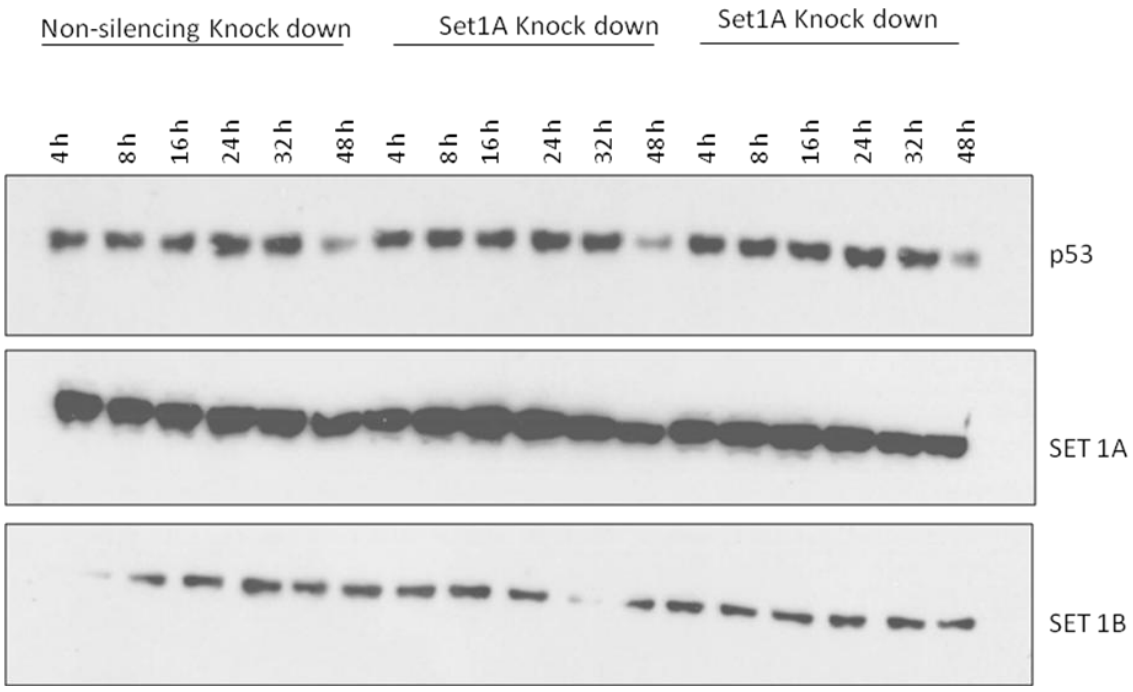


Figure 4. Recruitment of E1A, Set1A and Set1B to Ad early region gene promoters *in vivo.*
Cross-linked chromatin, prepared from *wt.* Ad 5 [1 pfu/cell] infected A549 cells or uninfected "mock" cells, was immunoprecipitated with IgG, E1A [M58 and M73], Set1A or Set1B antibodies 12 hours post-infection. Following reversed of cross-links, phenol/chloroform extraction and ethanol precipitation, immunoprecipitated DNA was subjected to PCR analysis using primer pairs that corresponded to E1A, E1B, E2e, E3, E4 promoter regions.

5. Investigating the role of Set1A and Set1B in viral early gene expression.

After looking at the interactions of Set1A and Set1B with E1A, and determining that Set1A and Set1B associate with early region gene promoters, I wanted to investigate whether Set1A and Set1B were required for efficient early region viral gene regulation after the infection of A549 with Adv5. To do this A549 cells were treated with predesigned silencer select siRNA (Ambion) directed against Set1A and Set1B (or non-silencing controls) for 48h, and then infected with wild type Adv 5 at 1 pfu/cell for 4, 8, 16, 24, 32

and 48 hours. At these times post-infection cells were harvested, and protein concentration determined. Proteins were then resolved by SDS-PAGE and western blotted using antibodies for p53, SET1A and SET1B, as well as early region proteins, E1A, E1B-55K, E2A, E4Orf3 and E4Orf6 (Figure 5). Unfortunately, despite the guarantee from Ambion about the efficacy of the siRNAs, Set1A and Set1B knockdown was not successful (Figure 5). Therefore not surprisingly, there were no differences in the levels of the E1A, E1B-55K, E2A, E4orf3 and E4orf6 proteins (Figure 5). Unfortunately, therefore, no conclusions about the role of Set1A and Set1B in early region gene expression can be made.



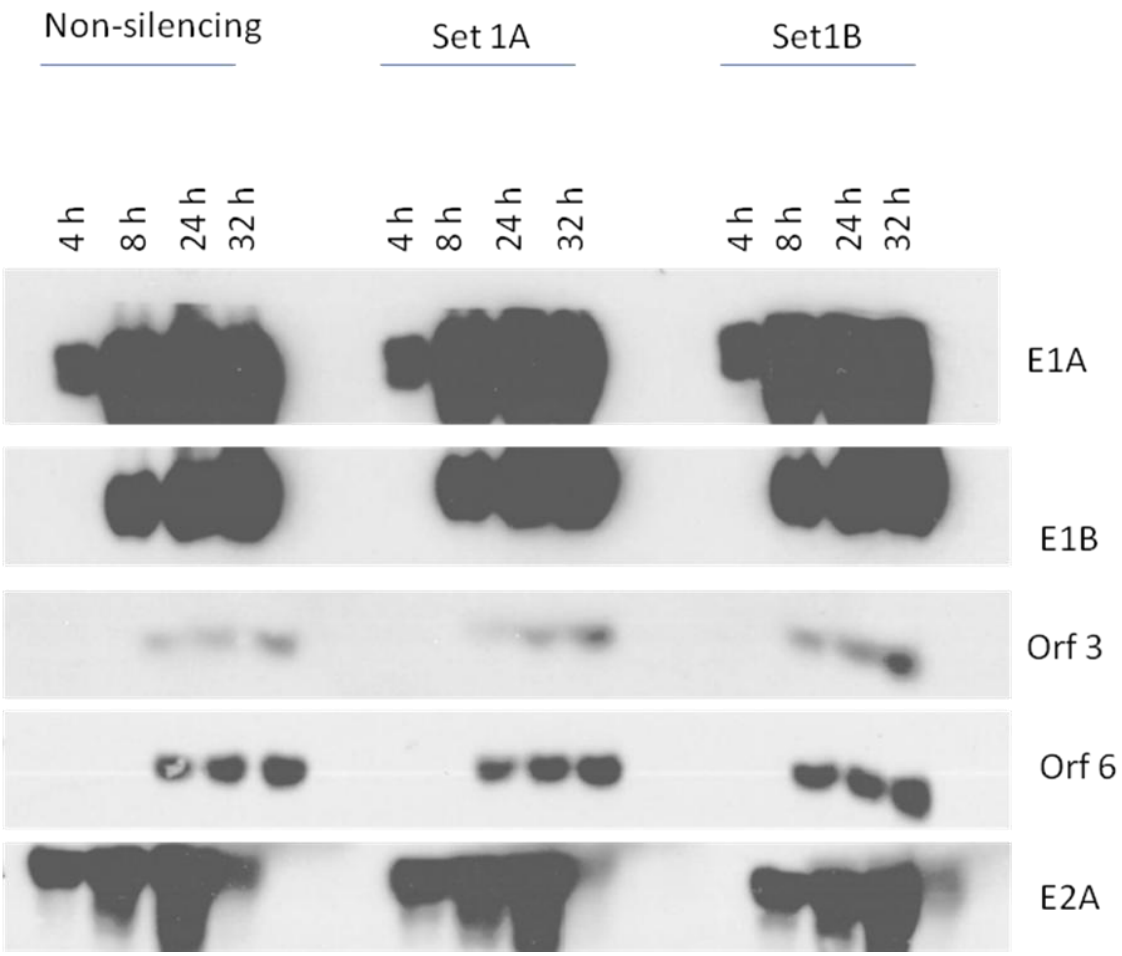


Figure 5. Investigating the role of Set1A and Set1B in viral early gene expression.

A549 cells were transfected with siRNAs directed against Set1A and Set1B, or non-silencing controls and subsequently infected with Adv5. Cells were harvested at the appropriate times and protein concentration determined. Proteins were resolved by SDS-PAGE and the level of knockdown was assessed by western blotting with anti- Set1A and Set1B antibodies to determine knockdown efficiency. The whole cell extract was also analysed by western blotting to evaluate levels of E1A, E1B-55K, E4Orf3, E4Orf6 and E2A.

6. Mass Spectrometry identification of E1A-binding proteins from HEK293 cells.

After looking at the interactions of Set1A and Set1B with E1A, I found that the M37 E1A antibody was the most efficient for co-immunoprecipitation of Set1A

and Set1B, and association with active histone-directed methyltransferases. Therefore, I decided to perform mass spectrometry analyses upon anti-M37 immunoprecipitates from HEK293 cell lysates to investigate whether I could identify novel E1A-interacting proteins. I therefore prepared HEK293 cell lysates using either stringent, HiLo buffer or, less stringent NETN buffer, and incubated lysates with E1A M37 or IgG, as a control. After isolation of the immunocomplexes upon protein G, I processed the immunoprecipitates for mass spectrometry (Refer to materials and methods section) Proteins that were found to associate with E1A in the HiLo buffer are presented in Table 1 and Figure 6.1. Proteins that were found to associate with E1A in the NETN buffer are presented in Table 2 and Figure 6.2. The proteins in the tables highlighted in yellow shows previously identified E1A binding proteins; adenovirus E1A is highlighted in red. These data are summarized as string diagrams to show the relationship between the proteins identified (Figures 6.1 and 6.2). In general more E1A-interacting proteins were identified in the less stringent NETN IP conditions compared to the more stringent HiLo IP conditions, although reassuringly a very similar set of E1A interacting proteins were identified. From the results obtained, some novel E1A interacting proteins were identified, like the MCM proteins, which are essential for pre replication complex formation and DNA replication. Proteins important for transcription and replication, like PRMT5, Fibrillarin and TIF1β were also identified. Despite the anti-E1A, M37 antibody being able to immunoprecipitate Set1A and Set1B from HEK293 cells, we did not identify these proteins in the mass spectrometry screen. This probably reflects the greater sensitivity of a western blot compared to mass spectrometry.

Protein	MW [kDa]	Scores	Peptides	SC [%]
CBP_HUMAN	265.2	786.0 (M:786.0)	20	9.7
EP300_HUMAN	264.0	621.4 (M:621.4)	13	6.0
E1BL_ADE05	55.0	584.4 (M:584.4)	12	30.0
ELYS_HUMAN	252.3	538.0 (M:538.0)	13	8.0
CTBP1_HUMAN	47.5	346.3 (M:346.3)	8	24.5
ADT3_HUMAN	32.8	289.6 (M:289.6)	7	27.2
IRS4_HUMAN	133.7	278.5 (M:278.5)	6	6.4
E1A_ADE05	31.8	272.6 (M:272.6)	4	27.3
ODPB_HUMAN	39.2	256.7 (M:256.7)	5	19.5
PRKDC_HUMAN	468.8	251.7 (M:251.7)	7	2.4
KPYM_HUMAN	57.9	240.5 (M:240.5)	5	13.2
ADT2_HUMAN	32.8	228.2 (M:228.2)	6	18.5
CTBP2_HUMAN	48.9	216.7 (M:216.7)	4	13.0
DCAF7_HUMAN	38.9	216.4 (M:216.4)	6	26.0
MCM7_HUMAN	81.3	210.9 (M:210.9)	6	11.8
FBRL_HUMAN	33.8	195.2 (M:195.2)	5	17.8
PRDX1_HUMAN	22.1	168.5 (M:168.5)	4	20.1
ABCF2_HUMAN	71.2	166.2 (M:166.2)	4	8.3
ATPO_HUMAN	23.3	149.0 (M:149.0)	3	21.1
ZMY11_HUMAN	70.9	147.2 (M:147.2)	4	7.6
HNRPU_HUMAN	90.5	146.5 (M:146.5)	3	5.5
HNRH2_HUMAN	49.2	142.2 (M:142.2)	4	12.9
PYRG1_HUMAN	66.6	141.7 (M:141.7)	3	6.4
SENP3_HUMAN	65.0	132.7 (M:132.7)	2	4.9
MCM6_HUMAN	92.8	129.3 (M:129.3)	3	5.6
UBP11_HUMAN	109.7	114.6 (M:114.6)	3	3.3

Table 1: Proteins identified in anti-E1A immunoprecipitates from HEK293 cells isolated in HiLo buffer.
HEK 293 cells were lysed in HiLo buffer (50mM Tris (pH 7.4), 0.825M NaCl, 1% (v/v) NP-40) and subject to sonication and centrifugation. Supernatants were then incubated overnight with either 20 μg normal mouse IgG (control), or 20 μg of the anti-E1A monoclonal antibody, M37. Samples were subsequently incubated with 20 μl of Protein G-Sepharose for 3h. The IPs were washed, denatured in 9M urea, 50mM ammonium bicarbonate and processed for mass spectrometric analysis as detailed in materials and methods section. Tryptic peptides were analysed using a Bruker Impact Time of Flight mass spectrometer, and identified using Proteinscape (Bruker) and the Mascot database (Matrix Science). The table shows proteins that specifically immunoprecipitated with the M37 antibody. Proteins highlighted in yellow represent previously identified E1A-binding proteins; adenovirus E1A is highlighted in red. MW, molecular weight; SC, sequence coverage.

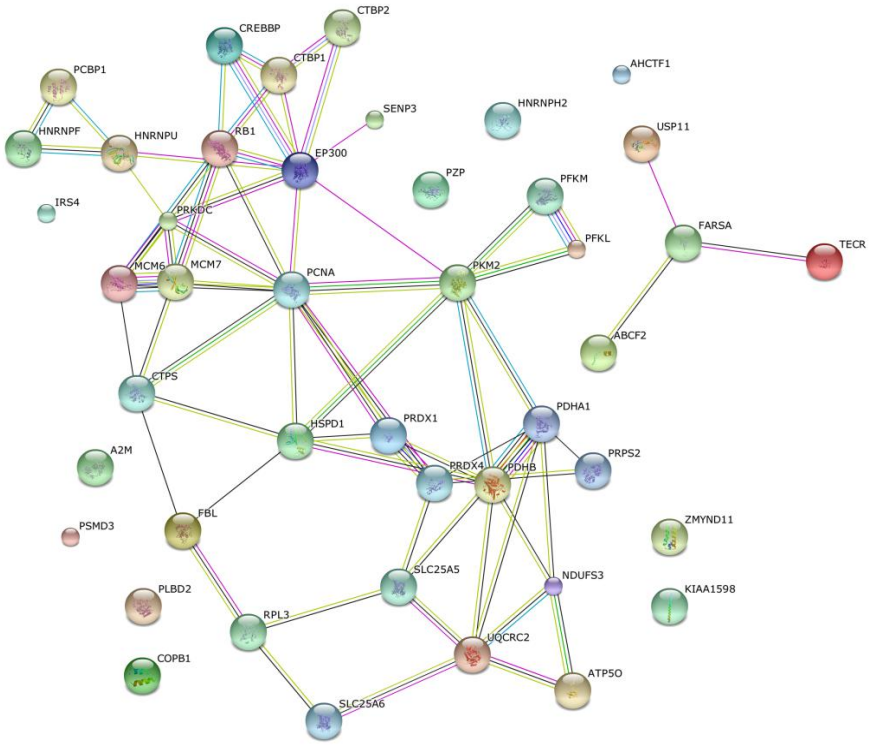


Figure 6.1 String diagram showing proteins identified in anti-E1A immunoprecipitates from HEK293 cells isolated in HiLo buffer. http://string-db.org/

Protein	MW [kDa]	Scores	Peptides	SC [%]
EP300_HUMAN	264.0	1360.2 (M:1360.2)	28	13.7
CBP_HUMAN	265.2	1185.0 (M:1185.0)	26	14.0
IRS4_HUMAN	133.7	502.3 (M:502.3)	10	9.9
MCM7_HUMAN	81.3	494.1 (M:494.1)	13	25.7
ACTG_HUMAN	41.8	484.2 (M:484.2)	8	33.1
E1BL_ADE05	55.0	477.5 (M:477.5)	10	20.8
HNRPM_HUMAN	77.5	471.7 (M:471.7)	12	20.1
SHOT1_HUMAN	71.6	468.6 (M:468.6)	11	25.2
HS90B_HUMAN	83.2	465.9 (M:465.9)	9	14.8
ATD3A_HUMAN	71.3	459.9 (M:459.9)	11	19.2
RB_HUMAN	106.1	432.7 (M:432.7)	8	10.1
ATD3B_HUMAN	71.3	385.3 (M:385.3)	10	16.8
E1A_ADE05	31.8	371.0 (M:371.0)	6	37.7
ATPA_HUMAN	59.7	329.2 (M:329.2)	6	15.0
KPYM_HUMAN	57.9	318.7 (M:318.7)	7	17.1
ADT3_HUMAN	32.8	311.2 (M:311.2)	9	32.2
CTBP1_HUMAN	47.5	305.3 (M:305.3)	7	24.1
PRKDC_HUMAN	468.8	304.3 (M:304.3)	8	2.6
ATPO_HUMAN	23.3	265.8 (M:265.8)	6	35.2
COF1_HUMAN	18.5	263.4 (M:263.4)	6	48.8
HS90A_HUMAN	84.6	238.4 (M:238.4)	5	8.7
ODPB_HUMAN	39.2	226.0 (M:226.0)	5	19.5
GBLP_HUMAN	35.1	223.5 (M:223.5)	5	18.0
CH60_HUMAN	61.0	218.8 (M:218.8)	4	10.3
HNRH1_HUMAN	49.2	209.3 (M:209.3)	5	16.7

Table 2: Proteins identified in anti-E1A immunoprecipitates from HEK293 cells isolated in NETN buffer.
HEK 293 cells were lysed in NETN buffer (50mM Tris (pH 7.4), 0.25M NaCl, 1% (v/v) NP-40, 1mM EDTA) and subject to dounce homogenization and centrifugation. Supernatants were then incubated overnight with either 20 μg normal mouse IgG (control), or 20 μg of the anti-E1A monoclonal antibody, M37. Samples were subsequently incubated with 20 μl of Protein G-Sepharose for 3h. The IPs were washed, denatured in 9M urea, 50mM ammonium bicarbonate and processed for mass spectrometric analysis as detailed in materials and methods section. Tryptic peptides were analysed using a Bruker Impact Time of Flight mass spectrometer, and identified using Proteinscape (Bruker) and the Mascot database (Matrix Science). The table shows proteins that specifically immunoprecipitated with the M37 antibody. Proteins highlighted in yellow represent previously identified E1A-binding proteins; adenovirus E1A is highlighted in red. MW, molecular weight; SC, sequence coverage.

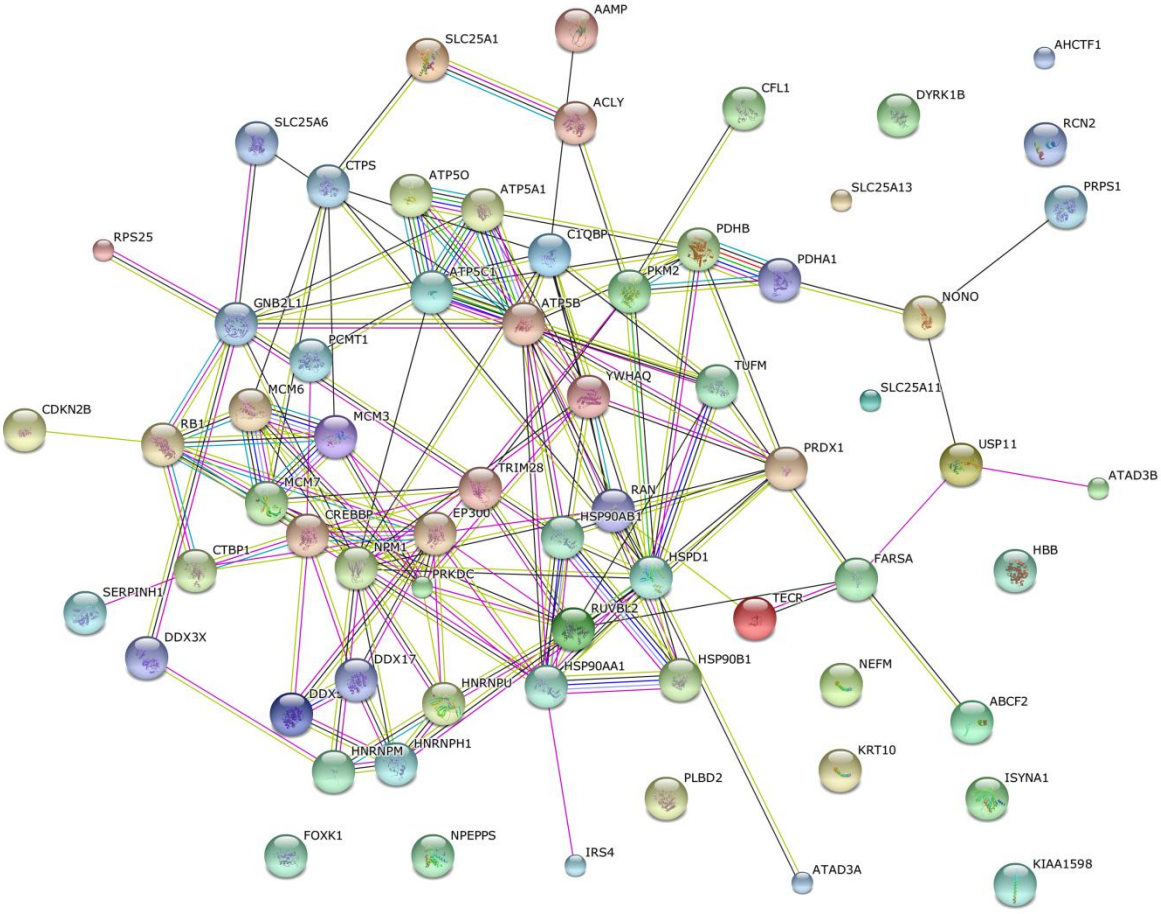


Figure 6.2. String diagram showing proteins identified in anti-E1A immunoprecipitates from HEK293 cells isolated in HiLo buffer. http://string-db.org/

7. Validation of MCM interaction with E1A.

Following the results obtained from Mass Spec, I carried out co-immunoprecipitation studies with some of the E1A-interacting cellular proteins like the MCM proteins to validate the interaction. The mini-chromosome maintenance proteins (MCMs) are 6 structurally similar family of proteins MCM 2 to 7 which forms a hexameric complex which serves as an essential component of the pre replication complex that helps the replication origins to assemble during replication. 293 cell lysate in Hi Lo buffer was co-immunoprecipitated with MCM 2 to 7 and the immunocomplexes were separated on urea-PAGE and western blotted for (Figure 7). From the results it was confirmed that MCM proteins interact with E1A. The functional

significance of this interaction requires further investigation.

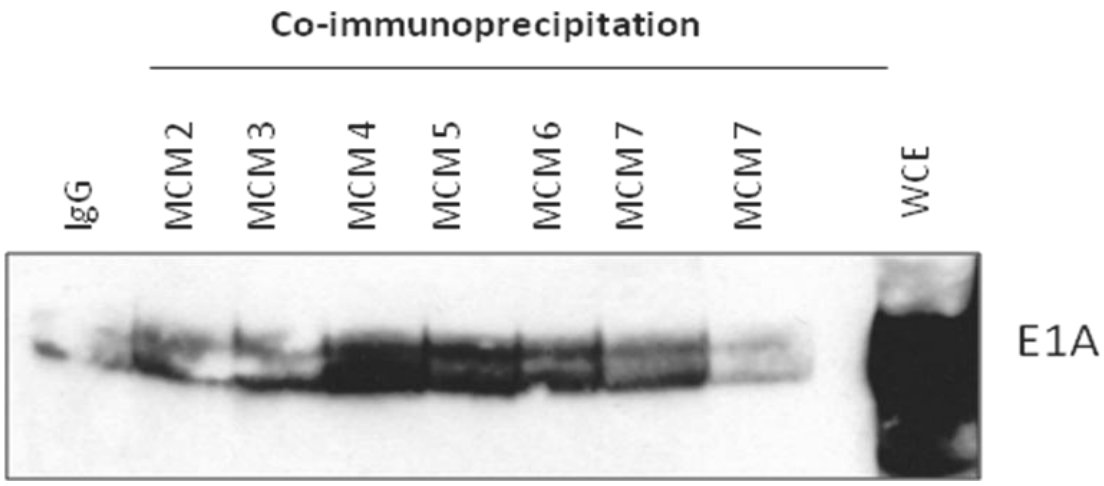


Figure 7. MCM proteins associate with E1A in HEK293 cells.
293 cell lysate was co-immunoprecipitated with MCM 2 to MCM 7 [MCM 7 (Santa Cruz) and (Millipore)] and IgG was used as negative control. Imunocomplexes along with 10% whole cell extract (WCE), as a positive control, were resolved on urea-PAGE and western blotted for E1A using primary antibody M58 (1:2000) and horseradish peroxidase anti-mouse secondary antibody (Dako) (1:2000).

IV. Discussion

Adenoviruses are typically used as a model system to investigate the role of viral early region proteins in the transcription of viral and cellular genes, and moreover, investigate the molecular basis of cellular transformation and tumourigenesis (Gallimore and Turnell, 2001; (Turnell, 2008). In this regard, Adv E1A has been studied in great detail to understand viral replication and cellular transformation as E1A associates with numerous cellular proteins that regulate transcription and function as tumour suppressor proteins. Adv E1A CR3 is very important for viral replication as it interacts with several cellular transcriptional factors, and transcriptional regulators, that serve to transactivate different cellular and viral promoters (Turnell, 2008). It has also been determined that the temporal regulation of transcription by E1A promotes the infected cell to enter the S-phase (Dery et al., 1985). The ability of E1A to regulate transcription programmes is achieved through its capacity to interact with factors that regulate transcription, such as: CBP/p300 (Co-activators); pRB, p107 and p130 (repressors); CtBP (co-repressors); TBP and TAFs (basal transcriptional machinery); Sur2 (ancillary co-activators-Mediator); and SWI and SNF (nucleosomal remodelling factors) (Gallimore, 2001, Turnell, 2008).

To understand fully the functional ability of Adv E1A to regulate viral gene regulation, it is important to identify specific, cellular binding partners that associate with the CR3 region of E1A, and then determine the consequences of the interaction upon transcription and viral replication. Interestingly, it is becoming clear that individual proteins serve as components of larger macromolecular complexes, and previous work in the Turnell laboratory has

determined the role of the proteasome and other CR3-associated proteins, in CR3-dependent transcription. Indeed, Rasti *et al* (2006) determined that 19S proteasomal ATPases, the 20S proteasome, and the 26S proteasome all interacted with CR3 of E1A to regulate viral transcription programmes. Later, unpublished work, conducted in this laboratory determined that the histone-directed methyltransferases Set1A and Set1B bind to the CR3 domain of E1A.

The primary aim of the present study was to investigate further the relationship between of Adv E1A and Set1A and Set1B in viral gene regulation. We initially started to investigate the specific roles of 12S and 13S E1A gene products in viral gene regulation following Adv infection. From the results obtained it was clear that CR3 was essential for E4 Orf6 and E4 Orf3 expression (Figure 1.A), whilst E1B-55K and E2A expression was also mainly dependent on 13S E1A expression though 12S E1A also contributed towards their expression (Figure 1.A). Consistent with earlier studies my work confirmed the important role 13S E1A plays in transactivation. Presumably, the ability of 12S to transactivate the E1B-55K and E2A genes, resides in its capacity to interact with proteins such as the pRB family, CBP/p30, and other transcriptional regulators.

To validate the functionality of E1A in HEK 293 cells, we next decided to investigate the ability of E1A to bind cellular proteins in these cells. To do this we immunoprecipitated the 293 cell lysate with antibodies specific for cellular proteins like CBP (N-terminal and CR1 binding protein), CtBP (C-terminal binding protein), pRB (CR1 and CR2 binding protein), p107 and p130 (CR1 and CR2 binding protein) and TBP (Binding to N-terminus and CR3) and then

Western blotted for E1A. From the results, it was clear that E1A associates strongly with the pRB family of Pocket proteins, pRB, p107 and p130, when compared to C-terminal and N-terminal binding proteins CtBP, CBP and TBP. To confirm these results we performed reciprocal immunoprecipitations using anti-E1A antibodies. From the results obtained it was confirmed that all pRB family proteins interact with E1A *in vivo.* These pRB proteins acts as transcriptional repressors and normally serve to inhibit E2F-dependent transcription programmes; E1A interaction with pRB promotes pRB dissociation from E2F, which results in the activation of E2F-dependent transcription. E2F drives cell cycle progression, and inhibits cellular differentiation and tumour suppression (Gallimore and Turnell, 2001).

After validating the functionality of E1A in HEK 293 cells, we investigated the interaction between the histone H3K4-directed methyltransferases, Set1A and Set1B and E1A *in vivo*. Previous work had determined that Set1A and Set 1B interact with Adv5 E1A, in both Adv5 E1-transformed cells and Adv5-infected cells (Munir and Turnell, unpublished observations). To confirm this, we immunoprecipitated Set1A and Set1B from Adv5 HEK 293 cell lysate, and Ad12 HER2 cell lysate, and resolved the immunocomplexes on an urea gel and Western blotted for E1A; we determined that Set1A and Set1B interacted with E1A in both Adv5- and Adv12- transformed cells (Figure 2C) and therefore confirmed the previous observations that E1A associates with Set1A and Set1B *in vivo*. It had not previously been determined however that Set1A and Set1B interact with E1A in Adv12-transformed cells. My data suggest that the interaction between E1A and Set1 proteins is conserved amongst divergent Adv types and that Set1 might play an important role in transcription

programmes regulated by different E1A species.

After showing that Set1A co-precipitated E1A from HEK293 and HER2 cells, we next investigated whether anti-E1A antibodies co-immunoprecipitated Set1A and Set1B from these cells. Interestingly we was able to co-immunoprecipitate Set1A and Set1B from HEK 293 cells, but not from HER2 cells (Figure 2D). Set1A and Set1B were seen as two distinct bands upon Western blotting. Whether these bands correspond to different Set1A and Set1B isoforms, or reflects post-translational modifications needs to be clarified in future research. E1A interacted with Set1A and Set1B in all my three experimental repeats from HEK 293 cells, and from the results obtained we confirmed both Set1A and Set1B methyltransferases interact with E1A *in vivo* and these interactions might play a crucial role in promoting E1A dependent transcription. We were unable to co-immunoprecipitate Set1A and Set1B from HER2 cells (Figure 2D). As we were able to perform the reciprocal co-immunoprecipitation in these cells, it is possible that the anti-Adv12 E1A antibodies used in this study sterically prevented the co-immunoprecipiation of the Set1 proteins. In the future it would be important to generate new Adv12 E1A antibodies raised against different regions of the E1A protein and investigate whether they can co-precipiate Set1A and Set1B.

It has previously been shown that GST-Adv E1A 12S and 13S can immonoprecipiate histone H3- and H4- directed methyltransferase activities from A549 cells (Munir and Turnell, unpublished observations). We therefore, investigated whether E1A associated with functional histone-directed methyltransferases *in vivo* in HEK 293 cells by performing a methyltransferase

assay upon anti-E1A immunoprecipitates. We demonstrated that a number of different α-Adv5 E1A antibodies could immunoprecipitate histone-directed methyltransferase activity from HEK 293 cells, and found that α-E1A antibody; M37 was highly efficient compared to positive control α-S8 (19S proteasomal ATPase) at immunoprecipitating methyltransferase activity (Figure 3). The reasons for this are not immediately apparent, but probably reflect the avidity of the M37 antibody for E1A, and the specific antibody-antigen binding site on E1A. From the results obtained we confirmed that E1A associates with functional histone-directed methyltransferase activities *in vivo*, which might have implications for the ability of 13S E1A to regulate viral transcription programmes. Interestingly E1A co-precipitated both H3 and H4 histone-directed methyltransferase activity. As Set1A and Set1B are H3K4-directed methyltransferases it is not surprising that E1A co-precipitated H3 methyltransferase activity. In the future it will be important to establish which H4 methyltransferases E1A can associate with, and determine whether they are important for E1A-regulated transcription. It will also be important to investigate whether AdvE1A can regulate site specific methylation of histones associated with cellular and viral gene promoters.

Given these findings, we next proceeded to determine whether Set1A and Set1B associate with E1A on early region gene promoters. We performed a ChIP assay with antibodies against E1A, Set1A and Set1B. Interestingly Set1A and Set1B were recruited along with E1A to all the early viral promoters (E1A, E1B, E2e, E3 and E4) but at modest levels when compared to E1A (Figure 4). It is possible therefore that specific H3 K4 modification of chromatinized viral DNA at early region gene promoters by Set1A and Set1B

might facilitate transcription of viral genes. Therefore, from the data obtained we can speculate that Set1A and Set1B do regulate Adv early gene expression at the promoter level. It will be important in the future to determine whether the ability of Set1A and Set1B to interact with Adv5 early region gene promoters is dependent upon CR3. This could be achieved using Adv5 CR3 mutants that do not bind to Set1A and Set1B. It would also be interesting to see whether antibodies that recognize mono, di and tri-methylated histone H3 K4 can also chromatin immunoprecipitate early region promoters. This would establish further a role for Set1 proteins in E1A transcription programmes.

To investigate a specific requirement for Set1A and Set1B in regulating CR3-dependent transcription, we next attempted to knockdown Set1A and Set1B gene expression by siRNA and determine the consequences of knockdown upon the transcription of viral early genes following Adv5 infection. Unfortunately, the knock down was not successful in two experimental repeats (Figure 5). Due to lack of time I wasn't able to investigate this further. In future research, therefore, it would be important to optimise Set1A and Set1B knock down using different clones of A549 cells, and then determine whether Set1A and Set1B expression in cells is essential for regulating early gene expression. It would also be interesting to see if general protein methyltransferase inhibitors, like 5′-deoxy-5′-methylthioadenosine (MTA) can inhibit Adv gene transcription. MTAs are known to inhibit histone methyltransferases such as Set1A and Set1B, and alter gene expression patterns (Huang et al., 2006). Indeed MTA has previously been shown to reduce the trimethylation of histone H3K4 associated with HSV-1 genomes, and furthermore inhibit HSV-1 viral gene expression and viral replication

(Huang et al., 2006). It might be, therefore, that Set1 proteins are common targets for DNA viruses.

As we determined that the M37 E1A antibody was highly efficient in co-immunoprecipitating Set1A and Set1B, and also associating with active histone-directed methyltransferases, we next decided to perform mass spectrometry analyses upon anti-M37 immunoprecipitates from HEK293 cell lysates to investigate whether we could identify novel E1A-interacting proteins (Figure 6.1 and Figure 6.2). Interestingly, some novel E1A interacting proteins were identified, like the minichromosome maintenance (MCM) proteins. MCMs are 6 structurally related proteins, MCM2 to MCM7, which form a hexameric complex and serves as an essential component of the pre-replication complex during DNA replication to assemble the replication origins. Proteins important for transcription and replication, like PRMT5, Fibrillarin and TIF1β were also identified. We also determined that although the M37 antibody was able to immunoprecipitate Set1A and Set1B from HEK293 cells, we couldn't identify these proteins in the mass spectrometry analyses. This might be due to the greater sensitivity of western blots compared to mass spectrometry, or the failure to generate Set1 tryptic peptides of sufficient size and quality to be detected by the mass spectrometer.

We next validated our mass spectrometry results by performing co-immunoprecipitation experiments with MCM2 to MCM7 in HEK 293 cells to see whether E1A interacted with multiple MCMs *in vivo.* After resolving the immunocomplexes on a urea gel and western blotting for E1A, the results confirmed that E1A interacts with multiple MCM proteins *in vivo*. In future,

reciprocal immunoprecipitation should be performed to validate that E1A can immunoprecipitate MCM2 to MCM7 *in vivo.* Moreover, the biological significance of E1A interaction with MCM proteins should be investigated. Indeed, previous work has shown that, different DNA viruses target MCM proteins to inhibit host cell DNA synthesis in order to promote viral DNA replication (Roberts et al., 2008); HPV E4 represses cellular DNA synthesis through interacting with MCM2 and MCM7 and inhibiting the cellular origins required for replication, and likewise playing a prime role in viral replication (Roberts et al., 2008). From my results, we can hypothesize that the association of Adv E1A with MCM proteins, akin to HPV, could inhibit the origins of replication required for cellular DNA synthesis in order to enhance viral replication. It will be important to investigate this possibility. As MCM proteins also participate in transcription it will also be important to investigate their requirement for Adv transcription.

In conclusion, work presented here has further defined E1A interaction with Set1 proteins, and established that Set1 proteins associate with viral early region promoters. Work presented here has also identified a number of potentially new E1A-interacting proteins, of which the MCM interaction with E1A has been validated. In the future it will be important to study the molecular roles of E1A-Set1 and E1A-MCM complexes, to see whether they function to regulate viral and/or cellular transcription, and DNA replication.

VI. Acknowledgements

I am very grateful to Dr. Andy Turnell, for giving me the oppurtunity to work on this project. I wish to express my gratitude and thanks to my supervisor Dr. Andy Turnell, for encouraging and supporting me through out my project work. I would also like to thank, Dr. Roger Grand, Paul Minshall, Jessica Foster and Fadi Qashqari for their kind help and support.

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