1.1 INTRODUCTION.

Carcinomas of the anogenital tract particularly cancer of the cervix, account for almost 12% of all cancers in woman and represent the second most common gynaecological malignancy in the world. Almost all squamous cell carcinomas and a vast majority of adenocarcinomas of the cervix are HPV positive. The development of cervical cancer has been linked to persistent infection with high risk HPV and is generally preceded by a long latency period. However, only minority of woman infected with HPV develop a cancer. These observations strongly suggest that accumulation of host genetic changes and predisposing host factors play a central role in malignant progression of high risk HPV associated lesions. Infection with the high risk HPV types 16 and 18 results initially in unobtrusive squamous intraepithelial lesions (SIL) in women. Most of the lesions are cleared 6-12 months after appearance, possibly due to the host immunological defence. A small percentage progress to high grade SIL (HSIL) carcinoma *in situ* and without surgical intervention to squamous-cell carcinoma or adenocarcinoma of the cervix (zur Hausen, 2000).

The human papillomaviruses (HPV) are responsible for some of the most common sexually transmitted infections in women. The HPVs belong to the *Papillomaviridae* family, which are non-enveloped viruses that contain a covalently closed-double stranded DNA genome. The 8-kbp HPV DNA molecule contains the early and late genes clustered in separate regions of the HPV genome. Early genes (E1, E2, E4, E5, E6 and E7) code for proteins involved in viral DNA replication, transcription control and cellular transformation, whereas late genes encode the major viral capsid protein (L1) and a minor capsid protein (L2).

HPV types that are associated with genital infections have been classified into two types according to their risk of causing cervical cancers; that is, high risk types (e.g.HPV16 and HPV18) and low risk types (e.g.HPV6 and HPV11). It appears that both high and low risk HPV-encoded E6 and E7 proteins show some ability to disrupt the normal control of cell proliferation, however, only E6 and E7 proteins encoded by high risk types are able to contribute to cell transformation. It is well-established that cell division follows a series of complex events which constitute the cell cycle. Actively dividing cells pass through distinct stages of DNA synthesis (S-phase) and mitotic division (M-phase), and these activities are separated by gap phases (G1 and G2). The main control of the cell cycle involves a family of kinases, known as cyclin-dependent kinases (CDKs) which control passage through both S and M phases.

The E6 and E7 genes of high risk HPV types are sufficient to immortalize human keratinocytes in tissue culture, and this activity requires inactivation of the cellular tumour suppressors p53 and retinoblastoma (RB). Whilst E6 interacts with p53, the E7 proteins from high risk HPVs bind the RB family of proteins. RB was identified as an important target of oncoproteins that are expressed by DNA tumour viruses, including the E7 proteins of human papillomaviruses. These findings indicated that RB is a regulator of cell proliferation. RB is closely related to two genes in mice and humans (p107 and p130), which are not commonly mutated in tumours. These proteins are referred to as the 'pocket proteins' because their main sequence similarity resides in a domain that mediates interaction with the viral oncoproteins. Interaction of E7 with the pocket proteins results, in the constitutive activation of E2F transcription factors. RB normally interacts with and inhibits the function of the activator E2Fs, E2F1-3, whereas p107 and p130 interact with the repressor E2Fs, E2F4 and E2F5.

Recently, it has been discovered that the pocket proteins are constituents of mammalian DREAM complexes. DREAM was first discovered in Drosophila embryonal cells and was named from its composition of Drosophila Rb, E2F and Myb-interacting proteins (Korenjak *et al*., 2004). Mammalian DREAM consists of a core LIN complex (LINC: comprised of Lin-9, Lin-37, Lin-52, Lin-54 and RBBP4) that associates with p130/E2F4 in G0/G1 to repress E2F-regulated genes (Litovchick et al., 2007, Mol. Cell 26:539-551) or with B-Myb in S/G2 to activate genes required for mitosis (Pilkinton et al., 2007, Oncogene 26:7535-7543; Schmit et al., 2007, Cell Cycle 6:1903-1913).

In this project, the effect of HPV E7 on mammalian DREAM complexes in HPV-transformed cell lines was conducted. In order to achieve the goal, firstly the DREAM complexes components in four different cell lines (T98G, C33a, SiHa and CaSki) were determined by protein co-immunoprecipitation and western blot. T98G glioblastoma cells were used as a control, as the DREAM complexes have been well-characterised in this cell line (Schmit et al., 2007). C33a is a cervical carcinoma cell line that does not carry any HPVgenes, whereas SiHa and CaSki cells are HPV 16 –positive, with two and 600 copies, respectively.

Following this, we designed and constructed 16E7 short hairpin RNAs (shRNAs) to identify the effect on HPV E7-depleted cell lines. The pLKO.1 vector system is employed for this purpose and two different constructs were generated, verified and used in transduction assays on SiHa and CaSki cells. Via the use of co-immunoprecipitation, western blot and flow cytometry methods, the effect of p130/DREAM and B-myb/DREAM complexes in E6/E7-depleted cells were analysed.

Ectopic expression of six different types of HA epitope-tagged HPV E7 proteins was performed in T98G cells to demonstrate the effect of various types E7 binding towards p130/DREAM complexes. The six types of HPV are, HPV 1E7, HPV 11E7 (low risk), HPV 16E7, HPV 18E7, HPV 33E7 (high risk) and HPV 48E7 (cutaneous) types. This assay is important in order to establish a rigorous assay for the effects of E7 proteins in DREAM complexes.

Consequently, four types of p130 plasmids were constructed consisting, wild type p130 (p130wt), phospho-site mutant p130 (p130PM22), E7 binding mutant p130 (p130mE7) and E7 binding mutant + phospho-site mutant p130 (p130mE7/PM22) to investigate the impact of E7 binding towards various types of p130 in CaSki cells. All p130 mutants plasmid were designed with HA epitope-tagged to differentiate between the endogenous and ectopic p130 in the transfected cell lines. The co-transduction assay with DREAM core components (Lin-9 and Lin-54) shRNA and flow cytometry analysis were carried out to demonstrate whether the disruption of p130/DREAM is really critical in Caski cells.

Knight A.S *et al.* (2009) have shown that B-myb/DREAM complex is required for transit through mitosis in embryonal stem cells, in which the retinoblastoma protein family is inactive. Therefore, we analysed the function of B-myb/DREAM complex in CaSki, since CaSki cells lacks of pocket protein function. The importance of B-myb/DREAM complex in CaSki is achieved through RNA interference and mitotic index analysis. In addition, we also studied the effect of several types of mitotic genes in Lin-54 (one of the core component in DREAM complex) depleted cells.

1.1.1 Objectives of the study

Recent evidence indicates that p130 regulates cell-cycle progression as part of a large protein complex termed DREAM. The composition of DREAM is cell cycle regulated, associating with E2F4 and p130 in G0/G1 and with the B-myb transcription factor in S/G2. In this study, we would like to address whether p130/DREAM is disrupted in HPV 16-transformed cervical cancer cells and whether this is a critical function for E6/E7.

Therefore, the main research objectives are:

1. To investigate whether E7 encoded by various types of HPV are able to disrupt the G0/G1 DREAM complex and thereby push the cell into cycle.
2. To distinguish whether p130 is protected from E7-mediated degradation by formation of DREAM complexes.
3. To demonstrate the presence of RB-associated DREAM complexes in primary keratinocytes.
4. To determine whether HPV E7 proteins from various HPV types will disrupt the RB-associated DREAM complexes interaction.

1.2 LITERATURE REVIEW

1.2.1. Papillomavirus research, a history

The viral aetiology of common warts in humans was first indicated in the turn of the 20th century by Ciuffo, who reported the transmission of common warts using intradermal inoculation of cell free filtrates (Ciuffo, 1907). The first papillomavirus was described in 1933, when Richard Shope identified the cottontail rabbit papillomavirus (CRPV) as the etiologic agent responsible for cutaneous papillomatos is in the cottontail rabbit (Shope, 1933). Following the demonstration that papillomas in domestic rabbit could undergo malignant conversion (Rous and Beard, 1934; Rous and Beard, 1935). Strauss *et al.* investigated the nature of the infectious agent responsible for the induction of warts in 1949 and were the first ones to detect the viral particles in human warts by electron microscopy

(Strauss *et al.*,1949). Another important step in PV research was made by Crawford and Crawford who characterized the physical properties of the viral DNA (Crawford and Crawford, 1963). However, the characterization of the viral life cycle and the natural history of virus infection were and still are hampered by the difficulty of amplifying the viruses in *in vitro* cell culture systems. An association between human papillomavirus infection and cervical cancer was first proposed in 1976 by H. zur Hausen (zur Hausen, 1976) , but was disputed due to the failure of regular skin warts to progress to malignancies. These inconsistencies were overcome by reports of the heterogeneity of HPVs (Gissman *et al.*, 1977) and by reports that sera from skin warts failed to react with virus particles from genital warts. In the beginning of the 1980s novel HPVs were identified in cervical cancer biopsies

(Boshart *et al.* , 1984; Dürs t *et al.* , 1983) using hybridization protocols described by Law *et al.* (Law *et al.*, 1979) . Transforming activity in cell culture was first demonstrated for the whole HPV genome (Dürst *et al.* , 1987) with the subsequent identification of the importance of the products of the early genes, E6 and E7, for maintaining the transformed phenotype.

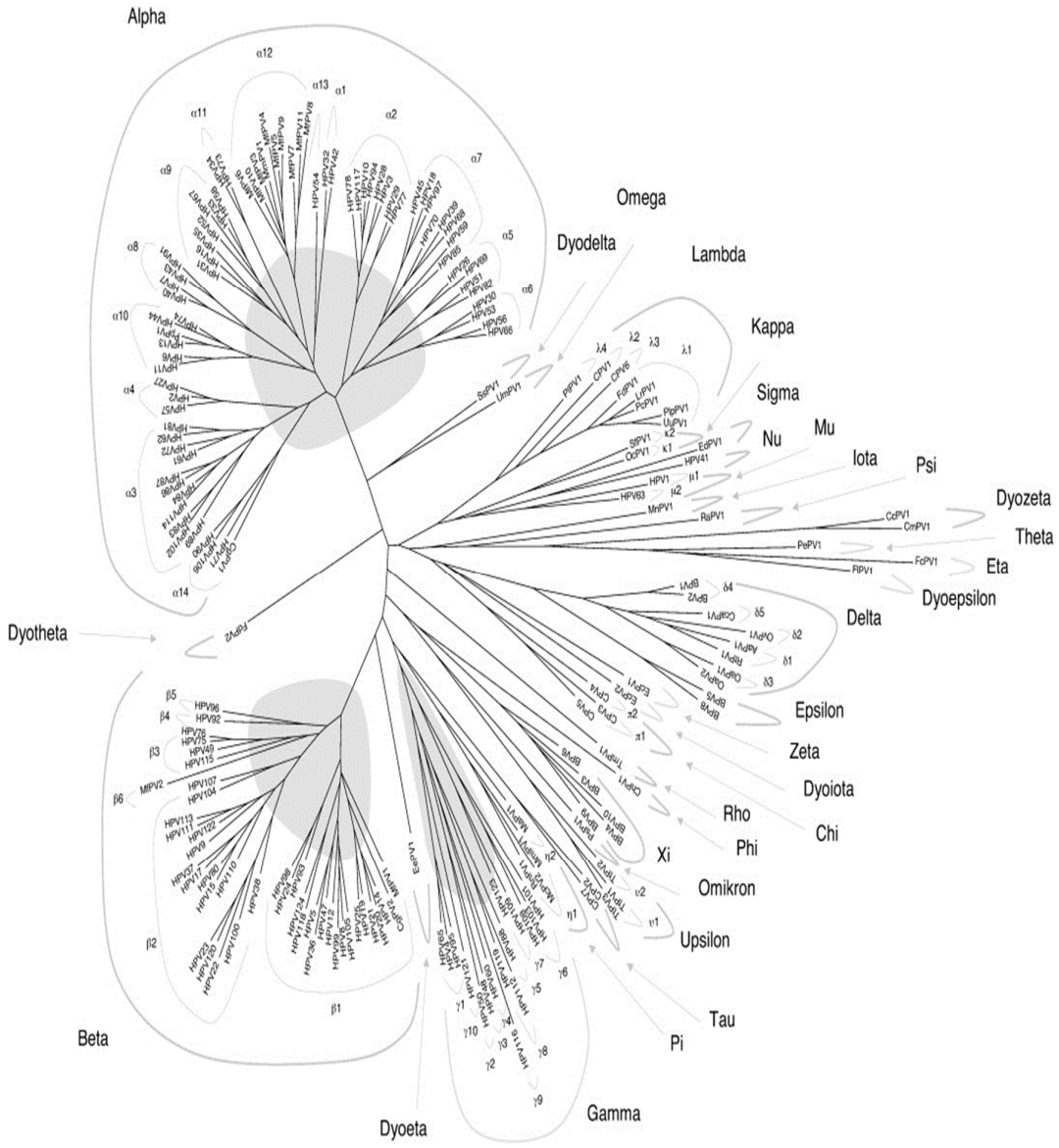
The characterization of the E6 and E7 protein facilitated the understanding of the role of tumour suppressor proteins in cell cycle and apoptosis control, such as p53 and pRb. In the last few decades the papillomavirus field has evolved rapidly, leading to confirmation the role of HPV in ano - genital cancers and identification of the high risk HPV types involved.

1.2.2. Human papillomavirus (HPV).

So far, 92 HPV types have been fully sequenced, of which 60 belong to the alpha genus and the other 32 belong to the beta and gamma genera (Figure 1.1). However, more than 120 putative novel types belonging to the beta and gamma genera have been partially characterized (Bernard *et al.*, 2010). HPVs are strictly epitheliotropic and infect epithelial cells of the skin or of the anogenital and oropharyngeal mucosa. Based on their tissue- tropism HPVs can be divided into cutaneous or mucosal HPV types. Examples of several HPV genotypes and their associated lesions and tissue tropism are given in (Table 1.1). The mucosotropic papillomaviruses make up most of the alpha genus, whereas the beta and gamma genera consist of cutaneous HPV types. The beta group consists of so-called EV-HPVs, which were first isolated from patients with Epidermodysplasia verruciformis (EV), a rare hereditary disease, and the gamma PV types consist mostly of PVs which are phylogenetically related to the beta HPV types.

Figure 1.1. Phylogenetic tree containing the sequences of 118 papillomavirus types. The L1 ORF sequences were used in a modified version of the Phylip version 3.572 and based on a weighted version of the neighbour-joining analysis. The tree was constructed using the Treeview program of the University of Glasgow. The numbers at the ends of each of the branches identify an HPV type; c-numbers refer to candidate HPV types. All other abbreviations refer to animal papillomavirus types. The outermost semicircular symbols identify papillomavirus genera, *e.g.* the genus alpha-papillomavirus. The number at the inner semicircular symbol refers to papillomavirus species. For instance, the HPV types 7, 40, 43, and 91 together form the HPV species 8 in the genus alphapapillomavirus.

Reprinted from Classification of papillomaviruses. Bernard et al., 2010


Alpha
Beta
Gamma
Delta
Epsilon
Zeta
Eta
Theta
Iota
Kappa
Lambda
Mu
Nu
Xi
Omikron
Omega
Phi
Pi
Rho
Sigma
Tau
Upsilon
Chi
Psi
Dyodelta
Dyoepsilon
Dyoiota
Dyotheta
Dyozeta
Dyoeta

HPV GENOTYPES ORIGINATED FROM	
Cutaneous HPV GENOTYPES	
1	plantar warts
2,4,26,27,29	common warts
3,10,28	flat warts
5,8,47	benign and malignant EV lesions
9,12,14,15,17,19-25, 50	EV lesions
38	melanoma; malignant cutaneous lesions
41, 48	cutaneous squamous cell carcinomas
49	flat warts under immuno-supression
75-77	common warts in renal allograft recipient
Mucosal HPV GENOTYPES	
6,11	genital warts, laryngeal papillomas
13	oral focal epithelial hyperplasia's
30	laryngeal carcinomas
32	oral focal epithelial hyperplasia's; oral papillomas
16,18,31,33,35,39,45,51,52,56, 58,59	anogenital intraepithelial neoplasia and cancer
34,40,42-44,53-55,61,62,64,67-69,71,74	anogenital intraepithelial neoplasia
72,73	oral papillomas (HIV patients); anogenital intraepithelial neoplasia's

EV= *Epidermodysplasia verruciformis*; HIV= human immunodeficiency virus

Table 1.1. Some examples of identified HPV genotypes and their origin.

Biological and epidemiological data from the mucosal HPV types have allowed the division of these types into two groups; the so- called low-risk HPV types, like HPV6 and 11, which are rarely associated with cancers and the high-risk HPV types, e.g. HPV16 or 18, which are preferentially associated with benign and malignant anogenital lesions (reviewed in zur Hausen, 1997) . Within the high-risk family, HPV16 and HPV18 are the most commonly detected genotypes in cervical lesions and are responsible for approximately 50% and 25% of the cervical cancer cases worldwide, respectively (Matsukura et al ., 1995; Zehbe et al ., 1996 and 1997). Penile, vulvar, anal and peri -anal carcinomas have been analysed only to a certain extent but it is conceivable that high risk mucosal HPVs also play a central role in the pathogenesis of these cancers (Melbye and Frisch, 1998). In addition, HPV infection of the upper airway appears to be associated with a subset of head and neck squamous cell carcinomas (HNSCC) (Gillison et al . , 2000) .

The low-risk types are mainly associated with genital warts or condylomas, but also with papillomas in the oral cavity or the larynx. Interestingly, some HPV types, like HPV32 or 13, appear to be confined to the oral cavity and not to the genital tract .Whereas most infections will result in a spontaneous clearance without any clinical manifestations a small fraction of the infected individuals will retain the virus and develop lesions that will progress to pre-invasive or invasive tumours.

Non -melanoma skin cancer (NMSC) is the most common cancer in the Caucasian adult populations (Pisani *et al*., 2002) which outnumbers all other cancers and comprises two main histological types; basal cell carcinoma (BCC) and squamous cell carcinoma (SCC). The incidence of NMSC is rising continuously across the globe mainly due to the aging of the population and the migration of people with sun-sensitive skin to subtropical regions. In the United States, approximately one million new NMSC cases are reported every year and increased NMSC incidence has been

observed in other regions such as Europe and Australia (Greenlee *et al.*, 2000; Kiviat, 1999; Marks, 1995).

1.2.3. HPV and cervical cancer

In women, cervical cancer is the second most frequent cancer worldwide and the leading cause of cancer-related deaths in women in developing countries (Drain et al., 2002; Snijders et al., 2006). In fact, cervical cancer ranks as the 2nd most frequent cancer among women in Malaysia, and the 2nd most frequent cancer among women between 15 and 44 years of age. Data is not yet available on the HPV burden in the general population of Malaysia (WHO, 2010). However, in South-Eastern Asia, the region Malaysia belongs to, about 8.4% of women in the general population are estimated to harbour cervical HPV infection at a given time (WHO, 2010).

Malaysia has a population of 8.70 millions women ages 15 years and older who are at risk of developing cervical cancer. Current estimates indicate that every year 2126 women are diagnosed with cervical cancer and 631 die from the disease (WHO, 2010). The incidence of cervical cancer has significantly decreased over the last 15 years in developed countries due to utilization of the Pap smear test, which allows for early detection (Singer, 1995). Nonetheless, cervical cancer is responsible for claiming 4,000 lives per year in the US and there are over 11,000 new cases yearly; therefore, it is not a trivial disease (Horppner et al., 2009). Further, in developed countries HPV infection occurs at alarming rates: HPV causing the most common viral sexually transmitted disease in the US, with 1 to 5 million new infections yearly (Burd, 2003). It is estimated that over 75% of sexually-active individuals have been infected with HPV (Cates, 1999).

1.2.4. HPV classification and genome organization

HPV are non-enveloped, double-stranded DNA viruses with a genome of approximated 7.9 kb (zur Hausen, 1996). The genome organization of HPV is illustrated in Figure 1.2. Transcription occurs from one of the two strands of the genome. The genome is divided into three segments; the early region (encoding proteins necessary for replication and transcription), the late region (encoding the L1 and L2 capsid proteins) and the long control region (containing *cis* regulatory elements necessary for replication and gene expression) (Longworth and Laimins, 2004; zur Hausen, 1996).

Sequence analysis has allowed the identification of more than 200 different types of HPV with differentiation of HPV types based on less than a 90% corresponding sequence of the L1 ORF with any previously identified HPV types (Burd, 2003; Delius and Hofmann, 1994). Two to ten percent similarity is recognized as a sub-type and <2% a variant (Munger and Howley, 2002). Ninety percent of HPV are classified into two major genera, Alpha and Beta. Alpha papillomaviruses include those HPV which cause genital/mucosal infections: HPV that infect the lining of the mouth, throat, respiratory tract, the anogenital or cervical epithelium (Burd, 2003; Doorbar, 2006). HPV 16 is the HR type of HPV responsible for more than 50% of cervical cancers (Hebner and Laimins, 2006). Beta papillomaviruses are associated with cutaneous infections, infecting the hands and feet. The remainder of the HPV are classified in Gamma, Mu and Nu genera and typically infect cutaneous sites (Doorbar, 2006).

HPV are associated with over 99% of all cervical cancer, 40-50% of penile and vulvar cancers and greater than 20% of head and neck cancers (D'Souza et al., 2007; Dillner et al., 2000; Hebner and Laimins, 2006; zur Hausen, 2002). HPV are classified as high-risk (HR) or low-risk (LR) depending on their ability to cause cancer. HPV 16, 18, 31, 33, and 45 and 59 are

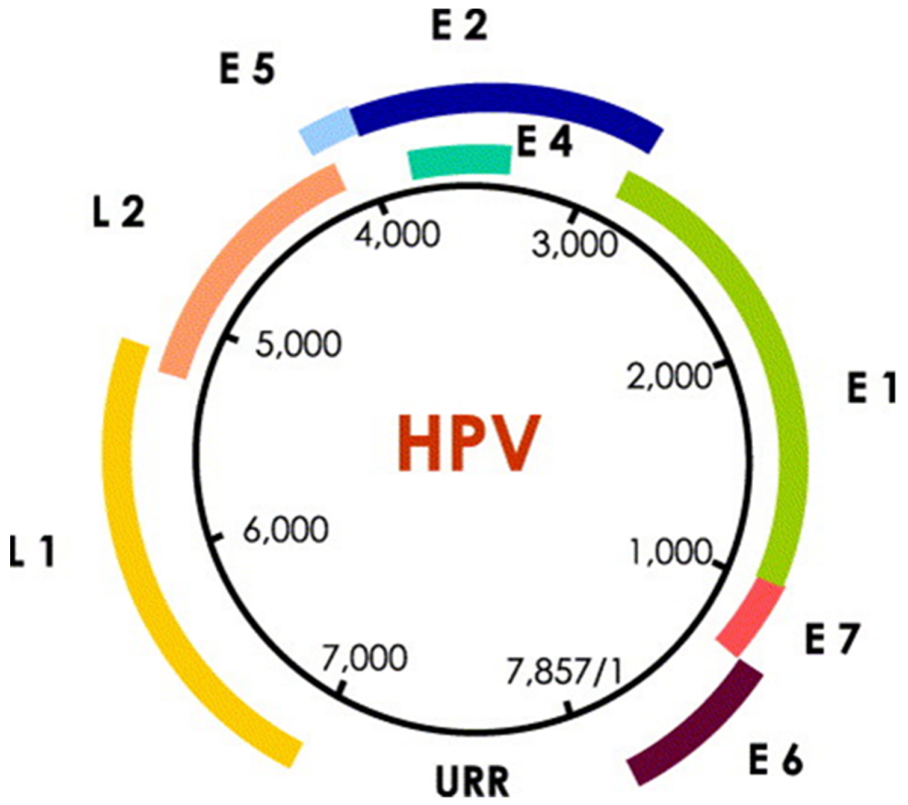


Figure 1.2. Schematic presentation of the HPV genome showing the arrangement of the early E or non-structural genes, the capsid genes (L1 and L2) and the upstream regulatory region (URR). Adapted from Munoz N. *et al.*, 2007

commonly associated with these malignancies (Burd, 2003; zur Hausen, 2000; zur Hausen, 2002). HPV 6, 11, 42, 43 and 44 are classified as LR, and can cause condyloma acuminata (genital warts) (Burd, 2003; Longworth and Laimins, 2004).

1.2.5. HPV life cycle

Viral infection occurs through a disturbed epithelial barrier, like a site of wounding. Only the basal, replicating keratinocytes are infected by HPVs (Olson, 1987). Infection of these actively proliferating cells results in a delay of differentiation and an expansion of the infected clone, due to the expression of the early proteins E6 and E7. During this phase, also called the non - productive stage, host cellular factors control transcription of early genes and viral DNA replication, while synthesis of structural proteins remains restricted and no virions are produced. Papillomaviruses modify the normal differentiation of the infected epithelium resulting in continued proliferation in the suprabasal layers (*stratum spinosum* and *stratum granulosum*) where replication of viral DNA occurs (Figure 1.3). Since the HPV genome does not encode for the enzymes required during DNA synthesis, the virus is completely dependent on the host replication machinery. As the DNA polymerases are usually only active in the S -phase of the cell cycle, the papillomaviruses encoded proteins (E6, E7 and probably E5) ensure continued growth and division of the cells after they have left the basal layers. Although the reason is not entirely clear, the productive stage of the viral lifecycle where the late genes are expressed and virions are assembled only occurs in the suprabasal differentiated keratinocytes. (Stoler *et al.,* 1990; Byrne, 1997; Howley, 1996; Laimins, 1993; Meyers and Laimins, 1996). Several findings indicate that differentiating keratinocytes produce differentiation specific factors that upregulate the viral late gene transcription (Collier *et al.*, 1998).

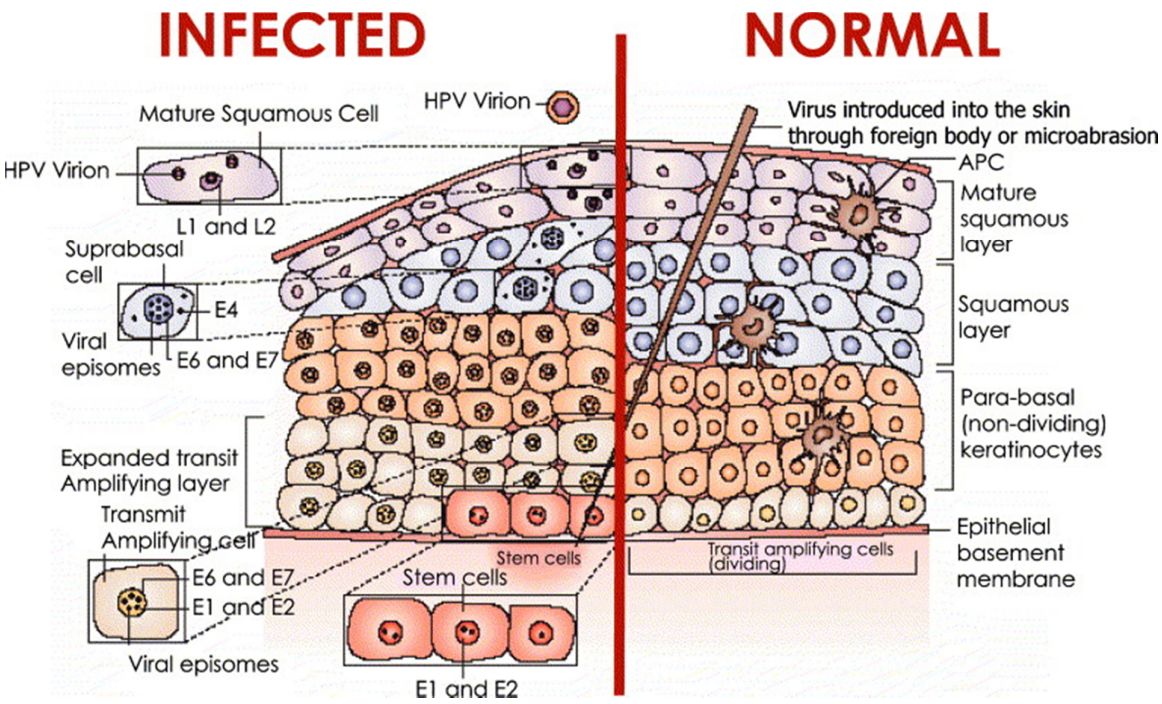


Figure 1.3. HPV life cycle in the epidermis. Infected basal cells migrate into the stratum spinosum and stratum granulosum still proliferating in order to replicate the viral genome. At the late stage of keratinocytes differentiation L1 and L2 are produced and viral assembly occurs. Progeny viruses are released upon normal shedding of epithelial cells. Adapted from Munoz N. *et al.*, 2007.

This absolute requirement for terminal differentiation of squamous epithelial cells for the expression of the late viral functions is responsible for the lack of success in propagating HPVs in culture. The mature viruses are released into the environment by shedding of the surface cell layers without the need for a lytic viral cycle for the dissemination of progeny. This mode of infection guarantees that a single basal cell, originally infected by HPV, may lead to the emergence of a field of virus producing cells at the surface of the developing lesion.

1.2.6.Function of the viral proteins

1.2.6.1. The early proteins

The proteins expressed early in the viral life -cycle are necessary for the initiation of viral protein expression, viral DNA replication and cell cycle proliferation even in presence of anti-proliferative factors. The **E6** and **E7** proteins play an important role in bypassing the cellular arrest signals, such as differentiation and immortalization of primary keratinocytes, to allow completion of the viral DNA replication. HPV 16 E7 for example has been shown to play a critical role in the DNA replicative stage of the viral life cycle by delaying the onset of differentiation (Flores *et al.*, 2000). In addition, these viral proteins can associate and alter, or completely neutralize, the normal functions of several cellular proteins (Scheffner *et al.* , 1990; Davies *et al.*, 1993) leading to deregulation of cell cycle, apoptosis and ultimately to transformation. In the early 1990's it was shown that E6 and E7 expression can lead to transformation of human cells and many other studies since then have contributed to the characterization of the individual properties of these proteins and how they affect the life of the infected cell.

The early proteins **E1** and **E2** play an important role in the initiation and regulation of viral DNA synthesis and gene expression. They can form a stable complex and bind to the replication origin of the HPV genome, where it recruits cellular factors essential for DNA replication (Yang *et al.*, 1991; Sedman and Stenlund, 1995). E1 has a cyclin -binding RXL motif and associates with cyclin E and A and is phosphor ylated by cyclin E or A- associated kinases . Mutation of E1 phosphorylation sites results in a reduction of HPV DNA replication, supporting the idea that the E1/c yclin association plays an important role in viral DNA replication (reviewed in Tommasino, 2001). Moreover, it has been shown that E1 has an ATPase and helicase activities (Hughes *et al* ., 1993; Lee *et al.* , 1999). It unwinds the origin of DNA replication (ori) (Seo *et al.*, 1993) and associates with human DNA polymerase ll primase *in vitro* (Park *et al.* , 1994).

The role of **E4** and **E5** in the viral life-cycle is not fully understood yet. In cutaneous HPV induced lesions, E4 is present at very high levels and sever al E4-isoforms have been detected. It has been shown that this protein associates with and disrupts the cytoplasmic keratin network (Door bar *et al.,* 1991). Doorbar *et al* (2000) have also reported that the E4 protein of HPV16 associates with a putative RNA helicase, suggesting a role for E4 in the productive phase of the infection establishing a favourable condition for viral maturation. Initial studies on bovine papillomavirus (BPV) have shown that E5 is a potent oncoprotein. It has been shown that HPV16 E5 is also able to induce cellular transformation, although with less efficiency than BPV E5, and can activate growth factor receptors such as epidermal growth factor (EGFR) (Martin *et al.,* 1989) and platelet derived growth factor receptor (PDGFR) (Petti *et al.*, 1991) . Interestingly, most of the cutaneous HPVs do not possess an E5 open reading frame (ORF). Since the integration of viral DNA, which occurs in tumour cells, results in a loss of E5 gene, it is probable that it is involved in early events during the multi –

step process of cervical carcinogenesis, and that its function is no longer required after the establishment of the transformed phenotype.

1.2.6.2. The late proteins L1 and L2

L1 and L2 are the major and minor capsid proteins, respectively. The capsid comprises 360 molecules of the major capsid protein L1, arranged as 72 pentamers. The number of L2 molecules per capsid has been estimated between 12 and 36 (Okun et al., 2001). The expression of these late genes occurs only in the terminally differentiated keratinocytes and it is regulated at both transcriptional and post- transcriptional levels (Tan et al., 1995; Zhao et al., 1996; Sokolowski et al., 1999).

1.2.7. HPV oncoproteins, E6 and E7

1.2.7.1 HPV E6

There are approximately 150 amino acids residues in the HPV E6 protein and its approximate size is 18 kDa. The E6 protein consists of two zinc-binding domains, each containing a C-X-X-C-X29-C-X-X-C sequence (Barbosa et al., 1989; Grossman and Laimins, 1989; Huibregtse et al., 1993). HR HPV E6 binds to E6-associated protein (E6-AP), a cellular ubiquitin-ligase, and targets the tumor suppressor p53 for degradation (Huibregtse et al., 1993; Scheffner et al., 1993). p53 plays many roles in cell-cycle regulation. It activates repair proteins in response to DNA damage, and if this damage is irreparable can induce cell arrest by activating p21, a cyclin kinase inhibitor (Hebner and Laimins, 2006; Levine, 1997).

The E6:E6-AP complex also targets NFX1-91 for degradation, enhances telomerase activity and increases cellular life-span (Gewin et al., 2004). Telomerase is

an enzyme that is responsible for extending telomeric ends by the addition of hexamer repeats. NFX1-91 transcriptionally represses telomerase reverse transcriptase (TERT) expression (Gewin et al., 2004). E6:E6-AP complex 16 has also been reported to target E6TP1, MCM7 (minichromosome maintenance 7) and Bak for degradation (Munger et al., 2004; Wise-Draper and Wells, 2008). Bak is a pro-apoptotic protein that is proteasomally degraded by HPV E6 (Thomas and Banks, 1999). MCM7 plays a role in guaranteeing that DNA replication occurs only once per cycle. Therefore, interaction of HPV E6 with MCM7 may result in over-duplication of chromosomes, contributing to genomic instability (Kukimoto et al., 1998). HR HPV E6 also binds directly to PDZ [PSD-95 (a 95 kDa protein involved in signaling), Dlg (the *Drosophila* discs large protein), and ZO1 (the zonula occludens 1 protein] domain-containing proteins via its C-terminal domain which is highly conserved amongst HR HPV types (Glaunsinger et al., 2000; Lee et al., 2000b; Nakagawa and Huibregtse, 2000; Thomas et al., 2001). LR HPV does not possess the PDZ binding motif (Pim et al., 2000). PDZ proteins are necessary for cell-cell adhesion and are implicated in cell signaling (van Ham and Hendriks, 2003). Binding of PDZ by HPV E6 is an important feature in progression to carcinogenesis.

HPV E6 mutants that can no longer bind PDZ are deficient for E6-induced transformation in rodent cells and reduction of tumor development in transgenic mouse models (James et al., 2006; Kiyono et al., 1997). There are other proteins that have been discovered to interact with HPV E6. Paxillin has been reported to bind to HR HPV E6, while both LR and HR HPV E6 bind to p300, MCM7 and Bak. HR HPV E6 has a higher affinity for p300, MCM7 and Bak (Kukimoto *et al.*, 1998; Patel *et al*., 1999; Thomas and Banks, 1999; Thomas and Chiang, 2005; Tong and Howley, 1997; Zimmermann *et al.*, 2000). p300 functions as a transcriptional coactivator and a histone acetyltransferase (Iyer *et al.*, 2004). Binding of p300 by HPV

E6 prevents acetylation of p53 at p300-dependent sites, down-modulating p53-mediated expression (Zimmermann *et al*., 2000).

1.2.7.2 HPV E7

E7 proteins consist of approximately 100 amino acid residues and can be divided into three regions: conserved region 1 (CR1, amino acids 2-15), CR2 (amino acids 16-38), and the C-terminal zinc-binding region (amino acids 39-98) containing two Cys-X-X-Cys motifs. CR1 and CR2 are conserved with adenovirus E1A and SV 40 large T antigen. The zinc-binding C-terminal domain of E7 oncoprotein is proposed to be involved in homodimerization (Gage et al., 1990; Jewers et al., 1992; Munger et al., 2004; Munger et al., 2001). E7 proteins of HR HPV 16 and LR HPV 6 share 50% amino acid sequence identity and 15% conservative changes (Armstrong and Roman, 1992; Gage et al., 1990). Both HR and LR HPV E7 proteins bind pRb family members through their LXCXE binding motif (Dyson et al., 1989) (Figure 1.4).

Furthermore, several *in vitro* studies have revealed that HPV 16 E7, as compared to HPV 6 E7, has a greater affinity for pRb, p107, and p130 (Ciccolini et al., 1994; Gage et al., 1990). HR HPV destabilize all pRb family members and this is a critical event that drives cellular transformation (Berezutskaya et al., 1997; Boyer et al., 1996; Davies et al., 1993; Gonzalez et al., 2001; Halbert et al., 1991; Helt and Galloway, 2001). The main contributing factor that results in enhanced binding of HR HPV E7 to pRb and its ability to target pRb for degradation is an aspartic acid versus glycine residue in 18 HR vs. LR E7 proteins at the position immediately before the LXCXE binding motif. Although HPV 6 E7 has a lower affinity for binding p130 than HPV 16 E7, it is as efficient in targeting p130 for degradation (Zhang et al., 2006).

Significant differences between the HPV 16 E7 and HPV 6 E7 proteins have been reported such as HPV 16 E7, but not HPV 6 E7, can cooperate with ras to transform primary rodent cells, immortalize primary keratinocytes, and abrogate growth arrest mediated by DNA damage (Demers et al., 1996; Halbert et al., 1991; Jewers et al., 1992; Matlashewski et al., 1987; Storey et al., 1988; Watanabe et al., 1992). The molecular basis for the transformation ability of HR HPV E7 has been mapped to the amino-terminal half of the E7 protein (Heck et al., 1992; Phelp et al., 1992). The amino-terminal halves of HR and LR E7 proteins contain consensus recognition sequences for casein kinase II (CK II) (Barbosa et al., 1990; Firzlaff et al., 1989; Massimi and Banks, 2000). There are 2 serines in E7 that are specifically phosphorylated by CKII. When these sites are mutated the transforming ability of HPV E7 decreases (Barbosa et al., 1990). The E7 proteins of the HR HPV are phosphorylated *in vitro* at a higher rate than the LR HPV-encoded E7 proteins (Storey et al., 1988).

HR HPV E7 proteins have a number of cellular binding partners other than the pocket proteins. HR HPV E7 but not LR HPV E7 has been reported to interact with cyclin A/cyclin dependent kinase (Cdk) 2, cyclin E/Cdk2, PCAF, TATA box-binding protein (TBP), histone deacetylases (HDAC), E2F1, p21CIP1 and p27KIP1 via its C-terminus (Dell and Gaston, 2001; Munger et al., 2004; Wise-Draper and Wells, 2008). pRb is a substrate of both the cyclin A/Cdk2 and cyclin E/Cdk2 complexes. HPV E7 interaction with these complexes results in reduction of Rb-associated transcriptional repression (McIntyre et al., 1996; Tommasino et al., 1993). Binding of HPV E7 to HDAC is indirect and is mediated by Mi2β (Brehm et al., 1999). p21CIP1 and p27KIP1 are cyclin kinase inhibitors and binding by E7 perturbs cell cycle inhibition (Funk et al., 1997; Zerfass-Thome et al., 1996). In differentiating cells, E7 binding to HDAC contributes to enhanced E2F-mediated transcription and increases proliferation (Hebner and Laimins, 2006).

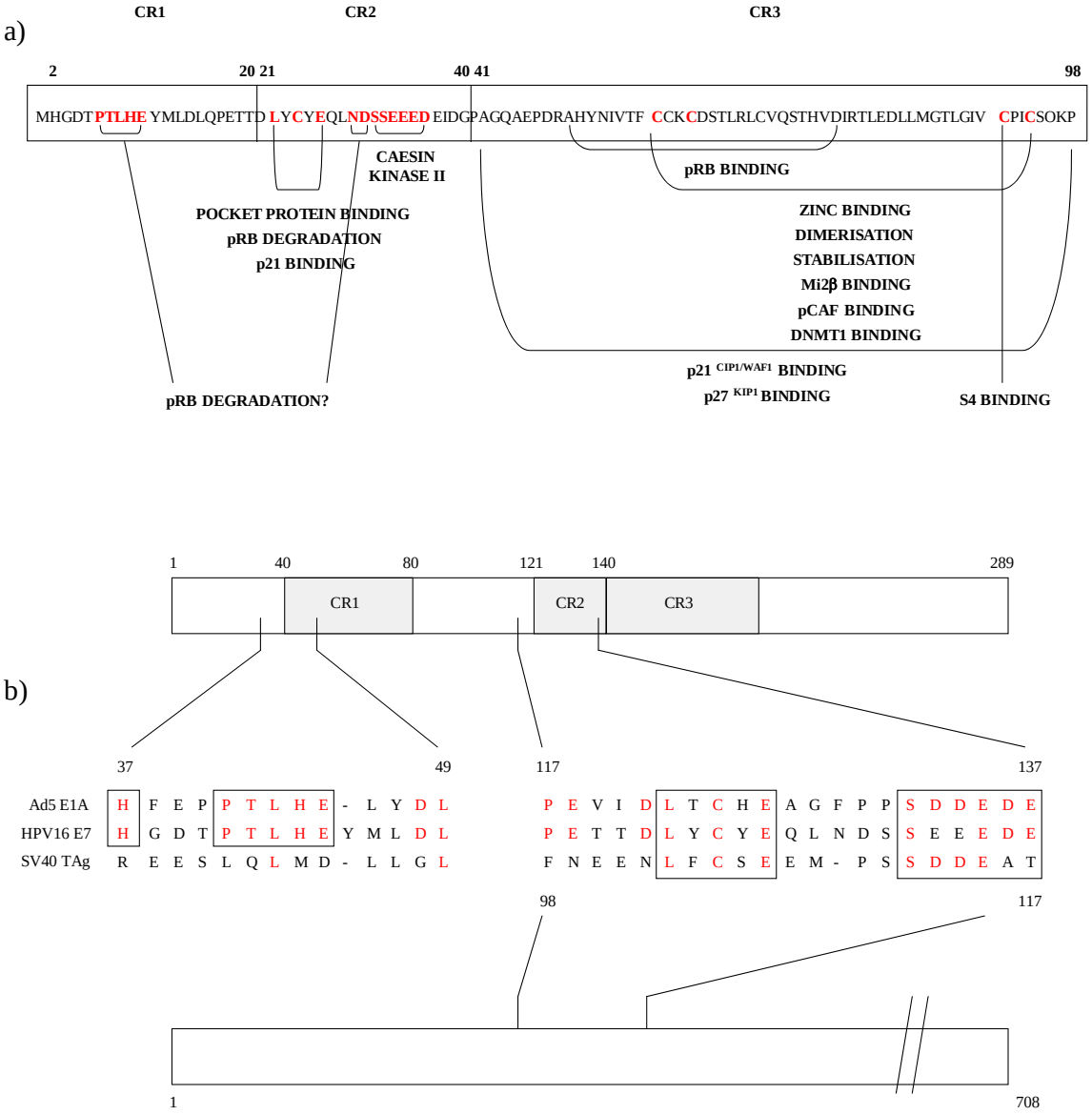


Figure 1.4. A schematic depiction of the prototypic HPV16 E7 protein. (a) The domain structure of the HPV16 E7 protein illustrating regions known to be involved in specific E7 functions. The primary amino acid sequence is given with the relevant residues shown in red. The functional interactions are discussed in Section 1.5. (b) Functionally conserved amino acid sequences within the Adenovirus type 5 E1A, HPV16 E7 and SV40 large T antigen proteins. Sequences obtained from the National Center for Biotechnology Information (NCBI)

1.2.7.3. E6 and E7 play a key role in cellular transformation

First indications that the E6 and E7 proteins were involved in the cervical cancer came from studies that showed the presence and expression of these two viral genes in cancer cells and cervical cancer tissue samples. In pre - malignant HPV infected lesions, the viral DNAs exist as extra chromosomal plasmids, mostly as monomeric circular molecules (Lambert, 1991). However, in the majority, if not all cancers, the viral DNA is usually integrated into the host cell genome. This integration appears to be a random event with regard to sites of host chromosomal integration, but most often results in disruption of the viral E2 open reading frame and the loss of E2 expression (Schwarz *et al.*, 1985, Yee *et al.* , 1985; Matsukura *et al.* , 1986; Smotkin and Wettstein, 1986; Baker *et al.* , 1987; Schneider - Maunour *et al.*, 1987). Since E2 is a regulator of the E6/E7 promoter, the loss of E2 results in deregulation of the expression of the E6 and E7 genes, which are consistently found expressed in HPV - associated cervical cancers (Schwarz *et al., 1985;* Smotkin and Wettstein, 1986; Baker *et al.*, 1987).

The first direct evidence for the transforming activity of the viral genes was obtained from studies performed in NIH3T3 cells (Tsunokawa *et al.*, 1986; Yasumoto *et al.*, 1986) and later it was shown that HPV16 E6 and E7 are able to immortalise primary human cells, including primary human keratinocytes, the natural host of the virus (Mansur and Androphy, 1993). However, E6 and E7 from low- risk viruses (1, 6 and 11) failed in immortalisation experiments (Woodworth *et al.* , 1989; Barbosa *et al*., 1991), providing a direct correlation between the *in vitro* properties and the potential carcinogenicity of the HPV. Transgenic mice coexpressing the E6 and E7 genes from the high risk HPV type 16 exhibit epidermal hyperplasia and various tumours (Lambert *et al.* , 1993).

Additional studies in human cells showed that expression of high- risk E6 and E7 proteins is required for the maintenance of the transformed phenotype (Yoshinouchi *et al.* , 2003; Yamato *et al.* , 2005). Consistent with this fact, inhibition of transcription of E6 or E7 genes in cervical carcinoma cell lines leads to a decrease in proliferation state and reversion of the malignant phenotype (Crook *et al.*, 1989; von Knoebel-Doeber itz *et al.*, 1992). In the last 10 -15 years, biochemical studies have provided further support to the carcinogenic functions of E6 and E7. Both viral oncoproteins are able to form stable complexes with cellular proteins and alter, or completely neutralise, their normal functions. These events lead to the loss of control of cell cycle checkpoints, of apoptosis and differentiation and eventually to transformation of the infected cell.

1.2.7.4. Control of cell cycle and its disruption by high-risk HPV E7.

Cell cycle control is regulated by the activity of cyclin-dependent kinases (CDKs). The activity of CDKs (CDK1, CDK2, CDK4, CDK6) is regulated by the abundance of their activating partner cyclins (cyclins A,B,D,E), phosphorylation by various kinases and interaction with CDK inhibitory proteins (CDKIs) (Sheer *et al*., 1995; Beijerbergen and Bernards 1996) (Figure 1.5). Two classes of mammalian cyclin-dependent kinase inhibitors have been described: The CIP/KIP family, comprised of p21,p27 and p57 and the INK4 family, comprised of p15, p16, p18 and p19 (Sheer and Roberts, 1995). Generally CDKs, cyclins and CDK inhibitors function within several pathways, including the $p16^{INK4A}$-cyclin D1- CDK4/6-pRb-E2F and $p21^{WAFI}$-$p27^{KIPI}$-cyclin E-CDK2 pathways (Kim and Zhao, 2005). The INK4 molecules specifically inhibit cyclin D complexes by interaction with CDK4 and CDK6 components. The KIP family affects cyclin E, cyclin A/CDK2, and cyclin B/CDK1 by

binding both the cyclin and CDK subunit. Alteration in CDKs, CDKIs and cyclins can lead to uncontrolled proliferation and might contribute to malignancy of the uterine cervix (Sheer and Roberts, 1995).

In many cancers and cancer cell lines the mechanisms which control the growth and differentiation mechanism are disrupted or at least impaired (Sheer *et al.*, 1996). In a dividing cell, mitogenic stimulation leads to synthesis and assembly of cyclin D/CDK4 complexes, which contribute to the phosphorylation and consequent inactivation of pRB, increased expression of cyclin E and sequestration of cdk2 inhibitors of the Cip/Kip family. Cyclin E/CDK2 continues to inactivate pRB and also phosphorylates substrates important for DNA synthesis and S phase entry. Cyclin A-CDK2 is assembled during S phase and remains active through G2 phase (Sheer *et al*., 1993; Sheer and Roberts, 1999).

HPV16 E7 (16E7) affects these cyclin-CDK complexes by deregulating transcriptional levels of cyclin E due to loss of E2F mediated repression; but also by affecting the posttranscriptional levels of cyclin E (Zerfass *et al*., 1995; Botz *et al*., 1996; Martin *et al*., 1998). 16E7 protein has been shown to bind to cyclinA-cdk2 in a cell cycle dependent manner, with maximal binding in the S and G2 phases (Tommasino *et al.*, 1993). Cyclin A-CDK2 interacts with and phosphorylates E2F, leading to loss of its DNA binding capability. Hence interaction of 16E7 with cyclin A/CDK2 prevents the inactivation of E2F and thereby permits the cell to bypass normal checkpoints with consequent loss of DNA replication fidelity, explaining increased chromosomal abnormalities (White *et al.*, 1994).

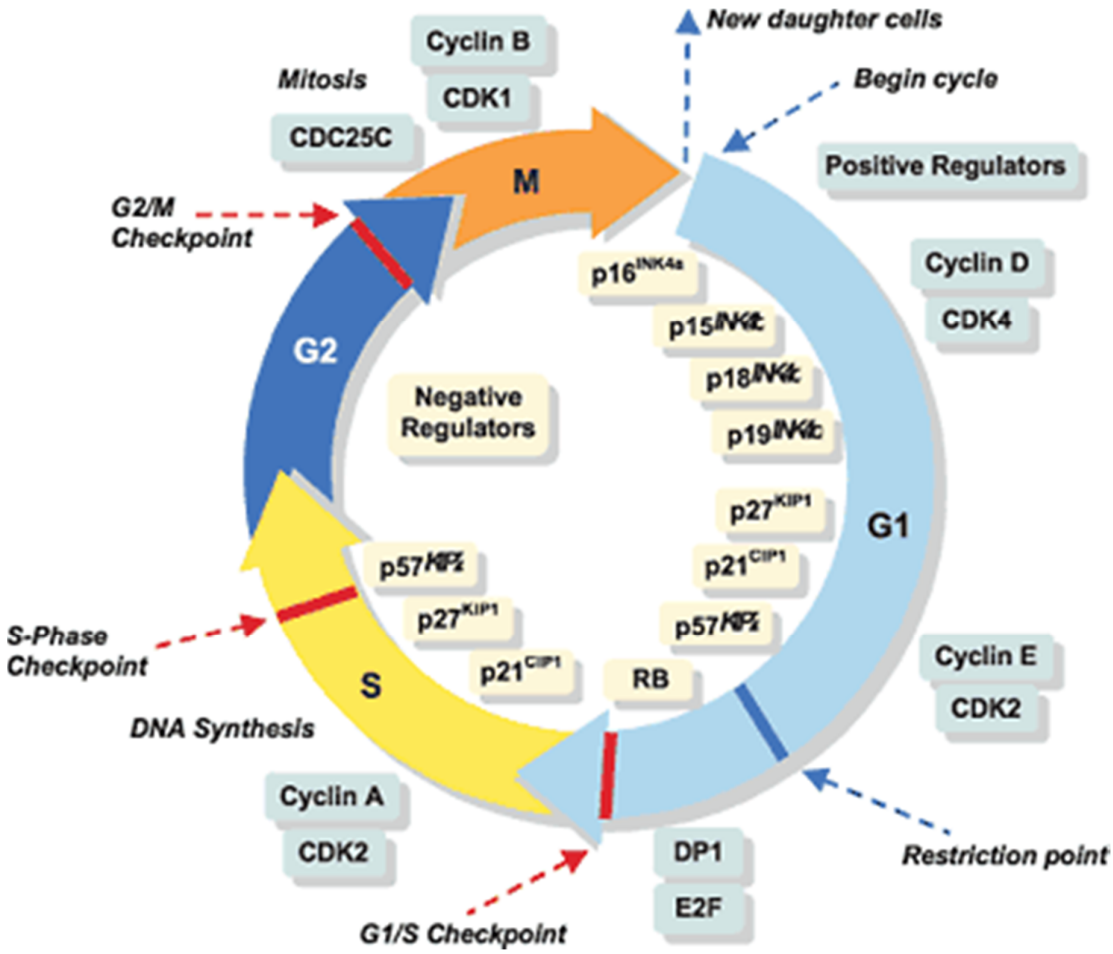


Figure 1.5. **The phases of the mammalian cell cycle and the cyclic activation of cyclin-dependent kinases**. The first cyclins to be synthesised following growth factor stimulation are the D-type cyclins. The D-type cyclins associate with cdk4 and 6 as the cell enters G_1 and are required for cyclin E expression. Cyclin E associates with cdk2 and drives the cell across the G_1/S checkpoint. Both cyclin D and cyclin E are required for expression of cyclin A. Cyclin A associates with cdk2 and cdk1 and is required for cyclin B expression. Cyclin B associates with cdk1 and drives the cell through the G_2/M checkpoint**.** Adapted from Kong *et al.*, 2003.

HPV16 E7 can override growth-inhibitory activities of cyclin dependent kinase inhibitors, including $p21^{CIPI/WAFI}$ and $p27^{KIPI}$ (Jones *et al*., 1997; Zerfass-Thome *et al*., 1996). Since these proteins are critical regulators of cell cycle arrest during keratinocyte differentiation (Missero *et al*., I996), their inhibition by E7 may also contribute to the maintenance of a replication-competent cellular milieu in differentiated host epithelial cells (Cheng *et al*., 1995). A carboxyl-terminal E7 domain that does not contribute to pRB binding and/or degradation is necessary for the ability of E7 to override p21- mediated growth arrest (Helt *et al*., 2002). The association with p21 abrogates its activity to inhibit cyclin/CDK activity as well as PCNA-dependent DNA replication (Funk *et al*., 1997; Jones *et al*., 1997). Since p27 has been implicated as a mediator of cellular growth inhibition by TGF-β in keratinocytes, this activity may contribute to the ability of E7 to prevent cellular growth inhibition by TGF-β (Pietenpol *et al*., 1990).

1.2.8. The tumor suppressor protein "Retinoblastoma Protein"

1.2.8.1 The tumor suppressor protein pRB and Cancer

pRB was the first tumor suppressor identified through human genetic studies. The retinoblastoma gene is mutated in many human cancers, including retinoblastoma, osteosarcoma and small cell lung cancer (Weinberg et al., 1995a). Mouse genetic studies revealed that pRB functions are essential for embryonic development. RB-/- null embryos die at 13.5 day gestation with prominent defects in the central nervous and hematopoietic system (Zhu et al., 2005; Jacks et al., 1992; Lee et al., 1992). To this day *RB1* remains the only single gene in which mutation is necessary and sufficient to cause human cancer.

Furthermore, pRB protein can be inactivated by phosphorylation through cyclin–dependent kinases (in particular CyclinD/ CDK4) (Du and Pogoriler,

2006). Overexpression of cyclin D1, activating mutations in CDK4/6, and inactivating mutations in the cyclin D/CDK4 inhibitor p16INK4a are frequent events in various human cancers (Kim and Zhao et al., 2005). Since most, if not all, human cancers have one or more of these events it has been proposed that disruption of pRb function is a general feature of cancer cells (Sherr et al., 1996). Some immunhistochemical studies of pRB in human cancer and in normal tissues, including uterine cervix, have shown that pRB is expressed in mature and in differentiated cells (Kim and Zhao et al., 2005).

1.2.8.2. pRB degradation.

HPV16 E7 is able to induce the proteasomal degradation of pRB and the related pocket proteins p130 and p107 in keratinocytes and a variety of other epithelial cell lines (Jones and Munger, 1997; Berezutskaya *et al.*, 1997; Gonzalez *et al*., 2001). This is a distinct function of the HPV16 E7 protein that is not shared by adenovirus E1A or SV40T antigen (Gonzalez *et al*., 2001). Degradation is necessary for the complete functional inactivation of pRB in epithelial cells. The low risk HPV6 E7 is not able to induce pRB destabilization (Demers *et al.*, 1994). HPV16 E7 induced pRB degradation overcomes both $p16^{INK4a}$- induced G1 arrest in keratinocytes and Saos-2 cell senescence and differentiation (Gonzalez et al., 2001; Giarre *et al.*, 2001).

The region of the HPV16 E7 protein required to mediate pRB destabilization is unclear. The integrity of the LXCXE motif and the ability to bind to pRB are necessary but not sufficient for destabilization (Jones and Munger, 1997; Gonzalez *et al.*, 2001; Giarre *et al.*, 2001). HPV16 E7 LXCXE point or deletion mutants that do not bind to pRB are not able to induce degradation (Munger *et al.*, 1989; Gonzalez *et al.*, 2001). Equally, the subcutaneous HPV1 E7 protein which binds to pRB with a similar affinity to the HPV16 E7 protein, does not induce pRB degradation

(Alunni-Fabbroni *et al*., 2000; Gonzalez *et al*., 2001). It is also partially able to overcome $p16^{INK4a}$-induced G1 arrest in keratinocytes and the effects of pRB in Saos-2 cells (Giarres *et al*., 2001; Gonzalez *et al*., 2001).

Interestingly, the low risk HPV6 E7 protein, which has a mid affinity for all of the pocket proteins, destabilizes p130 as efficiently as HPV16 E7 (Zhang *et al*., 2006). It does not induce the destabilization of either pRB or p107 (Zhang *et al*., 2006). The destabilization of p130 by both HPV6 and HPV16 E7 proteins is particularly interesting given that p130 is the predominant pocket protein in quiescent and differentiating cells. P130 plays a central role in the regulation of differentiation and its degradation delays the expression of differentiation markers in keratinocytes (Zhang *et al*., 2006). It is possible that p130 degradation simultaneously induces differentiated cells to re-enter the cell cycle while delaying the onset of differentiation and if this is the case, the degradation of p130 may be necessary for a productive viral infection, whereas pRB and p107 degradation simply further increases the selective growth advantage of the cell and enhances the oncogenic potential of the virus.

1.2.8.3. pRB belongs to the pocket protein family and exerts different function

The RB protein is a member of a family of three closely related mammalian proteins that includes p107 and p130. Together these are often referred to as the 'pocket proteins' because their main sequence similarity resides in a domain, which mediates interactions with viral and cellular proteins, namely the pocket domain (Classon and Dyson, 2001). Although pRB is the best characterised family member (Harbour and Dean, 2000a). The 105 kDa retinoblastoma protein (pRb) has been implicated in many cellular processes, such as regulation of the cell cycle, DNA-

damage responses, DNA-repair, DNA-replication, protection against apoptosis and differentiation (Classon and Harlow, 2002).

The most important function of pRB as a tumor suppressor is growth suppression which is mediated via interaction with other proteins (Qin et al., 1992; Chow and Dean 1996; Lee et al., 1998; Morris and Dyson 2001). pRB keeps cells in G1 by repressing the transcription of genes required for cell cycle transition into S phase, and those genes that encode other cell-cycle regulators or enzymes required for DNA synthesis.

The pRB protein is known to bind to more than 100 cellular proteins ranging from transcriptional regulators to enzymes involved in signal transduction and chromatin remodelling systems (Lee et al., 1998; Rubin et al., 1998; Morris and Dyson, 2001). The pRB contains several functional domains. The N-terminal region of pRb amino-acids 1-378, has been shown to be important for Sp-1 transactivation as it relieves repression by the inhibitor Sp1-I (Kim et al., 1992). Amino acids 379-772 are considered the small pocket domain, and are further subdivided by spacer region into A- (amino acids 394-571) and B- (amino acids 649-773) domains, while the large pocket includes A, B, and C domains. Domain A is required for an active conformation (Cho et al., 1997) while domain B binds to LXCXE motifs in interacting binding proteins like histone deacetylase (HDAC)-1 and HDAC-2, ATPase, BRG1, from the SDWI/SNF nucleasome remodelling complex (Brehm et al., 1998; Luo et al., 1998; Magnaghi-Jaulin et al., 1998), D-type cyclins (Weinberg et al., 1995b), the transcription factors UBF (Cavanaugh et al., 1995), Elf-1 (Wang et al., 1993) and the phosphatase PP1 a type 1 serine/threonine protein phosphatase (Durfee et al., 1993).

The large pocket of pRb is composed of the A/B region together with the C-pocket amino acids 773-870 which is most noted for its ability to bind to a class

of transcription factors known as E2F (Chellappan et al., 1991; Lees et al., 1993). The strongest growth suppressive effects are mediated through this interaction.

The C pocket of pRb is located in the carboxy-terminal region. This region contains binding sites for the c-Abl tyrosine kinase MDM2 which are distinct from the E2F site in the carboxy-terminal region (Welch and Wang, 1993; Xiao et al., 1995; Hsieh et al., 1999). Several of the reported pRb binding proteins have a pro-apoptotic function. For example the nuclear c-Abl tyrosine kinase can induce apoptosis due to its kinase activity (Welch and Wang 1993), which is inhibited by the interaction with pRb (Wang et al., 2000). Furthermore it can regulate the stability and apoptotic function of p53 via binding to MDM2 (Hsieh et al., 1999). pRb contains several consensus sites for caspase cleavage (Tan et al., 1998; Fattman et al., 2001 and 1997) on its carboxyl terminus. Degradation of is pRb due to caspase function observed in response to cell death signals and executing cell killing (Tan et al., 1997).

1.2.9 Pocket proteins

The principal substrates of the G_1 cdk-cyclins are the members of the pocket protein family, pRB/p105, p107 and p130/pRb2. The pocket proteins are a group of related nuclear phosphoproteins that each contain a conserved C-terminal bipartite 'pocket' that interacts with proteins containing a LXCXE motif. The pocket consists of two domains known as A and B that are separated by a spacer sequence. The pocket proteins are mediators of cell cycle progression, apoptosis, development, differentiation and senescence (reviewed in Classon & Dyson, 2001). In the active state, the pocket proteins negatively regulate gene expression by interacting with specific members of the E2F family of transcription factors (Bandara & LaThangue, 1991; Zamanian &

LaThangue, 1993; Vairo *et al.*, 1995; Chow and Dean, 1996). This interaction prevents E2F-mediated transcription by masking the transactivation domain and actively repressing the expression of target genes (Weintraub *et al.*, 1992; 1995; reviewed in Cobrinik, 2005). The pocket proteins orchestrate the repression of E2F-responsive genes by recruiting a variety of chromatin modifying activities to the promoter. It appears that the composition of these repressor complexes depends on the cellular context in which the gene is found. In cycling cells, pocket protein repressor complexes regulate reversible, cell cycle-mediated repression of genes required for cell cycle progression. These repressor complexes are known to contain the histone deacetylase HDAC1 (Brehm *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Ferreira *et al.*, 1998; Rayman *et al.*, 2002) and the nucleosome remodelling complex hSWI/SNF (Zhang *et al.*, 2000). hSWI/SNF complexes contain either the Brm-related gene product (BRG1) or the Brahma (BRM) ATPase (Narlikar *et al.*, 2002), either of which can interact with the pocket proteins and tether the complex to the promoter (Dunaief *et al.*, 1994; Strober *et al.*, 1996).

During cellular senescence or differentiation, pocket protein repressor complexes are associated with a more stable form of gene silencing that is associated with permanent exit from the cell cycle. This form of silencing is also thought to regulate the repression of differentiation and developmental genes in cycling cells. In senescent fibroblasts and differentiating myoblasts, the histone H3K9 methyltransferase SUV39H1 has been identified at the promoter of E2F-regulated genes in a pocket protein-dependent manner (Vandel *et al.*, 2001; Nielsen *et al.*, 2001; Narita *et al.*, 2003; Ait-Si-Ali *et al.*, 2004). This is thought to mediate a stable methylation pattern. E2F-regulated genes control the expression of a wide network of genes that include genes that are rate limiting for G_1/S progression and DNA synthesis, as well as genes required for mitosis, apoptosis, the DNA damage response, development and differentiation (Ishida *et al.*, 2001; Muller *et*

al., 2001; Polager *et al.*, 2002; Ren *et al.*, 2002; Weinmann *et al.*, 2002; Dimova *et al.*, 2003). Their controlled expression is therefore pivotal for the temporal control of a plethora of cellular functions.

p130 and p107 are thought to be important regulators of cell cycle progression, senescence, development and differentiation. Current models suggest that p130 and p107 are responsible for repressing most E2F-responsive promoters (Hurford *et al.*, 1997). Recent research has suggested that this might occur as part of a novel LINC complex (Osterloh *et al.*, 2007). LIN/E2F-4/p130 has been found to repress E2F-responsive genes required for G_1/S and G_2/M (Pilkinton *et al.*, 2007; Osterloh *et al.*, 2007). The predominant E2F repressor complex in G_0 and G_1 is thought to be LINC/E2F-4/p130 (Moberg *et al.*, 1996; Osterloh *et al.*, 2007). In contrast, the presence of native pRb repressor complexes on E2F target promoters in G_0/G_1 is debatable and pRb is only thought to have a role in repressing a small, very specific set of genes, most importantly the *cyclin E* gene (Black *et al.*, 2003; Herrera *et al.*, 1996; Hurford *et al.*, 1997). Instead, it primarily mediates exit from the cell cycle in times of cellular stress or prior to differentiation or senescence. ChIP data has identified pRb on E2F target promoters both in U2OS cells that have been growth arrested due to ectopic $p16^{INK4a}$ expression and in human fibroblasts upon the induction of senescence (Narita *et al.*, 2003).

1.2.9.1. Pocket proteins and cell cycle regulation

The inactivation of pocket proteins is regulated at the level of posttranslational phosphorylation by the G_1 cdk-cyclin complexes. Each cdk-cyclin complex

phosphorylates a set of specific serine and threonine residues on the protein. p130 is also regulated by unidentified G_0 kinases which phosphorylate a unique short B loop domain (Baldi *et al.*, 1995; Mayol *et al.*, 1995; 1996; Canhoto *et al.*, 2000). Expression of the G_1 cyclins leads to the gradual phosphorylation of active, hypophosphorylated pocket proteins into an inactive hyperphosphorylated form. Repressor E2F molecules are released upon pocket protein phosphorylation in late G_1 and are thought to be replaced on promoters by 'activator' E2Fs. pRb and p107 remain hyperphosphorylated until reactivation occurs by protein phosphatase 1 (Ludlow *et al.*, 1993).

Quiescent cells can be stimulated to re-enter G_1 by extracellular mitogenic stimuli such as growth factors. Recent evidence has suggested that exit from quiescence may be mediated by a novel cdk3-cyclin C complex which phosphorylates pRb in G_0 (Ren & Rollins, 2004). Growth factor receptor binding activates a range of intracellular signal transduction pathways that ultimately result in the transcriptional upregulation of the D-type cyclins, D1, D2 and D3 (Matsushime *et al.*, 1991; Won *et al.*, 1992). The D-type cyclins associate with cdk4 or cdk6 to form an active holoenzyme (Sherr, 1995). It is thought that association with a member of the CIP/KIP family of Cdkis promotes assembly of the cdk4/6-cyclin D complex (LaBaer *et al.*, 1997; Cheng *et al.*, 1999). Cdk4/6-cyclin D complexes phosphorylate the pocket proteins pRb, p107 and p130 through early G_1 (Weinberg *et al.*, 1995). In contrast to p107 and pRb, the phosphorylation of p130 renders it unstable and its protein levels sharply decline as the cell enters G_1 (Beijersbergen *et al.*, 1995; Baldi *et al.*, 1995; Dong *et al.*, 1998). Phosphorylation by cdk4/6-cyclin D complexes in early G_1 disrupts the interaction with HDAC and results in the partial relief of pocket protein repressor complexes present on E2F-regulated genes (Harbour *et al.*, 1999). This allows the expression of genes required to progress through G_1. These include *cyclin E, E2F-1, B-myb, dihydrofolate reductase* and *thymidine kinase* (Zhang *et al.*, 2000). The expression of cyclin E1 and

E2 promotes the formation of cdk2-cyclin E complexes which sequentially phosphorylate the pocket proteins on distinct residues. This second wave of phosphorylation is required for progression through the G_1/S checkpoint and entry into S phase. This stage of the cell cycle is known as the Restriction (R) point (Pardee, 1974). Once the cell passes through R, the cell cycle proceeds autonomously, impervious to extracellular growth factor withdrawal (Pardee, 1989). Cdk2-cyclin E phosphorylation disrupts the interaction between the pocket proteins and the chromatin remodelling BRG1/BRM complex, resulting in the expression of cyclin A and cdc2. This is required to push the cell through G_1/S (Zhang *et al.*, 2000). As the cell enters S phase, hyperphosphorylated pocket proteins dissociate from the repressor E2Fs. The repressor E2Fs disengage from the promoter and are replaced by the activator E2Fs. The presence of the activator E2Fs correlates with increased histone acetylation and the transcription of genes required for DNA replication such as *PCNA*, *Orc* and *cdc6* (Takahashi *et al.*, 2000; Taubert *et al.*, 2004).

It has recently become apparent that the dissociation of the LINC/p130/E2F-4 complex in G_1/S promotes the formation of a LINC/p107/B-myb complex that is responsible for promoting the expression of genes required for mitotic progression in an E2F-independent manner. These genes include *cyclin B1*, *cyclin A2* and *cdc2*, which are required for G_2/M progression, *BUB-1* and *CenPE*, which are required at the mitotic spindle checkpoint, *Aurora-A* and *Plk-1*, which are required for spindle assembly, and *UbCh10*, which is required for exit from mitosis (Pilkinton *et al.*, 2007; Osterloh *et al.*, 2007). The expression of cyclin A induces the formation of cdk2-cyclin A complexes in early S phase. These proteins bind to and deactivate activator E2Fs by phosphorylating DP. This prevents E2F-DP complexes from binding to DNA and thus promoting the continued synthesis of proteins that could lead to untimely DNA replication (Krek *et al.*, 1994; 1995; Dynlacht *et al.*, 1994).

1.2.10. dREAM and DRM complexes

1.2.10.1 The discovery of a novel *Drosophila* RBF/MIP130 repressor and *C.elegans* DRM complexes.

Recently, two novel RBF/E2F repressor complexes were isolated from *Drosophila* embryo nuclear extracts and S2 cells (Korenjak *et al*., 2004; Lewis *et al*., 2004). These multisubunit complexes were located on transcriptionally silent regions of polytene chromosomes and were found to repress the sex- and differentiation-specific dE2F target genes *ARP53D*, *CG17142*, *CG3505*, *RP49*, *CG3105* and *Vasa* (Dimova *et al.*, 2003; Korenjak *et al*., 2004; Lewis *et al*., 2004). The core complex was found to contain eight common subunits, either one of the pocket protein homologues RBF1 or RBF2, the transcriptional repressor dE2F2 and binding partner dDP, three Myb – interacting proteins MIP130, MIP120, MIP40, the Drosophila Myb homologue dMyb and the RbAp48 homologue CAF1p55 (Korenjak *et al*., 2004; Lewis, *et al*., 2004). The complexes were named dREAM (***D****rosophila* **R**BF, d**E**2F and **M**yb-interacting proteins) (Korenjak *et al.*, 2004) or Myb-MuvB (Lewis *et al*., 2004). Stoichiometric levels of the protein dLin-52 and substoichiometric levels of the histone deacetylase Rpd3, the NURF complex components ISWI, E(Bx) and NURF38, and the chromodomain-like L(3)MBT protein were also found in Myb-MuvB complexes (Lewis *et al*., 2004). The mechanism of gene repression is unclear. Although the complexes associate with unacetylated histone H4 tail peptides (Korenjak *et al*., 2004) and low levels of proteins required for chromatin modification and nucleosome remodelling co-purify with the complexes (Lewis *et al*., 2004; Taylor-Harding *et al*., 2004), these are not present in sufficient quantities to be considered core components of the complexes (Korenjak *et al.*, 2004; Lewis *et al*., 2004). In addition, inhibition of histone deacetylase activity was not found to affect the repression of target genes (Taylor-Harding *et al*., 2004). It is

thought that binding to deacetylated histone tails may be a prerequisite for the association of the complex with a specific promoter region, but active chromatin modification is not required to maintain the stable repression of target genes (Korenjak *et al.*, 2004). The dREAM complex was not found to have a role in regulating the expression of cell cycle dE2F target genes (Korenjak *et al*., 2004) and Myb-MuvB mediated gene repression was independent of *Drosophila* cdk2-cyclin E RBF1 phosphorylation (Lewis *et al*., 2004).

Each member of the core dREAM/Myb-MuvB complex, with the exception of dMyb, has a *C.elegans* homolog: RBF1/RBF2 (Lin-35), E2F2 (Efl-1), dDP (Dpl-1), MIP130 (Lin-9), MIP120 (Lin-54), MIP40 (Lin-37) and CAF1p55 (Lin-53). Interestingly, each homologue is a synMuv class B gene (Korenjak *et al*., 2004; Lewis *et al*., 2004). It has been proposed that the *C.elegans* homologues may form a core complex analogous to the *Drosophila* dREAM/Myb-MuvB complex in the worm that is responsible for the repression of genes required for vulval differentiation. This complex has been named DP, pRb, MuvB (DRM) (Harrison *et al.*, 2006). As yet, a *C.elegans* Myb homologue has not been identified. A second NURD-like complex, containing the synMuv class B homologues Hda-1 (Rpd3), Lin-53 (CAF1p55) and Let-418 (dMi2) is thought to mediate the activity of the DRM complex through histone deacetylation and nucleosome remodelling (Solari & Ahringer, 2000; Harrison *et al.*, 2006). Lin-9 has been shown to bind to Lin-53, an interaction that is proposed to tether the DRM complex to unmodified histones (Harrison *et al.*, 2006). Binding of the DRM complex to these regions would protect the histones from further modification and maintain a transcriptionally repressive state. This is similar to the proposed function of the dREAM/Myb-MuvB complex and would explain why enzymes such as Rpd3 were not found to be part of the core dREAM/Myb-MuvB complex.

1.2.10.2 The function of novel human Lin-9/pocket protein complexes

Human Lin-9 (*hLin-9),* a human homologue of the *lin-9, MIP130 and aly* genes has recently been identified (Korenjak *et al.*, 2004; Gagrica *et al.*, 2004). It has become apparent that hLin-9 functions both in the pRb tumour suppressor pathway (possibly through the activation of genes require for terminal differentiation) and in the activation of G_2/M specific genes, and is an integral component of both transcriptionally active and transcriptionally repressive pocket protein complexes (Gagrica *et al.*, 2004; Osterloh *et al.*, 2007; Schmit *et al.*, 2007).

hLin-9 is a nuclear, chromatin associated protein that has been shown to bind to pRb and the related pocket proteins p130 and p107 (Korenjak *et al.*, 2004; Gagrica *et al.*, 2004). hLin-9 was shown to bind to pRb via an interaction with the B, and to a lesser extent, the A pocket of the protein both *in vitro* and *in vivo* (Gagrica *et al.*, 2004). The B pocket contains the LXCXE-binding cleft. The interaction with pRb occurs via a region of the hLin-9 protein that includes an N-terminal domain known as Box 1 (Gagrica *et al.*, 2004). Box 1 and a C-terminal Box 2 are conserved in hLin-9 homologues of other species (Figure 1.6).

As hLin-9 mediates pRb tumour suppressor activity and the activation of genes required for differentiation through an interaction with the pocket domain, it is interesting to speculate as to whether viral oncoproteins could antagonise this interaction. Small DNA viruses such as HPV all encode viral oncoproteins that contain a conserved LXCXE domain. It is well established that this LXCXE domain interacts with the B pocket of the pocket proteins, prevents the association with E2F transcription factors and thus alters their tumour suppressor activity. It is possible that oncoproteins such as the E7 protein may also compete for binding with the hLin-9 protein, thus diminishing its functional activity. As the hLin-9 protein synergises with pRb in the

activation of genes required for differentiation, a situation can be envisaged whereby the E7 proteins could also prevent the expression of genes required for differentiation. If this scenario were true, high risk E7 proteins could simultaneously drive the cell through G_1/S and prevent the expression of genes required for terminal differentiation. This would result in a pool of continually dividing, undifferentiated cells similar to those seen in high grade lesions (Buckley *et al*., 1982).

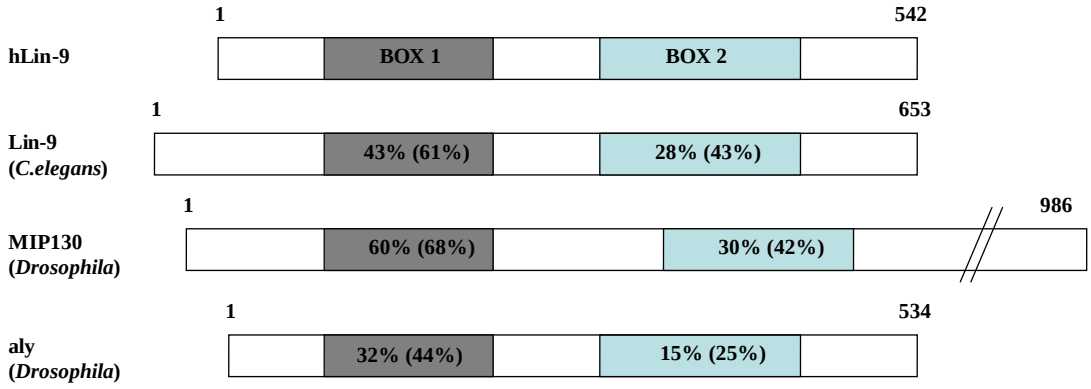


Figure 1.6. A schematic comparison of hLin-9 homologues from different species. All Lin-9 homologues contain two genetically conserved regions known as Box 1 and Box 2. The percentage identity and similarity in parenthesis to hLin-9 is shown with each box. Adapted from Gagrica *et al.*, 2004.

1.2.10.3. The DREAM/LINC complex

In addition to its role in the pRb tumour suppressor pathway, hLin-9 is also a core component of both transcriptionally active and transcriptionally repressive pocket protein complexes that function to control E2F-regulated genes (Osterloh *et al*., 2007; Schmit *et al*., 2007). hLin-9 has been isolated from lymphocytic MOLT-4 cells as part of a native multiprotein complex that has been named DREAM/LINC (Schmit *et al.*, 2007). DREAM contains at least five core components, all of which are homologues of proteins found within the *C.elegans* DRM and the *Drosophila* dREAM/Myb-MuvB complexes (Table 1.2). These are hLin-9 (Lin-9/MIP130), hLin-37 (Lin-37/MIP40), hLin-54 (Lin-54/MIP120), hLin-52 (Lin-52/dLin-52) and RbAp48 (Lin-53/CAFp55) (Schmit *et al.*, 2007). Although the DREAM complex is closely related to the DRM and dREAM/Myb-MuvB complexes, pocket proteins, B-Myb and E2F transcription factors do not form part of the stable core complex. Instead, the complex dynamically interacts with p130, p107, B-Myb and E2F4 in a cell cycle dependent manner (Schmit *et al.*, 2007). LINC has been found on the promoters of E2F-regulated genes both during quiescence and S phase (Pilkinton *et al*., 2007; Osterloh *et al*., 2007; Schmit *et al*., 2007).

During quiescence, DREAM is present on the promoters of E2F-regulated genes required for G_1/S and G_2/M progression in complex with p130 and E2F-4 (Pilkinton *et al*., 2007; Osterloh *et al*., 2007; Schmit *et al.*, 2007). Although the DREAM/p130/E2F4 complex represses transcription, an intact DREAM complex is not necessary for this function (Schmit *et al.*, 2007). p130 and E2F-4 are known to mediate gene repression through the recruitment of histone deacetylases and the modification of chromatin structure. In addition, RNAi studies have shown that hLin-9 is able to repress a group of genes typically required for development and/or

Drosophila melanogaster		***Caenorhabditis elegans***	**Humans**	
dREAM	**MMB**	**DRM**	**DREAM/LINC**	
RBF1,RBF2	RBF1,RBF2	LIN-35	p130,p107	-
dE2F2	dE2F2	EFL-1	E2F4/E2F5	-
dDP	dDP	DPL-1	DP1/DP2	-
P55/CAF1	P55/CAF1	LIN-53	RBBP4	RBBP4
MYB	MYB	-	-	B-MYB
MIP130	MIP130	LIN-9	LIN9	LIN9
MIP120	MIP120	LIN-54	LIN54	LIN54
MIP40	MIP40	LIN-37	LIN37	LIN37
-	LIN52	LIN-52	LIN52	LIN52
-	L(3)MBT	-	-	-
-	RPD3	-	-	-

Table 1.2: Comparison of dREAM (*Drosophila melanogaster*), DRM (*Caenorhabditis elegans)* and DREAM/LINC (human) complexes constituents. DREAM was first discovered in *Drosophila melanogaster* embryonal cells. *Drosophila* dREAM complex is resistant to dissociation by CDK-phosphorylation and they exist throughout cell cycle. The dREAM/MMB complexes are also highly conserved in evolution since they are related to the *Caenorhabditis elegans* (DRM). The homologs of all subunits of the DRM complexes have also identified in human complexes, named DREAM/LINC, whose composition is regulated at distinct phases of the cell cycle.

Adapted from (Heuvel and Dyson, 2008)

differentiation (Pilkinton *et al.*, 2007; Osterloh *et al.*, 2007). Little is known about hLin-9 mediated gene repression as yet, as hLin-9 has only been found as part of the DREAM complex on the promoters of genes required for cell cycle progression. It is possible that DREAM may also control the expression of genes required for development and/or differentiation in a manner similar to that of the dREAM/Muv/MybB complex. If this is the case, the protein composition of such a repressor complex may well be different.

During cell cycle re-entry, the promoter specificity of the DREAM complex changes. In late G_1, LIN, p130 and E2F4 dissociate from the promoters of genes required for G_1/S progression. This allows the activator E2F(1-3) transcription factors access to the promoter and results in the expression of genes required to drive the cell through G_1/S. As the cell progresses through G_1/S, LINC, p130 and E2F4 dissociate from the promoters of genes required for G_2/M progression and DREAM selectively interacts with p107 and B-Myb. hLin-9 interacts with the C-terminus of B-Myb and it is this interaction that is thought to tether DREAM to the promoter (Pilkinton *et al*., 2007; Osterloh *et al*., 2007). RNAi studies have shown that DREAM and B-Myb co-activate a specific cluster of genes required for G_2/M cell cycle progression. These include *cyclin B1*, *cyclin A2* and *cdc2*, which are required for G_2/M progression, *BUB-1* and *CenPE*, which are required at the mitotic spindle checkpoint, *Aurora-A* and *Plk-1*, which are required for spindle assembly, and *UbCh10*, which is required for exit from mitosis (Pilkinton *et al.*, 2007; Osterloh *et al*., 2007). RNAi suppression of either hLin-9 or B-Myb reduced cellular proliferation, delayed G_2/M progression and reduced expression of G_2/M genes (Pilkinton *et al.*, 2007; Osterloh *et al.*, 2007). These data suggest that B-Myb may function in a similar manner to dMyb, at least in the regulation of G_2/M gene expression. It is likely that cell cycle cues induce B-Myb to form a transcriptionally active hLin-9 protein complex during G_2/M. Activation

of the cyclin A promoter is enhanced by phosphorylation of B-Myb by cdk2-cyclin A (Pilkinton *et al*., 2007) but binding of hLin-9 to B-Myb was found to be phosphorylation-independent (Osterloh *et al*., 2007). Phosphorylation may therefore be a temporal cell cycle cue that is required for the activation of B-Myb. Interestingly, dMyb has been shown to regulate G_2/M progression in the Drosophila eye imaginal disc by inducing cyclin B expression (Okada *et al*., 2002). This suggests an evolutionarily conserved role in gene regulation, both in the repression of genes required for development and/or differentiation and cell cycle progression.