DISCUSSION

4.1 Investigation of the DREAM complexes in cervical cancer cells.

Overview

HPV 16E7 targets pRB family members for degradation. The ability of HPV 16E7 to target pRB for degradation is necessary for malignant transformation (zur Hausen., 2000). In contrast to HPV 16E7, HPV 6E7 is not transforming and does not affect the stability of pRB or p107 (Demers et al., 1994; Zhang et al., 2006); however it does target p130 for degradation (Zhang et al., 2006). pRB family members play a key role in regulating progression through the cell cycle. P130 is specifically up-regulated in G0/G1 and is responsible for keeping cells in a differentiated state (Cobrinik, 2005). The fact that both high risk and low risk E7 target p130 for degradation may indicate that p130 is important for the HPV life cycle. Targeting p130 for degradation may be conducive to creating an 'S phase-like' environment (Banerjee et al., 2006; Collins et al., 2005; Munger et al., 2001).

Recently, a multiprotein subunit has been identified in humans which is involved in cell cycle regulation. This complex is known as the DREAM or LINC complex. It was originally discovered in *Drosophila melanogaster*, in which it is involved in transcriptional repression (Korenjak et al., 2004; Lewis et al., 2004) The complex is known as dREAM (Drosophila, Rb, E2F and Myb-interacting proteins) (Korenjak *et al.*,2004) or Myb-MuvB (Lewis *et al.*, 2004). In addition to Rb/E2F this complex also contains a Drosophila MYB transcription factor, three Myb-interacting proteins (Mip40,Mip120 and Mip130) and a protein related to the mammalian pRB-binding protein RbAp48. It is also suggested that dREAM/Myb-MuvB complexes are highly conserved in evolution since they are related to the *Caenorhabditis elegans* synMuv class B genes, except dMYB. The synMuv class B proteins form a complex which is known as DRM (Harrison *et al.*, 2006). The homologs of all subunits of the invertebrate

complexes have also been identified in human complexes, named DREAM or LINC, whose composition is regulated at distinct phases of the cell cycle (Litovchick *et al*., 2007; Schmit *et al*., 2007 and Pilkinton *et al*., 2007). The core DREAM complex contains Lin9, Lin37, Lin54, Lin52 and RbAp48 (the human homologues of Drosophila Mip130, Mip40, Mip120, dLin52 and Caf1p55, respectively). The pRB family members, p130 and p107 were comprised in human DREAM complex as a transcriptionally repressive during the course of a cell cycle. The composition of DREAM is temporally regulated during the cell cycle, being associated with E2F-4 and either p107 or p130 in G0/G1 (Litovchick et al., 2007; Pilkinton et al., 2007; Schmit et al., 2007; Knight et al., 2009) and with the B-myb transcription factor in S/G2 (Litovchick et al., 2007; Pilkinton et al., 2007; Schmit et al., 2007; Knight et al., 2009).

Although the DREAM complex is closely related to the DRM and dREAM/Myb-MuvB complexes, pocket proteins, B-myb and E2F transcription factor do not form part of the stable core complex. The complex dynamically interacts with pocket proteins/E2F-4 or B-myb in a cell cycle-dependent manner (Schmit et al., 2007). During quiescence, DREAM is present on the promoters of E2F-regulated genes required for G1/S and G2/M progression in complex with p130 and E2F4 (Pilkinton *et al*,2007: Osterloh *et al.,* 2007). During cell cycle re-entry, the promoter specificity of the DREAM complex changes. In late G1, DREAM/p130/E2F4 complexes dissociate from the promoters of genes required for G1/S progression. This allows the activator E2F (1-3) transcription factors access to promoter and results in the expression of genes required to drive the cell through G1/S. On promoters of genes required for G2/M progression, DREAM selectively interacts with B-myb during S/G2. RNAi studies have shown that DREAM and B-myb co-activate a specific cluster of genes required for G2/M phase. These include cyclin B1, cyclin A2 and cdc2, which are required for G2/M progression, BUB-1 and CenPE, which are required at the mitotic spindle checkpoint,

Aurora kinase-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).

This thesis focused mainly on understanding whether p130 is a critical target for HPV E7. Initial studies were conducted to investigate the expression of DREAM complexes in HPV-transformed cell lines. Experiments were also conducted to determine the effect of p130/DREAM complex in HPV E7-depleted cells and whether the G1 arrest is dependent on the reformation of the p130/DREAM complex. Various types of HPV were also expressed ectopically in T98G glioblastoma cell lines and the effect of HPV E7 oncoproteins on p130 mutants was investigated. These findings will be discussed in detail in this chapter.

4.2 HPV16 E7 disrupts p130/DREAM complex.

Initial observations have provided evidence for the disruption of p130/DREAM complexes and p107/DREAM complexes in HPV16 E7 positive cells (Caski and SiHa). In both SiHa and CaSki cells, the lowering of p130 levels was shown by Western blot and is presumably due to E7-mediated degradation (Roman et al., 2006). HPV16 E7 is able to induce the proteasomal degradation of p130 and the related pocket proteins in keratinocytes and this is a distinct function of the HPV16 E7 protein that is not shared by adenovirus E1A or SV40T antigen (Gonzalez *et al*., 2001). The proteasome is a large 26S multisubunit complex that degrades polyubiquitylated proteins to small peptides. Proteasomes act on proteins marked specifically for degradation by a small protein called ubiquitin (Ciechanover et al., 2000). Ubiquitin is activated for transfer to substrate through the ATP-dependent formation of a thioester bond with the ubiquitin-activating (E1) enzyme and is subsequently transferred to a ubiquitin-conjugating (E2) enzyme. Finally, thioesterified ubiquitin is transferred to the

target protein with the assistance of a ubiquitin ligase (E3). E3s bind directly to substrate, suggesting that they provide specificity in ubiquitylation reactions. SCF complexes (E3 ubiquitin ligases) recognize and polyubiquitylate substrates in a phosphorylation-dependent manner, targeting them for degradation by the 26S proteasome (Deshaies, 1999). HPV 16 E7 and p130 both interact with and are ubiquitylated by SCFSkp2 complex (Oh et al., 2004a; Tedesco et al., 2002).

Furthermore, the results obtained also showed that p130/DREAM complex was disrupted, particularly in CaSki when compared to T98G cells in which of the p130/DREAM complex was expressed abundantly. This presumably reflects the binding of p130 to E2F4 by 16E7. Both HR (HPV16 E7) and LR HPV E7 proteins bind pRb family members through their LXCXE binding motif (Dyson et al., 1989). Furthermore, several *in vitro* studies have revealed that HPV 16 E7, in contrast to HPV 6 E7, has a greater affinity for pRb, p107, and p130 (Ciccolini et al., 1994; Gage et al., 1990). HR HPVs 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 HR vs. LR E7 proteins at the amino acid 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) The E7 proteins from the low risk HPV types bind to the pocket proteins with lower affinity than the high risk HPV E7 types.

B-myb is over-expressed in 16E7-containing cells, as the G0/G1 transcriptional repression (presumably mediated by p130/DREAM complex) is relieved (Lam *et al*., 1994). This is due to the inactivation of pRB family proteins by 16E7

protein which subsequently causes the G1 exit and cell cycle entry to S-phase. On the other hand, the higher expression of B-myb/DREAM complexes might be related to the interaction of 16E7 protein with the cyclin A/CDK2 complex (Tommasino *et al*., 1993) which will ensure the cells will remain in an S-phase like state where B-myb gene expression is maximal. In these experiments it was demonstrated that p130/DREAM complex was abundant in T98G cells (control cell line) (Figure 3.2). Claudio *et al* (1994) has demonstrated that in certain cell lines, such as T98G cells, which are deficient in the cdk inhibitor, p16, the p130 protein is the major cell cycle inhibitor instead of pRB and p107. However, in the C33A cell line there was a higher expression of p107 as indicated in the input control (Figure 6), This may be related to the lack of pRB as a cell cycle inhibitor in C33A cells (Chen *et al*., 2002).

4.3 Depletion of HPV E7 results in cell cycle arrest and reformation of the p130/DREAM complex.

In an effort to further understand the implications of HPV E7 towards p130/DREAM complex, the effect of HPV E7-depleted cells were investigated. The E7 expression was knocked down in SiHa and Caski cells using a short hairpin RNA (shRNA) directed against HPV16E7. Two sequences of HPV16E7 were customized, namely HPV16E7A and B and they were ligated into the pLKO.1 vector. Previously, difficulties were encountered in this part of the experiment due to low transfection efficiency, particularly in Caski cells. Therefore, a lentiviral system was employed in this experiment for a better uptake of shRNA genes into the cells. The cloned shRNA genes were designated as pLKO.1 sh.16E7A and pLKO.1 sh.16E7B, respectively, and were packaged in lentiviral particles. Lentiviruses carrying these shRNA genes were transduced into SiHa and Caski cells and the non-transduced cells were eliminated by

puromycin selection 24 hours post-transduction. Real-time PCR analysis, has demonstrated that E7 expression was decreased by 96.8% and 64% with 16E7B and 16E7A shRNAs respectively, when compared to control in Caski cells. Whereas in SiHa cells, the E7 mRNA expression was decreased by 72% and 98.8% with 16E7A and B, respectively. Next, the effects of E7 shRNA in cell cycle regulation was assessed. Propidium iodide staining showed that 16E7 depletion caused an accumulation of Caski cells in G1 with both 16E7 shRNAs. In addition to a delay in cell cycle entry into S phase, as shown by FACS analyses, the cells were decreased significantly in S/G2/M stages. This finding was consistent with results from Tang et al., (2006), which also found G1 arrest by suppressing E7 expression in Caski cells. The effect of p130/DREAM complex upon E7 suppression in CaSki cell was investigated by protein immunoprecipitation and western blot and it showed that the repressive p130/DREAM complex was reformed. Following this result, it was evident that the cell cycle arrest in CaSki cells is dependent upon disruption of p130/DREAM complex. It can therefore be inferred that the continued proliferation of HPV16-transformed cells is dependent upon disruption of p130/DREAM.

In undifferentiated cells, hypophosphorylated p130 is predominantly in the nucleus in the G0/G1 phase of the cell cycle. In S-phase, p130 is typically phosphorylated and transported to the cytoplasm where it is targeted for degradation Chestukhin et al., 2002; Tedesco et al., 2002). Therefore, the protein immunoprecipitation was carried out from nuclear extracts. P130/DREAM complex was reformed, and probably accounts for the loss of *B-myb* gene expression, as this complex is known to repress transcription through a promoter E2F-binding site (Lam and Watson, 1993;Lithovchick et al., 2007).

4.4 Re-expression of p53 tumour suppressor protein in E7 depleted-CaSki cells.

Depletion of E7 in CaSki cells inevitably suppressed E6 oncoproteins in this cell lines since all HPVs have a conserved structure of the early promoter, the E6 promoter or p97 including HPV 16, that initiates transcription of the E6/E7 polycistronic mRNA. Therefore, p53 was re-expressed upon E6 depletion which could also impact on DREAM complexes. Under these conditions, $p21^{Cip1}$ could inhibit cyclin E/A cdk2 complexes, which would preclude both activating phosphorylation of B-myb (Saville and Watson, 1998) and inactivating phosphorylation of p130 (Cheng *et al*., 2000).

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). High risk HPV E6 binds to E6-associated protein (E6-AP), a cellular ubiquitin-ligase, and targets the tumour suppressor p53 for degradation (Huibregtse et al., 1993; Scheffner et al., 1999). Activation of p53 occurs upon cellular stresses, such as DNA damage, oncogene activation, telomere erosion and hypoxia. It is mediated, at least in part, by inhibition of MDM2 and rapid stabilization of the p53 protein by post – translational modifications. E6 protein from the high -risk HPV type 16, has a higher affinity in binding towards p53 and by binding and targeting p53 for degradation, it prevents cell cycle arrest and apoptosis in stressed cells favouring accumulation of DNA damages and cellular transformation (Gu *et al.* , 1994) . Both viral oncoproteins (E6 and E7) 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 check points, of apoptosis and differentiation and eventually to transformation of the

HPV infected cell. As shown by the result, the p53 expression was increased upon E7 suppression in CaSki cells.

4.5 G1 arrest is dependent on DREAM complex reformation.

Experiments were designed to determine whether the profound G1 arrest in HPV E7-depleted cells is dependent on repressive DREAM complex reformation. One of the core DREAM components, Lin-54 was co- suppressed with 16E7 and the effect was tested by protein immunoprecipitation, western blotting and flow cytometry analysis. We found that reformation of p130/DREAM upon targeting E7 was diminished in cells co-transduced with Lin-54 shRNA. The G1 arrest was prevented completely by co-transduction with Lin-54 shRNA. Flow cytometry analysis also showed that the proportion of G1 cells is 83.5% with transduction on 16E7B shRNA alone, but this amount was decreased to 56.8% upon co-transduction with Lin-54 shRNA. In addition, CaSki cells were also transduced with 16E7 shRNA alone or either co-transduced with p130 to confirm whether the reformation of p130/DREAM is important for G1 arrest upon E7 suppresion. The cell cycle analysis showed that p130 depletion strongly overcame the G1 arrest caused by 16E7B shRNA. Moreover, qPCR analysis showed an increase in expression of S-phase genes, B-myb and cyclin-A, which are transcriptionally repressed by p130/DREAM (Lithovchick et al., 2007) upon co-transduction of p130 shRNA. This suggests that the continued proliferation of HPV 16-transformed cells is dependent upon disruption of p130/DREAM complex.

Other studies have found that targeting E6/E7 expression by RNA interference induces apoptosis or senescence (Jiang & Milner, 2002; Hall & Alexander, 2003; Johung et al., 2007; Sima et al., 2008; Yamato et al., 2008), in addition to effects on the cell cycle. We observed no obvious apoptosis in CaSki or SiHa cells during the course of our experiments, and there is no implication from our study that DREAM

complexes regulate cell survival. Recent studies do suggest however, that p130/DREAM is important for establishment of senescence, at least in context of the RAS oncogene (Tschop et al., 2011).

4.6 All HPV types are capable to degrade the p130/DREAM complex.

Both high risk and low risk HPV E7 proteins bind pRB family members through their LXCXE binding motif present in the CR2 domain (Dyson et al., 1989). This motif is necessary for binding to pRB and the related pocket proteins (Dyson et al., 1989; Davies et al., 1993; Hu et al., 1995). It is also necessary for the binding of the HPV 16E7 protein to the cyclin dependent kinase inhibitor (Cdki) p21 (Jones et al., 1997) and to mediate pocket protein destabilization (Berezutskaya et al., 1997; Jones and Munger, 1997). Furthermore, several *in vitro* studies have revealed that HPV 16E7 as compared to HPV 6E7, has a greater affinity for pRB, p107 and p130 (Ciccoloni et al., 1994; Gage et al., 1990). High risk 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).

To gain further insight into the relationship between HPV E7 proteins and DREAM complexes, various types of HPV E7 were expressed ectopically in T98G cells. T98G cell lines were employed since these cells have well characterized DREAM complexes. The results demonstrated that high risk (HPV33E7, HPV18E7 and HPV16E7) and low risk HPV1E7 efficiently decreased the p130/DREAM complexes in T98G cells. Whereas expression of high risk E7s greatly diminished p130 expression, presumably through degradation, 1E7 had little effect on p130 levels but was still able to prevent its interaction with DREAM. It is notable that strong affinity does not necessarily correlate with the ability to induce cellular transformation (Ciccolini *et al*.,

1994; Caldeira *et al*., 2000), since the E7 protein from the low risk HPV type I can associate strongly with p130, but fails to induce degradation and transformation (Alunni-Fabronni *et al*., 2000). The cutaneous HPV48 E7 and the low risk HPV11 E7 disrupted p130/DREAM complexes less efficiently than these other E7 types. HPV11 E7 expression nonetheless reduced p130 expression quite dramatically, and the lesser effect on complex formation probably relates to its lower affinity binding to pocket proteins. Comparison of the pocket protein binding domains of the high risk and low risk E7 proteins reveals a difference of one amino acid, aspartic acid 21 in HPV16 E7 versus glycine 22 in HPV11. Substitution of glycine 22 with an aspartic acid in HPV11 E7 confers greater affinity in binding to pocket proteins. However the correlation between the efficiency of binding to pocket protein does not hold for every HPV E7 types. In fact HPV 1, which has never been associated with cancer, has an aspartic acid in the pocket protein binding, like the high risk HPV E7s and binds to pocket protein with approximately the same affinity as HPV16 E7.

The result with HPV48 E7 was unexpected. Firstly, the HPV48 E7 has shown the ability to disrupt p130/DREAM complexes quite dramatically, in fact HPV48 E7 binds to pocket protein with a very low affinity (Caldeira *et al*.,2000;Dong *et al*., 2001). However, HPV48 E7 has an ability to bind to p21 and inactivate the E2F/p130-cyclin/cdk pathway which indirectly inhibits p130 to associate with E2F. This may explain how HPV48 E7 disrupted the p130/DREAM complex. Secondly, it is also apparent from the results that only T98G transfected with cutaneous HPV48E7 and high risk HPV16E7 expressed p107/DREAM complexes and they also expressed the B-myb/DREAM complex at the highest level compared to other HPV types. Schmit *et al*., (2007), have shown that p107 associates with the B-myb/DREAM complex in T98G cells. Therefore, the presence of p107 in DREAM complexes in HPV16 E7 and HPV48 E7 transfected cells may relate to this association rather than the formation of repressive

complexes. Since both HPV 16 E7 and HPV48 E7 disrupt the p130/DREAM complexes, it will then lead to the activation of S phase gene and the DREAM complex will associate with B-myb and p107.

4.7 HPV disrupts p130/DREAM complexes through different mechanisms.

From the preliminary results, they have provided us that the disruption of repressive p130/DREAM complexes by HPV16 E6/E7 oncoproteins is required for cell cycle progression in cervical cancer cells. Upon HPV E7 binding to the p130 LXCXE motif, this will cause an abrogation of cell differentiation since p130 functions has been interfered. Therefore, it is necessary for the HPV virus to disrupt the repressive p130/DREAM complex in host cells in order to continue their proliferation in S phase cycle. Taken these together, it can be deduced that p130 rather than pRB is the more significant cellular target of E7 during the normal replication cycle to establish conditions in suprabasal cells favouring HPV genome replication. This argument has been made in previous studies (Zhang *et al*., 2006; Genovese *et al*., 2008), however it is notable in one of these publications that p130 levels in submerged cultures of CaSki and SiHa cells were found to be rather similar to those of the control primary human keratinocytes, suggesting that E7 had little impact on p130 stability in these cervical cancer cells (Genovese et al., 2008). We also detected p130 by western blotting in SiHa and CaSki cells in our study, albeit at lower levels than in control T98G and C33A cells. Importantly, our work showed a profound p130/DREAM deficit in cervical cancer cells compared with control. This was particularly evident with CaSki cells, and suggest that the p130 detected in this system is not part of the DREAM complex. The residual p130 protein may be unable to bind to E7, presumably reflecting the complex

phosphorylation events that regulate its activity (Cobrinik, 2005), and is thus protected against degradation in CaSki cells.

E7 proteins could potentially target the p130/DREAM complex through two known mechanisms: direct interaction with p130 or induction of CDK2 phosphorylation via an interaction with p21. Therefore several types of p130 mutants were constructed and CaSki cells were used as they already expressed HPV16 E7 while HPV48 E7 was ectopically expressed in T98G cells to explore which of these mechanisms are responsible for the activities of 16E7 and 48E7 in disrupting of p130/DREAM and cell cycle progression.

Three types of p130 mutants have been constructed that were deficient in binding the E7 LXCXE motif (p130mE7), a mutant that could not be phosphorylated by cyclin-dependent kinase (CDK) (p130PM22) and a double mutant (p130PM22/mE7). The p130mE7 mutant was designed based on the work of Dick and Dyson (2002), who showed that a surface of the pRB B pocket was critical for binding LXCXE motif-containing proteins. The LXCXE motif was first identified in proteins encoded by small DNA tumor viruses (Stabel et al., 1985). The LXCXE motif allows the virus to bind to pocket proteins (p130 and p107) including pRB and it appears that viral proteins use the LXCXE motif to target and inactivate all three family members (Dyson and Harlow, 1992). The LXCXE binding site is one of the most highly conserved features in the pRB structures which are consistent with the viral evolution. This region of the pRB B pocket is partially conserved in p130 and two critical conserved amino acids (leucine and cysteine) in p130 were replaced with alanine by *in vitro* mutagenesis. Dick and Dyson indicated that mutation of the two amino acids severely reduced binding to E7 protein (Dick and Dyson, 2002).

The p130PM22 mutant harboured mutations at the 22 CDK phosphorylation sites by replacing the serines and threonines with alanine that could be phosphorylated (Farkas *et al*., 2002) (Figure 4.1). This mutant was designed in order to study one of the cutaneous HPV types, 48E7. Pearce J., (2007) in her Ph.D thesis previously showed that HPV48 E7 protein was competent to bind to the Cdk inhibitor, p21, *in vitro*. It is known that HPV48 E7 protein has an imperfect LXCXE motif (LXSXE), which is deficient in binding to p130 by the LXSXE motif but aligned with our results it showed that p130/DREAM was diminished by HPV48 E7. The Cdk inhibitor, p21 is known to be involved in E2F/pocket protein-cyclin E/A-CDK2 pathways in G1 and S phase. Therefore, upon HPV48 E7 binding to p21, this will abrogate its inhibition of cyclin/CDK activity and prevent the association of p130 with E2F4. This mutant was designed to explore the mechanism for HPV48 E7 in disrupting p130/DREAM complex.

The double mutant (p130PM22/mE7) was constructed to confirm the mechanism of each HPV type (16E7 and 48E7) for their role in proliferation in host keratinocyte cells. All p130 mutants and a control wild-type p130 were tagged with HA to differentiate between endogenous and ectopically expressed p130.

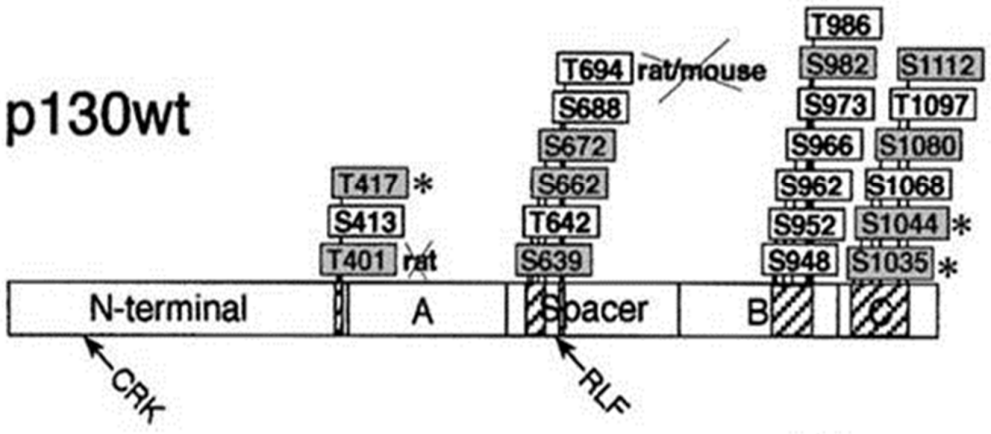

Figure 4.1: ***In vivo* phosphorylation sites in p130**. Schematic summary of the 22 mapped in vivo phosphorylation sites. Shaded boxes, residues conserved in p107; an asterisk indicates residues conserved in pRB. A,B and C and spacer refer to domains of p130. Among those, three were specifically targeted by (Cdk4/6), whereas most of the other phosphorylation sites were more general Cdk sites targeted by both Cdk4/6 and Cdk 2. The relative positions of the two cyclin A and E binding motifs (CRK and RLF) are indicated in the p130 constructed by arrows. CDKs phosphorylate serine and threonine by the proline site of serine and threonine at p130. (Adapted from K.Hansen *et al.*, 2001).

4.7.1. Defective in the B pocket of p130 abrogate the HPV-transformed cells proliferation

HPV16 E7 binds to a highly conserved shallow groove on the B pocket of hypophosphorylated pRb, an interaction that is mediated by the LXCXE motif (Huang *et al.*, 1993; Lee *et al.*, 1998). The CR2 region contains a conserved LXCXE sequence which interacts with the retinoblastoma tumor suppressor protein pRb and the related 'pocket proteins' p107 and p130 (Dyson *et al.*, 1989 and 1992). These proteins interact with the transcription factor E2F, which is able to regulate cell cycle transition (Chellapan *et al.*, 1991; Hiebert *et al.*, 1992; Zhu *et al.*, 1995). The primary activity of high risk E7 proteins is to inhibit members of the retinoblastoma (RB) tumor repressor family to induce progression into S phase (Vousden, 1994). In normal cells, pRB is hypophosphorylated in early G_1 and becomes increasingly phosphorylated towards S phase. In its hyphophosphorylated form, RB binds E2F transcription factors and actively represses transcription from promoters containing E2F sites. By binding pRB in a hypophosphorylated state, E7 prevents it from binding to E2F and thereby promotes cell cycle progression (Chellapan *et al.*, 1991). In normal epithelia, cell cycle exit occurs following differentiation. By binding to pRB, E7 promotes cell cycle progression in differentiated epithelial cells, allowing for replication of the HPV genome.

In this experiment, all mutants and p130wt were transfected into C33A and CaSki cells and western blotted using an anti-HA antibody. Our results showed that p130PM22 and p130wt were degraded in CaSki cells rather than in C33A cells. However, both p130mE7 and p130PM22/mE7 were expressed abundantly in CaSki cells. From this finding, it is strongly suggested that the p130mE7 mutation prevented binding to the 16E7 LXCXE motif since two critical amino acids in the B pocket had been changed to alanine and thus protected them from degradation.

In addition, since the p130mE7 and p130PM22/mE7 were protected from degradation, as anticipated, they were reformed into p130/DREAM complexes whereby the complex was completely diminished in p130PM22 and p130wt. From these results, it is shown that direct interaction of 16E7 with p130 through its LXCXE motif is the key determinant in promoting the S phase in CaSki cells, which is the conducive cycle for viral replication. Other than that, the differentiation marker for epithelial cells will no longer functioning since this property has been exert by HPV E7 proteins.

4.7.2 HPV48 E7 disrupts p130/DREAM via CDK2 phosphorylation

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). 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).

From the *in vitro* experiment that was done by Pearce J (2007), HPV48 E7 was competent to bind to the Cdk inihibitor, p21. Therefore, p130PM22 and

p130PM22/mE7 were designed to study the mechanism of HPV48 E7 in regulating continuous cell cycle in epithelial cells. The T98G human glioma cell line was used to express 48E7 and the p130 mutants including p130wt. Twenty-two phosphorylation sites in both p130 mutants were mutated by site-directed mutagenesis with alanine instead of serine and threonine. In normal cell cycle regulation, CDKs phosphorylate serine and threonine by the proline site of p130. From our results, it is likely that 48E7 reduced p130 expression via CDK phosphorylation since p130mE7 and p130wt were both reduced significantly when compared to p130PM22 and p130PM22/mE7. Therefore this result reflected on the p130/DREAM complex formation on T98G expressing p130PM22 and double mutant. As anticipated, both CDK deficient phosphorylation mutants were able to diminished the repressive DREAM formation but p130/DREAM complex was abundantly expressed in p130mE7 and p130wt. This was further confirmed by flow cytometry analysis, as cells expressing p130wt or p130mE7 were able to escape G1 arrest in the presence of 48E7. It can be concluded that continuous expression of 48E7 interferes with CDK inhibitor, p21 from binding to cyclin E.

4.8 B-myb/DREAM as an activating complex in cell cycle progression

Avian myeloblastosis viral oncogene homolog-2 (*mybl2*) is a member of a multigene family of transcription factors involved in control of cell cycle progression, differentiation and apoptosis (Oh et al., 1999; Sala , 2005). All members of this family, A-MYB, B-MYB (MYBL2) and C-MYB, contain conserved regulatory and transactivation domains that exhibit sequence-specific DNA-binding activity. Only B-MYB, the ancestral gene of this family, is expressed in all proliferating cells (Davidson et al., 2005).

As with many cell cycle associated transcription factors, B-Myb expression and function is dynamically regulated. The *mybl2* gene, which encodes B-Myb, is regulated directly by E2F transcription factors and is maximally induced at the G1/S boundary of the cell cycle (Sala , 2005). The *trans*-activation and gene regulatory potential of B-MYB is regulated by cyclin A/cdk2-mediated phosphorylation (Bessa et al., 2001), and B-MYB is degraded through a ubiquitin-mediated process late in S phase (Charrasse et al., 2000).

Recently, a multiprotein unit has been identified in *Drosophila melanogaster* which is involved in transcriptional repression. The complex is known as dREAM (Drosophila, Rb, E2F and Myb-interacting proteins) (Korenjak *et al*.,2004) or Myb-MuvB (Lewis *et al*., 2004). The homologs of all subunits of the invertebrate complexes have also been identified in human complexes, named DREAM or (LINC), whose composition is regulated at distinct phases of the cell cycle (Litovchick *et al*., 2007; Schmit *et al*., 2007 and Pilkinton *et al*., 2007). The core DREAM complex contains Lin9, Lin37, Lin54, Lin52 and RbAp48 (the human homologues of Drosophila Mip130, Mip40, Mip120, dLin52 and Caf1p55, respectively). The complex dynamically interacts with pocket peoteins/E2F-4 or B-myb in a cell cycle dependent manner. In G_0–G_1, DREAM binds to E2F4 and either p130 or p107 to repress transcription of E2F target genes regulating the G_1/S transition (Litovchick *et al.*, 2007). In S–G_2, DREAM switches to B-Myb to activate genes required for G_2/M transition and mitosis (Schmit *et al.*, 2007) (Figure 4.2).

In order for the HPVs to replicate their genome, the virus will interfere with repressive DREAM complex during G0-G1 to promote the cell cycle into S phase which require B-myb/DREAM complex to be activated to express the genes which are required for S/G2/M phases.

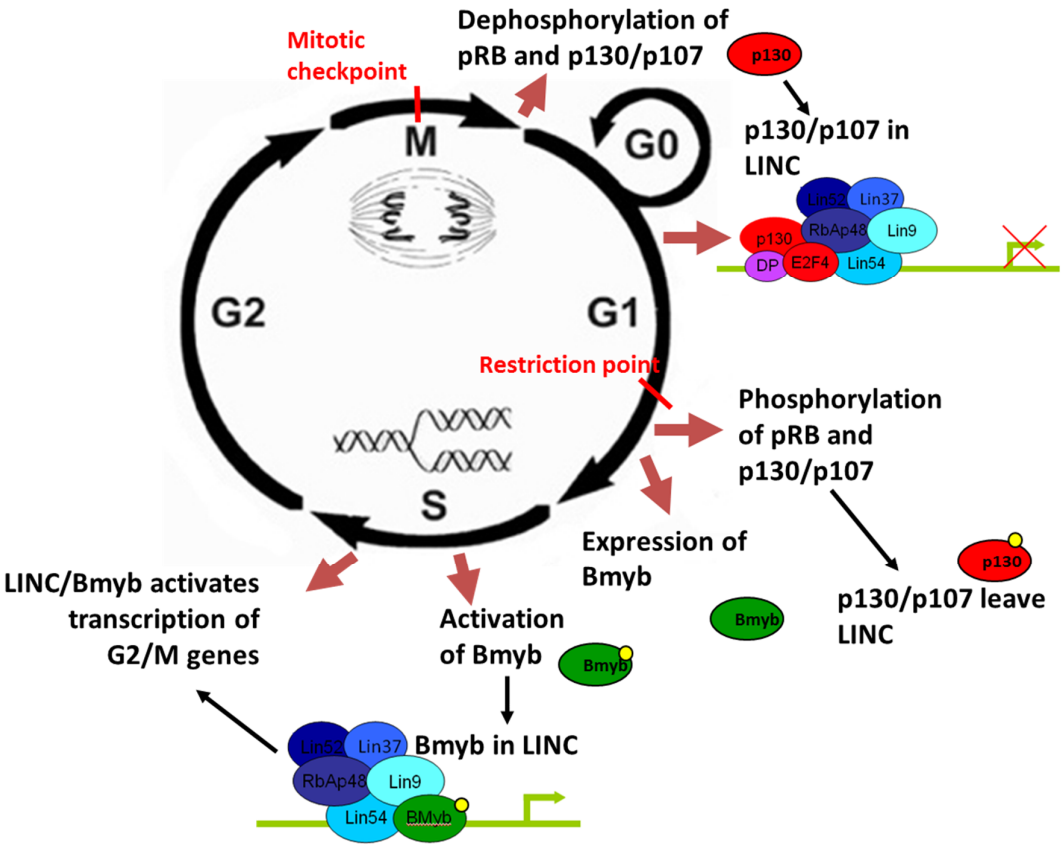

Figure 4.2: Diagram of repressive p130/DREAM and activating B-myb/DREAM complex localised during cell cycle progression.

4.8.1 B-myb/DREAM complex is not critical in CaSki to regulate the G2/M genes

Recently, 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. If the B-myb/DREAM complex is similarly required for mitosis in cervical carcinoma cells, particularly in Caski cells which also lacks pocket protein function, targeting the complex may arrest cells in mitosis. Depleting Lin-54 in embyonal stem cells had a particularly pronounced effect in cell cycle regulation.

Therefore, Caski cells were employed to investigate the effect of B-myb/DREAM complex by suppressing one of the DREAM components (Lin-54) since it expresses HPV16E7 at high levels. The preliminary results have provided evidence that B-myb/DREAM complex in Caski cell might not be as critical as in embryonal carcinoma stem cell, since the quantitative RT-PCR result shows in Caski Lin-54-depleted cell, the G2/M genes (cyclin B, aurora kinase A and polo-like kinase 1) level were not decreased by depleting Lin-54. However, the results were repeated by depleting Lin-9 and B-myb itself in CaSki cells. We showed that both results were consistent with Lin-54 suppression in CaSki cells and suggesting thatB-myb/DREAM complex is not critical in cell cycle regulation of HPV-transformed cell lines, CaSki.

Our results are contrary to those of Knight *et al.*, 2009 in which they showed that, in embryonal stem cells, B-Myb recruited Lin-9 to activate transcription of G_2/M genes in undifferentiated embryonal carcinoma cells. They also demonstrated that B-myb/DREAM complex is vital for progression through mitosis in cells lacking a G_1/S checkpoint. For cervical carcinomas, the B-myb/DREAM complex might not be as critical as p130/DREAM for the survival of the HPV virus.

4.9 p130 localization

p130 contains three nuclear localization signals (NLS), two in the C-terminus and one in the loop region (Chestukhin et al., 2002). In undifferentiated cells, hypophosphorylated p130 is predominantly in the nucleus in the G0/G1 phase of the cell cycle. In S-phase, p130 is typically phosphorylated and transported to the cytoplasm where it is targeted for degradation. Shuttling of p130 between the nucleus and the cytoplasm therefore provides a means of regulation (Chestukhin et al., 2002; Tedesco et al., 2002). p130 levels, like the levels of other pRb family members, are regulated in response to the proliferative state of cells and are controlled by Skp-Cullin-F-box (SCF) complexes which mediate proteolysis in a phosphorylation-dependent manner (Classon and Dyson, 2001); (DeCaprio et al., 1992; Tedesco et al., 2002); (Shirodkar et al., 1992). p130 has been shown to be phosphorylated in cycling cells by cyclin D/Cdk4 or Cdk6, cyclin A/Cdk2 and cyclin E/Cdk2 (Classon and Dyson, 2001; Cobrinik, 2005). Cdk4/Cdk6, not Cdk2, is responsible for targeting p130 for degradation in fibroblasts (Tedesco et al., 2002). In cycling cells Cdk4/ Cdk6 phosphorylates p130 on Ser 672, resulting in a hyperphosphorylated form of p130 that is targeted for degradation by an SCF 21 complex (Tedesco et al., 2002). In growth-arrested and terminally differentiated cells, p130 is phosphorylated by glycogen synthase kinase 3 (GSK3) in the loop region in the B subdomain and thus stabilized (Litovchick et al., 2004).

4.10. Disruption of the pRB/Lin-9 interaction by the E7 proteins

The Lin-9 protein is known to associate with pRb in mammalian cells, a function that appears to mediate a number of its known activities (Gagrica *et al*., 2004). As hLin-9 mediates pRb tumour suppressor activity through an interaction with the pocket domain, it is interesting to speculate as to whether the E7 proteins could antagonise this interaction. The high risk HPV16 E7 protein contains a LXCXE domain that is known to bind to the pocket domain of pRb and suppress its tumour suppressor activity. It is possible that oncoproteins such as the HPV16 E7 protein may compete for binding with the hLin-9 protein, thus diminishing its functional activity.

The strength of binding to pRb varies between different HPV types. Although generally speaking, the low risk and cutaneous HPV E7 proteins do not bind to pRb with a particularly high affinity and the high risk HPV E7 proteins do (Munger *et al*., 1989b), there is a grey area containing many in-between (Ciccolini *et al.*, 1994; Caldeira *et al*., 2000; Dong *et al*., 2001). In contrast to the high risk HPV types, low risk and cutaneous E7 proteins contain either a fully intact LXCXE motif, a partial LXCXE motif or no LXCXE motif (Caldeira *et al*., 2000). It is unclear why E7 proteins such as the cutaneous HPV1 E7 protein, which contains an intact LXCXE motif and is able to bind to pRb with a similar affinity to the high risk HPV16 E7 protein, does not have any *in vitro* transforming activity (Ciccolini *et al*., 1994). It is thought that the ability to target pRb for proteasomal degradation plays an important role, as the HPV1 E7 protein is not able to degrade pRb (Alunni-Fabbroni *et al*., 2000; Giarrè *et al*., 2001; Gonzalez *et al*., 2001), but this is not sufficient for cellular transformation (Balsisits *et al*., 2006). The additional functional activities of the high risk HPV E7 proteins that enable the proteins to efficiently overcome the G_1/S checkpoint and promote cellular transformation have not yet been fully characterised, but are thought to include the

ability to inactivate the Cdki $p21^{WAF1/CIP1}$ and induce genomic instability. Presumably the functional amalgamation of each of these activities defines the relative efficiency by which each E7 type may deregulate the cell cycle.

It would be interesting to see whether the affinity of the E7 type for pRb is directly comparable with an ability to antagonize pRb-hLin-9 binding, and whether this has any functional relevance. It is possible that the efficiency by which an E7 type disrupts the pRb-hLin-9 interaction is not just determined by its relative affinity for pRb. The cutaneous HPV1 E7 protein for example, may not be able to antagonize the pRb-hLin-9 interaction as efficiently as the high risk HPV16 E7 protein. In such a situation, the ability to prevent pRb-hLin-9 binding would be dependent on other unidentified activities or functional domains of the particular E7 type. If this were the case, it could mirror the disruption of the pRb-E2F interaction. Although the LXCXE motif is required to anchor the HPV16 E7 protein to pRb, an additional C-terminal region is required to disrupt E2F binding (Patrick *et al.*, 1994). By equal measure, it is possible that the efficiency by which an E7 type disrupts the pRb-hLin-9 interaction does not determine the functional activity of the pRb-hLin-9 complex. The HPV1 E7 protein may be able to stoichiometrically disrupt the pRb-hLin-9 complex in a similar manner to the HPV16 E7 protein, but this may not have the same functional effect. For example, as the HPV1 E7 protein is not able to degrade pRb, the E7 proteins may quickly become saturated.

Since the hLin-9 protein synergises with pRb in the activation of genes required for differentiation, a situation may be envisaged whereby the high risk E7 proteins could prevent the expression of these genes. 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 infections (Buckley *et al.*, 1982).