Mechanisms of embryonic stem cell division and differentiation

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Thesis Summary

The regulatory mechanisms governing dramatic proliferative changes during early mouse development are not well understood. This thesis aims to address this question using *in vitro* model systems of mouse embryogenesis. In particular, this thesis aimed to assess the function of the elevated, constitutive levels of cyclin dependent kinase 2 (CDK2) activity in embryonic stem (ES) and early primitive ectoderm-like (EPL) cells and the changes associated with differentiation into EPL embryoid bodies, *in vitro* equivalent of differentiation primarily to a mesodermal fate. It was determined that active CDK2 complexes associate with an increased proportion of substrates in pluripotent ES and EPL cells compared to EPL embryoid bodies. In addition, this thesis assessed the presence of other G1 CDK activity, determining that ES cells have high levels of constitutive CDK6 activity, which is refractory to inhibition by p16. Lineage specific decreases in CDK6 activity highlighted the complexities regulating cell proliferation during differentiation. Due to the reported constitutive E2F target gene expression in ES cells, this thesis also aimed to further analyse the regulation and activity of E2F transcription factors and pocket proteins in ES cells. It was demonstrated that constitutive phosphorylation of p107 and increased E2F-4 stability in ES cells contributes to increased levels of free E2F-4, that binds E2F target gene promoters *in vivo*. The importance of CDK regulation of p107 in ES cells was demonstrated by analysis of ectopic expression of phosphorylation-resistant mutant p107. The increased sensitivity of EPL cells to ectopic p107 highlighted differences in pluripotent cell populations. In addition, it was determined that differential regulation of p107 during differentiation was associated with increased p107 binding to E2F target gene promoters and decreased E2F target gene expression. Differentiation associated changes in regulation and activity of cell cycle regulators demonstrated in this thesis are important for understanding the pre-gastrulating mouse embryo and to enable regulation of pluripotent cell differentiation for therapeutic use.

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Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution. To the best of my knowledge, this thesis contains no material previously published or written by another person, except where due reference has been made in the text. All experiments were conducted by myself, except where due reference is made in the text.

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Josephine White
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Introduction

CHAPTER ONE

1.1 The mammalian cell cycle

Multiple tightly controlled molecular pathways coordinate cell cycle progression. The completion of each of the cell cycle phases, in the correct sequential order, is essential to ensure that DNA is faithfully replicated once and nuclei segregate their chromosomes through mitosis. A multitude of intracellular and extracellular signals must be incorporated throughout the cell cycle. Checkpoints are the surveillance mechanisms, which ensure that the cell responds to these signals before entering or continuing the cell cycle. Prior to entry into S phase, during G1 phase, checkpoint mechanisms assess whether to continue dividing or withdraw from the cell cycle, according to nutrient availability, cell size and cell density. During G2 phase, which intervenes between S phase and mitosis, checkpoint controls ensure the completion of DNA synthesis, fidelity of DNA replication and integrity of mitotic spindle formation prior to chromosome segregation. While the length of these phases may vary, their order is strictly maintained to ensure the integrity of the DNA is transmitted across successive generations (Elledge, 1996).

Regulation of cell cycle progression is coordinated by the activity of cyclin dependent kinases (CDKs). The catalytic functions of CDKs are tightly regulated to ensure CDK activation occurs only during the specific phases of the cell cycle. It is CDK activity that controls the timing of cell cycle events, by targeting proteins and complexes involved in cell cycle progression. When a checkpoint has been activated, the primary target is CDK activity. Inhibition of CDK activity leads to cell cycle arrest. Subsequently, a cascade of events is activated by the checkpoint to ensure that the cell responds to the intracellular or extracellular changes (Bartek and Lukas, 2001; Malumbres and Barbacid, 2001).

1.2 Regulation of cyclin dependent kinase activity

1.2.1 Cyclin Binding

The primary level of control of CDK activity is the association with the cyclin subunit. There are primarily four CDKs, CDK2, CDK4, CDK6 and CDC2, which associate with specific cyclins to regulate the mammalian cell cycle. The best characterised cyclins are cyclins D, E, A and B, each with multiple isoforms (Morgan, 1997). While CDK levels remain constant throughout the cell cycle, cyclins are unstable proteins that are synthesised and degraded throughout the cell cycle, thus generating waves of CDK activity (Fig 1.1) (Breeden, 2003; Tyers and Jorgensen, 2000). The conserved 100-residue cyclin domain, known as the cyclin box, is required for cyclin binding to CDKs

Figure 1.1

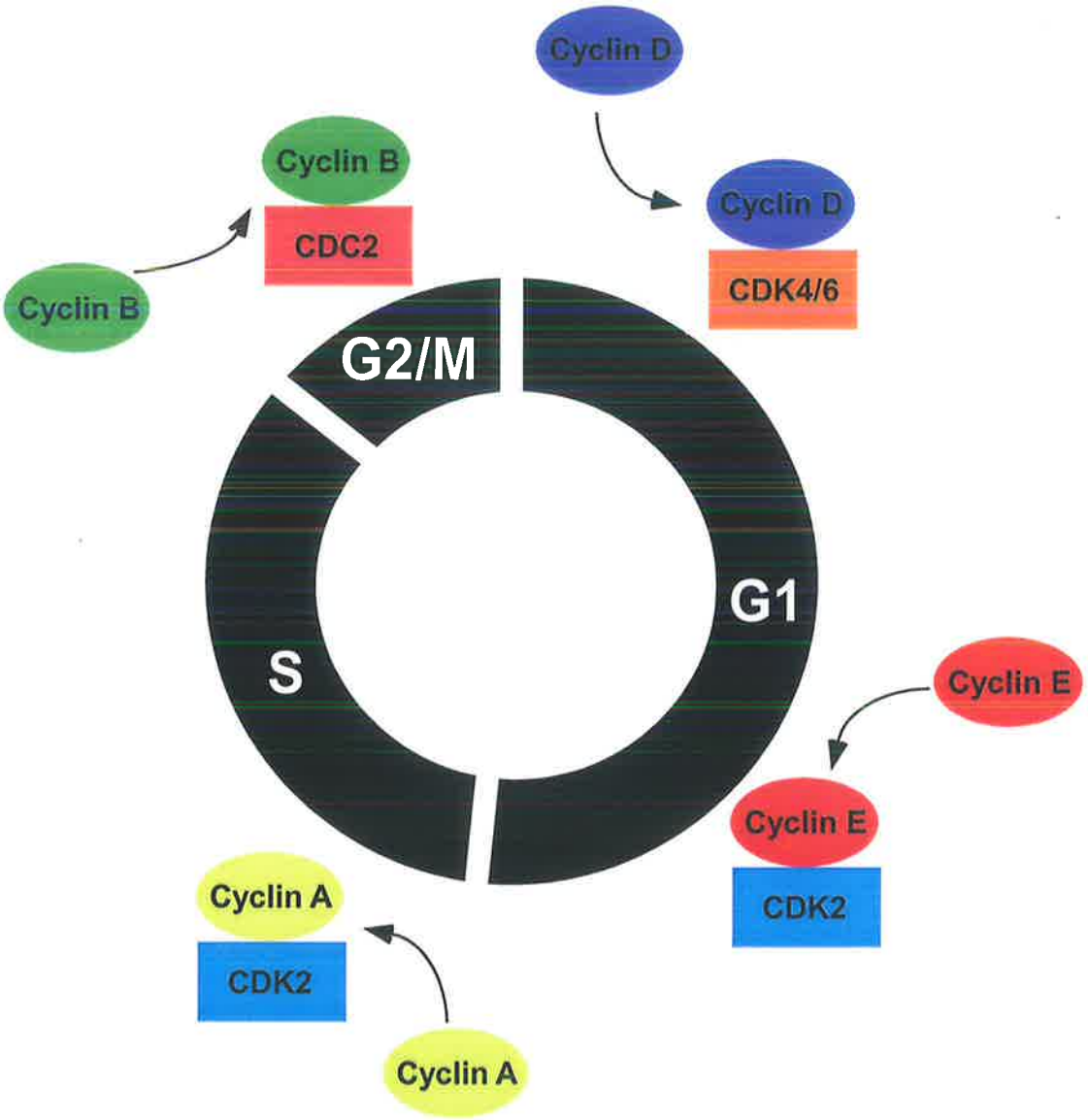


Figure 1.1 Regulation of cell cycle progression by the activity of cyclin dependent kinases (CDKs). The primary level of control of CDK activity is the specific association with cyclin subunits. CDK levels remain constant throughout the cell cycle. In contrast, cyclins are unstable proteins that are synthesised and degraded throughout the cell cycle, generating waves of CDK activity. Cyclin D-CDK4/6 and cyclin E-CDK2 complexes enable progression from G1 phase into S phase. The activity of cyclin A-CDK2 increases at the beginning of S phase. Progression into G2/M phase is associated with increased cyclin B-CDC2 activity.

(Horton and Templeton, 1997; Kobayashi *et al.*, 1992; Lees and Harlow, 1993). Upon cyclin binding, the CDK subunit undergoes a conformational change to its catalytically active form (Jeffrey *et al.*, 1995).

Regulation of cyclin availability occurs at many levels, such as transcription, localisation and degradation. The continued regulation of cyclin concentrations is essential. This is demonstrated by ectopic expression of cyclins causing premature entry into the next phase (Quelle *et al.*, 1993; Resnitzky *et al.*, 1994). Transcription of the D-type cyclins, cyclin D1, D2 and D3, is primarily regulated by extracellular signals transduced via the Ras/Raf-1/Mek/ERK pathway (Filmus *et al.*, 1994; Hitomi and Stacey, 1999; Lavoie *et al.*, 1996; Winston *et al.*, 1996). Cyclin D3 is also a target of E2F transcription factors (Ma *et al.*, 2003). Each of the D-type cyclins are differentially expressed in a tissue-specific manner, however, there is a high degree of functional redundancy as suggested by analysis of cyclin D nullizygous mice (Ciemerych *et al.*, 2002; Fantl *et al.*, 1995; Sicinski *et al.*, 1995). The timing of cyclin E expression in G1 phase is regulated by E2F transcription factors and their associated repressors (Botz *et al.*, 1996; Geng *et al.*, 1996; Le Cam *et al.*, 1999; Polanowska *et al.*, 2001). Cyclin A levels begin to increase from the beginning of S phase, however, while CREB, ATF-2, JunB, E2F and Sp1 have been implicated, the mechanism of *cyclin A* gene expression is not clearly defined (Andrecht *et al.*, 2002; Beier *et al.*, 2000; Desdouets *et al.*, 1995; Schulze *et al.*, 1995). The expression of B-type cyclins, from late S phase until mitosis, is regulated by the transcription factor NF-Y and the co-activator p300 (Bolognese *et al.*, 1999; Salsi *et al.*, 2003; Wasner *et al.*, 2003a; Wasner *et al.*, 2003b). In addition, c-myc has also been shown to regulate cyclin B1 expression (Farina *et al.*, 1996; Yin *et al.*, 2001).

Subcellular localisation also regulates cyclin availability for CDK binding. Cyclin D1 accumulates in the nucleus during G1 phase and is then shuttled to the cytoplasm during S phase (Baldin *et al.*, 1993). With no defined nuclear import or export signals and the dependence on phosphorylation for export, it is likely that cyclin D localisation is dependent on other proteins (Diehl *et al.*, 1998; Diehl and Sherr, 1997). Cyclin E is predominantly nuclear, entering the nucleus via the importin–alpha/beta heterodimer (Moore *et al.*, 1999; Ohtsubo *et al.*, 1995). Similarly, cyclin A concentrates in the nucleus, with its localisation signal overlapping with the ability of cyclin A to bind CDK (Jackman *et al.*, 2002; Maridor *et al.*, 1993; Pines and Hunter, 1991; Pines and Hunter,

1994). In contrast, cyclin B is cytoplasmic until just before nuclear envelope breakdown, when it is N-terminally phosphorylated and rapidly nuclear localised (Pines and Hunter, 1994; Yang *et al.*, 1998).

After CDK binding and activation, cyclins are degraded by the ubiquitin dependent-proteolytic machinery (Tyers and Jorgensen, 2000). Following S phase, D-type cyclins bound to CDK4/6 are inactivated by phosphorylation, leading to shuttling to the cytoplasm and ubiquitin dependent degradation (Baldin *et al.*, 1993; Diehl *et al.*, 1998; Diehl *et al.*, 1997). In contrast, ubiquitination and degradation of cyclin D1 that is not bound to CDK does not require phosphorylation (Germain *et al.*, 2000). Similarly, cyclin E degradation varies due to association with CDKs. When bound to CDK2, cyclin E is autophosphorylated and phosphorylated by other kinases, leading to dissociation of the complex and ubiquitin dependent degradation, via the Fbw7 pathway (Clurman *et al.*, 1996; Welcker *et al.*, 2003). In contrast, free cyclin E is not protected by association with CDK2 and is readily degraded by the proteasome, via the Cul-3 pathway (Clurman *et al.*, 1996; Singer *et al.*, 1999). While varying in the timing and mechanism, both A and B-type cyclins are degraded in an APC-dependent manner (Chang *et al.*, 2003; Morgan, 1999; Sorensen *et al.*, 2001).

1.2.2 Phosphorylation of cyclin dependent kinases

As for most kinases, the conformational changes required for CDK activity depends upon the phosphorylation of a threonine or tyrosine residue in the T loop (Marshall, 1994). The nuclear CAK phosphorylates the Thr-172 of CDK4, Thr-160 of CDK2 and Thr-161 of CDC2 increasing the affinity for ATP and substrate (Matsuoka *et al.*, 1994; Poon *et al.*, 1993). CDK2 is the only CDK shown to be phosphorylated by CAK in the absence of cyclin binding (Brown *et al.*, 1999; Fisher and Morgan, 1994; Kaldis *et al.*, 1998; Matsuoka *et al.*, 1994; Poon *et al.*, 1993). However, full activity of each CDK still requires cyclin binding (Jeffrey *et al.*, 1995; Russo *et al.*, 1996).

CDK activity is further regulated by inhibitory phosphorylations located in the active site of CDKs (Gu *et al.*, 1992; Krek and Nigg, 1991a; Krek and Nigg, 1991b). Complete inactivation of CDK4 and CDK2 involves phosphorylation on Tyr-17 and Tyr-15 respectively by Wee1 (Chow *et al.*, 2003; Gu *et al.*, 1992; Jinno *et al.*, 1999; Terada *et al.*, 1995; Watanabe *et al.*, 1995). At least for CDK2, this has been shown to be independent of cyclin binding (Coulonval *et al.*, 2003). In particular, inhibitory phosphorylation plays

an important role in controlling CDC2 activity in a cycling cell (Chow *et al.*, 2003). As cyclin B-CDC2 complexes form during S phase, the Tyr-15 and Thr-14 residues of CDC2 are phosphorylated by Wee1 and Myt1 (Booher *et al.*, 1997; Liu *et al.*, 1997a; Parker *et al.*, 1995; Watanabe *et al.*, 1995). These phosphorylations interfere with ATP binding and the ability of the γ phosphate of ATP to be transferred to the substrate (Endicott *et al.*, 1994). Three main phosphatases act in mammalian cells to remove the inhibitory phosphorylations. CDC25A is thought to be the regulator of G1/S phase CDK activity, since ectopic CDC25A expression causes premature activation of cyclin E-CDK2 and progression into S phase (Blomberg and Hoffmann, 1999; Vigo *et al.*, 1999). In comparison, CDC25B and CDC25C are key activators of CDC2 (Gabrielli *et al.*, 1996; Karlsson *et al.*, 1999; Lammer *et al.*, 1998; Millar *et al.*, 1991). While the role of CDC25B is confined to mitosis entry, CDC25C also plays a role in S phase entry (Karlsson *et al.*, 1999; Turowski *et al.*, 2003). The intricate systems that govern the activity of these inhibitory kinases and activating phosphatases provides the cell with another mechanism to tightly control cell cycle progression.

1.2.3 Cyclin dependent kinases inhibitors

Further control of CDK activity is provided by CDK inhibitors (CKIs). These proteins are the key transducers of checkpoint control mechanisms. Upon checkpoint activation, CKIs are expressed, leading to the inhibition of CDKs and cell cycle arrest. The mechanism of CKI action is dependent upon the cyclin-CDK complex and the CKI. Steric inhibition of CDK activity and blocking access to substrates and ATP are the main mechanisms (Pavletich, 1999). According to their structures and the CDK inhibited, the CKIs are divided into two families. The Ink4 family, which includes p16Ink4a, p15Ink4b, p18Ink4c, p19Ink4d, specifically inhibits cyclin D-CDK4/6 complexes. The Cip/Kip family, which includes p21Cip1, p27Kip1, p57Kip2, can inhibit all CDK complexes (Vidal and Koff, 2000). Ectopic expression of any CKI leads to G1 arrest in most cell types (Schreiber *et al.*, 1999; Thullberg *et al.*, 2000b). However, the expression patterns, regulation and activity of each CKI are different.

INK4 proteins are expressed in a cell-type specific manner and depending upon the stimulus. The G1 arrest induced by ectopic expression of the INK4 proteins is dependent on the presence of the retinoblastoma protein (RB) (Guan *et al.*, 1996; Lukas *et al.*, 1995b; Medema *et al.*, 1995; Thullberg *et al.*, 2000b). The best characterised INK protein is the tumour suppressor p16Ink4a (henceforth p16). p16 has been shown to be

frequently inactivated in human tumours (Kamb *et al.*, 1994) and p16-/- mice have an increased predisposition to tumour development (Serrano *et al.*, 1996). Anti-mitogenic signalling pathways induce p16 expression to induce senescence (Alcorta *et al.*, 1996; Passegue and Wagner, 2000; Zhu *et al.*, 1998). Similarly, transcriptional activation of p15Ink4b, in response to TGFβ in epithelial cells, induces cell cycle arrest (Hannon and Beach, 1994; Li *et al.*, 1995a; Li *et al.*, 1995b; Rich *et al.*, 1999). Both p18Ink4c and p19Ink4d are proposed to have roles in proliferating cells. E2F transactivation of p18Ink4c expression is thought to enable tight regulation of CDK4/6 activity (Blais *et al.*, 2002; DeGregori *et al.*, 1997). Similarly, cell cycle regulation of p19Ink4d transcription and protein abundance ensures cyclin D associated complexes are able to be active in G1 (Thullberg *et al.*, 2000a). INK4 proteins bind to the non-catalytic part of the kinase, leading to a conformational change that stops catalysis and weakens the binding to cyclins (Brotherton *et al.*, 1998; Jeffrey *et al.*, 2000; Russo *et al.*, 1998). Accordingly, most reports suggest that INK4 proteins compete with D-type cyclins for binding to CDK4 and CDK6, forming stable, inactive INK4-CDK complexes (Hall *et al.*, 1995; Jeffrey *et al.*, 2000).

The regulation and activity of the Cip/Kip family also varies in a cell-type, stimulus dependent manner. p21Cip1 is mostly regulated at the transcriptional level, but also at the level of mRNA stability (Macleod *et al.*, 1995). It is expressed in response to DNA damage (Dulic *et al.*, 1994; el-Deiry *et al.*, 1994), cytokines (Bottazzi *et al.*, 1999; Datto *et al.*, 1995) and differentiation (Cheng *et al.*, 2000b; Macleod *et al.*, 1995; Missero *et al.*, 1995). p27Kip1 levels are thought to be regulated primarily by post-transcriptional mechanisms. While translational mechanisms (Millard *et al.*, 1997) and subcellular localisation (Ishida *et al.*, 2002; Wang *et al.*, 1999) are involved, the best characterised control of p27Kip1 levels is by CDK-dependent proteolytic degradation (Ishida *et al.*, 2000; Malek *et al.*, 2001; Montagnoli *et al.*, 1999). Regulation of the levels of p27Kip1 is dependent upon a variety of anti-mitogenic signals, including contact inhibition (Polyak *et al.*, 1994), mitogen withdawal (Coats *et al.*, 1996), transforming growth factor-β (TGF-β) (Polyak *et al.*, 1994) and cAMP (Friessen *et al.*, 1997). Inhibition mediated by the Cip/Kip family requires the N-terminal inhibitory domain of the Cip/Kip to bind to the cyclin, subsequently altering the conformation of the CDK active site (Fotedar *et al.*, 1996; Hashimoto *et al.*, 1998). However, Cip/Kip proteins are inefficient inhibitors of

cyclin D-CDK complexes, as they appear to be involved in cyclin D-CDK4/6 complex formation (Cheng *et al.*, 1999; LaBaer *et al.*, 1997; Sugimoto *et al.*, 2002).

Interplay between the two families of CKIs enables further levels of control of CDK activity (Fig 1.2). The ability of p16 to inhibit cell cycle progression requires the inhibition of cyclin E-CDK2, which cannot be achieved directly by p16 (Jiang *et al.*, 1998; Mitra *et al.*, 1999). Redistribution of CKIs between cyclin-CDK complexes ensures inhibition of all CDK activity. Expression of p16 is associated with redistribution of p21Cip1 and p27Kip1 from CDK4/6 to CDK2 complexes (McConnell *et al.*, 1999; Mitra *et al.*, 1999). It is therefore suggested that cyclin E can bypass a p16 induced growth arrest by the number of cyclinE-CDK2 complexes exceeding that of Cip/Kip molecules (Alevizopoulos *et al.*, 1997; Jiang *et al.*, 1998).

1.3 Functions of cyclin dependent kinase activity

1.3.1 Cyclin D-CDK4/6

The fundamental role of cyclin D-CDK4/6 activity is the integration of extracellular signals into control of the cell cycle. Primarily this occurs at the transcriptional level with growth factor signals transduced via a Ras signalling cascade (Filmus *et al.*, 1994; Liu *et al.*, 1995; Winston *et al.*, 1996). However, Ras signalling pathways also govern cyclin D-CDK4 complex assembly (Cheng *et al.*, 1998) and cyclin D degradation (Diehl *et al.*, 1998). Cyclin D-CDK4/6 complexes are essential and rate-limiting for cell cycle progression in most cell types, demonstrated by experiments inhibiting cyclin D production or activity (Baldin *et al.*, 1993) and by the ectopic expression of cyclin D (Quelle *et al.*, 1993; Resnitzky *et al.*, 1994). The main proposed function of cyclin D-CDK4/6 activity is in the inactivation of the retinoblastoma protein (pRB). This is clearly demonstrated by the observation that cyclin D is dispensable in cells lacking pRB (Lukas *et al.*, 1995a; Medema *et al.*, 1995). Inactivation of pRB by cyclin D1-CDK4 enables gene expression by releasing E2F transcription factors (Harbour and Dean, 2000) and initiation of DNA replication by releasing MCM7 at replication origins (Gladden and Diehl, 2003; Sterner *et al.*, 1998). However, catalytic activity of CDK4 may not be essential. Overexpression of catalytically inactive CDK4 does not cause cell cycle arrest, since pRB is still inactivated by cyclin E-CDK2 activity (Jiang *et al.*, 1998; van den Heuvel and Harlow, 1993). This suggests a compensatory increase in other CDK complexes is equivalent to loss of CDK4/6 complexes. Similarly, when cyclin D1 is replaced with cyclin E in a mouse model and all developmental defects associated with

Figure 1.2

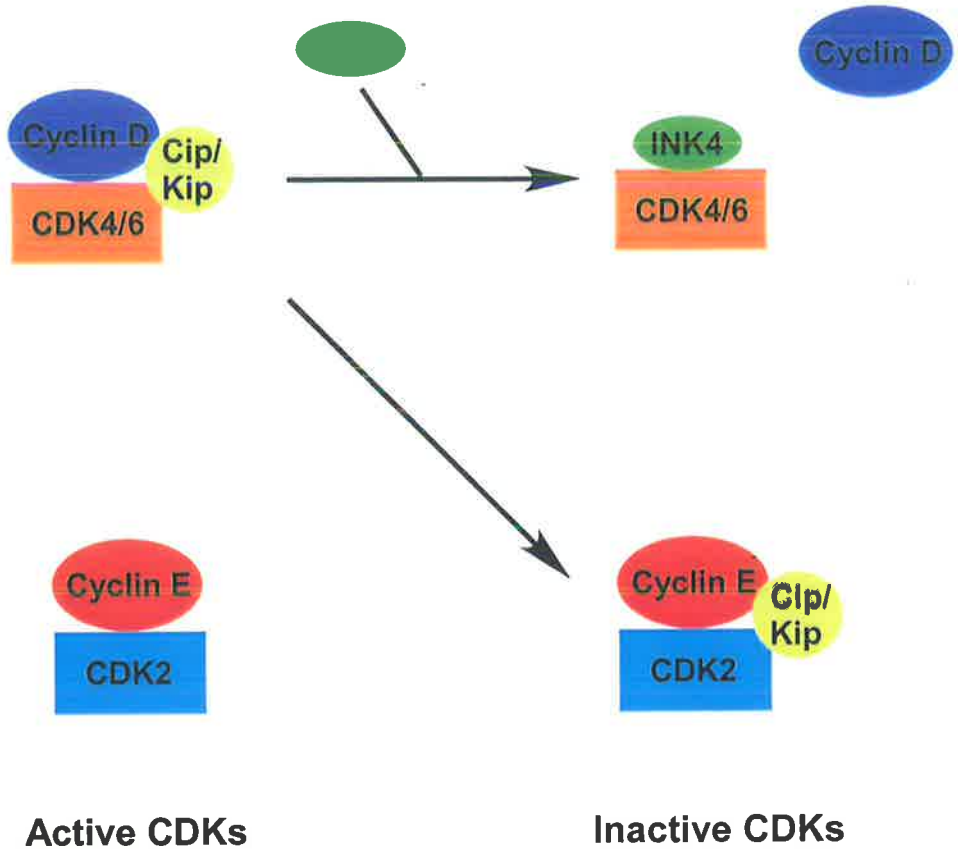


Figure 1.2 Redistribution of CKIs between cyclin-CDK complexes for inhibition of all CDK activity. Increased expression of INK4 proteins results in dissociation of Cip/Kip proteins from cyclin D-CDK4/6 complexes, formation of CDK4/6-INK4 complexes and cyclin D destabilisation. Association of Cip/Kip proteins with cyclin E-CDK2 complexes inhibits its kinase activity. (Adapted from Sherr and Roberts, 1999)

loss of cyclin D1 are rescued (Geng *et al.*, 1999). These observations suggest that it is mainly the presence of cyclin D-CDK4/6 complexes, not their catalytic activities that are required for G1 progression. This is likely to be related to the more recently recognised essential function of cyclin D-CDK4/6 complexes - CKI sequestration to enable CDK2 complex activation (McConnell *et al.*, 1999; Mitra *et al.*, 1999).

1.3.2 Cyclin E-CDK2

Activation of cyclin E-CDK2 is rate-limiting for the G1/S phase transition (Duronio *et al.*, 1996; Hua *et al.*, 1997; Krude *et al.*, 1997). The best characterised role of cyclin E-CDK2 is in the inactivation of pRB (Hatakeyama *et al.*, 1994; Hinds *et al.*, 1992; Kelly *et al.*, 1998; Lundberg and Weinberg, 1998). However, cyclin E-CDK2 is required in both pRB-positive and pRB-null cells (Ohtsubo *et al.*, 1995). Similarly, S phase entry does not often occur with overexpression of E2F transcription factors, as CDK2 activity is still required (Alevizopoulos *et al.*, 1997; Hofmann and Livingston, 1996). This suggests that cyclin E-CDK2 has roles that extend the regulation of the pRB/E2F pathway. These roles are distinct from those of cyclin D-CDK4/6 complexes, since ectopic co-expression of cyclin E and cyclin D1 has a combinatorial effect on the rate of cell cycle progression (Resnitzky *et al.*, 1994; Resnitzky and Reed, 1995).

The fact that cyclin E can induce S phase without activation of E2F transcription suggests cyclin E has essential targets involved in replication (Leng *et al.*, 1997; Lukas *et al.*, 1997). One such identified target is a component of the pre-replicative complex, CDC6 (Jiang *et al.*, 1999). Phosphorylation of CDC6 before the G1/S transition is essential for initiation of DNA replication (Herbig *et al.*, 2000). Cyclin E-CDK2 cooperates with CDC6 to promote formation of pre-replicative complexes (Coverley *et al.*, 2002; Furstenthal *et al.*, 2001). Also, the ability of CDC45 to bind chromatin and to load DNA polymerase onto chromatin is dependent on cyclin E-CDK2 (Arata *et al.*, 2000; Mimura and Takisawa, 1998). These targets delineate the direct role cyclin E-CDK2 plays in initiation of DNA replication. By stimulating activation of histone gene transcription via phosphorylation of NPAT, cyclin E-CDK2 is also involved in increasing the number of histones to enable chromatin packaging (Ma *et al.*, 2000; Zhao *et al.*, 1998; Zhao *et al.*, 2000). Cyclin E-CDK2 also binds and phosphorylates p300/CBP, transcriptional co-activators with intrinsic histone acetyltransferase (HAT) activity. The association leads to a peak of HAT activity in late G1 phase, enabling modification of chromatin structure at promoters and access for transcription factors (Ait-Si-Ali *et al.*, 1998). Modulation of the

chromatin state is also achieved by cyclin E-CDK2 binding and phosphorylating the SWI/SNF chromatin remodelling components, BRG1 and BAF155 (Shanahan *et al.*, 1999). While cyclin A-CDK2 appears to be more effective in centrosome duplication (Meraldi *et al.*, 1999), specific phosphorylation of nucleophosmin by cyclin E-CDK2 in late G1 is required for initiation of centrosome duplication (Okuda, 2002; Okuda *et al.*, 2000; Tokuyama *et al.*, 2001). Phosphorylation of E2F-5 by cyclin E-CDK2 stimulates E2F-5 transactivation by aiding recruitment of p300 (Morris *et al.*, 2000). Cyclin E-CDK2 can stimulate its own activity by binding and activating CDC25 phosphatase (Hoffmann *et al.*, 1994) and by triggering degradation of p27Kip1 (Sheaff *et al.*, 1997; Swanson *et al.*, 2000; Vlach *et al.*, 1997; Xu *et al.*, 1999). However, the roles of cyclin E in actively proliferating cells must be able to be carried out by other proteins as embryonic development can occur normally when deficient for both cyclin E isoforms or for CDK2 (Geng *et al.*, 2003; Ortega *et al.*, 2003; Parisi *et al.*, 2003). In contrast, cyclin E has an essential role in cell cycle reentry after quiescence (Geng *et al.*, 2003).

1.3.3 Cyclin A-CDK2

Numerous roles for the activity of cyclin A-CDK2 have been described. Cyclin A-CDK2 initiates DNA synthesis by the phosphorylation of unknown substrates after replication complexes are assembled (Coverley *et al.*, 2002). Also, the interaction between cyclin A-CDK2 complexes and Proliferating Cell Nuclear Antigen (PCNA) during DNA synthesis is thought to enable regulation of other factors, such as DNA ligase 1 (Koundrioukoff *et al.*, 2000). While involved in DNA synthesis, cyclin A-CDK2 also has an essential role in preventing reinitiation of replication. Disruption of complexes at replication origins occurs when CDC6 is phosphorylated by cyclin A-CDK2, leading to the translocation of CDC6 to the cytoplasm (Petersen *et al.*, 1999). Cyclin A-CDK2 phosphorylation of Mcm proteins, inactivates their DNA helicase activity and prevents their use in replication (Ishimi and Komamura-Kohno, 2001; Ishimi *et al.*, 2000). Similarly, DNA replication fork progression is regulated by the ability of cyclin A-CDK2 to bind to and phosphorylate Flap endonuclease 1 (Fen1) (Henneke *et al.*, 2003). Other roles are found in the regulation of mitotic entry and progression. Cyclin A-CDK2 phosphorylates the ubiquitin ligase Cdh1, inactivating APC-dependent degradation and enabling the accumulation of cyclin B1 (Sorensen *et al.*, 2001). Centrosome duplication is also regulated by cyclin A-CDK2 (Matsumoto *et al.*, 1999; Meraldi *et al.*, 1999). Prior to mitosis, phosphorylation of lamin B by cyclin A-CDK2 is thought to be required for nuclear envelope breakdown (Horton and Templeton, 1997). Also, cyclin A-CDK2

promotes DNA binding and transcriptional activation of Sp1 (Haidweger *et al.*, 2001). Similarly, B-myb transactivation potential is enhanced by cyclin A-CDK2 phosphorylation (Lane *et al.*, 1997; Sala *et al.*, 1997; Saville and Watson, 1998).

1.4 Regulation of G1/S phase progression

According to nutrient availability, cell size and cell density, cells continue through G1 phase. Progression from G1 into S phase normally requires the activation of a family of genes under control of E2F transcription factors. To enable E2F transactivation, the pocket protein family (pRB, p107, p130) of transcriptional repressors must be inactivated by the coordinated activity of cyclin D-CDK4/6 and cyclin E-CDK2. If the G1 checkpoint is activated by mitogen withdrawal or CKI expression, in response to DNA damage or anti-mitogenic signals, it is E2F transactivation that is targeted. CDK activity is inhibited, leading to the hypo-phosphorylation of the pocket protein family, repression of E2F transcription factors and no expression of many genes required for cell cycle progression.

1.4.1 E2F Transcription Factors

Seven members of the E2F transcription factor family have been identified in mammalian cells (E2F-1 to –7). The E2F proteins act as a heterodimeric complex with one of the DP family members (DP-1 and –2). The best characterised function of E2F transcription factors is in regulating the expression of many genes required for cell cycle progression, such as *cyclin E, cyclin A, cdc2, B-myb, DNA polymerase* α and *PCNA* (DeGregori *et al.*, 1995). However, recent microarray analysis suggests that the range of E2F regulated genes is much broader than previously thought, including genes involved in differentiation, development, mitosis, DNA damage and repair checkpoints and mitotic spindle checkpoints (Muller *et al.*, 2001; Polager and Ginsberg, 2003; Polager *et al.*, 2002; Ren *et al.*, 2002; Weinmann *et al.*, 2002).

Regulation of E2F activity

Expression of E2F transcription factors varies. E2F-1, -2 and –3 are expressed in a cell cycle regulated manner, with the peak of expression in mid-late G1 (Kaelin *et al.*, 1992; Leone *et al.*, 1998; Sears *et al.*, 1997; Slansky *et al.*, 1993). In comparison, E2F-4 and –5 and DP1 and DP2 expression does not vary significantly throughout the cell cycle (Bandara *et al.*, 1994; Ginsberg *et al.*, 1994; Moberg *et al.*, 1996). The specificity of expression of E2F proteins is dependent upon the cell-type, proliferation and differentiation (Kusek *et al.*, 2000).

Each of the E2F transcription factors are phosphoproteins, however, it is not entirely clear how phosphorylation regulates their activity. E2F-1 to –3 have a cyclin A binding domain, through which active cyclin A-CDK complexes can bind and phosphorylate E2Fs (Dynlacht *et al.*, 1994; Leone *et al.*, 1998; Xu *et al.*, 1994). Phosphorylation of these E2Fs by cyclin A-CDK complexes is associated with reduced DNA binding ability and subsequent decreased transactivation (Dynlacht *et al.*, 1994; Kitagawa *et al.*, 1995; Krek *et al.*, 1994). Cyclin E-CDK2 can also phosphorylate E2Fs, although DNA binding is not affected (Dynlacht *et al.*, 1994). E2F-4 and –5 do not have a cyclin A binding domain, although they can associate with cyclin A through p107 and p130 (Verona *et al.*, 1997; Woo *et al.*, 1997). However, cyclin A does not efficiently phosphorylate E2F-4 or influence its function (Dynlacht *et al.*, 1997). The protein responsible for and the function of the phosphorylation of E2F-4 and E2F-5 has not been identified (Ginsberg *et al.*, 1994; Vaishnav *et al.*, 1998). In contrast to phosphorylation, DNA binding and transactivation are increased by acetylation of E2F-1 (Martinez-Balbas *et al.*, 2000; Marzio *et al.*, 2000). While E2F-2 and –3 have also been shown to be acetylated, E2F-4 and –5 are not (Marzio *et al.*, 2000).

Nuclear localisation signals present within the sequences of E2F-1 to –3 enable these E2Fs to be constitutively nuclear (Allen *et al.*, 1997; Muller *et al.*, 1997; Verona *et al.*, 1997). In contrast, E2F-4 and –5 lack consensus nuclear localisation signals (de la Luna *et al.*, 1996), although an alternative N-terminal localisation signal has been proposed (Apostolova *et al.*, 2002). The nuclear localisation of E2F-4 and -5 is cell cycle regulated and mostly dependent upon binding to pocket proteins or DP2 (de la Luna *et al.*, 1996; Lindeman *et al.*, 1997; Magae *et al.*, 1996; Verona *et al.*, 1997). Both E2F-4 and –5 are exported from the nucleus via CRM1-mediated transport (Apostolova *et al.*, 2002; Gaubatz *et al.*, 2001). Subcellular localisation of these E2Fs enables a further mechanism of tightly controlling their activity.

Regulation of E2F protein abundance provides another level of control over E2F activity. E2F-1 and –4 are ubiquitinated near their C-terminus and degraded by proteasome machinery. These unstable proteins are protected from degradation when bound to pocket proteins (Campanero and Flemington, 1997; Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). Degradation of E2F-1 is linked to binding of Skp2, thus likely to be degraded via this

SCF pathway (Marti *et al.*, 1999). The signals for degradation have not been identified, however, dissociation from pocket proteins may be sufficient as turnover of "free" E2Fs ensures no excess of expression of E2F target genes.

E2F transactivation and repression

The strong, C-terminal transactivation domain of E2Fs is combined with different mechanisms by which E2Fs activate transcription depending upon the promoter, cell type and E2F. Binding of the E2F/DP heterodimer to promoters alters the DNA structure, bending the DNA to promote transcriptional activation (Cress and Nevins, 1996; Huber *et al.*, 1994). Differences in the ability of E2F/DP heterodimers to bend DNA and distinct properties of E2F sites within promoters suggests that the effects on target gene expression will vary (Tao *et al.*, 1997). E2Fs are also able to directly interact with basal transcription factors, TFIIH and TBP (Emili and Ingles, 1995; Hagemeier *et al.*, 1993; Pearson and Greenblatt, 1997; Ross *et al.*, 1999). This association is not required for E2F transactivation but it does enhance the level of transcription (Pearson and Greenblatt, 1997). Similarly, the transcription factor Sp1 can bind to and enhance the transcriptional activity of E2F-1 to –3, but not E2F-4 and –5 (Karlseder *et al.*, 1996; Lin *et al.*, 1996). E2Fs have also been shown to bind transcriptional co-activators CBP and p300, leading to a subsequent increase in E2F transactivation (Lee *et al.*, 1998a; Trouche *et al.*, 1996). The role of E2F in transactivation is clear. Ectopic expression of E2F-1 to –4 in quiescent cells induces aberrant expression of many E2F target genes and ectopic DNA replication (DeGregori *et al.*, 1997). Also, mouse embryonic fibroblasts (MEFs) isolated from E2F-3 knockout mice and MEFs lacking E2F-1 to –3 have reduced E2F target gene expression and severe proliferative defects (Humbert *et al.*, 2000b; Wu *et al.*, 2001). Similarly, inducible expression of an E2F-1 mutant, which binds DNA but has no transactivation domain, reduces E2F target gene expression and the number of S phase cells (Fan and Bertino, 1997). Also, DNA binding mutants of DP proteins sequester E2Fs in transcriptionally inactive complexes and halt S phase progression (Wu *et al.*, 1996). Therefore, in a cell-type dependent manner, E2Fs act as transcriptional activators.

However, it is often difficult to distinguish between the relative importance of E2F transactivation and repression. As cells exit the cell cycle or a checkpoint is activated, E2Fs associate with pocket proteins and repress target gene promoters. Importantly, checkpoints, activated in response to p16Ink4a, TGFβ or contact inhibition, require pRB-E2F complexes to arrest in G1, as expression of a mutant E2F-1, without the ability to

transactivate or bind pRB, leads to derepression of E2F target genes and no checkpoint arrest (Zhang *et al.*, 1999). This suggests that active repression of E2F target gene promoters in quiescence may transcend the transactivation functions of E2F. The importance of E2F-mediated repression of target genes in cycling cells is clearly evident in E2F-1 knockout mice, which are characterised by hyperproliferation of the thymus and tumour formation (Field *et al.*, 1996; Yamasaki *et al.*, 1996). However, the relative functions of E2Fs as transactivators or repressors of transcription in cycling cells is still reasonably controversial. This is mainly due to the fact that most research has been conducted upon cells re-entering the cell cycle after quiescence. Distinguishing between transactivation and repression is also difficult as multiple E2F complexes can act on promoters and there are many other transcription factor binding sites within promoters of E2F target genes.

It is commonly thought that E2F-1 to –3 are transactivators, whereas E2F-4 and –5 are repressors. However, this is mainly derived from analysis of quiescent populations. p130-E2F-4/5 complexes accumulate in response to mitogen deprivation (Moberg *et al.*, 1996; Tommasi and Pfeifer, 1997; Zwicker *et al.*, 1996) or TGF-β stimulation (Iavarone and Massague, 1999; Yagi *et al.*, 2002). Similarly, either E2F-4 or E2F-5 are required for cells to arrest in response to p16Ink4a (Gaubatz *et al.*, 2000). Analysis of E2F target gene promoters by chromatin immunoprecipitation (ChIP) assays also supports this hypothesis. When serum starved cells were restimulated, E2F-4 levels decreased on each promoter with concomitant increases in other E2Fs and subsequent gene expression (Takahashi *et al.*, 2000; Wells *et al.*, 2000). However, ChIP assays also show that in normal cycling cells E2F-4 is found on all promoters analysed and E2F-1, -2, -3 and -5 on the majority of promoters analysed (Takahashi *et al.*, 2000; Wells *et al.*, 2000). In particular, in cycling cells E2F-4 may activate transcription of certain genes as it is bound to promoters in late G1/S (Wells *et al.*, 2000). The ability of E2F-4 to act as a transactivator is supported by reporter assays and its ability to promote growth when ectopically expressed in cycling cells (Beijersbergen *et al.*, 1994b; Ginsberg *et al.*, 1994). Also, ectopic expression of E2F-4 in stratified epithelial tissue in the mouse is as effective as E2F-1 at inducing proliferation (Wang *et al.*, 2000). The association of E2F-4 with the acctyltransferase GCN5 stimulates transcriptional activation (Lang *et al.*, 2001) and in other cell types an E2F-4/5-p300 association stimulates transcription (Morris *et al.*, 2000; Yagi *et al.*, 2002),

suggests that the role of E2F-4 and –5 in transcriptional activation may be more prevalent than previously thought.

1.4.2 The pocket protein family

The tumour suppressor protein pRB has two identified structural and functional homologues, p107 and p130. Functional similarities are most evident with each of the pocket proteins binding to E2F and inhibiting E2F-responsive promoters (Chellappan *et al.*, 1991; Cobrinik *et al.*, 1993; Flemington *et al.*, 1993; Vairo *et al.*, 1995; Zamanian and La Thangue, 1993). However, p107 and p130 appear more similar to each other than pRB as demonstrated by analysis of MEFs isolated from knockout mice. p107-/- or p130-/- MEFs do not have deregulated expression of E2F target genes, whereas pRB-/- and p107-/-/p130-/- cells do (Hurford *et al.*, 1997). Similarities are also found in the mode of regulation of activity of pocket proteins. The phosphorylation status of the pocket proteins varies in a cell cycle dependent manner, enabling timely derepression of E2F transcription factors (Beijersbergen *et al.*, 1995; DeCaprio *et al.*, 1992; Mayol *et al.*, 1996; Suzuki-Takahashi *et al.*, 1995; Xiao *et al.*, 1996; Zhu *et al.*, 1995b). Accordingly, when overexpressed each can cause cell cycle arrest (Claudio *et al.*, 1994; Hinds *et al.*, 1992; Starostik *et al.*, 1996; Zhu *et al.*, 1993). However, there are differences in the response to ectopic expression in a cell-type, cell-context dependent manner, suggesting they have variant functions.

Inhibition of E2F activity

Different modes of inhibition of E2F-mediated transcription by pocket proteins occur depending on the cell type and promoter (Fig 1.3). Both the pocket domain and the C-terminus of the pRB family are involved in E2F binding (Huang *et al.*, 1992; Lee *et al.*, 1998b). By binding to the transactivation domain of E2F (Helin *et al.*, 1993), repression of transcription may occur by inhibiting the association with basal transcription machinery, without the presence of other repressors (Ross *et al.*, 1999). Binding of pocket proteins is also suggested to inhibit the ability of the E2F/DP heterodimer to bend DNA, therefore decreasing its transactivation ability (Cress and Nevins, 1996; Huber *et al.*, 1994). Pocket proteins can also actively inhibit E2F-mediated transcription by recruiting chromatin remodelling complexes. Histone deacetylases (HDACs) facilitate the removal of acetyl groups from core histones, promoting nucleosome formation, thus inhibiting transcription due to an inability of transcription factors to access DNA. HDAC associates with the pocket region, via a LxCxE-like motif (Brehm *et al.*, 1998; Ferreira *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998). Pocket proteins are able to

Figure 1.3

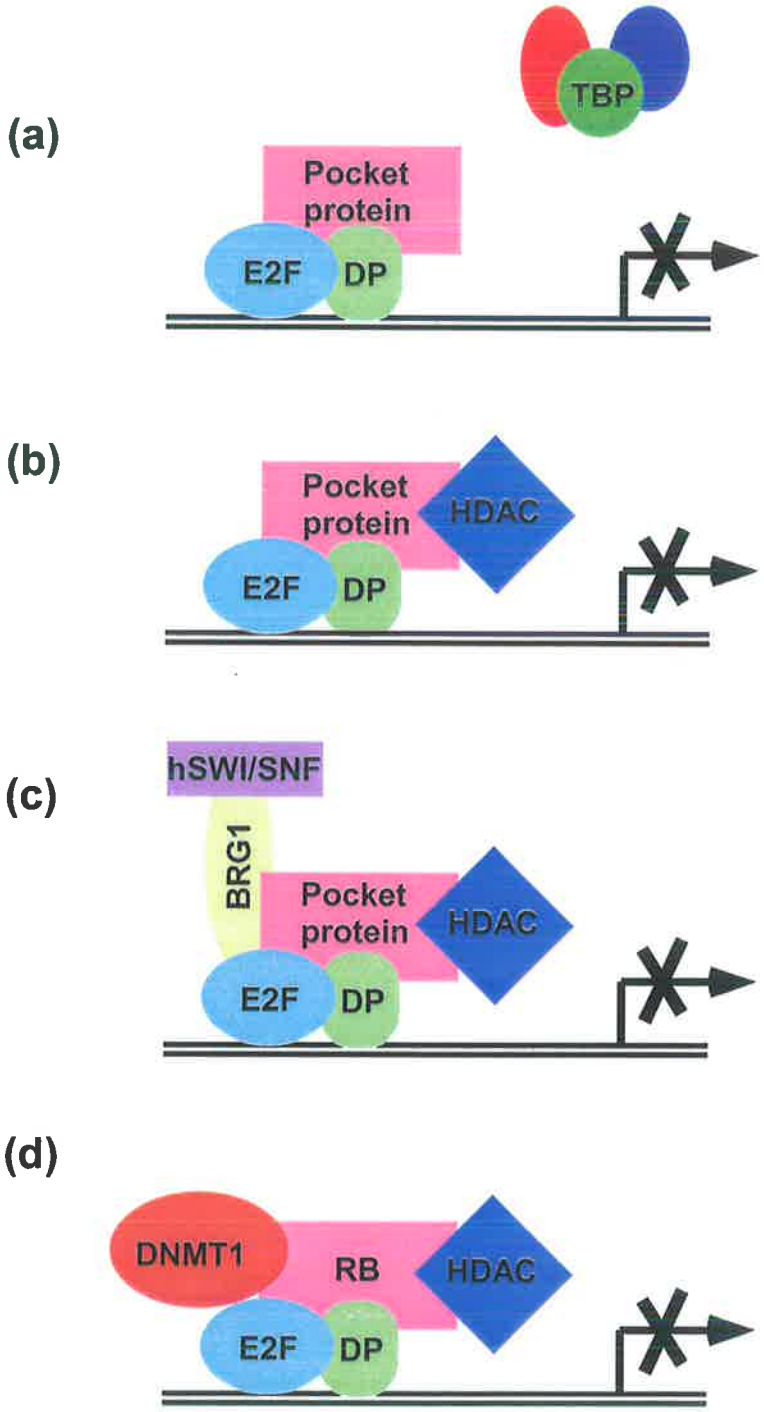


Figure 1.3 Different modes of inhibition of E2F transcription by pocket proteins. (a) Binding of pocket proteins to the E2F transactivation domain is proposed to inhibit association with basal transcription machinery and inhibit the ability of the E2F/DP heterodimer to bend DNA. (b) Pocket proteins recruit histone deacetylase activity (HDACs) to E2F target gene promoters for active repression of transcription. (c) Pocket proteins recruit both SWI/SNF and HDAC to actively inhibit E2F-dependent transcription by chromatin remodelling. (d) pRB recruits the DNA methyltransferase DNMT1, resulting in promoter methylation and repression E2F-dependent transcription, aided by HDAC activity.

simultaneously bind HDACs and E2Fs, thus enabling the recruitment of histone deacetylase activity to promoters of E2F-regulated target genes for active repression of transcription (Brehm *et al.*, 1998; Ferreira *et al.*, 1998; Luo *et al.*, 1998; Magnaghi-Jaulin *et al.*, 1998; Rayman *et al.*, 2002). ATP-dependent chromatin remodelling complexes, such as SWI/SNF, alter chromatin structure by changing the location or conformation of the nucleosome. pRB interacts with the SWI/SNF complex via association with the ATPase components BRM and BRG1 (Dunaief *et al.*, 1994) and is simultaneously able to interact with E2F (Trouche *et al.*, 1997). Expression studies suggest that BRG1 is important for inhibition of E2F-mediated transcription and for pRB induced growth arrest (Dunaief *et al.*, 1994; Strobeck *et al.*, 2000; Zhang *et al.*, 2000). Both p107 and p130 are also able to associate with BRG1 (Strober *et al.*, 1996), although the cellular function of this interaction is yet to be investigated. pRB can also recruit the DNA methyltransferase DNMT1, or SUV39H1 histone methylase to aid repression of E2F transcription by methylation of promoters (Nielsen *et al.*, 2001; Robertson *et al.*, 2000).

In normal cells, inactivation of pRB involves sequential phosphorylation by cyclin D-CDK4/6 then cyclin E-CDK2 complexes (Harbour *et al.*, 1999; Lundberg and Weinberg, 1998). This enables relief of direct pRB repression of E2F transcription as well as relief of active transcriptional repression for G1/S phase progression (Fig 1.4). Upon cyclin D-CDK4/6 phosphorylation of pRB, HDAC is released from pRB and repression of *cyclin E* transcription is alleviated (Bremner *et al.*, 1995; Harbour *et al.*, 1999; Lundberg and Weinberg, 1998; Zhang *et al.*, 2000). HDAC activity is not required to repress the transcription of S phase genes, *cyclin A* and *cdc2*, however BRG1 is required for their repression (Strobeck *et al.*, 2000; Zhang *et al.*, 2000). Thus, pRB-SWI/SNF is likely to remain bound to and actively repress *cyclin A* and *cdc2* promoters (Zhang *et al.*, 2000). Phosphorylation of pRB and BRG1 by cyclin E-CDK2 relieves active repression and enables transcription of other E2F-target genes required for cell cycle progression (Bremner *et al.*, 1995; Harbour *et al.*, 1999; Lundberg and Weinberg, 1998; Shanahan *et al.*, 1999; Zhang *et al.*, 2000). This tightly controlled switch between transcriptional repression and activation ensures the temporal order of cyclin expression is maintained. Similarly, binding of p130 to E2F is regulated by both CDK4/6 and CDK2 activity (Cheng *et al.*, 2000a). In contrast, release of E2F from p107 requires phosphorylation by CDK4/6 (Beijersbergen *et al.*, 1995; Cheng *et al.*, 2000a; Leng *et al.*, 2002; Xiao *et al.*, 1996).

Figure 1.4

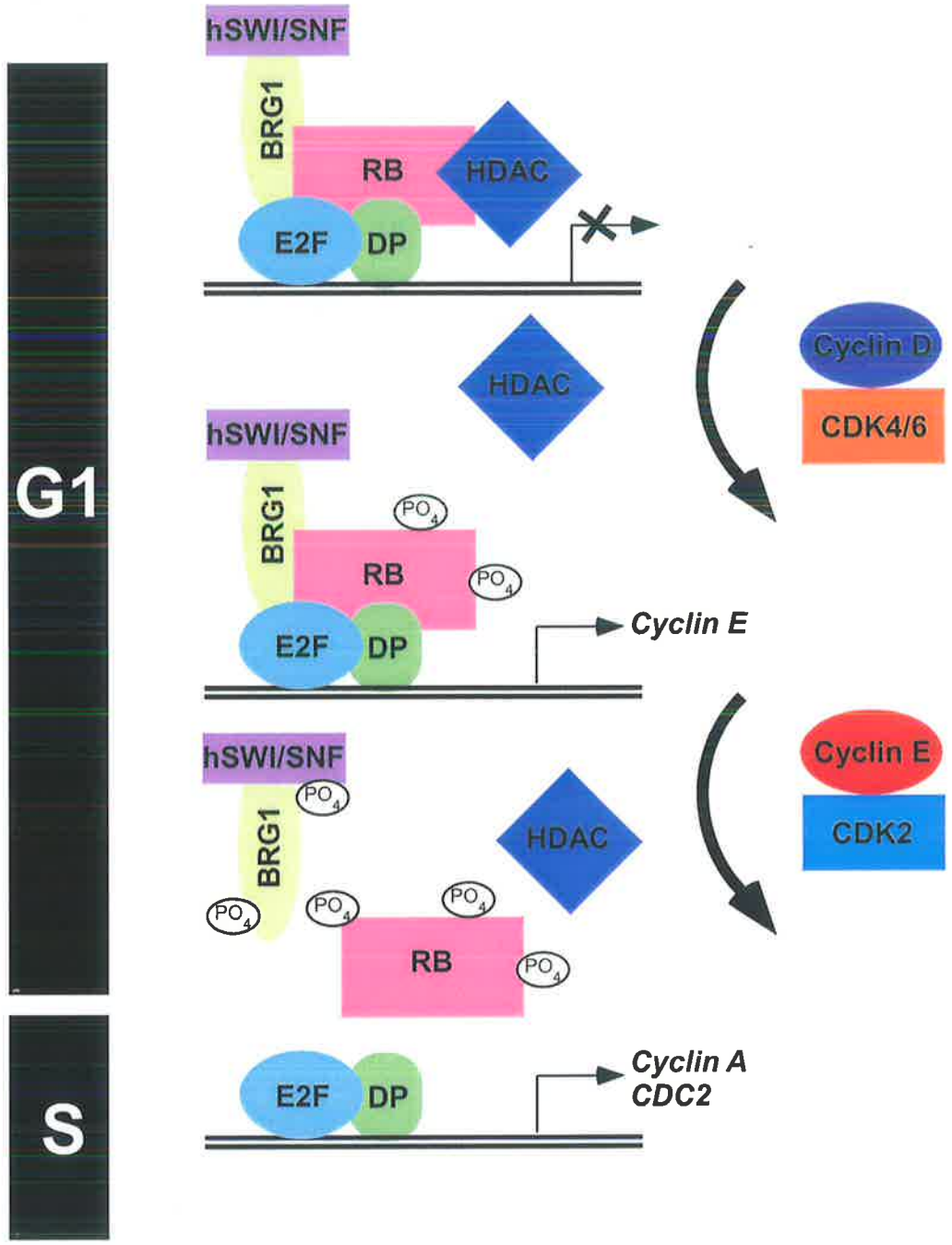


Figure 1.4 Regulation of E2F-mediated transcription by pocket proteins. Early in G1, E2F target gene promoters are actively repressed by the HDAC-pRB-SWI/SNF complex. Phosphorylation of pRB by cyclin D-CDK4/6 releases HDAC and enables *cyclin E* transcription. Other E2F target gene promoters, such as cyclin A and CDC2, are still repressed. Phosphorylation of pRB and BRG1 by cyclin E-CDK2 releases E2F-DP complexes and enables transcription of *cyclin A* and *CDC2*.

The relative roles of the pocket proteins in cycling cells and in quiescence is difficult to determine. Each of the pocket proteins appear to have redundant roles in both cycling and quiescent cells, as demonstrated by analysis of pRB-/-;p107-/-;p130-/- cells (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). However, analysis of pocket proteins binding to target gene promoters by ChIP assays suggests that in a normal cycling cell p107 and p130 are the main regulators of E2F activity (Takahashi *et al.*, 2000; Wells *et al.*, 2000). While p107 and p130 preferentially bind E2F-4 and –5 (Hijmans *et al.*, 1995; Sardet *et al.*, 1995), each of the other E2Fs are found on promoters in normal cycling cells (Takahashi *et al.*, 2000; Wells *et al.*, 2000), suggesting that p107 and p130 may be important in normal cycling cells to regulate all E2F activity. In contrast to analysis of cycling cells, pRB binds to *cyclin E* and *cdc2* promoters as cells are restimulated after serum deprivation (Wells *et al.*, 2000). This corresponds with deregulated *cyclin E* expression in pRB-/- MEFs upon cell cycle re-entry (Hurford *et al.*, 1997). These observations are concurrent with more recent suggestion that the role of pRB in control of E2F activity may be more relevant in senescing cells or during terminal differentiation (Dahiya *et al.*, 2001; Thomas *et al.*, 2001). This is supported by the observation that pRB, but not p107, can induce accumulation and phosphorylation of p130 (Jiang *et al.*, 2000). While pRB can be found in quiescent and cycling cells, p130 is highly expressed in quiescent and differentiated cells and only at lower levels in some cycling cells (Chen *et al.*, 1989; Cobrinik *et al.*, 1993; DeCaprio *et al.*, 1989; Garriga *et al.*, 1998; Smith *et al.*, 1996). In comparison, p107 is mainly expressed in proliferating tissues, such as the spleen and thymus (Garriga *et al.*, 1998).

E2F-independent functions of pocket proteins

A function of p107 and p130 not shared by pRB appears to be in the binding of cyclin E-CDK2 and cyclin A-CDK2 complexes (Adams *et al.*, 1996; Ewen *et al.*, 1992; Lacy and Whyte, 1997; Zhu *et al.*, 1995b). The precise function of this association is unknown, although titration of these active cyclin-CDK complexes is particularly important in growth suppression by p107 (Zhu *et al.*, 1995a). p107 has also been shown to bind to the N-terminal transactivation domain of c-Myc and inhibit its transcriptional activation (Beijersbergen *et al.*, 1994a; Gu *et al.*, 1994; Hoang *et al.*, 1995). The biological function of this interaction is unknown, although it is suggested that it is involved in p107 growth suppression. Another transcription factor regulated by p107 is B-myb – at the transcriptional level (Catchpole *et al.*, 2002; Hurford *et al.*, 1997; Lam *et al.*, 1995) and functional activity (Joaquin *et al.*, 2002; Sala *et al.*, 1996b). The direct interaction

between B-myb and p107 also regulates p107 function, since ectopic B-myb expression overcomes a p107-induced growth arrest (Bessa *et al.*, 2001; Joaquin *et al.*, 2002; Sala *et al.*, 1996a; Sala *et al.*, 1996b). This interaction inhibits B-myb transcriptional activity (Sala *et al.*, 1996b). Similarly, both pRB and p107 bind Sp1 and inhibit its transcriptional activity (Datta *et al.*, 1995). The association between pocket proteins and MCM7 is thought to prevent premature initiation of DNA replication, as suggested by the timely binding of pRB to MCM7 at replication origins (Gladden and Diehl, 2003; Sterner *et al.*, 1998). Also, p130 and pRB regulate rRNA synthesis by binding upstream binding factor (UBF) and repressing transcription of RNA polymerase 1 (Ciarmatori *et al.*, 2001; Hannan *et al.*, 2000). pRB is also implicated in the cell cycle regulation of histone gene expression (Gupta *et al.*, 2003).

Pocket proteins play an important role in the coordination of terminal cell cycle exit and differentiation. Partial regulation of this function is due to the association of pocket proteins with Id2, an inhibitor of terminal differentiation (Iavarone *et al.*, 1994; Lasorella *et al.*, 1996). During muscle terminal differentiation, hypophosphorylated pRB or p130 binds HBP1 and transcriptionally represses N-myc and cyclin D1 leading to cell cycle exit (Lavender *et al.*, 1997; Tevosian *et al.*, 1997). This allows Myf5 to activate transcription of MyoD and myogenin (Shih *et al.*, 1998). Skeletal myogenesis also involves pRB binding to and aiding transcriptional activity of MyoD and myogenin (Gu *et al.*, 1993; Schneider *et al.*, 1994). Loss of pRB leads to compensation by p107, however, p107 cannot maintain the terminally differentiated state (Schneider *et al.*, 1994). This highlights important differences between the pocket proteins. Roles for pRB have been implicated in neuronal differentiation, due to binding NRP/B (Wiggan *et al.*, 1998), and in adipocyte differentiation, due to binding and regulation of transcription factors C/EBP and NF-IL6 (Chen *et al.*, 1996a; Chen *et al.*, 1996b). The pocket proteins may also be involved in cell fate determination, since they are able to bind to a family of transcription factors containing paired-like homeodomains, Mhox, Chx10 and Pax-3 (Wiggan *et al.*, 1998). Transcriptional activity of Pax-3 is directly repressed by pRB (Wiggan *et al.*, 1998). Similarly, association of Pax-5 and Pax-6 with pRB is thought to regulate their transcriptional activity (Cvekl *et al.*, 1999; Eberhard and Busslinger, 1999). This suggests that the pocket proteins may integrate cell cycle regulation with developmental cues controlling tissue specific gene expression.

1.5 Deregulation of the cell cycle in cancer

Genomic instability is fundamental to the cellular transformation associated with cancer. Primary to the development of cancer is the loss of growth control. This is in part due to the propensity of oncogenes to mimic normal growth signals. However, this can also be attributed to abnormalities in one or more cell cycle components, enabling tumour cells to evade anti-proliferative signals (Hanahan and Weinberg, 2000). In particular, deregulation of the cyclin-CDK/pRB/INK4 pathway occurs in almost all human tumours (Carnero, 2002).

Overexpression of cyclin E and increased associated kinase activity, detected in many tumour types, is mainly due to gene amplification and increased protein accumulation (Gray-Bablin *et al.*, 1996; Schraml *et al.*, 2003). Also, while mutations in the p27Kip1 gene are rare, p27Kip1 is inactivated in many human tumours due to decreased expression, increased degradation and/or nuclear exclusion (Slingerland and Pagano, 2000). The consequence is deregulated CDK2 activity, which bypasses the requirement for cyclin D activities and inactivating pRB (Geng *et al.*, 1999; Gray-Bablin *et al.*, 1996; Jiang *et al.*, 1998; Nielsen *et al.*, 1997). However, since cyclin E can bypass the pRB/E2F pathway (Leng *et al.*, 1997; Lukas *et al.*, 1997) and cyclin E-CDK2 has many targets involved in DNA replication (Arata *et al.*, 2000; Coverley *et al.*, 2002; Jiang *et al.*, 1999; Mimura and Takisawa, 1998), cyclin E overexpression in tumour cells is likely to promote proliferation beyond its involvement in pRB/E2F regulation.

Similar to cyclin E, gene amplification or alteration of cyclin D1 and cyclin D2 has been observed in many human tumours (Sellers and Kaelin, 1997; Sherr, 1996). Also, a CDK4 mutant, refractory to p16 binding, has been isolated from hereditary melanoma (Wolfel *et al.*, 1995; Zuo *et al.*, 1996). This mutant leads to multiple tumour development when expressed in mouse models (Sotillo *et al.*, 2001). Aberrant CDK4/6 activity observed in cancer cells is also due to the prolific loss of INK4 proteins through deletion, mutation and/or promoter methylation (Ortega *et al.*, 2002). p16 inactivation is the most common, occurring in approximately 80% of cases in certain tumour types (Caldas *et al.*, 1994). These alterations can be inherited, as in familial melanoma (Hussussian *et al.*, 1994; Kamb *et al.*, 1994) or occur somatically in sporadic tumours (Caldas *et al.*, 1994). This is consistent with the spontaneous tumours observed at a young age in p16-/- mice (Serrano *et al.*, 1996). Each of these alterations result in the hyperphosphorylation and functional

inactivation of pRB, anomalous E2F transactivation and aberrant proliferation. However, genetic mutation of the tumour suppressor pRB is also very common in human cancer (Halaban, 1999; Kaye, 2002). In contrast mutations in p107, p130 and E2Fs are rare (Classon and Dyson, 2001; Claudio, 2000; Helin *et al.*, 1997; Mulligan *et al.*, 1998; Sellers and Kaelin, 1997).

1.6 The cell cycle and embryogenesis

Early embryonic development of many organisms is characterised by exceptionally short cell cycles. The early cell cycles of *Danio rerio* (zebrafish) are 15 minute cycles of consecutive S and M phases (Kimmel *et al.*, 1995; Kimmel and Law, 1985). As gastrulas develop, the constitutive cyclin E-CDK2 activity of these early cycles is downregulated and cell division rates decrease (Kane and Kimmel, 1993; Kane *et al.*, 1992; Yarden and Geiger, 1996). Post fertilization, the cell cycle length in *Xenopus Laevis* is approximately 30 minutes and characterised by consecutive S and M phases (Newport and Kirschner, 1982; Newport and Kirschner, 1984). Regulation of these cell cycles is dependent upon the periodic expression and degradation of B type cyclins and their association with cdc2 (Hartley *et al.*, 1996; Murray and Kirschner, 1989). During this time, cyclin E protein levels remain constant, but cyclin E-CDK2 activity oscillates (Hartley *et al.*, 1996; Rempel *et al.*, 1995). At the midblastula transition (MBT), maternal transcripts are degraded, including cyclin E, cyclin E-CDK2 activity is downregulated and gap phases are incorporated into the cell cycle (Hartley *et al.*, 1996; Hartley *et al.*, 1997; Howe *et al.*, 1995; Rempel *et al.*, 1995).

Similarly the first 8 cycles in *Drosophila* embryogenesis consist of only S and M phases. These are extraordinarily rapid, with an approximate duration of 9 minutes, and are controlled by maternal pools of cyclin-cdc2 complexes (Edgar and Schubiger, 1986; Edgar *et al.*, 1994; Foe and Alberts, 1983; Stiffler *et al.*, 1999). When the maternal transcript pools are depleted at mitosis 14, the cell cycle slows to 17.5 minutes and a G2 phase of variable length is observed (Edgar and O'Farrell, 1990; Reed, 1995). At this time, zygotic *cyclin E* expression is not cell cycle regulated (Richardson *et al.*, 1993). Then after mitosis 16, upon establishment of G1 phase, regulation of *cyclin E* expression and cyclin E-CDK2 activity becomes essential (Knoblich *et al.*, 1994; Li *et al.*, 1999; Richardson *et al.*, 1995; Secombe *et al.*, 1998). It is thought that CDK1 and CDK2 complexes are essential for *Drosophila* embryogenesis, whereas CDK4 complexes are dispensable (Meyer *et al.*, 2000). *Drosophila* CDK activity appears to be regulated

similarly to mammalian cells, with reported regulation at the level of expression and degradation of cyclins, phosphorylation of CDKs and CDK inhibitors reported (Knoblich *et al.*, 1994; Lane *et al.*, 1996; Richardson *et al.*, 1993; Sauer *et al.*, 1995; Sprenger *et al.*, 1997).

1.6.1 Early mouse embryogenesis

Precisely regulated events characterise mouse embryonic development. In contrast to *Drosophila* and *Xenopus*, the first few cell divisions of the mouse embryo are very slow, averaging 16 hours. Each of these cell cycles is characterised by four phases, although the length of the gap phases is extremely short (1-2 hours) (Bolton *et al.*, 1984; Chisholm, 1988; Howlett and Bolton, 1985; Smith and Johnson, 1986). Activation of zygotic transcription is also much earlier and is concurrent with most murine maternal transcripts being degraded at the second cell division (Clegg and Piko, 1983; Flach *et al.*, 1982; Giebelhaus *et al.*, 1983; Howlett and Bolton, 1985). Following division into 8 cells, the embryo compacts, resulting in outer cells differentiating into extraembryonic trophectoderm surrounding a pluripotent inner cell mass (ICM) (Gardner, 1983; Sutherland and Calarco-Gillam, 1983) (Fig 1.5). Pluripotence is defined by the ability to contribute to all embryonic and adult cell lineages (Hogan, 1994) (Fig 1.6).

After formation of the blastocoelic cavity, at approximately 4.0 days *post coitum* (dpc), ICM cells adjacent to the cavity differentiate into primitive endoderm, which later forms extraembryonic tissues (Fig 1.5). Implantation of the embryo into the uterine wall (4.5 - 5.0 dpc) coincides with a dramatic change in the rates of proliferation. The 20-25 ICM cells of the pre-implantation embryo increase to approximately 660 cells by 6.5dpc, with an average cell cycle time of 10 hours (Snow, 1977). These cell cycles are characterised by a very short G1 phase (Solter *et al.*, 1971; Stead *et al.*, 2002). During this period of rapid proliferation, the proamniotic cavity is established when inner pluripotent cells undergo apoptosis (Coucouvanis and Martin, 1995). The outer pluripotent cells, the primitive ectoderm, are reorganised to form a pseudo-stratified epithelium (Snow, 1977). Pluripotent cells of the post-implantation embryo are restricted, compared to those from the pre-implantation embryo, in that they do not differentiate or contribute to development following blastocyst injection (Brook and Gardner, 1997; Gardner, 1971). However, heterotopic transplantation of primitive ectoderm cells into 6.5dpc embryos demonstrates their pluripotent nature (Parameswaran and Tam, 1995; Tam and Zhou, 1996). The primitive endoderm cells bordering the pluripotent cells form the visceral

Figure 1.5

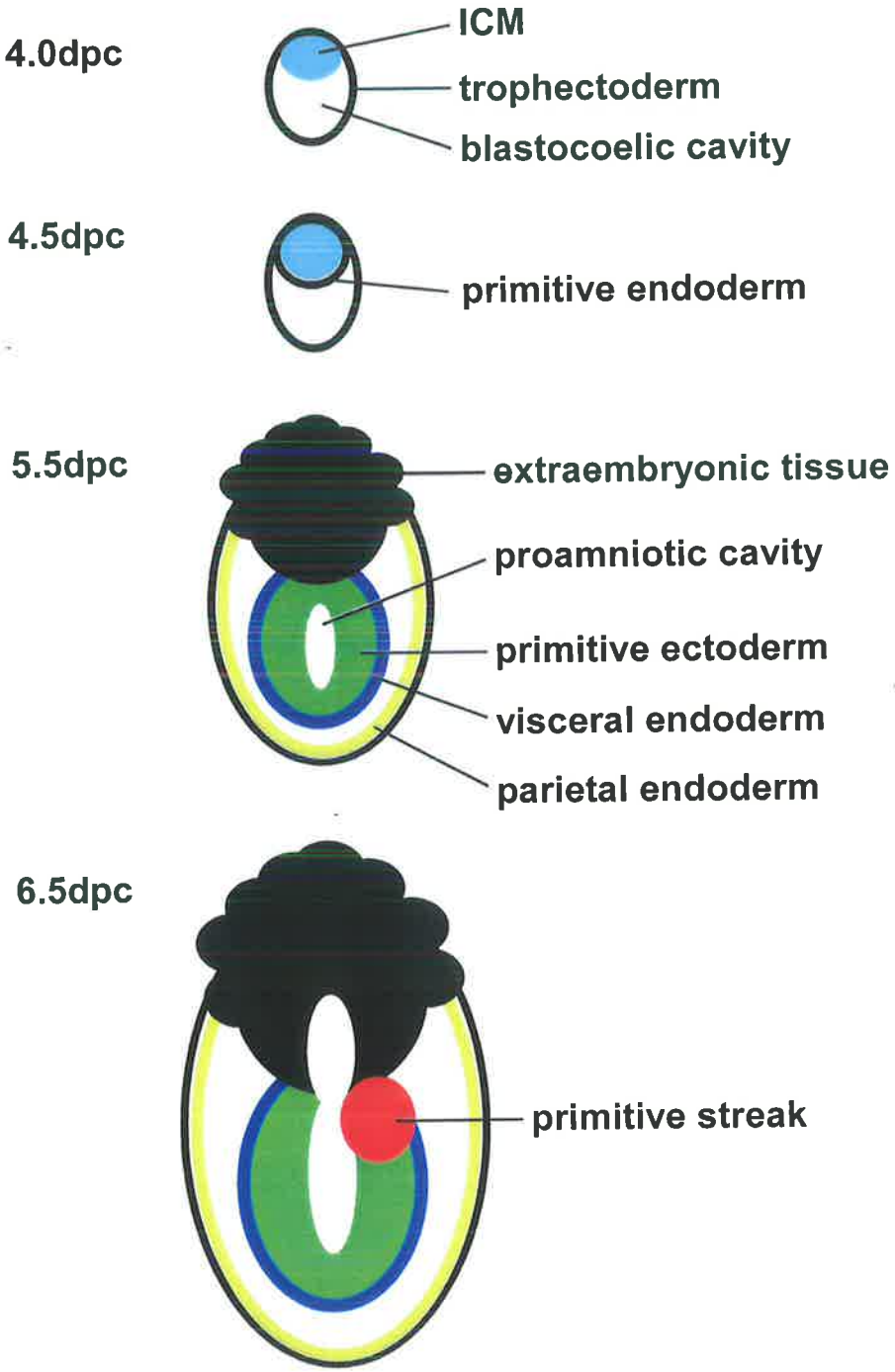


Figure 1.5 A schematic representation of early mouse embryogenesis. Prior to implantation at 4.0dpc, the blastocyst is characterised by pluripotent inner cell mass (ICM) cells and a blastocoelic cavity surrounded by an outer layer of trophectoderm. ICM cells adjacent to the cavity differentiate into primitive endoderm at 4.5dpc. After implantation by 5.5dpc, ICM cells have differentiated into primitive ectoderm, formed a pseudo-stratified layer and proamniotic cavity. Primitive endoderm, contacting primitive ectoderm, differentiates to form visceral endoderm. Primitive endoderm, contacting trophectoderm, differentiates to form parietal endoderm. Gastrulation begins at approximately 6.5dpc, with the emergence of nascent mesoderm through the primitive streak. (Adapted from Pelton *et al.*, 1998)

Figure 1.6

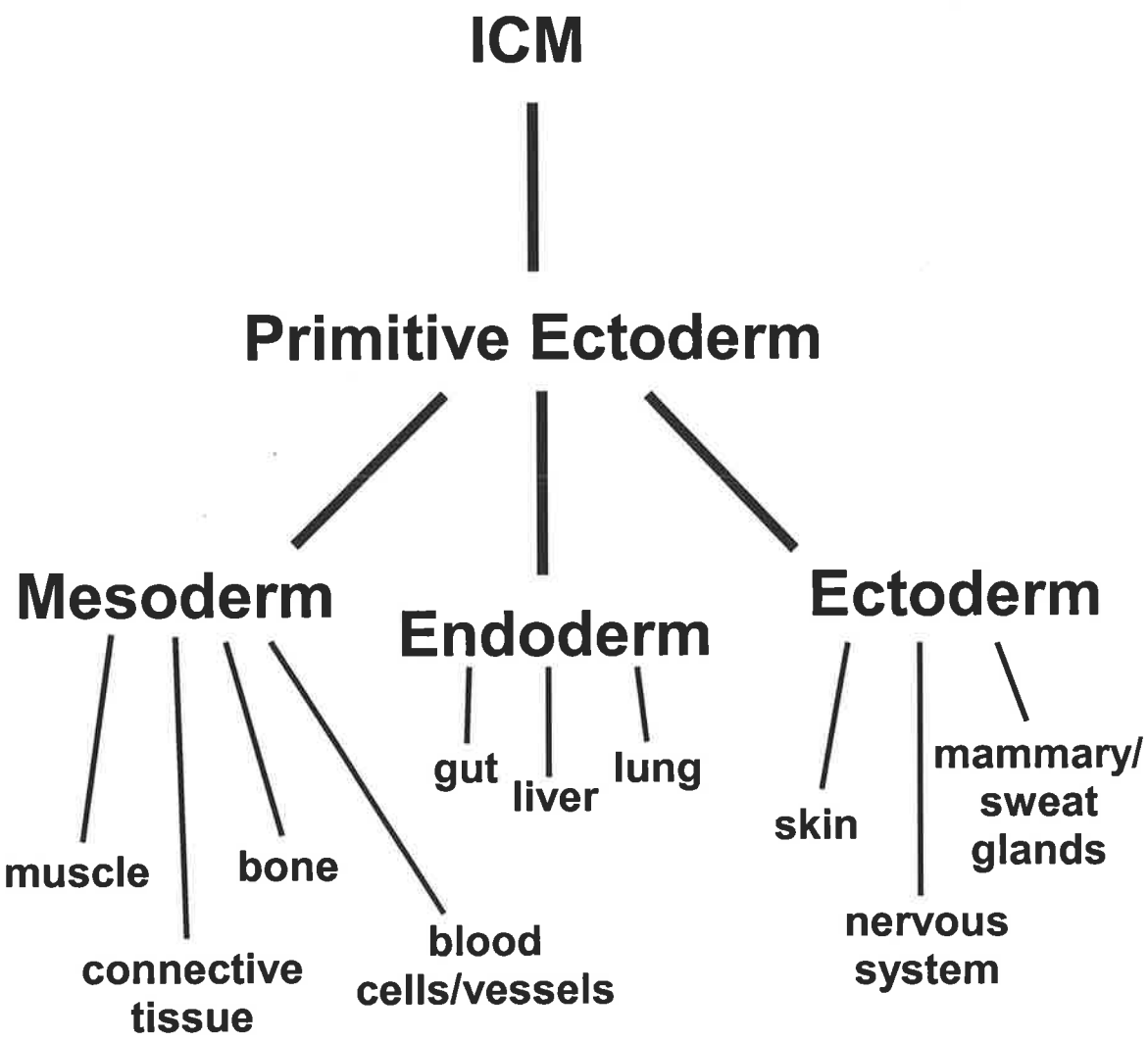


Figure 1.6 Pluripotent cells of the inner cell mass (ICM) differentiate into an intermediate, pluripotent cell population, primitive ectoderm. During gastrulation, primitive ectoderm differentiates into the three primary germ layers, ectoderm, endoderm and mesoderm, which are fated to give rise to all of the tissues that comprise the adult mouse.

endoderm, separated by a basement membrane (Hogan, 1994). In contrast, the primitive endoderm that migrates along the blastocoelic surface of the trophectoderm forms parietal endoderm (Gardner, 1983).

Gastrulation is the developmental period in which primitive ectoderm cells differentiate into the three primary germ layers – mesoderm, endoderm and ectoderm. At approximately 6.5dpc, primitive ectoderm cells migrate through the basement membrane to form a transient population of cells, termed the primitive streak (Fig 1.5). The migration of these cells to this region defines the anterior–posterior axis of the embryo (Tam and Behringer, 1997). Regionalisation of the embryo at this stage is demonstrated by the relative fates of primitive ectoderm cells (Lawson *et al.*, 1991; Quinlan *et al.*, 1995; Tam, 1989) but also by proliferation rates. At the onset of gastrulation, the cell cycle length of most primitive ectoderm cells is 4.8-5.1 hours, whereas cells of the primitive streak cycle as rapidly as 2.2 hours (Snow, 1977). Nascent mesoderm emerges from the primitive streak distally and laterally between primitive ectoderm and visceral endoderm layers (Nakatsuji *et al.*, 1986; Tam and Meier, 1982). Upon reaching the anterior pole and spanning the length of the embryo, definitive embryonic mesoderm is formed. The formation of mesoderm is accompanied by decreased rates of proliferation (Snow, 1977). The cells that migrate through the primitive streak and are incorporated into the layer of visceral endoderm form the definitive endoderm (Tam and Behringer, 1997).

1.6.2 Cell cycle regulation and early mouse embryogenesis

Cell cycle regulation is essential during development, since the direct consequence of proliferation is increased cell number. Cell number is obviously a determinant in the onset of differentiation, since gastrulation is delayed when primitive ectoderm cells are removed (Power and Tam, 1993; Rands, 1986b). However, cell number is not the only determinant, demonstrated by aggregate chimeric embryos that do not enter gastrulation prematurely despite possessing critical cell numbers (Lewis and Rossant, 1982; Rands, 1986a). These embryos downsize by not entering the rapid proliferative phase at the onset of gastrulation (Lewis and Rossant, 1982). This demonstrates that regulation of cell proliferation during early mouse development is essential.

Analysis of regulators of the cell cycle in mouse embryogenesis is difficult, mainly due to the inaccessibility of embryos in comparison to other model organisms. However, some

knowledge can be gained from *in situ* hybridisation analysis and from knockout studies. While there is differential expression of the three D-type cyclins during early mouse development, both *cyclin D1* and *cyclin D3* mRNAs can be detected in the ICM of blastocysts 3.5-4.5dpc (Faast *et al.*, 2004; Wianny *et al.*, 1998). In contrast, it is not until gastrulation that cyclin D1/D2–CDK4 complexes can be immunoprecipitated from embryos, whereas cyclin D3-CDK4 complexes are only detected after gastrulation (Savatier *et al.*, 1996). Analysis of the expression and activity of other cell cycle regulators has only been conducted from 8.5dpc, when most regulators appear to be present (Jiang *et al.*, 1997; Kusek *et al.*, 2000). Most cyclins and CDKs are functionally redundant during mouse development as demonstrated by knockout studies (Brandeis *et al.*, 1998; Ciemerych *et al.*, 2002; Fantl *et al.*, 1995; Geng *et al.*, 2003; Liu *et al.*, 1998a; Ortega *et al.*, 2003; Parisi *et al.*, 2003; Sicinski *et al.*, 1995). The exceptions are cyclin A2, with homozygous null mutants surviving only until 5.5dpc (Murphy *et al.*, 1997) and cyclin B1, with undetermined survival of homozygous null embryos prior to 10dpc (Brandeis *et al.*, 1998). While compensation may occur in the first cell divisions, these proteins become essential early in development. Similar functional redundancies are evident for the E2F and pocket protein families (Clarke *et al.*, 1992; Cobrinik *et al.*, 1996; Field *et al.*, 1996; Humbert *et al.*, 2000a; Humbert *et al.*, 2000b; Jacks *et al.*, 1992; Lee *et al.*, 1992; Lee *et al.*, 1996; Lindeman *et al.*, 1998; Rempel *et al.*, 2000; Yamasaki *et al.*, 1996). The importance of pocket proteins for early embryogenesis is most evident with the loss of pRB, 107 and p130. Ablation of these three genes led to limited contribution to differentiated cell types, suggesting that while being essential for the formation of the some cell types, pocket proteins are dispensable for others (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). A complete analysis of the contribution of the E2F family to mouse development has not been conducted, although combinations of E2F-1-/-;E2F-3-/- and E2F-2-/-;E2F-3-/- do not survive past 9.5dpc (Wu *et al.*, 2001).

1.6.3 In vitro model systems of early mouse embryogenesis

In vivo analysis of the molecular events occurring during early mouse embryogenesis are difficult to characterise due to the relative inaccessibility and limited size of embryos. *In vitro* model systems have been employed to overcome these problems. Embryonic stem (ES) cells can be isolated from the ICM of the pre-implantation blastocyst and cultured *in vitro*, retaining their pluripotency in the presence of leukaemia inhibitory factor (LIF) (Evans and Kaufman, 1981; Martin, 1981). ES cells can be reintroduced into the blastocyst and still contribute to normal development (Beddington and Robertson, 1989;

Robertson *et al.*, 1986). Their morphology, gene expression and differentiation potential demonstrates the equivalence of ES cells to ICM cells of the embryo (Nichols *et al.*, 1990; Pease *et al.*, 1990; Smith *et al.*, 1988; Williams *et al.*, 1988).

Removal of LIF from the culture medium induces spontaneous differentiation of ES cells (Smith *et al.*, 1992). ES cell differentiation can be partially controlled by a variety of chemicals, for example dimethyl sulfoxide induces mesoderm formation (Dinsmore *et al.*, 1996), whereas retinoic acid induces neurectoderm formation (van Inzen *et al.*, 1996). However, recapitulation of the differentiation of ICM cells prior to and during gastrulation can be conducted *in vitro* by culturing these cells in the absence of LIF as non-adherent aggregates, embryoid bodies (Beddington and Robertson, 1989; Evans and Kaufman, 1981). Differentiation of ES cells as embryoid bodies (ESEBs) occur in the same temporal order, with the same potential of ICM cells, although embryoid bodies do not have the spatial organisation of the embryo (Beddington and Robertson, 1989; Rathjen *et al.*, 1999). Ordered gene expression enables close comparison to the embryo (Fig 1.7) (Pelton *et al.*, 1998; Rathjen *et al.*, 1998). Primitive endoderm cells form around the ES cell aggregates relative to those in the embryo that form next to the blastocoelic cavity. The expression of *Rex1* by the pluripotent ICM and ES cells is downregulated as cells differentiate into primitive ectoderm. This is coincident with the upregulation of *Fgf5* expression in both primitive ectoderm cells of the embryo and the embryoid body. As in the embryo, extra-embryonic lineages of parietal and visceral endoderm are formed at this time and cavitation occurs (Doetschman *et al.*, 1985; Shen and Leder, 1992). Coincident with the downregulation of the marker for pluripotency, *Oct4*, genes expressed in the embryonic nascent mesoderm, *Brachyury* and *Goosecoid*, are also expressed in ESEBs. These differentiation events mimic those of gastrulation, resulting in the detection of derivatives of all three definitive cell types (Doetschman *et al.*, 1985).

In order to accurately recapitulate differentiation of ICM cells into embryonic lineages, pluripotent primitive ectoderm cells must be formed (Shen and Leder, 1992). These cells are restricted in that they cannot form primitive endoderm but can contribute to all embryonic lineages (Beddington, 1981; Gardner and Rossant, 1979). The formation of early primitive ectoderm-like (EPL) cells *in vitro* can be achieved with the growth of ES cells in MEDII, a medium conditioned by the human hepatocarcinoma cell line HepG2 (Rathjen *et al.*, 1999). Primitive ectoderm induction is thought to require signalling from

Figure 1.7

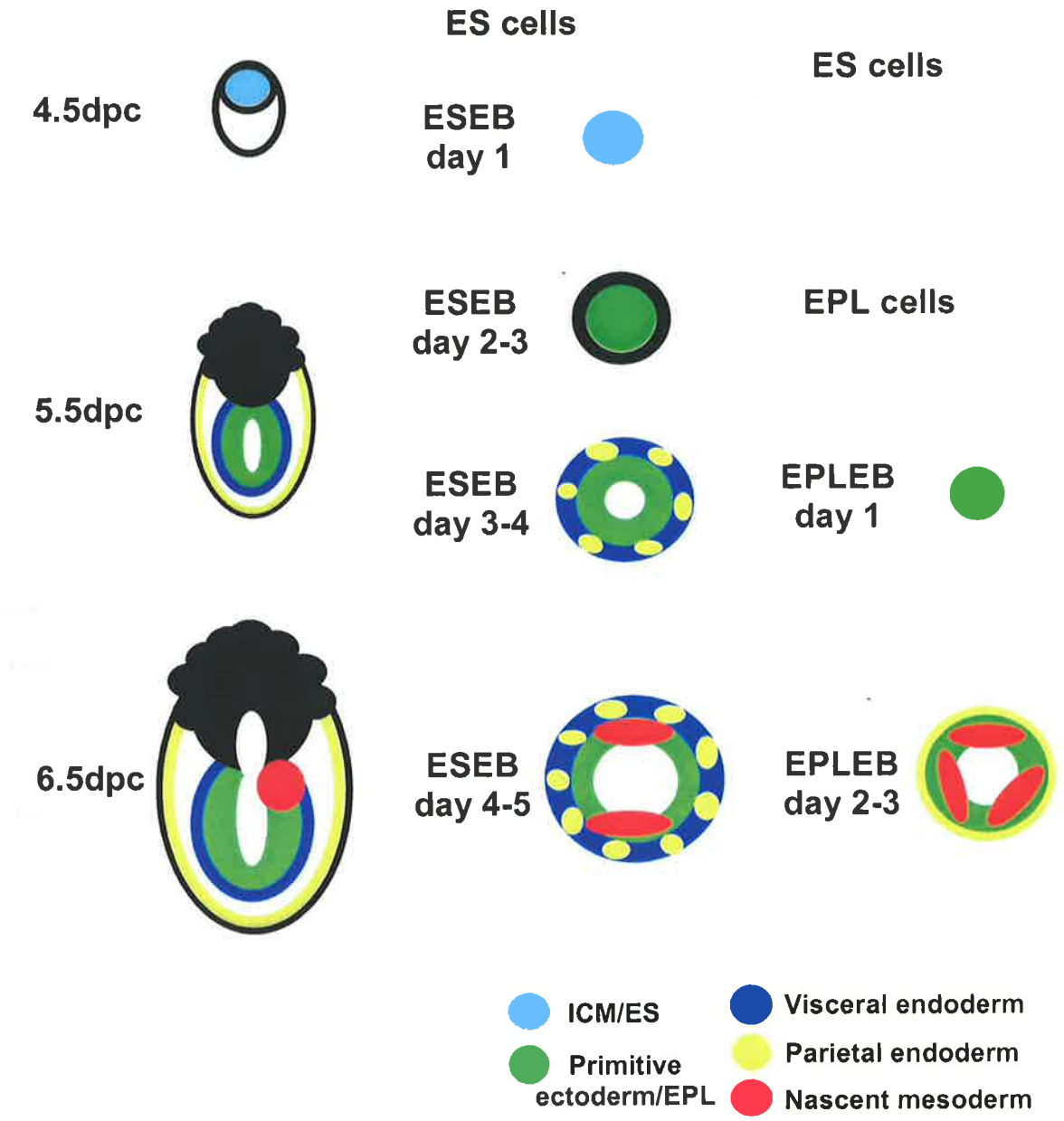


Figure 1.7 A schematic representation of embryoid body differentiation with the equivalent stages of embryogenesis. Mouse embryogenesis. At 4.5dpc, pluripotent inner cell mass (ICM) cells, primitive endoderm and a blastocoelic cavity are surrounded by an outer layer of trophectoderm. By 5.5dpc, ICM cells have differentiated into primitive ectoderm and a proamniotic cavity has formed. Primitive endoderm differentiates to form visceral and parietal endoderm. Gastrulation begins at approximately 6.5dpc with the formation of nascent mesoderm. ES embryoid body (ESEB) differentiation. Around aggregated pluripotent ES cells, an outer layer of primitive endoderm forms within 2-3 days, which differentiates into parietal and visceral endoderm between days 3-4 of culture. By this stage, primitive ectoderm and inner cavity are formed. Nascent mesoderm formation follows on days 4-5. EPL embryoid body (EPLEBs) differentiation. ES cells are differentiated into EPL cells as adherent cultures. Around aggregated pluripotent EPL cells, an outer layer of parietal endoderm forms within 2-3 days, however no visceral endoderm is detected. By 2-3 days in culture, an inner cavity has formed and nascent mesoderm has formed. (Adapted from Rathjen *et al.*, 1998)

visceral endoderm. Factors within MEDII mimic visceral endoderm, therefore providing a similar environment to that of the embryo for primitive ectoderm induction (Rathjen *et al.*, 2001; Rathjen *et al.*, 1999). These cells are equivalent to the pluripotent primitive ectoderm cells at approximately 5.25dpc, in accordance with their gene expression and differentiation potential (Lake *et al.*, 2000; Pelton *et al.*, 2002; Rathjen *et al.*, 1999).

Lineage specific differentiation can be accomplished by culturing EPL cells in different conditions. Culturing EPL cells as embryoid bodies (EPLEBs) in the absence of LIF or MEDII mimics differentiation of primitive ectoderm cells of the embryo, according to the temporal order of gene expression (Fig 1.7). However, EPLEBs are accelerated in timing and restricted in differentiation in comparison to EPLEBs. Compared to the maximal expression of *Brachyury* of 16% in day 5 ESEBs, day 3 EPLEBs 98% express *Brachyury* (Lake *et al.*, 2000). In conjunction with the primarily mesodermal fate, EPLEBs are unable to form visceral endoderm and embryonic ectoderm lineages (Lake *et al.*, 2000). It is the inability to form visceral endoderm that is thought to specify the mesodermal fate of EPLEBs (Lake *et al.*, 2000). In the embryo, mesoderm forms from cells that detach from the extracellular matrix, separate from visceral endoderm and migrate through the primitive streak (Nakatsuji *et al.*, 1986; Tam and Meier, 1982). In comparison, primitive ectoderm cells that remain associated with visceral endoderm form neurectoderm (Ciruna *et al.*, 1997; Quinlan *et al.*, 1995). By continuous culture of EPL cells in MEDII as embryoid bodies (EBMs), cells are fated to form neurectoderm (Rathjen *et al.*, 2002). This is consistent with the conclusion that factors within MEDII mimic visceral endoderm signalling (Rathjen *et al.*, 2001; Rathjen *et al.*, 1999).

1.6.4 Cell cycle regulation of embryonic stem cells

Similar to the pluripotent cells of the embryo, ES cells proliferate very rapidly, with cycling times of between 8-12 hours (Savatier *et al.*, 1994; Stead *et al.*, 2002). In contrast to differentiated cell types, the cell cycle structure of ES cells is characterised by a very short G1 phase with the majority of the cell cycle associated with DNA synthesis (Savatier *et al.*, 1994; Stead *et al.*, 2002). This cell cycle structure is similar to that of pluripotent cells of the embryo (Solter *et al.*, 1971; Stead *et al.*, 2002). Analysis of the regulators of the ES cell cycle has enabled an understanding of cell cycle control in these rapidly proliferating cells (Fig.1.8). Similar to ICM cells of the embryo, cyclins D1, D3 and their catalytic partners CDK4/6 are expressed in ES cells, however no cyclin D-CDK4 activity has been detected (Faast *et al.*, 2004; Savatier *et al.*, 1996). Accordingly,

Figure 1.8

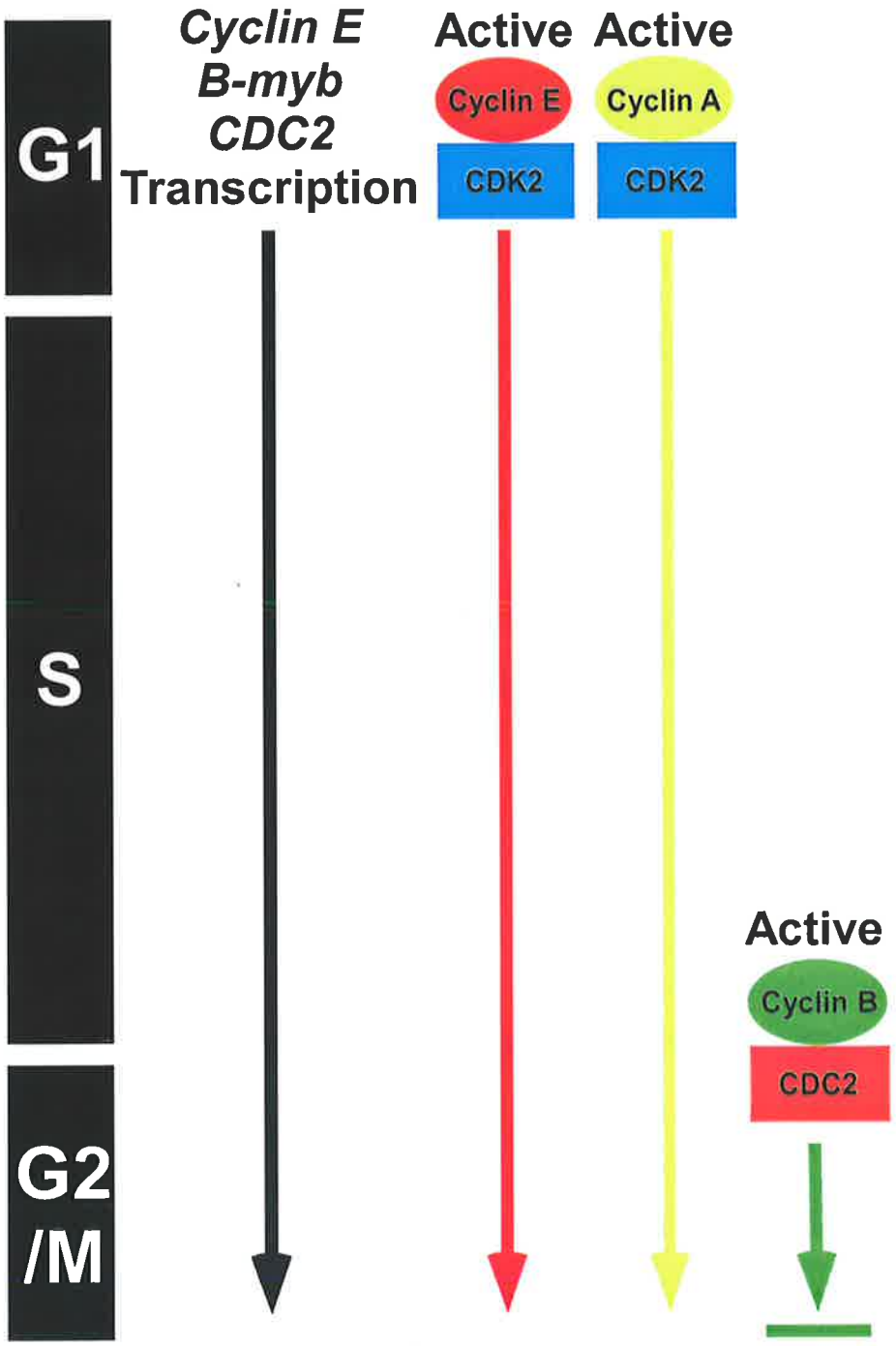


Figure 1.8 Regulation of cell cycle progression in mouse embryonic stem (ES) cells. The ES cell cycle is characterised by a very short G1 phase, with the majority of the cell cycle associated with DNA synthesis. In contrast to differentiated cells, expression of E2F target genes, *cyclin E*, *B-myb* and *CDC2*, lacks cell cycle periodicity in ES cells. Similarly, there are high levels of cell cycle independent cyclin E-CDK2 and cyclin A-CDK2 kinase activity in ES cells. Cyclin B-CDC2 kinase activity is the only identified cell cycle regulated CDK activity in ES cells .

ES cells are resistant to the growth inhibitory functions of p16 (Faast *et al.*, 2004; Savatier *et al.*, 1996). High levels of cyclin E are constitutively expressed leading to subsequent high, deregulated cyclin E-CDK2 activity (Stead *et al.*, 2002). Similarly, cyclin A and B protein levels and associated kinase activities are also high in ES cells (Stead *et al.*, 2002). However, cyclin B-CDC2 kinase activity is the only identified cell cycle regulated CDK activity in ES cells (Stead *et al.*, 2002). In accordance with these kinase activities, there are undetectable levels of the CDK inhibitors, p21, p27 and p16 in ES cells (Savatier *et al.*, 1996; Stead *et al.*, 2002). Specific inhibition of CDK2 activity in ES cells demonstrates that CDK2 activity levels determine the rate of ES cell division (Stead *et al.*, 2002).

While present in ES cells, pRB is predominantly hyperphosphorylated and thus inactive (Savatier *et al.*, 1994). While there is no detectable p130 in ES cells (LeCouter *et al.*, 1996), p107 is expressed and represents the small proportion of pocket protein associated with DNA-bound E2F complexes (Humbert *et al.*, 2000a; Stead *et al.*, 2002). Importantly, this p107-E2F DNA binding activity is independent of the cell cycle (Stead *et al.*, 2002). Consequentially, the expression of E2F target genes is not cell cycle regulated in ES cells (Stead *et al.*, 2002). The decreased dependence on pocket proteins for ES cell cycle control is highlighted by the fact that ES cells proliferate normally without the presence of all three pRB family members (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). These characteristics are likely to contribute to the rapid proliferation of ES cells. Importantly, ES cell cycle regulation resembles that described for many tumour types (Halaban, 1999). Similarities between ES cells and cancer cells are consistent with the observation that if introduced to a somatic location, ES cells will spontaneously form tumours (Martin, 1981).

1.6.5 Cell cycle regulation during differentiation

ES cells induced to differentiate by withdrawal of LIF and addition of retinoic acid show increased cell cycle length and dramatic cell cycle remodelling after 4 days of differentiation (Savatier *et al.*, 1996). As in the embryo, differentiation into ESEBs has shown upregulation of *cyclins D1*, *D2* and *D3* and cyclin D-CDK4 activity with or immediately following the expression of mesodermal markers, *Brachyury* and *Goosecoid* (Faast *et al.*, 2004; Savatier *et al.*, 1996; Wianny *et al.*, 1998). Cell cycle remodelling is associated with decreased expression of cyclin E and cyclin E-CDK2 activity (Kelly and Rizzino, 2000; Savatier *et al.*, 1996, E.Stead, PhD thesis). In contrast, *p21* and *p27*

expression is upregulated (Kelly and Rizzino, 2000; Savatier *et al.*, 1996). This suggests that the increased length of the cell cycle is likely to be in part due to the increased complexity of regulation.

Analysis of lineage-specific differentiation of ES cells into EPLEBs also demonstrates dramatic remodelling of cell cycle structure. In particular, the proportion of cells in G1 phase increasing from 25% in ES cells to 50% in EPLEB day 5 (E. Stead, PhD thesis). These changes correspond to those that occur during gastrulation (Snow, 1977). While their catalytic partners are constant, D-type cyclin expression increases as nascent mesoderm markers are expressed (Faast *et al.*, 2004). The decreased cyclin E expression and associated kinase activity also corresponds to the loss of pluripotency (E. Stead, PhD thesis). In contrast, cyclin A and B protein levels and associated kinase activities remain reasonably constant over this time, while CDK inhibitors are upregulated (E. Stead, PhD thesis, R. Faast, unpublished data). Decreases in levels of hyperphosphorylated pRB with concomitant increases in hypophosphorylated pRB are observed during EPLEB differentiation (S. Conn., Honours thesis).

1.7 Aims

Pluripotent cells of the early mouse embryo proliferate extremely rapidly. Differentiation of pluripotent cells during gastrulation is associated with significant proliferative changes. Due to practical limitations in analysis of the mouse embryo, most understanding of these early cell cycles has come from analysis of *in vitro* pluripotent ES cells. Given the role of CDK activity in governing many proliferative events, an aim of this thesis is to further analyse CDK activity in ES cells and during differentiation. In particular, the function of the high levels of CDK2 activity in ES cells and the changes associated with differentiation is assessed. With G1 regulation displaying the most dramatic differences as ES cells differentiate, this thesis also aims to assess the kinase activity of CDK4 and CDK6 in ES cells and differentiation. Additionally, this thesis aims to further analyse the deregulated E2F target gene expression in ES cells by analysis of the activity and function of E2F transcription factors and pocket proteins. Characterisation of the differences in expression and regulation of E2F target genes as ES cells differentiate is also conducted. It is hoped that this thesis will provide an understanding of how cell cycle regulatory mechanisms govern the rapid ES cell cycle and the proliferative changes associated with differentiation.

Materials and Methods

2.1 Abbreviations

Ab	Antibody
AMP	Ampicillin
APS	Ammonium persulphate
ATP	Adenosine tri-phosphate
bp	Base pairs
β-Me	β-Mercaptoethanol
BSA	Bovine Serum Albumin
C	Celsius
cDNA	Complementary DNA
CDK	Cyclin dependent kinase
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylene Diamine Tetra Acetic Acid
EGTA	Ethylene G Tetra Acetic Acid
EPL	Early Primitive Ectoderm-like
ES	Embryonic Stem
EtOH	Ethanol
FCS	Foetal Calf Serum
g	Gram
GST	Glutathione S-Transferase
HA	Haemagglutinin
Hepes	N-2-Hydroxyethyl-piperazine-N-2-ethansulphonic acid
HRP	Horseradish Peroxidase
ICM	Inner Cell Mass
IP	Immunoprecipitation
IPTG	Isopropyl-B-D-thiogalactopyranoside

Kb	Kilobase pair
kD	Kilodalton
L	Litre
LB	Luria broth
M	Moles per litre
mM	Millimoles per litre
µM	Micromoles per litre
µg	Microgram
µl	Microlitre
mA	Milliamperes
MEF	Mouse Embryonic Fibroblast
mLIF	Mouse Leukaemia Inhibitory Factor
MOPS	Morpholinopropanesulfonic acid
MQ	Milli-Q
mRNA	Messenger RNA
NP-40	Nonidet P-40
OD	Optical Density
PAS	Protein A Sepharose
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
PMSF	Phenylmethylsulphonylflouride
RNA	Ribonucleic Acid
rpm	Revoloutions per minute
RT	Room temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	SDS - Polyacrylamide Gel Electrophoresis
TBE	Tris Borate EDTA
TE	TRIS EDTA
TEMED	N, N, N', N'- Tetramethyl-Ethenediamine
TLCK	Nα-P-Tosyl-L-Lysine Chloromethyl Ketone
TPCK	N-Tosyl-L-phenylalanine Chloromethyl Ketone
TRIS	Tris (hydroxymethyl) amino methane

Tween-20	Polyoxyethylene-sorbitan-Monolaurate
UV	Ultraviolet
V	Volts

2.2 Bacterial Manipulations

2.2.1 Materials

Chemicals and reagents were obtained from Sigma, except those specified below.

Acetic acid	BDH Chemicals
Chloroform	BDH Chemicals
Ethanol	BDH Chemicals
IPTG	Diagnostic Chemicals
Glutathione-Agarose	Zymatrix
Phenol	Wako Pure Chemicals
Potassium acetate	BDH Chemicals
Sodium acetate	BDH Chemicals
Sodium hydroxide	BDH Chemicals

2.2.2 Buffers and Solutions

NETN buffer	20mM Tris pH 8.0, 100mM NaCl, 1mM EDTA, 0.5% NP-40, 1mM DTT, 5μg/ml aprotinin, 5μg/ml leupeptin, 5μg/ml pepstatin
Solution 1	50mM glucose, 10mM EDTA, 25mM Tris pH 8.0
Solution 2	1% SDS, 0.2M sodium hydroxide
Solution 3	2.5M potassium acetate, 45% acetic acid

2.2.3 Bacterial Strains and Media

E.coli DH5α

L+Amp plates	1% sodium chloride, 1% trytone, 0.5% yeast extract, 1.5% bacto-agar, 100μg/ml ampicillin

2.2.4 Bacterial Transformations

DNA was added to 45μl of $CaCl_2$ competent DH5α cells. Cells were incubated on ice for 20 minutes, 42°C for 2 minutes, then on ice for 2 minutes. Cells were resuspended in 1ml of LB and incubated at 37°C for 20 minutes. Cells were plated onto L+AMP agar plates and incubated overnight at 37°C.

2.2.5 Mini Plasmid Preparation from Bacteria

A single bacterial colony was used to inoculate a 2ml LB + AMP (0.1mg/ml) culture and grown overnight at 37°C. 1ml of culture was centrifuged at 14000rpm for 15 seconds, the supernatant discarded and cells resuspended in 100µl of solution 1. 200µL of solution 2 was added, mixed gently and 150µl of solution 3 was added. 400µL of 5M LiCl was added and centrifuged at 14000rpm for 10 minutes. DNA was precipitated from the supernatant by incubation on ice for 15 minutes with 800µl of 95% ethanol and collected by centrifugation at 14000rpm for 10 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol and resuspended in 30µl of MQ water. Plasmid preparations were digested in the presence of RNAse.

2.2.6 Maxi Plasmid Preparation from Bacteria

A single bacterial colony was used to inoculate a 2ml LB + AMP (0.1mg/ml) culture and grown overnight at 37°C. 200µl of this culture was used to inoculate a 200ml LB + AMP (0.1mg/ml) culture and grown overnight at 37°C. Cultures were centrifuged at 6000rpm for 10 minutes, the supernatant discarded and cells resuspended in 5ml of solution 1. 10ml of solution 2 was added, mixed gently and left at RT for 10 minutes. 7.5ml of solution 3 was added, mixed gently and incubated on ice for 15 minutes. Following centrifugation at 3000rpm for 15 minutes, DNA was precipitated from the supernatant by incubation on ice for 15 minutes with 1 volume of cold isopropanol. Following centrifugation at 3000rpm for 10 minutes, the DNA pellet was resuspended in 1.5ml MQ water. High molecular weight RNAs were precipitated by incubation on ice for 5 minutes with 2ml of cold 5M LiCl. Following centrifugation at 3000rpm for 10 minutes, DNA was precipitated from the supernatant by incubation on ice for 15 minutes with 2 volumes of cold 95% ethanol. DNA was pelleted by centrifugation at 3000rpm for 10 minutes, air dried and resuspended in 800µl of MQ water. RNA was removed with 40µg/ml RNase for 15 minutes at 37°C. DNA was precipitated by addition of 0.5 volumes of 20% PEG8000, 2.5M sodium chloride solution and incubation on ice for 5 minutes. Following centrifugation at 14000rpm for 5 minutes, the DNA pellet was resuspended in 600µl MQ water, left for at least 30 minutes, then extracted twice with 600µl of phenol/chloroform. Purified DNA was precipitated with 0.1 volumes of 3M sodium acetate pH 5.2 and 2.5 volumes of cold 95% ethanol and harvested by centrifugation at 14000rpm. DNA pellets were washed with 70% ethanol, air dried and resuspended in MQ water.

2.2.7 Preparation of bacterially expressed GST fusion proteins

For use in CDK4/6 kinase assays, a C-terminal fragment of human RB (amino acids 773 to 928) fused to GST was utilized as an *in vitro* substrate (gift from Boris Sarcevic from Ed Harlow). Human p16 fused to GST and human p21 fused to GST was utilised as an inhibitor of cyclin D associated kinase activity (kind gifts from Boris Sarcevic).

200ml cultures of DH5α *Escherichia coli* transformed with vectors encoding fusion proteins, were induced at 37°C with 1mM IPTG for 3 hours. Bacterial pellets were lysed by the addition of 10ml of ice-cold NETN buffer, followed by sonication. Soluble fusion proteins were purified by incubation with 250µl glutathione-agarose and elution with 500µl 20mM reduced glutathione. Fusion proteins were pooled and stored as aliquots at -80°C. Quantitation of fusion proteins was conducted by comparison to BSA standards and markers upon SDS-PAGE and Coomassie analysis.

2.3 Tissue Culture Methods

2.3.1 Materials

Reagents were obtained from Sigma, with the exception of those specified below.

Bacteriological petri dishes	Techno-plas
Bottle top filters (0.2µm)	Corning
Dissecting Microscope	Leica
DMEM	Gibco BRL
FCS	Commonwealth Serum Laboratories
Flasks	Falcon
Freezing vials	Nunclon
Laminar Flow Hood	Gelman Sciences
MG132	Biomol
Microscopy	Nikon ELW D0.3 phase contrast
mLIF (ESGRO)	Chemicon
Plugged pipettes (25 ml/10 ml)	Falcon
Plates	Falcon
Trays	Falcon
Trypsin-EDTA	Gibco BRL

2.3.2 Buffers and Solutions

Aphidicolin	Stock: 5 mg/ml aphidicolin in DMSO
L-glutamine	100mM L-glutamine in MQ H_20, filter sterilised
MG132	Stock: 50mM MG132 in DMSO
Nocodazole	Stock: 10mg/ml Nocodazole in DMSO
PBS/β-ME	100 mM β-ME in sterile PBS. (fresh fortnightly)
PBS/Gelatin	0.2% (w/v) gelatin in PBS. Sterilised by autoclaving
Puromycin	Stock: 10mg/ml Puromycin in MQ H_20

2.3.3 Cell Lines

D3 ES: Derived from ICM of the preimplantation 129 strain mouse embryo Kindly donated by Lindsay Williams, Ludwig Institute, Melbourne.

HepG2: Human hepatocellular carcinoma (ATCC HB-8065).

2.3.4 Derivation of MEFs

Time-mated outcrossed Swiss mice (Robertson, 1987) were euthanasied to harvest 13.5dpc embryos. Embryos were be-headed and eviscerated. The outer body, spine and limbs were collected in DMEM and passed through an 18 gauge needle 10-20 times. Cells were centrifuged at 1200rpm for 4 minutes, resuspended in 90% DMEM, 10% FCS and plated (3-4 embryos per 75cm^2 flask). Cells were passaged once prior to freezing in 90% FCS, 10% DMSO at 5x10^{6}cells/ml.

2.3.5 Media

DMEM	high glucose, L-glutamine, 25mM HEPES buffer, pyridoxine hydrochloride (Gibco, BRL)
DMEM (ES DMEM)	high glucose, L-glutamine, 100mg/L sodium pyruvate, pyridoxine hydrochloride (Gibco, BRL)
ES Complete	ES DMEM, supplemented with 10% FCS, 0.1 mM β-ME, 1000U/ml of mLIF
ES Incomplete	ES DMEM, supplemented with 10% FCS, 0.1 mM β-ME.
HepG2	90% ES DMEM, 10% FCS.
MedII	Conditioned medium produced by culturing HepG2 cells to subconfluency. Medium was harvested and filter sterilised (0.22 μm) and stored at 4°C for up to 14 days
MEF	90% DMEM, 10% FCS.

2.3.6 Gelatinised Tissue Culture plates

All tissue culture plates used for ES and EPL cells were gelatinised with 0.2% (w/v) gelatin in PBS. Plates were covered with gelatin solution and left for at least 30 minutes at room temperature. The gelatin solution was removed and the plate washed in PBS immediately before use.

2.3.7 Maintenance of Cell Cultures

ES cells were maintained on gelatinised petri-dishes in ES complete medium at 37°C in 10% CO_2. Every 2-3 days cells were passaged as follows: cells were washed with PBS, incubated with 1ml trypsin-EDTA for 2 minutes, added to 4ml ES complete medium, gently pipetted and centrifuged at 1200rpm for 4 minutes. Cells were gently resuspended in 10ml ES complete medium and re-seeded at $1x10^5$ or $5x10^4$ cells/cm^2.

EPL cells were induced and maintained by culturing $1x10^5$ ES cells/cm^2 on gelatinised petri-dishes in 50% ES incomplete medium and 50% MedII medium at 37°C in 10% CO_2. After 2 days of culture, EPL cells were harvested

MEFs were maintained in 175cm^2 flasks in MEF medium at 37°C in 5% CO_2. Every 4-5 days cells were passaged as follows: cells were washed once with PBS, incubated with 4ml trypsin-EDTA, added to 6ml MEF medium and centrifuged at 1400rpm for 2 minutes. Cells were resuspended in 10ml MEF medium and re-seeded at dilutions of 1:20 to 1:40 (approximately $5x10^4$ cells/cm^2).

HepG2s were maintained in 175cm^2 flasks in HepG2 medium at 37°C in 5% CO_2. Every 3-4 days cells were passaged as follows: cells were washed once with PBS, incubated with 4ml trypsin-EDTA, pipetted vigorously, added to 6ml HepG2 medium and centrifuged at 1400rpm for 2 minutes. Cells were resuspended in 10ml HepG2 medium and re-seeded at $5x10^4$ cells/cm^2.

2.3.8 Preparation of Embryoid Bodies

Single cell suspension of ES or EPL cells were plated into bacteriological dishes at a density of $1x10^5$ cells/ml in ES incomplete medium. Medium was replaced every 2 days by harvesting medium and bodies, centrifuging at 1200rpm for 10 seconds and resuspending in fresh medium.

2.3.9 Harvesting cells and embryoid bodies

All cells were removed from adherent culture with trypsin/PBS. Following dissociation, trypsin was inactivated with 5-10ml of medium containing 10% FCS. Embryoid bodies

were harvested into 30ml tubes, without trypsinisation. Cells were centrifuged at 1200 rpm for 4 minutes and washed 3 times in PBS. Cell pellets were used immediately or stored at –80°C.

2.3.10 Thawing cell lines

A vial of cells was removed from liquid nitrogen, thawed in a 37°C water bath and immediately diluted in 10 ml of fresh culture medium.

ES cells were centrifuged at 1200 rpm for 4 minutes, plated at $1x10^5$ cells/cm^2 on a gelatinised plate and incubated at 37°C, 10% CO_2 in a humidified incubator.

HepG2s and MEFs were plated immediately after thawing at $5x10^4$ cells/cm^2 and incubated at 37°C, 5% CO_2 in a humidified incubator. Medium was replaced the following day.

2.3.11 Stable ES Cell Line Establishment Via Electroporation

Cells were harvested to a single cell suspension and resuspended to $3\text{-}5x10^7$ cells in 900µl of cold PBS. 10µg of linearised DNA was added and the mix was transferred to an electroporation cuvette. The cells were subjected to 500µF/0.2kV, then rapidly transferred to 10ml of complete ES medium. Cells were plated into four gelatinised 10cm (10cm or 55cm^2) plates and grown overnight. The following day 1µg/ml puromycin was added to the medium. Everyday for 7-10 days, the medium was aspirated, cells washed with PBS and fresh medium was added until resistant colonies were large enough for picking. At this stage, a 96 well tray with 50µl aliquots of trypsin was prepared. Medium was aspirated from cells and PBS added. Under an inverted microscope, colonies were dislodged and aspirated in a 20µl volume. The colony was incubated with trypsin, dispersed by pipetting and transferred to a gelatinised 24 well tray to grow overnight in ES complete medium. The following day the cells were returned to selection medium containing 1µg/ml puromycin. Cells were then maintained as previously described (2.3.7) with 1µg/ml puromycin added.

2.3.12 Static Cell Synchronisation

ES cells were statically synchronised in mitosis by incubating cells in medium containing 45ng/ml nocodazole for 10-12 hours. Cells were statically synchronised in S phase by incubating cells in medium containing 5µg/ml aphidicolin for 10 –12 hrs.

2.3.13 Block/Release ES Cell Synchronisation

ES cells were synchronised in mitosis by incubating cells in medium containing 45ng/ml nocodazole for 10 –12 hours. Suspended and adherent cells were washed 3 times in PBS. Medium was then replaced with fresh ES complete medium containing 5μg/ml aphidicolin for 10 hours. Cells were harvested, washed 3 times with PBS, cultured in fresh medium and harvested every hour for 8 hours and then every 45 minutes for 5 subsequent hours.

2.3.14 Proteasome Inhibitor treatment of ES cell and embryoid bodies

ES cells and embryoid bodies were grown as described previously (2.3.7 and 2.3.8). Prior to harvesting, 5μM MG132 was added to cells/bodies. After 3 hours, both treated and untreated samples were harvested as described previously (2.3.9).

2.4 Molecular Methods

2.4.1 Materials

2.4.1.1 Chemicals and Reagents

Chemicals and reagents were obtained from Sigma, except those specified below.

Acrylamide	BioRad
BSA	Biolab
Bradford Reagent	Bio-Rad
Chloroform	BDH Chemicals
Formaldehyde	BDH Chemicals
Formamide	BDH Chemicals
Fugene	Roche
Glycerol	BDH Chemicals
Glycogen	Boehringer Mannheim
Herring sperm DNA	Gibco Life Technologies
Histone H1	Calbiochem
Hydrochloric acid	BDH Chemicals
Isopropanol	BDH Chemicals
Phenol	Wako Pure Chemicals
Protein A Sepharose	Amersham
Restriction Enzymes	New England Biolabs
RNAzol	Tel-test
SDS	Fisher Scientific

Skim Milk Powder	Diploma
Sodium Citrate	Fisher Scientific
Sodium hydroxide	BDH Chemicals
Taq Polymerase	Geneworks
TEMED	BioRad
Triton X-100	Fisher Scientific
tRNA	Roche
Tween-20	Fisher Biotech
Ultrahyb	Amersham

2.4.1.2 Buffers and Solutions

10x PCR buffer (promega)	500mM KCl, 100mM Tris pH9, 1% Triton X-100
10x PCR buffer (RB)	250mM Tris/HCl pH9.1, 160mM $(NH_4)_2SO_4$
ATP	220mM in MQ, neutralized with NaOH. Aliquot and store at -20°C
Cell Lysis Buffer	50mM Hepes pH7.9, 250mM KCl, 0.1mM EDTA, 0.1mM EGTA, 0.4mM NaF, 0.4mM $NaVO_4$, 10% glycerol, 0.1% NP40, 0.5mM PMSF, 50µg/ml TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
ChIP dilution buffer	0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris pH8, 176mM NaCl, 0.5mM PMSF, 50µg/ml TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
ChIP low salt wash buffer	0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH8.1, 150mM NaCl, 0.5mM PMSF, 50µg/ml TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
ChIP high salt wash buffer	0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris pH8.1, 500mM NaCl, 0.5mM PMSF, 50µg/ml TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
ChIP LiCl wash buffer	0.25M LiCl, 1% NP-40, 1% deoxycholate, 1mM EDTA, 10mM Tris pH 8.1, 0.5mM PMSF, 50µg/ml

	TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
ChIP SDS lysis buffer	1% SDS, 10mM EDTA, 50mM Tris pH8, 0.5mM PMSF, 50µg/ml TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
Complete Luciferase Buffer	48mM phosphate buffer, 6mM ATP, 3mM DTT in Luciferase Buffer
Coomassie Stain	0.03% Coomassie, 8.75% Acetic acid, 50% MeOH
Coomassie Destain 1	50% MeOH, 8.75% Acetic acid
Coomassie Destain 2	5% MeOH, 7% Acetic acid
50x Denhardt's solution	0.1%(w/v) Ficoll, 0.1%(w/v) polyvinylpyrrolidine, 0.1%(w/v) BSA
Farnham dialysis buffer	2mM EDTA, 50mM Tris pH8, 0.5mM PMSF, 50µg/ml TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
Farnham IP buffer	100mM Tris pH9, 500mM LiCl, 1% NP-40, 1% deoxycholate, 0.5mM PMSF, 50µg/ml TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
Gel Filtration Buffer	50mM HEPES pH7.5, 10mM $MgCl_2$, 150mM NaCl
Hybridisation buffer	50% formamide, 5x SSC, 50mM $NaPO_4$ pH6.5, 5x Denhardt's, 0.1% SDS, 0.2mg/ml yeast tRNA, 0.1mg/ml ssDNA
IP buffer	250mM NaCl, 20mM HEPES pH8, 0.1% NP-40, 1mM EDTA
Kinase Buffer	50mM HEPES pH 7.5, 10mM $MgCl_2$, 1mM DTT
Luciferase Buffer	1% Triton-X 100, 25mM glycyl glycine pH7.8, 15mM $MgSO_4$, 4mM EGTA, 1mM DTT
1000x Luciferin (10mM)	2mg in 720ml Luciferase buffer with 10mM DTT, stored in aliquots at -20°C
Lysis Buffer A	10mM HEPES pH8.0, 80mM KCl, 1mM DTT, 1.5mM $MgCl_2$
Lysis Buffer B	0.3mM HEPES pH8.0, 1.4mM KCl, 1mM DTT, 30mM $MgCl_2$
10x MOPs	200mM MOPS pH 7.0, 50mM NaOAc,

	10mM disodium EDTA
PBS	130mM NaCl, 2.5mM KCl, 10mM Na_2HPO_4, 30mM NaH_2PO_4
PBT	PBS with 0.1% Tween-20
Phosphate Buffer	100mM stock: 0.27g monobasic potassium phosphate, 1.74g dibasic potassium phosphate in 100ml, pH to 7.8
Ponceau S Stain	1% Ponceau S stain, 7% TCA
5x RNA load buffer	0.5% SDS, 25% Glycerol, 25mM EDTA, 0.025% Bromophenol Blue
RIPA buffer	0.5M LiCl, 50mM HEPES pH7.5, 1mM EDTA, 1% NP-40, 0.7% deoxycholate, 0.5mM PMSF, 50µg/ml TPCK, 50µg/ml TLCK, 1µg/ml leupeptin, 1mM DTT
2x SDS Load Buffer	100mM Tris pH6.8, 200mM DTT, 4% SDS, 0.2% Bromophenol Blue, 20% glycerol
SDS-PAGE Running Buffer	25mM Tris, 250mM glycine, 0.1% SDS
20x SSC	3M NaCl, 300mM sodium citrate
TE	10mM Tris pH8.0, 0.1mM EDTA
TNM	30mM TrisHCl pH7.6, 150mM NaCl, 15mM $MgCl_2$, 0.4% NP40
Tris-Tricine Running Buffer	100mM Tricine, 100mM Tris, 0.1%SDS
TUNES	10mM TrisHCl pH8.0, 7M Urea, 0.35M NaCl, 1mM EDTA, 2% SDS
Western Transfer Buffer	50mM Tris, 380mM glycine

2.4.1.3 Miscellaneous Materials

Broad Range Rainbow Markers	Amersham Biosciences
Chemiluminescent Substrate kit	Pierce
DLR luciferase kit	Promega
Flow cytometer EPICS XL-MCL	Beckman Coulter
HMV Calibration Kit	Pharmacia
Megaprime DNA Labelling kit	Amersham Biosciences
Nitrocellulose	Schleicher and Schuell
Nylon Membranes (Hybond-N)	Amersham Biosciences

PAGE mini-gel apparatus	Biorad
Phosphorimager	Molecular Imager FX, BioRad
Protein A Sepharose	Amersham Biosciences
ProbeQuant G-50 Micro Columns	Amersham Biosciences
Sonicator (Sonifier Cell Disruptor B-30)	Branson
E-max precision microplate reader	Molecular Devices
Western Transfer Wet Transfer Apparatus	Biorad
Whattman Paper	Whattman
X-ray Film	AGFA
X-ray developer	AGFA, Curix 60

2.4.1.4 Plasmids

pCAGIPuro – Vector containing CAG promoter (Pratt *et al.*, 2000), used for gene expression in ES and differentiated cells.

pCAG-E2F4 – Constructed by subcloning an EcoR1 fragment from pCMV-HA-E2F4 (Dr P. Cartwright) into pCAGIPuro.

pCAG-DP2 – Constructed by subcloning an EcoR1 fragment from pCMV-HA-DP-2 (Dr P. Cartwright) into pCAGIPuro.

pCAG-p107WT – Constructed by subcloning a Xho1 fragment from pSRα-p107WT-HA (Ashizawa *et al.*, 2001) into pCAGIPuro.

pCAG-p107ΔS/T-P – Constructed by subcloning a Xho1 fragment from pSRα-p107ΔS/T-P-HA (Ashizawa *et al.*, 2001) into pCAGIPuro.

pRL-TK – encodes the luciferase gene from Renilla reniformis (Promega), used as an internal control in transient transfection experiments.

pGL3TATAbasic-6xE2F luciferase reporter – pGL3 (Promega) containing a TATA box and six E2F binding sites 5'-TTTCGCGCTTAA-3'. Kindly provided by Dr P. Cartwright.

pGL3promoter-4xCCC luciferase reporter – four wildtype E2F binding sites upstream of a luciferase reporter (Cartwright *et al.*, 1998).

pGL3promoter-4xCAT luciferase reporter - four mutated E2F binding sites upstream of a luciferase reporter (Cartwright *et al.*, 1998).

2.4.1.5 Antibodies

Anti-Acetyl Histone H4 rabbit polyclonal	Upstate Biotechnology
Anti-E2F4 mouse monoclonal 2.12E8	Patrick Humbert
Anti-E2F4 mouse monoclonal TFE42	Kristian Helin
Anti-E2F4 rabbit polyclonal 144	Santa Cruz Biotechnology
Anti-p27 rabbit polyclonal N-20	Santa Cruz Biotechnology
Anti-p107 rabbit polyclonal C-18	Santa Cruz Biotechnology
Anti-cdc25A rabbit polyclonal C-20	Santa Cruz Biotechnology
Anti-CDK2 rabbit polyclonal M2	Santa Cruz Biotechnology
Anti-CDK4 goat polyclonal H-22	Santa Cruz Biotechnology
Anti-CDK4 goat polyclonal C-22	Santa Cruz Biotechnology
Anti-CDK6 rabbit polyclonal C-21	Santa Cruz Biotechnology
Anti-cyclin A rabbit polyclonal C-19	Santa Cruz Biotechnology
Anti-cyclin E rabbit polyclonal M-20	Santa Cruz Biotechnology
Anti-DP1 rabbit polyclonal K-20	Santa Cruz Biotechnology
Anti-DP2 rabbit polyclonal C-20	Santa Cruz Biotechnology
Anti-HA mouse monoclonal	Roche
Anti-HDAC rabbit polyclonal SSH9	Zymed Laboratories
Anti-α-Tubulin rat polyclonal	Serotec
Anti-mouse - Horseradish Peroxidase conjugated	DAKO
Anti-rabbit - Horseradish Peroxidase conjugated	DAKO
Anti-rat - Horseradish Peroxidase conjugated	DAKO

2.4.1.6 Oligonucleotides

Primer cyclin E.1:	5'-dCGTAAAAGAACACGCCCCCCG-3'
Primer cyclin E.2:	5'-dAAGCTGTGTCCGCCGCAGGCAGGCG-3'
Primer B-myb.1:	5'-dCAGAGCCAGGCCTCGCGCCTCATT-3'
Primer B-myb.2:	5'-dTCAGGACTCAGGCTGCTCGAGCCGC-3'
Primer cyclin A.1:	5'-dCCGCCCTGTAAGATTCCCGTCG-3'
Primer cyclin A.2:	5'-dAGCGTAGAGCCCAGGAGCCG-3'
Primer CDC2.1:	5'-dGGTAAAGCTCCCGGGATCCGCCAAT-3'
Primer CDC2.2:	5'-dGTGGACTGTCACTTTGGTGGCTGGC-3'
Primer Albumin.1:	5'-dGGTAAAGCTCCCGGGATCCGCCAAT-3'
Primer Albumin.2:	5'-dGTGGACTGTCACTTTGGTGGCTGGC-3'

2.4.1.7 Radiochemicals

[α-P^{32}] ATP - Geneworks (10mCi/ml, specific activity 3000Ci/mmol)

[γ-P^{32}] ATP - Geneworks (10mCi/ml, specific activity 3000Ci/mmol)

2.4.1.8 cDNA Fragments Used For Probe Synthesis

Fgf5: 800bp EcoR1/BamH1 fragment from a pBluescript KS+ plasmid containing full length mouse Fgf5 cDNA, kindly provided by Dr G. Martin.

Rex1: 848bp EcoR1 fragment from pCRTMII, kindly provided by Dr Neil Clarke.

Oct 4: 484bp Xho1/HindIII fragment from a pBluescript KS+ plasmid, kindly provided by Dr Hans Scholer.

Brachyury: 1700bp EcoR1 fragment from a pSK75 plasmid, kindly provided by Dr Bernhard G. Herrmann.

GAPDH: 300bp HindIII/Pst1 fragment from a pBluescript KS+ plasmid, kindly provided by Dr P. Rathjen.

Cyclin E: A plasmid containing a 1.8kb EcoR1 fragment of cyclin E cDNA was kindly provided by Nick Dyson.

Cyclin A: A plasmid containing a 1.7kb EcoR1 fragment of cyclin A cDNA in pBluescript SK+ was kindly provided by Dr DJ Wolgemuth.

CDC2: A plasmid containing a 1.2kb EcoR1 fragment of CDC2 cDNA was kindly provided by Nick Dyson.

B-myb: A plasmid containing a 3kb EcoR1/HindIII fragment of B-myb cDNA was kindly provided by Nick Dyson.

E2F4: A plasmid containing a 463bp Pst1 fragment of E2F4 cDNA kindly provided by Dr Joe Nevins.

2.4.2 Preparation of whole cell protein extracts

Cells were harvested and washed twice with ice-cold PBS. Cells were resuspended in 7 volumes of Cell Lysis Buffer and incubated on ice for 30 minutes with repeated pipetting. Lysates were then centrifuged at 13000rpm for 10 minutes at 4°C. After protein concentration was determined, extracts were snap frozen on dry ice and stored at -80°C.

2.4.3 Preparation of nuclear and cytoplasmic fractionated protein extracts

ES cells were grown to sub-confluence on 25cm diameter gelatinised plates (4 per prep). Cells were harvested and washed with ice-cold PBS. Twice the pellet volume of Lysis

Buffer A was carefully added to the pellet, typically 500µl Lysis Buffer A to 250µl pellet. Incubation time on ice, typically 35 minutes, was determined by the percentage of lysis by trypan blue staining, which was checked every 5 minutes after 20 minutes incubation. Lysates were then centrifuged at 1500rpm for 4 minutes at 4°C in 10ml tubes. The supernatant was retained as the cytoplasmic fraction. The pellet was washed three times with Lysis Buffer A. The pellet was resuspended in the original pellet volume (250µl) of Lysis Buffer B and incubated on ice for 30 minutes. Lysates were centrifuged at 13000rpm for 30 minutes at 4°C. The supernatant was centrifuged again at 13000rpm for 30 minutes at 4°C. The supernatant was retained as the nuclear fraction. After protein concentration was determined, extracts were snap frozen on dry ice and stored at -80°C.

2.4.4 Determination of Protein Concentration

A standard curve, consisting of 0.5, 1, 3, 4 and 5µg samples of BSA, was analysed at the same time as 1µl of each protein extract. Samples were dispensed into a 96 well tray and 200µl Bradford Reagent added. Absorbance at 595nm was determined using a UV spectrophotometer. Protein concentration was then determined using the standard curve.

2.4.5 Fractionation of whole-cell protein extracts by gel filtration

Column, buffers and equipment were equilibrated at 4°C. The Superdex 200 HR 10/30 column (Pharmacia Biotech) was calibrated using standards from a HMV Calibration Kit (10µg each protein – thyroglobulin 669kD, ferritin 440kD, catalase 232kD, lactate dehydrogenase 140kD, BSA 67kD) at a flow rate of 0.5ml/min. The void volume was 8ml. Whole cell lysates were prepared as in 2.4.2, then ultra-centrifuged at 50000rpm for 30 minutes at 4°C. 7mg lysate was fractionated in gel filtration buffer at a flow rate of 0.5ml/min, collecting 500µl fractions. In most experiments, every second fraction was analysed, except in figure 3.3.1.

The column was cleaned at room temperature with 1mg/ml trypsin in 50mM TrisHCl pH7.5 and 20mM $CaCl_2$, which was left overnight for digestion. Trypsin was washed from the column with 50ml MQ at a flow rate of 0.7ml/min. At the same flow rate, the column was washed with 50ml 1M NaOH, 50ml MQ, 30ml 0.1N HCl and 60ml MQ. The column was stored in 20% EtOH.

2.4.6 SDS-PAGE analysis

The percentage of the SDS-polyacrylamide separating gel was varied from 8-12% by altering the amount of 40% stock acrylamide used. The separating gel was composed of:

40% stock Bis-acrylamide solution (29:1); 325mM Tris pH8.8; 0.1% SDS; 0.03% APS; 0.125% TEMED. The separating gel was left to polymerise, beneath an overlay of butanol, before washing and applying the stacking gel. The stacking gel was composed of: 4% Bis-acrylamide (29:1); 125mM Tris pH6.8; 0.1% SDS; 0.06% APS; 0.1% TEMED. Minigels were run at 20mA per 0.75mm gel. Large 12% gels were run overnight at 66V in SDS-PAGE running buffer.

2.4.7 Tris-Tricine PAGE analysis

The percentage of the polyacrylamide separating gel was varied from 10-12% by altering the amount of 40% stock acrylamide used. The separating gel was composed of: 40% stock Bis-acrylamide solution (29:1); 1M Tris.Cl pH8.3; 0.075% APS; 0.125% TEMED. The separating gel was left to polymerise, beneath an overlay of butanol, before washing and applying the stacking gel. The stacking gel was composed of: 5% Bis-acrylamide (29:1); 975mM Tris.Cl pH8.3; 0.125% APS; 0.188% TEMED. Minigels were run at 80 Volts (0.75mm gels) in Tris-tricine running buffer.

2.4.8 Coomassie Gel Analysis

PAGE gels were incubated in Coomassie stain at room temperature overnight. Gels were washed for 1 hour in Coomassie destain 1, then incubated in Coomassie destain 2 until proteins were clearly visible, with low levels of background staining in the gel.

2.4.9 Western Analysis

Proteins were transferred to a nitrocellulose membrane for 1 hour at 250mA using Wet Western Transfer apparatus in Western Transfer Buffer. Upon completion of transfer, membranes were blocked in 5% non-fat milk powder in PBT for at least 1 hour at RT or overnight at 4°C. Membranes were incubated for 2 hours at RT or overnight at 4°C with primary antibody, diluted to the required concentration in 5% non-fat milk powder in PBT. Membranes were washed for at least half an hour in PBT, then incubated with the appropriate dilution of HRP-linked secondary antibody in 5% non-fat milk powder in PBT for one hour at RT. Membranes were washed for at least half an hour in PBT and then incubated for 1-3 minutes in Pierce Chemiluminescent Substrates. Membranes were then exposed to X-ray film and developed using an X-ray developer machine.

2.4.10 Immunoprecipitations

Cell lysates were adjusted to a volume of 400µl with IP buffer, then incubated with 2µg primary antibody and 25 µl 50% PAS beads for 3-4 hours at 4°C. The beads were

washed four times in IP buffer, resuspended in 30µl 2x SDS load buffer and incubated at 95°C for 2 minutes just prior to PAGE analysis

2.4.11 Kinase Assays

Cell lysates were adjusted to a volume of 400µl with cell lysis buffer and pre-cleared with 30µl of a 50 % suspension of PAS, for 30 minutes at 4°C. Cleared supernatants were incubated with 2µg primary antibody for 2-3 hours at 4°C, then with 25µl of fresh 50% PAS beads for 1 hour. The beads were washed four times in cell lysis buffer, then twice with 50mM HEPES pH7.5; 1mM DTT. Immune complexes bound to PAS were resuspended in 30µl kinase buffer, containing 10µCi of [γ-^{32}P]ATP and either 5µg of purified GST-Rb or 10µg of histone H1, and incubated at 30°C for 30 minutes. The reactions were terminated by the addition of 2x SDS load buffer, heated at 95°C for 2 minutes and resolved on a 12% SDS-PAGE gel. The activity of the immunoprecipitated kinases was quantitated from dried gels using a phosphorimager.

For experiments involving roscovitine treatment, post-washing, immune complexes were incubated with or without roscovitine in 30µl kinase buffer at 30°C for 30 minutes, prior to the kinase reaction, followed as above.

For experiments involving inhibitor treatment, post-washing, immune complexes were incubated with or without GST-p21, GST-p16 or GST in 30µl kinase buffer at 30°C for 1 hour, prior to the kinase reaction followed as above. Baculovirus-expressed, purified cyclin-CDK complexes (donated by Boris Sarcevic) were incubated with or without purified recombinant GST-p16 or GST in 20µl lysis buffer at 30°C for 1 hour. The kinase reaction was then followed as above.

2.4.12 Luciferase Assays

For transient transfections, ES cells were seeded at $7x10^4$ cells/ml ($3.5x10^4$cells/well of a 24 well tray) and grown for 8-10 hours. Triplicate wells were transfected with 100ng pGL3TATAbasic-6xE2F luciferase reporter, pGL3promoter-4xCCC luciferase reporter or pGL3promoter-4xCAT luciferase reporter and 25ng pRL-TK using the Fugene transfection method, following manufacturer's instructions. When expression vectors were included in transfections, the concentration of transfected DNA was equalised with empty pCAGiP vector (Pratt *et al.*, 2000). After 40 hours, cells were assayed for luciferase activity using the DLR luciferase kit.

For stably integrated reporters, cell pellets were incubated with Luciferase buffer (5 volumes of cell pellet) for 10 minutes on ice, with repeated pipetting. After

centrifugation at 4°C, 14000rpm, 10 minutes, 50µl lysate was dispensed into an eppendorf tube on ice. 50µl of Complete Luciferase buffer was added to each tube. Immediately prior to analysis, 50µl of 1x luciferin was added to samples. Luciferase readings were equalised according to the protein concentration of each sample.

2.4.13 Chromatin Immunoprecipitations (ChIPs)

2.4.13.1 Sonication optimisation

ES cells were grown for 2 days (approximately 10^7 cells/plate) as described in 2.3.7. Protein-protein and protein-DNA complexes were crosslinked by addition of 272µl formaldehyde to 10ml medium and incubation for 10 minutes at RT, rocking. The reaction was terminated by the addition of 1.25ml 1M glycine and incubation for 5 minutes at RT. Cells were washed twice with cold PBS, scraped from the plate and pelleted by centrifugation at 1000rpm for 4 minutes at 4°C. Cells were lysed with 200µl ChIP SDS lysis buffer for 10 minutes at 4°C. 2ml ChIP dilution buffer was then added. Samples were sonicated 4-10 times for 30 seconds each at half maximum power, incubating at 4°C for at least 1 minute between sonications. The supernatant was separated from cell debris after centrifugation at 14000rpm for 10 minutes at 4°C. 120µl 5M NaCl and 1µl 10mg/ml RNase was added to each sample, prior to incubation at 65°C for at least 4 hours. 60µl 0.5M EDTA, 120µl 1M Tris HCl pH6.5 and 12µl 10mg/ml Proteinase K was added and samples were incubated at 45°C for 1 hour. Following a phenol/chloroform extraction, DNA was precipitated with ethanol and 20µg glycogen. DNA was resuspended in 30µl MQ and 2µl of each chromatin sample was analysed on a 1% TBE-agarose gel with ethidium bromide to assess the lengths of chromatin fragments produced. As an average of 600bp fragments is optimal, sonication conditions (7x 30 seconds) producing the most consistent fragment lengths (500bp-1.5kb) was chosen.

2.4.13.2 PCR optimisation

PCRs were optimised using 0.5µl DNA from 2.4.13.1. Buffers tested were 10x PCR buffer (promega) and 10x PCR buffer (RB). PCR conditions tested included the addition of 10% glycerol or 1M betaine; altering $MgCl_2$ concentrations from 1mM to 3mM and annealing temperatures from 53°C to 65°C.

Optimised conditions: 20µl reaction containing PCR buffer (RB), 1.5mM $MgCl_2$ (except 2.5mM $MgCl_2$ for cyclin E primers), 200µM dNTPs, 50ng each primer, 1M betaine and 1.5 units of Taq polymerase (Geneworks). PCR conditions: 1 cycle: 95°C, 5 minutes;

60°C, 5 minutes; 72°C, 3 minutes, then 30-39 cycles: 95°C, 1 minute; 60°C, 2 minutes; 72°C, 1.5 minutes.

2.4.13.3 Preparation of blocked Protein A Sepharose

Protein A Sepharose was solubilised according to manufacturer's instructions and washed with 300ml MQ and 50 ml TE. 5ml 50% PAS slurry was incubated with 2.5mg sonicated herring sperm DNA and 5mg BSA overnight at 4°C. Blocked PAS was stored at 4°C.

2.4.13.4 ChIP washing optimisation

Chromatin was collected as in 2.4.13.1, fragmenting DNA with 7x 30 second sonications. Chromatin samples (700µg) were pre-cleared with 60µl blocked PAS for 30 minutes at 4°C, then incubated with 2µg primary antibody overnight at 4°C. Immune complexes were collected with 60µl blocked PAS for 1 hour at 4°C. Washing conditions tested were (1) 2x Farnham dialysis buffer, 4x Farnham IP buffer, 3 minutes each at RT (Wells *et al.*, 2000) (2) 7x RIPA buffer, 3 minutes each at 4°C (Takahashi *et al.*, 2000) (3) 1x ChIP low salt wash buffer, 1x ChIP high salt wash buffer, 1x ChIP LiCl wash buffer, 2x TE, 5 minutes each at 4°C (Upstate Biotech). Immune complexes were eluted with 250µl 0.1M $NaHCO_3$ in 1% SDS twice for 15 minutes each at RT. Eluants were combined.

To reverse crosslinks, 20µl 5M NaCl was added to each sample, prior to incubation at 65°C for at least 4 hours. 10µl 0.5M EDTA, 20µl 1M Tris HCl pH6.5 and 2µl 10mg/ml Proteinase K were added and samples were incubated at 45°C for 1 hour. Following a phenol/chloroform extraction, DNA was precipitated with ethanol and 20µg glycogen. DNA was resuspended in 30µl MQ. PCRs were conducted on 1µl DNA as in 2.4.13.2 (34 cycles) with each of the primer sets. PCR products were separated by electrophoresis on 1.5% TBE-agarose gels and visualised with ethidium bromide.

2.4.13.5 Equalising chromatin input

DNA concentrations were determined using a Varian Cary 3 Bio UV-Visible spectrophotometer. 50µg of each chromatin sample was dispensed and the volume increased to 500µl with 1% SDS. Crosslinks were reversed, protein extracted and DNA precipitated as in 2.4.13.4. DNA was resuspended in 500µl MQ (0.5µg/µl). PCRs were conducted on 1µ1 of each DNA sample as in 2.4.13.2 (30 cycles) using 2 different sets of primers. PCR products were separated by electrophoresis on 1.5% TBE-agarose gels and visualised with ethidium bromide.

2.4.13.6 ChIP assays

ES cells and EPL cells were grown for 2 days (approximately 10^7 cells/plate) as described in 2.3.7. Embryoid bodies were grown as described in 2.3.8. Approximately 8-10 x 10^7 cells (as ES and EPL cells and embryoid bodies) were harvested to collect enough chromatin to conduct 5 sets of ChIP assays. Protein-protein and protein-DNA complexes were crosslinked by addition of 272µl formaldehyde to 10ml medium and incubation for 10 minutes at RT rocking. The reaction was terminated by the addition of 1.25ml 1M glycine and incubation for 5 minutes at RT. Cells were washed twice with cold PBS. Adherent cells were scraped from the plate and pelleted by centrifugation at 1000rpm for 4 minutes at 4°C. Cells (approximately $2x10^7$ cells per tube) were lysed with 200µl SDS lysis buffer for 10 minutes at 4°C. 2ml ChIP dilution buffer was then added. Samples were sonicated 7 times for 30 seconds each at half maximum power. The supernatant was separated from cell debris after centrifugation at 14000rpm for 10 minutes at 4°C. Chromatin extracts were stored at -20°C until used. When all chromatin extracts were collected, the chromatin input was equalised as described in 2.4.13.5. Chromatin extracts (700µg) were pre-cleared with 60µl blocked PAS for 30 minutes at 4°C, then incubated with 2µg primary antibody overnight at 4°C. Immune complexes were collected with 60µl blocked PAS for 1 hour at 4°C. Immune complexes bound to PAS were washed at 4°C (5 minutes each) once with ChIP low salt wash buffer, once with ChIP high salt wash buffer, once with ChIP LiCl wash buffer and twice with TE. Immune complexes were eluted with 250µl 0.1M $NaHCO_3$ in 1% SDS twice for 15 minutes each at RT. 20µl 5M NaCl was added to combined eluants and incubated at 65°C overnight. 10µl 0.5M EDTA, 20µl 1M Tris HCl pH6.5 and 2µl 10mg/ml Proteinase K were added and samples were incubated at 45°C for 1 hour. Following a phenol/chloroform extraction, DNA was precipitated with ethanol and 20µg glycogen. DNA was resuspended in 30µl MQ. PCRs were conducted on 1µl DNA as in 2.4.13.2 . To ensure the PCR was exponentially increasing, PCRs were conducted for 30, 33, 36, 39 cycles with the first and last sample of the differentiation before conducting PCRs on all precipitated products using the primers described in 2.4.1.6. PCR products were separated by electrophoresis on 1.5% TBE-agarose gels and visualised with ethidium bromide.

2.4.14 Flow Cytometry

In order not to lose any apoptotic cells, the cell medium and PBS wash were retained. Cells were incubated with 2ml of Trypsin-EDTA, then centrifuged at 1200rpm for 4

minutes. Cell pellets were resuspended in 300µl PBS with 1% FCS. Gradually, while vortexing, 900µl ice cold 100% ethanol was added to the cell suspension. Cells were left at 4°C for at least 10 minutes for fixation, then centrifuged at 2000rpm for 5 minutes. Cell pellets were washed twice with PBS, counted and cell numbers were equalised. Cells were resuspended in 500µl PBS. Prior to flow cytometric analysis, cells were incubated with 500µg/ml RNase A and 2µg/ml propidium iodide for 15 minutes. Flow cytometric analysis was performed according to the manufacturer's specifications.

2.4.15 RNA extraction

Cells were resuspended in RNAzol (1ml per 10^7 cells), then disrupted by manual pipetting. 200µl chloroform was added, tubes shaken vigorously, then stored on ice for 5 minutes before centrifugation at 12000 rpm for 10 minutes. The top layer was transferred into a new tube with 2 volumes of isopropanol and incubated at 4°C for at least 30 minutes. Precipitated RNA was collected by centrifugation, washed with 70% ethanol, air dried at room temperature and resuspended in sterile MQ. The RNA concentration was determined using a Varian Cary 3 Bio UV-Visible spectrophotometer and equalised for each sample.

2.4.16 Northern Transfer

Gel tanks and trays were sterilised by incubation 0.1% SDS; 0.02M NaOH in water. 1% agarose gels were set containing 1x MOPS and 7.2% formaldehyde. Prior to electrophoresis, 20µg RNA (volume no greater than 10µl) was denatured in 3µl 10x MOPS, 10µl formamide, 3.5µl formaldehyde, 0.5µl ethidium bromide and 7µl 5x RNA load buffer at 65°C for 5 minutes. Gels were run at 70V for 2 hours in 1 x MOPS running buffer. RNA was briefly visualised under UV light to ensure RNA remained intact. Prior to transfer, gels were washed twice for 10 minutes in sterile MQ. RNA was then transferred to Hybond-N nylon Membrane using capillary transfer in 20x SSC overnight. RNA was then covalently cross-linked to the membrane by irradiation with 1200mJoules of UV radiation in a Stratagene UV Stratlinker 1800.

2.4.17 Preparation of [^{32}-P]-Labelled DNA Probes

Isolated cDNA sequences were labelled with [α-^{32}P]-dATP using the Megaprime DNA labelling kit. Approximately 150ng DNA, in a volume of 21µl, was incubated with 5µl Primer Solution at 95°C for 5 minutes. At room temperature, 4µl of each of dGTP, dCTP, dTTP; 5µl 10x Reaction Buffer; 2µl Klenow enzyme and 5µl [α-^{32}P]-dATP were

added, then incubated at 37°C for 10 minutes. Unincorporated nucleotides were removed using ProbeQuant G-50 micro columns. Probes were denatured at 95°C for 5 minutes, then incubated on ice for 5 minutes prior to addition to the pre-hybridisation solution.

2.4.18 Hybridisation and washing

Membranes were prehybridised in Ultrahyb or Hybridisation buffer (2.4.1.2) for at least 2 hours at 42°C. [^{32}-P]-labelled DNA probe was added to the hybridisation mixture (10ng probe/ml hybridisation buffer) and incubated overnight at 42°C. Membranes were washed four times for 20 minutes in 2 x SSC, 0.1% SDS at 42°C or twice for 20 minutes in 2 x SSC, 0.1% SDS at 42°C and twice for 20 minutes in 0.2 x SSC, 0.1% SDS at 42°C. Membranes were sealed in plastic, exposed to phosphorimager screens, scanned and quantitated using a phosphorimager and Imagequant software. Membranes were stripped by covering in boiling 0.01% SDS, and left to reach room temperature.

CHAPTER THREE

Reassortment of active cyclin-CDK2 complexes during differentiation

3.1 Introduction

Dramatic proliferative changes are associated with early mouse development (Snow, 1977). Mean generation times required to account for changes in cell number decrease from 10 hours for pluripotent cells of the implanting blastocyst, to 4.4 hours as gastrulation begins (Hogan, 1994; Snow, 1977). Subsequent differentiation to mesodermal derivatives is associated with increased cell cycle length (Snow, 1977; Solter *et al.*, 1971). In particular, the decreased length of G1 phase in pluripotent cells isolated from the pre-gastrulation mouse embryo, in comparison to differentiated derivatives, suggests that one major difference is in the regulation of the G1/S transition (Solter *et al.*, 1971; Stead *et al.*, 2002). However, the regulatory mechanisms governing the mammalian embryonic cell cycle are not well understood.

The small size and relative inaccessibility of early mouse embryos leads to practical limitations in their analysis, therefore *in vitro* models systems are utilised. In terms of both gene expression and differentiation potential, embryonic stem (ES) cells are equivalent to cells of the ICM of the 4.5dpc embryo (Nichols *et al.*, 1990; Pease *et al.*, 1990; Smith *et al.*, 1988; Williams *et al.*, 1988). The rapid cell division of ES cells is associated with high levels of cell cycle-independent cyclin E and cyclin A associated kinase activity (Stead *et al.*, 2002). The decreased rates of cell division when CDK2 activity is inhibited, suggests that the rapid proliferation of ES cells may be a consequence of elevated CDK2 kinase activity (Stead *et al.*, 2002). Early primitive ectoderm-like (EPL) cells, equivalent to the primitive ectoderm of the 5.5dpc embryo (Lake *et al.*, 2000), have similarly high levels of constitutive CDK2 activity (Stead *et al.*, 2002). Differentiation of EPL cells into embryoid bodies (EPLEBs) accurately and temporally recapitulates the events associated with differentiation of ICM cells into primitive ectoderm and their subsequent differentiation, primarily to a mesodermal fate, during gastrulation (Lake *et al.*, 2000). Coincident with changes in rates of cell division and increased length of G1 phase during mouse embryogenesis (Snow, 1977; Solter *et al.*, 1971), the loss of pluripotency and formation of nascent mesoderm in EPLEB differentiation is associated with an increased proportion of cells in G1 phase and down-regulation of cyclin E-associated and overall CDK2 kinase activity (E. Stead, PhD thesis). This further supports the hypothesis that elevated CDK2 activities are involved in the rapid proliferation of pluripotent cells and that regulation of CDK2 kinase activity is associated with differentiation.

To gain some understanding of the changes in regulation and function of CDK2 activity in response to different stimuli, gel filtration analysis has been conducted (McConnell *et al.*, 1999; Musgrove *et al.*, 1998; Prall *et al.*, 1998; Prall *et al.*, 1997; Steiner *et al.*, 1995). In particular, this technique identifies the proportions of cyclins associated with active CDK complexes and enables an understanding of how the composition of active CDK complexes changes in response to different stimuli (McConnell *et al.*, 1999; Musgrove *et al.*, 1998; Parry *et al.*, 1999; Prall *et al.*, 1998; Prall *et al.*, 1997; Steiner *et al.*, 1995; Swarbrick *et al.*, 2000) or in different cell types (Sweeney *et al.*, 1998). In order to further understand the role of CDK2 activity in ES cells and to characterise changes in the biochemistry of cyclin-CDK2 complexes during differentiation, gel filtration analysis of whole cell protein from ES cells and EPLEBs was conducted.

3.2 Analysis of G1/S regulatory proteins in fractionated extracts

The increased expression of cyclin E and cyclin A in ES cells (Stead *et al.*, 2002) stimulated an interest in understanding the differences in distribution of these proteins in complexes in ES cells compared to differentiated cells. Therefore, a comparison of the elution profiles of cyclin E, cyclin A and CDK2 was conducted upon fractionation of protein extracts. ES cells were converted to EPL cells, from which EPLEBs were generated as described in 2.3.8. Northern analysis demonstrated that cells differentiated according to that previously described (Lake *et al.*, 2000) (Fig 3.2.1). ES cells, EPLEBs day 5 and mouse embryonic fibroblasts (MEFs) (2.3.4) were harvested and the whole cell protein extracted was fractionated on a Superdex 200 size exclusion column (2.4.5). Due to differences in levels of cyclin A, cyclin E and CDK2 and their associated kinase activities in ES cells, EPLEBs day 5 and MEFs (E. Stead, PhD thesis), conditions were optimised to ensure that, with equal concentrations of input protein, sufficient levels of protein and stable complexes were eluted to detect in western analysis and kinase assays in all cell types. Calibration of the column with protein standards enabled approximate molecular weights to be assigned to fractions collected. Equal volumes of fractions were used for western analysis, to analyse the protein levels of cyclins and CDKs (Fig 3.2.2). While some cyclin E protein was detected in the higher molecular weight fractions (>160kD), the majority of cyclin E was found in the lower molecular weight fractions (<160kD) in each cell type (Fig 3.2.2a). Cyclin A protein was detected in similar fractions in each cell type, with most cyclin A protein eluting in lower molecular weight fractions (<160kD) (Fig 3.2.2b). Some CDK2 protein was detected in the higher

molecular weight fractions (>160kD), however, the majority of CDK2 was associated with lower molecular weight fractions (<160kD) (Fig 3.2.2c). The elution profile of CDK2 most closely resembled that of cyclin E (Fig 3.2.2a and 3.2.2c). The CDK inhibitor p27 could not be detected in ES cells, as previously reported (Savatier *et al.*, 1996; Stead *et al.*, 2002). In EPLEB day 5 and MEFs, p27 was detected in the lower molecular weight fractions (<160kD) (Fig 3.2.2d). These data suggest that cyclin E, cyclin A and CDK2 generally associate with complexes of similar molecular weight in all cell types analysed.

3.3 Analysis of kinase activity in fractionated extracts

Due to high levels of constitutive CDK2 activity in ES cells (Stead *et al.*, 2002), it was of interest to analyse changes in composition of the active CDK2 complexes formed in ES cells compared to differentiated cells. Therefore, a comparison of the elution profiles of cyclin E, cyclin A and CDK2 associated kinase activity was conducted upon fractionation of protein extracts. Equal volumes of protein fractions collected from the size exclusion column were subjected to immunoprecipitation using antibodies directed against either the cyclin or CDK subunit and assayed for kinase activity *in vitro* using histone H1 as a substrate (Fig 3.3.1 and 3.3.2). Consistent with cyclin E protein, the majority of the cyclin E-associated kinase activity eluted in low molecular weight fractions (<160kD) in ES cells (Fig 3.3.1). In contrast to ES cells, most of the cyclin E-associated kinase activity in EPLEB day 5 and MEFs eluted in higher molecular weight fractions (>160kD) (Fig 3.3.1). In the higher molecular weight fractions, there was no significant difference in the sizes of complexes associated with cyclin E-CDK2 activity between cell types, however, there was significantly more activity eluting with these fractions in ES cells (Fig 3.3.1). This suggests that there is an increased association of active cyclin E-CDK2 complexes with higher order complexes in ES cells. The overall decrease in cyclin E-associated activity over differentiation is consistent with that previously reported (E. Stead, PhD thesis).

Consistent with cyclin A protein (Fig 3.2.2b), the majority of cyclin A-associated kinase activity eluted in lower molecular weight fractions (<160kD) in ES cells (Fig 3.3.2a). This suggests that there is a higher proportion of active cyclin A-CDK that is not associated with higher order complexes in ES cells compared to differentiated cell types. In both ES cells and EPLEB day 5, a broad peak of cyclin A-associated kinase activity

Figure 3.2.1

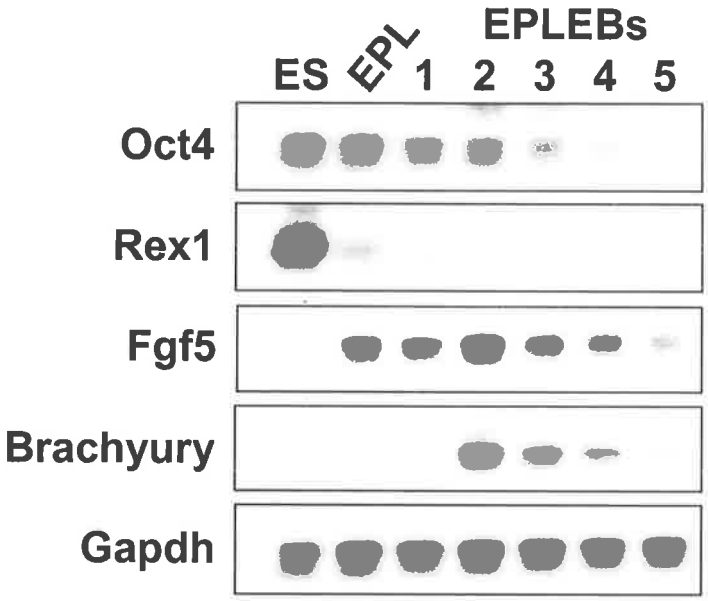


Figure 3.2.1 ES cells were differentiated into EPL cells and then EPL embryoid bodies (EPLEBs) and harvested for RNA. Total cell RNA (20μg) was resolved on a formaldehyde-agarose gel, blotted and probed with ^{32}P-labelled Rex1, Oct4, Fgf5, Brachyury and Gapdh probes, utilising Gapdh as a loading control.

Figure 3.2.2

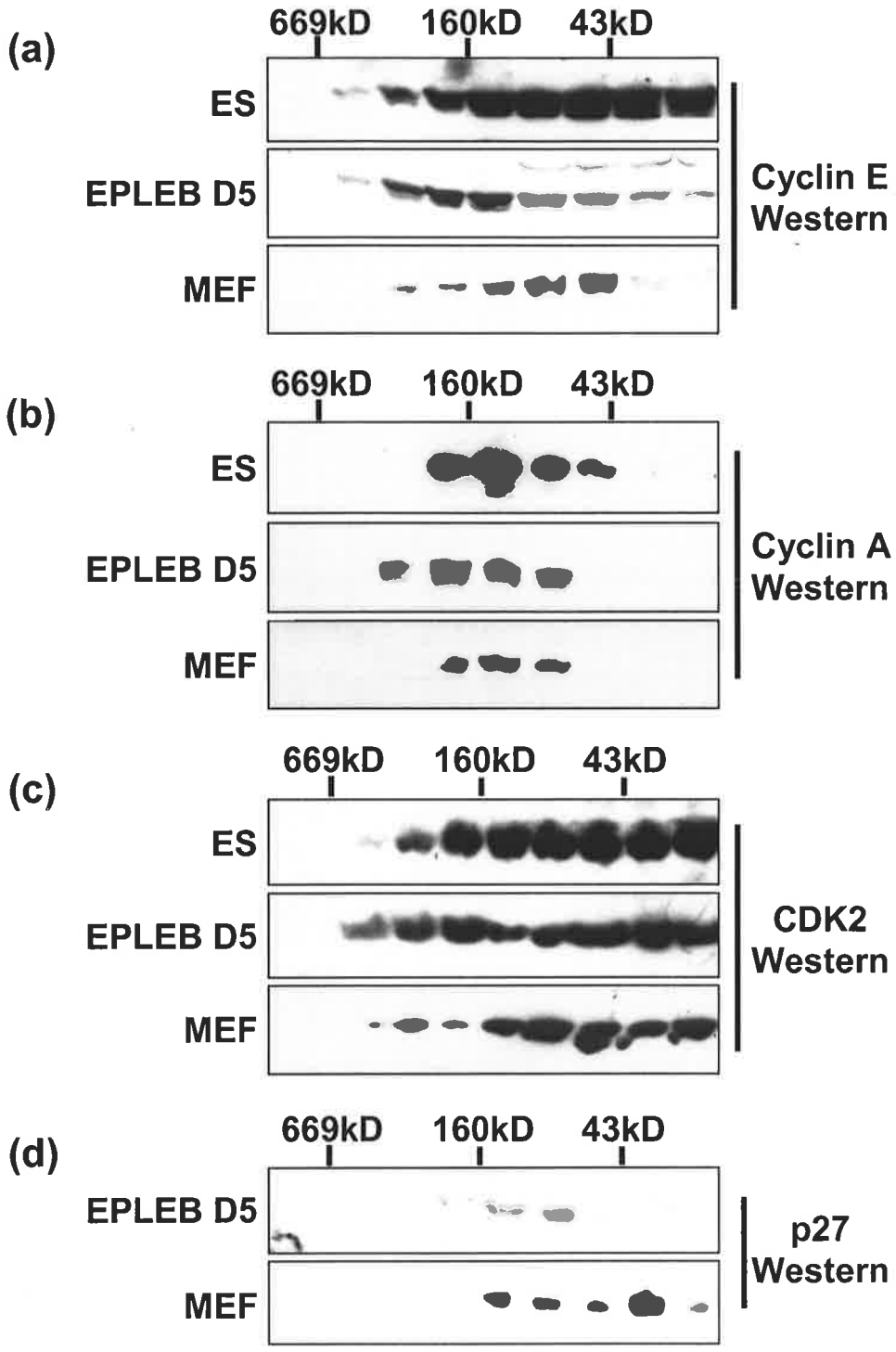


Figure 3.2.2 ES cells were differentiated into EPLEBs. ES, EPLEB day 5 and MEFs were harvested and the whole cell protein extracted was fractionated on a Superdex 200 size exclusion column. The fractions collected were allocated approximate molecular weights according to protein standards. Equal volumes of every second protein fraction was resolved on 12% SDS-PAGE gels and subjected to western analysis. Blots were probed with antibodies for cyclin E (a), cyclin A (b), CDK2 (c) and p27 (d).

Figure 3.3.1

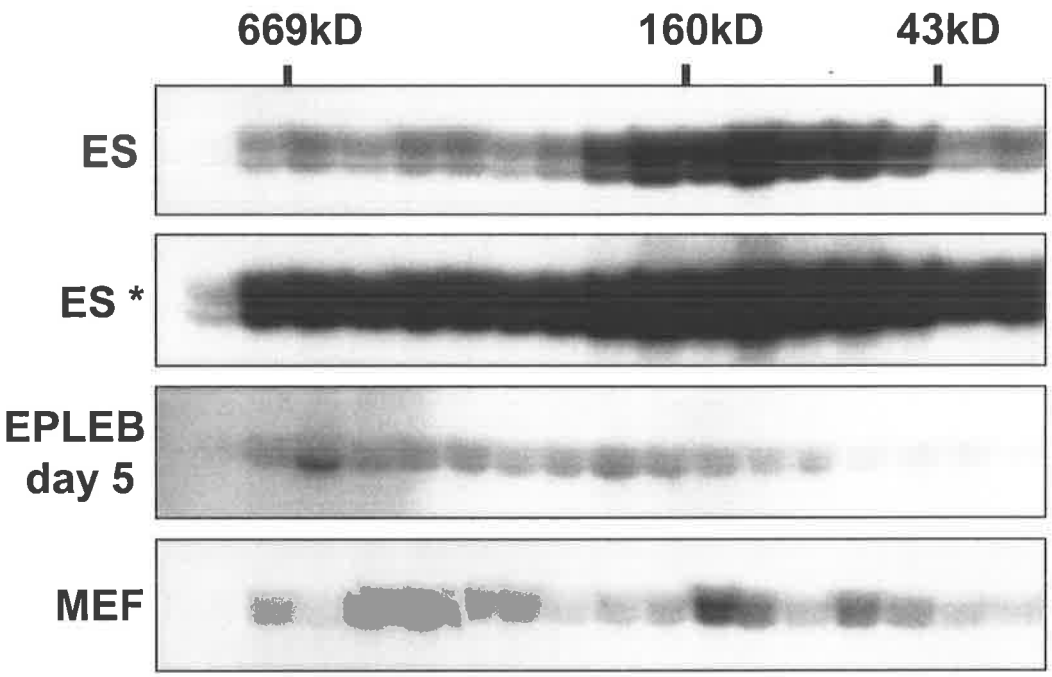


Figure 3.3.1 ES cells were differentiated into EPL embryoid bodies. ES, EPLEB day 5 and MEFs were harvested and the whole cell protein extracted was fractionated on a Superdex 200 size exclusion column. The fractions collected were allocated approximate molecular weights according to protein standards. Equal volumes of every protein fraction was subjected to immunoprecipitation with cyclin E antibodies and assayed for kinase activity *in vitro* using histone H1 as a substrate. The equivalent exposure to the EPLEB day 5 and MEF activity for ES cells is labelled ES *.

Figure 3.3.2

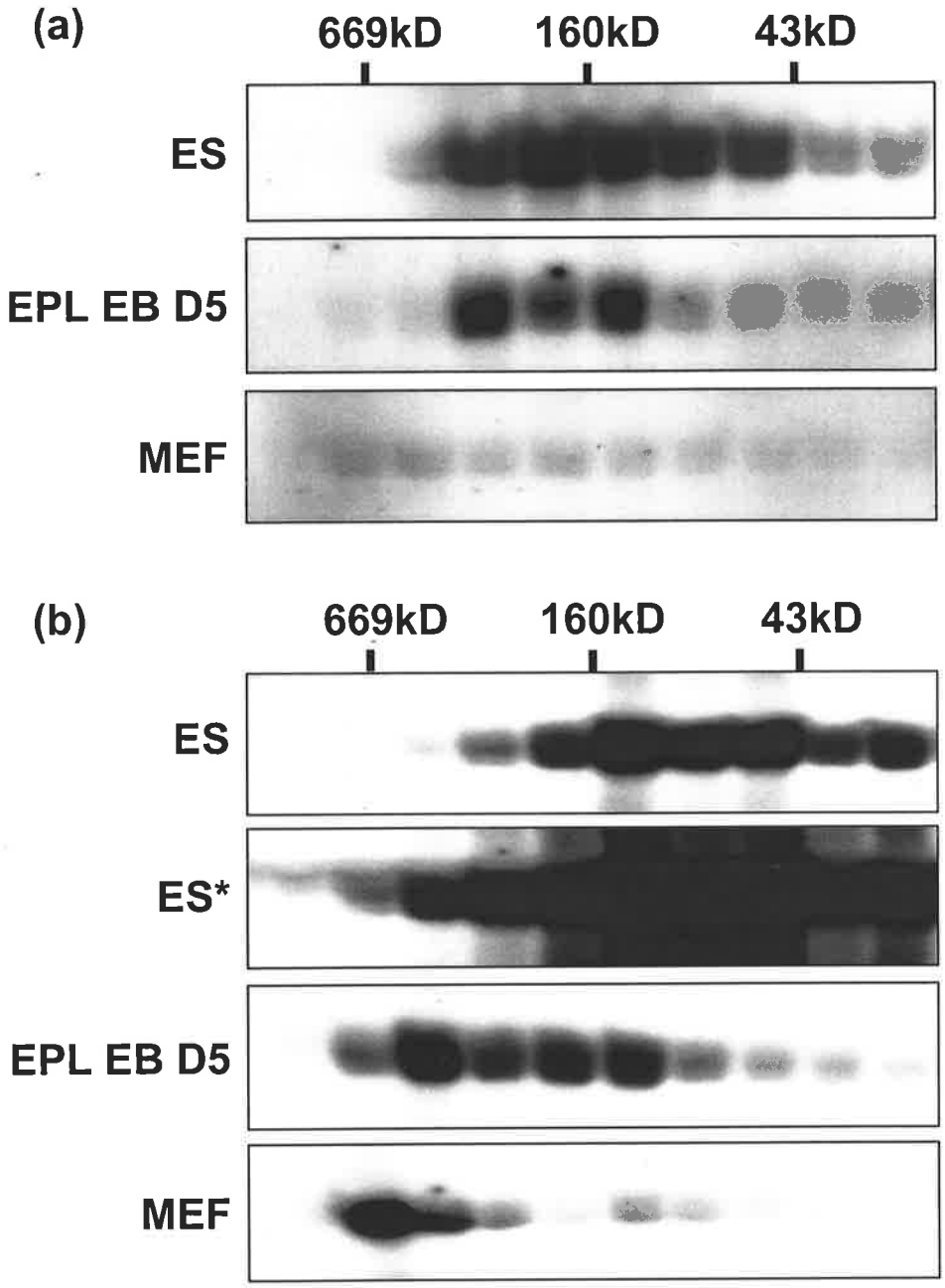


Figure 3.3.2 ES cells were differentiated into EPL embryoid bodies. ES, EPLEB day 5 and MEFs were harvested and the whole cell protein extracted was fractionated on a Superdex 200 size exclusion column. The fractions collected were allocated approximate molecular weights according to protein standards. Equal volumes of every second protein fraction was subjected to immunoprecipitation and assayed for kinase activity *in vitro* using histone H1 as a substrate. Antibodies used for immunoprecipitation were directed against cyclin A (a) and CDK2 (b). The equivalent exposure to the EPLEB day 5 and MEF activity for ES cells in (b) is labelled ES *.

was detected (approximately 160kD) (Fig 3.3.2a). In comparison, the cyclin A-associated activity detected in MEFs was similar in all fractions (Fig 3.3.2a).

In ES cells, the majority of CDK2 activity eluted in the lower molecular weight fractions (<160kD) (Fig 3.3.2b). However, there is also an increase in CDK2 eluting in high molecular weight fractions (>160kD) compared to differentiated cell types (Fig 3.3.2b). This closely corresponded with cyclin E-associated kinase activity (Fig 3.3.1 and Fig 3.3.2b), suggesting that the majority of active CDK2 is bound to cyclin E in ES cells. In both EPLEB day 5 and MEFs, CDK2 activity eluted in the higher molecular weight fractions (>160kD) (Fig 3.3.2b). This was directly comparable to the elution profiles of kinase activity associated with the CDK2 regulatory subunits (Fig 3.3.1 and Fig 3.3.2a).

3.4 Analysis of cell cycle independent cyclin E-associated kinase activity in pluripotent cells

In contrast to differentiated cell types, there is cell cycle-independent cyclin E-associated kinase activity in ES and EPL cells (Stead *et al.*, 2002). To initiate an understanding of the function of cyclin E-CDK2 throughout the ES cell cycle, changes in the biochemistry of cyclin E-CDK2 complexes in mitosis, a phase when cyclin E-CDK2 is usually inactivated, was analysed. ES and EPL cells were statically blocked in G2/M using nocodazole, a spindle depolymerisation agent. Cell cycle arrest was confirmed by flow cytometry (Fig 3.4.1a). Asynchronous and nocodazole blocked cells were harvested and the whole cell protein extracted was fractionated on a Superdex 200 size exclusion column (2.4.5). Equal volumes of protein fractions collected were subjected to immunoprecipitation using antibodies directed against cyclin E and assayed for kinase activity *in vitro* using histone H1 as a substrate (Fig 3.4.1b). Consistent with reported similarities (Stead *et al.*, 2002), the elution profiles of cyclin E-associated kinase activity were analogous between ES and EPL cells (Fig 3.4.1b). The majority of active cyclin E-CDK2 eluted in lower molecular weight fractions (<160kD) in both cell types, for asynchronous and nocodazole blocked cells (Fig 3.4.1b). However, there were differences in the elution of cyclin E-CDK2 kinase activity between asynchronous and nocodazole blocked samples for both ES and EPL cells, particularly in the higher molecular weight fractions (>160kD) (Fig 3.4.1b). This suggests that there may be specific functions for the mitotic cyclin E-CDK2 activity in pluripotent cells.

Figure 3.4.1

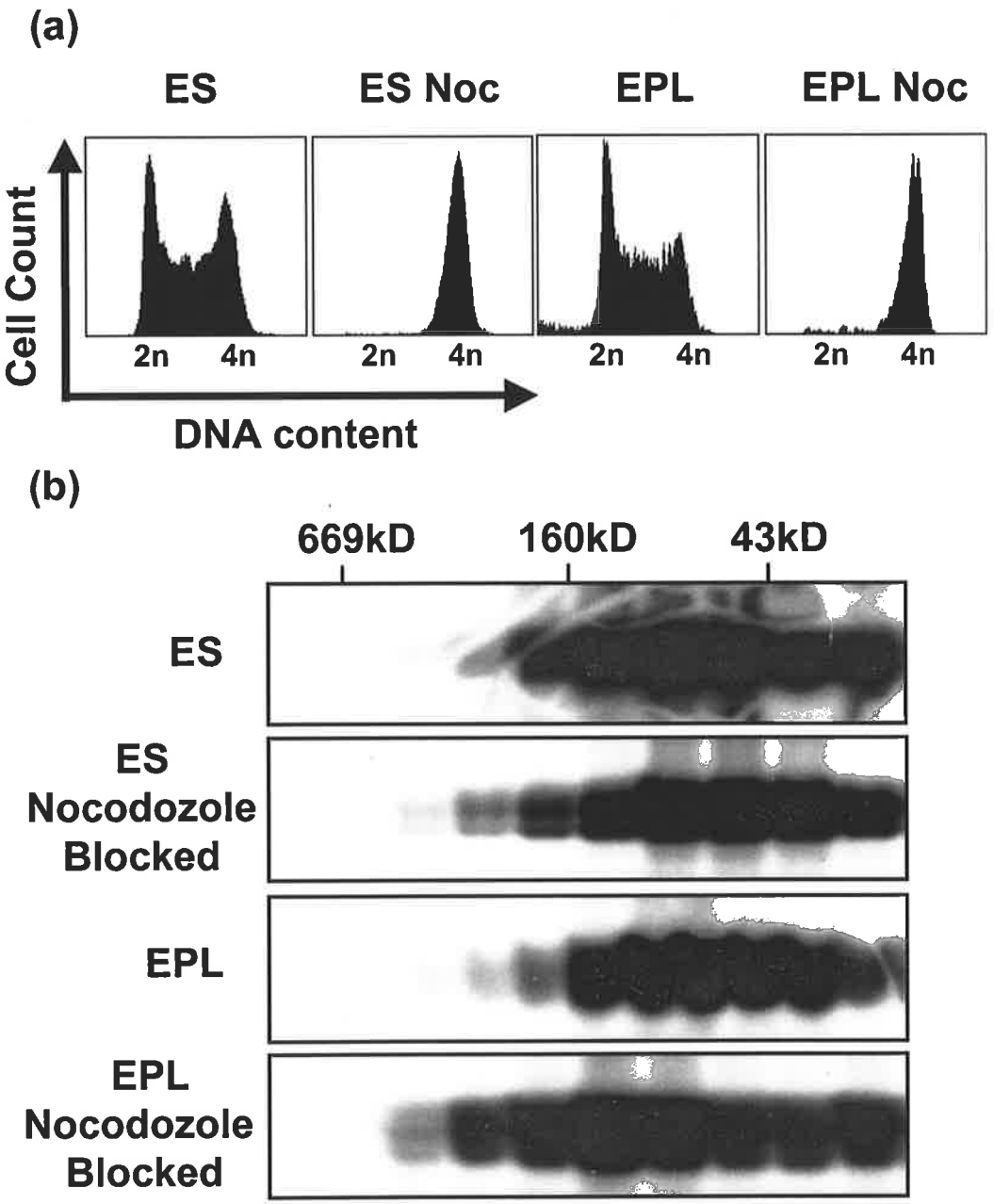


Figure 3.4.1 ES and EPL cells were statically blocked in mitosis using nocodazole. (a) Asynchronously growing and nocodazole blocked cells were harvested, stained with propidium iodide and analysed by flow cytometry. (b) Asynchronous and nocodazole blocked ES and EPL cells were harvested and the whole cell protein extracted was fractionated on a Superdex 200 size exclusion column. The fractions collected were allocated approximate molecular weights according to protein standards. Equal volumes of every second protein fraction was subjected to immunoprecipitation using antibodies directed against cyclin E and assayed for kinase activity *in vitro* using histone H1 as a substrate.

3.5 Analysis of cyclin E-associated kinase activity during differentiation

Differences detected in the elution profiles of cyclin E-associated kinase activity between ES cells and EPLEBs day 5 (Fig 3.3.1) promoted an interest in a more detailed analysis of changes in biochemistry of cyclin E-CDK2 over EPLEB differentiation. ES cells were differentiated into EPLEBs as described in 2.3.8. Northern analysis demonstrated that cells differentiated according to that previously described (Lake *et al.*, 2000) (Fig 3.2.1). Cells and bodies were harvested and the whole cell protein extracted was fractionated on a Superdex 200 size exclusion column (2.4.5). Equal volumes of protein fractions collected were subjected to immunoprecipitation using antibodies directed against cyclin E and assayed for kinase activity *in vitro* using histone H1 as a substrate (Fig 3.5.1). To clearly identify differences in the elution profiles of cyclin E-CDK2 activity, equivalent exposures are not represented. ES cells, EPL cells and EPLEBs day 2 had very similar elution profiles, with the majority of active cyclin E-CDK2 eluting in the lower molecular weight fractions (<160kD) (Fig 3.5.1). Concomitant with decreased cyclin E-CDK2 activity in EPLEB differentiation (E. Stead, PhD thesis), active cyclin E complexes were reassorted (Fig 3.5.1). The overall proportion of lower molecular weight (<160kD), active cyclin E-CDK2 complexes decreased on EPLEB day 3. By EPLEB day 4, there was a dramatic decrease in the proportion of lower molecular weight active cyclin E-CDK2 complexes. By EPLEB day 5, active cyclin E-CDK2 complexes were almost exclusively observed in higher molecular weight fractions (>160kD) (Fig 3.5.1). These data show that differentiation of ES cells is associated with reassortment of cyclin E-CDK2 complexes.

3.6 Summary

The high levels of cyclin E-CDK2 activity and the role of this activity in driving the rapid proliferation of ES cells (Stead *et al.*, 2002) directed an interest in analysing the role of cyclin-CDK2 activity in ES cells. The work presented in this chapter is an initial characterisation by gel filtration analysis of the biochemistry of cyclin-CDK2 complexes in ES cells and during differentiation. Limitations of this work come mainly from the potential disruption of complexes during extraction and fractionation of proteins. However, many attempts to stabilise complexes were taken, such as mild buffers and all steps conducted at 4°C. Also, to attain sufficient concentrations of active complexes for westerns and kinase assays in all cell types, a high concentration of protein was required. Since CDK2 kinase activity is detected in fractions with apparent molecular weights lower than that of active CDK2 complexes, it is likely that the column has been

Figure 3.5.1

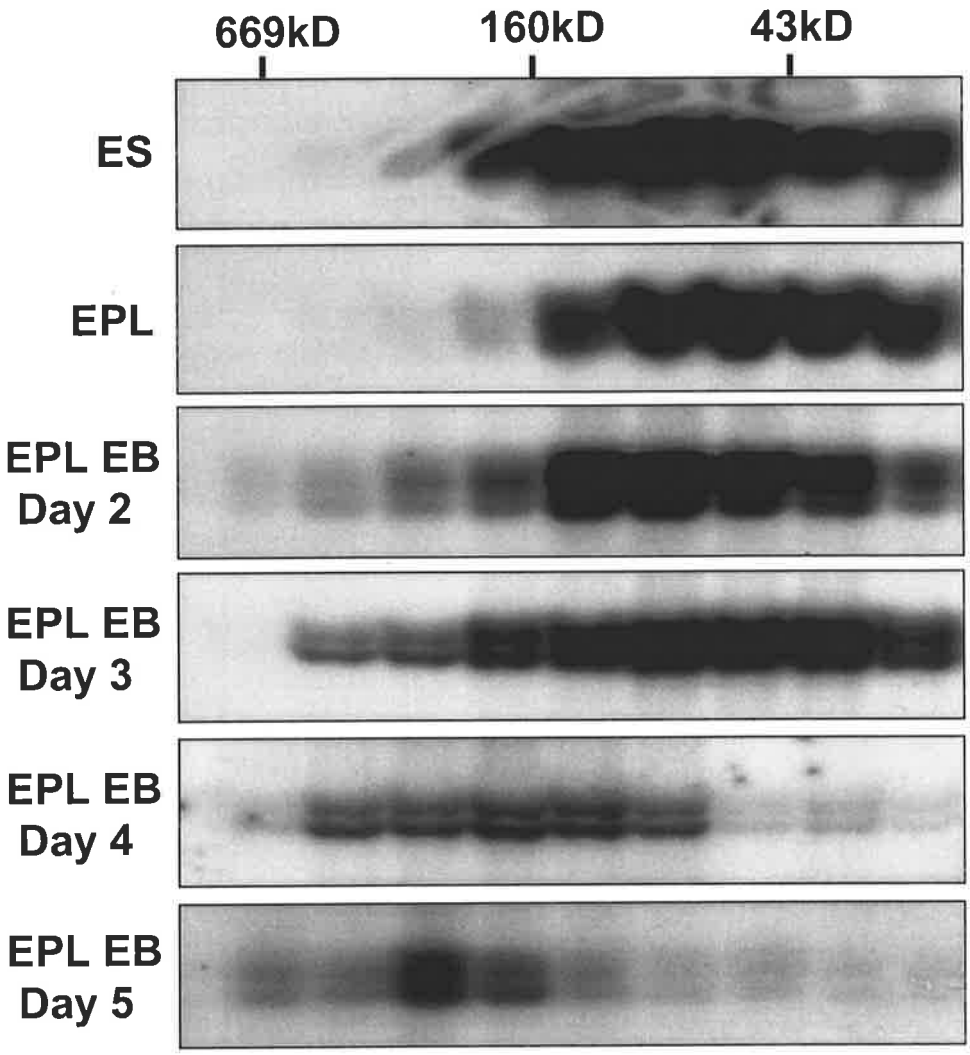


Figure 3.5.1 ES cells were differentiated into EPL embryoid bodies. ES, EPL and EPLEBs were harvested and the whole cell protein extracted was fractionated on a Superdex 200 size exclusion column. The fractions collected were allocated approximate molecular weights according to protein standards. Equal volumes of every second protein fraction collected from the size exclusion column were subjected to immunoprecipitation using antibodies directed against cyclin E and assayed for kinase activity *in vitro* using histone H1 as a substrate. To highlight differences in elution profiles equivalent exposures are not represented - ES, EPL and EPLEB days 2 and 3 are similar exposures, whereas EPLEBs days 4 and 5 are darker exposures.

overloaded. Therefore, the protein standards only provide an estimate of molecular weights eluting in each fraction. In order to combat this problem, a larger column, such as a Superdex 200 16/60, could be used.

These data indicate that the majority of cyclin E and CDK2 protein and cyclin E-CDK2 activity elutes in similar, lower molecular weight fractions in ES cells, suggesting that it is likely that most cyclin E and CDK2 protein is in discrete, active complexes in ES cells. However, not all cyclin E-CDK2 complexes are active in ES cells, as determined by Cdc25 activation assays (Stead *et al.*, 2002). In contrast, the majority of cyclin E-CDK2 activity was found in high molecular weight fractions in differentiated cell types, whereas the majority of cyclin E was found in lower molecular weight fractions. This suggests that in differentiated cells the majority of cyclin E protein was either not complexed to CDK2 or within inactive cyclin E-CDK2 complexes. This is consistent with reports from other cell types, indicating that a small proportion of cyclin E accounts for most of the associated kinase activity (Musgrove *et al.*, 1998; Prall *et al.*, 1997). Therefore, in addition to the increased levels of cyclin E protein compared to differentiated cell types (E. Stead, PhD thesis), there is an increase in the overall proportion of cyclin E in active complexes in ES cells. This may be due to increased activation of cyclin E-CDK2 by phosphorylation or the lack of CKIs in ES cells. Accordingly, the elution of CKIs in the lower molecular weight fractions of differentiated cell types suggests that the decreased CDK activity in these fractions may be due to association with a CKI, as the apparent molecular weight of a cyclin E-CDK2-CKI complex would be in these fractions (<160kD). The function of the high levels of discrete, active cyclin E-CDK2 complexes in ES cells may be to enable phosphorylation of any substrates with which active cyclin E-CDK2 complexes only transiently associate. Alternatively, it may provide a pool of active cyclin E-CDK2 complexes enabling rapid association with any newly synthesised substrates.

In addition, these data indicate that there is increased cyclin E-CDK2 activity associated with higher order complexes in ES cells compared to differentiated cell types. This suggests that cyclin E-CDK2 is acting on a greater proportion of its targets in ES cells. Since the primary function of cyclin E-CDK2 is to associate with substrates, modify their activity and promote proliferation, an increased association with substrates would aid in the rapid proliferation of ES cells. Further analysis should be conducted to identify

proteins in these active complexes and assess their role in ES cell maintenance and proliferation. Candidates include the RB family, with which cyclin E-CDK2 associates and enables E2F-dependent transcription of many genes involved in cell cycle progression, differentiation and development (Hatakeyama *et al.*, 1994; Hinds *et al.*, 1992; Kelly *et al.*, 1998; Lundberg and Weinberg, 1998; Muller *et al.*, 2001; Polager and Ginsberg, 2003; Polager *et al.*, 2002; Ren *et al.*, 2002). Also, active cyclin E-CDK2 associates with CDC6 and CDC45 to regulate initiation of DNA replication (Arata *et al.*, 2000; Coverley *et al.*, 2002; Herbig *et al.*, 2000; Jiang *et al.*, 1999; Mimura and Takisawa, 1998). Similarly, active cyclin E-CDK2 associates with proteins involved in the modulation of chromatin, such as p300/CBP (Ait-Si-Ali *et al.*, 1998), SWI/SNF (Shanahan *et al.*, 1999) and p220/NPAT (Ma *et al.*, 2000; Zhao *et al.*, 1998; Zhao *et al.*, 2000). Identification of novel higher order complexes containing cyclin E-CDK2 should also be conducted to increase the understanding of the function of cyclin E-CDK2 activity.

In contrast to cyclin E-associated kinase activity, the elution profiles of cyclin A-associated kinase activity were similar between ES cells and EPLEB day 5. Therefore, in addition to similarities in the overall levels of cyclin A-associated activity (E. Stead, PhD thesis), the proportion of higher order complexes with cyclin A-associated activity are likely to be similar between ES cells and EPLEB day 5. Minor differences in elution of cyclin A-associated activity may be due to some reassortment of complexes or to changes in activity of complexes, due to activation by phosphorylation or CKIs, or due to changes in cyclin A-CDC2 activity. In comparison, while the complexes formed are likely to be similar, there is less cyclin A-associated kinase activity in higher order complexes in MEFs, suggesting that proportionally fewer cyclin A targets are regulated in MEFs. This highlights differences in the regulation of the cell cycle between early embryonic and more differentiated cell types. Similar to cyclin E-CDK2, cyclin A-associated kinase activity is involved in regulating cell cycle progression, therefore increased association with targets is likely to aid in proliferation. Candidates include proteins involved in DNA synthesis, such as Proliferating Cell Nuclear Antigen (PCNA) (Koundrioukoff *et al.*, 2000) and regulators of transcription, such as p107 and p130 (Adams *et al.*, 1996; Ewen *et al.*, 1992; Lacy and Whyte, 1997; Zhu *et al.*, 1995b), Sp1 (Haidweger *et al.*, 2001) and B-myb (Lane *et al.*, 1997; Sala *et al.*, 1997; Saville and Watson, 1998). Also, cyclin A-CDK2 regulates proteins involved in preventing reinitiation of replication, such as

inactivation of CDC6 (Petersen *et al.*, 1999), Mcm proteins (Ishimi and Komamura-Kohno, 2001; Ishimi *et al.*, 2000) and Flap endonuclease 1 (Henneke *et al.*, 2003). Additionally, cyclin A-CDK2 regulates mitotic targets, such as the ubiquitin ligase Cdh1 (Sorensen *et al.*, 2001) and lamin B (Horton and Templeton, 1997). With inhibition of CDK2 activity in ES cells leading to decreased progression through all cell cycle phases (Stead *et al.*, 2002), it is likely that regulation of these mitotic cyclin A-CDK2 targets is increased in ES cells. Confirmation of the presence of these proteins in complexes with active cyclin A should be conducted by immunoprecipitation followed by western analysis and kinase assays.

Interestingly, the elution of CDK2 activity mirrors that of cyclin E not cyclin A, suggesting that most CDK2 activity in ES cells is attributable to cyclin E association. This may be due to increased complex formation between cyclin E-CDK2 compared to cyclin A-CDK2 or due to a specific increase in the activity of cyclin E-CDK2 complexes. Depletion analysis should be used to assess differences in the association of CDK2 with cyclin subunits. Differences in association with CDK2 would be expected, since cyclin A also associates with CDC2, which would decrease the pool of cyclin A to complex with CDK2. Importantly, these data suggest that in ES cells there is an increase in CDK2 activity associating with higher order complexes. This would lead to increased regulation of substrates by CDK2 and further supports the hypothesis that it is increased levels of CDK2 activity that is driving the rapid proliferation of ES cells.

The cell cycle-independent cyclin E-associated kinase activity detected in ES and EPL cells (Stead *et al.*, 2002) suggests that this activity may have functions throughout the cell cycle. This is supported by the observation that specific inhibition of CDK2 activity inhibits all phases of the cell cycle, including mitosis (Stead *et al.*, 2002). Differences in elution of active cyclin E-CDK2 in asynchronous and nocodazole blocked fractionated extracts of both ES and EPL cells suggests that there may be specific higher order complexes formed in mitosis. This supports the hypothesis that cyclin E-CDK2 has a role in mitosis in these pluripotent cells. As suggested by analysis of knockout animals, cyclin A and cyclin E are likely to have overlapping roles at least in early embryogenesis (Geng *et al.*, 2003; Murphy *et al.*, 1997; Parisi *et al.*, 2003). While there is high cyclin A-associated activity in ES cells (Stead *et al.*, 2002), there still may be some overlap in the function of active cyclin A and cyclin E complexes in ES cells. This may include specific

functions of cyclin A-associated activity or an increase in cyclin E-CDK2 regulation of functions that are known to overlap. For example, both act to regulate centrosome duplication (Matsumoto *et al.*, 1999; Meraldi *et al.*, 1999; Okuda, 2002; Okuda *et al.*, 2000; Tokuyama *et al.*, 2001). Confirmation of the role of cyclin E-CDK2 in mitosis should be conducted, by analysis of the association between mitotic CDK targets and active cyclin E complexes.

Differentiation of ES cells into EPLEBs is characterised by reassortment of cyclin E-CDK2 complexes, suggesting that the function of cyclin E-CDK2 changes with differentiation. Coincident changes in cell cycle structure and decreases in cyclin E-CDK2 activity associated with differentiation suggest that cyclin E-CDK2 plays a role in regulating proliferative changes that occur with differentiation (E. Stead, PhD thesis). The decreased proportion of cyclin E-CDK2 activity in lower molecular weight fractions suggests there are less discrete, active cyclin E-CDK2 complexes available as ES cells differentiate. Accordingly, decreasing the pool of active cyclin E-CDK2 to transiently associate with substrates or enable rapid association with substrates is likely to decrease the rapid progression through the cell cycle. These differences may be attributed to decreased cyclin E transcription (E. Stead, PhD thesis), increased cyclin E degradation, increased CKI expression (R. Faast, unpublished data) or increased association of cyclin-CDK complexes with CKIs. While there may not be different higher order complexes formed in ES cells compared to differentiated cells, the overall decrease in the levels of cyclin E-CDK2 associating with these complexes suggests there is decreased regulation of cyclin E-CDK2 substrates in differentiated cells. Accordingly, since most identified cyclin E-CDK2 substrates are involved in the G1/S transition, delays in progression into S phase would be predicted. This is reflected in the cell cycle structure, which is remodelled at this time with an increase in the length of G1 phase (E. Stead, PhD thesis).

CHAPTER FOUR

Analysis of CDK4 and CDK6 activity in embryonic stem cells and differentiation

The results presented in this chapter are represented in the published work entitled, Cdk6-cyclin D3 activity in murine ES cells is resistant to inhibition by p16(INK4a). Oncogene, 2004 Jan; 23 (2): 491-502. Faast, R., White, J., Cartwright, P., Crocker, L., Sarcevic, B. and Dalton, S.

4.1 Introduction

The fundamental role of cyclin D-CDK4/6 activity is the integration of extracellular signals into control of the cell cycle. Growth factor and anti-mitogenic signalling pathways control most levels of regulation of cyclin D-CDK4/6 activity, including cyclin D transcription and degradation, cyclin D-CDK4 complex assembly and INK4 protein expression (Alcorta *et al.*, 1996; Cheng *et al.*, 1998; Diehl *et al.*, 1998; Filmus *et al.*, 1994; Liu *et al.*, 1995; Passegue and Wagner, 2000; Winston *et al.*, 1996; Zhu *et al.*, 1998). One of the key functions of cyclin D-associated kinase activity is the inactivation of pRB to enable E2F-dependent gene expression (Harbour and Dean, 2000). This is supported by the fact that while cyclin D-CDK4/6 complexes are essential and rate-limiting for cell cycle progression in most cell types (Baldin *et al.*, 1993; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994), cyclin D is dispensable in cells lacking pRB (Lukas *et al.*, 1995a; Medema *et al.*, 1995). Inactivation of pRB and p130 involves both cyclin E-CDK2 and cyclin D-CDK4/6 activities, whereas, while p107 is phosphorylated by CDK2 complexes, only cyclin D-CDK4/6 acts to release p107 from E2F (Beijersbergen *et al.*, 1995; Harbour *et al.*, 1999; Lundberg and Weinberg, 1998; Xiao *et al.*, 1996). A secondary function of cyclin D-CDK4 complexes is in the sequestration of CKIs, p21 and p27, to enable CDK2 complex activation (McConnell *et al.*, 1999; Mitra *et al.*, 1999).

The rapid proliferation of pluripotent ES cells appears to be differentially regulated in comparison to differentiated derivatives. In particular, high levels of constitutive CDK2 activity enable rapid proliferation of ES cells (Stead *et al.*, 2002). While cyclin D1 and D3 are expressed in ES cells, no CDK4 activity has been detected in ES cells (Savatier *et al.*, 1996). Consistent with this, ES cell cycle progression is unaffected by p16 expression (Savatier *et al.*, 1996). The elevated CDK2 activity suggests that ES cells may have a reduced requirement for cyclin D-associated activity. This is consistent with the observation that pRB is constitutively hyper-phosphorylated in ES cells (Savatier *et al.*, 1994). However, some CDK4 is complexed to cyclin D1 and D3 and a high proportion of CDK6 is complexed to cyclin D1 and D3 in ES cells (Faast *et al.*, 2004; Savatier *et al.*, 1996). Since there is no p21 or p27 expression detected in ES cells (Savatier *et al.*, 1996), any CDK complexes formed in ES cells will not be inhibited by CKIs. It is essential to confirm whether there is CDK4/6 activity in ES cells to understand the regulation of pocket proteins, E2F transcription factors and cell cycle progression in ES cells.

As ES cells differentiate by the withdrawal of LIF and the addition of retinoic acid, CDK4 activity is rapidly upregulated (Savatier *et al.*, 1996). There are no reports of analysis of the changes in CDK4/6 activity associated with culturing ES cells in the absence of LIF as embryoid bodies, which is a more accurate recapitulation of the differentiation of ICM cells prior to and during gastrulation. Predictions can be made on when CDK4 activity will be present in embryonic development from immunoprecipitation experiments from whole embryos showing the presence of cyclin D1/D2–CDK4 complexes at gastrulation and cyclin D3-CDK4 complexes after gastrulation (Savatier *et al.*, 1996). This suggests that it may be at gastrulation that cell cycle regulation is integrated with extracellular signalling pathways. In comparison to *in vitro* model systems, this coincides with a down-regulation of cyclin E-associated and overall CDK2 kinase activity and an elongation of G1 phase (E. Stead, PhD thesis). Characterising the changes in CDK4/6 activity during differentiation will elucidate differences in cell cycle regulation associated with pluripotent cell differentiation and should indicate whether this hypothesis is likely to be correct.

4.2 Analysis of CDK4/6 activity in ES cells

In order to assess CDK4/6 activity in ES cells, whole ES cell extracts were subjected to immunoprecipitation, using antibodies directed against CDK4 or CDK6, and assayed for kinase activity *in vitro* using GST-RB$^{773\text{-}928}$ as a substrate (Fig 4.2.1). CDK4 and CDK6 kinase activity was detected in ES cells (Fig 4.2.1). These data suggest that, despite previous reports (Savatier *et al.*, 1996), there is in fact CDK4 and CDK6 activity in ES cells. Due to this discrepancy, a series of inhibition experiments were conducted to confirm this observation. To assess whether the precipitated activity was CDK activity, the CKI p21, a universal inhibitor of CDK activity, was used (Harper *et al.*, 1993; Xiong *et al.*, 1993). To find the appropriate concentration of GST-p21 to use, titrations were conducted. CDK2 was immunoprecipitated from ES cell extracts and treated with 1-4μg of GST-p21 or 4ug GST, prior to assaying for *in vitro* kinase activity using GST- RB$^{773\text{-}928}$ as a substrate (Fig 4.2.2a). Inhibition of CDK2 activity was most efficient with 4μg of GST-p21. These assays were repeated with CDK2, CDK4 and CDK6 immunoprecipitations from ES cells and 4μg GST-p21 or 4μg GST. GST-p21 effectively inhibited the kinase activity of CDK2, CDK4 and CDK6 (Fig 4.2.2b). This shows that the precipitated activity is in fact CDK activity.

Figure 4.2.1

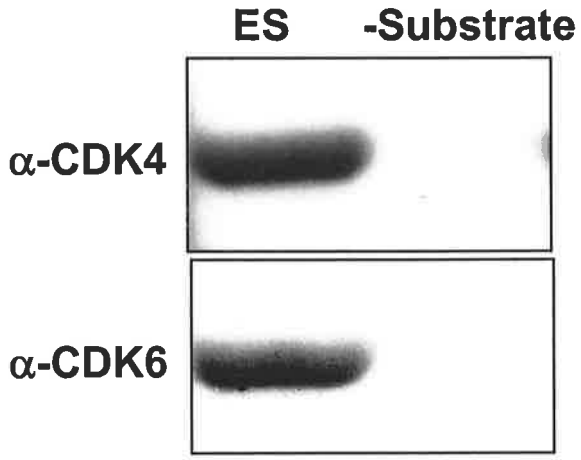


Figure 4.2.1 CDKs were immunoprecipitated from whole-cell ES lysates (400μg total protein), utilising antibodies for CDK4 and CDK6. *In vitro* kinase assays were conducted using GST-$RB^{773-928}$ as a substrate. ^{32}P-labelled GST-$RB^{773-928}$ was resolved on SDS gels, dried and exposed to X-ray film.

Figure 4.2.2

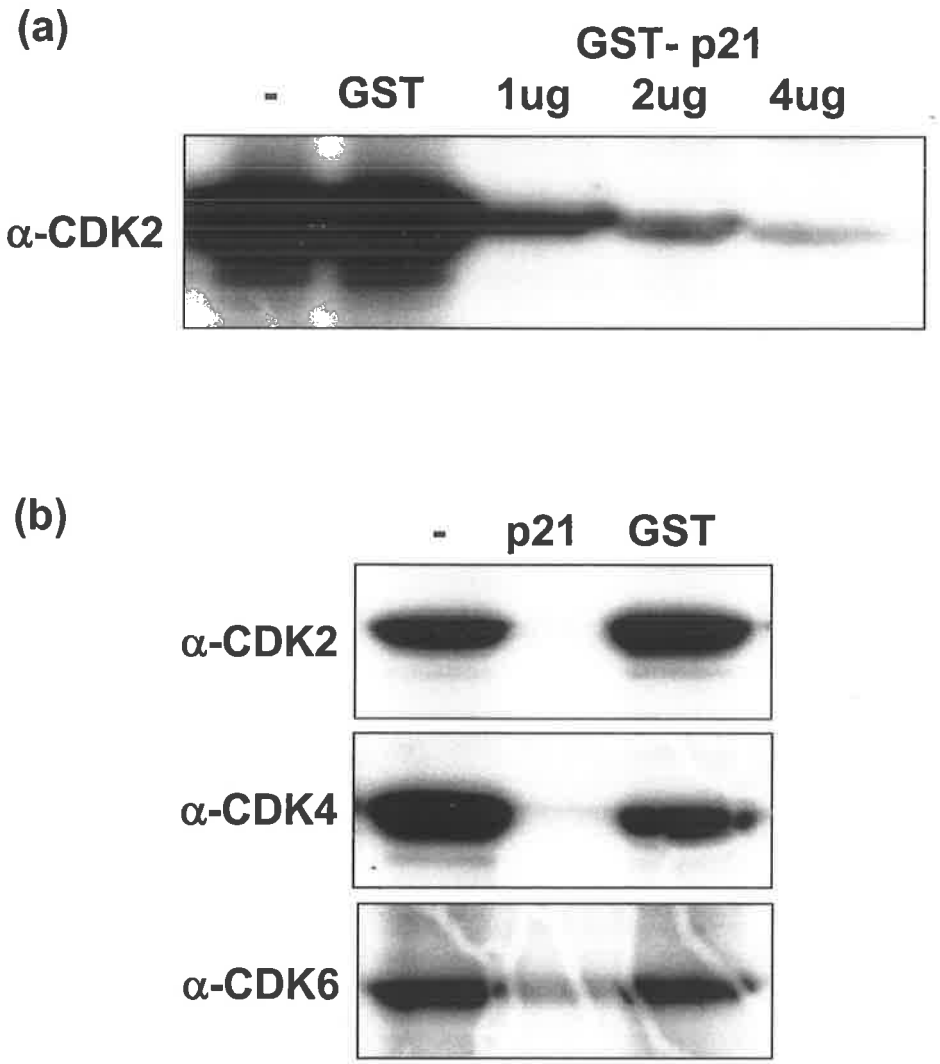


Figure 4.2.2 (a) CDK2 immunoprecipitated from whole-cell ES lysates (400μg total protein) was treated with or without 1, 2 or 4μg GST-p21 or 4μg GST. *In vitro* kinase assays were conducted using GST-$RB^{773-928}$ as a substrate. (b) CDK2, CDK4 and CDK6 were immunoprecipitated from whole cell ES lysates (400μg total protein). IPs were incubated with or without 4μg GST-p21 or GST prior to *in vitro* kinase assays using GST-$RB^{773-928}$ as a substrate. ^{32}P-labelled GST- $RB^{773-928}$ was resolved on SDS gels, dried and exposed to X-ray film.

With high levels of CDK2 activity in ES cells (Stead *et al.*, 2002), it was important to ensure that the activity immunoprecipitated with CDK4 and CDK6 antibodies was specific. The purine derivative CDK inhibitor Roscovitine specifically binds in the ATP binding pocket of the CDK (Meijer and Kim, 1997). Roscovitine inhibits CDK2 activity (IC_{50}=700nM) and CDC2 activity (IC_{50}=650nM), whereas has little effect on cyclin D1/CDK4 or cyclin D3/CDK6 activity (IC_{50}=>100μM) (Meijer and Kim, 1997). A μM range of roscovitine was analysed to distinguish between CDK2 and CDK4/6 activity. CDK2, CDK4 or CDK6 was immunoprecipitated from whole ES cell extracts. Immunoprecipitations and purified cyclin-CDK complexes produced in insect cells were treated with varying concentrations of roscovitine and assayed for *in vitro* activity using GST- $RB^{773-928}$ as a substrate (Fig 4.2.3). Immunoprecipitated CDK2 and CDK4 activity was significantly inhibited with 1μM roscovitine (Fig 4.2.3). However, purified cyclin D1-CDK4 complexes were only significantly inhibited with 10μM roscovitine (Fig 4.2.3). This suggests that the activity detected in the CDK4 immunoprecipitations is not CDK4 and may be CDK2. This was confirmed by western analysis conducted upon CDK4 immunoprecipitations (Appendix 1, R. Faast), showing cross-reactivity of the CDK4 antibody between CDK2 and CDK4. In contrast, CDK6 immunoprecipitated from ES cells was not significantly inhibited until at 25μM roscovitine (Fig 4.2.3). This was consistent with inhibition of purified cyclin D3-CDK6 complexes (Fig 4.2.3). This suggested that the CDK6 activity immunoprecipitated from ES cells was in fact attributable to CDK6. In contrast to CDK4 antibodies, there was no CDK2 immunoprecipitated with CDK6 antibodies (Appendix 1, R. Faast), however, CDK6 was efficiently immunoprecipitated (Faast *et al.*, 2004). In these assays, activity of all CDK4/6 complexes assessed was inhibited more than the reported IC_{50} values, this is likely to be due to differences in the experimental design. These experiments have shown that there is CDK6 activity in ES cells (Fig 4.2.1), however, these experiments are unable to confirm the status of CDK4 activity in ES cells.

4.3 Analysis of CDK6 activity in ES cells

Upon confirmation of the presence of CDK6 kinase activity in ES cells, it was of interest to characterise the nature of this activity. In particular, given the importance of CDK6 activity in the cell cycle dependent regulation of the pRB family, it was of interest to analyse CDK6 activity throughout the ES cell cycle. ES cells were statically blocked in G1 and S phases using aphidicolin, a DNA polymerase alpha inhibitor, and in G2 phase and mitosis using nocodazole, a spindle depolymerisation agent. Cell cycle arrest was

Figure 4.2.3

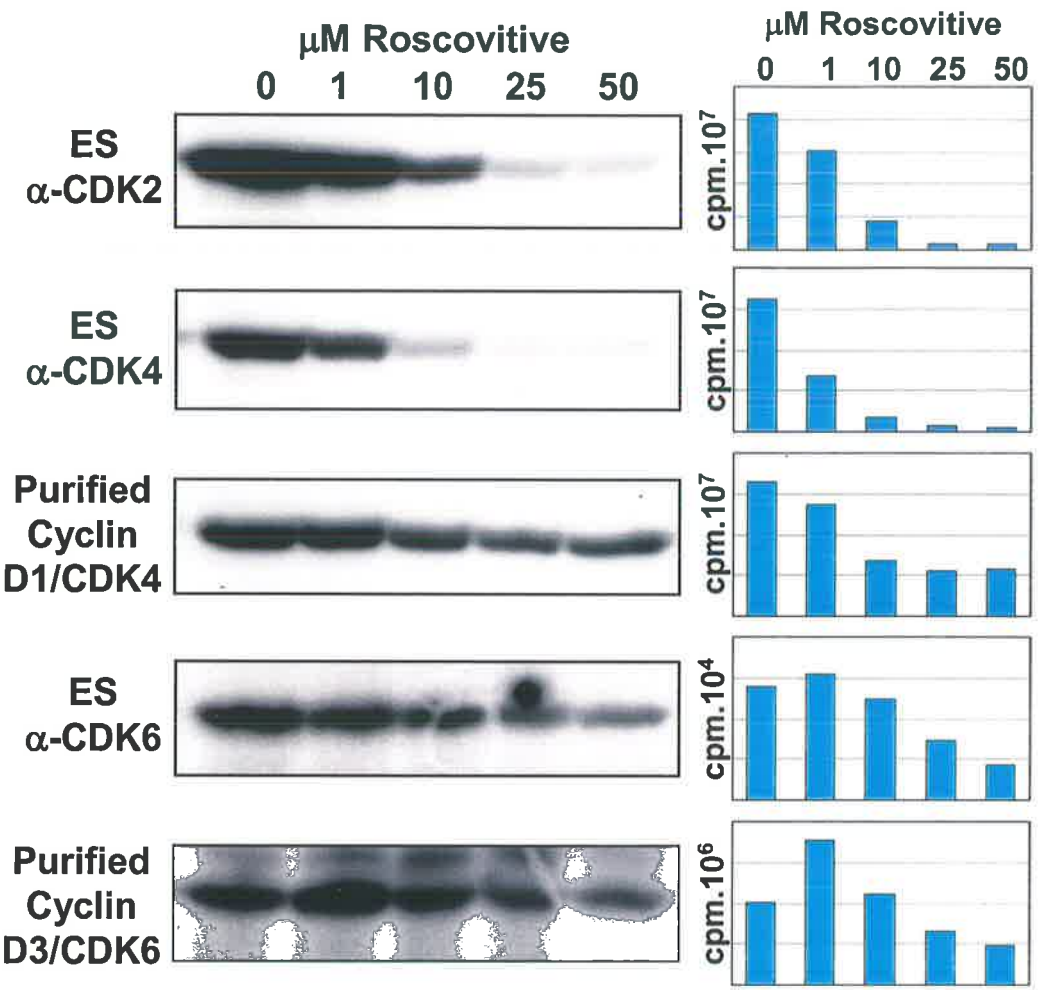


Figure 4.2.3 Recombinant cyclin-CDK complexes produced from recombinant baculoviruses in *Spodera frugiperda 9* (Sf9) insect cells were kindly donated by Boris Sarcevic. CDK2, CDK4 and CDK6 were immunoprecipitated from whole cell ES lysates (400μg total protein). Recombinant cyclin-CDK complexes and IPs were treated with a μM range of roscovitine in lysis buffer for 1 hour at 30°C. *In vitro* kinase assays were conducted using GST-$RB^{773-928}$ as a substrate. ^{32}P-labelled GST- $RB^{773-928}$ was resolved on SDS gels, dried and exposed to X-ray film. Quantitation of CDK6 kinase activity was determined by phosphorimager analysis.

confirmed by flow cytometry (Fig 4.3.1a). Whole cell protein was extracted for immunoprecipitations with CDK6 antibodies and subsequent *in vitro* kinase assays were performed, using GST- $RB^{773-928}$ as a substrate (Fig 4.3.1b). There was no significant reproducible difference in CDK6 activity between ES cells blocked in G1 and S or G2 and M phases. This suggests that CDK6 is active throughout the ES cell cycle.

In an attempt to further elucidate the role of CDK6 activity in ES cells, alternate substrates of CDKs were analysed. Previous reports demonstrate that CDK2 phosphorylates histone H1 and pRB *in vitro*, whereas CDK4/6 phosphorylates pRB with a much higher efficiency than histone H1 (Dulic *et al.*, 1992; Hinds *et al.*, 1992; Kato *et al.*, 1993; Koff *et al.*, 1991; Koff *et al.*, 1992; Matsushime *et al.*, 1994). Consistent with this, purified cyclin E-CDK2 efficiently phosphorylated both GST-$RB^{773-928}$ and histone H1, whereas purified cyclin D1/D3-CDK4 and cyclin D1-CDK6 complexes were only able to phosphorylate pRB (Fig 4.3.2). In contrast, purified cyclin D3-CDK6 complexes and CDK6 immunoprecipitated from ES cells were able to phosphorylate both pRB and histone H1 (Fig 4.3.2). This suggests that cyclin D3-CDK6 has alternate substrates compared to other CDK4/6 complexes. The similarity between purified cyclin D3-CDK6 and CDK6 immunoprecipitated from ES cells is consistent with the majority of CDK6 complexing with cyclin D3 in ES cells (Faast *et al.*, 2004). This suggests that the association of CDK subunits with different cyclins determines substrate specificity. In particular, cyclin D3 specifically alters CDK6 activity, enabling it to phosphorylate alternate targets with increased efficiency.

4.4 Analysis of p16 inhibition of CDK6 activity in ES cells

These data show that ES cells have robust, cell cycle-independent CDK6 activity. This is consistent with the observation that pRB is constitutively hyper-phosphorylated in ES cells (Savatier *et al.*, 1994). However, this is inconsistent with ES cells being insensitive to transient expression of p16 (Savatier *et al.*, 1996). To assess this discrepancy, the sensitivity of CDK complexes to *in vitro* p16 treatment was analysed. To ensure equal molar concentrations of all constituents, purified cyclin-CDK complexes were assessed on coomassie gel (data not shown). Titrations were conducted on purified complexes to assess sensitivity to GST-p16. Purified cyclin D3-CDK4, cyclin D3-CDK6 and cyclin E-CDK2 complexes were treated with 1μg or 4μg of GST-p16 or 4ug GST, prior to assaying for *in vitro* kinase activity using GST-$RB^{773-928}$ as a substrate (Fig 4.4.1). As expected, purified cyclin D3-CDK4 activity was efficiently inhibited by GST-p16,

Figure 4.3.1

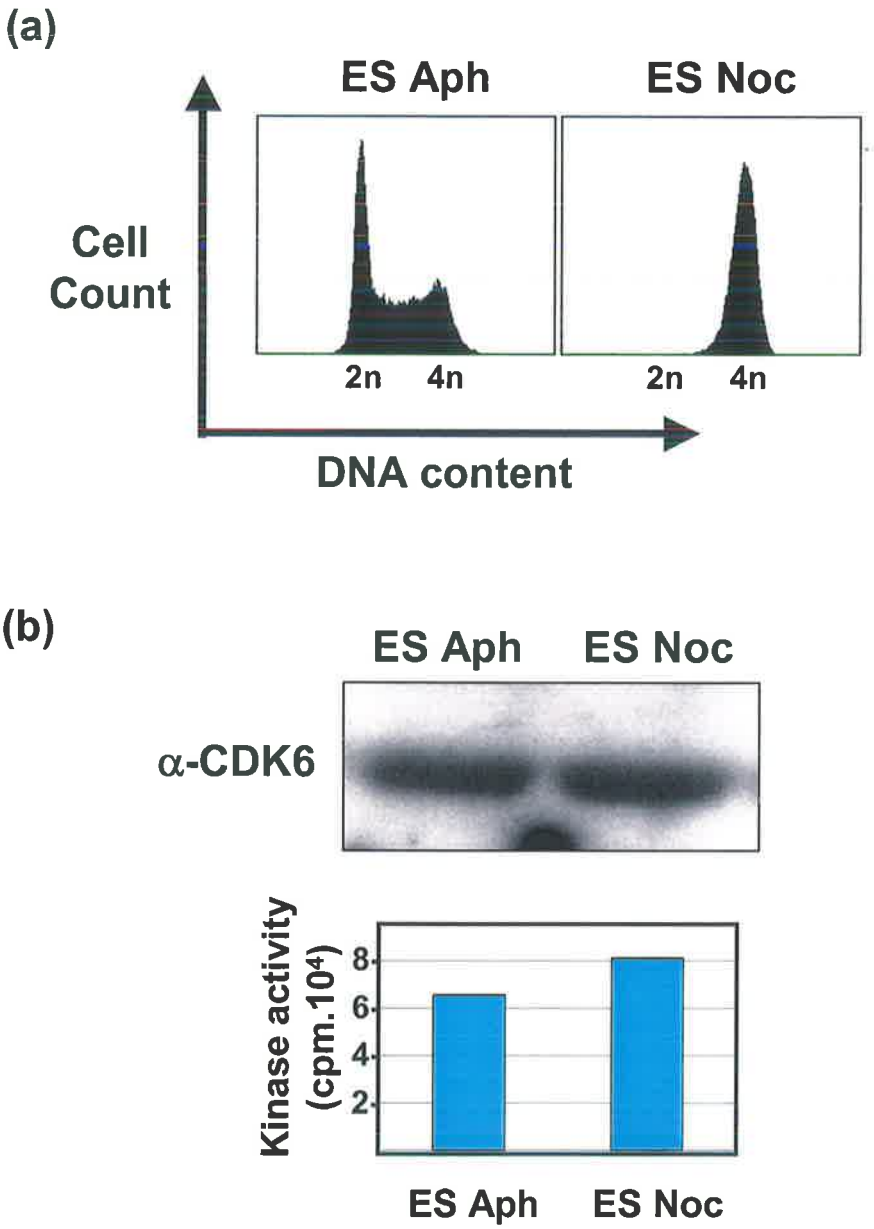


Figure 4.3.1 ES cells were statically blocked in G1/S phase using aphidicolin (Aph) and in mitosis using nocodazole (Noc). (a) Aphidicolin blocked and nocodazole blocked cells were harvested, stained with propidium iodide and analysed by flow cytometry. (b) CDK6 was immunoprecipitated from whole cell lysates (400μg total protein) and used in *in vitro* kinase assays with 5μg of purified GST-RB$^{773\text{-}928}$ as a substrate. ^{32}P-labelled GST-RB$^{773\text{-}928}$ was resolved on SDS gels, dried and exposed to X-ray film. Quantitation of CDK6 kinase activity was determined by phosphorimager analysis.

Figure 4.3.2

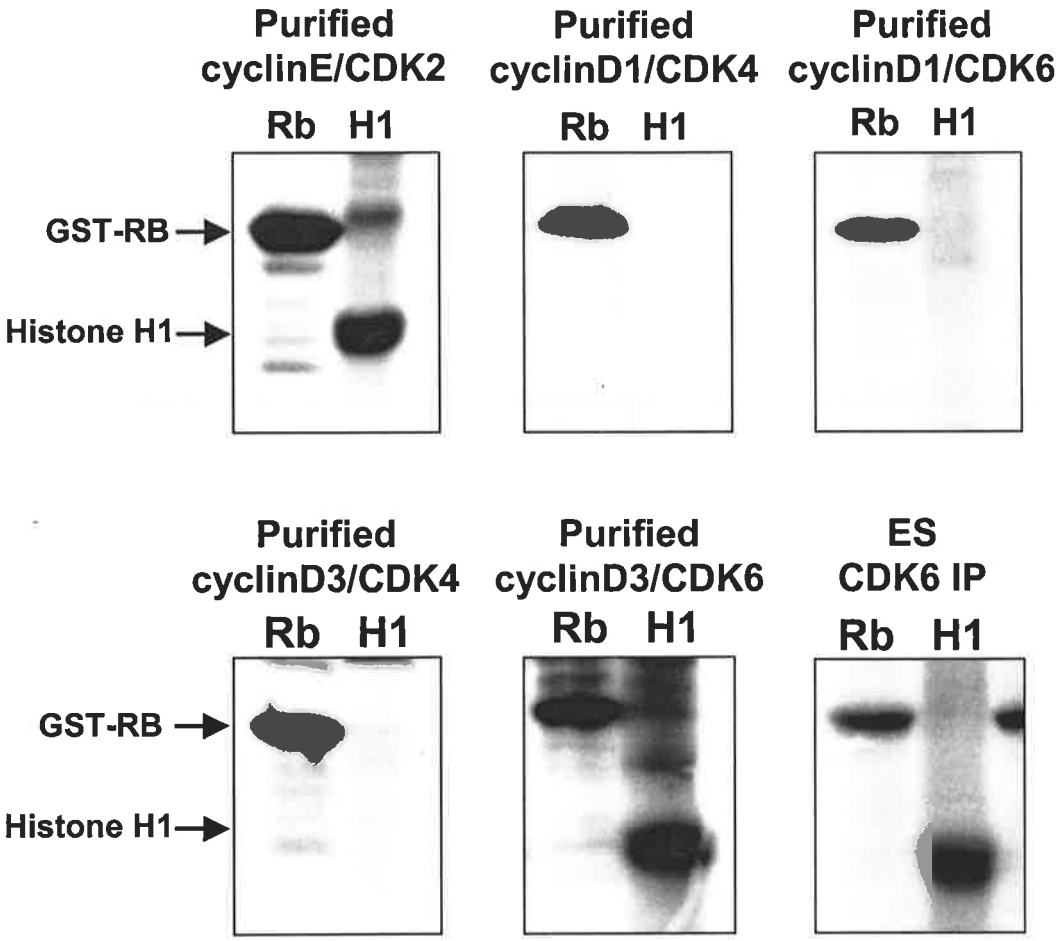


Figure 4.3.2 Recombinant cyclin-CDK complexes produced from baculoviruses in *Spodera frugiperda 9* (Sf9) insect cells were kindly donated by Boris Sarcevic. Recombinant cyclin-CDK complexes and CDK6 immunoprecipitated from ES whole-cell lysates (400μg total protein) were used in *in vitro* kinase assays. 10μg of histone H1 or 5μg of purified GST-RB$^{773\text{-}928}$ was used as a substrate. ^{32}P-labelled substrates were resolved on SDS gels, dried and exposed to X-ray film. Quantitation of CDK6 kinase activity was determined by phosphorimager analysis.

Figure 4.4.1

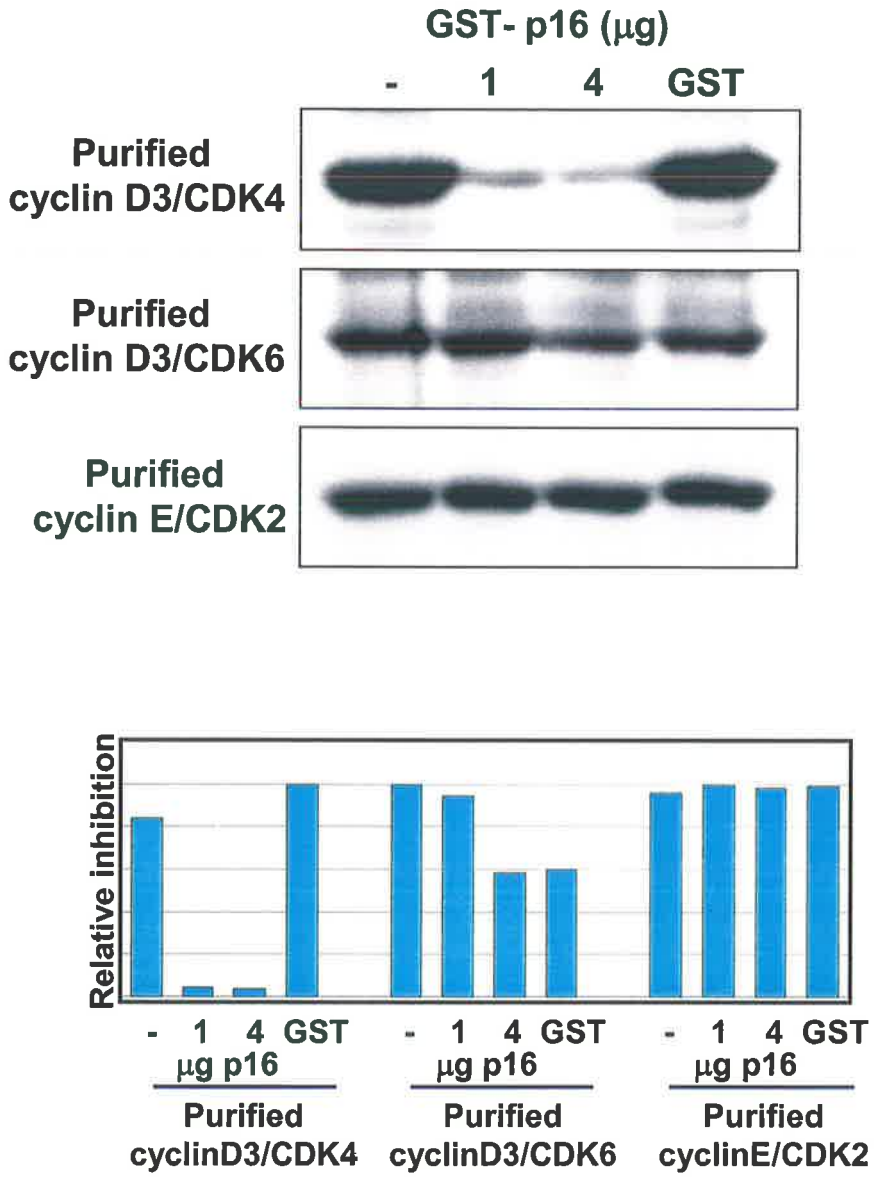


Figure 4.4.1 Recombinant cyclin-CDK complexes produced from recombinant baculoviruses in *Spodera frugiperda 9* (Sf9) insect cells were kindly donated by Boris Sarcevic. Recombinant cyclin-CDK complexes were incubated with or without GST-p16 or GST in lysis buffer for 1 hour at 30°C. I*n vitro* kinase assays were conducted using GST-$RB^{773-928}$ as a substrate. ^{32}P-labelled GST-$RB^{773-928}$ was resolved on SDS gels, dried and exposed to X-ray film. Quantitation of CDK6 kinase activity was determined by phosphorimager analysis.

whereas cyclin E-CDK2 was not. However, there was significantly less inhibition of purified cyclin D3/CDK6 complexes. These assays were repeated, treating a spectrum of purified cyclin-CDK complexes and CDK6 immunoprecipitations with 4µg GST-p16 or 4µg GST (Fig 4.4.2). As expected, cyclin E-CDK2 complexes were insensitive to p16 treatment and cyclin D1-CDK4, cyclin D3-CDK4 and cyclin D1-CDK6 activities were specifically inhibited by GST-p16. In comparison, cyclin D3-CDK6 was insensitive to GST-p16. This shows that cyclin D3-CDK6 evades inhibition by p16 *in vitro*. Similarly, CDK6 immunoprecipitated from ES cells is insensitive to GST-p16 (Fig 4.4.2). This is consistent with the observation that the majority of CDK6 is complexed to cyclin D3 in ES cells (Faast *et al.*, 2004).

To assess the sensitivity of CDK6 to p16 *in vivo*, clonal ES cell lines were used, which had stably integrated wildtype p16 or a mutant form $p16^{P114L}$, defective for CDK4/6 binding (Faast *et al.*, 2004). No effect on CDK6 activity in these cell lines was detected (Fig 4.4.3). These data show that cyclin D3-CDK6 is not inhibited by p16 *in vitro* or *in vivo*, which is consistent with ES cells being insensitive to transient expression of p16 (Savatier *et al.*, 1996).

4.5 Analysis of CDK6 activity as ES cells differentiate

ES cells were differentiated into ES and EPL embryoid bodies as described in 2.3.8. Northern analysis demonstrated that cells differentiated according to that previously described (Lake *et al.*, 2000) (Fig 4.5.1). Whole cell protein was extracted for immunoprecipitations with CDK6 antibodies and subsequent *in vitro* kinase assays were performed, using GST-$RB^{773-928}$ as a substrate (Fig 4.5.2). In both embryoid body systems, CDK6 activity was down regulated over differentiation (Fig 4.5.2). The kinetics and magnitude of down-regulation was different between the two systems. CDK6 activity in ESEBs was decreased initially between days 2-3, coinciding with increased expression of the marker for primitive ectoderm, *Fgf5* (Fig 4.5.1a and Fig 4.5.2a). The decline of CDK6 activity, by day 5 of ESEB differentiation, to the levels of MEFs coincides with the formation of nascent mesoderm, as indicated by the expression of *Brachyury* (Fig 4.5.1a and Fig 4.5.2a). Similar to ESEBs, the initial decline in CDK6 activity in EPLEB differentiation coincided with the formation of primitive ectoderm, as indicated by the expression of *Fgf5* (Fig 4.5.1b and Fig 4.5.2b). However, in contrast to ESEBs, CDK6 activity declined to the levels of MEFs by day 1 of EPLEB differentiation (Fig 4.5.1b and Fig 4.5.2b). These magnitude and temporal differences highlight the

Figure 4.4.2

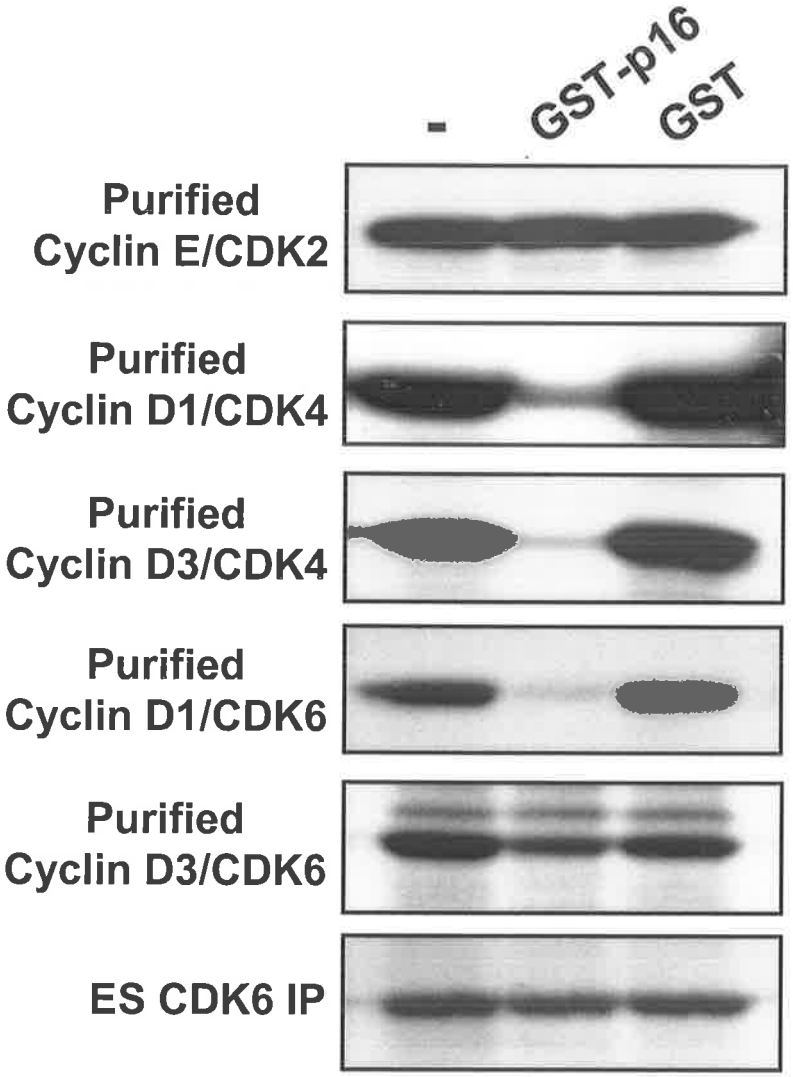


Figure 4.4.2 Recombinant cyclin-CDK complexes produced from baculoviruses in *Spodera frugiperda 9* (Sf9) insect cells were kindly donated by Boris Sarcevic. CDK6 was immunoprecipitated from whole cell ES lysates (400μg total protein). Recombinant cyclin-CDK complexes and CDK6 IPs were incubated with or without 4μg GST-p16 or 4μgGST in lysis buffer for 1 hour at 30°C. *In vitro* kinase assays were conducted using GST-$RB^{773\text{-}928}$ as a substrate. ^{32}P-labelled GST- $RB^{773\text{-}928}$ was resolved on SDS gels, dried and exposed to X-ray film.

Figure 4.4.3

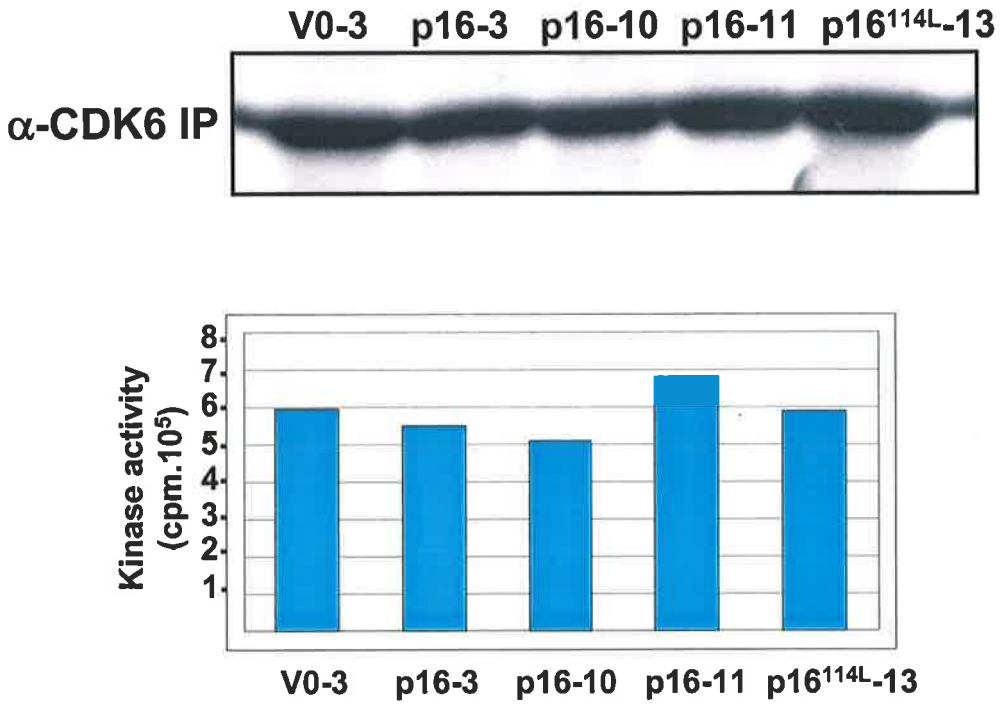


Figure 4.4.3 CDK6 kinase activity analysed in stable, puromycin-resistant p16 cell lines - vector only (vo-3), wildtype p16 (clones 3,10, 11) and the CDK4/6 binding mutant p16 ^{p114L} ($p16^{114L}$-13). CDK6 was immunoprecipitated from whole-cell lysates (400μg total protein) and used in *in vitro* kinase assays using GST-$RB^{773-928}$ as a substrate. ^{32}P-labelled GST- $RB^{773-928}$ was resolved on SDS gels, dried and exposed to X-ray film. Quantitation of CDK6 kinase activity was determined by phosphorimager analysis.

Figure 4.5.1

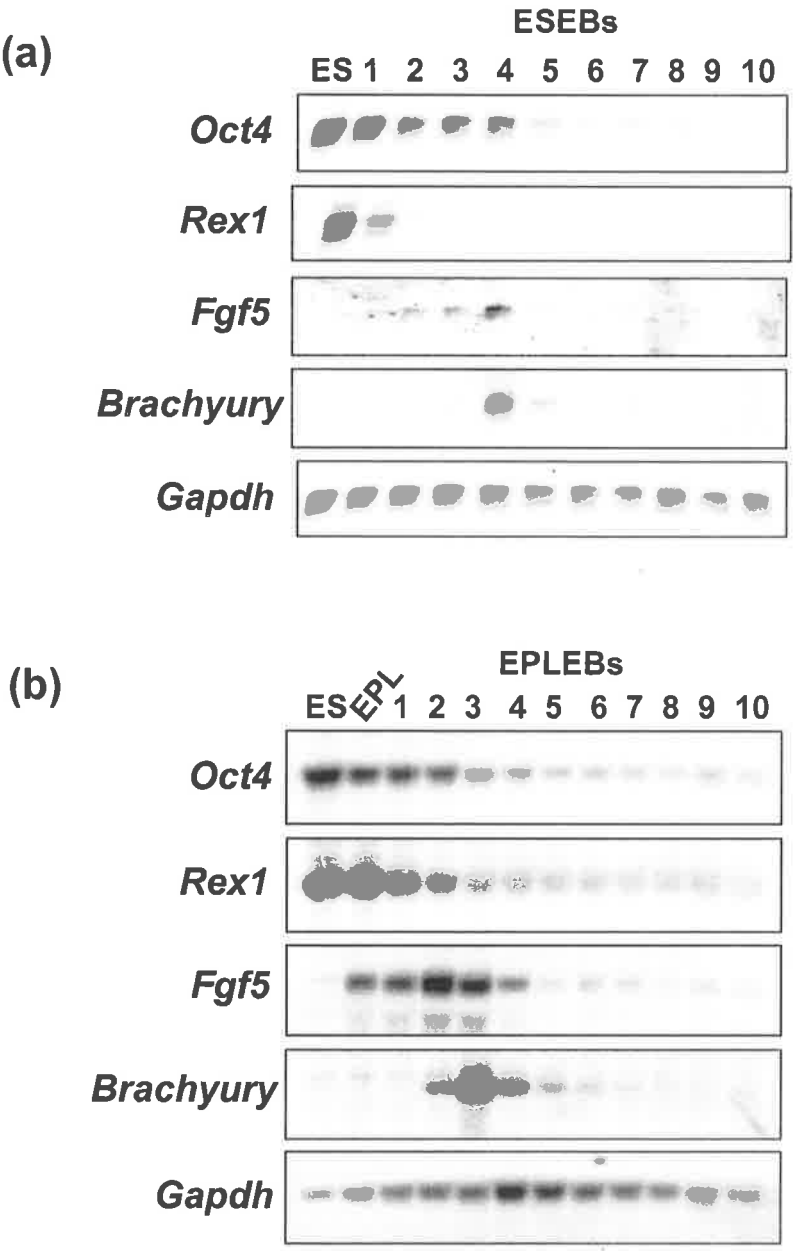


Figure 4.5.1 (a) ES cells were differentiated as embryoid bodies (ESEBs). (b) ES cells were differentiated into EPL cells then EPL embryoid bodies (EPLEBs). For both experiments, total cell RNA (20μg) was resolved on a formaldehyde-agarose gel, blotted and probed with ^{32}P-labelled Rex1, Oct4, Fgf5, Brachyury and Gapdh probes, utilising Gapdh as a loading control.

Figure 4.5.2

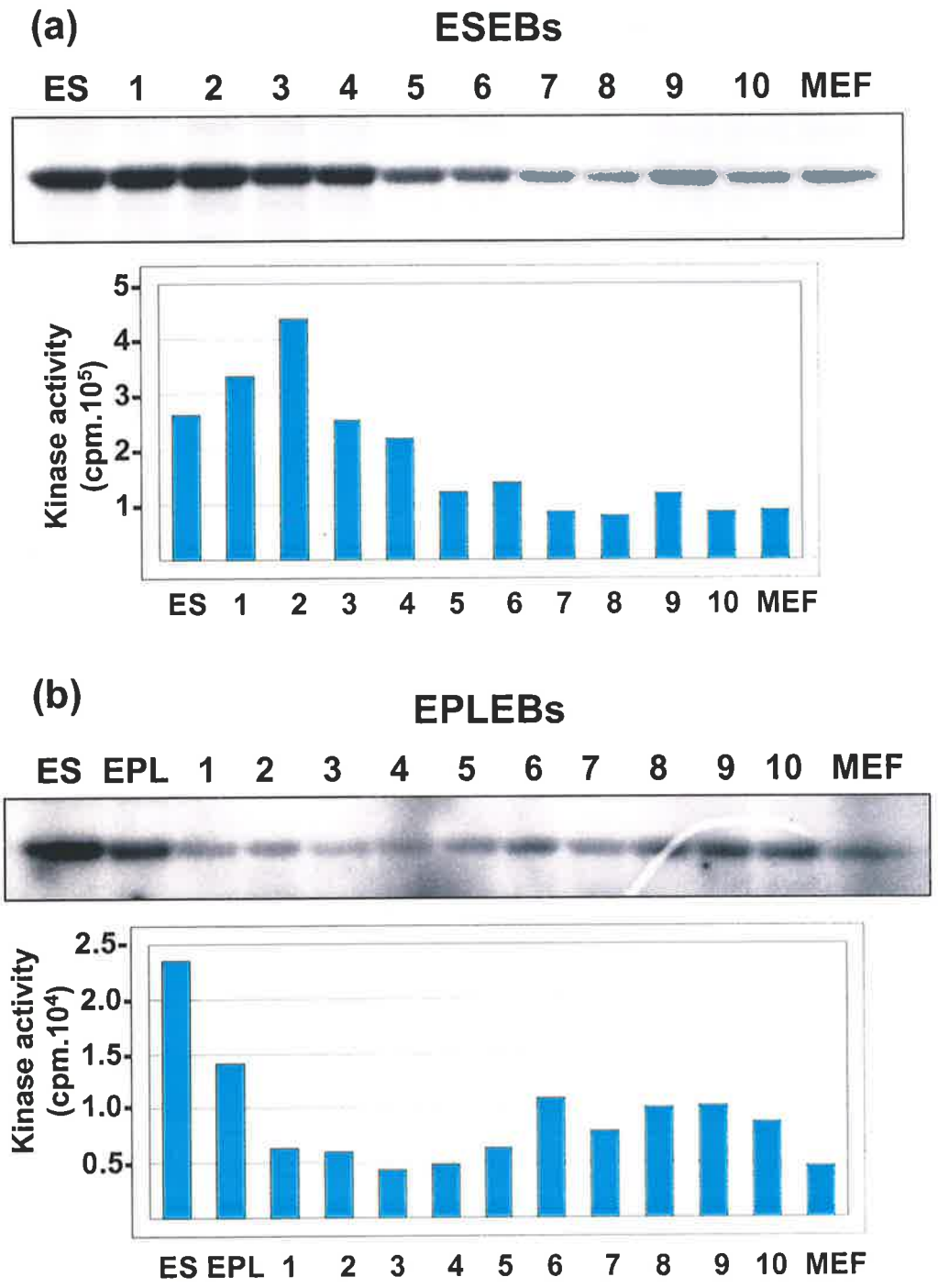


Figure 4.5.2 (a) Protein was extracted from ES cells, ESEBs (from figure 4.5.1a) and MEFs. (b) Protein was extracted from ES and EPL cells, EPLEBs (from figure 4.5.1b) and MEFs. CDK6 was immunoprecipitated from whole cell lysates (400μg total protein) and used in *in vitro* kinase assays using GST-$RB^{773\text{-}928}$ as a substrate. ^{32}P-labelled GST- $RB^{773\text{-}928}$ was resolved on SDS gels, dried and exposed to X-ray film. Quantitation of CDK6 kinase activity was determined by phosphorimager analysis.

divergent differentiation of these two model systems and suggest that there may be lineage specific differences in regulation of CDK6 activity.

4.6 Summary

This analysis has identified robust CDK6 kinase activity in ES cells. The kinase activity precipitated with the CDK6 antibody was confirmed to be CDK6 by a series of inhibitor experiments, in concert with analysis of immunoprecipitations (Appendix 1, R. Faast; Faast *et al.*, 2004). In contrast, no accurate analysis of CDK4 activity could be conducted due to antibody cross-reactivity between CDK2 and CDK4. However, expression of tagged CDK4 in ES cells demonstrated that there was no CDK4 activity in ES cells (P. Cartwright, unpublished data), consistent with that previously reported (Savatier *et al.*, 1996). This is also consistent with the low level of cyclin D-CDK4 complexes detected in ES cells (Faast *et al.*, 2004), which may be due to the lack of p21 and p27 to enable complex formation (Cheng *et al.*, 1999; LaBaer *et al.*, 1997; Sugimoto *et al.*, 2002). However, several independent mechanisms of cyclin D-CDK4/6 complex formation exist, as demonstrated by analysis of p21-/-;p27-/- MEFs (Sugimoto *et al.*, 2002). It is likely that it is one of these independent mechanisms that is aiding cyclin D-CDK6 complex formation in ES cells.

Constitutive CDK2 activity in ES cells is predominantly due to cell cycle-independent cyclin E and cyclin A expression (Stead *et al.*, 2002). These data show that CDK6 activity is also cell cycle-independent in ES cells. Accordingly, cyclin D1, cyclin D3 and CDK6 proteins are present throughout the cell cycle in ES cells (R. Faast, unpublished data). Strict regulation of the periodic synthesis and destruction of p18Ink4c and p19Ink4d is thought to control cell cycle-dependent CDK4/6 activity in proliferating cells (Blais *et al.*, 2002; DeGregori *et al.*, 1997; Thullberg *et al.*, 2000a). These proteins have not been detected in ES cells (R. Faast, unpublished data), which is likely to lead to no cell cycle regulation of CDK6 activity. Since CDK6 activity is known to inactivate pocket proteins (Meyerson and Harlow, 1994), it is predicted that this constitutive CDK6 activity, in concert with CDK2, may maintain constitutive E2F target gene transcription in ES cells by persistent inactivation of pocket proteins. This is consistent with the reported constitutive hyperphosphorylation of pRB (Savatier *et al.*, 1994) and cell cycle independent E2F target gene transcription in ES cells (Stead *et al.*, 2002). Since only CDK4/6 activity has been reported to release E2F from p107 (Beijersbergen *et al.*, 1995; Cheng *et al.*, 2000a; Leng *et al.*, 2002; Xiao *et al.*, 1996), the observed high levels of

deregulated CDK6 activity elucidates the high proportion of free E2F and very low proportion of p107-E2F complexes detected in ES cells in a cell cycle independent manner (Humbert *et al.*, 2000a; Stead *et al.*, 2002).

It is evident from the literature that the functions of CDK6 activity are not entirely understood. It was previously shown that CDK6, complexed with cyclin D2 and D3, phosphorylates pRB much more efficiently than histone H1 (Meyerson and Harlow, 1994). In this thesis, it was demonstrated that cyclin D3-CDK6 complexes, both purified and immunoprecipitated from ES cells, efficiently phosphorylate both histone H1 and pRB. Differences in these results may be due to experiment design or due to a specific modification in the transformed cell lines analysed in the previous report (Meyerson and Harlow, 1994). Importantly, these data suggest that the binding of a cyclin subunit can confer substrate specificity. This is reminiscent of viral cyclins, which preferentially associate with CDK6 and enable phosphorylation of both pRB and histone H1 (Godden-Kent *et al.*, 1997; Jung *et al.*, 1994). This suggests that cyclin D3-CDK6 complexes may have further, alternate roles within the cell. The identification of cytoplasmic substrates of CDK6 and cytoskeletal changes associated with ectopic CDK6 activity has led to the suggestion that CDK6 may couple cell cycle regulation and cell morphology (Ericson *et al.*, 2003; Kwon *et al.*, 1995; Mahony *et al.*, 1998). The differential CDK6 activity in the differentiation systems demonstrated here is associated with differences in morphological changes, particularly with EPL cell formation. Similarly, differentiation of ICM cells to primitive ectoderm is associated with reorganisation of cells to form a pseudo-stratified epithelium (Snow, 1977). This suggests that during early mouse development regulation of CDK6 activity may be involved in cell morphology changes. Further identification and analysis of CDK6 substrates and analysis of the function of CDK6 in regulating these substrates will need to be conducted. Analysis of the result of ectopic CDK6 and/or cyclin D3 and dominant negative CDK6 in ES cells would be particularly useful.

The CDK6 activity detected in ES cells is not inhibited by p16 *in vitro* or *in vivo*. This is consistent with previous reports showing ES cell cycle progression is not inhibited by transient p16 expression (Savatier *et al.*, 1996). The major CDK6 complex formed in ES cells is with cyclin D3 (Faast *et al.*, 2004). The activity of purified cyclin D3-CDK6 complexes was shown to be refractory to inhibition by p16 *in vitro*. Taken together, this suggests that CDK6 activity is not inhibited in ES cells because cyclin D3-CDK6 is the

major active complex and it is insensitive to p16 inhibition. However, a proportion of cyclin D1-CDK6 complexes are detected in ES cells (Faast *et al.*, 2004). Analysis of purified cyclin D1-CDK6 complexes shows their inhibition by p16 expression. Thus, if these complexes are active in ES cells their activity would be predicted to be inhibited by p16 expression, although there is no significant inhibition of total CDK6 activity in ES cells. The cyclin D1-CDK6 complexes formed in ES cells may not represent a high enough proportion of CDK6 complexes to show significant differences if inhibited by p16. Alternatively, cyclin D1-CDK6 complexes are not active, which is possible due to the observations that there is no nuclear cyclin D1 in ES cells (Savatier *et al.*, 1996) and only nuclear CDK6 is active in most cell types (Mahony *et al.*, 1998; Nagasawa *et al.*, 1997). Further analysis has shown a lack of recruitment of p16 into complexes with CDK6 in ES cells (Faast *et al.*, 2004). This observation is similar to the decreased efficiency of cyclin D3-CDK6 binding to p21 and p27, leading to cyclin D3-CDK6 evading inhibition by p27, during anchorage deprivation imposed G1 arrest (Lin *et al.*, 2001). Consistent with this, CDK6 kinase activity in ES cells, contributed mainly to by cyclin D3-CDK6 complexes (Faast *et al.*, 2004), was less efficiently inhibited by p21 than CDK2 activity (Fig 4.2.2). However, due to potential differences in the efficiency of immunoprecipitation, a more complete analysis of this effect in ES cells should be conducted.

According to the role of CDK6 in integrating extra-cellular signals to control of the cell cycle, differences in CDK6 activity associated with ES cell differentiation are likely to reflect changes in mitogenic signals during embryonic development. While in both systems differentiation led to decreased CDK6 activity, down-regulation of CDK6 activity occurred more rapidly as ES cells differentiated into EPLEBs than ESEBs. This is consistent with the temporally advanced nature of EPLEBs. Primitive ectoderm formation, as determined by marker gene expression, is associated with decreased CDK6 activity in both systems. Differences in the magnitude of this reduction are likely to be due to the increased heterogeneity of ESEBs compared to EPLEBs. After formation of primitive ectoderm (days 1-2 EPLEBs and days 3-4 ESEBs), the main difference between embryoid bodies is the presence of visceral endoderm in ESEBs and not EPLEBs (Lake *et al.*, 2000). This suggests that visceral endoderm or its associated signalling may be involved in the regulation of CDK6 activity. This hypothesis is supported by the fact that EPL cells are formed in the presence of MEDII, which mimics visceral endoderm

signalling (Rathjen *et al.*, 2001; Rathjen *et al.*, 1999), and the formation of EPLEBs requires the withdrawal of MEDII and the associated visceral endoderm-like signalling. While there are no reports relating cell cycle regulation of pluripotent cells to visceral endoderm, there are many reports suggesting the involvement of visceral endoderm in pluripotent cell maintenance, survival, apoptosis and differentiation (Rathjen *et al.*, 2001). Therefore, this would be a powerful mechanism for integration of developmental cues with proliferative controls.

Alternatively, differences in CDK6 activity may be due to the increased specification for mesoderm formation in EPLEBs. In the mouse embryo at gastrulation, primitive ectoderm cells of the primitive streak are fated to form mesoderm (Lawson *et al.*, 1991; Quinlan *et al.*, 1995; Tam, 1989). Cells of the primitive streak proliferate more rapidly than other primitive ectoderm cells, with cell cycle times estimated between 2.2-3.6 hours compared to 4.8-8.1 hours (6.5-7.5dpc) (Snow, 1977). Accordingly, it is likely that the complexity of cell cycle regulation is further decreased to enable these decreased proliferation rates. These changes may be reflected in the decreased CDK6 activity observed in EPLEBs. Also, lineage specific differences in regulation of CDK6 activity *in vitro* are consistent with the lineage specific, non-redundant functions of D-type cyclins delineated upon analysis of mice with ablation of combinations of D-type cyclins (Ciemerych *et al.*, 2002). Given the differences in expression of D-type cyclins over differentiation, changes in CDK6 activity may be related to changes in D-type cyclin availability. This is consistent with coincident decreased CDK6 activity and decreased cyclin D3 protein levels in EPL cells (Faast *et al.*, 2004). However, other factors must contribute to differences in CDK6 activity, since decreased cyclin D3 levels in ESEBs day 1 (Faast *et al.*, 2004) is not associated with decreased CDK6 activity. The relative contributions of each of the D-type cyclins to CDK6 activity in ES cells or over differentiation could not be investigated due to cross-reactivity upon immunoprecipitation with each of the antibodies to D-type cyclins tested. However, analysis of the contribution of cyclin D3 to changes in CDK6 activity should be conducted with ES cells ectopically expressing cyclin D3. Importantly, differentiation of murine erythroleukemia (MEL) cells requires downregulation of CDK6 activity (Matushansky *et al.*, 2000; Matushansky *et al.*, 2003). Further analysis of CDK6 and its involvement in differentiation will be conducted by analysis of the effects of ectopic CDK6 expression on differentiation potential of ES cells.

CHAPTER FIVE

Analysis of the p107/E2F-4 pathway in embryonic stem cells

5.1 Introduction

The primary level of regulation of E2F activity is the binding of pocket proteins (Chellappan *et al.*, 1991; Cobrinik *et al.*, 1993; Flemington *et al.*, 1993; Vairo *et al.*, 1995; Zamanian and La Thangue, 1993). In ES cells, RB is mainly hyperphosphorylated, thus inactive (Savatier *et al.*, 1994), and there is no detectable p130 (LeCouter *et al.*, 1996). In contrast, p107 is active in ES cells, representing the small proportion of pocket protein associated with DNA bound E2Fs (Humbert *et al.*, 2000a; Stead *et al.*, 2002). This suggests that p107 is the sole pocket protein regulator of E2F activity in ES cells. The function of p107 in controlling cellular proliferation is clearly demonstrated by the G1 arrest caused by overexpression of p107 in most cell types (Claudio *et al.*, 1994; Starostik *et al.*, 1996; Zhu *et al.*, 1993). p107 growth arrest correlates with decreased E2F transactivation and E2F target gene expression (Jiang *et al.*, 2000; Zhu *et al.*, 1993). Regulation of p107 is primarily due to differential phosphorylation by cyclin A/E-CDK2 and cyclin D-CDK4/6 complexes, leading to periodic phosphorylation of p107 (Beijersbergen *et al.*, 1995; Cheng *et al.*, 2000a; Leng *et al.*, 2002; Xiao *et al.*, 1996). However, it is CDK4/6 kinase activity, not CDK2, that releases p107 from E2F, enabling the cell cycle-dependent derepression of E2F target genes (Beijersbergen *et al.*, 1995; Xiao *et al.*, 1996). The importance of p107 regulation by phosphorylation is highlighted by the ability of ectopic cyclin D-CDK4, but not cyclin A/E-CDK2, to overcome a p107 growth arrest (Beijersbergen *et al.*, 1995; Xiao *et al.*, 1996). This is further evident from analysis of cells that are impaired in p107-induced growth arrest (Zhu *et al.*, 1993), as efficient growth arrest occurs when CDK4 phosphorylation sites or all putative CDK phosphorylation sites of p107 are mutated to non-phosphorylatable residues (Ashizawa *et al.*, 2001; Farkas *et al.*, 2002; Leng *et al.*, 2002). Significantly, in ES cells the presence of DNA binding E2F-p107 complexes is independent of cell cycle position (Stead *et al.*, 2002). This suggests that there is an absence of periodicity in the regulation of p107 activity in ES cells, which is likely to contribute to the constitutive E2F target gene expression in ES cells (Stead *et al.*, 2002). Therefore, it was of great interest to gain a further understanding of the regulation and role of p107 in ES cells.

E2F-4 represents approximately 95% of the DNA-binding activity in ES cells (Humbert *et al.*, 2000a). In addition to its regulation by pocket proteins, E2F-4 activity is also regulated by nuclear localisation and proteolytic degradation. Without its own consensus nuclear localisation signal, nuclear localisation of E2F-4 is mostly dependent upon

binding to RB family members or DP2 (de la Luna *et al.*, 1996; Lindeman *et al.*, 1997; Magae *et al.*, 1996; Verona *et al.*, 1997). Importantly, the ability of E2F-4 to act as a transactivator is dependent upon nuclear localisation (Muller *et al.*, 1997; Verona *et al.*, 1997). Similarly, association with RB family members increases the stability of E2F-4, from a half life of 2-3 hours to 8 hours, by protecting it from proteasome-mediated degradation (Campanero and Flemington, 1997; Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). The constitutive E2F target gene expression in ES cells and predominance of E2F-4 in DNA binding assays (Humbert *et al.*, 2000a; Stead *et al.*, 2002) promoted an interest in understanding the regulation and activity of E2F-4 in ES cells.

5.2 p107 and E2F4 regulation in ES cells

To further understand why there is a lack of periodic expression of E2F target genes observed in ES cells, the regulation of E2F-4 and p107, key regulators of the E2F pathway in ES cells, were investigated. Since E2F-4/p107 complex formation is regulated by CDK phosphorylation (Beijersbergen *et al.*, 1995; Cheng *et al.*, 2000a; Leng *et al.*, 2002; Xiao *et al.*, 1996) and DNA binding E2F-4/p107 complexes are present throughout the ES cell cycle (Stead *et al.*, 2002), it was of interest to assess p107 phosphorylation over the cell cycle in ES cells. One technique utilised to assess pocket protein phosphorylation is mobility changes on polyacrylamide gels (Beijersbergen *et al.*, 1995; Calbo *et al.*, 2002; Cheng *et al.*, 2000a). ES cells were synchronised at the G1/S boundary using a double block method and released to follow a complete cell cycle as described in 2.3.13. Flow cytometric analysis confirmed the synchrony of the ES cell population (Fig 5.2.1a). Western analysis indicated that there is no significant variation in the mobility of p107 over an ES cell cycle (Fig 5.2.1b). This is most clearly demonstrated by comparing Aphidicolin (G1 and S) and Nocodazole (G2 and M) blocked samples (Fig 5.2.1b). This suggests that there is little difference in the phosphorylation status of p107 over an ES cell cycle. Since most of the functions of p107 are regulated by phosphorylation, this suggests that there is no cell cycle regulation of p107 activity in ES cells.

With E2F-4 constituting the majority of the DNA binding activity, it was also of interest to investigate the regulation of E2F-4 in ES cells. In addition to its regulation by pocket proteins, E2F-4 activity is regulated by subcellular localisation. Despite attempts with different antibodies, analysis of E2F-4 localisation by immunofluorescence was unsuccessful, mainly due to the domed shape of the ES cell colony and the very high

Figure 5.2.1

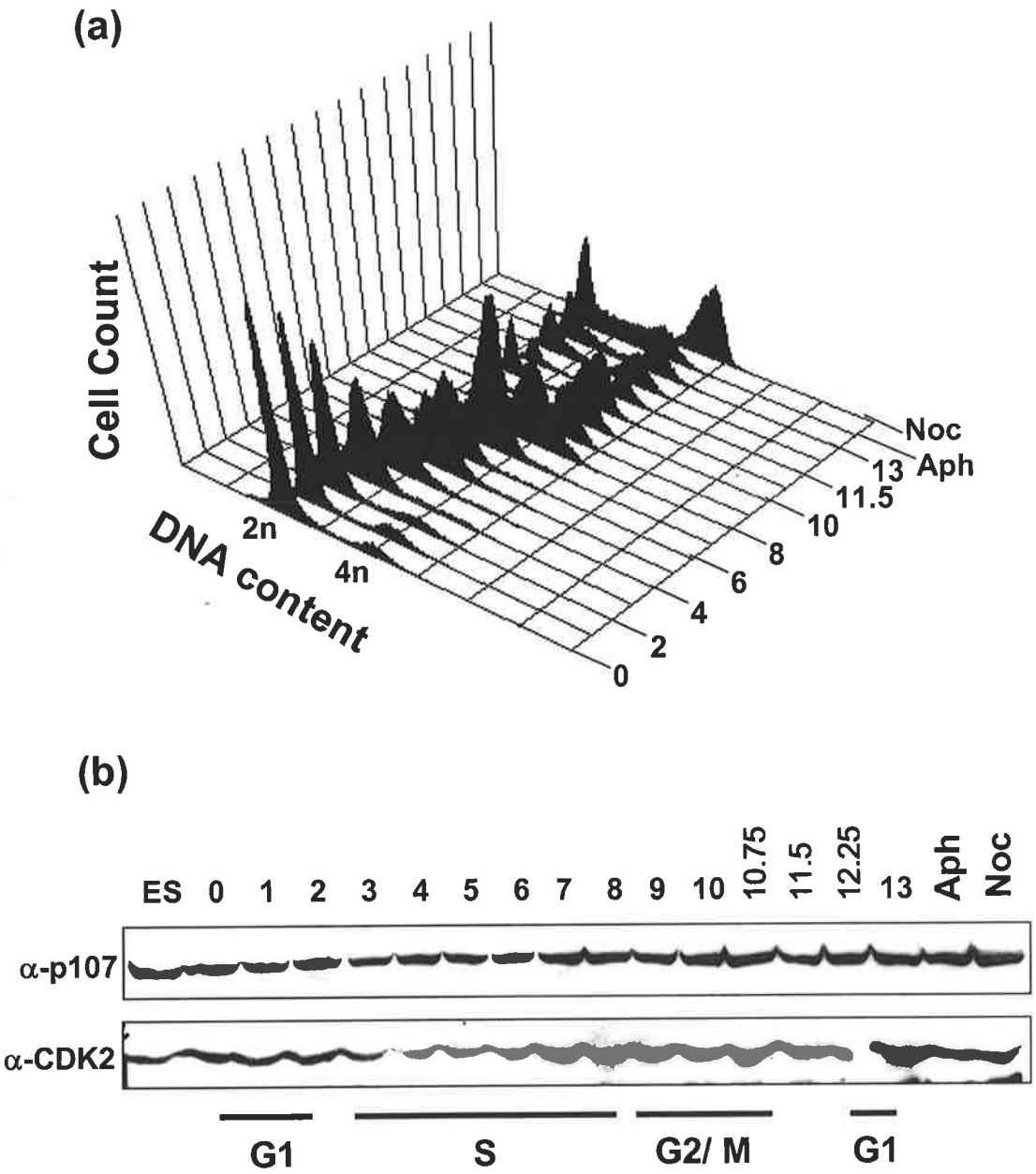


Figure 5.2.1 Asynchronously cycling ES cells were statically arrested in mitosis using nocodazole. Washing cells from this block, cells were released into fresh media containing aphidicolin, which statically synchronised cells at the G1/S border. Synchronous cells were followed through an entire cell cycle, harvesting cells at defined intervals. (a) Synchronous cells were harvested, stained with propidium iodide and analysed by flow cytometry using WinMDi software. (b) Synchronous cells were harvested, whole cell protein extracted, run on an 8% SDS-PAGE gel and analysed by Western analysis, utilising p107 and CDK2 antibodies.

nuclear to cytoplasmic ratio (data not shown). Therefore, ES cells were fractionated into nuclear and cytoplasmic fractions as described in 2.4.3 and equal concentration of each fraction and whole cell protein were separated by electrophoresis and analysed by western analysis. Cytoplasm was absent in the nuclear fraction, as indicated by the lack of tubulin (Fig 5.2.2a). p107, E2F-4 and DP2 were in both the nucleus and cytoplasm, however, each were repeatedly found at higher levels in the cytoplasm compared to the nucleus (Fig 5.2.2a). In particular, approximately 40% of E2F-4 was found in the nucleus compared to approximately 60% in the cytoplasm (Fig 5.2.2a). A higher form of E2F-4, potentially phosphorylated E2F-4, was detected in the whole cell extract and cytoplasm but not in the nuclear extract (Fig 5.2.2a). Since E2F-4 activity is dependent on its nuclear localisation (Muller *et al.*, 1997; Verona *et al.*, 1997), this suggests that a proportion of E2F-4 is likely to be active, as indicated by its nuclear localisation. However, it is likely that E2F-4 activity is limited in ES cells by its ability to translocate to the nucleus.

A further level of regulation of E2F-4 activity is proteolytic degradation. To assess whether E2F-4 stability is differentially regulated between ES cells and differentiated ES cells (EPLEBs day 7), both were treated with the proteasome inhibitor MG132 for 3 hours prior to harvesting. Western analysis was conducted, utilising a-tubulin as a control. This indicates that E2F-4 stability is enhanced in ES cells compared to differentiated cells (Fig 5.2.2b). Therefore, subcellular localisation of E2F-4 is regulated in ES cells, however, E2F4 stability is increased in ES cells, suggesting that its regulation by proteolytic degradation is decreased.

5.3 E2F4 activity in ES cells

While E2F-4 constitutes the majority of the DNA binding activity *in vitro* (Humbert *et al.*, 2000a), it was important to confirm that E2F-4 associated with E2F target gene promoters *in vivo*. The Chromatin Immunoprecipitation (ChIP) assay enables an analysis of the transcription factors and complexes bound to promoters, thus identifying proteins that contribute to promoter activity. This technically challenging assay required many levels of optimisation (2.4.13). The specificity of antibodies were confirmed by immunoprecipitation (IP) and western analysis. Crosslinking of protein-protein and protein-DNA complexes had to be tightly controlled, as it was found that under-crosslinking led to unstable complexes while over-crosslinking led to complexes being too tightly bound disabling antibody access. The number of sonication steps was adjusted

Figure 5.2.2

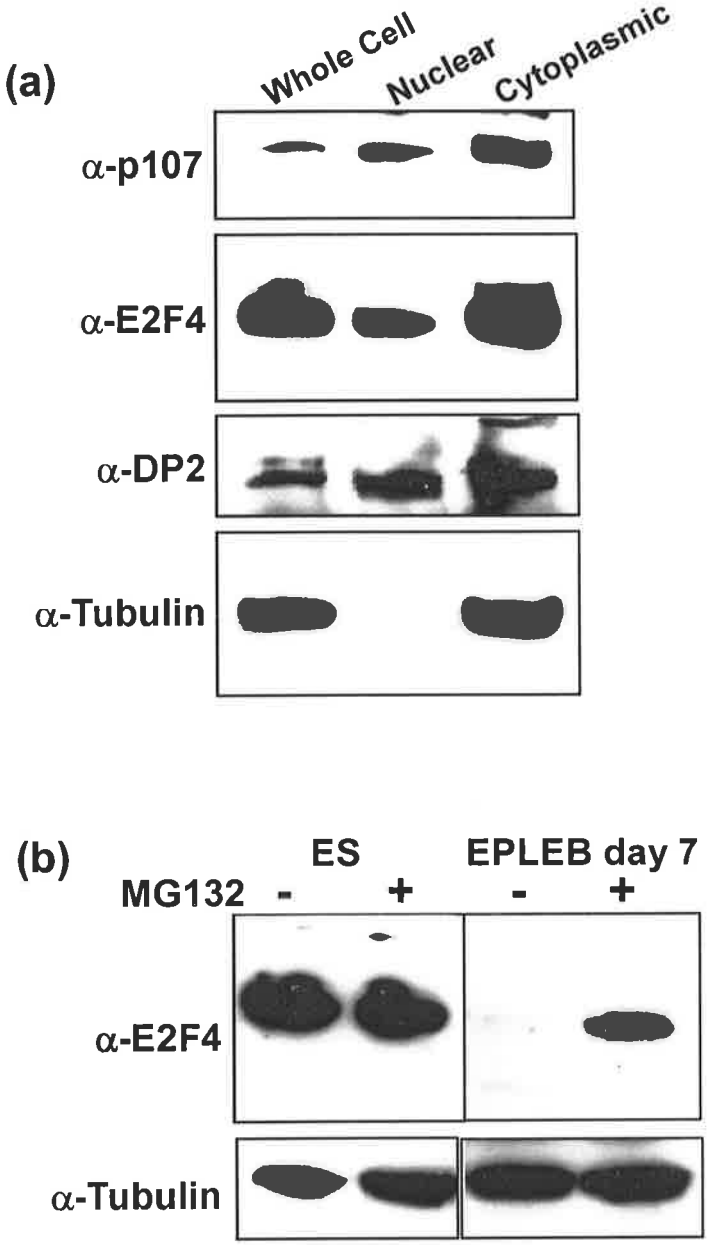


Figure 5.2.2 (a) ES cells were fractionated into nuclear and cytoplasmic fractions. 50μg of whole cell, nuclear and cytoplasmic proteins were separated on a 10% Tris-Tricine gel and subjected to western analysis, utilising p107, E2F-4, DP2 and α-Tubulin antibodies. (b) ES cells were differentiated into EPL embryoid bodies. ES cells and EPLEBs day 7 were treated with or without 5μM MG132 for 3 hours prior to harvesting. 50μg of whole cell proteins for each treatment were separated on a 10% Tris-Tricine gel and subjected to western analysis, utilising E2F-4 and α-Tubulin antibodies.

to ensure that fragments were of similar length. The amount of input chromatin and antibody concentration for the IPs were optimised to ensure the best yield of precipitated chromatin. The IPs were optimised, by testing various washing conditions. PCR conditions were optimised, by altering buffers, polymerase and adding 1M Betaine to attain the best yields.

To control for non-specific binding to antibodies and contamination, the level of immunoprecipitated promoters was compared to a non-specific antibody IP. The antibody chosen for this IP was a commercial rabbit polyclonal antibody cdc25a, since the E2F-4 antibody used was also a commercial rabbit polyclonal (2.4.1.5). Also, binding to the Albumin promoter was analysed, since it has no consensus E2F binding site and no reported binding of proteins in the p107-E2F pathway. These controls enabled the level of background to be identified. Importantly, ChIPs were conducted at the same time to ensure that they were as comparable as possible to the controls. PCR analysis could then be conducted as described in 2.4.13.

The binding of E2F-4 to E2F target gene promoters was then analysed. Chromatin from asynchronous ES cells was collected. IPs were conducted with an E2F-4 antibody and the control antibody. The DNA precipitated was then analysed for the presence of the promoters of interest. PCRs indicated that E2F-4 associated with the cyclin E, B-myb, cyclin A and CDC2 promoters (Fig 5.3.1). This was a specific interaction, as there was no Albumin promoter present in the E2F-4 IP (Fig 5.3.1). Also, there was very little contamination in the assay, as indicated by the lack of product in the control antibody IP (Fig 5.3.1). This shows that E2F-4 binds *in vivo* to E2F target gene promoters analysed.

These data demonstrate that E2F-4 is bound to E2F target gene promoters in ES cells, therefore E2F-4 may enable the constitutive expression of these genes detected in ES cells (Stead *et al.*, 2002). To ensure that there are no modifications of E2F-4 rendering it transcriptionally inert in ES cells, luciferase reporters under the control of E2F sites or mutated E2F sites were utilised. Transient transfection of ES cells was optimised using transfection of pCAG-GFP, with the Fugene transfection reagent having the highest transfection efficiency (data not shown). E2F-4 or DP2 was transiently transfected with the E2F or mutant reporter and a renilla control for 40 hours. Both increased the activity of the E2F reporter, however there was a greater increase in activity when DP2 was

Figure 5.3.1

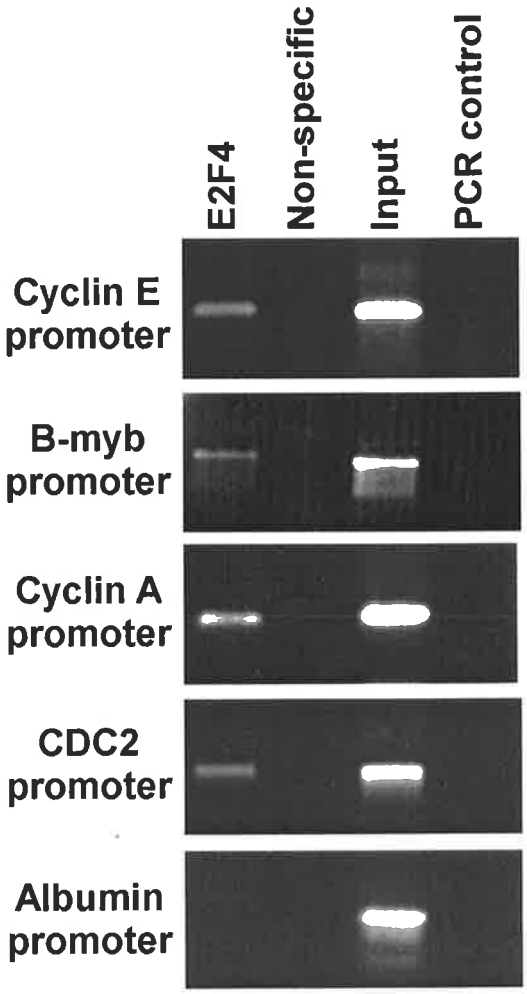


Figure 5.3.1 Crosslinked chromatin (700μg) from asynchronous ES cells was incubated with E2F4 or cdc25a (Non-specific) antibodies. Immunoprecipitated DNA was analysed by PCR using primers specific for different promoters. PCR controls included analysis of 0.03% of the total input chromatin (Input) and the addition of water instead of input DNA (PCR control).

transfected (Fig 5.3.2a). This suggests that DP2 may be limiting in ES cells. When both E2F-4 and DP2 were transfected into ES cells, there was a cooperative increase in the activity of the E2F-responsive luciferase reporter (Fig 5.3.2b). While this does not show that endogenous E2F-4 is acting as a transactivator, it shows that E2F-4 is competent to activate E2F promoters when it is translocated to the nucleus.

5.4 *In vitro* functional analysis of p107 in ES cells

The constitutive E2F activity and decreased p107 activity in ES cells (Humbert *et al.*, 2000a; Stead *et al.*, 2002) is likely to be due to the high levels of constitutive CDK6 (this thesis) and CDK2 (Stead *et al.*, 2002) kinase activity in ES cells, which leads to cell cycle independent phosphorylation of p107 (Fig 5.2.1). It was therefore of interest to analyse the role of p107 and the contribution of phosphorylation to the regulation of its function in ES cells. Accordingly, the effects of expression of p107 and a phosphorylation defective mutant p107 was analysed in ES cells. The phosphorylation resistant mutant (p107ΔS/T-P) has been generated by mutation of the 18 putative CDK phosphorylation sites (Ser/Thr-Pro) in human p107 (Ser/Thr to Ala) by the Hatakeyama lab (Fig 5.4.1a) (Ashizawa *et al.*, 2001; Kondo *et al.*, 2001). Constructs were sub-cloned into the pCAGiP vector, with expression controlled by the CAG promoter. ES cells were transiently transfected with luciferase reporters driven by E2F or mutated E2F promoters and with p107 or mutant p107ΔS/T-P. p107 effectively inhibited endogenous E2F activity in ES cells (Fig 5.4.1b). While the E2F reporter used for these assays was different to that for previous assays, due to delays in its availability, experiments have been conducted with both E2F-responsive reporters and the inhibition of E2F activity by p107 is comparable (data not shown). An average of three experiments shows a 2.5 fold decrease in endogenous E2F activity with p107-WT compared to a 3.7 fold inhibition by p107ΔS/T-P. Since ectopic p107-WT is capable of inhibiting a high percentage of E2F activity, it is likely that the levels of p107 are too high to be completely inhibited by CDK6 kinase activity. The increased inhibition by p107ΔS/T-P supports the hypothesis that p107 activity is regulated by phosphorylation in ES cells. However, western analysis to analyse the levels of p107-WT and p107ΔS/T-P expressed were unsuccessful, due to the low transfection efficiency, therefore, potential differences in expression may lead to inaccuracies in direct comparisons of fold inhibition.

Figure 5.3.2

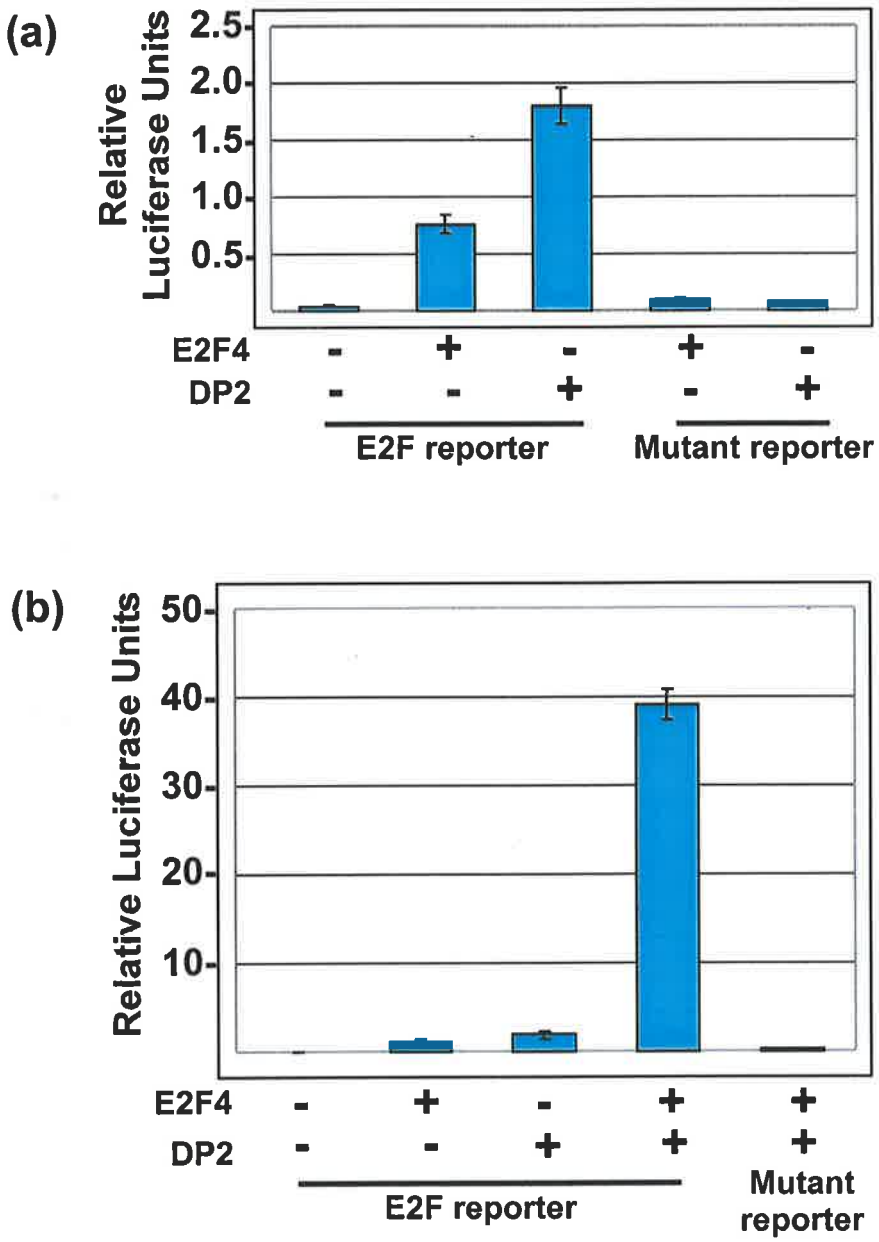


Figure 5.3.2 ES cells were transfected for 40 hours with the E2F-luciferase reporter (pGL3TATAbasic-6xE2F) or mutated E2F-luciferase reporter, renilla control and 10ng pCAG-E2F4 or 10ng pCAG-DP2 (a) or both (b). Firefly luciferase activity was normalised to renilla luciferase activity and data are presented as relative activity. Experiments were performed in triplicate and data represents a typical experiment.

Figure 5.4.1

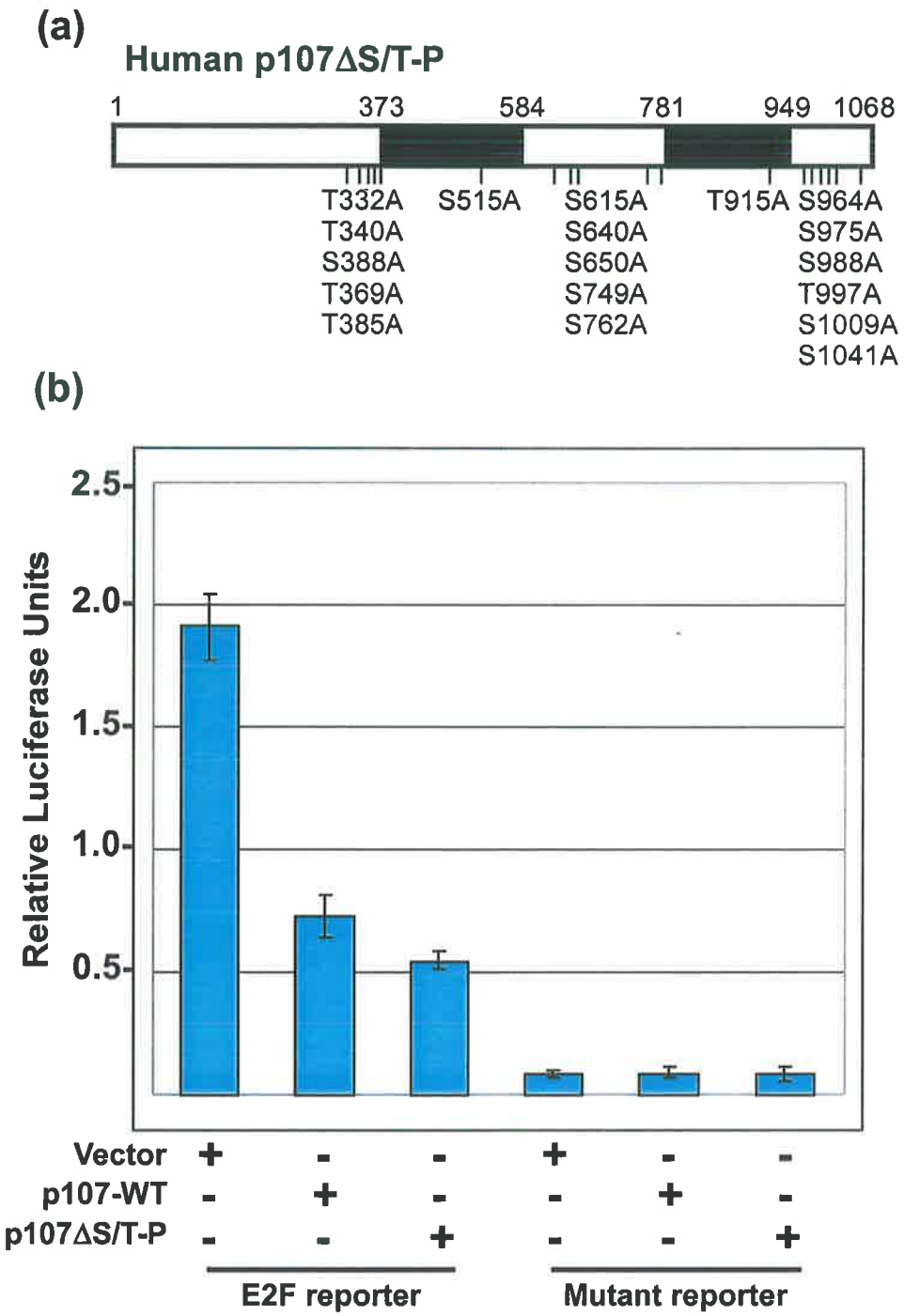


Figure 5.4.1 (a) All 18 putative CDK phosphorylation sites of p107 were mutated to alanine residues to make a non-phosphorylatable mutant p107 (p107ΔS/T-P) (Ashizawa *et al*, 2001). (b) ES cells were transfected for 40 hours with the E2F-luciferase reporter (pGL3promoter-4xCCC) or mutated E2F-luciferase reporter, a renilla control and 50ng of empty pCAG expression vector (Vector), wildtype p107 (p107-WT) or mutant p107 (p107ΔS/T-P). Firefly luciferase activity was normalised to renilla luciferase activity and data are presented as relative activity. Experiments were performed in triplicate and data represents a typical experiment.

The ability of p107 to specifically inhibit E2F-4 activity was also tested. A titration of p107-WT and p107ΔS/T-P was analysed for the ability to inhibit transiently transfected E2F-4 and DP2 (Fig 5.4.2a). Increasing amounts of DNA transfected, decreased the transfection efficiency, as indicated by the decreased E2F-4/DP2 activity in the vector alone transfections (Fig 5.4.2a). As expected for a specific interaction, increasing the concentration of p107-WT or p107ΔS/T-P increased the fold repression of E2F-4/DP2 activity (Fig 5.4.2a). Importantly, with sufficient p107 and p107ΔS/T-P transfected, the majority of the E2F activity could be inhibited (Fig 5.4.2a). The inhibition of E2F-4/DP2 activity by p107 in ES cells was shown to be specific by comparison to a mutated E2F reporter (Fig 5.4.2b). An average of three experiments shows a 2-3 fold increase in level of inhibition of exogenous E2F-4/DP2 by p107ΔS/T-P compared to p107-WT. This suggests that p107 can specifically inhibit E2F-4 activity and that this inhibition is subject to regulation by phosphorylation in ES cells.

5.5 *In vivo* functional analysis of p107 in ES cells

To analyse the effects of p107 on endogenous E2F targets, clonal, puromycin-resistant ES cell lines were generated, which had stably integrated wildtype p107 (p107-WT) or the mutant p107 (p107ΔS/T-P). Transfection efficiency was lower for both p107 and p107ΔS/T-P compared to the vector alone control, however, morphologically the stable cell lines were normal, with similar growth rates (data not shown). Expression was detected via a HA tag incorporated into the constructs (Fig 5.5.1a). As was the case for all the cell lines generated, p107ΔS/T-P clones had lower expression levels than p107-WT (Fig 5.5.1a). Given the fact that expression was under the control of the same promoter and the high number of clones assessed decreased the probability that the integration site determined expression levels, this suggests that ES cells are more sensitive to higher levels of expression of p107ΔS/T-P than p107-WT. In p107ΔS/T-P-7 cell line, there was an increase in a higher form of E2F-4, which may indicate increased phosphorylation of E2F-4 in this cell line (Fig 5.5.1a). To assess the ability of ectopic p107-WT and p107ΔS/T-P to bind E2F-4 and to gain an understanding of the differences in E2F-4 binding, levels of ectopic p107-WT and p107ΔS/T-P were approximately equalised in the input for a HA IP (Fig 5.5.1b). Both p107-WT and p107ΔS/T-P bound E2F-4, however there were higher levels of E2F-4 bound to p107ΔS/T-P than p107-WT (Fig 5.5.1b). The increased binding of E2F-4 to p107ΔS/T-P supports the hypothesis that in ES cells p107 association with E2F-4 is regulated by phosphorylation.

Figure 5.4.2

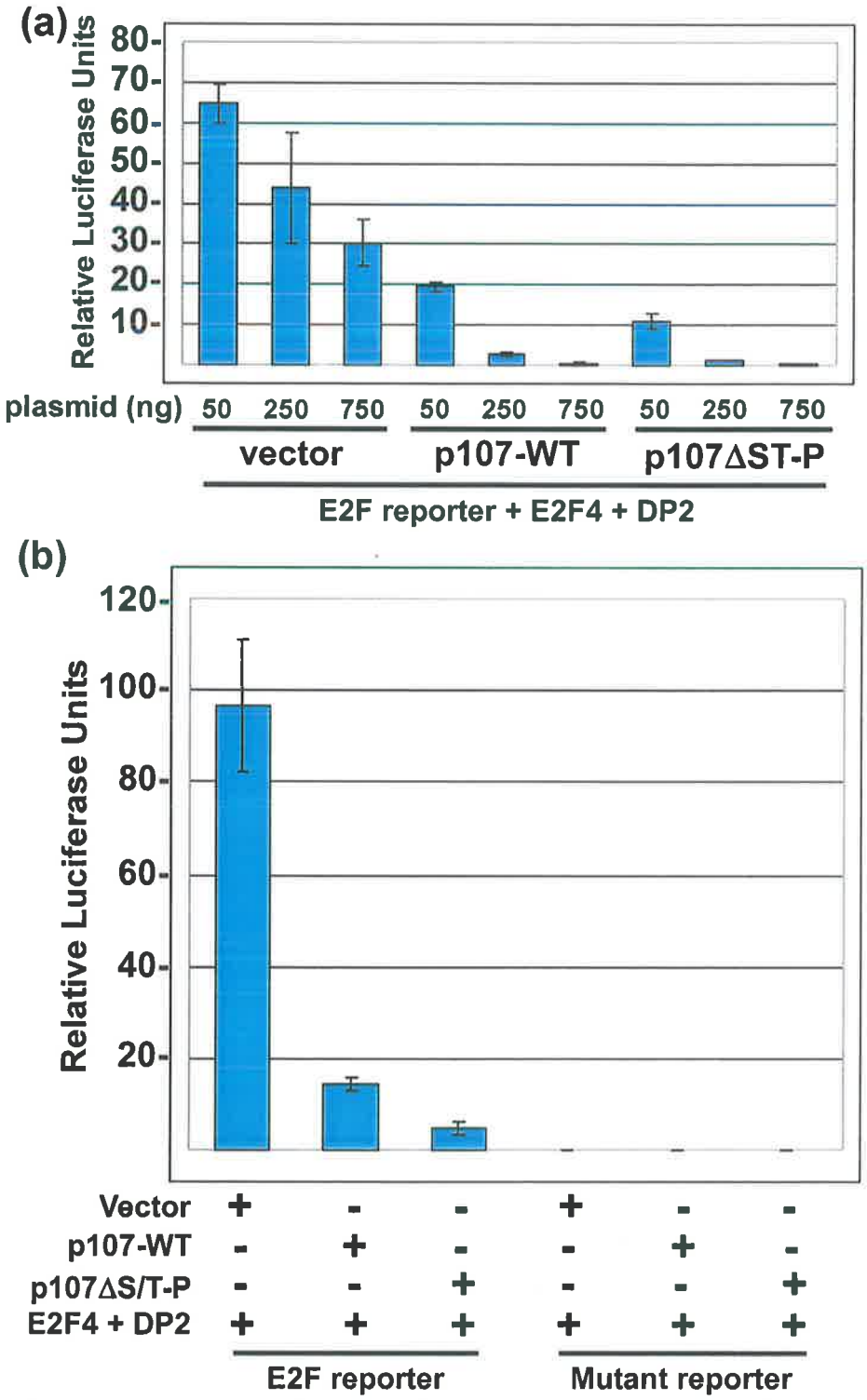


Figure 5.4.2 ES cells were transfected for 40 hours with the E2F-luciferase reporter (pGL3TATAbasic-6xE2F) or mutated E2F-luciferase reporter, renilla control and 10ng pCAG-E2F4 and 10ng pCAG-DP2. (a) Increasing amounts of pCAG (Vector), pCAG-p107-WT or pCAG-p107ΔS/T were also transfected. (b) 50ng of pCAG (Vector), pCAG-p107-WT or pCAG-p107ΔS/T were also transfected. Firefly luciferase activity was normalised to renilla luciferase activity and data are presented as relative activity. Experiments were performed in triplicate and data represents a typical experiment.

Figure 5.5.1

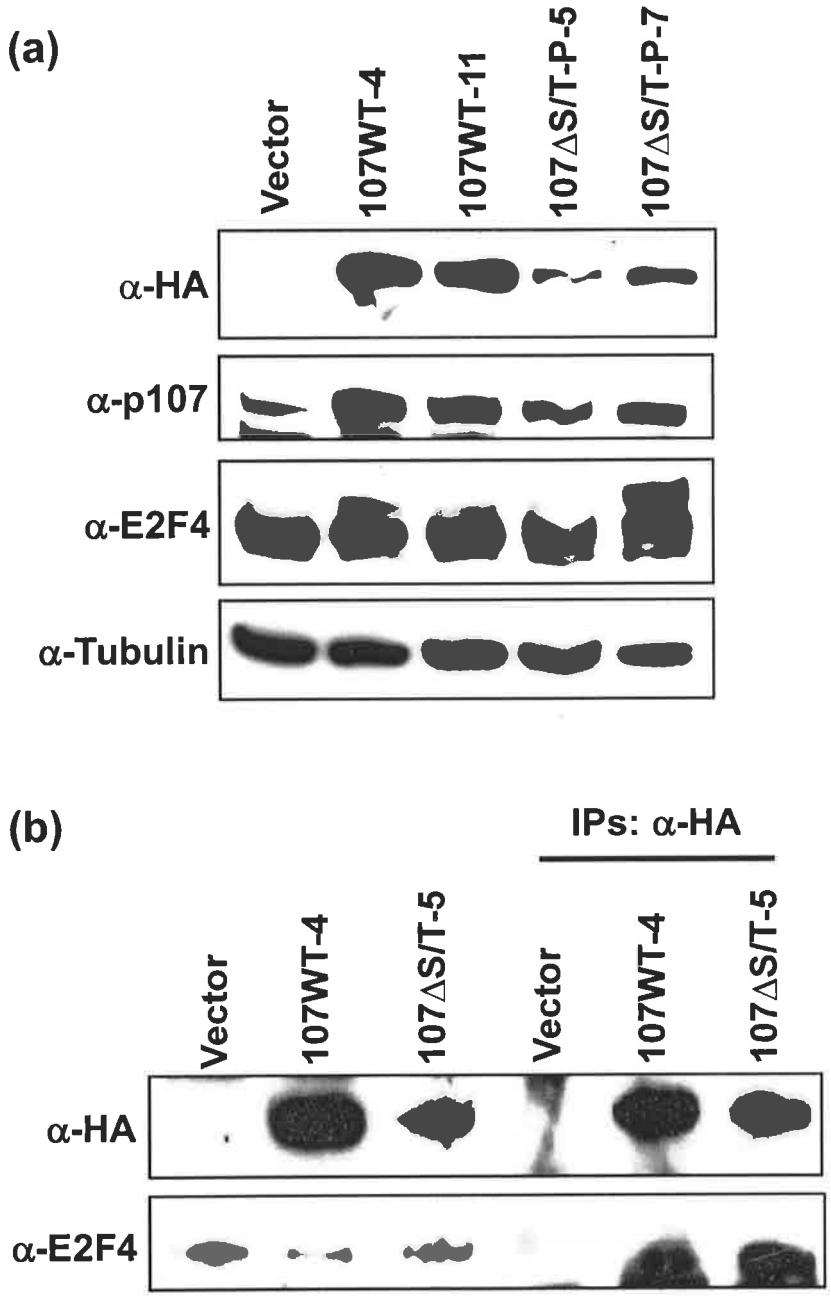


Figure 5.5.1 ES cells were stably transfected with pCAG vector (vector), pCAG-p107WT or pCAG-p107ΔS/T-P. ES cells from different cell lines were harvested and whole cell protein extracted. (a) 50μg protein was separated on a 8% SDS-PAGE gel and subjected to Western analysis, utilising HA, p107, E2F-4 and α-Tubulin antibodies. (b) 1mg vector, 100μg p107WT-4 and 1mg p107ΔS/T-P proteins were used for immunoprecipitations (IPs) with a HA antibody. 50μg whole cell extract and IPs were separated on a 8% Tris-Tricine gel and subjected to Western analysis, utilising HA and E2F-

Figure 5.5.2

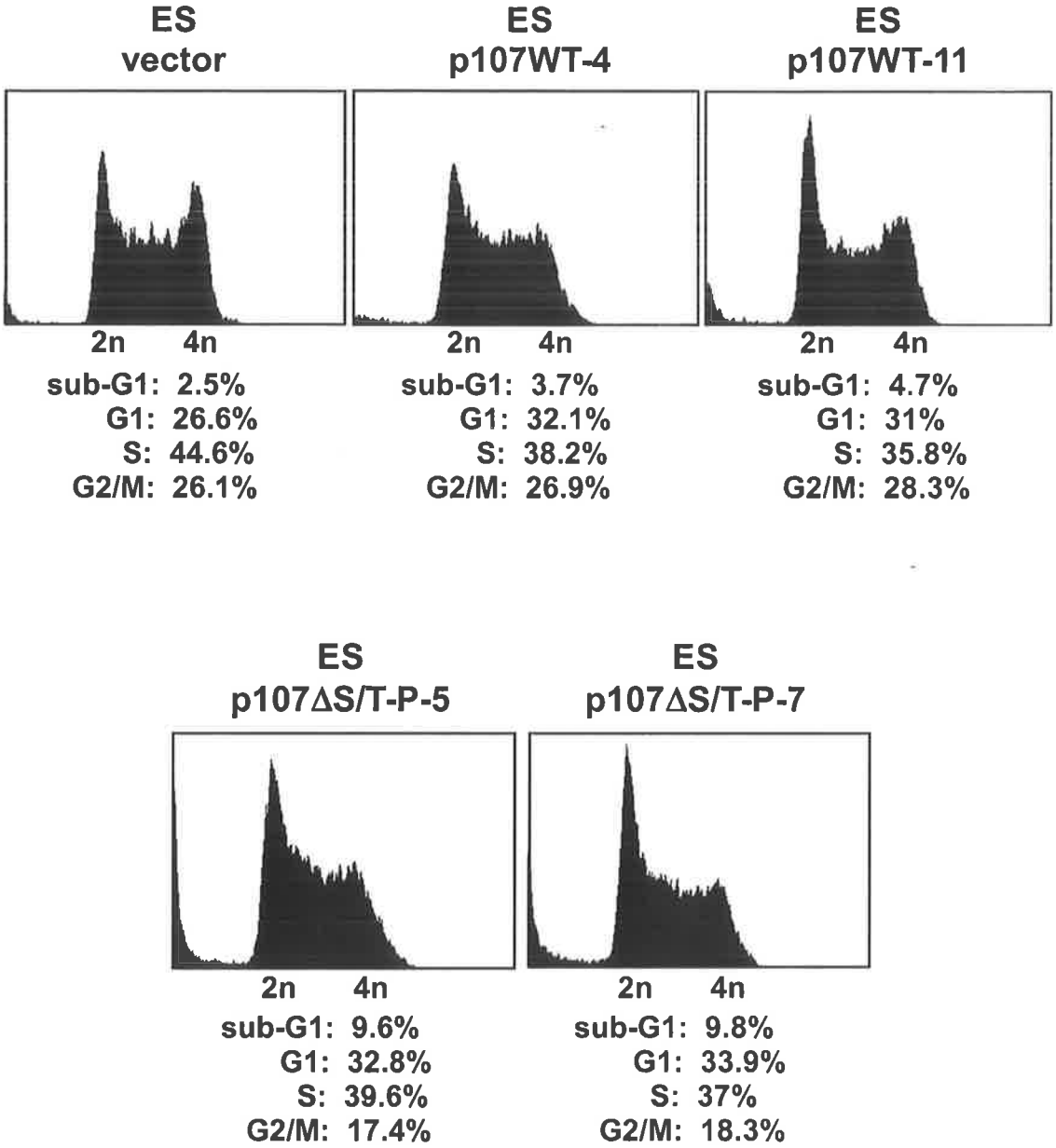


Figure 5.5.2 ES cells were stably transfected with pCAG vector (vector), pCAG-p107WT or pCAG-p107ΔS/T-P. Asynchronously growing ES cells from different cell lines were fixed and stained with propidium iodide. The proportion of cells in each phase of the cell cycle was analysed using a Beckman Coulter flow cytometer

Figure 5.5.3

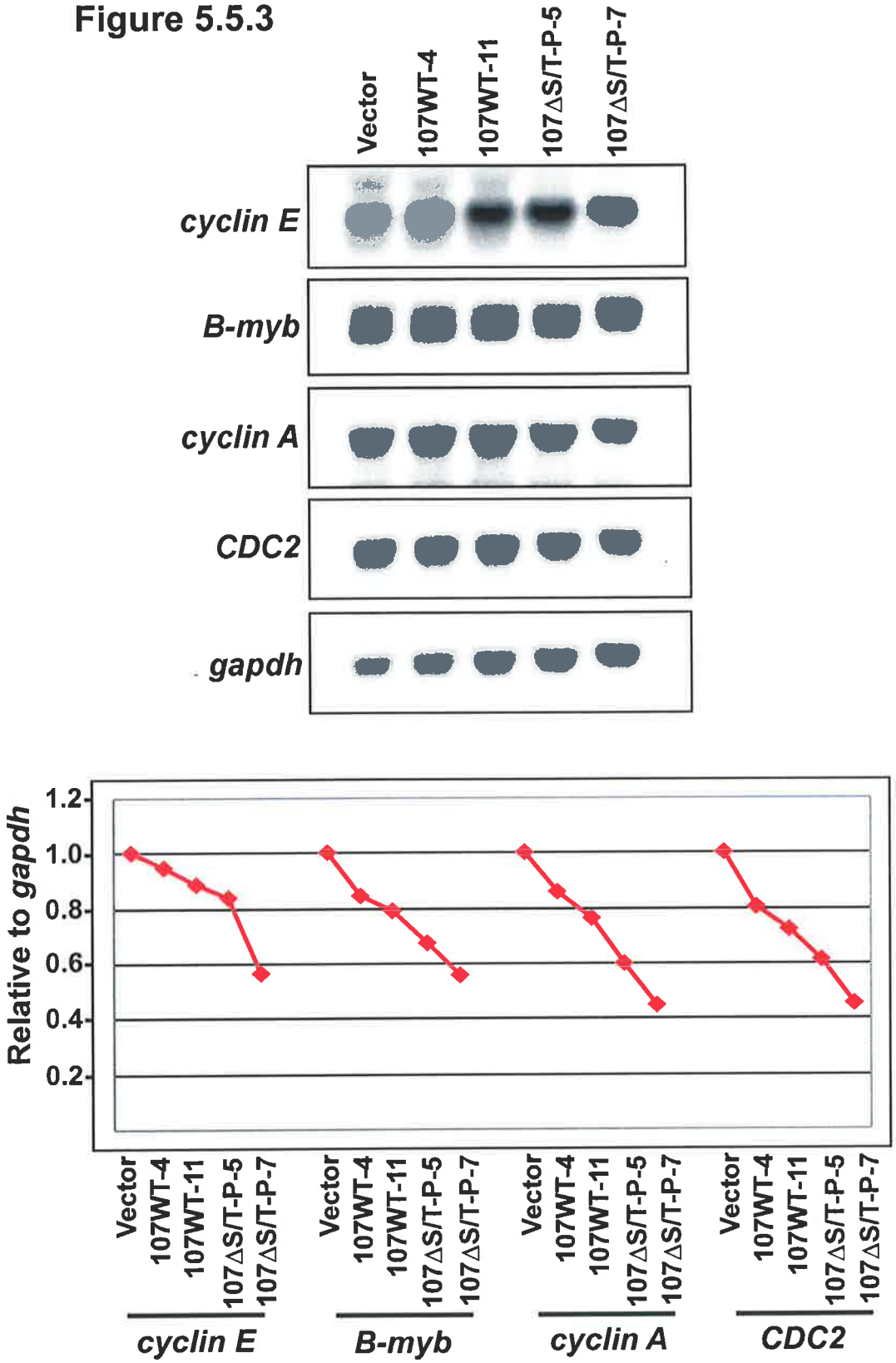


Figure 5.5.3 ES cells were stably transfected with pCAG vector (vector), pCAG-p107-WT or pCAG-p107ΔS/T. Total cell RNA (20μg) was resolved on a formaldehyde -agarose gel, blotted and probed with ^{32}P-labelled cyclin E, B-myb, cyclin A, CDC2 and gapdh probes. Transcript levels were quantitated by phosphorimaging and shown relative to the gapdh control.

While the levels of p107-WT and p107ΔS/T-P expressed did not cause a growth arrest, it was of interest to analyse any changes in the structure of the cell cycle. Flow cytometry was conducted on ES cells from the stable cell lines. Both p107-WT and p107ΔS/T-P ES cells had a significant increase in the proportion of cells with a 2n DNA content, indicative of increased number of cells in G1 phase (Fig 5.5.2). An increase in the sub-2n (sub-G1) population, indicative of cell death, was also evident, particularly in the p107ΔS/T-P cells (Fig 5.5.2). An increased proportion of p107ΔS/T-P cells with a DNA content just greater than 2n was also evident (Fig 5.5.2). This suggests that cells are slower to enter S phase than control ES cells. Significantly, p107ΔS/T-P cells had a decreased proportion of cells with a 4n DNA content, indicative of cells in G2/M phase (Fig 5.5.2), suggesting either a specific loss of G2/M phase cells or highlighting the delays in progression through other phases of the cell cycle.

The effect of ectopic p107-WT and p107ΔS/T-P on E2F target genes was then analysed. RNA was extracted from ES cells and analysed by Northern analysis. Northern blots were probed for E2F target genes – *cyclin E*, *B-myb*, *cyclin A* and *CDC2* and normalised according to *gapdh* levels (Fig 5.5.3). Each of the target genes showed decreased expression in p107-WT and p107ΔS/T-P cell lines compared with control ES cells (Fig 5.5.3). The effect was comparable between genes and cell lines, suggesting that the ectopic p107-WT and p107ΔS/T-P acts similarly on all the genes analysed. Interestingly, decreased E2F target gene expression was only noted in cell lines of early passage. As cells were continually passaged, there were differences in which target genes were effected, until after 5 or 6 passages there was no decrease in E2F target gene expression (data not shown). This may be due to the observation that transgenes are progressively silenced in ES cells (Smith, 2001) or may indicate that ES cells are capable of compensatory increases in other pathways governing the expression of these genes.

5.6 Discussion

The constitutive E2F target gene expression and DNA-binding E2F-4/p107 complexes detected in ES cells (Stead *et al.*, 2002) led to the hypothesis that there are differences in the regulation of the p107-E2F pathway in ES cells compared to differentiated cells. Since the regulation of p107 activity is primarily due to CDK phosphorylation (Beijersbergen *et al.*, 1995; Cheng *et al.*, 2000a; Leng *et al.*, 2002; Xiao *et al.*, 1996), the phosphorylation status of p107 in ES cells was investigated, by assessing mobility

changes on polyacrylamide gels. While this technique has been previously utilised to show cell cycle dependent phosphorylation of p107 (Beijersbergen *et al.*, 1995; Calbo *et al.*, 2002; Cheng *et al.*, 2000a), further confirmation requires analysis with phospho-specific antibodies or by metabolic labelling. Still, the similar mobility of p107 over an ES cell cycle suggests that there is a lack of periodic phosphorylation of p107 in ES cells. Constitutive phosphorylation of p107 is consistent with the low proportion of cell cycle independent p107-E2F complexes in ES cells (Humbert *et al.*, 2000a; Stead *et al.*, 2002). This is likely to be due to the constitutive CDK2 activity (Stead *et al.*, 2002) and constitutive CDK6 activity (this thesis) in ES cells. From this analysis, the activity of phosphatases acting upon p107 in ES cells remains undetermined, however, the invariability of p107 phosphorylation status suggest that if phosphatases are active in ES cells, p107 is rapidly re-phosphorylated. Importantly, the likely consequence of constitutive p107 phosphorylation is cell cycle independent p107-E2F complexes and repression of E2F activity, which is consistent with published data (Stead *et al.*, 2002). This demonstrates major differences in the regulation of p107 in ES cells compared to differentiated cell types. These differences may aid the rapid cell cycle progression that is characteristic to the ES cell.

Since E2F-4 constitutes the majority of E2F DNA binding activity in ES cells (Humbert *et al.*, 2000a; Stead *et al.*, 2002), it was important to analyse the regulation of E2F-4 activity in ES cells. In addition to regulation by pocket proteins, E2F-4 activity is regulated by nuclear localisation, which is dependent upon binding DP2 or RB family members (de la Luna *et al.*, 1996; Lindeman *et al.*, 1997; Magae *et al.*, 1996; Verona *et al.*, 1997). The distribution of E2F-4 in both the nucleus and cytoplasm suggests that E2F-4 activity is regulated by subcellular localisation in ES cells. However, in comparison to other cell lines (Muller *et al.*, 1997), there is proportionally more E2F-4 located in the nucleus than the cytoplasm in ES cells. Since the nuclear localisation of E2F-4 is controlled in a cell cycle dependent manner (Lindeman *et al.*, 1997; Verona *et al.*, 1997), this difference may indicate that nuclear localisation of E2F-4 may be cell cycle independent in ES cells. Assessment of this hypothesis requires analysis of E2F-4 subcellular localisation over the ES cell cycle.

Importantly, there is still a high proportion of E2F-4 in the cytoplasm in ES cells. Nuclear localisation of E2F-4 requires binding DP2 or RB family members (de la Luna *et*

al., 1996; Lindeman *et al.*, 1997; Magae *et al.*, 1996; Verona *et al.*, 1997). With no p130 (LeCouter *et al.*, 1996) and constitutive phosphorylation of RB (Savatier *et al.*, 1994), p107 is the sole pocket protein enabling E2F-4 nuclear localisation. Decreased p107/E2F-4 association (Humbert *et al.*, 2000a; Stead *et al.*, 2002), likely due to the constitutive CDK activity in ES cells (Stead *et al*, 2002, this thesis), suggests that there is limited nuclear localisation of E2F-4 by p107. Also, transient transfection analysis suggests DP2 is limiting for E2F-4 activity, therefore there may be insufficient DP2 to nuclear localise all of the E2F-4 in ES cells. However, there is only a small increase in luciferase activity when DP2 is transfected compared to transfected E2F-4. This is inconsistent with the high proportion of E2F-4 in the cytosol in ES cells. This suggests that E2F-4 is retained in the cytosol in ES cells by binding DP1 or an alternatively spliced form of DP2, lacking a nuclear localisation signal (de la Luna *et al.*, 1996; Ormondroyd *et al.*, 1995; Rogers *et al.*, 1996). Alternatively, the presence of E2F-4/DP2 complexes in the cytosol may be due to CRM-1 nuclear export, as it is CRM-1 that is thought to maintain the low levels of free E2F-4 in the nucleus (Gaubatz *et al.*, 2001; Verona *et al.*, 1997). Consistent with a suggested link between phosphorylation of E2F-4 and nuclear localisation (Gaubatz *et al.*, 2001), the absence of phosphorylated E2F-4 in the nuclear fraction suggests that E2F-4 may be exported to or retained in the cytoplasm due to differential phosphorylation. Also consistent with this, there is increased inhibition of E2F target genes and increased phosphorylated E2F-4 in the p107ΔS/T-P-7 cell line, suggesting that this may also be linked to cytoplasmic retention. This hypothesis requires further investigation with phospho-specific antibodies and analysis of a phosphorylation resistant mutant E2F-4.

Similarly, association with pRB family members increases the stability of E2F-4, from a half life of 2-3 hours to 8 hours, by protecting it from proteasome-mediated degradation (Campanero and Flemington, 1997; Hateboer *et al.*, 1996; Hofmann *et al.*, 1996). Despite decreased regulation of E2F-4 activity by pocket proteins in ES cells (Humbert *et al.*, 2000a; LeCouter *et al.*, 1996; Savatier *et al.*, 1994), E2F-4 stability is enhanced in ES cells compared to differentiated cell types. This suggests that the pathway governing E2F-4 stability is impaired in ES cells. C-terminal ubiquitination regulates E2F-4 turnover (Hateboer *et al.*, 1996), however, the degradation pathway has not yet been reported. Since E2F-1 and E2F-4 degradation has some similarities (Hateboer *et al.*, 1996; Hofmann *et al.*, 1996), some indication comes from analysis of E2F-1 degradation.

Skp2 and cullin ubiquitin ligases (Cul 1-3) are involved in E2F-1 turnover, most likely by separate mechanisms (Marti *et al.*, 1999; Ohta and Xiong, 2001). Analysis of knockout studies show Cul3 and Skp2 are not essential for the normal proliferation of ES cells (Nakayama *et al.*, 2000; Singer *et al.*, 1999). In conjunction, the degradation of another Skp2 target, c-Myc (Kim *et al.*, 2003; von der Lehr *et al.*, 2003), is impaired in ES cells (P. Cartwright, unpublished data), suggesting that this pathway may not be active in ES cells. If E2F-4 degradation is similar to E2F-1, decreased activity of Skp2 in ES cells would explain why there is no efficient degradation of E2F-4 in ES cells. Accordingly, deregulation of E2F-4 turnover may increase the levels of free E2F-4 and its ability to transactivate E2F target genes.

Data presented here suggests that E2F-4 may be acting as a transactivator in ES cells. ChIP assays indicate that E2F-4 binds to cyclin E, B-myb, cyclin A and CDC2 promoters in ES cells. This suggests E2F-4 has a role in regulation of these E2F target genes in ES cells. However, since several different E2Fs can be associated with the same promoter in the same cell cycle phase (Takahāshi *et al.*, 2000; Wells *et al.*, 2000), this does not exclude the possibility that other E2Fs are binding and activating transcription. Alternatively, transcription of these genes may be regulated by E2F-independent mechanisms. The ability of transiently transfected E2F-4 to activate transcription in ES cells suggests that E2F-4 can act as a transactivator in ES cells. Also, the fact that E2F-4 constitutes approximately 95% of the DNA-binding E2F activity in ES cells (Humbert *et al.*, 2000a) suggests that ectopic p107 is inhibiting E2F-4 activity in ES cells, as indicated by the inhibition of endogenous E2F activity and E2F target genes upon p107 expression. This further supports the hypothesis that E2F-4 is acting as a transactivator in ES cells. However, there is still the potential that exogenous p107 is inhibiting alternate E2Fs in this assay or is restricting association with basal transcription machinery. Further evidence for E2F-4 acting as a transactivator would come from the association of E2F-4 with histone acetyltransferase (HAT) activity, since E2F-4 transactivation is increased by its ability to recruit the acetyltransferase GCN5 and its cofactor TRAAP (Lang *et al.*, 2001) or p300 (Yagi *et al.*, 2002). Attempts to assess E2F-4 association with HAT activity in ES cells were unsuccessful, however, this activity may be only detectable when E2F-4 is overexpressed, as in published reports (Lang *et al.*, 2001; Yagi *et al.*, 2002). Importantly, while E2F-4 may have a role in transactivation in ES cells, it is not essential

for proliferation, since E2F-4-/- ES cells proliferate normally with no compensatory increase in E2F DNA binding activity (Humbert *et al.*, 2000a).

Alternatively, E2F-4 may be important for target gene repression rather than activation. In this model, E2F binding recruits pocket proteins and chromatin remodelling complexes, such as HDAC, to actively repress promoters, whereas free E2F is transcriptionally inactive (Muller and Helin, 2000). Therefore in ES cells, decreased E2F target gene expression with p107 expression may be due to active repression of promoter activity, rather than inhibition of E2F activity. Attempts to analyse the level of p107-associated HDAC activity were unsuccessful, however, it is of great interest to analyse the contribution of HDAC activity to the repression of E2F target genes by p107 in ES cells. Distinguishing between these models requires analysis of dominant negative E2F-4, as, without the ability to transactivate or bind pocket proteins, an indication of whether endogenous E2F-4 normally activates or represses transcription in ES cells would be evident.

Since E2F-5 constitutes the remainder of the E2F DNA binding activity in ES cells (Humbert *et al.*, 2000a), E2F-5 may be involved in E2F-dependent transcription in ES cells. Increased cyclin E-CDK2 kinase activity stimulates E2F-5 phosphorylation and the formation of p300/E2F-5 complexes, enabling E2F-5 dependent transcription (Morris *et al.*, 2000). The high, constitutive cyclin E-CDK2 kinase activity in ES cells (Stead *et al.*, 2002) may lead to increased, constitutive levels of p300/E2F-5 complexes, enabling the stimulation of constitutive E2F target gene transcription. Further analysis of the activity and function of E2F-5 in ES cells should be conducted.

In contrast to the majority of cell types analysed (Claudio *et al.*, 1994; Starostik *et al.*, 1996; Zhu *et al.*, 1993), the growth of ES cells with stably transfected p107 suggests that there is sufficient CDK activity in ES cells to overcome increased levels of p107. This is consistent with the high levels of CDK2 activity (Stead *et al.*, 2002) and CDK6 activity (this thesis) in ES cells. Also, ES cells do not express p16 and are not sensitive to p16 expression, suggesting that CDK6 activity is not regulated by p16 in ES cells (Faast *et al.*, 2004; Savatier *et al.*, 1996; Stead *et al.*, 2002, this thesis). Similarly, p107 expression does not cause growth arrest in the human tumour cell line U2-OS, which is thought to be due to the lack of p16, enabling uncontrolled cyclin D-CDK4/6 activity (Zhu *et al.*, 1993).

Alternatively, the lack of p107 growth arrest may be due to the high levels of B-myb expression in ES cells, as B-myb binding to p107 has been shown to overcome a p107 growth arrest (Bessa *et al.*, 2001; Joaquin *et al.*, 2002; Sala *et al.*, 1996a; Sala *et al.*, 1996b).

p107 expression in ES cells leads to significant inhibition of E2F activity and E2F target gene expression. This is consistent with the ability of ectopic p107 to bind E2F-4 in ES cells, suggesting that p107 is repressing E2F-4 activity. Importantly, stably transfected p107 does not entirely inhibit E2F target gene expression in ES cells. However, transient transfection analysis shows that with sufficient expression of p107, E2F activity can be entirely inhibited in ES cells. This suggests that the inhibition of E2F target genes in stably transfected ES cells is dependent upon the level of p107 expression. It still remains to be determined whether ES cell growth would be arrested with increased p107 expression and the consequent increased inhibition of E2F activity. Analysis of the cell cycle response to p107 in transiently transfected ES cells with complete inhibition of E2F activity is required, although this is technically difficult due to the low transfection efficiency of ES cells. In U2-OS cells, p107 expression decreases E2F activity, due to limiting cyclin D-CDK4/6 activity, however, p107 is unable to cause a growth arrest (Farkas *et al.*, 2002; Leng *et al.*, 2002; Xiao *et al.*, 1996; Zhu *et al.*, 1993). Therefore, it is also likely that the inhibition of E2F activity by ectopic p107 is due to limiting CDK6 activity in ES cells. Accordingly, coexpression of cyclin D and p107 in ES cells is predicted to rescue the inhibition of E2F activity observed.

Mutational analysis suggests that two functional domains of p107 can act independently to cause growth arrest. The N-terminal E2F binding domain inhibits E2F activity, while the C-terminal cyclin/CDK binding domain sequesters cyclin A/E-CDK2 complexes, blocking access to alternate substrates (Castano *et al.*, 1998; Woo *et al.*, 1997; Zhu *et al.*, 1995b). The ability of p107 to sequester cyclin A/E-CDK2 complexes may lead to decreased cyclin A/E-CDK2 activity in p107 expressing ES cells. However, the continued growth of p107 expressing ES cells suggests that there are free cyclin A/E-CDK2 complexes. Potentially, due to the high levels of cyclin E-CDK2 activity in ES cells, cell cycle progression may continue despite inhibition of E2F activity by p107, since cyclin E can induce S phase without activation of E2F transcription (Leng *et al.*, 1997; Lukas *et al.*, 1997). However, long term compensation for E2F-dependent

transcription by cyclin E has not been demonstrated. Still, in SAOS-2 cells, cyclin E-CDK2 coexpression partially rescues the p107 growth arrest (Beijersbergen *et al.*, 1995; Zhu *et al.*, 1993) and reduces flat cell formation induced by p107 expression (Ashizawa *et al.*, 2001). This suggests that high levels of cyclin E-CDK2 activity override some of the E2F-independent functions of p107 enabling cell cycle progression. To assess whether the high levels of CDK2 activity in ES cells affects p107 function, analysis of ES cells expressing dominant negative CDK2 should be conducted.

Functional regulation of p107 by phosphorylation in ES cells is further supported by analysis of expression of the phosphorylation-resistant mutant p107ΔS/T-P. Lower levels of p107ΔS/T-P expression compared to p107-WT were detected in stable ES cell lines, suggesting that ES cells are more sensitive to p107ΔS/T-P than p107-WT. Also, coincident with increased levels of E2F-4 binding, there was increased repression of E2F target genes upon stable transfection of p107ΔS/T-P compared to p107-WT. This effect was comparable to that of the transiently transfected reporter, however, there was a decreased fold inhibition of endogenous E2F target genes compared to the luciferase reporter. This is likely to be due to decreased levels of expression of p107-WT and p107ΔS/T-P in the stable cell lines. These data support the hypothesis that the ability of p107 to repress E2F activity in ES cells is regulated by phosphorylation. Also, these data support the presence of CDK6 kinase activity in ES cells, since only CDK4/6 kinase activity releases p107 from E2F (Beijersbergen *et al.*, 1995; Xiao *et al.*, 1996). Since E2F target gene expression is not entirely repressed, it is likely that there is still sufficient free E2F-4 in ES cells to enable transcription. A comparison of the levels of free E2F-4 in the stable cell lines by gel shift analysis would assess this hypothesis. A major limitation of the analysis of stable cell lines is that it does not enable identification of a specific growth arrest in response to p107ΔS/T-P activation. This would require inducible expression of p107ΔS/T-P. This proved difficult, however, would be worthwhile pursuing further. Due to the significantly lower levels of expression of p107ΔS/T-P compared to p107-WT, it is predicted that with sufficient p107ΔS/T-P a growth arrest would be observed.

Despite the propagation of ES cell stably expressing p107 and p107ΔS/T-P, significant cell cycle structure changes were noticed. The increased proportion of G1 and early S phase cells suggests that ES cells expressing p107ΔS/T-P are delayed in S phase entry. This is similar to that observed in the 6-1 mouse lymphoid cell line (Kondo *et al.*, 2001).

Many E2F target genes are important for entry into S phase, thus delayed G1/S progression may be linked to increased inhibition of E2F activity. Also, since cyclin A/E-CDK2 activity is important for DNA synthesis initiation and progression, delays in G1/S progression may be linked to sequestration of cyclin A/E-CDK2 complexes by p107ΔS/T-P. Alternatively, delayed G1/S progression may be linked to other E2F-independent functions of p107. Ectopic p107 may inhibit DNA replication by its ability to bind MCM7 at replication origins (Gladden and Diehl, 2003; Sterner *et al.*, 1998). Alternatively, delays in DNA replication may come from the ability of p107 to bind and inhibit the transcriptional activity of transcription factors involved in cell proliferation, c-Myc (Beijersbergen *et al.*, 1994a; Gu *et al.*, 1994; Hoang *et al.*, 1995) and B-myb (Joaquin *et al.*, 2002; Sala *et al.*, 1996b).

The decreased proportion of p107ΔS/T-P cells with a 4n DNA content suggests a specific loss of G2/M phase cells. Recent evidence suggests that E2F transcription factors and the RB family regulate genes involved in G2/M progression and checkpoint control (Lukas *et al.*, 1999; Polager and Ginsberg, 2003; Polager *et al.*, 2002; Ren *et al.*, 2002; Weinmann *et al.*, 2002). Therefore, this effect may be due to decreased E2F-dependent transcription of genes involved in G2/M progression. Importantly, G2/M checkpoints can be activated in ES cells, demonstrated by the response to DNA damage (Schmidt-Kastner *et al.*, 1998). Therefore, the specific loss of G2/M phase cells in p107ΔS/T-P cells may indicate cells undergoing apoptosis in response to a G2/M checkpoint. This is supported by the increased cell death observed in p107ΔS/T-P ES cells. ES cells undergoing cell death rather than growth arrest is consistent with the ES cell response to other growth limiting situations, such as serum withdrawal and DNA damage (E. Stead, unpublished data, Aladjem *et. al.*, 1998). These data suggest that ES cells may have deficiencies in some levels of checkpoint control or rely on alternatives, whereby rather than arresting and manage the problem, ES cells undergo cell death.

Analysis of the p107-E2F pathway in differentiation

CHAPTER SIX

6.1 Introduction

In order to understand the dramatic proliferative changes associated with early mouse development, *in vitro* model systems are utilised. Characteristic to ES cells is rapid cell cycle progression, with minimal gap phases (Stead *et al.*, 2002). This is altered dramatically as ES cells differentiate with increased proportions of cells in G1 phase coincident with a loss of pluripotency in ESEBs and EPLEBs (E. Stead, PhD thesis). Concurrent downregulation of cyclin E-CDK2 activity is likely to be involved in decreases in cell proliferation rates (E. Stead, PhD thesis). Differentiation of ES cells with retinoic acid indicates that as cyclin E-associated kinase activity decreases, CDK4 activity increases (Savatier *et al.*, 1996). This is consistent with the observation that it is not until gastrulation that cyclin D1/D2–CDK4 complexes can be immunoprecipitated from embryos (Savatier *et al.*, 1996). This suggests that as ES cells differentiate there is increased complexity of regulation of p107 by CDK phosphorylation.

While ES cells proliferate normally without the presence of all three pocket proteins, teratocarcinomas formed from these cells are predominantly relatively undifferentiated neuronal cells, without any muscular differentiation (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). This is in contrast to other ES cell teratocarcincomas, which contain differentiated mesodermal, endodermal and ectodermal cells (Evans and Kaufman, 1983). This suggests pocket protein regulation may be essential in mesodermal differentiation. Similarly, E2F-4 appears to be important for the development of mesodermal lineages, with high levels of expression in spleen, thymus and bone marrow and defective hematopoiesis in E2F-4 deficient mice (Humbert *et al.*, 2000a; Rempel *et al.*, 2000). Also, activating mutations of E2F-4 have been found in leukemia and gastrointestinal cancers (Komatsu *et al.*, 2000; Takashima *et al.*, 2001). It was therefore of interest to analyse changes in the p107/E2F-4 pathway in the EPLEB system, as these cells have a primarily mesodermal fate (Lake *et al.*, 2000).

6.2 Characterisation of p107/E2F-4 pathway as ES cells differentiate

To characterise the p107/E2F-4 pathway in EPLEBs, analysis of the expression of key regulators of this pathway was required. ES cells were differentiated into EPL embryoid bodies as described in 2.3.8. Western analysis conducted on protein extracted from EPLEBs shows that p107, DP2 and HDAC levels do not change significantly as ES cells differentiate (Fig 6.2.1a). The mobility change of p107 from ES to EPL cells suggests that there is a decrease in phosphorylation of p107 in EPL cells (Fig 6.2.1a). Decreased

Figure 6.2.1

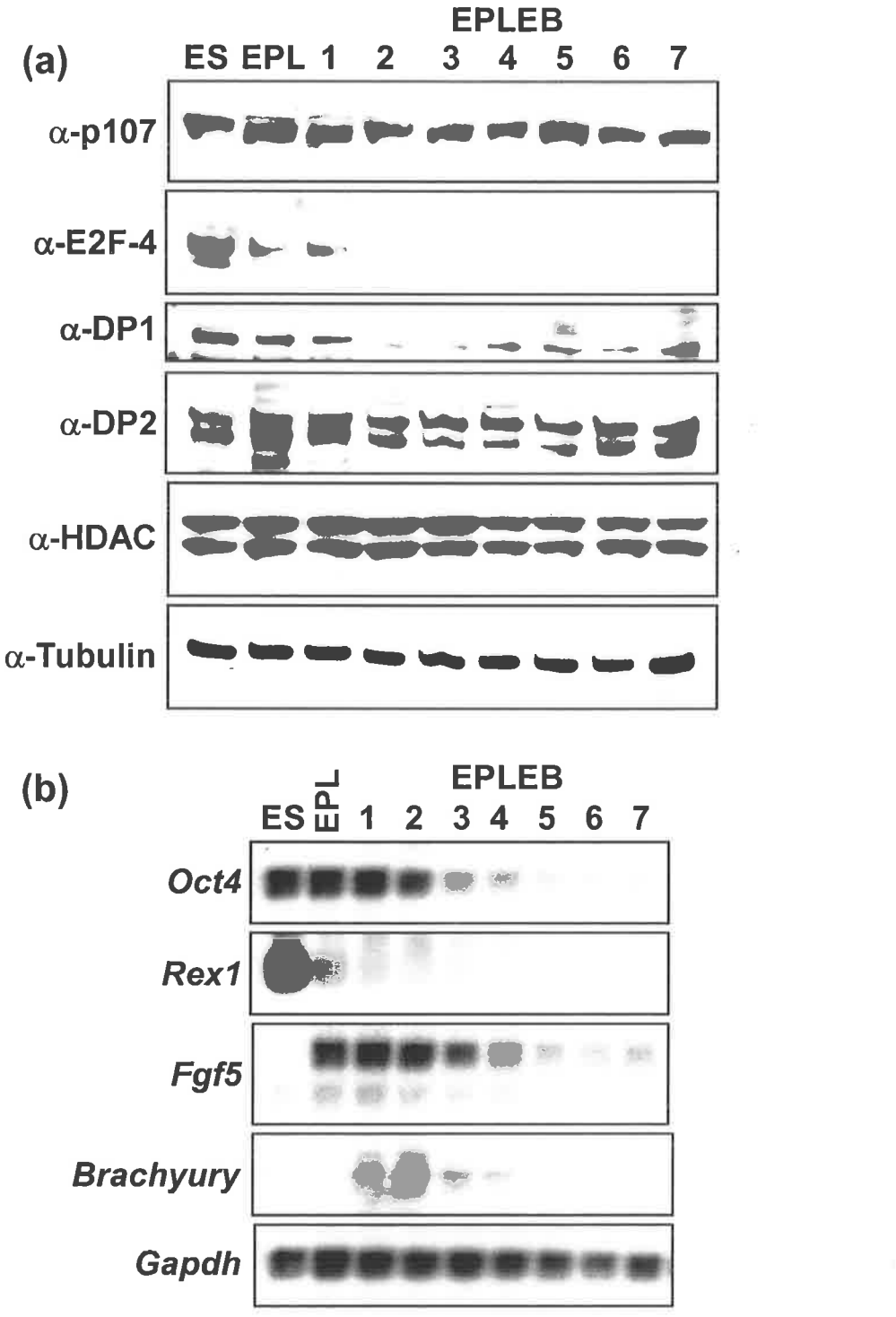


Figure 6.2.1 ES cells were differentiated into EPL embryoid bodies (EPLEBs). Cells were harvested for protein and RNA. (a) Two identical sets of 50μg aliquots of whole cell protein were run on an 8% and a 10% SDS-PAGE gel and subjected to western analysis. Blots from the 8% gel were probed with the p107 and HDAC antibodies. Blots from the 10% gel were probed with the E2F-4, DP1, DP2 and Tubulin antibodies. (b) Total cell RNA (20μg) was resolved on a formaldehyde-agarose gel, blotted and probed with ^{32}P-labelled Rex1, Oct4, Fgf5, Brachyury and Gapdh probes, utilising Gapdh as a loading control.

DP1 levels over differentiation were detected (Fig 6.2.1a). Also, two forms of DP2 were detected, these are likely to be different splice variants of DP2 (Fig 6.2.1a). Significantly, the levels of E2F-4 decrease in EPL cells and are barely detectable by day 2 of EPLEB differentiation (Fig 6.2.1a). Northern analysis demonstrated that cells differentiated according to that previously described (Lake *et al.*, 2000) (Fig 6.2.1b).

To determine if the apparent differences in E2F-4 levels was due to transcriptional regulation, northern analysis was conducted. There was no significant difference in *E2F-4* RNA levels as ES cells differentiate to EPLEBs (Fig 6.2.2a). This suggested that E2F-4 protein abundance was differentially regulated as ES cells differentiate, thus, the turnover of E2F-4 was analysed. ES cells, EPL cells and EPLEBs were treated with or without the proteasome inhibitor MG132 for 3 hours and harvested for protein extraction. Western analysis indicated there was no apparent degradation of E2F-4 in ES cells, however, E2F-4 became unstable in EPL cells (Fig 6.2.2b). In contrast, p107, CDK2 and tubulin were stable (Fig 6.2.2b). Minor differences in the levels of p107 were specific to this experiment and may be due to differences in transfer of higher molecular weight proteins from the Tris-Tricine gel or to western blotting procedure. Similarly, the detection of a higher non-specific band by p107, with increased protein loading, was consistent with batch variation in commercial antibodies. This suggests that as ES cells differentiate E2F-4 activity becomes regulated by proteolytic degradation.

6.3 Changes in E2F activity as ES cells differentiate

Given the differential regulation of E2F-4 and phosphorylation of p107 detected as EPLEBs differentiate, it was hypothesised that differences in E2F target gene expression would be detected. ES cells were differentiated into EPL embryoid bodies (2.3.8) and northern analysis was conducted. *Cyclin E* expression was down regulated gradually from ES cells to EPLEB day 2, reaching a constant level at day 3 (Fig 6.3.1). These changes are similar to that previously reported (E.Stead, PhD thesis). In comparison, *B-myb* expression was rapidly downregulated in EPL cells, reaching a consistent level in EPLEBs day 1. Expression of *CDC2* and *cyclin A* was similar as ES cell differentiate (Fig 6.3.1). These differences suggest that there are further complexities to the regulation of E2F target gene expression as ES cells differentiate.

To gain a further understanding of E2F activity in EPLEB differentiation, ES cells were stably transfected with a luciferase reporter driven by an E2F responsive promoter. Cell

Figure 6.2.2

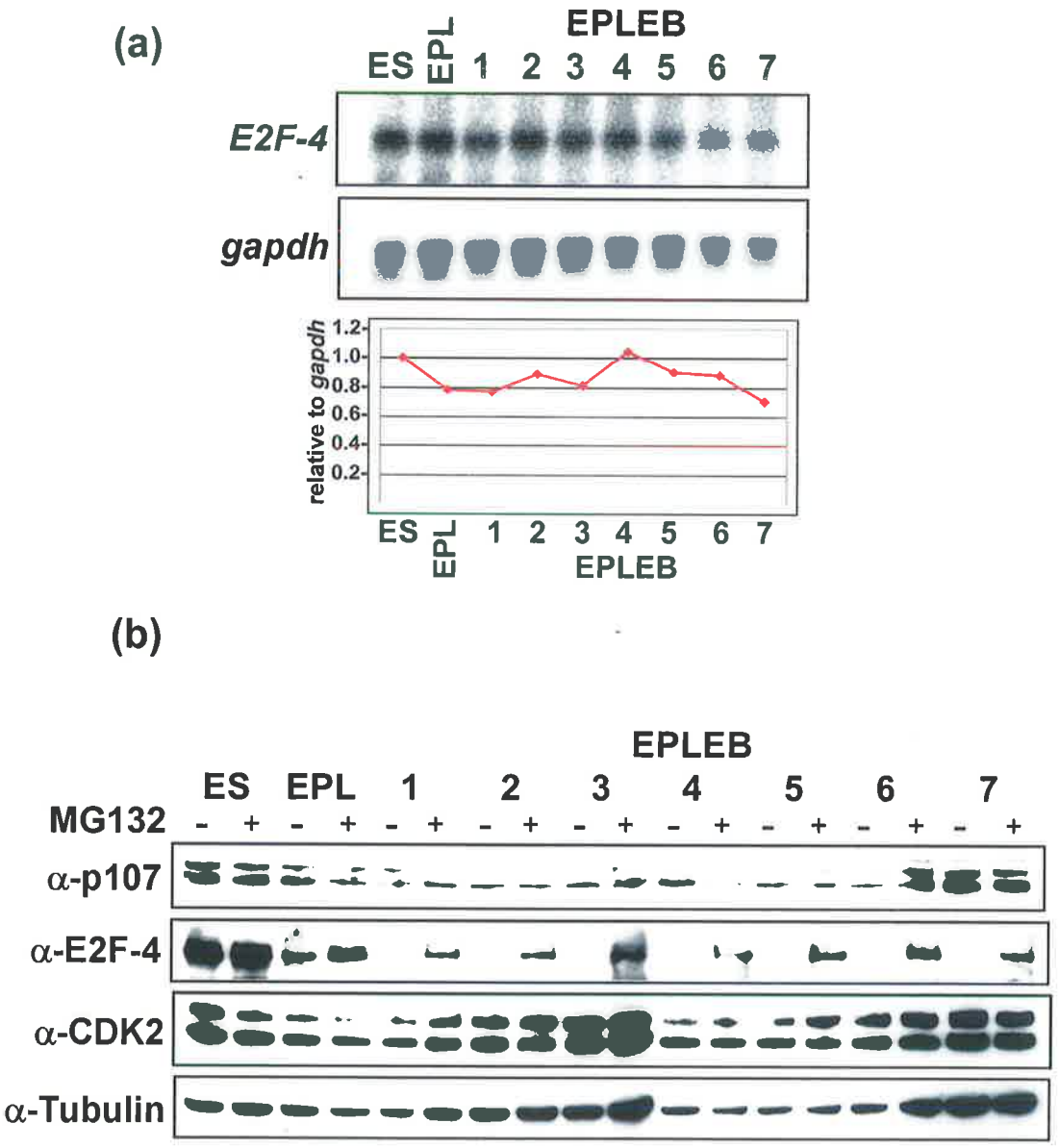


Figure 6.2.2 (a) ES cells were differentiated into EPLEBs. Total cell RNA (20μg) was resolved on a formaldehyde-agarose gel, blotted and probed with ^{32}P-labelled E2F-4 and Gapdh probes. Transcript levels were quantitated by phosphorimaging, utilising *Gapdh* as a loading control. (b) ES cells were differentiated into EPLEBs. Prior to harvesting, cells and bodies were treated with or without 5μM MG132 for 3 hours. 50μg of whole cell protein was run on 10% Tris-Tricine gels and subjected to western analysis, utilising p107, E2F-4, CDK2 and α-Tubulin antibodies.

Figure 6.3.1

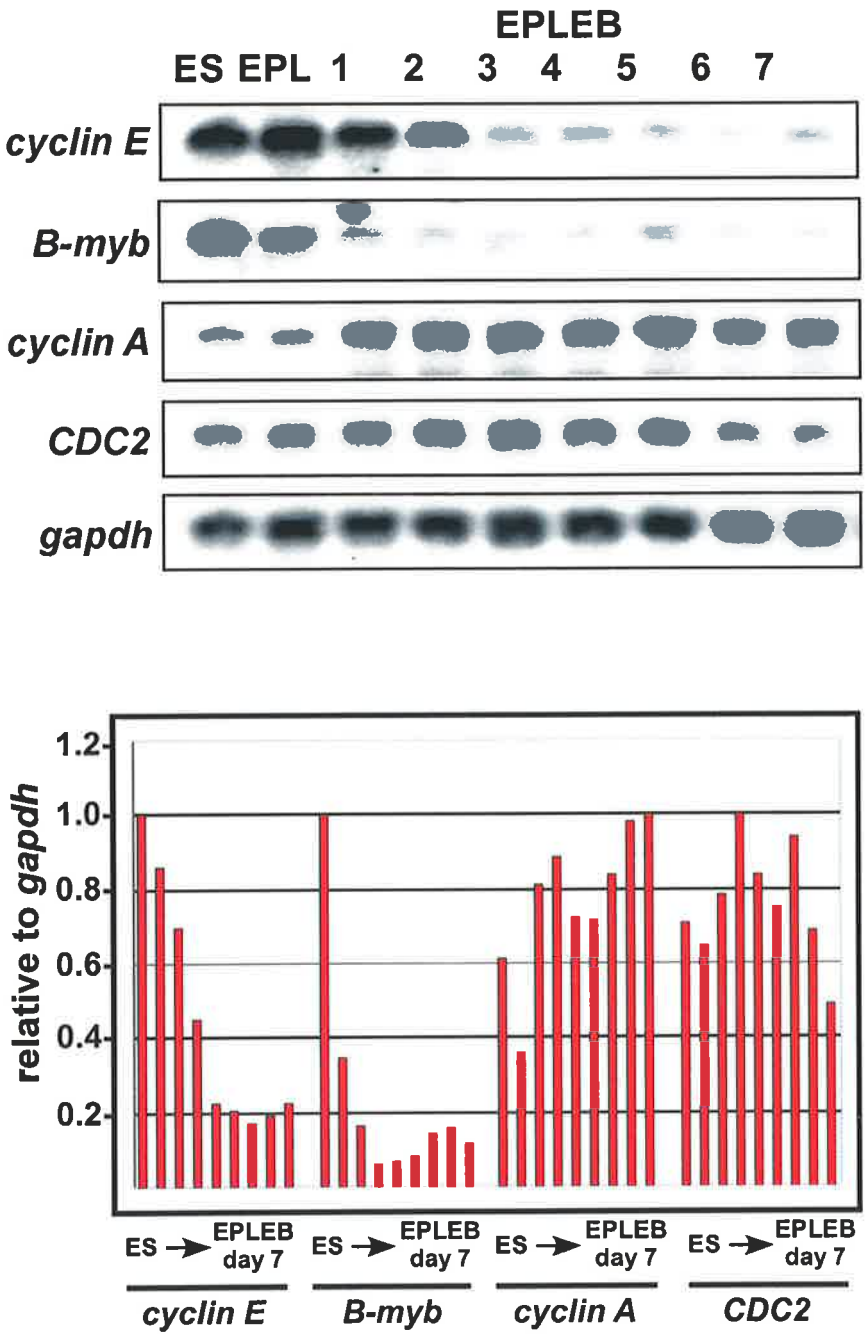


Figure 6.3.1 ES cells were differentiated into EPLEBs. Total cell RNA (20μg) was resolved on a formaldehyde-agarose gel, blotted and probed with ^{32}P-labelled cyclin E, B-myb, cyclin A, CDC2 and Gapdh probes. Transcript levels were quantitated by phosphorimaging, utilising Gapdh as a loading control.

lines were differentiated and luciferase activity in these lines was analysed. The luciferase activity decreased in EPL cells and reached basal levels by EPLEB day 1 (Fig 6.3.2a). To further confirm differences in E2F activity between ES and EPL cells, cells were transiently transfected with luciferase reporters driven by E2F or mutated E2F promoters. While the fold difference was not as great, the E2F activity in EPL cells was lower than that of ES cells (Fig 6.3.2b). This suggests that global E2F activity decreases upon differentiation of ES cells.

6.4 Association with E2F target gene promoters as ES cells differentiate

To further investigate how E2F target genes are regulated as ES cells differentiate, Chromatin Immunoprecipitation (ChIP) assays were employed (2.4.13). This assay enables an analysis of the transcription factors and complexes bound to promoters. It also enables an analysis of the relative state of the promoter, assessed by the level of acetylation of histones. Chromatin was collected from ES cells, EPL cells and EPLEBs. To ensure equal input DNA was used in the IPs, DNA concentrations were equalised, as assayed on a spectrophotometer. Equal proportions of each chromatin sample was used to make DNA preparations and conduct PCRs to ensure the spectrophotometer readings were accurate and equal proportions of input chromatin was attained. IPs were conducted at the same time to ensure that they were as comparable as possible to the controls. Upon completion of each set of IP's, it was essential to ensure results were obtained when the PCR was exponentially increasing for each sample. Therefore, the first and last sample of the differentiation was analysed over numerous cycle numbers to ensure the PCR was in its linear phase.

To gain an impression of the activity of each promoter, the acetylation status of histone H4 at promoters was analysed. ChIP assays were conducted on ES, EPL and EPLEB chromatin extracts with an anti-acetyl Histone H4 antibody. For cyclin E, B-myb and CDC2 promoters, the high levels of acetylated histone H4 in ES cells gradually declined as ES cells differentiated (Fig. 6.4). In comparison, the acetylation status of histone H4 at the cyclin A and Albumin promoters remained reasonably constant (Fig. 6.4). High levels of acetylation at E2F target gene promoters suggests that the chromatin is in an "open" state in ES cells, thus likely to be easily accessible for transcription factors. Accordingly, as ES cells differentiate, the decreased acetylation of promoters suggests that transcription factor accessibility decreases or becomes more tightly regulated.

Figure 6.3.2

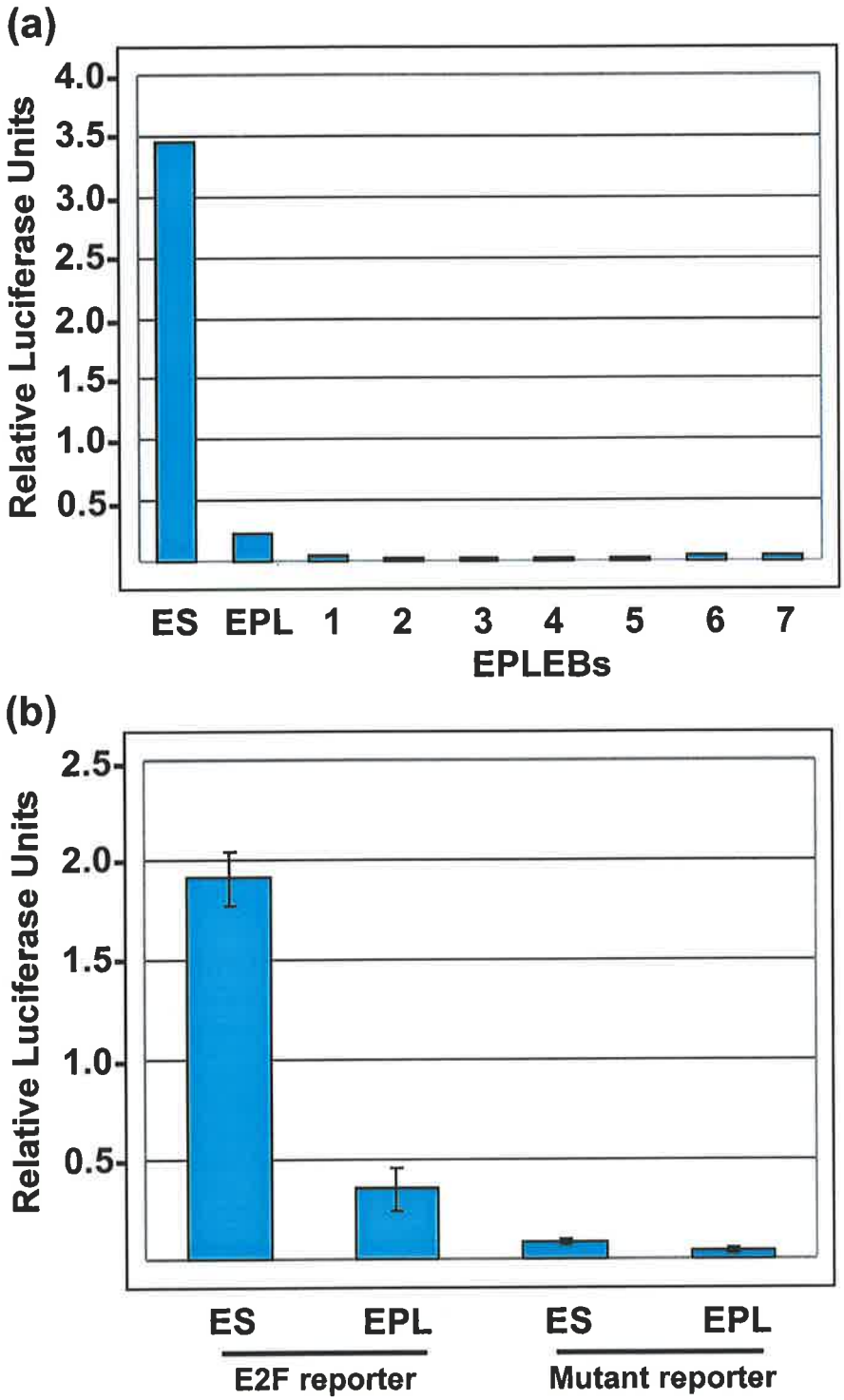


Figure 6.3.2 (a) ES cells were stably transfected with the E2F-luciferase reporter. ES cells were differentiated into EPLEBs. Protein harvested from cells and bodies was analysed for luciferase activity. Luciferase units were normalised using protein concentrations. Data represents a typical experiment. (b) ES and EPL cells were transfected for 40 hours with the E2F-luciferase reporter or mutated E2F-luciferase reporter and a renilla control. Firefly luciferase activity was normalised to renilla luciferase activity and data are presented as relative activity. Experiments were performed in triplicate and data represents a typical experiment.

Figure 6.4

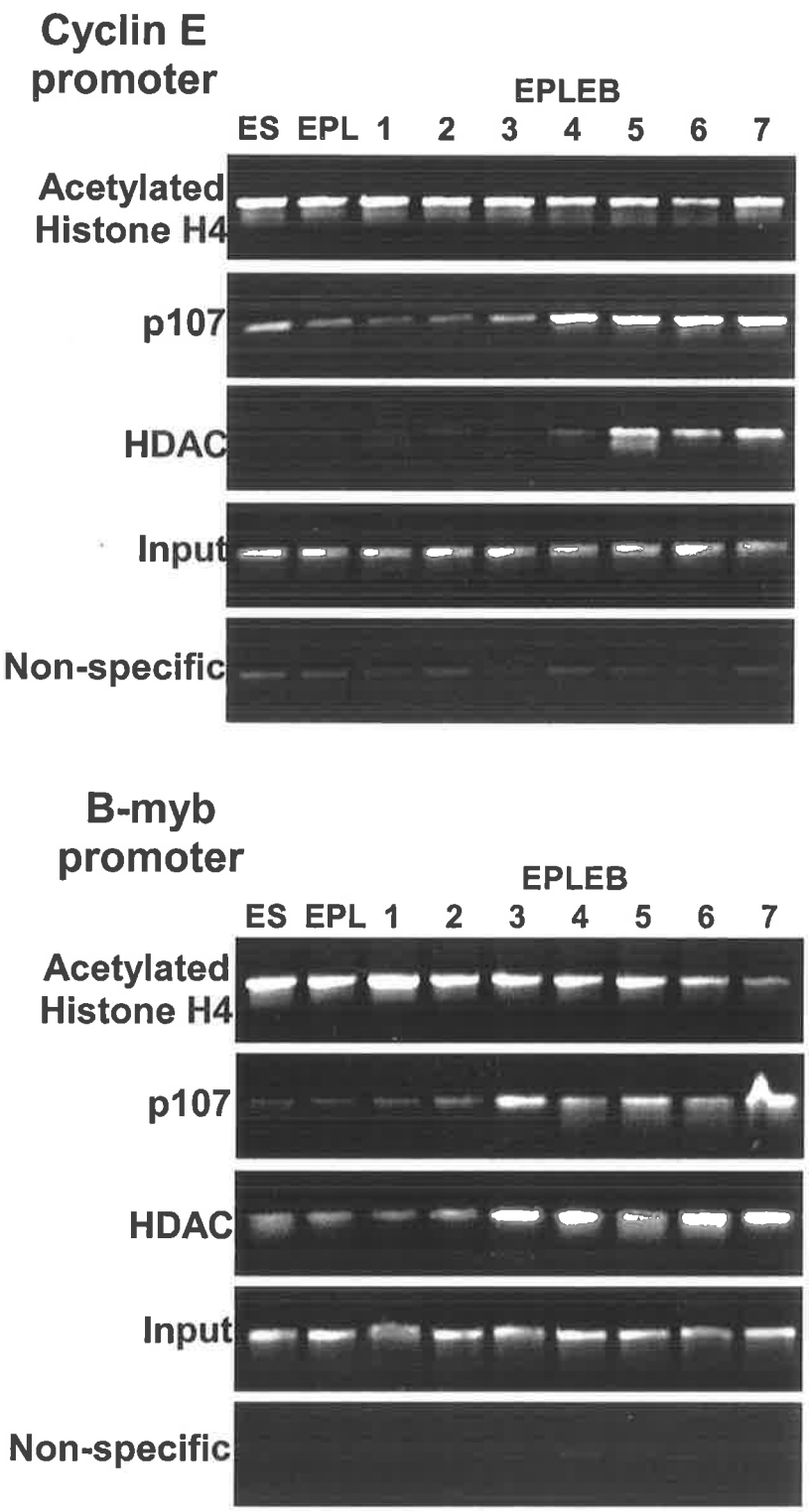

Cyclin E promoter
EPLEB
ES EPL 1 2 3 4 5 6 7
Acetylated Histone H4
p107
HDAC
Input
Non-specific
B-myb promoter
EPLEB
ES EPL 1 2 3 4 5 6 7
Acetylated Histone H4
p107
HDAC
Input
Non-specific

Figure 6.4

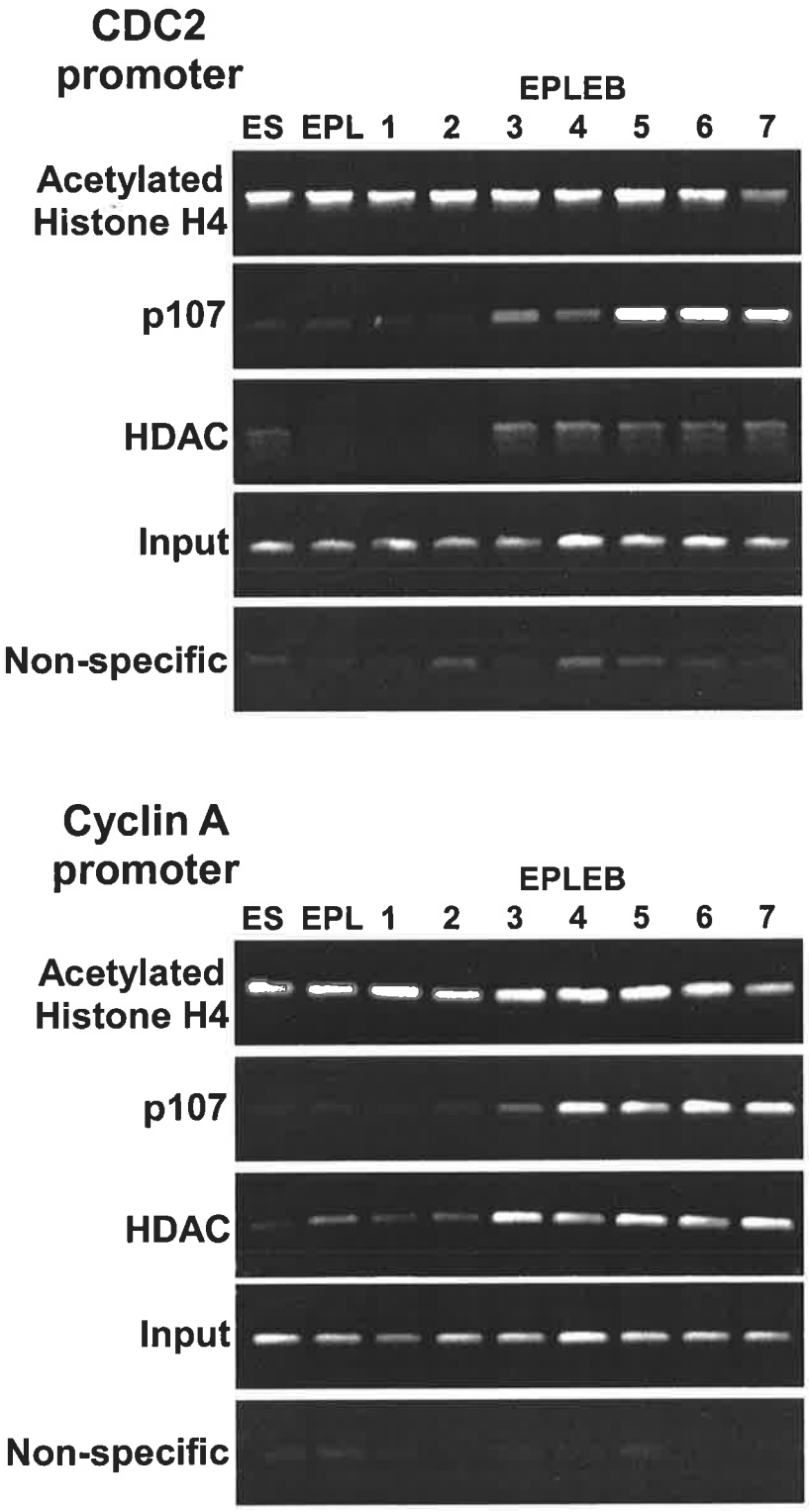

CDC2
promoter
EPLEB
ES EPL 1 2 3 4 5 6 7
Acetylated
Histone H4
p107
HDAC
Input
Non-specific
Cyclin A
promoter
EPLEB
ES EPL 1 2 3 4 5 6 7
Acetylated
Histone H4
p107
HDAC
Input
Non-specific

Figure 6.4

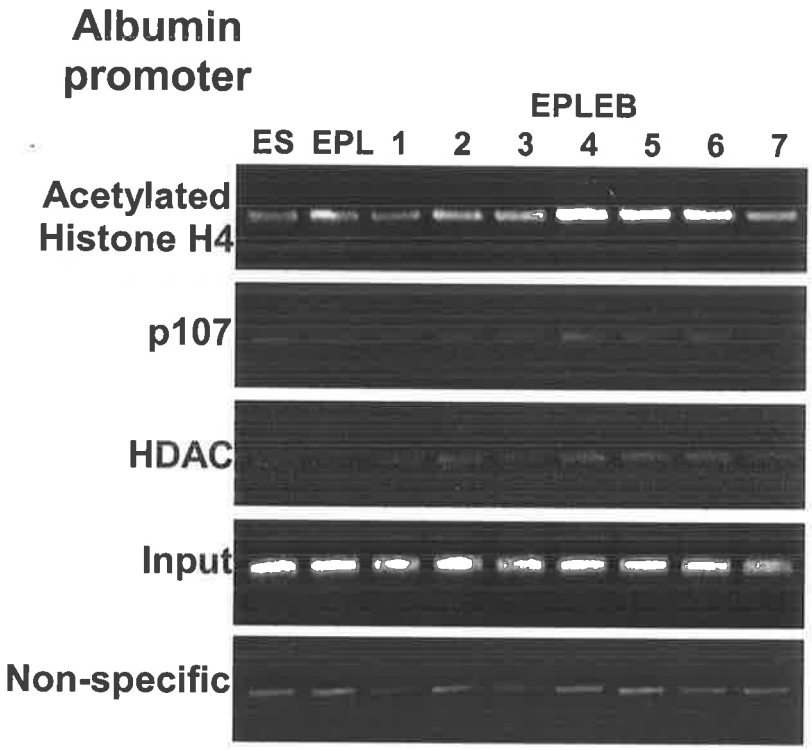


Figure 6.4 Crosslinked chromatin (700μg) from asynchronous ES cells, EPL cells and EPLEBs was incubated with Acetylated histone H4, p107, HDAC or cdc25a (Non-specific) antibodies. Immunoprecipitated DNA was analysed by PCR using primers specific for different promoters (cyclin E, B-myb, cyclin A, CDC2 and Albumin). To ensure equal concentrations of chromatin was analysed, PCRs on 0.03% of the total input chromatin (Input) were conducted.

Association of p107 to these promoters was also analysed. The general trend for p107 binding was an increase as ES cells differentiate (Fig 6.4). Levels of p107 increased on days 3-4 on the cyclin E, B-myb, cyclin A and CDC2 promoters (Fig. 6.4). Low levels of the Albumin promoter was detected in DNA immunoprecipitated by p107 antibodies, however, this did not follow the same trend as the other promoters analysed (Fig. 6.4). Since there is no reported binding of E2F or p107 to the Albumin promoter and the product was much less than for other promoters, it is likely that this indicates the level of background in the ChIP assays. HDAC was also analysed due to its ability to bind p107 and aid in the repression of E2F target genes. The general trend of HDAC binding was similar to p107, increasing as ES cells differentiate (Fig. 6.4). Similar to that detected in p107 IPs, there were low levels of the Albumin promoter detected in DNA immunoprecipitated by HDAC antibodies. Since this was at much lower levels than for the other promoters analysed, it is also likely that this indicates the level of background in the ChIP assays. In accordance with its role in deacetylation, HDAC association with promoters directly opposed that of histone H4 acetylation status (Fig. 6.4), suggesting that increased HDAC activity at promoters is directly associated with decreased acetylation of promoters.

6.5 Function of p107-E2F pathway as ES cells differentiate

The differences in phosphorylation of p107 and binding to E2F target gene promoters as ES cells differentiate stimulated an interest in analysing the role of p107 and the contribution of phosphorylation to the regulation of its function as ES cells differentiate. Initial *in vitro* analysis was conducted to assess the effects of expression of p107 and a phosphorylation defective mutant p107 in EPL cells. EPL cells were transiently transfected with luciferase reporters driven by E2F or mutated E2F promoters and with p107 or mutant p107ΔS/T-P. p107 effectively inhibited E2F activity in EPL cells (Fig 6.5.1). An average of three experiments shows a 2.2 fold decrease in endogenous E2F activity with p107-WT compared to a 2.7 fold inhibition by p107ΔS/T-P. This difference supports the hypothesis that p107 activity is regulated by phosphorylation in EPL cells. However, there is less difference in EPL cells between p107-WT and p107ΔS/T-P inhibition of E2F activity (1.2 fold) in comparison to ES cells (1.5 fold) (Fig 5.4.1b). This suggests that there is less regulation by phosphorylation of p107 inhibition of E2F activity in EPL cells compared to ES cells. Technical difficulties in transient transfection of embryoid bodies prevented further analysis of the effects of p107 in the EPLEB system.

Figure 6.5.1

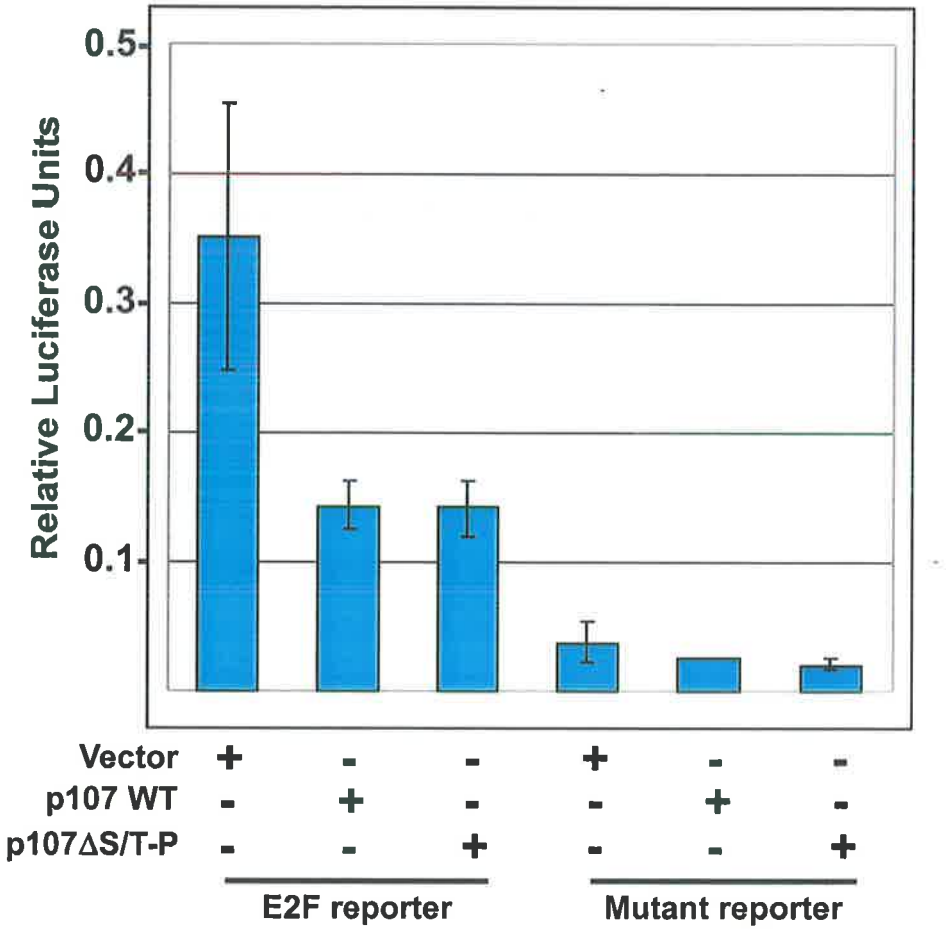


Figure 6.5.1 EPL cells were transfected, 8 hours after plating, for 40 hours with the E2F-luciferase reporter (pGL3promoter-4xCCC) or mutated E2F-luciferase reporter, a renilla control and 50ng of vector, wildtype p107 (p107-WT) or phosphorylation-resistant p107 (p107ΔS/T-P). Firefly luciferase activity was normalised to renilla luciferase activity and data are presented as relative activity. Experiments were performed in triplicate and data represents a typical experiment.

To further analyse the effect of p107 as ES cells differentiate, stable p107-WT and p107ΔS/T-P clones were differentiated as EPLEBs. Morphologically cells and bodies appeared relatively normal (data not shown). Northern analysis showed that, by expression of marker genes, cells differentiated appropriately (Fig 6.5.2a). However, analysis of expression of p107-WT and p107ΔS/T-P by Western analysis showed that in EPL cells expression of p107-WT and p107ΔS/T-P was undetectable (Fig 6.5.2b). These differentiation experiments were conducted in the absence of puromycin, suggesting that the expression of both p107-WT and p107ΔS/T-P transfected genes were silenced upon differentiation.

In order to maintain expression, ES cells were differentiated in the presence of puromycin. On day 1 of EPL cell formation, p107-WT and p107ΔS/T-P cells showed increased levels of cell death compared to control cell lines, as indicated by the number of floating cells (data not shown). By day 2 of EPL cell formation, there were very few cells remaining attached to the dish in p107-WT and p107ΔS/T-P lines (data not shown). These differences were specific for p107 expressing cells, as vector only cells were also maintained in puromycin. These EPL cells and the remaining floating cells were harvested and analysed by flow cytometry. Some differences in cell lines were observed, which may be attributed to differential expression or silencing of the transfected genes. An increased proportion of cells in G1 and a loss of cells from S phase was observed in p107WT-4 EPL cells (Fig 6.5.3). Other cell lines were decreased in all cell cycle phases with a greater proportion of cells lost from S phase (Fig 6.5.3). In each case there was an increase in the sub-G1 population in p107-WT and p107ΔS/T-P EPL cells, which is indicative of cell death (Fig 6.5.3). This suggests that with the continued expression of p107-WT and p107ΔS/T-P EPL cells are not able to survive. Attempts to culture remaining p107 expressing cells as EPLEBs were unsuccessful, resulting in death of all cells.

6.6 Discussion

Constitutive CDK activity and E2F target gene expression in ES cells (Stead *et al.*, 2002) is associated with differential regulation of the p107/E2F-4 pathway. Changes in CDK activity associated with differentiation of ES cells into EPLEBs (E. Stead, PhD thesis and this thesis) led to the hypothesis that activity of the p107-E2F pathway would change as ES cells differentiate. This is reflected in the changes in mobility of p107 with

Figure 6.5.2

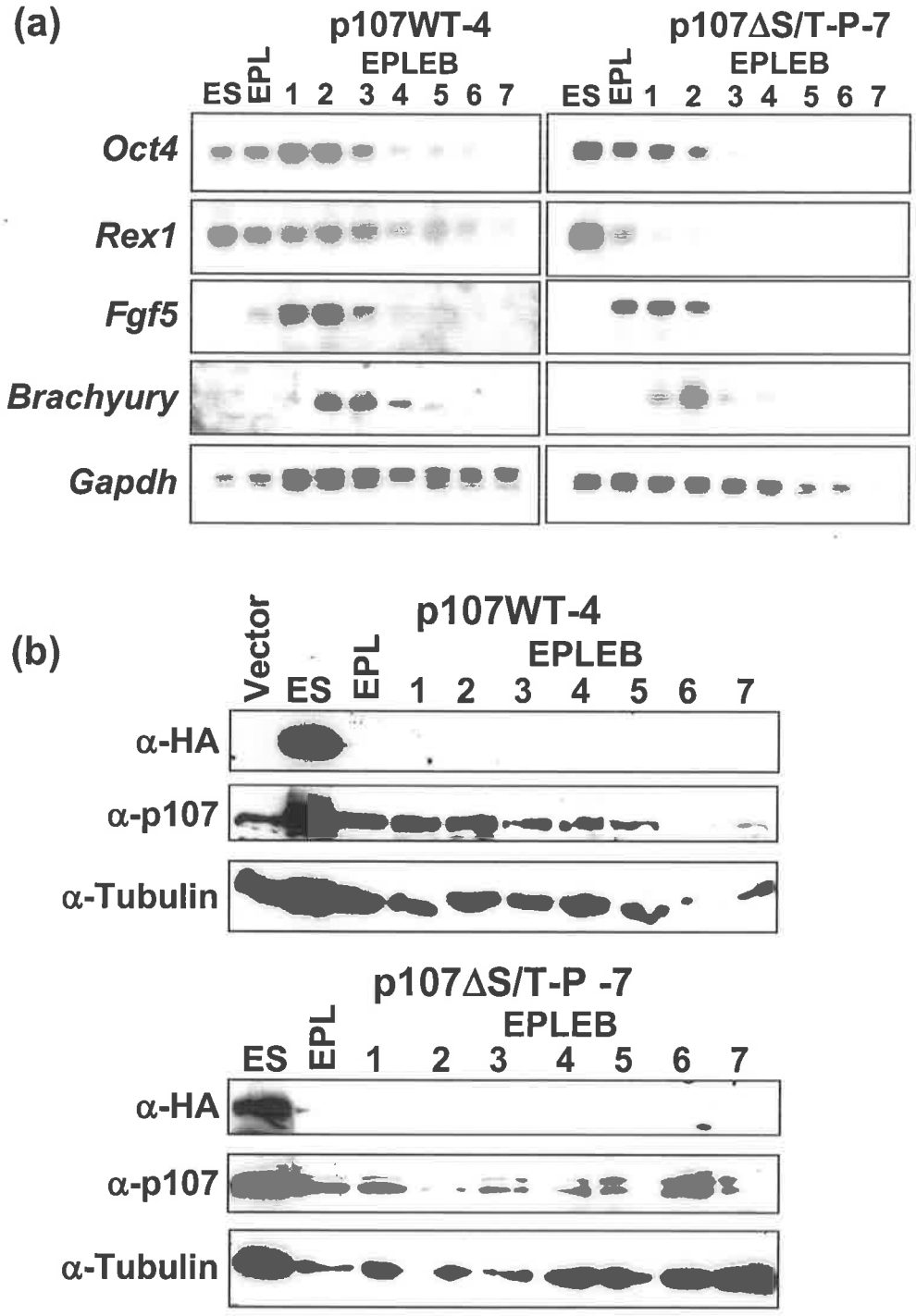


Figure 6.5.2 ES cells were stably transfected with pCAG vector (Vector), pCAG-p107-WT or pCAG-p107ΔS/T-P. ES cells from different cell lines were differentiated into EPLEBs. (a) Total cell RNA (20μg) was resolved on formaldehyde -agarose gels, blotted and probed with ^{32}P-labelled Rex1, Oct4, Fgf5, Brachyury and Gapdh probes, utilising Gapdh as a loading control. (b) Whole cell protein (50μg) was resolved on a 10% SDS-PAGE gel and subjected to western analysis, utilising HA, p107 and Tubulin antibodies.

Figure 6.5.3

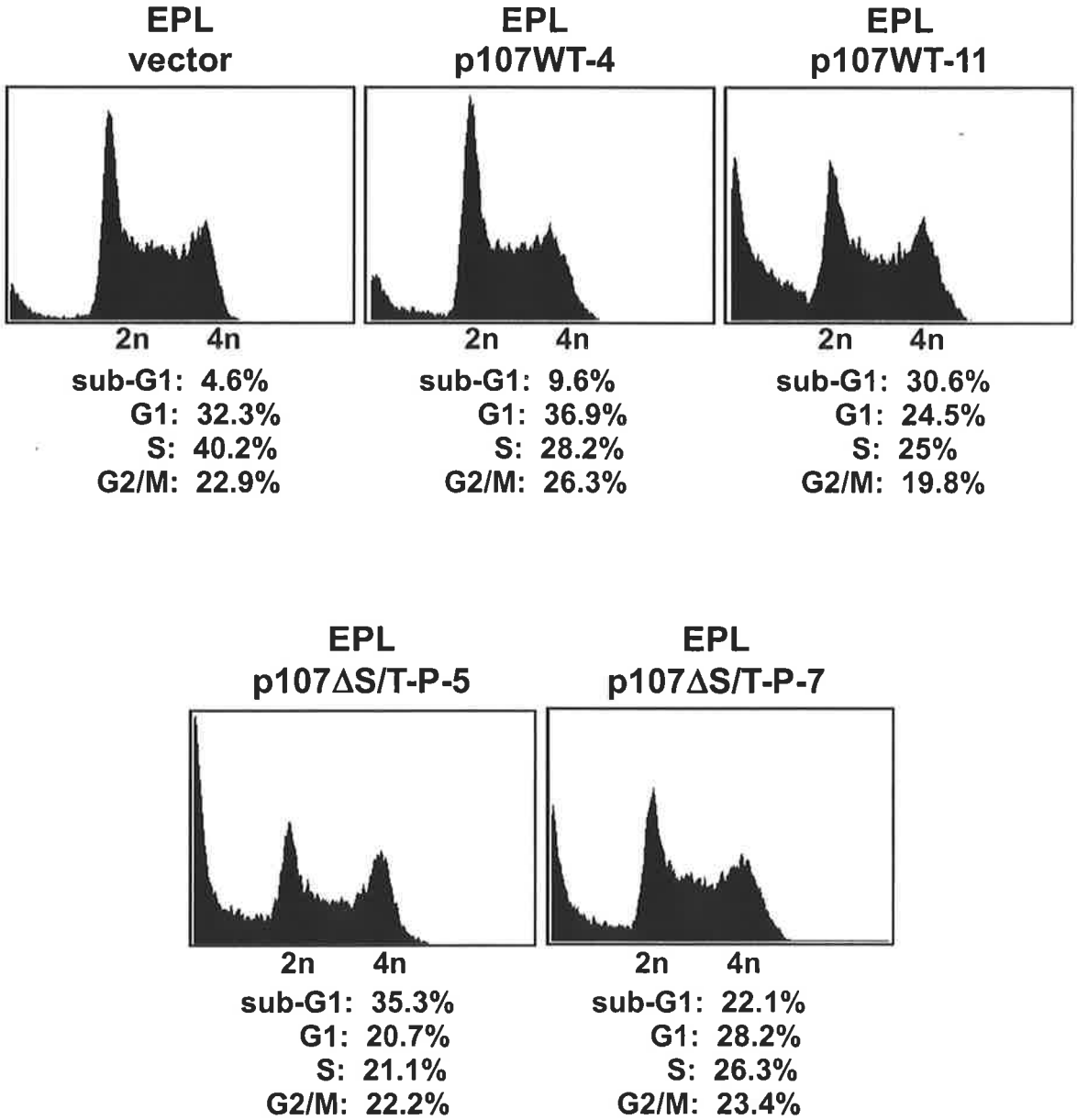


Figure 6.5.3 ES cells were stably transfected with pCAG vector (vector), pCAG-p107WT or pCAG-p107ΔS/T-P and differentiated into EPL cells. Asynchronously growing EPL cells from different cell lines were fixed and stained with propidium iodide. The proportion of cells in each phase of the cell cycle was analysed using a Beckman Coulter flow cytometer and WinMDI 2.8 software.

differentiation of ES cells into EPL cells. In particular, differences in p107 phosphorylation status between ES and EPL cells may reflect the decreases in CDK6 activity observed in EPL cells (this thesis, chapter 4), as CDK2 activity is constant (E. Stead, PhD thesis). It would be predicted that this would lead to increased association of p107 with E2F-4 in EPL cells. However, analysis of this hypothesis was technically challenging due to increased E2F-4 proteolysis in EPL cells, decreasing the proportion of E2F-4 available and making direct comparisons between ES and EPL cells difficult. Also, if this hypothesis were correct, expression of E2F target genes would decrease in EPL cells. With the exception of *B-myb*, there was no significant decrease in the expression of E2F target genes analysed in EPL cells, suggesting that further mechanisms may regulate their expression. Further changes in p107 phosphorylation status as ES cells differentiate are difficult to estimate by mobility shift alone. More accurate predictions require the use of phospho-specific antibodies or metabolic labelling. However, the decreases in cyclin E-CDK2 activity at EPLEB day 3 would suggest that p107 phosphorylation would also decrease (E.Stead, PhD thesis). Comparatively, it is also at this differentiation stage in other *in vitro* systems that CDK4 activity is increased (Savatier *et al.*, 1996), suggesting that the overall phosphorylation state of p107 may not change. However, p107 regulation of E2F activity would change dramatically, as it is only CDK4/6 activity that can release p107-E2F complexes (Beijersbergen *et al.*, 1995; Cheng *et al.*, 2000a; Leng *et al.*, 2002; Xiao *et al.*, 1996). Therefore, it is predicted that as ES cells differentiate there is an increase in p107 regulation of E2F activity. Interestingly, while p107 phosphorylation status has not been investigated in the gastrulating embryo, similar changes in phosphorylation of p107, also assessed by mobility changes on polyacrylamide gels, were detected upon analysis of whole mouse embryo lysates from 9dpc to 18 dpc (Leezer *et al.*, 2003). This suggests that the regulation of p107 changes throughout mouse embryonic development.

In addition to the decreased regulation of E2F-4 activity by p107 (Humbert *et al.*, 2000a; Stead *et al.*, 2002), E2F-4 stability is increased in ES cells compared to differentiated cells (this thesis). Similar to the changes in phosphorylation of p107 in EPL cells, E2F-4 stability is decreased in EPL cells. While E2F-4 is protected from degradation when bound to pRB family members (Campanero and Flemington, 1997; Hateboer *et al.*, 1996; Hofmann *et al.*, 1996), the high proportion of free E2F-4 in ES cells suggests that the differences in stability between ES and EPL cells is not due to differences in binding to

pocket proteins. Instead, it is likely that the pathway that governs the proteasome-mediated degradation of E2F-4 becomes activated as EPL cells are formed. Further analysis requires the identification of the pathways governing E2F-4 turnover. While E2F-4 is not essential for the differentiation of ES cells (Humbert *et al.*, 2000a), it would be of interest to see whether decreased E2F-4 activity is required for differentiation by analysis of the effects on differentiation when a non-degradable mutant of E2F-4 is expressed.

The likely consequence of differential regulation and activity of the p107/E2F-4 pathway as ES cells differentiate is changes in E2F target gene expression. Each of the E2F target genes analysed displayed differential expression as ES cells differentiate. This may be due to differential activity of E2F family members. Similar to that observed here, differentiation of the embryonal carcinoma cell line, P19, with retinoic acid is associated with downregulation of E2F-4 levels (Kusek *et al.*, 2001). E2F-1 to -3 are also present in undifferentiated P19 cells but are dramatically upregulated upon differentiation with retinoic acid (Kusek *et al.*, 2001). Thus, changes in E2F target gene expression with differentiation may be related to differences in expression of E2F family members. It is difficult to determine which E2Fs regulate these E2F target genes, since E2F-1 to -4 are associated with each of the promoters analysed in cycling cells (Takahashi *et al.*, 2000; Wells *et al.*, 2000). Also, each of the genes analysed are upregulated in response to ectopic E2F-1 to -4 expression (DeGregori *et al.*, 1995; DeGregori *et al.*, 1997; Muller *et al.*, 2001; Polager *et al.*, 2002), suggesting that each can be transcriptionally activated by many E2F family members. However, the decreased expression of each of the genes analysed in E2F-3-/- MEFs, suggests that E2F-3 may be essential for their expression (Humbert *et al.*, 2000b). Further analysis of the presence and activity of other E2F family members in ES cells and differentiation is required to assess the contribution of E2F transactivation to differences in E2F target gene expression.

Alternatively, differences in E2F target gene expression as ES cells differentiate may reflect differences in regulation by pocket proteins. Decreased *cyclin E* expression on day 3 is concomitant with increased association of p107 with the cyclin E promoter, suggesting that inhibition of E2F activity by p107 is involved in the downregulation of *cyclin E* expression. p107 recruits HDAC to actively repress E2F activity (Ferreira *et al.*, 1998). The importance of this association to enable repression of E2F activity has not

been investigated, however, repression of *cyclin E* expression by pRB requires HDAC activity (Zhang *et al.*, 2000). The increased association of HDAC with the cyclin E promoter from day 3 suggests that it may be instrumental for decreased expression of *cyclin E*. Analysis of the effects of the HDAC inhibitor Trichostatin A (TSA) upon *cyclin E* expression was conducted. However, EPLEBs were sensitive to TSA, such that even analysis of changes in E2F responsive reporter activity was unsuccessful (data not shown). Alternatively, since *cyclin E* expression is strongly derepressed in pRB-/- MEFs (Hurford *et al.*, 1997), pRB may be involved in the regulation of *cyclin E* expression as ES cells differentiate. The hyper-phosphorylation of pRB in ES cells (Savatier *et al.*, 1994) and constitutive expression of *cyclin E* (Stead *et al.*, 2002) suggest that ES cells may mimic pRB-/- cells. During EPLEB differentiation, increases in hypo-phosphorylated pRB (S. Conn., Honours thesis) are concomitant with decreases in *cyclin E* expression, suggesting that increases in active pRB may regulate *cyclin E* expression. ChIP assays were conducted to assess changes in binding of pRB to E2F target gene promoters. Unfortunately, all attempts were unsuccessful, including positive controls of MEFs, asynchronous and growth arrested by serum starvation (data not shown). This suggests that the antibody utilised may not recognise chromatin associated pRB.

In contrast to *cyclin E*, *B-myb* expression is strongly derepressed in p107-/-;p130-/- MEFs (Hurford *et al.*, 1997), suggesting that p107 and p130 are essential in regulation of *B-myb* regulation. ES cells are similar to p107-/-;p130-/- MEFs, in that there is no p130 (LeCouter *et al.*, 1996) and p107 is highly phosphorylated (this thesis) and only a small proportion is bound to DNA bound E2Fs (Humbert *et al.*, 2000a). This is consistent with the high levels of *B-myb* expression in ES cells. Decreases in *B-myb* expression in EPL cells may reflect increased p107 activity. Given the decreased CDK6 activity and decreased phosphorylation of p107 in EPL cells (this thesis), it is likely that there is increased inhibition of E2F activity by p107 in EPL cells. However, there is no significant increase in p107 binding to the B-myb promoter in EPL cells compared to ES cells, as indicated by ChIP assays. Still, while there may be no change in p107 binding to the B-myb promoter, decreased E2F-4 levels in EPL cells suggest that there may be a proportional increase in p107 inhibition of E2F activity in EPL cells, leading to inhibition of *B-myb* expression. The timing of decreased *B-myb* expression does not correlate with increased HDAC association, suggesting active repression may not be a key regulator of *B-myb* expression. However, changes in the association of HDAC with the B-myb

promoter inversely correlates with histone H4 acetylation, suggesting that HDAC recruitment may be important in B-myb promoter activity as ES cells differentiate. Interestingly, a significant correlation between decreased E2F-4 levels and decreased *B-myb* expression, suggests that E2F-4 may be transactivating *B-myb* expression. Unfortunately, upon analysis of E2F-4 binding to target gene promoters over differentiation, batch variation in commercial antibodies led to unsuccessful ChIP assays and the loss of equivalent EPLEB chromatin extracts for analysis. Further analysis with ectopic expression of E2F-4 should be conducted to assess its role in *B-myb* expression in pluripotent cells.

Both *cyclin A* and *CDC2* are also derepressed in p107-/-;p130-/- MEFs (Hurford *et al.*, 1997), suggesting that p107 and p130 are essential in regulation of their expression. While there is no change in levels of expression of these genes as ES cells differentiate, there is increased association of p107 with cyclin A and CDC2 promoters. This suggests that p107 association is not repressing transcription of these genes. This may be related to the observation that repression of *cyclin A* and *cdc2* by pRB requires the recruitment of BRG1 (Strobeck *et al.*, 2000; Zhang *et al.*, 2000). p107 is also able to associate with BRG1 (Strober *et al.*, 1996), however, the requirement for p107-BRG1 association for repression of *cyclin A* and *cdc2* has not been reported. Still, the lack of downregulation of *cyclin A* and *cdc2* over EPLEB differentiation may be due to a lack of BRG1 recruitment by pocket proteins. Analysis of the binding of BRG1 to p107 and to E2F target gene promoters over EPLEB differentiation should be investigated.

Alternatively, there is potential that these genes are regulated by E2F-independent mechanisms. While E2F-binding sites control cell cycle regulated expression of *cyclin E* (Botz *et al.*, 1996; Geng *et al.*, 1996; Le Cam *et al.*, 1999; Polanowska *et al.*, 2001), binding sites for other transcription factors, such as Sp1, are also present in the cyclin E promoter (Geng *et al.*, 1996). Also, cell cycle dependent *B-myb* expression is regulated by E2F sites (Liu *et al.*, 1996; Lucibello *et al.*, 1997), however, transcriptional activation also involves Sp1 and MRG15 (Leung *et al.*, 2001; Pardo *et al.*, 2002; Sala and Watson, 1999; Tominaga *et al.*, 2003). Regulation of *cyclin A* expression also involves a range of transcription factors, such as Sp1, CREB/ATF, NF-Y, ets-2 and JunB (Desdouets *et al.*, 1995; Liu *et al.*, 1998b; Liu *et al.*, 1997b; Wen *et al.*, 1995). Similarly, *CDC2* expression

is regulated by NF-Y, Sp1, ets-2 and upstream stimulatory factors (Jun *et al.*, 1998; Liu *et al.*, 1998c; North *et al.*, 1999; Shimizu *et al.*, 1995).

By comparison to marker gene expression, there is an increase in p107 bound to E2F target gene promoters just after the formation of nascent mesoderm. This suggests that E2F target genes become more tightly regulated by pocket proteins upon mesoderm formation. The coincident increased G1 phase and decreased cyclin E-CDK2 activity (E. Stead, PhD thesis) suggest that these changes may be linked to overall changes in cell cycle regulation. The lineage specific importance of pocket proteins is evident from analysis of pRB-/-;p107-/-;p130-/- ES cells, which exhibit no muscular differentiation when teratocarcinomas are formed (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). It is of interest to further analyse the role of p107 in mesodermal lineage differentiation by analysis of the effects of inducible p107 expression as ES cells differentiate into EPLEBs.

The high levels of histone acetylation in ES cells compared to differentiated cells suggests that ES cells may have an overall, relaxed chromatin structure. This would be consistent with the inactivity of pRB in ES cells, as pRB-/- MEFs have a more relaxed chromatin structure (Herrera *et al.*, 1996). The consequences would be increased accessibility for transcription factors. The acetylation of E2F target gene promoters generally decreased with differentiation. This may reflect the recruitment of histone acetylase activity by E2Fs or other transcription factors during early differentiation, which is decreased upon increased p107 binding. Alternatively, decreased acetylation of histones at E2F target gene promoters may be due to the increases in binding of HDAC to promoters. This correlation is similar to that of cell cycle dependent acetylation of E2F target gene promoters and HDAC binding to promoters (Ferreira *et al.*, 2001).

The similarities between ES and EPL cells are abundant, however the difference in response to p107 expression highlights the fact that there are differences. While the level of E2F activity in EPL cells is lower, the average fold inhibition of E2F activity by p107-WT and p107ΔS/T-P was more comparable in EPL cells compared to ES cells. This suggests that there is less regulation of p107 function by phosphorylation in EPL cells compared to ES cells. While there is no change in the levels of CDK2 activity, EPL cells have less CDK6 activity than ES cells (this thesis, chapter 4). This suggests that the levels of CDK6 activity in EPL cells is limiting for inactivation of ectopic p107. With

limiting CDK6 activity, an increase in the association of p107 with E2F would be predicted. However, there is no increase in the levels of p107 on E2F target gene promoters in EPL cells as analysed by ChIP assay. This is likely to be linked to the decreased levels of E2F-4 in EPL cells compared to ES cells. Analysis of the effects of ectopic CDK6 expression upon the regulation of p107 and E2F activity in EPL cells should be conducted to assess this hypothesis.

Accordingly, the cell proliferation response in stable p107-WT and p107ΔS/T-P EPL cells was comparable. In comparison to ES cells, EPL cells expressing p107-WT or p107ΔS/T-P undergo cell death, without cell cycle phase specific arrest in proliferation. Cell line specific differences in the response to ectopic p107 may be due to differential expression of p107. However, due to the silencing of transgenes, a more accurate analysis of the effects of p107 on the cell cycle structure of EPL cells requires sorting cells ectopically expressing p107 or analysis of the cell cycle structure of EPL cells transiently transfected with p107 expression vectors. While unlikely to be related to the ability to sequester cyclin A/E-CDK2 complexes, as the levels of these complexes are comparable between ES and EPL cells, this response may be due to the decreased levels of E2F-4 in EPL cells and decreased levels of CDK6 activity in EPL cells. Maintaining the levels of ectopic p107 in EPL cells would lead to relatively less free E2F-4, which would further decrease E2F transcription. To confirm this hypothesis, coexpression of a non-degradable E2F-4 could be analysed, which would increase levels of E2F-4 to potentially maintain E2F-dependent transcription. Importantly, the response of EPL cells to ectopic p107 is similar to primitive ectoderm cells of the pre-gastrulating mouse embryo upon DNA damage, in that they undergo cell death rather than growth arrest (Heyer *et al.*, 2000).

Alternatively, differences in the effects of ectopic p107 between ES and EPL cells may be related to E2F-independent functions of p107. p107 binds to the N-terminal transactivation domain of c-Myc and inhibits its transcriptional activation (Beijersbergen *et al.*, 1994a; Gu *et al.*, 1994; Hoang *et al.*, 1995). c-Myc protein levels decrease as ES cells differentiate (P. Cartwright, unpublished data), therefore there may be proportionally more inhibition of c-Myc activity and termination of cell proliferation. More significantly, B-myb is required for inner cell mass formation and the proliferation of ES cells (Tanaka *et al.*, 1999). The direct interaction between B-myb and p107 inhibits B-

myb transcriptional activity (Sala *et al.*, 1996b). Importantly, there is a large proportion of p107 associated with B-myb in ES cells (Joaquin and Watson, 2003). With the high levels of expression of B-myb in ES cells, it is unlikely that in stable p107-WT and p107ΔS/T-P ES cells there is complete inhibition of B-myb activity by ectopic p107. However, since the expression of *B-myb* decreases in EPL cells, ectopic p107 may be binding and inhibiting all B-myb activity in EPL cells, mimicking the B-myb knockout and disabling proliferation. Further analysis of the associations between p107 and B-myb in these cell systems would enable a deeper understanding of p107 activity in early development

CHAPTER SEVEN

Final Discussion

The aim of this thesis was to further understand how cell cycle regulatory mechanisms govern the rapid ES cell cycle and the proliferative changes associated with differentiation. Specifically this project, characterised the function of CDK2 activity, established the status of CDK4/6 activity and characterised the role and regulation of p107 and E2F-4 in ES cells and differentiation.

7.1 Roles of CDK2 activity in ES cells and differentiation

In ES cells, high, constitutive levels of cyclin E-CDK2 activity contributes to rapid ES cell cycle progression (Stead *et al.*, 2002). This thesis demonstrated that there is increased cyclin E-CDK2 kinase activity associated with higher order complexes in ES cells compared to differentiated cells. Accordingly, this suggests that cyclin E-CDK2 is acting on a greater proportion of targets in ES cells. Importantly, the available pools of proteins forming complexes with cyclin E-CDK2 may be saturated, since the majority of cyclin E-CDK2 activity is found in lower molecular weight complexes, unlikely to be associated with other proteins. Since most of the identified cyclin E-CDK2 targets are involved in proliferation (Ait-Si-Ali *et al.*, 1998; Arata *et al.*, 2000; Coverley *et al.*, 2002; Hatakeyama *et al.*, 1994; Herbig *et al.*, 2000; Hinds *et al.*, 1992; Jiang *et al.*, 1999; Mimura and Takisawa, 1998; Shanahan *et al.*, 1999), enhancing their activity is likely to be increasing the rate of proliferation. Further analysis is required to identify proteins in these higher order complexes and assess the effects of cyclin E-CDK2 phosphorylation upon their activity. Similarly, the massive pool of free, active cyclin E-CDK2 in ES cells, but not in differentiated cells, is likely to enable rapid phosphorylation of any substrates with which cyclin E-CDK2 phosphorylates but does not stably associate, promoting their activity and aiding the rapid proliferation of ES cells. With most cyclin E-CDK2 targets involved in the preparation for and initiation of DNA replication, it is understandable why there is little delay in G1 phase in ES cells. However, the decreases in all cell cycle phases upon inhibition of CDK2 activity (Stead *et al.*, 2002) and the changes in complexes formed with active cyclin E-CDK2 in G2/M phase (this thesis) suggests that cyclin E-CDK2 may have functions in all phases of the ES cell cycle. Similarly, cyclin E-CDK2 is thought to function during mitosis in Xenopus embryogenesis prior to the MBT (Hartley *et al.*, 1996). Importantly, these data demonstrate that increased regulation of substrates is likely to be enabling cyclin E-CDK2 to drive the rapid progression of the ES cell cycle.

In accordance with the decreased cyclin E-associated kinase activity as ES cells differentiate into EPLEBs (E. Stead, PhD thesis), this thesis demonstrated reassortment of active cyclin E-CDK2 complexes during differentiation. In particular, there are decreased levels of cyclin E-CDK2 kinase activity in higher order complexes and, even more strikingly, a decrease in the proportion of free, active cyclin E-CDK2 complexes. With the majority of the identified cyclin E-CDK2 targets involved in the G1/S phase progression and the coincident cell cycle structure remodelling (E. Stead, PhD thesis), it is likely that the decreased association of active cyclin E-CDK2 with targets leads to delays in progression through the cell cycle. Importantly, these changes also occur concomitantly with a loss of pluripotency and after formation of nascent mesoderm. Equivalent cells of the mouse embryo have passed through the primitive streak, formed mesoderm and have increased cell cycle length (Lawson *et al.*, 1991; Snow, 1977). This suggests that the rapid proliferation associated with the early gastrulating mouse embryo is likely to be due to increased activation of CDK2 targets by high constitutive cyclin E-CDK2. After formation of embryonic mesoderm, the decreased, regulated CDK2 activity leads to decreased association of active cyclin E-CDK2 with targets, increased delays in activation of targets and subsequently increasing the length of the cell cycle. While functional redundancies exist, as mouse embryonic development can occur normally when deficient for both cyclin E isoforms or for CDK2 (Geng *et al.*, 2003; Ortega *et al.*, 2003; Parisi *et al.*, 2003), the requirement for decreased cyclin E-CDK2 activity to enable differentiation in both *Drosophila* and *Xenopus* embryogenesis (Hartley *et al.*, 1996; Hartley *et al.*, 1997; Howe *et al.*, 1995; Knoblich *et al.*, 1994; Li *et al.*, 1999; Rempel *et al.*, 1995; Richardson *et al.*, 1995; Secombe *et al.*, 1998), suggests that regulation of this activity is also required for differentiation in mouse development. Analysis of the developmental potential of ES cells ectopically expressing cyclin E should be conducted.

7.2 Putative roles of CDK6 activity in ES cells and differentiation

Similar to the high levels of constitutive CDK2 activity in ES cells (Stead *et al.*, 2002), this thesis demonstrated that ES cells have elevated, cell cycle independent CDK6 activity. This implies that, like CDK2 activity, CDK6 activity may be involved in driving the rapid proliferation of ES cells. Previous reports have demonstrated that ectopic CDK6 expression shortens G1 phase (Grossel *et al.*, 1999; Nagasawa *et al.*, 2001), which is consistent with the shortened G1 phase of ES cells (Savatier *et al.*, 1994; Stead *et al.*, 2002). While in some cell types increased CDK6 activity promotes rapid proliferation (Grossel *et al.*, 1999; Lucas *et al.*, 1995), in others the rate of proliferation is reduced

(Nagasawa *et al.*, 2001). However, a role for CDK6 in promoting proliferation is further supported by its ectopic expression in many cancers (Brito-Babapulle *et al.*, 2002; Costello *et al.*, 1997; Hayette *et al.*, 2003; Lien *et al.*, 2000). In particular, ES cell proliferation would certainly be promoted by the ability of CDK6 activity to release E2F from complexes with p107 in a cell cycle independent manner. This function is consistent with the lack of cell cycle periodicity of E2F-4/p107 DNA binding complexes and deregulated E2F transcription in ES cells (Stead *et al.*, 2002). Overall, this suggests that CDK6 activity may be involved in the rapid proliferation of ES cells. However, downregulation of CDK6 activity observed here is uncoupled from changes in cell cycle structure in ESEBs and EPLEBs (E. Stead, PhD thesis), suggesting the involvement of CDK6 kinase activity in determining proliferation rates may only be in pluripotent cell types.

In both systems, changes in CDK6 kinase activity occurred temporally earlier than cyclin-E associated or overall CDK2 kinase activity (E. Stead, PhD thesis). This demonstrates that regulation of CDK activity occurs by alternate mechanisms during differentiation. Importantly, due to its role in the integration of extra-cellular signals into cell cycle regulation, changes in CDK6 kinase activity with differentiation are likely to reflect changes in mitogenic and anti-mitogenic signals in early mouse development. The lineage specific differences in CDK6 kinase activity are likely to highlight the differences in extra-cellular signalling in the formation of different cell types. In particular, coincident differences in the presence of visceral endoderm and its associated signalling (Lake *et al.*, 2000) suggests that it may be involved in the regulation of CDK6 kinase activity, enabling integration of developmental cues for survival, apoptosis, differentiation and cell cycle regulation. Lineage specific differences in changes in cell cycle length during mouse embryogenesis (Snow, 1977) and changes in CDK6 kinase activity during differentiation of *in vitro* model systems (this thesis) highlights the importance of understanding signalling mechanisms regulating proliferation in a lineage specific manner if ES cells are to be used in a therapeutic manner. Analysis of the effects of ectopic CDK6 expression on the differentiation potential of ES cells should be conducted to determine whether this downregulation of CDK6 kinase activity is required for differentiation.

7.3 p107 and E2F-4 ES cells and differentiation

Similar to pRB (Savatier *et al.*, 1994), this thesis demonstrated that cell cycle independent phosphorylation of p107 in ES cells is likely to be the major contributing factor to the lack of periodicity of DNA-binding p107-E2F complexes and E2F target gene expression (Stead *et al.*, 2002). This is likely to be a consequence of the constitutive CDK activity in ES cells, supported by the increased sensitivity to a CDK phosphorylation resistant mutant p107 demonstrated in this thesis. In addition to decreased regulation by pRB family members, the increased stability and nuclear localisation of E2F-4 in ES cells (this thesis) is also likely to contribute to the increased E2F target gene expression in ES cells (Stead *et al.*, 2002). However, confirmation of the role of E2F-4 in transactivation of E2F target genes requires analysis of dominant negative E2F-4. Still, the functional consequence of decreased regulation of E2F activity by p107 is increased expression of genes involved in cell cycle progression, which aids the rapid cell cycle progression observed in ES cells. Importantly, the normal proliferation of ES cells lacking all three members of the pRB family suggests that these proteins are not required for ES cell proliferation (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). However, delays in progression through cell cycle phases upon expression of ectopic p107 in ES cells (this thesis) highlight the importance of p107 regulation to enable cell cycle progression in ES cells.

Differences between pluripotent cell populations were highlighted by work presented in this thesis. Previous work demonstrated that EPL cells have similar levels of CDK2 activity and similar deregulated expression of E2F target genes (Stead *et al.*, 2002). This thesis demonstrated that EPL cells have less CDK6 activity than ES cells. Additionally, there is less E2F-4 in EPL cells, due to increased turnover of E2F-4, which is reflected in the decreased levels of E2F reporter activity and *B-myb* expression in EPL cells. Collectively, this leads to an increased sensitivity to p107 in EPL cells compared to ES cells. Importantly, expression of other E2F target genes is not decreased in EPL cells, suggesting that other cell cycle regulatory mechanisms have not changed in EPL cells. Therefore, differences in mechanisms of cell cycle regulation exist between pluripotent cell populations. Since primitive ectoderm induction is thought to require signalling from visceral endoderm, mimicked in the formation of EPL cells (Rathjen *et al.*, 2001; Rathjen *et al.*, 1999), it is likely that this signalling also alters cell cycle regulation. These changes highlight the importance of forming pluripotent primitive ectoderm cells to

accurately recapitulate differentiation of ICM cells into embryonic lineages. Due to the inseparable links between cell cycle regulation and differentiation, recapitulation of these signalling pathways is essential to enable lineage specific differentiation of pluripotent cells for therapeutic use.

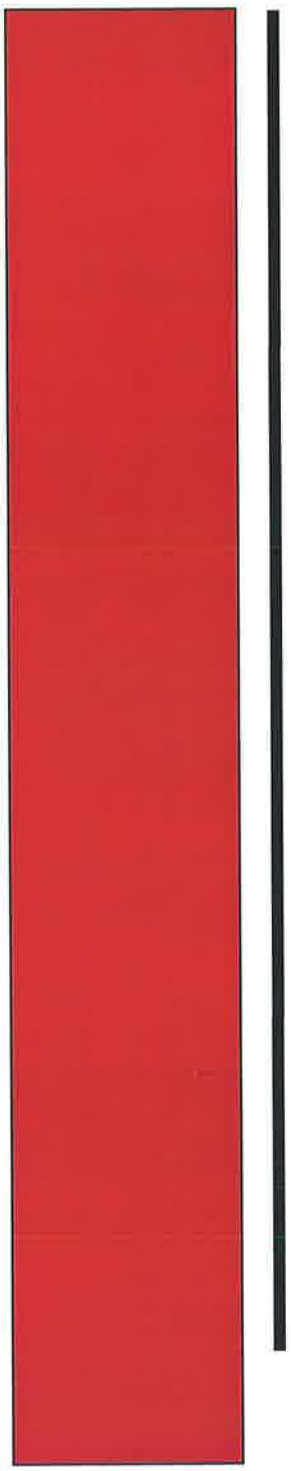
The formation of mesoderm is associated with increased p107 activity, demonstrated by increased p107 association with E2F target gene promoters. The timing of these changes is coincident with increased length of G1 phase and decreased CDK2 kinase activity in EPLEBs (E. Stead, PhD thesis). It is also equivalent to when CDK4 kinase activity increases when ES cells were differentiated with retinoic acid (Savatier *et al.*, 1996). This suggests that the formation of mesoderm is associated with increased complexity of cell cycle regulation, in particular G1 regulation. Analysis of *Drosophila* embryogenesis suggests that incorporation of G1 regulation into the cell cycle is associated with a requirement for the activity of pRB family members (Du and Dyson, 1999; Duronio and O'Farrell, 1994). Accordingly, it is proposed that at gastrulation, regulation of the cell cycle by pRB family members is essential. This is consistent with the limited differentiation of pRB-/-;p107-/-;p130-/- teratocarcinomas, in particular the lack of muscular differentiation (Dannenberg *et al.*, 2000; Sage *et al.*, 2000). It is of interest to further analyse the role of p107 in mesodermal lineage differentiation by analysis of the effects of inducible p107 expression as ES cells differentiate into EPLEBs. It is also of interest to analyse the signals leading to these cell cycle regulatory changes in a developmental context and to enable lineage specific differentiation of ES cells for therapeutic use.

7.4 Similarities between ES cells and tumour cells

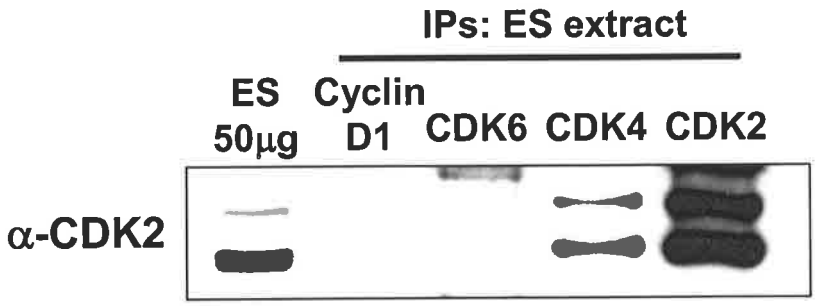
The spontaneous formation of tumours when ES cells are introduced to a somatic location (Martin, 1981) and their indefinite proliferation potential (Smith, 2001) suggests that there may be some properties that are similar between ES cells and cancer cells. The high levels of *cyclin E* expression and constitutive cyclin E-associated kinase activity in ES cells (Stead *et al.*, 2002) is found in many cancer types, particularly breast cancer (Gray-Bablin *et al.*, 1996; Keyomarsi *et al.*, 1995; Keyomarsi and Pardee, 1993; Schraml *et al.*, 2003; Spruck *et al.*, 1999). Similar to ES cells (Stead *et al.*, 2002), the proposed function of the increased cyclin E-associated activity in tumour cells is to aid rapid cell cycle progression. Deregulation of the cyclin-CDK/pRB/INK4 pathway occurs in almost all human tumours (Carnero, 2002) and in ES cells (Faast *et al.*, 2004; Savatier *et al.*, 1994;

Savatier *et al.*, 1996; Stead *et al.*, 2002). In particular, the insensitivity to p16 expression is similar for both ES cells and cancer cells (Faast *et al.*, 2004; Lukas *et al.*, 1995b; Medema *et al.*, 1995; Savatier *et al.*, 1996). Similarly, increased levels of CDK6 activity in ES cells (this thesis) are also observed in many cancers (Brito-Babapulle *et al.*, 2002; Costello *et al.*, 1997; Hayette *et al.*, 2003; Lien *et al.*, 2000). Also, the cell cycle independent phosphorylation of p107 in ES cells (this thesis) has been observed in tumour cell lines (Cheng *et al.*, 2000a). Consequentially, both ES cells (Humbert *et al.*, 2000a; Stead *et al.*, 2002) and some tumour cell types (Dhillon and Mudryj, 2002; Gray-Bablin *et al.*, 1996; Halaban *et al.*, 2000) have high levels of free E2F DNA binding activity independent of cell cycle position. Each of these similarities confer a growth advantage, which is intrinsic to both ES cells and tumour cells. The extent of the similarities between ES cells and some tumour cell types may enable the use of the understanding of cell cycle regulation in ES cells to be exploited for new mechanisms for combating cancer.

Appendix



Appendix 1



Appendix 1 The CDK4 antibody (Santa Cruz H-22) cross-reacts with CDK2, however, the CDK6 antibody (Santa Cruz C-21) does not. Cyclin D1, CDK6, CDK4 and CDK2 were immunoprecipitated from whole ES cell lysates (400µg total protein). Western analysis was conducted using a CDK2 antibody. Experiments conducted by Renate Faast.

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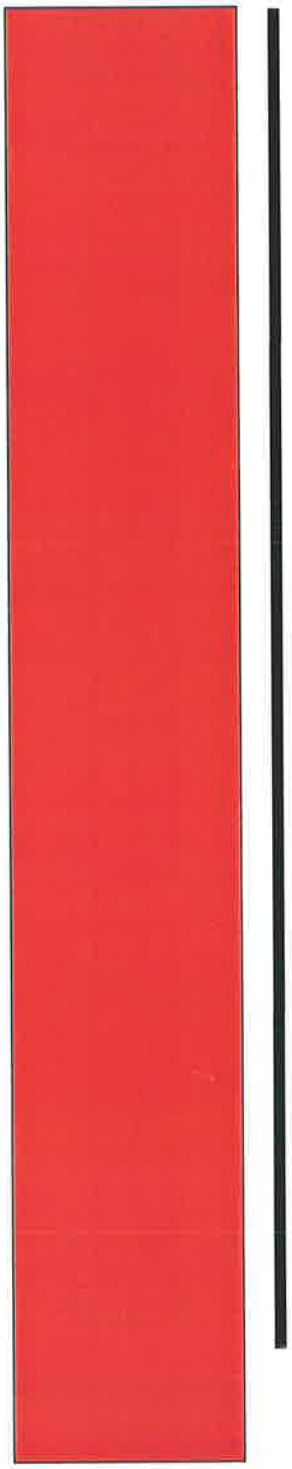

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Thesis Ammendments

Chapter 1	Page 5: line 17	should read " key transducers of G1 checkpoint."
Chapter 1	Page 20: line 9	should read "RNA polymerase I"
Chapter 1	Page 21: line 9	add "leading to genomic instability"
Chapter 1	Page 30: line 10	should read "in comparison to ESEBs"
Chapter 2	Page 40: line 14	should read "euthanised"
Chapter 3	Fig 3.2.1: legend	add "Equivalent samples collected for protein extraction for fractionation experiments."
Chapter 4	Fig 4.4.3: legend	"$p16^{p114L}$" should read "$p16^{114L}$"
Chapter 4	Page 93: line 14	should read "decreased cell cycle times."
Chapter 5	Page 98: line 18	should read "α-Tubulin"
Chapter 5	Fig 5.5.1: legend	last line missing "4 antibodies."
Chapter 5	Fig 5.5.2: legend	last line missing "and WinMDI 2.8 software."
Chapter 5	Page 111: line 14	should read "constitutive E2F activity"

Response to "Specific Questions for candidate"

Figure 3.4
Proportional to lower molecular weight fractions, there is more cyclin E-CDK2 activity eluting in higher molecular weight fractions for nocodazole blocked ES cells compared to asynchronous ES cells. Scans of this activity are not available for quantitation.

Figure 4.4.3
The stable cell lines represented in this figure had p16 levels both comparable to and greater than that of $p16^{114L}$. Reference to this fact should have been made in the text. The blot was conducted by Renate Faast and is presented in the paper (Faast et al, 2004).

Figure 5.2.1
The order the data was presented in the thesis did not enable the reference to figure 6.2.1, which shows the decreased mobility of p107 in ES cells compared to differentiated cells.

Figure 5.2.2(a)
The cytoplasmic fraction may be contaminated with nuclear proteins, however, conclusions were only made regarding nuclear localisation. Accordingly, the percentage of E2F-4 in the nucleus was estimated in comparison to the whole cell extract.

Figure 5.4.2(a)
Due to the significant decreased efficiency of transfection with high concentrations of DNA, a comparison of the ratio of inhibition of E2F-4/DP2 activity by p107 compared to vector control was conducted.

Figure 5.5.3
Other loading controls were not examined in these experiments. However, the difference in which target genes were affected by ectopic p107 upon continued passaging, decreases the likelihood that this was due to an effect on GAPDH rather than the E2F target genes.

Figure 6.4
Precipitation of Acetylated Histone H4 indicates that the region of the promoter analysed is acetylated in each sample. Differences in promoter acetylation status between samples may be more evident upon analysis of other regions of promoters.