

**TUMOURIGENIC AND TUMOUR SUPPRESSIVE PROPERTIES OF
KSHV CYCLIN *IN VITRO* AND *IN VIVO***

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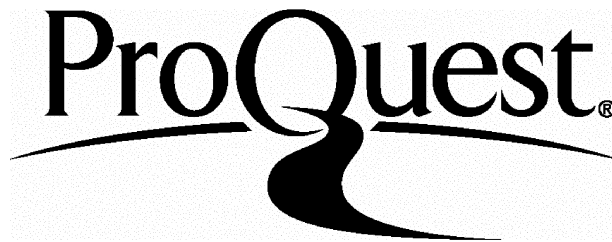
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ABSTRACT

Expression of the Kaposi's sarcoma-associated herpesvirus (KSHV or HHV-8) cyclin D homologue, K cyclin, may contribute to viral oncogenesis. This thesis shows that ectopic expression of K cyclin also triggers an apoptotic response. Apoptosis is caspase-dependent and is partially inhibited by expression of either a dominant negative (dn) caspase 9 or the KSHV Bcl-2 homologue, indicating involvement of the mitochondrial apoptotic pathway. Furthermore, even when K cyclin expression does not kill cells directly, it sensitises to induction of apoptosis by various stress signals.

In primary fibroblasts, K cyclin expression induces accumulation of transcriptionally active p53 with consequent apoptosis and growth arrest in wild type but not in p53-deficient cells. In contrast, apoptosis is not accompanied by induction of p19^{ARF} protein and requires neither E2F1 nor p19^{ARF}. Interestingly, K cyclin-expressing wt and p53-deficient cells become multinucleated and polyploid. Centrosome analysis of such cells shows that K cyclin expression is associated with centrosome amplification and aneuploidy, which is further exacerbated by p53 loss. I hypothesise that K cyclin expression leads to abortive cytokinesis and polyploidy, ultimately triggering p53-dependent apoptosis and growth arrest.

The *in vitro* data suggest that K cyclin expression is tumourigenic in a p53 null background. To corroborate this, I developed a E μ K cyclin transgenic mouse model which expresses K cyclin protein in B and T cells. These mice develop lymphomas at a low incidence (10%) and long latency (5-9 months). Tumours often contain mutations in the p53 pathway. E μ K cyclin mice were crossed with p53 null mice. All K cyclin transgenic p53^{-/-} mice developed B and/or T cell lymphoblastic lymphomas extremely rapidly (around 85 days). These data show that p53 acts as a potent suppressor of K cyclin-induced tumours *in vivo*.

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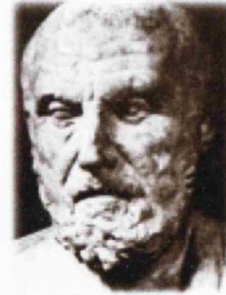
ABBREVIATIONS

7-AAD	7-aminoactinomycin D	FLICE	FADD interleukin-1 β - converting enzyme
AIDS	acquired immunodeficiency syndrome	GSK-3 β	glycogen synthase kinase 3 β
AIF	apoptosis inducing factor	GST	glutathione-S-transferase
APC	anaphase-promoting	HcC	holocytochrome C
complex		H&E	haematoxylin and eosin
APS	ammonium persulfate	HEPES	N'-(2-hydroxyethyl) piperazine
Apaf-1	apoptotic protease-activating factor-1		N'-(2-ethanesulphonic acid)
ARF	alternate reading frame	HHV-8	human herpesvirus 8
ATP	adenosine triphosphate	HIV	human immunodeficiency virus
ATM	ataxia telangiectasia mutated		
ATR	ATM-related kinase	hTERT	human telomerase repeat transcriptase
Bcl-2	B cell lymphoma 2		
BH domain	Bcl-2 homology domain	HVS	herpesvirus saimiri
BrdU	5'-bromodeoxyuridine	ICRF	Imperial Cancer Research Fund
BSA	bovine serum albumine		
CAK	cdk activating kinase	IP	immunoprecipitation
Cdk	cyclin dependent kinase	IPTG	isopropyl β -D- thiogalactopyranoside
CKI	cyclin dependent kinase inhibitor	IRF	interferon regulatory factor
CLN	cyclin-like protein	KS	Kaposi's sarcoma
CMV	cytomegalovirus	KSBcl-2	Kaposi's sarcoma Bcl-2
DD	death domain	KSHV	Kaposi's sarcoma-associated herpesvirus
DED	death effector domain		
DMSO	dimethylsulfoxide	LANA	latency-associated nuclear antigen
DNA	deoxyribonucleic acid		
DSB	double strand break	laser	light amplification by stimulated emission of radiation
DTT	dithiothreitol		
EBV	Epstein-Barr virus		
EDTA	ethylenediaminetetraacetic acid	MCD	multicentric Castleman's disease
EHV-2	equine herpesvirus-2	MCM	minichromosome maintenance
EtOH	ethanol		
FBS	foetal bovine serum	MEF	mouse embryo fibroblast
FADD	Fas-associated death domain	MEN	mitotic exit network
FITC	fluorescein diacetate	MHC	major histocompatibility complex
FLIP	FLICE inhibitory protein		

MHV68	mouse herpesvirus 68	TAg	SV40 large T antigen
MRC-5	human embryonic lung fibroblast	TCR	T cell receptor
NES	nuclear export signal	TEMED	N, N, N', N' - tetramethylethylenediamine
NLS	nuclear localisation signal	TNF- α	tumour necrosis factor- α
NP40	Nonidet- P40	Tween	polyoxyethylene-sorbitan monolaurate
ORC	origin recognition complex		
ORF	open reading frame	UV	ultraviolet
PAA	polyacrylamide	wt	wild type
PALS	periarterial lymphatic sheath	X-Gal	5-bromo-4-chloro-3-indolyl β -D-galactosidase
PCNA	proliferating cell nuclear antigen		
PCR	polymerase chain reaction	bp	base pair
PEL	primary effusion lymphoma	$^{\circ}$ C	degrees Centigrade
PI	propidium iodide	g	gram
PFA	paraformaldehyde	h	hour(s)
PMSF	phenyl-methyl-sulphonyl fluoride	kb	kilobase
Rb	retinoblastoma	KDa	kilodalton
RNA	ribonucleic acid	Kg	kilogram
RPMI	Roswell Park Memorial Institute	M	moles per litre
SA- β -gal	senescence-associated β -Galactosidase	m	milli
SIN	septation initiation network	ml	milli litre
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis	ON	overnight
		rpm	revolutions per minute
		RT	room temperature
		μ	micro

CHAPTER 1:
INTRODUCTION

The way in which a tumour spreads through the human body is best described by the ancient Greek physician Hippocrates: a tumour looks like a crab (“karkinoma” in Greek) in that there is a central body to the tumour and the tumour extension appear as the legs of a crab, that adhere to anything it seizes upon. The Latin word for crab, “cancer”, later became integrated in the English language. Cancer is a complex disease in that multiple gene products can contribute to the malignancy in a multistep manner. Recently, the aim has been to simplify this complexity by drawing the circuitry of “molecular switches” (Hahn and Weinberg, 2002) by defining events that are “mission critical” (Evan and Vousden, 2001) or by categorising typical “cancer cell traits” such as the evasion of cell death (Hanahan and Weinberg, 2000). Central to this comprehensive understanding is still an old and simple concept: cancer cells undergo uncontrolled division and escape the negative regulatory signals that are fired to kill or stop the division of such malignant cells.



Many viral proteins engage the cell division machinery, named the cell cycle, presumably to ensure the replication of the viral genome. Host cell survival is essential for successful viral reproduction. Paradoxically, deregulation of the cell division by some viruses is associated with the induction of neoplasia and death of the host. Studies that address viral oncogenesis have been instrumental in the identification of key cell cycle regulators that determine the fate of a virally infected cell. This thesis identifies some of the factors that determine the balance between cell death and cellular transformation upon expression of a cell cycle regulator encoded by the oncogenic Kaposi’s sarcoma-associated herpesvirus.

1.1 An introduction to the cell cycle

1.1.1 Cell cycle phases and controls

The most fundamental function of the cell division cycle is to accurately duplicate the DNA in its S (Synthesis) phase, and then segregate the copies precisely into two genetically identical daughter cell during the M phase (Mitosis). These phases are separated by two gap phases in which cells grow and double their mass of proteins and organelles: G1 phase between the M and S phases and G2 phase between M and the next S phase. G1, S and G2 phase together are called interphase, which in a typical human cell growing in culture occupies around 23 hours, compared to 1 hour for M phase (Hagting et al., 2002). M phase consists of two events: nuclear division (mitosis) and cytoplasmic division (cytokinesis). Mitosis starts with chromosome condensation in prophase, followed by nuclear envelope

breakdown in prometaphase. The pairs of sister chromatids become attached to the microtubules of the mitotic spindle and align at the equator of the spindle in metaphase. In anaphase sister chromatids separate and move to opposite poles of the spindle, where they decondense in telophase. M phase ends with the division of the cytoplasm during cytokinesis.

When quiescent cells are stimulated to enter the cell cycle, they undergo a period of mitogen dependence in late G1. This transition period, called START in yeast and the Restriction point (R) in mammalian cells, represents a point of no return after which cells become refractory to mitogenic stimuli and are committed to complete the remaining phases of the cell cycle (Pardee, 1974). When extracellular conditions are less favorable, which is the case for most cells in the human body, cells delay their G1 phase or enter a specialised rest phase called G0.

Cells that have passed through the restriction point can only be blocked in one way: upon the execution of so-called cellular checkpoints (Hartwell and Weinert, 1989). These are intracellular control mechanisms that monitor the integrity of the DNA, the DNA replication process, metabolic distress and proper attachment of chromosomes to the mitotic spindle. Cells are transiently arrested before mitosis (G2/M), during mitosis (M) or at the next restriction point (G1/S) itself to allow time for the damage or deficiency to be corrected. An appreciation of the molecular processes that govern the cell cycle and its checkpoint controls is central to our understanding of how and why cancer cells continuously cycle.

1.1.2 Identification of cyclins and Cdks: a bit of history

Genetic complementation studies in yeast have been instrumental in the identification of the key regulators of the cell cycle. Using budding yeast *Saccharomyces cerevisiae* mutants with a defective cell division cycle (*cdc*), *Cdc28* was identified as a critical regulator of G1/S progression (Hartwell et al., 1974). Similar screens in fission yeast *Schizosaccharomyces pombe*, showed that *Cdc2* was essential for entry into mitosis and was homologous to *Cdc28* (Nurse and Thuriaux, 1980). The proteins encoded by these genes were characterised as Serine/Threonine kinases whose activity oscillates during the cell cycle. The importance of this kinase as a universal cell cycle regulator was emphasised upon the cloning of human *Cdc2* by complementation of a fission yeast *Cdc2* mutant with a human cDNA library (Lee and Nurse, 1987).

“Cyclins” were originally discovered by scientists searching for proteins that fluctuated in level through the cell cycle of the sea urchin *Arbacia* (Evans et al., 1983). It was reasoned that proteins behaving in this manner were important for controlling cell division. Their importance was underscored by the finding that cyclins were part of a protein complex that promoted the onset of M phase in *Xenopus* oocytes, called maturation-promoting factor

(MPF) (Lohka et al., 1988). Intriguingly, the other protein in the complex cross-reacted with antibodies raised against yeast Cdc2 protein (Nurse, 1990). Further studies in fission yeast revealed the interaction and cooperation in between Cdc2 and the cyclin-like protein cdc13 (Booher and Beach, 1987; Hagan et al., 1988). When cyclin binding was found to be essential for kinase activity of Cdc2, the name cyclin-dependent kinase (Cdk) was eventually adopted for Cdc2- family proteins.

1.1.3 Mammalian cyclin/Cdk complexes

While a single Cdk (Cdc2 or Cdc28) triggers the major transitions of the yeast cell cycle, mammalian cells accommodate multiple *Cdc*-related genes. After identification of the human Cdc2 homologue, called Cdk1, other human Cdks were identified by low stringency hybridisation or by redundant PCR using oligonucleotides corresponding to conserved regions (Elledge and Spottswood, 1991; Grana et al., 1994; Hanks, 1987; Johnson and Smith, 1991; Matsushime et al., 1992; Meyerson and Harlow, 1994; Tassan et al., 1995; Tsai et al., 1991; Wu et al., 1994). At present, 9 different Cdks with known functions have been identified, only four of which (Cdk1, 2, 4, 6) are involved directly in cell cycle control. Cdk7 contributes indirectly by acting as a Cdk-activating kinase (CAK) that phosphorylates other Cdks (see section 1.2.4). Several Cdks act as components of the machinery that controls basal transcription by RNA polymerase II (Cdk7, 8, 9). Cdk5 is expressed in post-mitotic cells of the central nervous system and is required during neural differentiation (reviewed in Morgan, 1997).

Mammalian G1 cyclins D and E were cloned by their ability to complement conditionally defective G1 cyclin (CLN) deficiency in budding yeast (Koff et al., 1991; Lew et al., 1991; Xiong et al., 1992a). Three D-type cyclins exist (cyclin D1, D2 and D3), which are differently expressed in various cell lineages, with most cells expressing cyclin D3 and either D1 or D2 (Sherr, 1993). No differential roles for these cyclins have thus far been recognised. Recently, a gene similar to cyclin E in sequence and biochemical properties was cloned and called cyclin E2 (Lauper et al., 1998). Cyclins E1 and E2 have overlapping expression patterns in mice tissues and are suggested to be co-overexpressed in human tumours (Geng et al., 2001). Future research will elucidate whether any of their functions are distinct. The human cyclins A and B were discovered by virtue of their homology with *Xenopus* cyclins (Pines and Hunter, 1989; Pines and Hunter, 1990). Mammalian cells contain two types of cyclin A and B. Cyclin A1 is only expressed in meiosis and early in embryogenesis, whereas cyclin A2 is found in proliferating somatic cells (Yang et al., 1997). Cyclin B2 probably plays a role in Golgi remodelling during mitosis (Jackman et al., 1995),

while cyclin B1 controls all other roles of this cyclin type. Thus far at least 16 cyclins exist for many of which binding partners and functions have yet to be identified.

The coordinated activation of cyclin/Cdk complexes is tightly regulated throughout the cell cycle. Cdk expression levels are more or less constant throughout the cell cycle. The primary regulation of Cdk activity is therefore governed by sequential synthesis, post-translational modification and degradation of cyclins (Fig. 1.1). D-type cyclins are short-lived proteins (see section 1.2.9) and their synthesis and assembly with their catalytic partners Cdk4 or Cdk6 in late G1 is dependent on mitogenic signalling and, specifically, the activation of Ras/Raf/MAPK pathway (Hitomi and Stacey, 1999). In addition, expression of Myc, a protein that is rapidly induced upon mitogenic signalling (Kelly and Siebenlist, 1986), leads to the transcriptional activation of cyclin D2 and increased cyclin D1 protein synthesis rates (Bouchard et al., 1999; Perez-Roger et al., 1999). Cyclin D/Cdk activity persists through the first and subsequent cycles as long as mitogenic stimulation continues (Matsushime et al., 1991). Cyclin E protein levels peak at the G1/S progression, followed by an increase of cyclin A levels in S phase. Both cyclin E and A interact with and activate Cdk2, while cyclin A can also form complexes with Cdk1 (Lees and Harlow, 1993). At the G2/M boundary the levels of cyclin B increase, resulting in the activation of its Cdk partner Cdk1. Fluctuations in cyclin expression and the resultant oscillation in Cdk activity forms the basis for a coordinated cell cycle progression and a platform for more subtle regulatory controls.

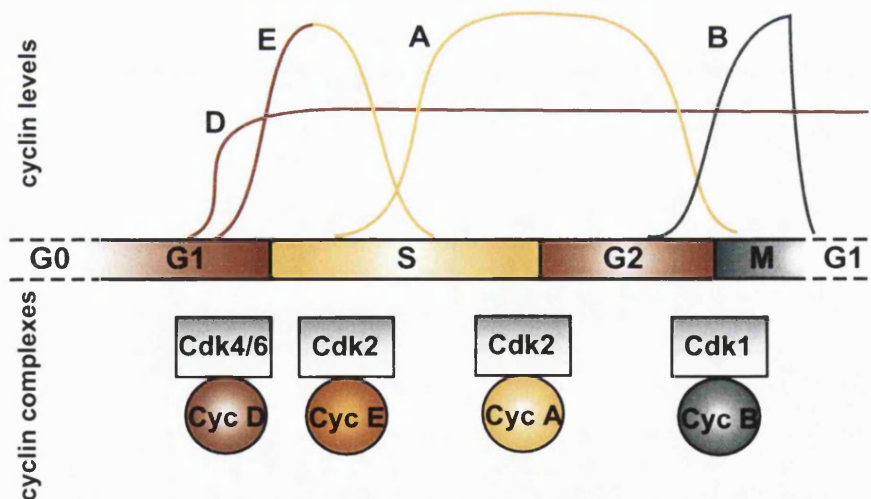


Fig. 1.1 Oscillation of cyclin expression and cyclin/Cdk complex formation through phases of the cell cycle. See text for details.

1.2 Regulatory mechanisms governing cyclin/Cdk activity

The primary mechanism of Cdk activation is the binding of a cyclin subunit. The availability of cyclins is not only determined by expression patterns, as described above, but

also by cyclin stability and localisation. Complete activation of most Cdks also requires phosphorylation of the Cdk at a positive regulatory residue by CAK and the dephosphorylation of inhibitory residues by members of the *cdc25* phosphatase family. Negative regulation, on the other hand, is mediated by binding of Cdk inhibitors (CKIs) directly to the Cdk subunit or to the cyclin/Cdk complex. Knowledge of the cyclin and Cdk structures helps to understand the basis of these regulatory mechanisms.

1.2.1 Structure of the cyclin subunit

Cyclins are a remarkably diverse family of proteins, ranging in size from about 35 to 90 kDa. Sequence homology is concentrated in a section of around 100 amino acids long, known as the “cyclin box”, which is necessary for Cdk binding and activation (Kobayashi et al., 1992; Lees and Harlow, 1993). Thus far, cyclin structures of bovine and human cyclin A, cyclin H and three viral cyclin D homologues have been described (Brown et al., 1995; Card et al., 2000; Jeffrey et al., 1995; Jeffrey et al., 2000; Kim et al., 1996; Schulze-Gahmen et al., 1999). A comparison of these structures suggests that the core of all cyclins contains two compact domains of five helices each, called the “cyclin fold” (Fig. 1.2). The first 5-helix bundle corresponds to the conserved cyclin box. This central fold is flanked by additional helices at the N and C termini, whose position and secondary structure vary in different cyclins.

1.2.2 Structure of the Cdk catalytic subunit

Cdks are closely related in size (35-40 kDa) and sequence (>40% identity) and have the same overall fold as other eukaryotic protein kinases. Monomeric Cdk2 consists of an N-terminal lobe rich in β -sheets (N lobe), a larger C-terminal lobe rich in α -helices (C lobe) and a deep catalytic cleft at the junction of the two lobes (De Bondt et al., 1993). Crystallised cyclin/Cdk6 complexes show a very similar Cdk structure, suggesting that the major structural features of Cdk2 are probably conserved in all Cdks (Jeffrey et al., 2000; Schulze-Gahmen and Kim, 2002). The catalytic core provides highly specific binding sites that allow the two substrates, ATP and protein, to be appropriately positioned to allow the γ -phosphate of ATP to face the hydroxylated side chain on the protein substrate. Catalytic residues promote the transfer of the phosphate to the oxygen residue of the hydroxyl group.

The catalytic activity of the Cdk subunit is restrained by two mechanisms. First, a large, somewhat flexible loop (called T-loop) on the C-terminal lobe blocks protein substrate binding at the entrance of the catalytic cleft. Second, a stretch of helical amino acids in the N-terminal lobe (PSTAIRE helix) that contains key residues required for ATP phosphate binding is directed away from the cleft (De Bondt et al., 1993) (Fig. 1.2).

1.2.3 Cdk activation by cyclin binding

The crystal structure of the human Cdk2-cyclin A1 complex has significantly contributed to the identification of mechanisms underlying the activation of Cdks (Jeffrey et al., 1995). Two helices in the cyclin box of cyclin A bind the PSTAIRE helix of Cdk2 and the N-terminal helix of cyclin A interacts with residues on the N-terminal lobe of Cdk2. Although this interaction does not affect the cyclin structure, major conformational changes occur in the Cdk catalytic cleft (Fig. 1.2). Cyclin binding changes the T loop structure and position such that it does not obstruct the catalytic cleft anymore. Cyclin binding also moves the PSTAIRE helix into the catalytic cleft and rotates it by 90°, resulting in the correct positioning of the PSTAIRE Glu 51 residue with Asp145 and Lys33 side-chains that together catalyse the phosphotransfer reaction. The net result is an increase in the Cdk kinase activity by several orders of magnitude as measured *in vitro* (Connell-Crowley et al., 1993).

Why do cyclins only bind selective Cdks? Although the major cyclin/Cdk binding interactions and structural changes are conserved in all studies described thus far, variabilities in the length of the PSTAIRE helix and the position of the cyclin N-terminal helix have been found (Jeffrey et al., 2000; Kim et al., 1996). This may underlie the specificity of Cdk4/6 for D-type cyclins, Cdk2 for cyclin A and B and Cdk1 for cyclin B.

1.2.4 Regulation of Cdk activity by phosphorylation

Full activation of Cdk activity is mediated by phosphorylation of a conserved Threonine residue (Thr160 in Cdk2, Thr161 in Cdk1) in the T loop by the CAK enzyme (Fig. 1.2). CAK phosphorylation may be required for activation of cyclin B/Cdk1 complexes, where cyclin binding alone has little effect in *in vitro* Cdk activity assays (Desai et al., 1995). The major candidate for higher eukaryotic CAK found in *Xenopus*, starfish and mammalian cell lysates is the cyclin H-Cdk7-Mat1 complex (Nigg, 1996). Immunodepletion of Cdk7 removes the majority of CAK activity from these sources, but additional CAK enzymes may exist. CAK phosphorylation moves the T loop outwards, resulting in additional Cdk-cyclin contacts and probably increased potential for substrate binding (Russo et al., 1996b). Although CAK activity is constant through the cell cycle, Thr160 phosphorylation of Cdks is cell cycle-regulated, raising the possibility that cyclin binding is required for efficient Cdk-directed CAK activity. Dephosphorylation of this residue can be achieved by the Cdk associated phosphatase KAP (Poon and Hunter, 1995).

In addition, Cdks can undergo inhibitory phosphorylation on Thr14 and Tyr15 residues that are located in the roof of the ATP phosphate-binding site. It is likely that this reduces the affinity for ATP thereby preventing Cdk catalytic activity (Endicott et al., 1999). Phosphorylation of these sites is mediated by the Wee1 and Myt1 protein kinases (Lew and

Kornbluth, 1996). Dephosphorylation is carried out by phosphatases of the Cdc25 family, which release the inhibitory action. Cdc25A and B are expressed during G1 or at the G1/S boundary and Cdc25C is the mitotic isoform. Complicating things further, Cdc25, Myt1 and Wee1 itself are phosphorylated too, further enhancing Cdc25 activity but inhibiting Wee1 activity. Interestingly, part of the mitotic phosphorylation on Wee1 and Cdc25 is catalysed by cyclin B/Cdk1 and Cdc25A can be phosphorylated by cyclin E/Cdk2 (Hoffmann et al., 1994; Mueller et al., 1995), providing the potential for positive feedback. Not surprisingly, this extensive phosphorylation network is amenable to regulation by various stimuli, which is central to the execution of cell cycle checkpoints.

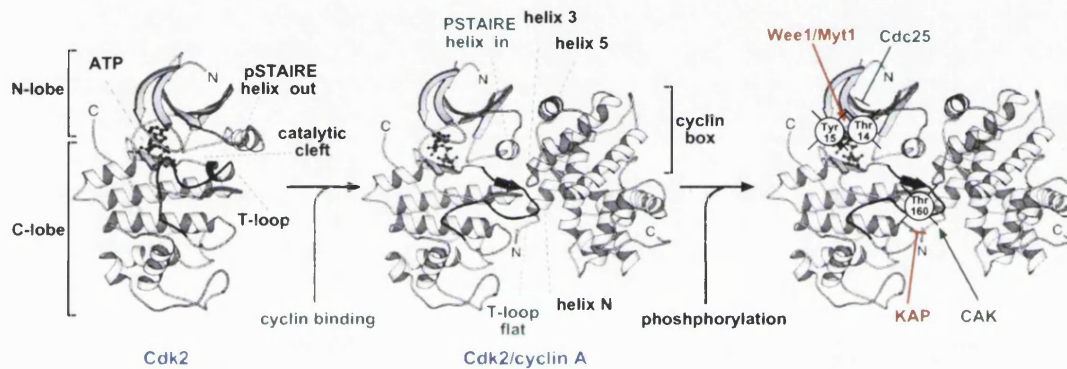


Fig. 1.2 Structural basis of the modulation of Cdk activity by cyclin binding and phosphorylation. Structure of human monomeric Cdk2 or cyclin A/Cdk2/ATP complex. Pictures are adapted from (Morgan et al., 1997) and (Jeffrey et al., 2000). Modifications in green represent activation events, modifications in orange represent inhibitory events. See text for details.

1.2.5 The INK4-family of Cdk inhibitors

The Ink4 family of Cdk inhibitors (CKI) contains four members that are named according to their molecular weights, p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d} (p16, p15, p18, p19) and their ability to inhibit Cdk4. These proteins specifically inhibit Cdk4 and Cdk6 and do not bind Cdk2 or Cdk1. The prototype member of this family, p16, was first identified in a yeast two-hybrid screen using Cdk4 as a bait (Serrano et al., 1993). The locus that encodes p16 also directs the expression of a second unrelated protein designated as p19^{ARF} (p14^{ARF} in humans) via an alternate reading frame (Quelle et al., 1995). This locus and its product will be introduced in section 1.8.2. The second Ink4 family member, *p15*, was cloned using the *p16* coding sequence to probe a library from TGF- β arrested cells. *p15* is located on chromosome 9, adjacent to the *p16* gene, suggesting that this locus may have undergone gene duplication (Hannon and Beach, 1994). Two additional members of this gene family, *p18* and *p19*, were cloned by PCR-based strategies or identified by two-hybrid screening (Chan et al., 1995; Guan et al., 1994; Hirai et al., 1995).

The mechanisms regulating the expression of these Cdk inhibitors remain elusive and differ between family members. Furthermore, promoter elements are not well conserved

between human and mice and transcriptional regulation may therefore vary between species. Different from other Ink4 proteins, p19 expression in human cells is periodic through the cell cycle, with higher expression levels during S and G2 phases (Thullberg et al., 2000). Oscillations in p19 levels may thus ensure cell cycle-regulated inhibition of cyclin D/Cdk. In mice, p18 and p19 are primarily expressed during embryonic development, whereas p16 and p15 are only be detected after birth (Zindy et al., 1997). In both cultured mouse and human fibroblasts, p16 and p18 expression are progressively upregulated upon increased cell doublings. However, only in human cells does p16 loss seem to correlate with immortalisation (Brookes et al., 2002), and references therein. In addition, oncogene expression or genotoxic stress specifically induces the expression of p16 in cultured cells, although recent evidence suggests that this may also occur in mice (Schmitt et al., 2002b) and recently reviewed in (Serrano and Blasco, 2001). Not much is known about the transcription factors that activate Ink promoters. Roles for JunB, AP-1 or Ets have been suggested in p16 promoter activation (Ohtani et al., 2001; Passegue and Wagner, 2000), while the growth-inhibitory cytokine TGF- β specifically activates the p15 promoter via SMAD transcription factors (Hannon and Beach, 1994).

INK4 proteins are composed of multiple ankyrin repeats, that consists of pairs of antiparallel helices, stacked side by side and connected by a series of intervening hairpin motifs (Russo et al., 1998). This classical protein-protein interaction domain is involved in binding to the non-catalytic side of Cdk4 and Cdk6, opposite to the cyclin D binding site. Binding of Ink4 inhibitors to Cdk4 or Cdk6 kinases prevents their interaction with their cognate D-type cyclins. However, the Ink4 and cyclin D binding sites do not overlap. Instead, Ink4 binding induces allosteric changes by rotating the two Cdk4/6 lobes by 15° in the vertical axis, thus mislocating the cyclin-interacting sequences, and distorting the ATP binding site (Jeffrey et al., 2000) (Fig. 1.3). Moreover, by disrupting the formation of cyclin D/Cdk complexes, Ink4 proteins also force the redistribution of Cip/Kip inhibitors from cyclinD/Cdk4/6 to cyclin E/Cdk2 complexes, indirectly causing a downregulation of Cdk2 kinase activity (McConnell et al., 1999).

Until recently, it was much debated whether Ink4 proteins act by dissociating cyclin/Cdk complexes or by forming inhibitory ternary Ink4/Cdk/cyclin complexes. While p15 or p19/cyclin D/Cdk4/6 ternary complexes could be formed in reconstitution assays, the majority of p16 is found in binary complexes with Cdk4/6 *in vivo* (Hall et al., 1995; Parry et al., 1995). The crystal structure of the p18/viral cyclin/Cdk6 complex supports a model in which Ink4 binding weakens the affinity between the cyclin and the Cdk (Jeffrey et al., 2000). This, together with the short half-life of cyclin D protein, probably explains why Ink4/Cdk binary complexes are more abundant in the cell. In addition, other proteins such as the

recently identified p34^{SEI-1} protein can modulate the interaction between cyclin D and its Cdk binding partners, rendering the complex more resistant to inhibition by p16 (Sugimoto et al., 1999).

1.2.6 The Cip/Kip family of Cdk inhibitors

Cdk inhibitors of the Cip/Kip family are more broadly acting, universal Cdk inhibitor. This CKI family consists of three members, p21^{Cip1}, p27^{Kip1} and p57^{Kip1} (p21, p27, p57). p21, the first mammalian CKI to be identified, was initially noticed as a protein forming quaternary complexes with a cyclin/Cdk pairs and proliferating cell nuclear antigen (PCNA) (Xiong et al., 1992b). It was later identified as a Cdk2 interacting protein (Cip1) in a Cdk2 two-hybrid screen (Harper et al., 1993) and independently cloned by other groups that together established p21 to be a potent inhibitor of cyclin D- E- and A- and weak inhibitor of cyclin B-associated kinase activity (el-Deiry et al., 1993; Gu et al., 1993; Noda et al., 1994; Xiong et al., 1993a). The findings that p21 mRNA expression was induced upon p53 induction (el-Deiry et al., 1993), and that p21 was absent from cyclin/Cdk complexes in cells lacking functional p53 (Xiong et al., 1993b), were crucial in deciphering the pathway of p53-mediated cell cycle arrest (see section 1.8.3). Substantiating its role as a cell cycle inhibitor, p21 also directly inhibits DNA replication via its interaction with PCNA, an essential DNA replication protein, in a manner independent of cyclin/Cdks (Waga et al., 1994; Warbrick, 2000) Of note, p21 does not affect PCNA-mediated nucleotide-excision repair upon UV-induced DNA damage, consistent with its induction as a checkpoint protein upon DNA damage (Li et al., 1994a). In addition, p21 may play a role in terminal differentiation of cells as a strong correlation between p53-independent p21 induction and terminal differentiation has been noted in cells of various origins (Halevy et al., 1995; Macleod et al., 1995; Noda et al., 1994; Steinman et al., 1994).

The p27 CKI was cloned in a two-hybrid screen using cyclin D1 and Cdk4 as a bait (Toyoshima and Hunter, 1994) and was independently isolated as a protein that is highly expressed in TGF- β or cell-cell contact-arrested cells (Polyak et al., 1994a; Polyak et al., 1994b). Like p21, p27 is a potent inhibitor of cyclin D-, E- and A- associated Cdks and a weaker inhibitor of cyclin B/Cdk1 complexes (Toyoshima and Hunter, 1994). Numerous studies since have shown that p27 accumulates in quiescent and density-arrested cells and that mitogen-triggered decreases in its levels are required for exit out of G0 (Coats et al., 1999; Millard et al., 1997; Nourse et al., 1994; Poon et al., 1995; Rivard et al., 1996; Winston et al., 1996). In contrast, p21 levels are low in quiescent cells and increase in response to mitogenic signals (Li et al., 1994b). Of the Cip/Kip proteins, p27 is therefore thought to be the primary modulator of proliferative status. Intriguingly, cyclinE/Cdk2 complexes phosphorylate p27 on

a specific Threonine residue (Thr 187) and trigger the ubiquitin/proteasome-dependent degradation of this CKI, thus antagonising the action of its own inhibitor (Montagnoli et al., 1999; Pagano et al., 1995; Sheaff et al., 1997; Vlach et al., 1997) (see also section 1.2.9).

The third member of the Cip/Kip family, p57, was cloned using a cyclin D1 two-hybrid screen to isolate mouse p57, followed by a low stringency hybridisation of a human embryonic cDNA library to isolate the human counterpart (Matsuoka et al., 1995). A second group cloned p57 by screening a mouse cDNA library with p21 (Lee et al., 1995b). Overexpression of p57 causes a G1 arrest via the inhibition of G1/S Cdk (Lee et al., 1995b). Unlike p21 and p27, p57 has a more restricted tissue distribution during mouse embryogenesis and is expressed in heart, brain, lung, skeletal muscle, kidney, pancreas and testis (Matsuoka et al., 1995). In human tissues mRNA is mainly detected in the placenta, and at lower levels in skeletal muscle and heart (Lee et al., 1995b). Most of the p57 expressing cells are terminally differentiated. The defined spatial and temporal expression pattern is suggestive of a role for p57 in tissue development. Importantly, although p57 is thought to act functionally analogous other Cip/Kip proteins, no detailed comparisons have been performed and much less studies have focused on p57.

The Cip/Kip family inhibitors can bind isolated cyclin and Cdk subunits independently, although their binding affinity for cyclin/Cdk complexes is higher (Chen et al., 1996a; Harper et al., 1995; Lin et al., 1996; Toyoshima and Hunter, 1994). They contain an N-terminal region with significant homology (38-44%), while their carboxy-terminal portions are variable in length and divergent in sequence and function. Analysis of deletion mutants indicated that the N-terminal moiety is necessary and sufficient to bind to, and inhibit Cdk complexes (Chen et al., 1996a; Lee et al., 1995b; Luo et al., 1995; Polyak et al., 1994b). This region contains two subsections: a short motif required for cyclin binding (called Cyl, containing the ZRXL motif that is crucial for cyclin interaction) and a more complex segment that binds the Cdk subunit (Chen et al., 1996a; Fotadar et al., 1996). Again, the crystal structure of the 69 aa p27 N-terminal domain in complex with cyclin A/Cdk2 provided a breakthrough in our understanding of the Cip/Kip inhibitory mechanism (Russo et al., 1996a). The p27 peptide stretches across the top of the cyclin/Cdk complex in an extended conformation (Fig. 1.3). The most N-terminal part of the p27 peptide binds a conserved hydrophobic groove on the cyclin box. This p27/cyclin interaction is likely to serve as an anchor point that facilitates subsequent p27/Cdk binding, since it does not require any conformational changes. The more C-terminal part of the p27 peptide interacts with the Cdk upper N-lobe, disrupting the conformation of the Cdk subunit and the ATP binding site. Third, a small helix of p27 inserts in the catalytic cleft and prevents ATP binding. Together, these interactions fully disrupt any catalytic potential of the cyclin/Cdk complex.

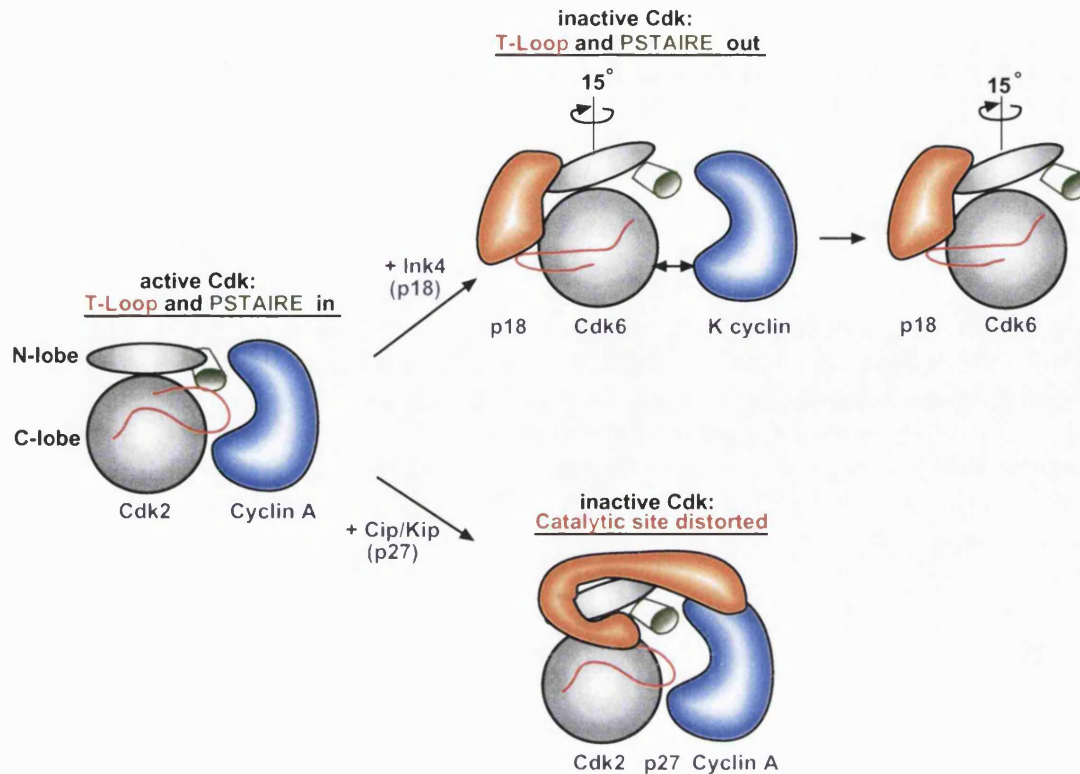


Fig. 1.3 Graphic representation of cyclin/Cdk inhibition by Cdk inhibitors. Based on crystal structures of the ternary p27/cyclin A/Cdk2 (Russo et al., 1996a) and p18/viral cyclin/Cdk6 (Jeffrey et al., 2000) complexes and reviewed in (Pavletich et al., 1999). The T-loop is indicated in red and the PSTAIRE helix in green. See text for details.

1.2.7 Cip/Kip proteins: inhibitors or assembly factors of cyclin D/Cdk complexes?

Although it is generally assumed that Cip/Kip inhibitors antagonise Cdk2 activity, experiments in recent years have indicated that p21 and sometimes p27 can be associated with active cyclin D/Cdk complexes in the cell (reviewed in Sherr and Roberts, 1999). More specifically, *in vitro* kinase activity of recombinant cyclin D/Cdk complexes was inhibited less by Cip/Kip proteins compared with cyclin A/Cdk2 complexes, and cyclin D-associated kinase activity was measured at sub-stoichiometric levels of Cip/Kip proteins (Blain et al., 1997; Soos et al., 1996; Zhang et al., 1994). Furthermore, p21 and p27 actually promoted the assembly of cyclin D/Cdk both *in vitro* and *in vivo* and, consistent with the crystal structure data (Pavletich, 1999), both cyclin and Cdk binding were required for complex assembly (LaBaer et al., 1997). Adding to these findings, immunodepletion of p21 resulted in a concomitant depletion of cyclin D-associated kinase activity (LaBaer et al., 1997). Finally, MEFs that lacked both p21 and p27 failed to assemble detectable amounts of cyclin D1 or D2/Cdk4 complexes and lacked any detectable cyclin D-dependent kinase activity (Cheng et al., 1999). Together, these data are supportive of a model in which Cip/Kip proteins act as assembly factors and activators of, specifically, cyclin D/Cdk complexes.

These data should however be taken with some caution. First, *p21/p27*^{-/-} proliferate and are susceptible to p16-mediated growth inhibition (Cheng et al., 1999), implying that p21 and p27 are not strictly required for cyclin D/Cdk activity. Second, the crystal structure reveals that p27 preferentially binds to the cyclinA/Cdk2 complex instead of the individual components (Russo et al., 1996a); if it is true that Cip/Kip proteins promote cyclin D/Cdk complex assembly, why then are p27/Cyclin D binary complexes rarely found? Third, the stability of cyclin and Cdks is significantly reduced in the absence of p21 and p27 (Cheng et al., 1999); detecting any remaining kinase activity in such cells may therefore be technically demanding. Indeed, contrasting the findings of Cheng *et al.*, a recent report claims that *p21/p27*^{-/-} MEFs exhibit detectable cyclin D3/Cdk4 complex formation and activity (Bagui et al., 2000). This group also argues that p21 or p27-containing cyclin D3/Cdk4 complexes lacked kinase activity both *in vitro* and *in vivo*. Thus, although the presence of Cip/Kip proteins clearly increases the stability of cyclin D and Cdks and therefore cyclin D-associated kinase activity, it is ambiguous how these CKIs would actively contribute to Cdk catalytic activity. Purification and crystallisation of cyclin D/Cdk/CKI complexes will be essential in clarifying the properties of the physiologic holoenzymes.

1.2.8 Cyclin/Cdk localisation

The critical cyclin/Cdk targets are nuclear proteins. Nuclear localisation of cyclin/Cdk complexes is therefore essential for its activity and nucleocytoplasmic shuttling is a means to regulate this activity (reviewed in Yang and Kornbluth, 1999). The G1/S cyclins A and E are expressed constitutively in the nucleus (Ohtsubo et al., 1995; Pines and Hunter, 1991) consistent with their roles in DNA replication and S phase progression (see section 1.3). In contrast, nuclear localisation of cyclin D and B are tightly regulated during cell cycle progression. The importance of nuclear cyclin D localisation is underlined by the observation that overexpression of a cyclin D1 mutant (Thr156A) that blocks the transport of cyclin D1/Cdk4 complexes into the nucleus can cause a G1 cell cycle arrest (Diehl and Sherr, 1997).

Neither cyclins nor Cdks contain canonical nuclear localisation sequences. Cyclin/Cdk nuclear import could potentially be mediated via binding to an NLS-containing protein, via a “piggyback” mechanism. Suggested candidates are the Cip/Kip proteins, which contain an NLS near their C-termini and have been proposed to enhance cyclin D/Cdk nuclear accumulation (Cheng et al., 1999; LaBaer et al., 1997). However, the cyclin D1 T156A mutant still interacts with p21 and overexpression of p21 overcomes the transport defect of this mutant (Diehl and Sherr, 1997). Furthermore, loss of both p21 and p27 does not fully abolish cyclin D1 nuclear import (Cheng et al., 1999). Results from the Diehl group recently suggested that, instead of enabling nuclear import, p21 and p27 directly inhibit GSK-3 β -

triggered cyclin D1 nuclear export (Alt et al., 2002). If Cip/Kip proteins do not act as nuclear import factors, what then could be the mechanism? One candidate is the p34^{SEI-1} protein that binds to cyclin D1/Cdk4 complexes and contains an NLS (Sugimoto et al., 1999). Yet another possibility is that the cyclin D protein itself contains a cryptic (as yet unidentified) NLS that regulates nuclear import via a classical pathway, as has been suggested for cyclin E (Moore et al., 1999). This pathway involves the binding of cargo protein to the soluble NLS receptor, importin- α , which interacts with the nuclear transport factor, importin- β , to form a trimeric complex that travels through the nuclear pore (Kuersten et al., 2001).

Signals regulating cyclin B localisation have been extensively studied because of the dramatic cyclin B relocation associated with mitotic entry (reviewed in Takizawa and Morgan, 2000). Similar to cyclin E, cyclin B contains a cryptic NLS that mediates nuclear import through a novel mechanism involving the direct interaction with importin- β nuclear transport factor (Moore et al., 1999; Takizawa et al., 1999). However, the major mechanism governing nuclear accumulation of cyclin B is via its cytoplasmic retention signal (CRS) at the N-terminus. The CRS contains an NES that mediates binding with the nuclear export mediator Crm1 (Yang et al., 1998). Phosphorylation of four or five serine residues within the CRS/NES region triggers a decrease in export and an increase in import, resulting in nuclear translocation (Hagting et al., 1999; Li et al., 1997a; Yang et al., 1998). Although little is known about the kinases that act on cyclin B, phosphorylation by cyclin B/Cdc2 itself and by polo-kinases have been suggested (Izumi and Maller, 1991; Toyoshima-Morimoto et al., 2001).

1.2.9 Cyclin/Cdk/CKI stability

While Cdks are constitutively expressed and are relatively stable, cyclins are subject to regulated degradation. The degradation of cyclins and many other cell cycle components is regulated by the ubiquitination-proteasome system. Ubiquitination involves the covalent attachment of ubiquitin chains to lysine residues in the target protein, leading to their recognition and degradation by the proteasome (reviewed in Harper et al., 2002) (Fig. 1.4A). Two structurally and functionally similar ubiquitin ligases exist: the Skp-Cullin-F-box (SCF) complex and the anaphase-promoting complex (APC). These are multiprotein complexes, each consisting of at least 8-10 subunits, which target proteins at G1/S/G2 phases (SCF) or at G2/M (APC) (Fig. 1.4B). Target specificity is governed by factors that interact with the ligases. These comprise F-Box sequence motif-containing proteins, such as Skp2, in the case of SCF (SCF^{skp2}), or WD-40 repeat-containing proteins such as Cdh1 or Cdc20 in case of APC (APC^{Cdh1}, APC^{Cdc20}). APC targets usually contain defined destruction box (D-box, consensus RxxLxxxN) or KEN box (consensus KENxxxN/D/E/S) motifs that are thought to

mediate binding to Cdh1 or Cdc20 proteins, respectively. Additional elements are probably required for proper recognition, and motifs other than the D-box and KEN box may exist.

Many substrates of the SCF need to be phosphorylated prior to ligase recognition. In contrast, activation of the APC is mainly regulated by phosphorylation of the core APC complex itself (Harper et al., 2002). The degradation of cyclin D1 when bound to Cdk4 can be initiated upon phosphorylation of Thr286 by GSK-3 β (Diehl et al., 1998; Diehl et al., 1997). Mitogenic signalling via the Ras-PI3K-Akt pathway inhibits this phosphorylation, resulting in an increased half-life of cyclin D/Cdk complexes. Cyclin E/Cdk2 complexes can initiate cyclin E degradation via phosphorylation of Thr380, thus forming a negative feedback loop to control cyclin E expression in late G1 (Clurman et al., 1996; Won and Reed, 1996). Both cyclin D1 and cyclin E are also degraded as free proteins, which happens in the absence of Thr286 or Thr380 phosphorylation, respectively (Germain et al., 2000; Singer et al., 1999). Degradation of p27 is initiated by cyclin E/Cdk2-catalysed phosphorylation of Thr187 (Vlach et al., 1997). This reaction is thought to be mediated in trans by the phosphorylation of inactive ternary p27/cyclin E/Cdk2 complexes by active cyclin E/Cdk2 (Xu et al., 1999). The APC mediates degradation of cyclin A after nuclear envelope breakdown in prometaphase, while cyclin B1 degradation occurs during the metaphase to anaphase transition (Clute and Pines, 1999; den Elzen and Pines, 2001; Geley et al., 2001). Interestingly, the APC is thought to be activated upon phosphorylation of its Cdc20 subunit and/or APC core subunits by Cdk2 and Cdc2 itself (reviewed in Morgan, 1999). In conclusion, ubiquitination is often initiated by cell cycle proteins themselves, thus ensuring a tight temporal regulation of protein activity.

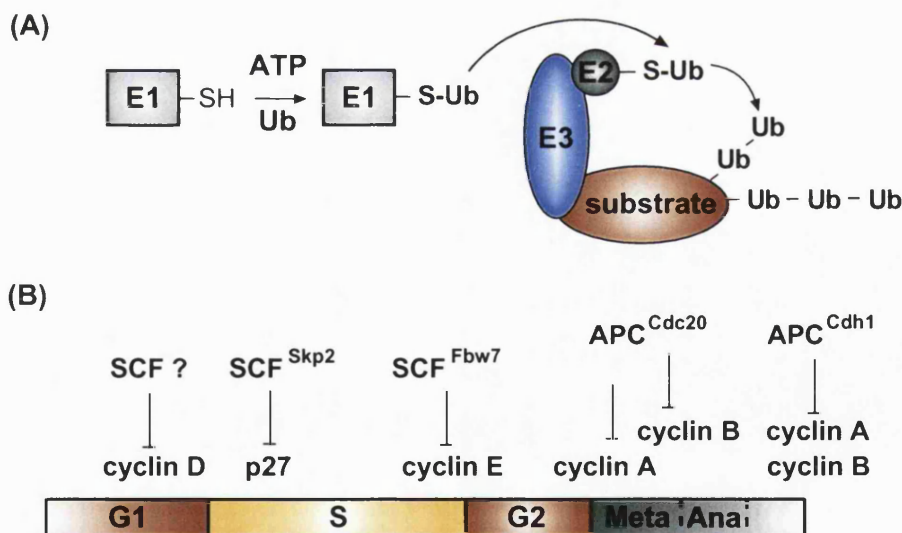


Fig. 1.4 Ubiquitination of cell cycle regulators. (A) Simplified scheme of the ubiquitination reaction. E1 uses ATP to form a high-energy thiol ester with ubiquitin (Ub), which is transferred to a cysteine residue on the E2 ubiquitin-conjugating enzyme. The E2 assembles with an E3 ubiquitin ligase to transfer ubiquitins to one or more lysines on the substrate. Multiple rounds of ubiquitination lead to the formation of poly-ubiquitin chains that are recognised by the proteasome. (B) Temporal control and cell cycle targets of the APC and SCF ubiquitin ligase complexes. The SCF specific for cyclin D degradation has not yet been identified. Adapted from (Harper et al., 2002).

1.3 G1/S progression

A coordinated progression through G1/S phases of the cell cycle is critical since passage through the restriction point commits cells to divide. During early G1 cells integrate information derived from mitogenic stimuli and nutrient availability. D-type cyclins act as the sensors, with cyclin D transcription, cyclin D/Cdk complex assembly, nuclear transport and stability each being mitogen-dependent steps (see previous sections). The most recognised function of cyclin D/Cdk activity is inactivation of the Rb tumour suppressor.

1.3.1 The retinoblastoma protein

The retinoblastoma gene, *Rb*, is the classical example of Knudson's "two-hit" hypothesis to explain cancer predisposition: germline mutation of one tumour suppressor allele predisposes to tumour formation, and when mutation of the second allele occurs during the lifetime of the individual this results in tumour initiation/progression (Knudson, 1971). Genetic linkage and cytogenic analysis of families predisposed to retinoblastoma led to the identification of the *Rb* gene (Friend et al., 1986). Although *Rb* gene mutations are rare and only found in retinoblastomas, small cell lung carcinomas, sarcomas and bladder carcinomas (Horowitz et al., 1990), the "Rb pathway" is disrupted either by misexpression of regulators of Rb or by the action of viral oncoproteins (see section 1.5.1).

Rb and its relatives p107 and p130 belong to a family of proteins called the "pocket proteins" (reviewed in Harbour and Dean, 2000). This name reflects the folding of the conserved C-terminal A and B domains around a central pocket, constituting a protein binding site. Many proteins bind the pocket via an LxCxE motif (Lee et al., 1998). Interestingly, cyclins contain an LxCxE motif in the same hydrophobic region that recruits Cip/Kip Cdk inhibitors and this motif thus mediates recruitment of both substrates and inhibitors (Endicott et al., 1999). Although numerous proteins interact with Rb, the most established function of Rb is interaction with and inhibition of the E2F family of transcription factors. E2Fs do not bind Rb via an LxCxE motif, thus allowing the binding of Rb in complex with other proteins to E2F promoter elements (Huang et al., 1992; Lee et al., 1998).

Rb is thought to repress E2F-dependent transcription by at least two mechanisms. First, Rb directly binds the transactivation domain of E2F, thus blocking recruitment of the transcription machinery (Helin et al., 1993; Helin et al., 1992). Second, Rb binding to E2F actively represses transcription via its interaction with factors that influence chromatin structure. These include histone deacetylases (HDACs), which remove acetyl groups from histone octamers, thereby facilitating condensation of nucleosomes into chromatin (reviewed in Kouzarides, 1999). Because E2F, like most transcription factors, interacts with the histone

acetyl transferases (HAT) p300/CBP and p/CAF (Trouche et al., 1996), HDAC activity counteracts E2F-associated nucleosome disruption. Rb also recruits the BRM and BRG proteins, the ATPase components of the human SWI/SNF chromatin-remodelling complex. Together, these factors cause the tight assembly of DNA and chromatin in nucleosomes, inhibiting access of the transcriptional apparatus (Dunaief et al., 1994; Singh et al., 1995; Zhang et al., 2000). It was recently shown that Rb recruits the histone methyltransferase SUV39H1 to E2F-responsive promoters, suggesting a third mechanism by which Rb represses transcription (Nielsen et al., 2001; Vandel et al., 2001).

Rb contains 16 potential Cdk phosphorylation sites, which regulate the binding of distinct proteins (Knudsen and Wang, 1997). Complete phosphorylation of Rb relieves its binding to E2F, triggering E2F-dependent transcription of critical G1/S genes such as cyclin E and A. Successive phosphorylation by cyclin D/Cdk complexes in early G1 and cyclin E/Cdk2 complexes in late G1 is necessary for complete hyperphosphorylation of Rb (Lundberg and Weinberg, 1998). In addition, phosphorylation by cyclin A/Cdk2 may maintain Rb hyperphosphorylation during S phase (Sherr, 1996). Rb can be dephosphorylated by the PP1 phosphatase, which restores Rb growth-suppressing function after mitosis (reviewed in Tamrakar et al., 2000). Although mainly based on overexpression studies, a likely mechanistic explanation for sequential Rb inactivation is that first, phosphorylation by cyclin D/Cdk4 disrupts the association of Rb with HDAC, thereby allowing cyclin E expression. Rb-SWI/SNF interaction is, however, maintained and is sufficient to repress the cyclin A promoter. Only upon phosphorylation by cyclin E/Cdk2, is Rb repression of the cyclin A promoter relieved (Harbour et al., 1999; Zhang et al., 2000). This would ensure the orderly activation of cyclin E and A in S phase.

1.3.2 The E2F family of transcription factors

E2F was originally identified as a factor that binds the adenoviral E2 promoter, which is activated upon viral E1A expression (reviewed in Nevins, 1992). This finding converged with the demonstration that Rb was inactivated upon E1A expression, establishing the first link between Rb and E2F (Whyte et al., 1988). E2F1 was later cloned by virtue of its ability to bind Rb (Helin et al., 1992; Kaelin et al., 1992; Shan et al., 1992), followed by the identification of other E2F family members using low-stringency hybridisation of cDNA libraries (Shan et al., 1992). Since then, E2F has been shown to regulate the transcription of multiple genes involved cell cycle progression (such as cyclin E, cyclin A, Cdc2, Cdc25A, Rb, E2F1), nucleotide biosynthesis (such as dihydrofolate reductase, thymidylate synthetase and thymidine kinase) and DNA replication (such as Cdc6, ORC1 and MCM proteins) (reviewed in Dyson, 1998; Helin, 1998).

To bind DNA, E2F proteins form obligate heterodimers with a related family of DP proteins (reviewed in Trimarchi and Lees, 2002). Six E2F family proteins (E2F1-6) and two DP proteins (DP1 and DP2) exist, which heterodimerise in all possible combinations. However, different pocket proteins preferentially bind to certain E2Fs *in vivo*, and this occurs at distinct times of the cell cycle. The exception is E2F6, which may repress genes by recruiting the polycomb complex (Trimarchi et al., 2001).

In quiescent cells, E2F4 and E2F5 expression levels are high and they are predominantly bound to p130 (Hijmans et al., 1995; Vairo et al., 1995). Cycling cells express E2F4 and E2F5 mainly in the cytoplasm, which correlates with their poor transcriptional activation capacity in overexpression assays and their inability to drive quiescent cells into S phase (Muller et al., 1997; Verona et al., 1997). Importantly, MEFs that lack E2F4 and E2F5 or p130 have defects in their ability to arrest upon growth-arrest signals (Gaubatz et al., 2000), and mice lacking E2F4 or E2F4 and E2F5 together exhibit developmental abnormalities as a result of aberrant differentiation (Humbert et al., 2000; Lindeman et al., 1998; Rempel et al., 2000). E2F4 and E2F5 are therefore thought to be crucial for mediating the transcriptional repression of E2F-responsive genes. This is confirmed by the presence of E2F4/p130 complexes on promoters of repressed genes in G0/G1, such as *Cdc2* and *cyclin A2* as measured by *in vivo* footprinting analysis (Huet et al., 1996; Tommasi and Pfeifer, 1995).

In contrast, Rb binds to E2Fs in both quiescent and cycling cells and p107 is predominantly associated with E2Fs during S phase. E2F1, E2F2 and E2F3 are specifically expressed in dividing cells where they interact with Rb (Ikeda et al., 1996). They are potent transcriptional activators and their overexpression drives quiescent cells to enter S phase (Lees et al., 1993). Furthermore, cells that lack expression of E2F1, E2F2 and E2F3 are unable to proliferate (Wu et al., 2001), confirming their role in S phase progression. In addition to mediating proliferation, overexpression of E2F1, but probably also E2F2 and E2F3, can trigger apoptosis (Trimarchi and Lees, 2002) This will be further discussed in section 1.8. Interestingly, the functional inactivation of Rb in either embryonic tissues or tumours is associated with inappropriate proliferation and apoptosis, effects that overlap exactly with the phenotypes noticed upon E2F overexpression (reviewed in Mulligan and Jacks, 1998). Further substantiating this observation, loss of E2F1 or E2F3 expression in Rb-deficient mice is sufficient to suppress these effects (Tsai et al., 1998; Ziebold et al., 2001). E2F1, E2F2 and E2F3 are therefore considered to be critical activators of genes required for G1/S progression, and their activity is counter-acted by the Rb pocket protein.

1.3.3 Orderly progression through G1/S cell cycle phases

A (simplified) picture of G1/S progression can be drawn based on the assimilation of the previous information (Fig. 1.5). Expression of critical cell cycle genes is suppressed in quiescent cells via the activity of p130/E2F4/E2F5 complexes. When cells are stimulated with mitogens, cyclin D levels rise, cyclin D/Cdk4/6 complexes become more abundant and cyclin D/Cdk activity increases. In addition, an increase in Cip/Kip protein levels promotes cyclin D nuclear accumulation and stability. Here, cyclin D/Cdk complexes triggers the hypophosphorylation of Rb, which increases the expression of cyclin E. Cyclin E/Cdk2 activity also increases because the Cip/Kip Cdk inhibitors are sequestered away from cyclin E/Cdk complexes towards cyclin D/Cdk complexes. This drives a positive feedback loop through further phosphorylation of Rb and consequent release of additional E2F activity. The feedback is amplified by the phosphorylation of p27 by cyclin E/Cdk2, which initiates its degradation. Together, these signals stimulate E2F-dependent expression of cycle regulatory and DNA replication genes, including cyclin A and E2F itself.

Interestingly, cyclin E, but not cyclin D, is required for G1/S progression in cells lacking Rb function (Ohtsubo et al., 1995) and overexpression of cyclin E is sufficient to overcome growth arrest induced by a phosphorylation-resistant Rb mutant (Lukas et al., 1997). This implies that the main downstream target of cyclin D/Cdk activity is Rb, while E/Cdk2 has critical S phase progression targets other than Rb. Indeed, cyclin E, like cyclin A, can trigger phosphorylation of proteins of the DNA replication machinery, such as Cdc6, directly activating DNA replication (Jiang et al., 1999; Krude et al., 1997; Petersen et al., 1999) (see also section 1.7.4). Furthermore, the finding that a knock-in of human cyclin E1 in *cyclin D1*^{-/-} mice restores most of the phenotypic effects of mice lacking cyclin D1 (growth

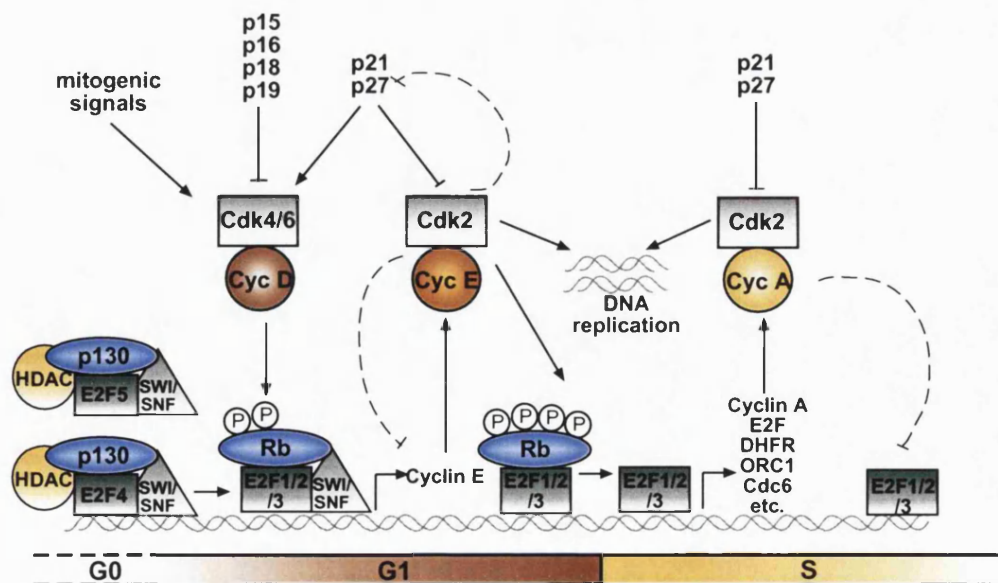


Fig. 1.5 Regulation of G1/S progression. Dashed lines represent feedback loops that consist of the activation of ubiquitin-dependent degradation. Many additional fine-tuning regulations exist and this diagram is therefore highly simplified. See text for further details.

retardation, underdeveloped mammary epithelium, hypoplastic retinal tissue) supports the idea that the primary role of cyclin D1 *in vivo* is to activate cyclin E/Cdk2 (Geng et al., 1999).

Active cyclin D/E and A-dependent kinases are regulated by negative feedback loops: cyclin E/Cdk2 complexes phosphorylate cyclin E, directing it to SCF-mediated ubiquitination. Cyclin A/Cdk2 phosphorylation of components of the APC may initiate proteolysis of cyclin A itself. Importantly, E2Fs are thought to be inactivated around S/G2 by at least two mechanisms. First, cyclin A/Cdk2 phosphorylation of DNA-bound E2F1 inhibits its DNA binding activity (Krek et al., 1994; Krek et al., 1995). Second, E2F1 undergoes SCF^{Skp2}-dependent ubiquitination and degradation (Marti et al., 1999). Negative regulation of G1/S progression can also be enforced upon Cdk inhibitor induction, for example upon the action of growth-inhibitory cytokines or the execution of checkpoints (see section 1.8.3).

1.4 G2/M progression

It is clear the E2Fs are essential regulators of G1/S progression. However, an emerging idea is that E2Fs are also able to affect mitosis. For example, the E2F-dependent increase in cyclin A levels is required for the rise in cyclin B expression levels during late S/G2 phases (Lukas et al., 1999). The complexity of E2F-triggered responses is best illustrated by results from micro-array analyses (Ishida et al., 2001; Muller et al., 2001). These studies identified clusters of E2F-induced genes that are known to function during mitosis (e.g. *Cdc2* and *cyclin B*), as well as genes involved in apoptosis (e.g. *Apaf-1*, *caspase 3*, *caspase 7*), development and differentiation (e.g. Polycomb group genes). However, these studies analysed transcriptional responses upon ectopic E2F expression by inducible systems or adenoviral transfer, which could have influenced target specificity. Furthermore, E2F-independent changes in gene expression upon cell cycle progression complicate the interpretation of these results. The Dynlacht lab used an alternative approach, which relied on the identification of E2F repressed genes in quiescent cells using chromatin IP of E2F4-bound promoter DNA, followed by DNA microarray analysis (Ren et al., 2002). Interestingly, a significant number of genes that function in mitosis were again identified. These included regulators of cytokinesis (e.g. *Polo kinase*), chromosome condensation and chromosome segregation (e.g. *securin*). In addition, many proteins involved in DNA repair and checkpoint controls were found. Although validation of individual E2F-dependent targets with regards to cell cycle-dependent expression is required, these results suggest a broader role for E2Fs in mitosis as well as G1/S. As in G1/S progression, the timed activation and degradation of cyclins is a key regulator of mitotic protein function (Fig. 1.6).

1.4.1 Mitotic entry

Early mitotic events in prophase, such as chromosome condensation, are initiated by nuclear cyclin A/Cdk2 activity (Furuno et al., 1999). However, major mitotic events such as nuclear envelope breakdown coincide with cyclin B/Cdk1 nuclear import in the last minutes before prophase (Hagting et al., 1999). Importantly, the relocation of cyclin B to the nucleus appears to be required for the initiation of mitosis: in *Xenopus*, the mitosis-promoting activity of cyclin B is blocked by mutations that prevent its nuclear localisation (Li et al., 1997a) and enforced expression of active cyclin B/Cdk1 in the cytoplasm of human cells does not induce premature chromosome condensation or nuclear envelope breakdown (Heald et al., 1993). Targets of cyclin B/Cdc2 include both structural proteins, such as the nuclear envelope protein lamin (Dessev et al., 1991), and regulatory proteins, such as the nucleolar proteins nucleolin and NO38 (reviewed in Nigg, 1993). Their phosphorylation is likely to be important for nuclear envelope breakdown, spindle assembly and other mitotic processes, although their regulation by cyclin B/Cdk1 phosphorylation has not been extensively studied.

1.4.2 Mitotic exit

After the initiation of mitotic events, degradation of key mitotic regulators is essential for mitotic exit. The first step in mitotic exit is triggered, at least in part, by the APC-dependent destruction of a protein generically referred to as securin (Pds1 in budding yeast, Cut2 in fission yeast, PTTG -for pituitary tumour transforming gene- in mammals). Securin plays a negative role in mitosis by binding and inhibition of separase, an endoprotease that disrupts chromatid cohesion by cleaving the cohesion complex protein Scc1 (reviewed in Nasmyth, 2002). Thus, sister chromatid separation depends on proper degradation of securin by APC^{Cdc20}. Consistent with this, anaphase is blocked in cells expressing non-destructible forms of securins (Zou et al., 1999).

Next, a timely degradation of cyclins A and B is essential for mitotic progression beyond anaphase. This is illustrated by findings in different model systems that non-degradable versions of cyclin A and B arrest cells in metaphase or in anaphase (Geley et al., 2001; Parry and O'Farrell, 2001; Sigrist et al., 1995). Mitotic cyclin degradation occurs in two phases that are coordinated by the sequential activation of the APC^{Cdc20} and APC^{Cdh1} (see also Fig. 1.4) and, again, oscillations in (Cdk) kinase activity appears to be a key regulator. First, the APC^{Cdc20} complex can be activated upon phosphorylation of APC core subunits by Polo-like kinase (see below) and cyclin B/Cdk1 complexes (Harper et al., 2002) and references therein). This is thought to increase the binding of the Cdc20 specificity subunit to APC, thereby activating APC^{Cdc20} activity and initiating the degradation of cyclins A and B (Fang et al., 1998). Cyclin A/Cdk2 phosphorylation of Cdh1 has been reported to inhibit the binding of

this specificity subunit to APC. The degradation of cyclin A therefore allows the activation of APC^{Cdh1} and consequent complete degradation of mitotic cyclins (Lukas et al., 1999).

1.4.3 Other mitotic kinases

Although securin and mitotic cyclins are the best understood APC substrates, a large number of other proteins display cell cycle regulated degradation via the APC. An intriguing example is the conserved family of recently characterised polo-kinases (Plks) (reviewed in Nigg, 1998). At least three Plks are expressed in mammalian cells (Plk1-3), of which only Plk1 has been extensively characterised. Plk1 localises to mitotic structures, including centrosomes, kinetochores and the spindle midzone (Golsteyn et al., 1995). Its activity peaks in M phase, where it has diverse functions in both mitotic entry and exit. As mentioned above, Plk1 can phosphorylate cyclin B and promote its nuclear accumulation (Toyoshima-Morimoto et al., 2001). Furthermore, Plk-mediated phosphorylation of Cdc25C activates its dephosphorylation of inhibitory phosphates on Cdk2, further enhancing cyclin B/Cdk1 activity (Kumagai and Dunphy, 1996; Qian et al., 2001). Recently, Plk1 was shown to mediate loss of chromatid cohesion in prophase in a separase/Scc1-independent manner (Sumara et al., 2002). This process, which only occurs in mammalian cells, is followed by a separase-dependent step at the metaphase anaphase transition. Mitotic exit can be promoted by Plk-mediated phosphorylation and activation of the APC (Harper et al., 2002), and references therein). Plk therefore triggers both the activation of cyclin B and its degradation, thus suggesting its role as a master regulator of mitosis. Indeed, cells expressing a dominant negative Plk1 are unable to exit mitosis or undergo cytokinesis (Seong et al., 2002). Interestingly, Plk1 can transform rodent cells and it was found to be amplified in squamous cell carcinomas (Knecht et al., 1999; Smith et al., 1997), implying that improper execution of mitosis may contribute to tumourigenesis.

Another APC^{Cdh1}-specific target is the Aurora family of protein kinases (Castro et al., 2002; Taguchi et al., 2002). The founding member of this family was first identified in budding yeast in a genetic screen for mutations that lead to increases in chromosome missegregation and was termed Ipl1p (for increases in ploidy 1) (Chan and Botstein, 1993). In vertebrates, this family contains three different members: Aurora -A, -B and -C (reviewed in Bischoff and Plowman, 1999). These kinases are localised to mitotic structures such as the mitotic spindles and centrosomes (spindle-pole bodies in yeast), which constitute the microtubule organising centers of the cell (reviewed in Doxsey, 2001; Hinchcliffe and Sluder, 2001b). Aurora A has been implicated in centrosome duplication and separation and in spindle assembly. Recent results in yeast imply that Ipl1 is crucial in promoting bi-orientation of sister chromosome attachment to the spindle (Biggins and Murray, 2001; Tanaka et al.,

2002), but this has not yet been shown in animal cells. Interestingly, aurora is amplified in some human cancers (colorectal and breast tumours and various tumour cell lines), and its overexpression induces cellular transformation (Bischoff et al., 1998; Zhou et al., 1998). However, high levels of aurora expressed in tissue culture cells does not alter centrosome duplication directly, but induces cells to undergo an aberrant mitosis without cytokinesis, producing tetraploid cells in G1 (Meraldi et al., 2002). A similar phenotype was seen upon Plk1 overexpression. Thus, the ability of APC^{Cdh1} to keep aurora and Plk1 levels in check would appear to be important in maintaining the proper execution of cytokinesis and ploidy.

1.4.4 The initiation of cytokinesis

During anaphase, molecules such as actin and myosin redistribute to the midzone region between the spindle poles, ultimately forming an actin ring. Contraction of the actin ring, also called cleavage furrow, initiates the process of cytokinesis. A post-mitotic bridge consisting of septins remains at the point where the two cells have separated, and cytokinesis is completed by the destruction of this bridge and restoration of an intact cell membrane around each of the resulting cells (Guertin et al., 2002). Although cytokinesis and mitotic exit are tightly linked, many aspects with regards to the coordination of cytokinesis and mitotic progression are obscure. In budding yeast, a regulatory network of kinases regulates late mitotic events such as spindle disassembly and cytokinesis. This signalling network is called the mitotic exit network (MEN) in budding yeast or the septation initiation network (SIN) in fission yeast (McCollum and Gould, 2001).

It is not clear whether a similar kinase cascade is operative in mammalian cells and few homologous mammalian proteins, of which Plk is one, have been identified. These

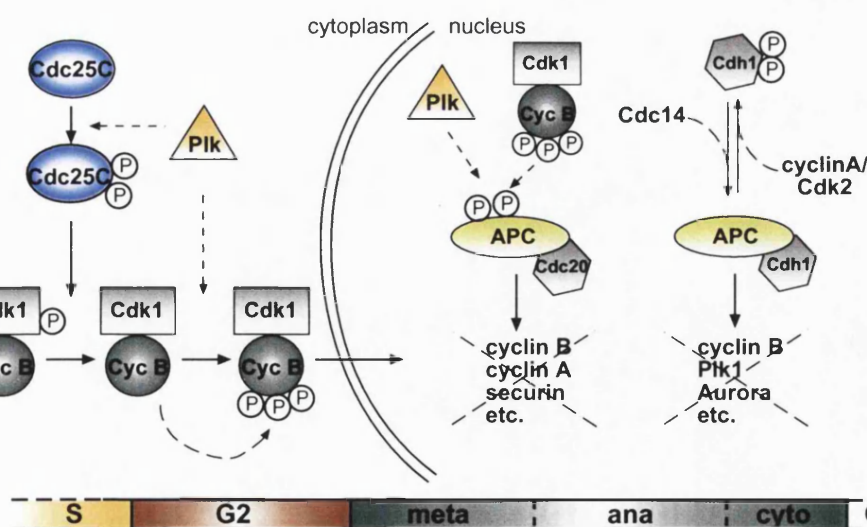


Fig. 1.6 Schematic representation of major events in G2/M progression. This model is based on data obtained from various overexpression studies in different model systems. Dashed arrows denote potential (de)phosphorylation events, for which the *in vivo* evidence in human cell systems remains scarce. Meta= metaphase, ana= anaphase, cyto= cytokinesis. See text for details.

networks converge to activate the Cdc14p phosphatase, generally considered to be the masterswitch that reverses cyclin B/Cdk-dependent phosphorylation (Visintin et al., 1998). Yeast Cdc14 phosphatase activity switches on APC^{Cdh1} by dephosphorylating Cdh1, stabilises the Cdk1-inhibitor Sic1p and enhances Sic1p expression via stabilising the Swi5p transcription factor. This promotes both the inactivation and degradation of cyclin B. Human cells possess two Cdc14 orthologs (Cdc14A and B), which can dephosphorylate Cdh1 (Kaiser et al., 2002) *in vitro*, but its *in vivo* functional properties remain to be established. Cdc14A overexpression leads to premature centrosome splitting and formation of supernumery mitotic spindles, whereas inactivation of Cdc14A by siRNA leads to failure of centrosomes to split and defects in cytokinesis (Kaiser et al., 2002; Mailand et al., 2002). Thus, it appears that the basic mechanism of coupling mitotic exit and cytokinesis via Cdk1 inactivation is conserved. The big remaining question is which Cdk1 and/or Cdc14 substrates in cytokinesis and in particular, actin-myosin ring formation, are crucial for the execution of cytokinesis.

1.5 Cell cycle deregulation in mouse models and human cancer

The majority of cells in the human body reside in a non-dividing, terminally differentiated or quiescent state. Cancer cells have acquired properties that allow them to survive, divide and expand as a population, at a higher rate than normal cells. Perhaps surprisingly, the lengths of tumour cell cycles measured *in vivo* and *in vitro* are not significantly shorter than those of normal cells (Ho and Dowdy, 2002). Consequently, the ability of a cell to escape cell death and avoid cell cycle arrest or G0 entry are likely to contribute more to tumour growth than events that regulate the rate of individual cell division.

How do cell cycle proteins fit in the tumourigenesis picture? Classically, studies to address the oncogenic or tumour-suppressive functions of cell cycle regulators have relied on overexpression or inhibition of candidate genes. This has provided important insights in the molecular circuitry of gene interactions. For instance, the finding that microinjection of antibodies against cyclin D blocks cells during G1 was crucial to ascertain the importance of D-type cyclins in G1 progression (Baldin et al., 1993; Quelle et al., 1993). In addition, the observation that serum-deprived quiescent *Rb*^{-/-} MEFs show an increase in S-phase reentry upon serum stimulation, implies that Rb regulates the G0-G1 transition (Herrera et al., 1996). However, whether D-type cyclins or Rb promote or inhibit tumourigenesis, respectively, depends on influences of the *in vivo* physiological environment on processes like quiescence, differentiation and survival. The development of mouse models, genetic screening for tumour-associated mutations in man and the identification of proteins that are often targeted

by oncogenic viruses, together have aided in the identification of cell cycle regulatory pathways that are commonly affected in cancer.

1.5.1 Mouse models translated to human cancer and vice versa

Cell cycle regulators are frequently mutated in human neoplasias. Specifically, overexpression of cyclin D1, E, Cdk4 and Cdk6 is associated with numerous types of cancer, and these genes are classed as bona fide human proto-oncogenes (Cordon-Cardo, 1995; Malumbres and Barbacid, 2001). Tumour-associated changes involve gene amplifications in the case of cyclin D1 or Cdk4 or translocation in case of Cdk6 (Sherr, 1996). In agreement with their oncogenic potential in human tumours, transgenic mice that express cyclin D1 or cyclin E in mammary tissue develop mammary hyperplasia and carcinomas (Bortner and Rosenberg, 1997; Wang et al., 1994). Oncogenes often act in cooperation with each other, an example of which is the finding that transgenic expression of cyclin D1 in lymphocytes requires a cooperating oncogene (c- N, or L-myc) to reveal its oncogenicity (Bodrug et al., 1994; Lovec et al., 1994).

Remarkably, genetic or epigenetic alterations (such as promoter methylation) in Cdk2 or its regulators have rarely been found in human tumours. However, low expression of p27 or overexpression of cyclin E1 in tumour samples occurs often and correlates with poor prognosis in breast carcinoma, colon carcinoma, prostate tumours and other malignancies (Cordon-Cardo, 1995). How cyclin E and p27 levels are altered is not yet understood, although one possibility is that such tumours contain mutations in proteins that regulate their stability (Loda et al., 1997). Interestingly, the mouse *p27* gene also does not behave as a classical tumour suppressor, because tumours do not delete or silence both copies of the gene. Yet, there is evidence that p27 is haplo-insufficient for tumour suppression, with loss of only one copy of the gene being sufficient to contribute to cancer: *p27*^{+/-} mice were significantly more susceptible than wild type mice to γ -irradiation and chemical carcinogen-induced tumourigenesis of the lung, intestine and pituitary. In addition, these mice spontaneously develop pituitary tumours (Fero et al., 1998).

Loss of the *Rb* gene or methylation of the *Rb* promoter is a frequent event in human cancers, while *p130* is less frequently lost and *p107* mutations have not yet been found (Mulligan and Jacks, 1998). This emphasises a different role for these pocket proteins in tumour suppression, which is supported by analysis of mutant mice. Whereas mouse *Rb* is essential for embryonic development, mutation of either *p107* or *p130* in the same genetic background has no effect on viability (Cobrinik et al., 1996; Lee et al., 1996). Moreover, *Rb*^{+/-} mice develop tumours of endocrine origin (pituitary, thyroid and adrenal medulla) while there is no detectable increase in the tumour incidence of *p107*^{-/-} or *p130*^{-/-} mice.

Instead, p107 and p130 appear more important for differentiation. In particular, *p107;p130*^{-/-} mice show defects in limb development due to deregulated chondrocyte growth (Cobrinik et al., 1996). Interestingly, combined deficiency of Rb and p107 causes retinoblastoma in chimeric mice, showing that pocket proteins may have redundant functions and can partially overlap in tumour suppression (Robanus-Maandag et al., 1998).

There is compelling evidence that *p16* loss of function occurs frequently in human cancers, in a pattern consistent with *p16* being a tumour suppressor (Ruas and Peters, 1998). In familial melanomas, for example, one defective copy of *p16* is inherited, whereas the second is lost in tumour cells. The *p16* function is also lost in many sporadic cancers, such as pancreatic carcinomas, often involving p16 promoter methylation. The adjacent *p15* locus is only inactivated concomitant with loss of the p16 gene. No evidence for mutations in p18 and p19 exists in human cancers and it is unclear why Ink4 family members other than p16 are not similarly targeted (Cordon-Cardo, 1995; Sherr and Roberts, 1999). In agreement with human tumour development, gene disruption of the p16 gene in mice predisposes to the development of melanoma, although the effect was only subtle (Krimpenfort et al., 2001; Sharpless et al., 2001). In addition, no overt effects of p15 or p19 loss were detected (Zindy et al., 2000). In contrast, mice lacking p18 resemble those that lack p27 as they exhibit organomegaly and pituitary tumours (Franklin et al., 1998). Mice lacking p21 also develop a variety of tumours (mainly sarcomas and B cell lymphomas) (Martin-Caballero et al., 2001). However, such tumours have a long latency period (around 16 months), suggesting that acquisition of cooperating mutations may be required for tumour development.

As an alternative route to p16 inactivation, some cancer cells have acquired miscoding mutations in Cdk 4 or Cdk6, resulting in loss of Ink4 binding (Easton et al., 1998; Wolfel et al., 1995). Importantly, mice carrying a knock-in Cdk4 mutant that was originally identified in human melanoma (Wolfel et al., 1995) develop multiple tumours with almost complete penetrance (mainly endocrine tumours and hemangiosarcomas) and display high susceptibility to melanoma development after carcinogenic treatment (Sotillo et al., 2001a; Sotillo et al., 2001b). These results highlight the central role of Cdk4 regulation in cell transformation.

Mutations in the E2F family of transcription factors have not been found in human tumours. This may reflect the importance of “normal” E2F activity in the regulation of processes important for cell metabolism, division and survival. Implicit in this would be that unrestrained E2F activity is disadvantageous. E2F1 may even act as a tumour suppressor in some circumstances: *E2F1*^{-/-} mice have an excess of T cells, resulting from a defect in thymocyte apoptosis, and develop testicular atrophy. Most surprisingly, the *E2F1*^{-/-} mice develop a broad spectrum of tumours that arise between 8 and 18 months of age (Field et al.,

1996; Garcia et al., 2000; Yamasaki et al., 1996; Zhu et al., 1999). Loss of E2F2 cooperates with E2F1 in the regulation of lymphocyte proliferation and also in tumour suppression (Zhu et al., 2001). This shows that E2F1 and E2F2 can function as tumour suppressors *in vivo*, presumably because of their intrinsic ability to sensitise cells to apoptosis (see section 1.8). In contrast, E2F3 loss did not cooperate with E2F1 loss in tumour development (Cloud et al., 2002), suggesting that different E2Fs may have non-overlapping functions.

Taken together, disruption of the p16/cyclinD/Rb and p27/cyclinE/Rb pathways often occurs in human tumours. This suggests that deregulation of the G0/G1/S transition is important for unrestricted cell division. In addition, many cancers harbour mutations in upstream signalling pathways leading to cyclin D activation, such as *Ras* mutations. Importantly, recent research has revealed the many tumours also exhibit mutations in mitotic regulators, such as Plk and Aurora as mentioned above, and in genes involved in the execution of checkpoints (see section 1.8.3), emphasising that factors other than deregulation of proliferation can contribute to tumourigenesis.

1.5.2 Small DNA tumour virus-associated deregulation of the cell cycle

Given that Rb is a key negative regulator of cellular proliferation, the low frequency of Rb mutations may seem surprising. One explanation is that *in vivo* Rb loss is associated with pronounced apoptosis, at least in part mediated by the induction of E2F-dependent induction of apoptosis (Yamasaki et al., 1998). Thus, only the simultaneous inactivation of Rb and inhibition of apoptosis would provide a strong tumourigenic stimulus. Studies of DNA tumour virus oncogenesis have been crucial in the substantiation of this paradigm.

Small DNA tumour viruses such as simian virus 40 (SV40), papilloma viruses and adenoviruses rely heavily on the host cell for the synthesis of ribonucleotides to build their viral genome. Because these viruses primarily infect quiescent cells, early viral gene expression is critical to stimulate host cells to enter G1/S phases. This is achieved by the expression of large T antigen in the case of SV40, the E7 protein of human papilloma virus (HPV) and the E1A protein of adenovirus (Fig. 1.7). These proteins all inactivate Rb by binding directly to the pocket region via their LxCxE motifs (DeCaprio et al., 1988; Dyson et al., 1989; Lee and Cho, 2002; Whyte et al., 1988), thereby liberating the E2F transcription factor (Chellappan et al., 1992). Importantly, regions that sequester Rb protein are necessary for cellular transformation (reviewed in Fattaey et al., 1993; Nevins, 1992). In addition to binding Rb, SV40 large T, E1A and E7 interact with a plethora of other host proteins, some of which are involved in transcriptional activation (e.g. p300/CBP) (recently reviewed in Ali and DeCaprio, 2001; Gallimore and Turnell, 2001; Munger et al., 2001).

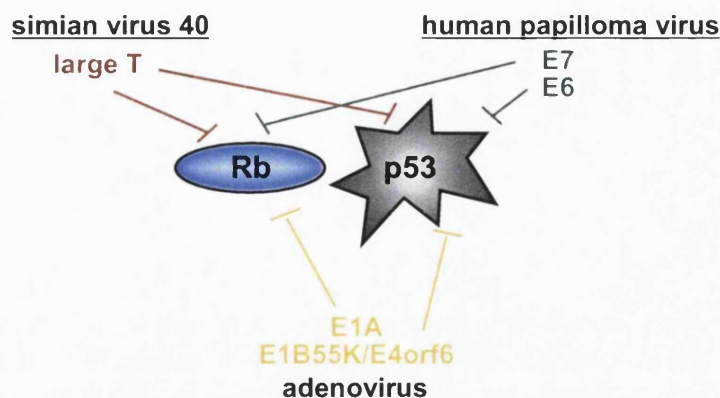


Fig. 1.7 Small DNA tumour virus proteins targeting Rb or p53.

The transforming ability of these viruses also depends on the inactivation of the p53 tumour suppressor, an important downstream target of E2F-induced apoptosis (Fig. 1.7, see section 1.8.1). SV40 large T binds the DNA binding domain of p53, thereby inhibiting p53-mediated transcriptional activation (Mietz et al., 1992). HPV and adenoviruses encode proteins (E6 and a combination of E1B55K and E4orf6, respectively) that specifically bind p53 and target it for degradation (Querido et al., 2001; Scheffner et al., 1990). Thus, inactivation of Rb concomitant with inhibition of p53 tumour suppressor functions is a recurring theme in viral transformation. In support of this, expression of E1A induces p53-dependent apoptosis and this correlates with Rb binding (Samuelson and Lowe, 1997). Still, these functions are not necessarily interdependent; the activation of p53 could also be initiated as a DNA damage response to viral infection.

1.6 Kaposi's sarcoma associated herpesvirus

Small DNA tumour viruses target crucial host cell pathways with few viral gene products. In contrast the large DNA tumour herpesviruses show much greater genetic complexity and encode for example viral DNA polymerase and accessory factors involved in generating nucleotide pools. Herpesviruses cause chronic latent infections that recur when immune surveillance is compromised, which explains the derivation of their name from the Greek word 'herpein' – meaning 'to creep'. They have a characteristic virion architecture consisting of a DNA/protein core surrounded by an icosahedral capsid, amorphous tegument and an envelope with numerous glycoproteins (Wu et al., 2000) (Fig. 1.8). Three subfamilies exist that have been classified based on their genomic structure and sequence similarities, named alpha-, beta-, and gammaherpesviruses (Roizman and Baines, 1991). To date, eight human herpesviruses have been identified, of which Kaposi's sarcoma-associated herpesvirus (KSHV) is the eighth and most recent one (HHV-8) (Table 1.1).

Table 1.1 Classification of the herpesvirus family. Family names are indicated in bold, subfamily names in the left column and genus names in the right column. HHV= human herpes virus.

Alphaherpesviridae	
Simplexvirus	herpes simplex virus 1 (HSV-1; HHV-1) herpes simplex virus 2 (HSV-2; HHV-2)
Varicellavirus	varicella zoster virus (VZV; HHV-3)
Betaherpesviridae	
Cytomegalovirus	human cytomegalovirus (HCMV; HHV-5)
Roseolovirus	human herpesvirus 6 (HHV-6) human herpesvirus 7 (HHV-7)
Gammaherpesviridae	
Lymphocryptovirus	Epstein-Barr virus (EBV; HHV-4)
Rhadinovirus	Kaposi's sarcoma-associated herpesvirus (KSHV; HHV-8)

1.6.1 The discovery of KSHV (a.k.a. HHV-8)

KSHV was identified by Chang and colleagues by using representational difference analysis, a PCR-based subtractive hybridisation method, on Kaposi's sarcoma (KS) samples of acquired immunodeficiency syndrome (AIDS) patients (Chang et al., 1994). KS, the most common neoplasm in people with AIDS, was expected to have an infectious etiology, as gay and bisexual individuals were approximately 10 times more likely to develop KS (Archibald et al., 1992; Beral et al., 1992). It is now established that KSHV carriers are uncommon in the general population of the USA, UK and northern Europe (around 3-10%), but much more frequent in Mediterranean countries (4-35%) and Africa (30-60%) (Sarid et al., 1999).

Sequence analysis classified KSHV as a gammaherpesvirus (Moore et al., 1996) with sequence similarity to several other oncogenic gammaherpesviruses, including EBV. The genome of KSHV was subsequently mapped and sequenced by Russo and colleagues using cosmid and lambda phage genomic libraries from a primary effusion lymphoma (PEL; see

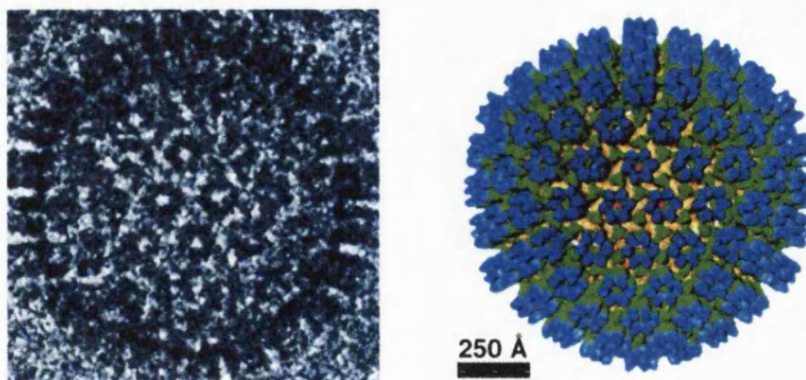


Fig. 1.8 Three-dimensional structure of the KSHV capsid. Left panel shows a cryo-EM image of an ice-embedded KSHV capsid purified from the BCBL-1 cell line. Right panel shows the 3D reconstruction of the same capsid. The capsid is an icosahedral protein shell made up of pentons (blue) and triplexes (green). Adapted from (Wu et al., 2000).

below) -derived cell line, stably co-infected with KSHV and EBV (Russo et al., 1996c). The KSHV genome was found to encode at least 81 open reading frames (ORFs), of which 66 showed significant sequence similarity to ORFs of the prototype rhadinovirus herpesvirus saimiri (HVS), a T cell tropic virus of squirrel monkeys. This classified KSHV as a herpesvirus, subfamily gammaherpesviridae, genus *Rhadinovirus*. The ORFs with HVS homology were numbered based on the HVS nomenclature and ORFs thought to be unique for KSHV were named K1-K15; additional unique ORFs have since been included.

Like other Rhadinoviruses, the KSHV genome consists of a linear double stranded DNA unique region (~ 140 kb) flanked by multiple GC rich 801 bp long terminal repeats (Russo et al., 1996c), giving a total estimated size of around 170 kb (Renne et al., 1996a). Besides HVS and KSHV, known members of this family include the mouse herpesvirus 68 (MHV68) and equine herpesvirus 2 (EHV-2) and viruses in chimpanzees and gorillas (Lacoste et al., 2000). All Rhadinoviruses contain ORFs that show striking homology to known cellular genes. Moreover, these genes are unspliced, implying that these viruses may have captured host cell genes at a spliced RNA stage during their evolution. They encode proteins that either directly affect DNA synthesis or are involved in immune-regulation, cell cycle or apoptosis control (Moore and Chang, 1998).

1.6.2 KSHV-associated Kaposi's sarcoma

Since the discovery of KSHV in KS samples, the virus has been shown to be associated with all forms of this disease and is now accepted to be the transmissible agent of KS (Sarid et al., 1999). KS is a skin tumour of endothelial origin that is most frequently found in immunosuppressed patients, such as transplant recipients and AIDS patients. In addition, a form called classic KS exists as an indolent disease that affects the lower extremities, most often in elderly HIV-negative patients of Mediterranean, Middle Eastern or eastern European men. A more aggressive form of KS that often affects lymph nodes and occurs in both children and adults, called endemic KS, exists mainly in equatorial countries of Africa (reviewed in Boshoff and Weiss, 2001).

KS skin lesions start as a patch of small endothelial lined spaces around blood vessels, which are infiltrated with inflammatory cells. The disease progresses to a plaque stage of spindle-celled vascular processes filled with red blood cells throughout the dermis. Late stage nodular lesions consist of highly vascularised sheets of spindle cells, some of which are undergoing mitosis (Boshoff and Weiss, 2001). Only around 10% of spindle cells in early KS lesions are KSHV-positive (Dupin et al., 1999) and paracrine mechanisms may therefore be involved in disease progression. In late stage nodular lesions, around 90% of the spindle cells are KSHV-infected, suggesting that the virus provides a growth advantage to

infected cells (Boshoff and Weiss, 2001). Indeed, studies of viral terminal repeat (TR) sequence variability or X-chromosome inactivation patterns support the idea that KS begins as a polyclonal hyperplasia that develops through oligoclonality into a monoclonal tumour (Gill et al., 1998; Judde et al., 2000).

1.6.3 KSHV-associated lymphoproliferative disorders

Soon after the discovery of KSHV in KS, KSHV was also found to be associated with a rare form of lymphoma, primary effusion lymphoma (PEL), also called body cavity based lymphoma (BCBL) (Cesarman et al., 1995). PEL manifests itself as malignant effusions in the pleural, pericardial or peritoneal cavities. The malignant cells are differentiated B cells that are thought to be clonal expansions, based on the presence of clonal immunoglobulin gene rearrangements and monoclonal viral TRs (Judde et al., 2000). The majority of PEL patients also carry HIV and EBV, suggesting that cooperation between viruses during pathogenesis may occur (Boshoff and Weiss, 2001).

Another lymphoproliferative disorder that is associated with KSHV is multicentric Castleman's disease (MCD) (Soulier et al., 1995). MCD is an unusual disease of B cell-lineage plasmablasts and is characterised by lymphadenopathy and splenic infiltration. MCD is more common in HIV-infected individuals, although EBV co-infection has not been detected. Studies examining the number of KSHV repeats (Judde et al., 2000) or immunoglobulin rearrangements (Du et al., 2001) indicate that MCD is polyclonal in origin.

1.6.4 Latent and lytic gene KSHV gene expression

The majority of tumour cells in KSHV-associated diseases are latently infected with virus, which persists as episomal DNA (around 40-50 copies per cell), with only 1-3% of the cells showing lytic replication (Boshoff et al., 1995; Zhong et al., 1996). This suggests that latent genes may be important for viral pathogenesis. Lytic replication is required to maintain viral infection, and treatment of HIV carriers with gancyclovir, a drug that blocks lytic KSHV replication, markedly reduces the incidence of KS development (Martin et al., 1999).

The infection of primary human endothelial cells with KSHV virus has been shown to induce cellular transformation (Flore et al., 1998). However, only 1-5% of the cells actively produced virus in this system, suggesting that infected cells might support the growth of uninfected cells via paracrine mechanisms (Ensoli and Sturzl, 1998). No KSHV-infected endothelial cell line exists that can be serially passaged without losing the virus (Lagunoff et al., 2002). This, together with the difficulty to achieve efficient de novo infection and serial propagation of KSHV virus (Renne et al., 1998), have complicated studies to address kinetics of gene expression in KS-like spindle cells. Most such studies therefore use lymphocytic PEL

cell lines that are latently infected with KSHV. The treatment of these cells with phorbol esters (such as TPA) or sodium butyrate induces lytic viral replication, leading to the release of viral progeny (see for example Miller et al., 1997; Renne et al., 1996b).

A combination of mRNA expression patterns (Sarid et al., 1998; Sun et al., 1999; Zhong et al., 1996), protein detection (Low et al., 2001; Platt et al., 2000; Rainbow et al., 1997) and more recently, microarray studies with PEL cell lines (Jenner et al., 2001; Paulose-Murphy et al., 2001), has shown that latent gene expression is highly restricted and mainly encompasses an “oncogenic cluster” of three genes: latency-associated nuclear antigen (LANA), K cyclin and viral FLICE inhibitory protein (v-FLIP) (Fig. 1.9). These genes are localised adjacently in the genome and are co-transcribed on two polycistronic, alternatively transcribed mRNAs transcripts, LT1 (encoding LANA/K cyclin/v-FLIP) and LT2 (encoding K cyclin and v-FLIP) (Dittmer et al., 1998; Sarid et al., 1998; Talbot et al., 1999). It is thought that translation initiation of the 3' v-FLIP ORF is governed by an internal ribosome entry site (IRES) within the K cyclin ORF (Grundhoff and Ganem, 2001; Low et al., 2001).

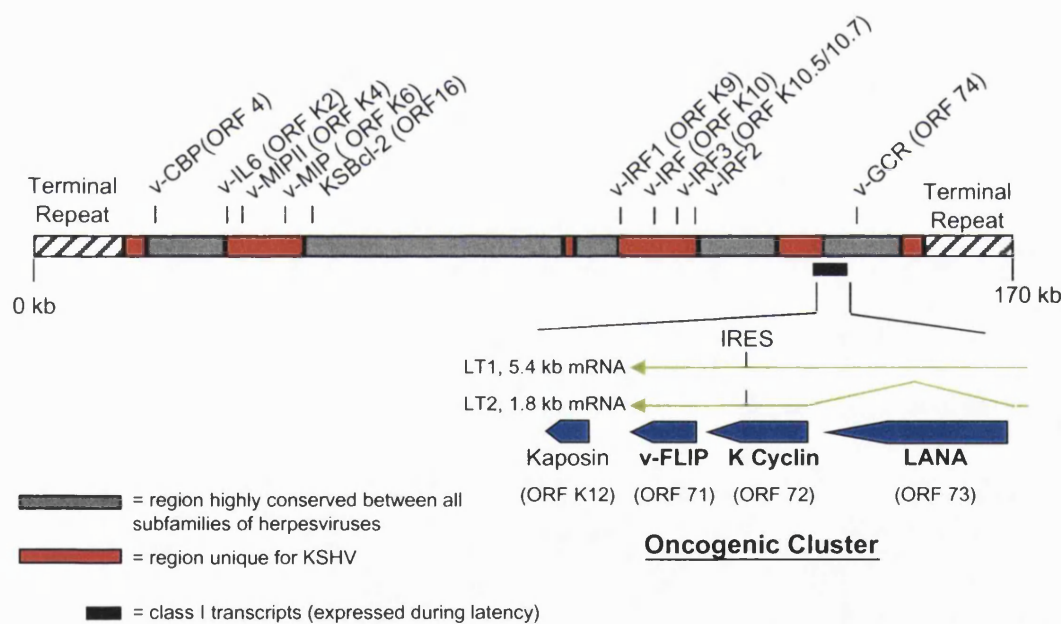


Fig. 1.9 Genetic map of KSHV ORFs. Indicated are the genomic localisation of various latently or lytically expressed ORFs that have mammalian homologues, such as the immune regulatory proteins viral complement binding protein (v-CBP), chemokines (v-MIP and v-MIP11), a cytokine (IL-6). Other homologues include signalling molecules such as a viral G-protein coupled receptor (v-GCR) and interferon regulatory factor (v-IRF), and the viral apoptosis inhibitor Bcl-2 (KS-Bcl2). An oncogenic cluster of three genes that are localised on overlapping LT1 or LT2 mRNA transcripts, is expressed during latency. Expression of Kaposin, a membrane protein that regulates actin remodelling and cellular adhesion and can transform NIH3T3 fibroblasts (Kliche et al., 2001), has been detected in latently infected cells, but its expression pattern is distinct from v-FLIP, LANA and K cyclin. In addition, some RNA transcripts encoding putative interferon regulatory factors (v-IRF2, v-IRF3 and K10) have been suggested as latent genes (Jenner et al., 2001). It is unclear whether the four IRF-related genes have different or overlapping functions.

1.6.5 KSHV-encoded v-FLIP

The v-FLIP protein is a homologue of cellular FLIP, a protein that is thought to inhibit CD95 (Fas) death receptor-induced apoptosis via inhibiting the recruitment and activation of FLICE (caspase 8) protease to the death receptor cytoplasmic domain (Irmeler et al., 1997; Krueger et al., 2001) (see section 1.8.5). Cellular FLIP expression is detected during T cell development at a time when Fas-induced apoptosis is inhibited and its expression is upregulation in some melanoma tumours (Irmeler et al., 1997). This suggests an anti-apoptotic role for FLIP under physiological conditions. However, certain splice forms of cellular FLIP are described to have pro-apoptotic activity, at least in transient overexpression studies (Goltsev et al., 1997; Han et al., 1997; Inohara et al., 1997; Shu et al., 1997). One report describes KSHV v-FLIP-mediated protection from Fas-induced apoptosis (Djerbi et al., 1999). In this study, v-FLIP expression in B cells promoted tumour formation when such cells were injected into immunocompetent mice. In addition, v-FLIPs from other gammaherpesviruses (EHV-2 and HVS) were shown to protect cells from death receptor-induced apoptosis, together implying that v-FLIPs inhibit death receptor signalling (Bertin et al., 1997; Thome et al., 1997).

1.6.6 KSHV-encoded LANA

LANA (also called LNA-1) is abundantly expressed in all stages of KSHV-associated disorders. This antigen was first identified based on its reactivity with sera from KS patients in immunofluorescence assays on latently infected PEL cell lines (Kellam et al., 1997). Anti-LANA antibodies have since been used in epidemiological studies of KSHV infection (Kellam et al., 1999). LANA is a nuclear protein that shows a speckled expression pattern, which colocalises with KSHV episomal DNA as measured by fluorescence in situ hybridisation (FISH) (Ballestas et al., 1999; Rainbow et al., 1997). Analogous to the function of EBV nuclear antigen-1 (EBNA1) protein, LANA is thought to tether the KSHV genome to host chromosomal DNA during mitosis and is required for segregation of KSHV episomes to progeny cells (Ballestas et al., 1999; Cotter and Robertson, 1999).

The LANA protein has a predicted molecular mass of around 132 kDa and contains of three distinct domains. 1) An N-terminal proline rich domain that may be involved in protein-protein interaction. 2) A central domain with highly repetitive blocks of acidic residues (EEDD DEQQQ and LEEQEQL); similar domains often function in transcriptional activation. 3) A C-terminal region that contains an NLS and a putative leucine zipper domain, which may be involved in homo- or heterodimerisation (Komatsu et al., 2002). This sequence therefore predicts that LANA interacts with multiple proteins and regulates transcription. Indeed, transient transfection studies have shown that LANA can enhance expression from

cellular promoters such as the telomerase promoter (Radkov et al., 2000; Renne et al., 2001), enhance expression of its own viral LT1/LT2 promoter (Jeong et al., 2001; Renne et al., 2001) or repress transcription, especially of NF- κ B responsive promoters (Krithivas et al., 2000; Renne et al., 2001). This is likely to involve the interaction of LANA with transcriptional regulators such as RING3 (Mattsson et al., 2002; Platt et al., 1999), activating transcription factor 4 (ATF-4)/cAMP response element binding protein (CREB) (Lim et al., 2000), the CBP transcriptional coactivator (Lim et al., 2001) and the chromatin remodelling protein mSin3A (Krithivas et al., 2000).

Interestingly, LANA has also been reported to bind p53 and inhibit its transcriptional activation function, thereby protecting cells from p53-induced apoptosis (Friborg et al., 1999). In addition, LANA was shown to bind the Rb protein and activate E2F-dependent transcription. Importantly, LANA alleviated a growth inhibitory response induced by ectopic Rb expression and could transform primary rodent fibroblasts together with H-Ras (Radkov et al., 2000). Thus, LANA functionally resembles the SV40 Large T oncogene of small DNA tumour viruses. Taken together, LANA appears to be a multifunctional protein, although the relevance of the protein interactions and transcriptional effects with regards to viral pathogenesis remain to be established.

1.7 KSHV-encoded viral cyclin (K cyclin)

One way in which EBV herpesvirus may promote cell cycle progression is via the induction of cellular cyclin D2 by the action of a latent membrane protein (LMP-1) (Arvanitakis et al., 1995). In contrast, some gammaherpesviruses such as HVS, MHV68 and KSHV encode their own viral cyclin D homologue (which I will refer to as V cyclin, M cyclin and K cyclin, respectively). K cyclin, like other viral cyclins, exhibits several properties that are different from D-type cyclins, which may function to deregulate host cell G0/G1/S control.

1.7.1 K cyclin is a cyclin D homologue

The realisation that KSHV ORF72 encodes a cyclin D homologue was interesting since genomic sequencing had failed to identify sequence similarity to EBV-encoded oncogenic LMP or EBV-encoded nuclear antigen (EBNA) genes (Chang et al., 1996). It was demonstrated that K cyclin-associated kinase was capable of phosphorylating Rb *in vitro*, and could overcome an Rb-induced cell cycle arrest in Rb-negative SAOS-2 cells. These initial

experiments suggested that K cyclin expression could contribute to KSHV-induced cell cycle progression and set the stage for subsequent K cyclin functional studies.

The K cyclin sequence shows around 53% sequence similarity with cyclin D2, with the highest level of homology around the cyclin box (Li et al., 1997b) (Fig. 1.10). This, together with the ability of K cyclin to bind and activate Cdk4 and Cdk6 and direct their kinase activity towards Rb (Chang et al., 1996; Godden-Kent et al., 1997; Li et al., 1997b), shows that K cyclin resembles D-type cyclins both at the structural and functional level. Binding and kinase assays with extracts of baculovirus-infected insect cells or Cos cells indicate that K cyclin mainly forms active complexes with Cdk6 (Godden-Kent et al., 1997; Li et al., 1997b). The preference for Cdk6 is remarkable in light of the KSHV host cell repertoire, since lymphoid cells express relatively high levels of Cdk6 and cyclin D/Cdk6 activity predominates in such cells (Meyerson and Harlow, 1994).

K cyclin/Cdk6 complexes show enhanced kinase activity towards its substrates as measured by *in vitro* kinase assays when compared with cyclin D/Cdk complexes (Jeffrey et al., 2000; Li et al., 1997b; Swanton et al., 1999). This could be the result of increased interactions between K cyclin and Cdk, with the more stable active conformation accounting for an increased kinase activity (Jeffrey et al., 2000). Another contributing factor could be a potential increased stability of K cyclin compared with cyclin D. Indeed, K cyclin does not contain residues homologous to the C-terminal cyclin D1 Thr286 residue or cyclin E Thr380 residue that have been implicated in the destruction of these cyclins (Clurman et al., 1996; Diehl et al., 1998; Diehl et al., 1997; Won and Reed, 1996) (see section 1.2.9). However, a direct comparison of the stability of K cyclin compared with cyclin D1 has not yet been performed.

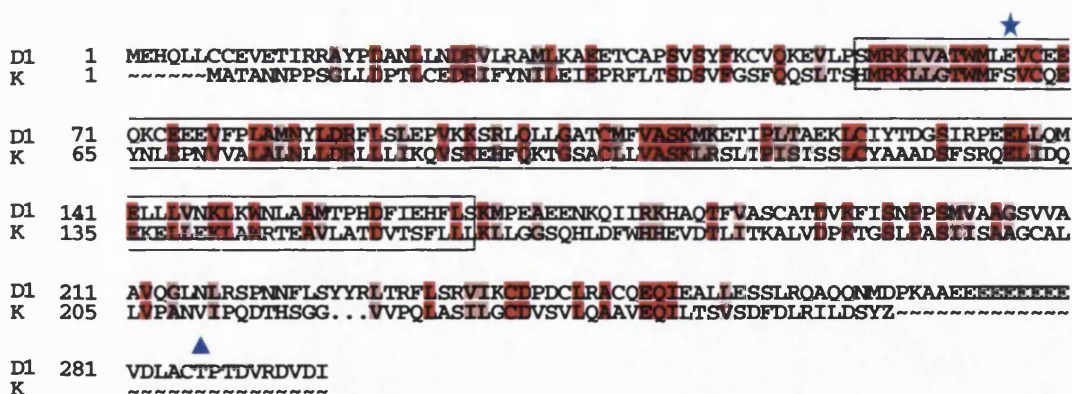


Fig. 1.10 Alignment of the amino acid sequences of cyclin D1 and K cyclin. Residues shaded in red represent identical amino acids; residues highlighted pink indicate similar amino acids. The highest similarity is seen in the cyclin box (boxed region). The blue star marks Glu66 of cyclin D1/Ser60 of K cyclin, a residue localised in the hydrophobic p27-binding groove on the cyclin surface. The blue triangle marks Thr286 of cyclin D1, the phosphorylation of which is thought to trigger the degradation of cyclin D1.

1.7.2 K cyclin/Cdk complexes are not inhibited by CKIs

K cyclin/Cdk6 complexes are refractory to inhibition by both the Cip/Kip and Ink4 families of Cdk inhibitors, as shown by the inability of CKIs to inhibit kinase activity towards Rb in protein extracts from baculovirus-infected Sf9 cells (Swanton et al., 1997; reviewed in Swanton et al., 1999). The resistance to Cip/Kip proteins correlated with the lack of p21/p27 binding to K cyclin/Cdk complexes. This conclusion is supported by the superimposition of the cyclin A/p27 structure over the HVS viral cyclin structure (Schulze-Gahmen et al., 1999). While the p27 binding pocket of cellular cyclins is highly conserved (see section 1.2.6), viral cyclins contain substitutions in electronegative residues that are crucial for p27 binding. Notably, Glu66 of cyclin D (Glu220 of cyclin A), which forms a hydrogen bond and a salt bridge with p27, is substituted for Ser60 in K cyclin (Swanton et al., 1999). This results in the loss of the electronegative character of the p27 binding groove and reduces the number of possible contacts between K cyclin and p27, thereby decreasing the likelihood of p27 binding (Schulze-Gahmen et al., 1999; Swanton et al., 1999).

Insight in the lack of inhibition by Ink4 proteins was provided by the crystallisation of the K cyclin/Cdk6/p18 ternary complex (Jeffrey et al., 2000) (section 1.2.5). The authors showed that only when Cdk6 is CAK phosphorylated, are Cdk6/K cyclin complexes resistant to inhibition by p18. They suggest that CAK phosphorylation of Cdk6 increases the interactions between the N and C lobes of Cdk6. This, together with increased interactions between K cyclin and the Cdk6 C-lobe could stabilise the active arrangement of the catalytic cleft, and prevent its disruption upon Ink binding. In agreement with this, the HVS viral cyclin/Cdk6 crystal structure reveals that the interface of this complex is 20% larger than in the cyclin A/Cdk2 complex. Interestingly, Jeffrey and colleagues did not succeed in creating cyclin D crystals. This might be because of the intrinsic instability of the cyclin D protein, which, if true, would be suggestive of an enhanced stability of K cyclin protein.

1.7.3 K cyclin/Cdk6 complexes phosphorylate Cdk2-type substrates

Another unique feature of K cyclin/Cdk6 complexes is that they phosphorylate an extended array of substrates that are not normally targets of cellular cyclin D/Cdk6. These include the cyclin E/Cdk2 targets p27, histone H1, inhibitor of DNA binding protein 2 (Id-2) and Cdc25A (Ellis et al., 1999; Mann et al., 1999) and the cyclin A/Cdk2 targets of the replication machinery Cdc6 and Orc1 (Laman et al., 2001a) (see below). Thus, the activity of the K cyclin/Cdk6 complex in part mimics the combined activities of G1 and S phase cyclin/Cdk complexes.

What explains the expanded substrate specificity of K cyclin/Cdk6 complexes? The substrate specificity of Cdk2 differs depending on its binding to cyclin E or cyclin A,

implying that the cyclin subunit is likely to contribute (Sarcevic et al., 1997; Zarkowska et al., 1997). Indeed, substrates bind to a conserved binding groove on the cyclin. An additional determinant is the conformation of the catalytic site: phosphorylation of the Cdk2 T loop organises and stabilises the active Cdk2 conformation, thereby contributing to binding and phosphorylation of a peptide substrate (Brown et al., 1999). By inference, the effect of K cyclin binding on the conformation of the Cdk6 catalytic cleft is likely to govern kinase substrate specificity. Further support for this is the finding that non-CAK-phosphorylated Cdk6 in complex with K cyclin targets only one Rb site, while CAK-phosphorylated kinase targets multiple sites (Child and Mann, 2001).

1.7.4 Initiation of DNA replication upon K cyclin expression

The finding that K cyclin/Cdk6 complexes phosphorylate replication proteins that are also targeted by mammalian Cdk2, suggests that they may control replication in a manner analogous to cellular cyclin/Cdks. Little is known about the regulation of DNA replication in human cells, but it is likely to overlap with the proposed mechanism based on studies in mainly yeast and *Xenopus* (Fig. 1.11). At the heart is the tightly regulated assembly of pre-replicative complexes (pre-RCs) at replication origins in a reaction known as “licensing” (reviewed in Bell and Dutta, 2002). Pre-RCs assembly is initiated at the M/G1 transition of the cell cycle and occurs in a stepwise manner. First, a 6-unit protein complex called the origin recognition complex (ORC) binds to origins. This leads to the recruitment of additional DNA replication factors including Cdc6, Cdt1 and a group of six closely related proteins known as the Mcm2-7 complex. After this licensing phase, origins are activated to “fire” as

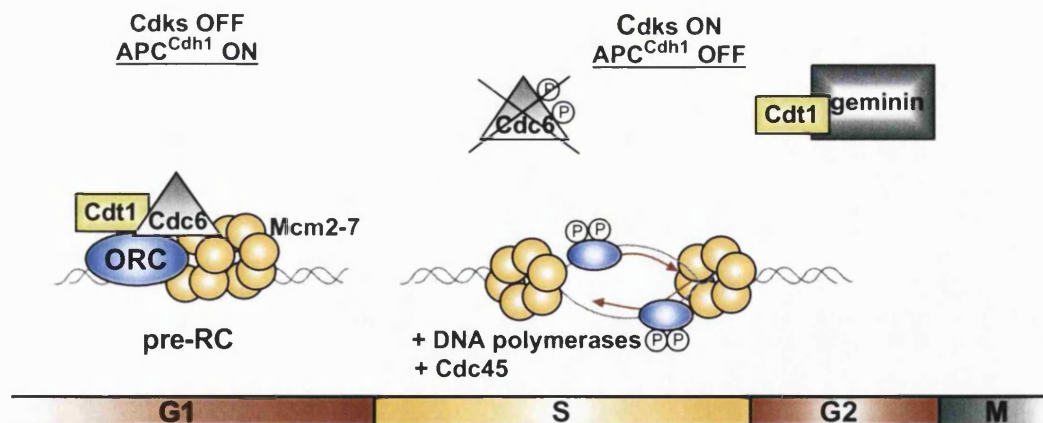


Fig. 1.11 Regulation of DNA replication licensing in human cells. Cells are competent to licence origins and form pre-RCs in G1, when S/G2/M Cdk activity is low. High APC activity in M/early G1 degrades the Cdt1 inhibitor geminin. ORC binding to origins is followed by the recruitment of Cdt1, Cdc6 and Mcm replication proteins. S phase Cdk activity is required to fire origins to initiate replication, although the Cdk substrate(s) involved in this are unknown. Origin firing involves the recruitment of proteins such as Cdc45 and DNA polymerases to the origins. Re-licensing is inhibited in S/G2/M because of the combined actions of S phase and mitotic Cdks (see text for details).

cells pass through the G1 to S phase transition upon recruitment of other replication factors, including Cdk, Cdc45 and DNA polymerases.

Cdk activity is essential both for triggering the initiation of DNA replication from origins that contain pre-RCs, as well as preventing the assembly of new pre-RCs. Cdk activity is thought to prevent pre-RC formation subsequent to origin firing in S phase in multiple redundant ways (Fig. 1.11). First, phosphorylation by cyclin A/Cdk2 complexes in S phase promotes the nuclear export of Cdc6 to the cytoplasm (Jiang et al., 1999; Petersen et al., 1999; Saha et al., 1998), which is followed by APC-mediated degradation of Cdc6 in mitosis (Mendez and Stillman, 2000; Petersen et al., 1999). Second, S phase Cdk phosphorylate the largest ORC subunit, Orc1, thereby initiating its degradation by the SCF^{Skp2} complex (Mendez et al., 2002). Third, G1/S Cdk inhibition of the APC^{Cdh1} complex (see section 1.2.9) allows the accumulation of the Cdt1 inhibitor geminin, thereby inhibiting Cdt1 function (Dhar et al., 2001; Wohlschlegel et al., 2000). A potential fourth mechanism could be the phosphorylation and inhibition of Mcm proteins by cyclin A/Cdk2 or cyclin B/Cdk1 complexes, although no evidence for this has yet been shown in human cells (Bell and Dutta, 2002). In addition to G1/S, also mitotic kinase activity is thought to inhibit pre-RC formation, since Mcm chromatin association occurs as cyclin B is degraded and before the induction of cyclin E (Mendez and Stillman, 2000; Mendez et al., 2002) Thus, because Cdk activity remains high from the onset of S phase until the end of the following mitosis, re-licensing of origins cannot occur until the beginning of the next cell cycle. Surprisingly, although there is an absolute requirement for G1/S phase Cdk activity with regards to origin firing, the proteins that must be phosphorylated to promote replication in humans as well as in yeast systems remain unknown (Bell and Dutta, 2002).

Addition of K cyclin, but not cyclin D, to isolated human or mouse G1 nuclei triggered the initiation of replication in a manner analogous to cyclin A addition (Laman et al., 2001a). Whether origin firing depended on K cyclin-mediated phosphorylation of Orc1 or Cdc6 is unknown. In addition, the phosphorylation of Orc1 by K cyclin/Cdk resulted in the nuclear exclusion of Orc1 protein (Laman et al., 2001b), in agreement with its cytosolic ubiquitination and degradation at the S/G2 transition. The elucidation of mechanisms regulating DNA replication in mammalian cells will also clarify how K cyclin expression mediates origin firing and/or licensing inhibition.

1.7.5 K cyclin expression deregulates G0/G1/S control

How could K cyclin expression contribute to productive KSHV propagation? Viruses are likely to infect quiescent cells that contain high levels of Cdk inhibitors. In such an environment, K cyclin could still form active complexes with endogenous Cdk and stimulate

the exit from G0. In addition, its broadened substrate specificity to include G1 and S phase targets would allow progression through G1 and initiation of S phase. Functional studies support this hypothesis, as K cyclin expression induces S phase entry in serum starved (quiescent) NIH 3T3 cells and in p16 or p27 overexpressing, G1-arrested U2OS cells (Swanton et al., 1997). Furthermore, the evasion of Cdk inhibition by K cyclin/Cdk complexes would be beneficial in case of the activation of anti-viral tumour suppressor pathways by the host cell.

Importantly, cell cycle progression upon K cyclin expression still relies on the host cell cycle machinery. Cells expressing a p27 T187A mutant that cannot be phosphorylated and ubiquitinated cannot complete S phase even when K cyclin is expressed (Ellis et al., 1999; Mann et al., 1999). This implies that the activation of endogenous Cdks upon p27 degradation is required for full S phase progression. Complementing these data, K cyclin expression is unable to bypass a block on endogenous Cdk2 imposed by the chemical Cdk inhibitor roscovitine or by a dominant negative Cdk2 mutant (Ellis et al., 1999). Another factor is the dependence of K cyclin/Cdk activity on CAK phosphorylation by endogenous enzyme. Without CAK phosphorylation K cyclin/Cdk complexes can be inhibited by Ink proteins (Jeffrey et al., 2000) and have a more restricted substrate specificity (Child and Mann, 2001). In conclusion, K cyclin expression in KSHV-infected cell is likely to create an environment that is more conducive to (viral) DNA replication, but requires the cooperation of endogenous cyclin/Cdk activity for full cell cycle progression.

1.8 Oncogene-induced sensitisation to apoptosis

The correct regulation of cell cycle progression is essential for tumour cell proliferation. However, oncogenes characteristically drive proliferation of cells outside of their normal spatial and temporal environment. Perhaps not surprisingly therefore, such deregulation is not beneficial by default and oncogene expression trigger the activation of suppressor pathways that induce apoptosis and/or cell cycle arrest, thus maintaining cellular “fail-safe” mechanisms against uncontrolled cell proliferation (Evan and Littlewood, 1998). Apoptosis, defined as a programmed form of cell death that is accompanied by cellular shrinkage (Kerr et al., 1972), constitutes discrete molecular pathways that can converge to cooperatively induce cell death. During recent years some of the apoptotic pathways activated by oncogenes have been identified, in which the p53 tumour suppressor is a key player.

1.8.1 The p53 tumour suppressor protein

The p53 gene was first identified in 1979 as a protein that interacted with the DNA tumour virus SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). Although initially thought to be an oncogene, experiments 10 years later demonstrated that constitutive overexpression of p53 abrogated oncogene-induced transformation and inhibited the growth of tumour cells by triggering cell death (Baker et al., 1990; Diller et al., 1990; Eliyahu et al., 1989; Finlay et al., 1989), identifying p53 as the first tumour-suppressor gene. The importance of p53 in tumorigenesis is emphasised by the fact that the *p53* gene is mutated in approximately 50% of human cancers (Hollstein et al., 1999), and the p53 pathway is affected in many other cases.

p53 is best characterised as a transcriptional activator. The p53 protein binds as a tetramer to p53 response elements via its sequence-specific DNA-binding core domain (Pavletich et al., 1993). The majority of tumour-associated p53 mutations occur in this central region, and either alter the structural integrity of this domain or the amino acids that directly contact the DNA, thereby disrupting the ability of p53 to bind DNA (Cho et al., 1994; Kern et al., 1991). Tetramerisation is mediated by a C-terminal oligomerisation domain. This domain elicits the dominant negative effect of mutant p53 protein, as oligomerisation of tumour-derived mutants with wt p53 inhibits transcriptional activation by the wt protein (Kern et al., 1992). The actual transcriptional activation by p53 is regulated by an N-terminal transactivation domain that binds transcriptional coactivators such as CPB or PCAF (Gu et al., 1997) (Fig. 1.12).

The p53 protein is expressed at low levels in normally proliferating cells. Its levels are controlled mainly through the action of the Mdm2 ubiquitin ligase, which binds the transactivation domain of p53 and targets p53 for degradation by the proteasome (Haupt et al., 1997; Kubbutat et al., 1997). Since Mdm2 is a p53 transcriptional target, p53 signals its own destruction (reviewed in Kubbutat and Vousden, 1998)– a paradigm common to most cell cycle proteins. The importance of this feedback loop in tissue homeostasis is illustrated by the observation that *Mdm2*^{-/-} mice are only viable in a *p53*^{-/-} background (Jones et al., 1995; Montes de Oca Luna et al., 1995), suggesting that over-activation of p53 is deleterious. In addition, p53 deficiency rescues embryonic lethality in mice lacking the Mdm2 family member Mdmx, suggesting that also regulation of p53 function by Mdmx is crucial. However, Mdmx does not appear to exert its function by degrading p53 (Jackson and Berberich, 2000; Stad et al., 2001), but rather by inhibiting p53-mediated transactivation of target genes (Shvarts et al., 1996). Indeed, the fact that both *Mdm2*^{-/-} and *Mdmx*^{-/-} mice are embryonic lethal suggests that their role in controlling p53 function is non-redundant.

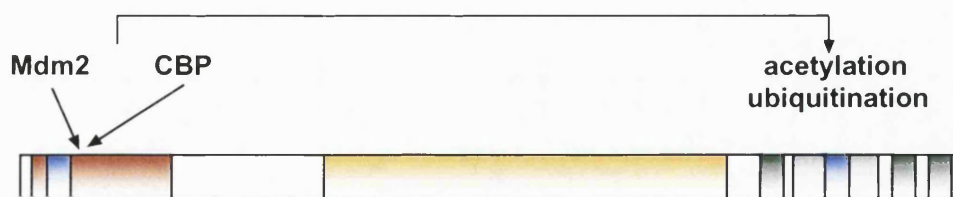


Fig. 1.12 Schematic structure of the main p53 domains. The N-terminal transactivation domain is shown in red, the central DNA-binding domain in yellow and the C-terminal oligomerisation domain in gray. Nuclear export signals are shown in blue and nuclear import signals in green. Interaction of proteins such as Mdm2 or CBP with the p53 N-terminus can lead to modification such as acetylation (by CBP) or ubiquitination (by Mdm2) of the p53 C-terminus. Adapted from (Vousden et al., 2002).

The activation of p53 is controlled by cellular stress pathways, such as genotoxic damage or oncogene expression. These signal the modulation of p53 function by regulating its stability, localisation and/or activation (reviewed in Vousden, 2002). The phosphorylation of amino terminal p53 residues can decrease its affinity for Mdm2 resulting in reduced p53 degradation. Various kinases have been implicated in the phosphorylation of selective N-terminal p53 residues (reviewed in Stewart and Pietenpol, 2001). The best-studied examples are the phosphorylation of Ser15 and Ser20 upon DNA damage. These sites are targeted by, respectively, ATM and ATM-activated Chk2 kinase upon ionising radiation, or ATR and ATR-activated Chk1 kinase upon UV irradiation (reviewed in Abraham, 2001). Importantly, stabilisation and activation of p53 in response to DNA damage requires the activity of Chk1 or Chk2 kinases (Chehab et al., 2000; Hirao et al., 2000; Shieh et al., 2000). N-terminal phosphorylation of p53 has also been suggested to promote p53 nuclear retention. In this model Mdm2-mediated ubiquitination of the p53 C-terminus unmasks the NES in this region, a process that is inhibited upon N-terminal phosphorylation of p53. Furthermore, N-terminal phosphorylation may also mask the NES that resides in the p53 N-terminus (Vousden, 2002), although this has not been fully tested at present.

In addition to stabilisation and correct localisation, the activation of p53 also relies on the modification of C-terminal residues. In particular, acetylation of lysine residues by N-terminally bound CBP or p/CAF can enhance the binding of p53 to the DNA (Gu and Roeder, 1997; Liu et al., 1999; Sakaguchi et al., 1998). This may happen secondary to the phosphorylation of N-terminal residues, as Ser15 and Ser33 phosphorylation has been shown to recruit CPB and p/CAF to the p53 N-terminus (Dumaz and Meek, 1999; Lambert et al., 1998). Acetylation is thought to disrupt the inhibitory folding of the C-terminal domain back onto the DNA binding domain. However, it has also been suggested that chromatin acetylation, but not p53 acetylation, is required for p53 transcriptional activation (Espinosa and Emerson, 2001), and the role of p53 acetylation therefore remains controversial.

Interestingly, cyclin dependent kinases have also been implicated in the modification of p53. Phosphorylation of C-terminal Ser315 by Cdk1 and Cdk2 may enhance p53 DNA binding in a sequence-specific manner (Price et al., 1995; Wang and Prives, 1995). In support of a physiological role for Cdk-mediated Ser315 modification, the Cdc14 phosphatase, which often dephosphorylates Cdk target proteins, was recently shown to interact with p53 and dephosphorylate this site (Li et al., 2000). However, an unphosphorylatable p53 Ser315→Ala mutant was equally effective at activating transcription as wt p53 in a transient transfection assay (Wang and Prives, 1995). The *in vivo* significance of Ser315 phosphorylation may therefore involve other cellular functions.

1.8.2 The ARF/p53 pathway

A different way in which p53 can be stabilised is upon inhibition of Mdm2 activity by the alternative reading-frame protein of the *INK4a/ARF* locus, human p14^{ARF} or mouse p19^{ARF} (ARF). The ARF protein is of particular interest because its expression is induced by oncogenes like E2F1, activated Ras, c-Myc or E1A (Bates et al., 1998; de Stanchina et al., 1998; Palmero et al., 1998; Zindy et al., 1998), or more general, by “hyperproliferative signals”.

The *ARF* gene was first identified by the detection of an alternative transcript transcribed from the *INK4a* locus (Mao et al., 1995; Stone et al., 1995), and was later appreciated to encode a functional protein (Quelle et al., 1995). The two transcripts (α or β) have different first exons (1 α or 1 β for *INK4* and *ARF*, respectively), but share their last two exons (Fig. 1.13). Because these exons are translated using different open reading frames, there is no amino acid identity between the p16 and ARF proteins. The *INK4A/ARF* locus is commonly altered in human cancer, with loss of both tumour suppressor genes by LOH or homozygous deletion occurring most frequently. However, specific methylation of the *p16* promoter is common in a wide panel of tumours such as myelomas, bladder tumours and colon cancer (Esteller et al., 2001; Ruas and Peters, 1998), while methylation of the *p14^{ARF}*

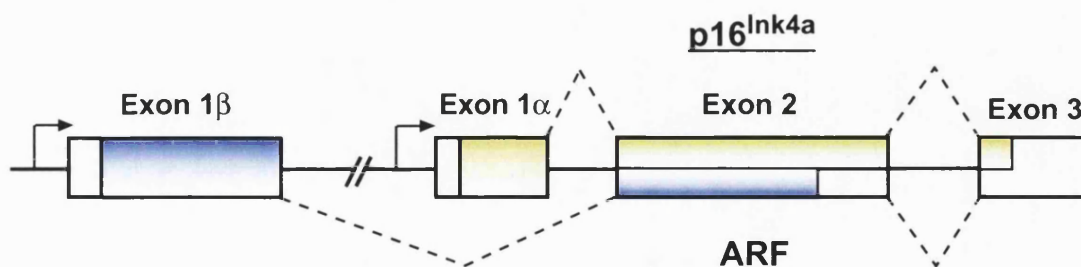


Fig. 1.13 Architecture of the *INK4a/ARF* locus. Transcription of the α and β mRNAs encoding p16^{Ink4a} and ARF, respectively, is initiated at different promoters. Exon 1 α and 1 β are spliced to the same exon 2 acceptor, but use alternative reading frames for translation. The p16 coding sequence is shown in green and the ARF coding sequence in blue. The locus is not drawn to scale. Adapted from (Quelle et al., 1995).

promoter only occurs in few malignancies including colon carcinomas (Esteller et al., 2001). One can only speculate on why one locus would encode two different tumour suppressors.

The expression of the ARF-encoding transcript is negatively regulated both by p53, through an as yet unknown mechanism, and by Rb, through its ability to repress E2F-dependent transcription (Sharpless and DePinho, 1999). This is illustrated by the observation that ectopic introduction of E2F1 induces the expression of ARF (Bates et al., 1998). ARF expression can interfere with all the known functions of Mdm2, including its ability to block p53 transcription (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998), ubiquitinate p53 (Honda and Yasuda, 1999) and enforce p53 nuclear export (Weber et al., 1999; Zhang and Xiong, 1999). Thus, ARF neutralises the ability of Mdm2 to promote the degradation of p53. Since ARF localisation is predominantly nucleolar, it was initially thought that ARF sequesters Mdm2 in the nucleolus and therefore away from p53 (Weber et al., 1999). However, ARF has also been shown to stabilise p53 without relocalising Mdm2 to the nucleolus (Korgaonkar et al., 2002; Llanos et al., 2001). In addition, ARF-induced p53 activation does not always correlate with p53 stabilisation (Korgaonkar et al., 2002).

Experiments performed with primary mouse embryo fibroblasts (MEFs) confirm that at least some of the ARF and p53 functions overlap. Introduction of *ARF* into wt MEFs induces a cell cycle arrest, while *p53*^{-/-} MEFs are refractory to such an arrest (Kamijo et al., 1997). Moreover, spontaneously immortalised cells contain p53 mutations or bi-allelic ARF loss in a mutually exclusive manner (Kamijo et al., 1997).

Several lines of evidence show that oncogenes induce ARF expression *in vivo* and that the *ARF* gene functions as a tumour suppressor. For instance, E2F1-dependent apoptosis in thymocytes correlates with an increase in ARF mRNA levels (Zhu et al., 1999). In addition, *ARF*^{-/-} mice are highly tumour prone and die of cancers within 15 months of age, and *ARF*^{+/-} mice develop tumours upon loss of the second allele (Kamijo et al., 1999). Moreover, loss of ARF expression cooperates with c-Myc or activated Ras expression in the development of tumours (Eischen et al., 1999; Lin and Lowe, 2001; Schmitt et al., 1999). More recent reports however revealed that oncogene-induced apoptosis can also occur in an ARF-independent manner. In particular, apoptosis in the lens or central nervous system of developing *Rb*^{-/-} mice (Tsai et al., 2002) or apoptosis upon SV40 Large T expression in choroid plexus epithelial cells (Tolbert et al., 2002) cannot be suppressed by ARF loss, although in both cases E2F1 and p53 are required. The latter results therefore highlight that oncogene-induced p53 activation does not exclusively involve ARF.

Conversely, it is also becoming clear that ARF has roles independent of p53. The best example of this is the finding that mouse B lymphocytes lacking both ARF and p53 proliferate at a faster rate than cells that lack either one of these genes (Eischen et al., 1999).

Furthermore, c-Myc transgenic mice that lack the pro-apoptotic Bax protein (see below) do not exhibit p53 mutations, but still contain ARF deletions. This implies that ARF loss has different consequences for tumourigenesis than lack of p53-induced apoptosis (Eischen et al., 2001). Importantly, triple knock-out mice lacking expression of Mdm2, ARF and p53 develop tumours at a frequency greater than those observed in animals lacking both p53 and Mdm2 or p53 alone (Weber et al., 2000). This shows that in the absence of Mdm2, ARF may interact with other proteins than p53 to inhibit cell proliferation. An unexpected but intriguing candidate may be the binding of ARF to E2Fs, which targets E2F for degradation and thereby blocks proliferation (Martelli et al., 2001; Mason et al., 2002).

1.8.3 Responses to p53 activation

The outcome of p53 activation, whether it is growth arrest, apoptosis, DNA repair or even cellular senescence depends on the cellular context. Furthermore, the actual way in which p53 is activated defines which target genes are regulated (Vousden and Lu, 2002). In addition to target gene regulation, either via transcriptional activation or repression, but transcriptionally-independent functions of p53 have also been described (Vousden, 2000). These attributes complicate predictions with regard to the outcome of p53 activation.

Increasing evidence supports the idea that p53 controls its cellular response by activation of either growth arrest or apoptosis genes (Vousden and Lu, 2002). Since high or low p53 expression levels have been described to preferentially trigger apoptosis or growth arrest, respectively (Chen et al., 1996b), one explanation could be that the promoters of apoptotic genes contain low-affinity p53 binding sites. However, although high- and low-affinity p53 binding sites have been reported (Szak et al., 2001), not all apoptotic genes contain low-affinity binding sites (Kaeser and Iggo, 2002). Another factor that determines promoter selectivity appears to be post-translational modification of p53, which has been suggested for Ser315 phosphorylation by Cdks (Wang and Prives, 1995) and for Ser46 phosphorylation (Oda et al., 2000b). A third selectivity determinant is the binding of adaptor proteins to p53. Examples are the binding of the transcriptional co-activators JMY or ASPP, which target p53 to induce apoptotic genes such as *Bax* (Samuels-Lev et al., 2001; Shikama et al., 1999). An intriguing recent finding is that the p53 family members p63 and p73 are important players in recruiting p53 to promoters of apoptotic genes. Consistent with this, p53-induced apoptosis required at least one of the other family members (Flores et al., 2002).

Although the human genome contains more than 4000 putative p53 binding sites (Wang et al., 2001), only a small number of genes have thus far been established as critical p53 targets. These include the growth arrest genes p21 (el-Deiry et al., 1993; Harper et al., 1993), Gadd45 (Kastan et al., 1992) and 14-3-3 σ (Hermeking et al., 1997). Interestingly, a

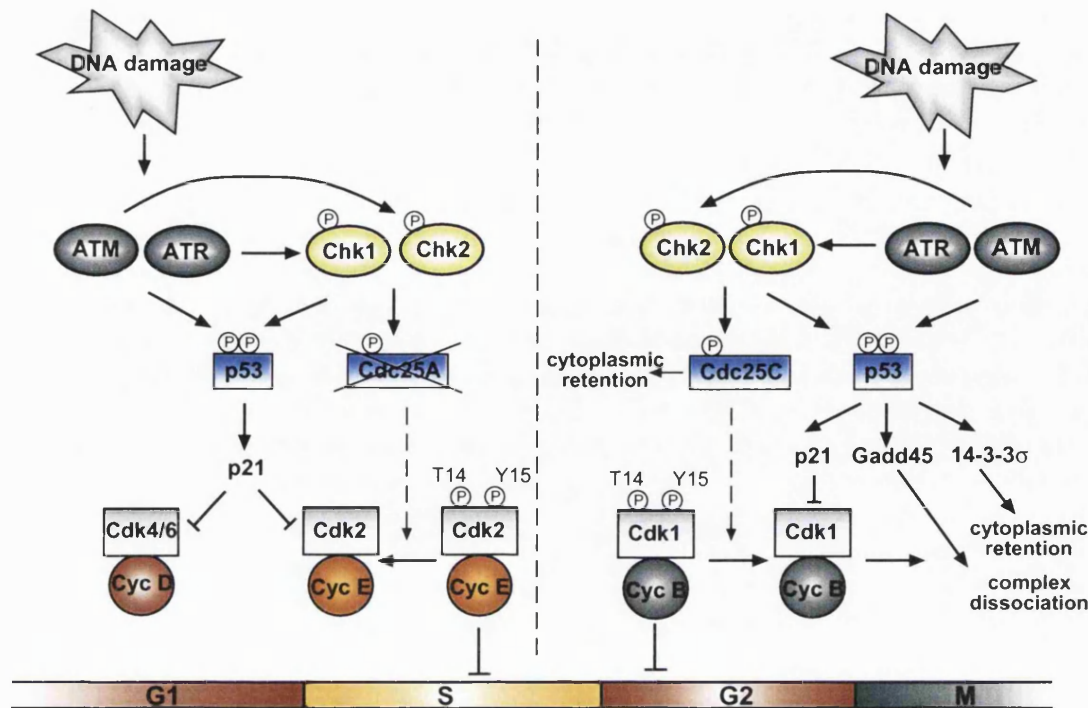


Fig. 1.14 Participation of p53 transcriptional target in G1/S and G2/M arrest in response to DNA damage. DNA damage activates the ATM and ATR kinases, which phosphorylate p53 and the Chk kinases. Chks phosphorylate and inactivate Cdc25 phosphatases, initiating the ubiquitination and degradation of Cdc25A or cytoplasmic retention of Cdc25C (via binding to 14-3-3 protein). This prevents the activation of cyclin E/Cdk2 and cyclin B/Cdk1 complexes. The p53 targets p21, 14-3-3 σ and Gadd45 maintain the cell cycle arrest via inhibition of cyclin/Cdk activity, cytoplasmic retention of cyclin B/Cdk1 complexes and possibly cyclin B/Cdk1 complex dissociation, respectively.

model for p53-induced G1 or G2 arrest, mainly based on studies of the p53-dependent DNA damage response, can be composed solely using these three p53 targets (Fig. 1.14). While p21 inhibits the activity of cyclin/Cdk complexes, 14-3-3 σ sequesters cyclin B/Cdk1 complexes in the cytoplasm preventing it from targeting critical mitotic substrates (Chan et al., 1999). Gadd45 has been suggested to inhibit cyclin B/Cdk1 complex formation, thereby further inhibiting mitotic cyclin activity (Jin et al., 2000; Zhan et al., 1999). Additional p53-independent, and therefore transcription-independent, pathways contribute to the cell cycle arrest. These pathways operate by inhibiting Cdc25 phosphatases, thereby directly blocking cyclin/Cdk activity (Fig. 1.14). It has been suggested that the latter response triggers an initial rapid response to DNA damage, which is followed by the p53-dependent maintenance of the cell cycle arrest (Bartek and Lukas, 2001).

Established apoptotic p53 target proteins include the death receptor Fas (Owen-Schaub et al., 1995) (see below) and various mitochondrial apoptotic pathway proteins such as Apaf-1 (Moroni et al., 2001), Bax (Miyashita and Reed, 1995), Noxa (Oda et al., 2000a) and PUMA (Nakano and Vousden, 2001; Yu et al., 2001) (see below). Indeed, the p53-

induced release of pro-apoptotic proteins from the mitochondria is a principal route towards apoptosis (reviewed in Schuler and Green, 2001).

1.8.4 The mitochondrial apoptotic pathway

The activation of p53 by oncogenes can trigger apoptosis via the upregulation of p53 target genes that are part of the mitochondrial apoptotic pathway. In addition, oncogenes may also initiate the mitochondrial apoptotic pathway independently of p53 (Evan and Vousden, 2001), an example of which is c-Myc (Juin et al., 1999). Mitochondria contribute to apoptosis largely through their ability to release pro-apoptotic proteins such as holo-cytochrome c (cyt c) and Smac/DIABLO from their intermembrane space into the cytosol (reviewed in Ravagnan et al., 2002). Once in the cytosol, Smac/Diablo promotes apoptosis by binding to and antagonising inhibitor of apoptosis (IAP) proteins, which constitute a conserved family of caspase inhibitors (Shi, 2002; Verhagen et al., 2000). Cytosolic cytochrome c forms a complex with Apaf-1 and the serine proteinase pro-caspase-9, called the apoptosome (Jiang and Wang, 2000). This results in the autocatalytic processing and activation of caspase 9, followed by the initiation a cascade of caspases cleaving and activating other family members. Active effector caspases such as caspase 3 and 7 ultimately cleave substrates that are critical for the execution of apoptosis, like the caspase-activated DNase (CAD) (Shi, 2002).

Important regulators of cytochrome c and Smac/Diablo release by the mitochondria are the Bcl-2 family of proteins (reviewed in Gross et al., 1999). This family consists of pro-apoptotic members, such as the p53 target Bax, and anti-apoptotic members such as Bcl-xl and Bcl-2. Bcl-2 family proteins contain up to four conserved Bcl-2 homology (BH) α -helical domains, designated BH1-4. Deletion and mutagenesis studies argue that the BH3 domain serves as the critical death domain. This is consistent with the existence of many BH3-only pro-apoptotic proteins, such as Bim. In addition, caspase cleavage of the amino terminus of Bcl-xl or Bcl-2, predicted to expose their BH3 domain, converts them into pro-apoptotic molecules (Cheng et al., 1997a). However, the mechanism by which the BH3 domain or Bcl-2 families in general mediate mitochondrial protein release is unclear. Bcl-2 family members can heterodimerise with each other and thereupon form pores in artificial membranes (Antonsson et al., 1997; Minn et al., 1997). One possibility therefore is that pro-apoptotic Bcl-2 proteins form selective channels in the mitochondrial membrane. An alternative model is that these proteins regulate the activity of pre-existing channels like the permeability transition pore (PTP) (Ravagnan et al., 2002). Disruption of the equilibrium between anti- and pro-apoptotic family members upon increased Bax expression by p53 or oncogene-induced signals could open this “mitochondrial gate”.

1.8.5 Death receptor-induced apoptosis

Apoptotic pathways activated by oncoproteins such as c-Myc or E2F1 can synergise with other apoptotic pathways. A prime example is the oncogene-induced sensitisation to apoptosis triggered upon ligation of TNF family death receptors CD95/Fas, TNF-R1 or TRAIL (Hueber et al., 1997; Klefstrom et al., 1997; Phillips et al., 1999). Cross-talk apparently does not require the upregulation of death receptors (Hueber et al., 1997), and these pathways rather seem to intersect downstream of the death receptor (Phillips et al., 1999).

Following engagement of death receptors with their ligand, a multiprotein complex of proteins is triggered, called the death-inducing signalling complex (DISC) (Medema et al., 1997) (Fig. 1.15). The CD95 DISC has been characterised most extensively. Trimerisation of CD95 allows the homotypic interaction of death domains (DDs) in the cytoplasmic tails of receptors with a DD domain in the adaptor protein FADD. FADD then recruits the initiator caspase 8 via their N-terminal death effector domain (DED). A high local concentration of

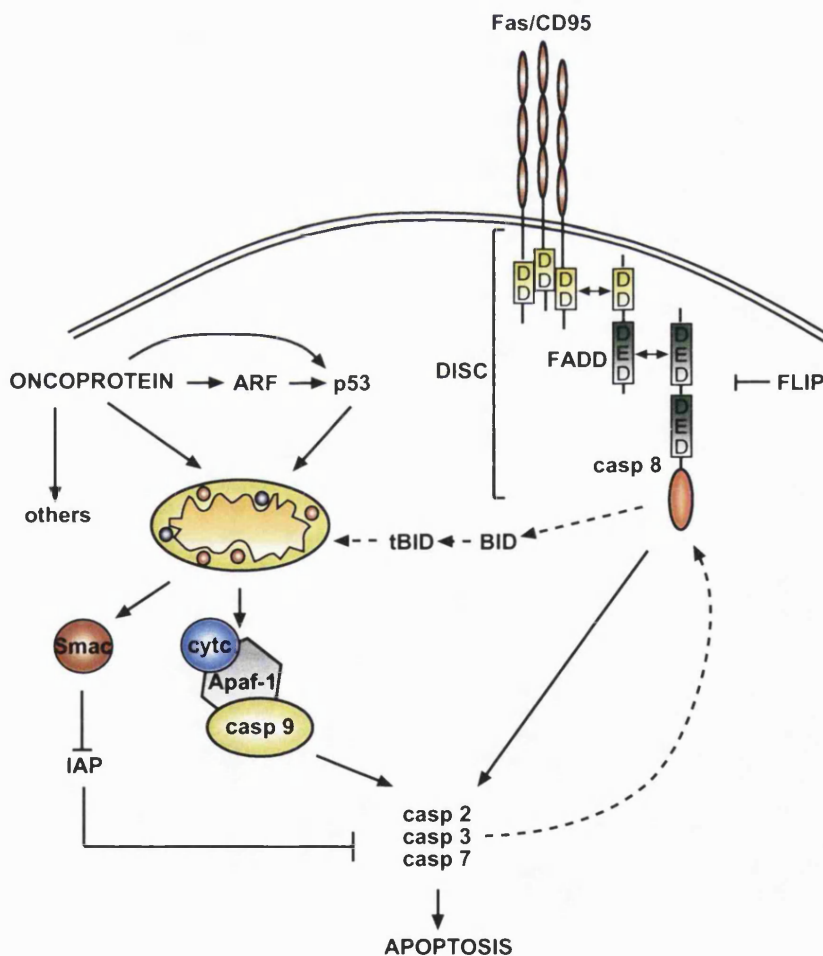


Fig. 1.15 Mitochondrial and death receptor signalling pathways cooperate to induce apoptosis. See text for details. Positive feedback loops are indicated with dotted lines. FLIPs contain two DED domains that bind to the FADD and inhibit the recruitment of caspase 8 to the DISC.

pro-caspase 8 is thought to facilitate self-processing and cleavage to the active enzyme. Activated caspase 8 then initiates apoptosis by cleavage of the downstream effector caspases 3, 6, 7 (Ashkenazi and Dixit, 1998).

In lymphocytes the death receptor pathway can trigger apoptosis without evoking a mitochondrial apoptotic response (Scaffidi et al., 1998). However, in most cells positive feedback loops between these pathways determine the apoptotic outcome. For example, active caspase 9 activates the executioner caspase 3, which in turn cleaves caspase 8 outside the Fas DISC, thereby linking the mitochondrial to the death receptor pathway (Krammer, 2000). In addition, following TNF or Fas treatment caspase 8 cleaves the BH3-only protein Bid, generating a truncated Bid fragment that is required for cyt c release from the mitochondria (Li et al., 1998; Luo et al., 1998). This links the death receptor pathway to the mitochondrial pathway. Taken together, p53-induced, mitochondrial and death receptor apoptotic pathways, either initiated directly by oncoproteins or by other factors such as death receptor ligands all converge to trigger the demise of the cell (Fig. 1.15).

1.9 Aim of this study

The aim of the experiments presented in this thesis is to identify and characterise factors that may counterbalance or promote oncogenesis by the KSHV-encoded viral cyclin, K cyclin. Specifically, chapters 3, 4 and 5 address whether K cyclin promotes apoptosis and/or growth arrest and the involvement of the E2F1/ARF/p53 pathway, using both established cell lines and primary mouse fibroblasts. Chapter 6 asks whether K cyclin promotes tumourigenesis in a transgenic mouse model that uses the E μ enhancer to drive K cyclin expression. This chapter aims to translate results from the *in vitro* experiments into the *K cyclin* transgenic model.

CHAPTER 2:
MATERIALS AND METHODS

2.1 Reagents

2.1.1. General reagents

All general laboratory reagents were from Sigma (Poole, Dorset UK or St. Louis, MO, USA), BDH (Poole, Dorset, UK), Fisher Scientific (Santa Clara, CA, USA) or Bio-Rad Laboratories (Hercules, CA, USA), unless stated otherwise.

2.1.2. Antibodies

The commercial primary antibodies used in this study are listed in Table 2.1.

Table 2.1. Commercial antibodies used in this study

Antibody	Type	Immunogen	Source
Anti- β -actin AC-15	Mouse ascites fluid	N-terminal peptide	Sigma, Saint Louis, MO, USA
Bcl-2	Mouse monoclonal	Full length human Bcl-2 protein	BD Transduction Laboratories, San Diego, CA, USA
DHA6 anti human Bcl-2	Rabbit polyclonal	Aa 41-54 of human Bcl-2	BCN lab, ICRF, London, UK
Cyclin B1 sc-245	Mouse monoclonal	Full length human cyclin B1	Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA
Mouse Fas (CD95), JO2	Hamster monoclonal	Mouse Fas expressing mouse lymphoma WR19L cells	BD Pharmingen, San Diego, CA, USA
Anti-centrin 20H5	Mouse ascites fluid		Gift from J.L. Salisbury, Rochester, MN, USA
Anti-cytochrome c, 6H2.B4	Mouse monoclonal	Rat cytochrome	BD Pharmingen
Anti-FLAG M2	Mouse monoclonal	FLAG octapeptide (DYKDDDDK)	Sigma
Anti- γ -tubulin	Mouse ascites fluid	Aa 38-53 of γ -tubulin	Sigma
Anti-p19ARF	Rabbit polyclonal	Aa 54-75 of mouse p19ARF	Novus Biologicals, Littleton, CO, USA
Anti-mouse p21	Rabbit polyclonal	Full length mouse p21	BD Pharmingen
Anti-p21	Mouse monoclonal	Aa 154-164 of human p21	BD Pharmingen
p53 Protein (CM5)	Rabbit polyclonal	Full length mouse p53 protein	Novocastra Laboratories Ltd, Newcastle upon Tyne, UK
Anti human Rb, G3-245	Mouse monoclonal	Aa 332-344 of human Rb	BD Pharmingen
Anti-ORF73	Rabbit polyclonal	Three LANA peptides	Gift from A. Grundhoff/D. Ganem, UCSF, USA
Anti-mouse CD45R/B220-PE	Rat monoclonal	AMLV-induced pre-B tumour cells	BD Pharmingen
Anti-mouse TCR β -FITC	Hamster monoclonal	TCR purified from hybridoma	BD Pharmingen
Anti-mouse CD8b.2-FITC	Rat monoclonal	Mouse thymus or spleen	BD Pharmingen
Anti-mouse CD4-PE	Rat monoclonal	Mouse CTL clone A15.1.17	BD Pharmingen
Anti-mouse IgM-FITC	Rat monoclonal	Mouse mu heavy chain	Biosource

2.1.3 Synthetic oligonucleotides

Salt free synthetic oligonucleotides were prepared by the ICRF Central Services (40 nM scale) or were purchased from Operon (50 nM scale, Alameda, CA, USA). The sequences of the oligonucleotide primers used in this study are depicted in Table 2.2.

Table 2.2 List of oligonucleotide primers used in this study

Primer	Usage	Sequence
Kcyc 5'	genotyping	GCG CGG ATC CTT AAT AGC TGT CCA GAA TGC GCA GAT C
Kcyc 3'	genotyping	ACG CCT CGA GAT GGA CTA CAA GGA CGA CGA C
ATF911 5'	genotyping	CTA ACC AAT CCA CTG CCA TGG C
ATF302.4 3'	genotyping	GCC TGA TAA GAG GTA TGG GCT TAG GGT ACG
p53exon6	genotyping	GTG GTG GTA CCT TAT GAG CC
p53exon7	genotyping	ATA GGT CGG CGG TTC AT
p53neo	genotyping	CAT CGC CTT CTA TCG CCT TC
ARF1	genotyping	AGT ACA GCA GCG GGA GCA TGG
ARF2	genotyping	TTG AGG AGG ACC GTG AAG CCG
ARFNEO2	genotyping	ACC ACA CTG CTC GAC ATT GGG
E2F1 5'	genotyping	GGA TAT GAT TCT TGG ACT TCT TGG
E2F1 3'	genotyping	CTA AAT CTG ACC ACC AAA CGC
PGKB	genotyping	CAA GTG CCA GCG GGG CTG CTA AAG
KBcl-2 5'BamFlag	cloning	GCG CGG ATC CAT GGA CTA CAA GGA CGA TGA CGA TAA GAT GGA CGA GGA CGT TTT GCC
KBcl-2 RI3'	cloning	GCG CGA ATT CTT ATC TCC TGC TCA TCG CGA CC
2xFlag-v-FLIP Kpn5'	cloning	CGG GTA CCA TGG ACT ACA AGG ACG ACG ACG ACA AGG ACT ACA AGG ACG ACG ACG ACA AGA ATG GCC ACT TAC GAG GTT CTC
v-FLIP Bam3'	cloning	GCG CGG ATC CCT ATG GTG TAT GGC GAT AGT GT
Flag Xho5'	cloning	ACG CCT CGA GAT GGA CTA CAA GGA CGA CGA C
Kcyc Bam3'	cloning	GCG CGG ATC CTT AAT AGC TGT CCA GAA TGC GCA GAT C
Kcyc 3'AS	cloning	TTA ATA GCT GTC CAG AAT GCG CAG
Kcyc 3'sense	cloning	CTG CGC ATT CTG GAC ACG TAT TAA

2.2 Solutions

2.2.1 Commonly used buffers

Phosphate buffered Saline (PBS)	Made from 10x stock solution (Gibco, Grand Island, N.Y., USA)
Tris buffered saline (TBS)	25 mM Tris-HCl 144 mM NaCl pH 8.1
Tris-EDTA (TE), pH 8.0	10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0

2.2.2 Protein extraction buffers

SDS boiling lysis buffer	250 mM Tris-HCl, pH 6.8 2.5% SDS boiled at 100°C for 5 minutes
Laemmli lysis/sample buffer	50 mM Tris-HCl, pH 6.8 10% glycerol 2% SDS 5% (w/v) 2-mercaptoethanol 0.2% bromophenol blue
RIPA buffer	1% (v/v) Triton X-100 0.5% (w/v) sodium deoxycholate 0.1% (w/v) SDS 50 mM HEPES, pH 7.4 150 mM NaCl 1 mM EDTA 100 mM PMSF 1 mM DTT 0.01 mg/ml Aprotinin 1 μ M E64

Hypotonic lysis buffer

- 10 mM HEPES, pH 7.5
- 10 mM KCL
- 3 mM MgCl₂
- 0.05% NP-40
- 1 mM EDTA, pH 8.0
- 10 mM NaF
- 1 mM DTT
- 1x Complete Protease Inhibitor Cocktail
(Boehringer Mannheim, Mannheim, GER)

NETN buffer

- 20 mM Tris, pH 8.0
- 100 mM NaCl
- 1 mM EDTA
- 0.5% (v/v) NP-40
- 10 μ M DTT

2.2.3 SDS-PAGE and Western blotting

Resolving gel solution

- 40 mM Tris-base, pH 8.8
- 0.1% (w/v) SDS
- 6, 10, 12 or 15% (w/v) acrylamide
- 0.16, 0.27, 0.32 or 0.4% (w/v) bisacrylamide
- 0.1% (w/v) APS
- 0.04% (v/v) TEMED

Stacking gel solution

- 125 mM Tris-HCl, pH 6.8
- 0.1% (w/v) SDS
- 5% (w/v) acrylamide
- 0.14% (w/v) bis-acrylamide
- 0.1% (w/v) APS
- 0.1% (v/v) TEMED

Running buffer

- 20 mM Tris-base
- 190 mM glycine
- 0.1% (w/v) SDS

4x gel sample buffer	8% (w/v) SDS 40% (w/v) glycerol 2% (v/v) 2-mercaptoethanol 250 mM Tris-HCl, pH 6.8 0.8% bromophenol blue
Western transfer buffer	20 mM Tris-base 190 mM glycine 20% (w/v) methanol
TBST washing buffer	1x TBS buffer 0.1% (v/v) Tween 20
Blocking buffer	1x TBST buffer 3% (w/v) BSA
2-Mercaptoethanol stripping buffer	62.5 mM Tris-HCl, pH 6.7 2% (w/v) SDS 0.7% (v/v) 2-mercaptoethanol heated to 65°C
Acid stripping buffer	200 mM glycine, pH 2.5 1% (w/v) SDS heated to 65°C
Ponceau S protein staining solution	0.1% (w/v) Ponceau S 5% (v/v) acetic acid

2.2.4 Molecular biology

Luria-Bertani (LB) broth	1% (w/v) bacto-tryptone 0.5% (w/v) yeast extract 1% (w/v) NaCl
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LB agar	1x LB broth 1.5% agar
Tris-boric acid-EDTA (TBE)	89 mM Tris-base 89 mM boric acid 2 mM EDTA
Tris-acetate-EDTA (TAE)	40 mM Tris-acetate 1 mM EDTA
20x SSC	3 M NaCl 0.3 M Na ₃ citrate
Church buffer	0.18 M NaH ₂ PO ₄ •2H ₂ O 0.35 M Na ₂ HPO ₄ 7% SDS 10 μM EDTA, pH 8.0
Transformation buffer	10 mM PIPES 15 mM CaCl ₂ •2H ₂ O 250 mM KCl
5x DNA gel electrophoresis loading buffer	30% (v/v) glycerol 0.25% (w/v) bromophenol blue
2x Transfection buffer	280 mM NaCl 10 mM KCl 1.5 mM Na ₂ HPO ₄ •2H ₂ O 12 mM dextrose 50 mM HEPES pH 6.95
Tail buffer (DNA extraction)	50 mM Tris, pH 8.0 100 mM EDTA 100 mM NaCl 1% SDS

ACK erythrocyte lysis buffer	155 mM NH ₄ Cl
	10 mM KHCO ₃
	0.1 mM EDTA
	pH 7.8

2.3 Tissue Culture

2.3.1 Tissue culture reagents

All media and tissue culture reagents were obtained as sterile solutions and were provided by the ICRF Central Services, the UCSF cell culture facility or were from Invitrogen (Carlsbad, CA, USA), unless stated otherwise. Media used in this study: Dulbecco's Modified Eagle Medium (DMEM) containing high (4.5 g/L) glucose and RPMI Medium 1640. Media for growth of adherent cells were supplemented with 10% FBS (certified US origin), 2 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin. Lymphocytes were grown in RPMI medium containing 50 µM 2-mercaptoethanol and 10% heat-inactivated FBS. Reconstituted complete media were stored at 4°C for up to 1 month.

Where indicated, transfected or infected cells were selected by virtue of their antibiotic resistance. Puromycin (Sigma) was typically used at 2.5 ng/µl, Hygromycin B (Invitrogen) at 100 µg/ml and Geneticin-418 (Fisher) at 800 µg/ml, unless stated otherwise.

Tissue culture plastics were from Becton Dickinson (Cowley, Oxford, UK), Nunc Life technologies Ltd (Paisley, Scotland, UK), Corning Incorporated (Corning, NY, USA) or from Fisher Scientific (Santa Clara, CA, USA).

2.3.2 Culture of mammalian cell lines

Mammalian tissue culture cell lines were cultured at 37°C in the presence of 7.5% CO₂. Cells were routinely passaged by washing the cells with PBS, trypsinisation in trypsin (0.05% w/v)/Versene (0.04% w/v) during incubation for 2-5 minutes at 37°C, and plating at an appropriate density in tissue culture dishes.

Stocks of the cell lines were cryopreserved in liquid nitrogen. Typically, cells grown until confluence in 15 cm dishes were trypsinised, pelleted and taken up in 3 ml of freeze medium (90% FBS, 10% DMSO). Cryopreservation vials containing 1 ml of cells in freeze medium were frozen slowly by placing them in Nalgene freezing containers at -80°C. The next day the vials were transferred to liquid nitrogen storage tanks. Cells were recovered from liquid nitrogen storage by rapidly thawing the cells in a 37°C water bath and transfer to pre-

warmed growth medium. The cells were pelleted, resuspended in fresh medium and typically plated in 10 cm tissue culture dishes.

The following tissue culture cell lines, obtained from the ICRF tissue culture facility unless stated differently, were used in this study:

Rat-1: rat fibroblasts, derived from Fischer rat embryos. Rat-1 cells do not express p21^{Cip1} because its promoter is methylated (Allan et al., 2000). In addition, no expression of p19^{ARF} has been detected (Lomax and Fried, 2001). Rat-1 cells were split every three days to a 1:5-1:10 dilution. Care was taken to prevent overgrowth to high confluence to prevent transformation of the cells.

293: primary human embryonal kidney cells transformed by sheared adenovirus type 5 DNA. Cells were split every three to four days to a 1:3-1:4 dilution.

293T: derivative of the 293 cell line in which the temperature sensitive gene for SV40 large T antigen was inserted. Cells were split every three to four days to a 1:3-1:4 dilution.

Phoenix: 293T cell-derived cell line that contains the gag-pol and envelope genes and allows for the production of helper free retroviruses that can deliver genes to dividing cells. Viruses containing ecotropic envelope proteins (capable of infecting mouse or rat cells) or amphotropic envelope proteins (capable of infecting human cells) were used, called Phoenix-Eco or Phoenix-Ampho. Original source: G. Nolan's lab, Stanford University, USA. Cells were split every three days to a 1:3-1:4 dilution.

U2OS: primary human osteogenic sarcoma cells. The American Type Culture Collection (ATCC) reports that this cell line is chromosomally highly altered, with chromosome counts in the hypertriploid range, although the karyotype of the ICRF cultures are unknown. U2OS cells express wt p53 and Rb, but do not express p16^{INK4} because its promoter is methylated (Stott et al., 1998). Cells were split every three to four days to a 1:5-1:8 dilution.

Swiss 3T3: mouse fibroblasts, derived from Swiss mouse embryos. These cells are highly sensitive to contact inhibition, which makes them valuable for transformation studies. They express high amounts of CD95 Fas receptor (Hueber et al., 1997). The karyotype is hypertriploid. Swiss 3T3 cells contain wt p53 (for example mentioned in (Mussman et al., 2000)). Cells were split every three to four days to a 1:3-1:5 dilution.

MRC-5: human lung fibroblasts, derived from normal lung fetal tissue. This is a normal diploid human cell line that, according to ATCC, shows 3.6% polyploidy. The estimated population doubling number of the used cells was 40. Cells were split every four days to a 1:3-1:4 dilution.

MRC-5hTERT: MRC-5 cells that were retrovirally infected with hTERT (human telomerase catalytic subunit) -encoding pWZLblast3 virus (gift from Dr. M. McMahon, who derived this vector from pCIhTERT that originated in Dr. R. Weinberg's lab). Infected cells were maintained as a pooled population of immortalised cells (courtesy of Beatrice Griffiths). Cells were split every four days to a 1:3-1:4 dilution.

A20: mouse B cell lymphoma cells, obtained from ATCC. This line is derived from a spontaneous reticulum cell sarcoma found in an old BALB/c mouse. Suspension cultures were maintained at around 2.5×10^5 – 1×10^6 cells per ml and cells were fed every day by adding RPMI (1:2). Cells were split every three days by spinning at 3,000 rpm and re-plating at 2.5×10^5 cells per ml.

2.3.3 Isolation and culture of primary mouse embryo fibroblasts

E2F1^{-/-} mice (strain C57Bl/6 x 129) (Field et al., 1996), *p53*^{-/-} mice (strain C57Bl/6) (Jacks et al., 1994) and *p21*^{-/-} mice (strain C57Bl/6 x 129) (Brugarolas et al., 1995) and matched wild type (wt) controls were obtained from Jackson Laboratories. *p19^{ARF}*^{-/-} mice (strain C57Bl/6 x 129) were a generous gift from Frank McCormick and were originally from C. Sherr (Kamijo et al., 1997). Nullizygous or wild type mice were bred and the females checked each morning for the presence of a vaginal plug to establish the day of conception. Plugged females were separated from the male and sacrificed 12-14 days later. Embryos were removed from the uterus and separated from their yolk sac and placenta. The head and liver were removed from each embryo. The embryos were mechanically sheared in a droplet of PBS using two needles. Single cell suspensions were generated by digestion in 5 ml trypsin while pipetting up and down for 1-2 minutes using a 2 ml pipet. Cells were taken up in growth medium and plated in 10 cm dishes (1 1/2 embryo per dish). MEFs were maintained on a 3T9 protocol, passaging 3×10^6 cells per 10 cm dish every 3 days (Todaro G.J. and Green H, 1963). If required, chromosomal DNA was prepared from embryo heads or MEFs by phenol/chloroform precipitation (see section 2.7.4.), and used to genotype individual embryos. Wt MEFs used in the experiments were typically derived from C57Bl/6 x 129 mice, unless it is stated otherwise.

The genotype of the MEFs used in this study was typically deduced from the genotype of the parents used for breedings. MEF genotypes were confirmed to be as expected. The absence of *p19^{ARF}* and *p53* expression in null MEFs was shown in the immunoblotting experiments (Fig. 4.3 and 4.4). However, immunoblotting of mouse *E2F1* was unsuccessful. The *E2F1*^{-/-} MEF genotype was therefore confirmed by PCR analysis as shown in Fig. 2.1 (see section 2.7.5 for genotyping protocol).

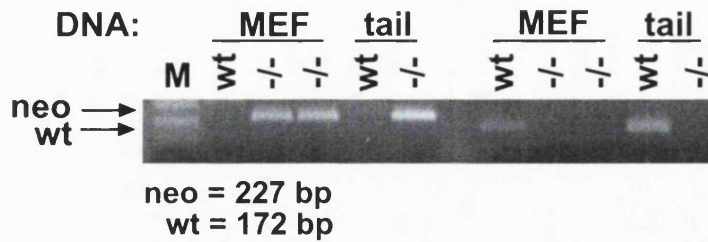


Fig. 2.1 Genotyping of E2F1^{-/-} MEFs. PCR analysis on DNA isolated from wt or E2F1^{-/-} (-/-) tailsnips or MEFs, depicting wildtype (wt) or E2F1^{-/-} (neo) alleles. M= 1kb DNA ladder.

2.3.4 Isolation of the IPTG-inducible K cyclin K18 Rat-1 cell line

IPTG inducible K cyclin expressing Rat-1 cells were prepared using the LacSwitch II inducible mammalian expression system (Stratagene Ltd., Cambridge, UK). This system provides a vector encoding the *Escherichia coli* lactose (lac) repressor (pCMVLacI), which suppresses the promoter activity of the lac operator in the second vector (pOPRSVI). A cell line is prepared that contains both expression vectors in each cell. Upon addition of 5 mM IPTG to the growth medium of these cells, IPTG enters the cells and binds to the LacI repressor resulting in a decreased affinity of the repressor for the operator. This results in the IPTG-inducible expression of the gene of interest.

Rat-1 cells were split at 7.5×10^5 cells per 10 cm dish and co-transfected the next day with 5 μ g of pOPRSVI-K vector and 5 μ g of pCMVLacI vector using CaPO₄ transfection (see section 2.6.1). 24 Hours after transfection the cells were split to 1:20 and plated in medium containing Hygromycin and Geneticin-418. Transfected cells were selected for by growth for 12 days in the presence of antibiotics, with medium changes every three days. A pooled population of cells was shown to express Flag-tagged K cyclin by immunoblotting with anti-Flag antibody. The pooled cells were split 1:30 and grown for one week. Single colonies were trypsinised using cloning rings. In this way, 18 clonal cell lines were derived, which were tested for IPTG-inducible expression of Flag-tagged K cyclin. Two clonal cell lines expressed K cyclin only upon growth in medium containing 5mM IPTG, of which the line with the highest levels of K cyclin expression (K18) was chosen to be used in further experiments. Whenever a new vial was thawed, the cells were tested for inducible K cyclin expression.

2.3.5 Exposure of cells to ionising radiation

Where indicated, cells were exposed to ionising radiation to stimulate apoptotic signaling involving p53. Cells were exposed to varying doses (1-16 Gy) of γ -irradiation using a Cesium irradiator (Mark 1, Model 68 SN.1019, J.L. Shepherd & Associates, Glendale, Ca.), based on an estimated radiation dosage of 2.26 Gy/minute in the center of the turntable.

2.4 Protein Biochemistry

2.4.1 Preparation of total protein extracts

Total protein extracts were prepared from confluent monolayers of cells. Cells were washed twice with PBS. If equal protein loading was not critical, lysates were prepared by direct addition of Laemmli sample buffer (around 500 μ l per 10 cm dish). If protein determination was critical, lysates were prepared using boiling lysis SDS buffer. The dishes were swirled to spread the buffer and DNA and proteins were collected using a cell scraper. Lysates were heated 5 minutes to 100°C, vortexed and DNA was sheared by microtip sonication for 8 seconds at 10-12 watts (RMS, 60 sonic dismembrator). Lysates were centrifuged to pellet debris and cleared lysates were stored at 4°C, for up to one year.

For immunoprecipitation assays, lysates were prepared using ice-cold 1x RIPA buffer, which contained freshly added protease inhibitors. Cells were washed twice with cold PBS and the dishes were placed on ice. RIPA buffer (300 μ l/10 cm dish or 500 μ l/50x10⁶ lymphocytes) was added and cells were lysed during incubation for 5 minutes on ice. Lysates were collected using a cell scraper and cleared by centrifugation. Protein concentrations were measured and lysates used directly for immunoprecipitations.

2.4.2 Preparation of nuclear protein extracts

Lysates were prepared from confluent monolayers of cells. Cells were washed with PBS and harvested by trypsinisation. Cells were washed once more and resuspended at around 1x10⁶ cells/100 μ l of ice-cold hypotonic lysis buffer. Cells were lysed during incubation for 20 minutes on ice and next spun at 3,000 rpm for 5 minutes. The nuclear pellet was washed twice with hypotonic lysis buffer and pelleted by centrifugation at 3,000 rpm for 5 minutes. Nuclei were resuspended in PI staining buffer and analysed by flow cytometry (see section 2.8.5).

2.4.3 Determination of the protein concentration in extracts

Protein concentrations of extracts were assessed using the Bio-Rad detergent compatible (DC) protein assay package (equivalent to Lowry assay). This assay measures the color obtained from the reaction of folin-ciocalteu phenol reagent with the tyrosyl residues of an unknown protein. Briefly, 1 or 2 μ l of protein sample was mixed with 25 μ l of reagent A' (containing sodium hydroxide and copper tartrate/carbonate) and 200 μ l of reagent B (containing folin-ciocalteu). Mixtures were incubated for 15 minutes at RT and the absorbance at 690 nm was measured using a plate-reader (Wallac 1420 multilabel counter, Perkin Elmer Life Sciences, Boston, MA, USA) or using a spectrophotometer (Ultrospec

2000, Amersham Pharmacia Biotech). Protein concentrations were estimated by comparison of the A_{690} values to a standard curve of known concentrations of BSA protein.

2.4.4 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Separation of proteins was achieved by gel electrophoresis according to the SDS-discontinuous system described by Laemmli (Laemmli, 1970). This system is based on a buffering system containing SDS to denature proteins, so that equal charge densities are conferred to all polypeptides and electrophoretic separation is on the basis of molecular weight alone. Gel mixtures were prepared by adding SDS, TEMED (Bio-Rad) and APS (Sigma) and variable amount of acrylamide (30% acrylamide/Bis solution 37.5:1, Bio-Rad) to Tris buffer (see section 2.2.3), according to table 2.3. Two-phase gels consisting of resolving and stacking gels were poured. First, six volumes of resolving gel were poured between two glass plates, which was overlaid with isobutanol and allowed to polymerise. After polymerisation, the top of the gel was rinsed with water. Next, one volume of stacking gel was poured above the resolving gel and allowed to polymerise with a comb in place. The polymerised gel was cast in an electrophoresis apparatus (Bio-Rad) that was filled with running buffer. Samples were prepared by addition of 4x gel sample buffer at a 1:4 final dilution and boiled for 3 minutes at 100°C. Samples were loaded into the wells left by the comb and overlaid with running buffer. Rainbow molecular weight markers (Amersham, Piscataway, NJ, USA) were run alongside samples to allow estimation of protein molecular weight. Gels were typically run at 30V for 1 hour, followed by 1-2 hours at 100 V (minigels) or 200 V for 3-4 hours (large gels).

Table 2.3 Recipes for SDS-PAGE gels (amounts in ml for 1 minigel or 1/3 large gel)

	Resolving gel				Stacking gel
	6%	10%	12%	15%	
H ₂ O	5.3	4	3.3	2.3	1.4
30% (v/v) Acrylamide/0.8% (v/v) bis	2	3.3	4	5	0.33
1.5 M Tris (pH 8.8)	2.5	2.5	2.5	2.5	0.25
10% (v/v) SDS	0.1	0.1	0.1	0.1	0.02
10% (v/v) APS	0.1	0.1	0.1	0.1	0.02
TEMED	0.008	0.004	0.004	0.004	0.002

2.4.5 Western Blotting

2.4.5.1 Protein transfer

Proteins separated by SDS-PAGE were subsequently transferred from the gel matrix onto a hydrophobic PVDF membrane (Immobilon-P, Millipore, Bedford, MA, USA). Membranes were pre-wetted in methanol, washed with Milli-Q water and soaked in transfer

buffer. Hydrated membranes were laid on top of the gel, and next ‘sandwiched’ between filter paper (Whatmann 3MM, Maidstone, England) and hydrated scotch brites in a transfer cassette. This cassette was placed in a Bio-Rad transfer chamber filled with cold transfer buffer. Immobilisation of the negatively charged SDS-coated proteins was achieved by electrophoresis at 4°C, typically at 250 mA for 1 hour. Longer transfer times (up to 2 hours) were applied when proteins larger than 80 kDa were transferred.

2.4.5.2 Ponceau S staining of membranes

Proteins immobilised on the PVDF membrane were visualised by staining with Ponceau S (Sigma) reagent to confirm equal protein loading in each of the lanes. Membranes were laid in Ponceau-S staining solution for around 2-5 minutes, under gentle agitation. Membranes were slightly destained by washing with Milli-Q water and Protein bands were photographed. Membranes were fully destained by washing in washing buffer for 10 minutes, with several changes of the buffer.

2.4.5.3 Immunoblotting

Membranes were blocked by incubation of the membrane in blocking buffer, typically for 1 h at RT. The membranes were next incubated in a solution of blocking buffer containing appropriately diluted primary antibody (usually 1:500-1:3000), under gentle agitation. Incubation times varied from 1 h at RT to 16 h at 4°C, dependent on the abundance of the protein and the affinity of the antibody. Primary antibody solutions containing sodium azide (NaN₃, 0.01% w/v, to prevent bacterial outgrowth) were stored at 4°C and re-used multiple times, if the affinity of the antibody allowed this. After primary antibody binding, membranes were washed for at least 45 minutes with 4 to 5 changes of washing buffer. Membranes were next incubated in blocking buffer containing horseradish peroxidase-linked secondary antibody (usually 1:3000-1:5000, Amersham Pharmacia Biotech) for 30 minutes at RT, under gentle agitation. Membranes were washed as above and bound antibody was visualised by enhanced chemoluminescence (ECL, Amersham Pharmacia Biotech), according to the manufacturer’s instructions. Membranes were exposed to Kodak XAR-5 film (Rochester, New York, USA) in a cassette containing intensifying screens (Amersham), and developed using an automatic film processor (Konica SRX-101A, Diagnostic Imaging Inc, USA).

2.4.5.4 Reprobing of membranes

Probed membranes were stripped from antibodies by incubation for 1 h at 65°C in pre-heated acid or 2-mercaptoethanol stripping buffer under gentle agitation. Membranes

were washed in washing buffer for around 30 minutes and blocked and immunoblotted with another antibody of interest as described in section 2.4.5.3. Membranes were re-probed up to a maximum of three times.

2.4.6 Immunoprecipitation

Immunoprecipitation was used to enrich for Flag-tagged K cyclin protein expressed in primary lymphocytes when determining the expression of the transgene in various founder lines (see section 2.7.2). This method relies on the binding of an antibody to the protein of interest, concomitant with the binding of protein A or G isolated from the cell walls of *S. aureus* and group G *Streptococci*, respectively, to the Fc portion of the antibody. Protein G Sepharose beads (Sigma) were equilibrated with RIPA lysis buffer by mixing them with 1 ml of buffer, centrifugation for 1 minute at 5,000 rpm, followed by another wash step. Beads (20 μ l per sample) were next mixed with equal amounts of RIPA protein lysates (250 μ g per sample, see section 2.4.1) and rotated for 1 h at 4°C to pre-clear the lysate from proteins that bind the beads non-specifically. This was repeated one with fresh beads. Next, lysates were incubated with 8.8 μ g of anti-Flag mouse monoclonal antibody, 20 μ l of fresh protein G Sepharose beads and lysis buffer to reach a final volume of 1 ml. Tubes were rotated overnight at 4°C. Beads were precipitated by centrifugation for 1 minute at 5,000 rpm, the supernatant was removed and beads were washed three times with 500 μ l lysis buffer to remove all unbound protein. Beads were finally boiled with gel sample buffer to dissociate the protein from the beads and proteins were analysed by SDS-PAGE and western blotting using rabbit polyclonal anti-K cyclin antibody.

2.4.7 Bacterial expression and purification of K cyclin protein

An overnight culture of DH5 α bacteria transformed with pGEX-KG plasmid was diluted 1:10 in to fresh LB plus 50 μ g/ml ampicillin and grown for at 37°C while shaking at 150 rpm until the OD₅₉₅ reached 0.6-0.7 (around 2-3 h). The cultures were placed at 25°C while shaking at 150 rpm until the OD₅₉₅ reached 1.3-1.4 (a further 3-4 h). IPTG was added to reach a final concentration of 25 μ M and cultures were induced to express protein during an overnight incubation at 18°C, while shaking at 150 rpm. The bacteria were harvested by centrifugation at 4,000 rpm for 10 minutes at 4°C. All subsequent steps were performed at 4°C, using pre-chilled solutions. The bacterial pellet was resuspended in 1/10th of the original culture volume of NETN lysis buffer. Another 1/10th volume of NETN buffer was added and the bacterial mixture was incubated on ice for 20-30 minutes with occasional mixing. Samples were sonicated four times at 14 microns for 30 seconds each time. Membrane debris was precipitated by centrifugation at 10,000 rpm for 30 minutes at 4°C. The supernatant was

incubated for 2 h with 2 ml glutathione sepharose suspension (1:1 glutathione sepharose 4B (Pharmacia) : NETN) per 40 ml of NETN lysis buffer, while rotating at 4°C. The resin was washed three times with 5 ml of NETN buffer. To the resin, 2 ml of thrombin (1 mM CaCl₂, 50 mM Tris, pH 8) and 5 units of thrombin enzyme were added and the mixture was incubated overnight at 16°C with gentle rotation. The resin was precipitated by centrifugation for 5 minutes at 1,700 rpm at 4°C and the supernatant containing recombinant K cyclin protein was stored with protease inhibitors (1 mM PMSF and 2 µg/ml Aprotinin A) at 4°C. Protein recovery was analysed by SDS-PAGE on a 12% gel, followed by staining of proteins during incubation of the gel for 30 minutes in coomassie staining solution (0.25% (w/v) coomassie brilliant blue R250, 45% (v/v) methanol, 10% (v/v) glacial acetic acid). The gel was destained during overnight incubation in 45% methanol, 10% acetic acid solution and subsequently dried onto whatmann paper. The protein concentration was estimated by comparison to a BSA standard.

2.4.8 Production of anti-K cyclin rabbit polyclonal antibodies

Rabbit polyclonal antibodies directed against Flag-tagged K cyclin were produced by the ICRF biological resources unit. 2 mg of purified K cyclin protein was handed over to their staff and was used in a standard rabbit immunisation protocol. A primary immunogen boost of 200 µg protein in complete Freund's adjuvant was injected subcutaneously, followed by 6 subsequent boosts at two-week intervals of 100 µg protein in incomplete Freund's adjuvant. Two animals were immunised. Final rabbit bleedouts, called EV-3 and EV-4, tested positive for K cyclin protein binding by western blotting analysis when used at a 1:200 dilution in blocking buffer.

2.4.9 In vitro transcription and translation

In vitro translation products were generated using the TNT T7 coupled reticulocyte lysate system (Promega, Madison, WI, USA). Standard reactions used 1 µg of plasmid DNA substrate carrying a T7 polymerase promoter, which was added to 25 µl reaction buffer containing 50% (v/v) rabbit reticulocyte lysate, 40 µM amino acids minus methionine, 40 µCi ³⁵S-Methionine/Cysteine (Promix, Amersham biosciences, Freiburg, GER), 40 units of Rnasin Ribonuclease inhibitor and 40 units of T7 polymerase. The mixture was incubated at 30°C for 1 hour. Translation was confirmed by denaturation of the sample in 1x Laemmli sample buffer and separation by SDS PAGE on 15% gels. The gels were dried using a BIO RAD Model 583 Gel dryer and ³⁵S-labelled proteins were visualised by autoradiography at room temperature using Kodak XAR-5 film.

2.5 Molecular Biology

2.5.1 Preparation of competent bacteria

A single colony of DH5- α bacteria was inoculated in 200 ml of LB medium contained in a 2 litre flask. Flasks were shaken at 150-250 rpm for around 48 h at 18°C until the OD₆₀₀ reached 0.4-0.6. Cells were centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was discarded and around 1/3 volume (70 ml) of ice-cold transformation buffer was added. Bacteria were resuspended by gently rotating the flask while keeping it on ice, and it was taken care that no air-bubbles were formed. Cells were centrifuged again and resuspended in 1/12.5 volume (16 ml) of ice-cold transformation buffer. DMSO was added till final concentration of 7% (1.3 ml) and cells were left on ice for another 10 minutes. Competent bacteria were aliquotted into 200 μ l Nunc freezing vials and snap-frozen in liquid nitrogen. Vials were stored at -80°C.

2.5.2 Transformation of competent bacteria

Ligated or supercoiled DNA was introduced into bacteria by heat shock transformation. Competent bacteria were thawed on ice and 4-5 μ l of a ligation mix or 10 pg of supercoiled plasmid DNA was added to 50 μ l bacteria in 1.5 ml microfuge tubes. The mixture was left on ice for 15-30 minutes. Tubes were placed in a water bath at 42°C for 90 seconds and transferred back on ice for 2 minutes. Bacteria were diluted with 1 ml LB and tubes were left at 37°C for 1 h to allow recovery of the cells. Bacteria were next spread onto LB-agar plates containing 50 μ g/ml of ampicillin. Plates were incubated at 37°C overnight and colonies were picked the next day.

2.5.3 DNA minipreps

DNA Minipreps were performed using the QIAprep spin miniprep kit (QIAGEN Ltd., Valencia, CA, USA). Single antibiotic-resistant bacterial colonies were picked, inoculated in 5 ml of LB medium containing 50 μ g/ml of ampicillin and grown overnight at 37°C with shaking. 1.5 ml of the culture was transferred to a microfuge tube and the cells were pelleted by centrifugation. Cells were lysed by standard alkaline lysis using the buffers provided. Protein in the lysates was precipitated by centrifugation for 10 minutes at 10,000 rpm and cleared lysate was applied to a QIAprep column. The column was centrifuged to load the DNA and, washed with column washbuffer (supplied with kit) and centrifuged twice to remove residual buffer. The DNA was eluted with 50 μ l of elution buffer (supplied with kit). Samples were analysed by restriction enzyme digestion an agarose gel electrophoresis.

2.5.4 DNA maxipreps

DNA maxipreps were performed using the QIAGEN maxiprep kit. 5 ml overnight cultures of antibiotic-resistant bacteria were used to inoculate 250 ml of LB medium containing 50 µg/ml of ampicillin. Cultures were incubated overnight at 37°C with shaking. The bacteria were harvested by centrifugation at 3,000 rpm for 10 minutes and the cells were lysed by standard alkaline lysis using the buffers provided. Lysates were cleared by centrifugation at 4,000 rpm for 30 minutes and any loose precipitate was removed by filtration through four layers of muslin cloth. Plasmid DNA was recovered from the filtrate by addition to a pre-equilibrated QIAGEN-tip 500 column. The column was washed twice with column wash buffer and plasmid DNA was eluted with 15 ml elution buffer (wash and elution buffer supplied with kit). 22.5 ml isopropanol was added to the eluate and the solution was firmly mixed and left on ice for 30 minutes. The DNA was precipitated by centrifugation for at 4,000 rpm for 30 minutes at 4°C and washed with 3 ml of 70% Ethanol. The DNA was dried by exposure to the air for around 20 minutes and finally dissolved in 200 µl of TE or MilliQ water.

2.5.5 Restriction enzyme digestion

All restriction digests were performed in the buffer supplied with each enzyme, or the buffer most compatible with both enzymes when double digests were performed. All enzymes were purchased from New England Biolabs (NEB, Hitchin, Herts, UK), Promega (Southampton, UK) or TaKaRa (Fisher Scientific, Pittsburgh, PA, USA). Digests were typically performed at 37°C for 1 hour using at least 3 units of enzyme per µg of DNA.

2.5.6 Phosphatase treatment of vector DNA

When preparing vector DNA for blunt-end ligation, vector DNA was dephosphorylated following restriction: at least 1 unit of calf intestinal alkaline phosphatase (CIAP, NEB) and 1/9th volume of 10x phosphatase buffer were added to the digest mixture and left for 30 minutes at 37°C, followed by incubation for 30 minutes at 56°C. A second volume of CIAP was then added and the incubations were repeated.

2.5.7 Agarose gel electrophoresis

Samples were prepared for electrophoresis by addition of a 1/4th volume of 5x gel electrophoresis loading buffer. Samples were analysed using horizontal 0.8-1.2% (w/v) agarose gels in 1x TBE buffer containing 0.5 µg/ml ethidium bromide (BioRad). The gel was submerged in 1x TBE buffer in an electrophoresis apparatus, samples were loaded into the wells and a molecular weight 1 kb ladder (Invitrogen) was loaded to provide size markers.

Samples were electrophoresed at 30-100V and DNA was visualised using a UV transilluminator. When required, bands of interest were excised for DNA purification.

2.5.8 Purification of DNA following electrophoresis

DNA was routinely purified using the Jetsorb Gel Extraction kit (Genomed, Witney, Oxon, UK). The agarose slice was incubated with 300 μ l of high salt buffer A1 per 100 mg of agarose and 10 μ l of resin at 50°C, until the agarose had melted. The suspension was vortexed, centrifuged and the resin was washed once with 300 μ l of high salt buffer A1. The resin was washed twice by centrifugation and resuspension in 300 μ l of low salt buffer A2. The resin was next pelleted, dried by exposure to the air and DNA was eluted by addition of 30 μ l of distilled water and incubation for 5 minutes at 50°C. Samples were centrifuged and the supernatant containing the DNA was collected. Samples were analysed by agarose gel electrophoresis and compared with a standard to estimate the DNA concentration for subsequent reactions.

2.5.9 DNA Ligations

Ligations were performed in the buffer supplied with the T4 DNA ligase (NEB). Reaction volumes were usually 10-15 μ l and contained 1 unit of ligase and around 50 ng of DNA. Typically, an insert:vector ratio of 4:1 was used and reactions were incubated at room temperature for 1-3 h or overnight at 16°C.

2.5.10 Polymerase Chain Reaction (PCR)

High fidelity VENT polymerase (NEB) was used to generate fragments for subcloning. 50 μ l reactions were performed in heat-resistant 0.2 ml thin-wall tubes (MJ Research, Waltham, MA, USA) and contained 1x VENT buffer, 10-100 ng of template DNA, 1 μ M of each primer, 250 μ M of each dNTP (Amersham Pharmacia) and 1.5 units of VENT polymerase. A standard program was used for fragments of 1 kb or less, which started with an initial denaturation step at 95°C for 5 minutes, followed by 30 cycles with the following parameters: 95°C for 1 minute, annealing for 1 minute at 50°C and 1 minute extension at 72°C. The extension time was increased for fragments larger than 1 kb and the annealing temperature was increased if the melting temperature of the primers was higher than 55-60°C. A final extension at 72°C for 10 minutes was applied and samples were cooled to 4°C. Reactions were performed in a MJ research PTC-225 thermal cycler. PCR products were analysed by agarose gel electrophoresis and restricted with the appropriate enzymes for further cloning.

2.5.11 DNA sequencing

A reaction mixture containing 500 ng of DNA, 3.2 pmoles of primer was taken up in a final volume of MilliQ water and handed over to the ICRF central services or UCSF Cancer Center genome analysis core. Sequencing was performed using the ABI prism dye terminator cycle sequencing kit using standard procedures and were analysed on an ABI3700 capillary DNA fragment analysis instrument (PE applied biosystems, Warrington, Chesire, UK or Foster city, CA, USA). DNA sequences were viewed using EditView software and cloned DNA fragments were confirmed to contain the expected sequence.

2.5.12 Southern blotting

Tailsnip DNA preps (see section 2.7.4) were restricted with PstI enzyme (100 units of enzyme per sample of around 20-35 µg DNA). Samples were run on large 0.7% 1x TAE gels containing 0.5 µg/ml Ethidium bromide, at 60-80 V for around 4 h in 1x TAE buffer. Gels were depurinated by incubation in 0.25 N HCl for 15 minutes at RT, with gentle shaking. The solution was poured off and gels were subsequently denatured by incubation in 1.5 M NaCl, 0.5 M NaOH for 30 minutes at RT. DNA was transferred to Hybond-N⁺ membrane (Amersham) by capillary blotting. Briefly, in a glass dish filled with denaturing solution a gel tray was placed upside down. The tray was overlaid with a wick made from three sheets of whatman 3 MM filter paper, saturated with denaturation solution. The gel was placed on the wick and air bubbles were removed. A saranwrap collar was put around the gel to prevent both evaporation and short-circuiting of the solution. A sheet of Hybond-N⁺ membrane, followed by 4 whatman 3MM sheets was laid on top of this, followed by stacks of absorbent paper towels (approximately 7 cm high). A glass plate was placed on top of the stack and a 0.75-1 kilogram weight was placed on top. Protein was transferred during overnight incubation. The membrane was removed from the stack and dried on a clean whatman sheet. The DNA was cross-linked to the membrane by baking the membrane in an oven at 80°C for 1 hour. Membrane was floated on 2x SSC buffer and when wet, submerged for 5 minutes before pre-hybridising. Membrane was pre-hybed in Church buffer for at least 30 minutes at 65°C, while rotating the hybe bottle. Denatured probe (see section 2.5.13) was added to the hybridisation solution and was incubated overnight at 65°C. The blot was washed twice for 10 minutes with 2xSSC/0.05% SDS at room temperature, followed by a 10 minute wash with 0.1xSSC/0.1%SDS at 65°C. Moist membrane was wrapped in saran wrap and exposed to Kodak XAR-5 film or a phosphorimager screen.

2.5.13 Random labelling and purification of DNA probes

DNA probes used for Southern blotting were labelled using the multiprime DNA labelling kit (Amersham). Around 100 ng of DNA was taken up in a final volume of 5 μ l MilliQ water. DNA was denatured by incubation for 5 minutes at 95°C. The tube was placed on ice and to the DNA solution 10 μ l labelling mix, 5 μ l of primer, 25 μ l of water, 2 μ l of Klenow enzyme and 3 μ l of dCTP- α^{32} P (30 μ Ci) was added. The mixture was incubated for 1 hour at 37°C, followed by a denaturing step for 3 minutes at 95°C.

The labelled probe was purified using Sephadex G50 NICK columns (Pharmacia). The columns were drained, and washed with 400 μ l of TE. The probe solution (50 μ l) was applied to the column and after it had entered the resin, 400 μ l of TE was added. The flowthrough was discarded and the fraction containing the probe was added to the membrane for hybridisation.

2.5.15 DNA constructs made during this study

A brief description of the cloning steps used to make DNA constructs that were used in this study are outlined below. All constructs were verified by restriction digests and ABI sequencing (see section 2.5.11).

hBcl-2pcDNA3: human Bcl-2 was subcloned from pBabepuro Bcl-2 (Fanidi et al., 1992) by EcoRI digestion and ligated into EcoRI-digested pcDNA3. This plasmid was used to express human Bcl-2 in microinjection and transfection studies.

KSBcl-2pcDNA3: KSBcl-2 DNA was amplified by PCR from a λ phage DNA clone (CBC0.5 vers. 56 SY, a generous gift from F. Neipl) using KBcl-2 5'BamFlag and KBcl-2 RI3', digested with BamHI/EcoRI and ligated into pcDNA3. This plasmid was used to express Flag-tagged KSBcl-2 in microinjection and transfection studies.

v-FLIPpcDNA3: v-FLIP DNA was amplified by PCR from a BAC clone (SY 3-7, a gift from F. Neipl) using 2xFlag-v-FLIP Kpn5' and v-FLIP Bam3' primers, digested with KpnI and BamHI and ligated into pcDNA3. This plasmid was used to express Flag-tagged v-FLIP in transfection studies.

KpHSE3': Flag-tagged K cyclin was amplified by PCR from 2FKpcDNA3 plasmid using Flag Xho5' and K cyc Bam3' primers, digested with XhoI and BamHI and ligated into SalI- and BamHI-restricted pHSE3' vector. This plasmid was linearised by restriction with XhoI and the fragment containing the E μ -K cyclin cassette was used for the generation of transgenic mice.

K/FpHSE3': K cyclin DNA was amplified by PCR from the SY 3-7 BAC clone (see above) using the Flag Xho5' and Kcyc 3'AS primers. Ires-v-FLIP DNA was amplified from

the same BAC clone using the Kcyc 3'sense and v-FLIP Bam3' primers. Products were annealed and extended to obtain full-length Kcyclin-Ires-v-FLIP DNA, which was restricted with XhoI and BamHI and ligated into Sall- and BamHI-restricted pHSE3' vector. This plasmid was linearised with XhoI and the fragment containing the E μ -Kcyclin/v-FLIP cassette was used for the generation of transgenic mice.

K/FpcDNA3: The XhoI- and BamHI-restricted Kcyclin-Ires-v-FLIP fragment (see above) was ligated into pBS-KS shuttle vector. The cassette was released using KpnI and XbaI restriction and ligated into pcDNA3. This plasmid was used for microinjection studies. The ATG startcodon of the v-FLIP gene was mutagenised to delete the Thymidine nucleotide using the QuikChange site-directed mutagenesis kit (Stratagene). This plasmid was called K/FmutpcDNA3.

KpBMN: double Flag-tagged K cyclin was subcloned from KpcDNA3 by restriction with BamHI and EcoRI and ligated into the pBMNIresEGFP retroviral expression vector.

LANApBabepuro: LANA DNA without the His-tag was subcloned from pcDNA/His ORF73 plasmid by restriction with EcoRI and ligation into CIP-treated pBabepuro vector. This plasmid was used for retroviral expression studies.

2.5.16 Donated DNA constructs

The following plasmids were generously provided by other lab members or the following investigators:

Table 2.4 Description of donated plasmids

Plasmid name	Protein	Usage	Source
pcDNA3	-	Expression vector	D. Mann
2FKpcDNA3	2xFlag-K cyclin	Transfection and microinjection assays	D. Mann
2FDpcDNA3	2xFlag-cyclin D1	Transfection and microinjection assays	D. Mann
E2F1pcDNA3	5'HA-tagged E2F1	Transfection assays	H. Laman
hBcl-2pBpuro	human Bcl-2	Subcloning	T. Littlewood
c.9DN pcDNA3	caspase 9 DN	Transfection assays	Y. Lazebnik
pEFLacZ	β -Galactosidase	Marker "blue cell assay"	R. Treisman
YFP	YFP	Microinjection marker for injected cells	R. Pepperkok
Rc/CMVCyclin E	myc-tagged cyclin E	Microinjection assays	J. Pines
PCMX-Cyclin A	human cyclin A	Microinjection assays	N. Perez-Roger
pGEX-KG	GST-tagged K cyclin	Recombinant K cyclin production	C. Swanton
pHSE3'	-	Transgene E μ cassette	R. Zinkernagel
pOPRSVI-K	2xFlag-K cyclin	IPTG-inducible expression, G418 ^r	H. Laman
pCMVLacI	LacI repressor	IPTG-inducible expression, Hygro ^r	H. Laman
pBabepuro	-	Retroviral expression vector	D. Mann

2FkpBpuro	2xFlag-K cyclin	Retroviral expression	D. Mann
CyclinD3pBpuro	2xFlag-cyclin D3	Retroviral expression	D. Mann
pcDNA/His ORF73	His-tagged LANA	Transfection/luciferase assays	P. Moore
pcDNA3/73	LANA	Transfection/luciferase assays	A. Grundhoff
ppGS-ORF73/LANA	LANA	Retroviral expression	J. Friborg
ppGS-Neo-Exp	-	Retroviral expression control	J. Friborg
pBMNIresEGFP	EGFP	Retroviral expression vector	J. Klefstrom
pBpurop53(302-390)	DNp53	Retroviral expression	T. Littlewood
pGDp65	p65	p65 pos. control NFκB luciferase assay	J. Klefstrom
pRL-TK	<i>Renilla</i> Luciferase	<i>Renilla</i> luciferase internal control	J. Klefstrom
pBIIXLuc	Firefly Luciferase	NFκB luciferase reporter	J. Klefstrom
pGL3cyclinGluc	Firefly Luciferase	p53 luciferase reporter	M. Oren
RGCluc	Firefly Luciferase	p53 luciferase reporter	M. Oren
p53luc	Firefly Luciferase	p53 luciferase reporter	S. Ried
BaMdm2luc	Firefly Luciferase	p53 luciferase reporter	S. Ried
p53SN3	human p53	Luciferase assays	B. Hahn

2.6 Transfection and transduction of mammalian cells

2.6.1 Calcium Phosphate (CaPO_4) method for transient transfection

Cells were grown in 10 cm dishes to reach subconfluency at the day of transfection (60-80%). The medium was changed for fresh medium and equilibrated during incubation for 30 minutes at 37°C. 400 µl 2x transfection buffer was placed in clear 5 ml FACS tubes. A DNA mix was prepared by diluting 10 µg of DNA in 360 µl sterile 0.1 M TE, to which 40 µl of CaCl_2 was added. The DNA mix was gradually dropped on the transfection buffer, which was continuously aerated to initiate the formation of a DNA precipitate. This mixture was incubated at RT for 10 minutes, and then dropwise added to the culture medium of the cells in the dish. The cells were incubated for 16 hours. Next, the precipitate was washed off the cells by rinsing the cells twice in serum free medium, and fresh growth medium was added. 24-48 hours later the cells were harvested or split to perform further assays.

2.6.2 Superfect method for transient transfection

Subconfluent monolayers of cells (60-80% confluent) grown in 6-well dishes were transfected using SuperFect reagent (Qiagen Inc., Chatsworth, CA, USA). Briefly, 95 µl of serum free DMEM medium was added to the DNA mixture, to which 10 µl of SuperFect reagent was added. The tubes were inverted three times to mix the contents. After incubation

for 5-10 minutes at RT to allow complex formation, 600 μ l of growth medium containing 10% FBS was added to each sample and transferred onto the cell monolayer, which had been washed once with PBS. After incubation of the cells for three hours, cells were washed with PBS, and 4 ml of fresh growth medium containing 10% FBS was added to each well. Amounts were scaled up 3-fold if cells grown in 10 cm plates were transfected.

2.6.3 Fugene method for transient transfection

Where indicated, subconfluent (60-80%) cell monolayers grown in 6-well dishes were transfected with Fugene 6 reagent (Roche Diagnostics Corporation, Indianapolis, IN, USA), according to the manufacturer's protocol. Briefly, transfection mixes were made by adding 6 μ l of Fugene reagent to 94 μ l of serum free DMEM medium, followed by addition of the DNA mixture. The tubes were tapped to mix the contents and incubated at RT for 15-30 minutes. The mixture was added dropwise to the growth medium (2 ml DMEM + 10% FBS) of the cells. After incubation of the cells for three hours, 2 ml of growth medium was added to each well. Cells were harvested 24-48 h after transfection.

2.6.4 Electroporation of A20 B cells

Cells were spun and resuspended in RPMI medium + 10% FBS (10×10^6 cells/500 μ l medium per sample). A titrated amount of plasmid DNA was added to the cells and the mixture was transferred to a 0.4 mm cuvette. Cells were electroporated at a voltage of 310 V, capacitance of 960 μ F using BioRad Gene PulserTM (time constants between 18.5-22.5). Cells were next transferred to 10 ml of pre-warmed medium and cultured until further analysis.

2.6.5 Lipofectamine Plus transfection of phoenix retroviral producer cells

Phoenix-Eco or Phoenix-Ampho retrovirus producer cells were split at 4×10^6 cells per 10 cm dish. The next day, cells were transfected using Lipofectamine Plus reagent (GibcoBRL). Specifically, 4 μ g of plasmid DNA was taken up in 560 μ l of serum- and antibiotic-free medium, to which 18 μ l of Plus reagent was added. The solution was mixed by flicking the tube several times and incubated for 15 minutes at RT. Next, a pre-mixed solution of 27 μ l Lipofectamine reagent in 560 μ l serum- and antibiotic-free medium was added to the DNA solution. The resulting suspension was mixed by inverting the eppendorf tube 3-4 times and incubated for another 15 minutes at RT. Phoenix cells were washed once with PBS and replenished with 5 ml of serum- and antibiotic-free medium, to which the DNA/lipofectamine solution was carefully added (phoenix cells are loosely adherent cells). Cells were cultured for 2-3 h at 37° and then replenished with fresh medium containing 10% FBS and antibiotics.

2.6.6 Retroviral infection of mammalian cells

Phoenix cell culture supernatants were harvested 40-48 h after transfection and purified over a 0.45 µm syringe filter. Polybrene (Sigma) was added to a final concentration of 10 µg/ml. Passage 2-4 MEFs were plated at 1×10^5 cells per well in a 6-well plate or 1×10^6 cells per 10 cm dish, and were infected the next day with 1 ml/well or 5.5 ml/dish of freshly harvested virus. For growth curve analysis or for Western blot analysis, MEFs cells were infected for 18-24 h and selected by growth for 48 h in medium containing 5 µg/ml puromycin (Sigma). For apoptosis assays, MEFs were infected for 8 h with fresh pBMNIresEGFP virus supernatant, followed by overnight incubation in normal growth medium. Phoenix cells were replenished with fresh medium to produce an additional 24 h viral supernatant, with which a second 8 h infection was performed the next day.

MRC-5 cells were plated at 1×10^6 cells per 10 cm dish and were infected the next day for 9 h with 5.5 ml of freshly harvested virus. Infected cells were trypsinised and seeded at 1:3 dilution in medium containing 5 µg/ml puromycin. The absence of reverse transcriptase (RT) activity in culture supernatants of infected MRC-5 cells was confirmed 72 h after antibiotic selection using the *Quan-T-RT* assay (Amersham). This assay makes use of the principle that ^3H needs to be in the proximity of scintillant in order to produce light. A DNA/RNA primer linked to beads with scintillant can be used as a template for reverse transcriptase (RT). A signal will therefore be measured only when RT-activity is present in the sample. Specifically, a tritium mixture of 25 nCi/µl ^3H was prepared in assay buffer. Cell culture supernatants (70 µl per sample) were incubated with 20 µl tritium mixture and 10 µl of primer for 4 h at 37°C. Next, 200 µl of stop buffer was added to each sample and samples were diluted with 650 µl TBE and 50 µl 20% SDS. The incorporation of ^3H was measured using a scintillation counter and compared to appropriate negative control and a positive control of 20 units of AMV reverse transcriptase (Promega).

2.7 Transgenic Mouse Techniques

2.7.1 Preparation of transgene DNA

Transgene DNA plasmid (KpHSE3' and K/FpHSE3') was linearised by restriction with XhoI. The transgene cassette-containing fragment (6kb or 6.7 kb, respectively) was gel-purified using a Genomed Jetsorb kit (Bad Oeynhausen, Ger), according to the manufacturer's protocol. DNA was taken up in a final volume of 30 TE. DNA was next purified using Wizard DNA clean-up columns (Promega). Briefly, DNA was mixed with 1 ml resin and the

mixture was loaded onto a magic cleanup column fixed to the end of a 2 ml syringe. The solution was pushed through the column, and the column was washed by with 2 ml of 80% isopropanol. The column was placed in an eppendorf tube and spun for 20 seconds at 12,000 rpm to remove residual isopropanol. To the column 40 μ l of hot (60-70°C) MilliQ water was added, left for one minute, and next spun for 20 seconds at 12,000 rpm to collect the eluant. DNA in the eluant was precipitated by adding 1/10 volumes of 3M sodium acetate (NaAc) and 2 volumes of 95% Ethanol, followed by incubation for 1 hour at -20°C. The DNA was pelleted by centrifugation for 10 minutes at 12,000 rpm, washed with 300 μ l of 70% Ethanol and dried by exposure to the air for around 20 minutes. DNA was dissolved in 20 μ l of injection buffer (10 mM Tris, 0.1 mM EDTA, pH 7.4) and the DNA concentration was determined by running it on an agarose gel parallel with a 2-fold dilution series of a reference sample. Transgene DNA was diluted to a final concentration of 5 ng/ μ l in injection buffer and handed over to the ICRF transgenic core unit.

2.7.2 Microinjection of oocytes and identification of founders

Linearised, purified plasmids were injected into the male pronucleus of fertilised CBA x C57BL/6 (F1 hybrid) oocytes by staff of the ICRF transgenic core unit, using standard techniques (Brinster et al., 1985). Injected oocytes were implanted in pseudopregnant foster mothers (around 25 oocytes per animal, 8 animals per transgene construct). The offspring was screened for integration of the transgene DNA by PCR and Southern analysis, and positive animals were bred with F1 mice. Transmission of the transgene was confirmed by PCR and Southern analysis of the progeny. Expression of transgene protein was established by immunoprecipitation and western blotting analysis. Two transgenic lines for *K cyclin* were generated (called E μ *K cyclin* 1996E-H (6) and E μ *K cyclin* 2269A.1 (20), or pair 6 and pair 20), and one founder line for *K cyclin/v-FLIP* was generated (called E μ *K cyclin/v-FLIP* 2284G (14), or pair 14).

2.7.3 Routine breeding of mice

Routine mouse breedings were performed by ICRF or UCSF animal house staff. Transgenic mice were mated with F1 strain mice (usually 2 females and one male per cage) and pregnant females were separated. When crossing mice with different strains (*p53*^{-/-}, *ARF*^{-/-}) no backcrossing was performed, and littermates were taken as controls. Newborn pups were weaned at three weeks of age and marked by ear-punching or toe-clipping. Tailsnips were clipped and used to genotype individual mice.

2.7.4 Tailsnip DNA preps

Tailsnips were taken up in 700 μ l of tailbuffer containing 40 μ l of a 10 mg/ml proteinase K solution and incubated overnight at 55°C. To this, 700 μ l of a 25:24:1 phenol:chloroform:Iso-amyl alcohol (pH 8.5) solution (Sigma) was added and tubes were rotated for 15 minutes at RT. Tubes were spun at 10,000 rpm for 10 minutes. Approximately 500 μ l of the upper aqueous phase was removed using a blunted 1 ml pipette tip and transferred to a tube containing 500 μ l of 100% ethanol. The solution was shaken until a DNA precipitate was visible. The DNA was pelleted by centrifugation for 5 minutes at 10,000 rpm and the DNA pellet was washed once with 300 μ l 70% ethanol. The ethanol was carefully removed and DNA was dried by exposure to the air for around 20 minutes at RT. The DNA was dissolved in 100 μ l of milliQ water and stored at RT until uses for genotyping.

2.7.5 Mouse genotyping

Genomic DNA isolated from tailsnips was genotyped by PCR analysis. In all cases 1 μ l (of 100 μ l) DNA was used as a template, reaction mixtures contained 250 μ M of each dNTP (Amersham Pharmacia) and MilliQ water was added to reach the final reaction volume. All PCR cycles were started with a denaturation step by incubation for 5 minutes at 95°C and were followed with a final extension step for 10 minutes at 72°C and cooling to 4°C. Reactions were performed in a MJ research PTC-225 thermal cycler (Waltham, MA, USA). See Table 2.2 for primer sequences.

2xFlag K cyclin in combination with ATF genomic DNA positive control: A 25 μ l reaction mixture contained 1x Promega DNA polymerase buffer, 2.5 mM MgCl₂, 0.5 μ M of each primer (Kcyc 5', Kcyc3', ATF911 5' and ATF302.4 3') and 1.5 units Promega Taq DNA polymerase in storage buffer A. The following PCR protocol was used: 30 cycles of 1 minute denaturation at 95°C, 1 minute annealing at 60°C and 1 minute extension at 72°C to obtain reaction products of around 800 bp for 2xFlag K cyclin and around 400 bp for ATF.

p53 wt: A 25 μ l reaction mixture contained 1x Promega DNA polymerase buffer, 10 mM MgCl₂, 0.75 μ M of each primer (p53exon6 and p53exon7) and 1.5 units Promega Taq DNA polymerase in storage buffer A. The same PCR protocol was used as described above for K cyclin in combination with ATF, to obtain reaction products of around 500 bp.

p53 neo: A 25 µl reaction mixture contained 1x Promega DNA polymerase buffer, 10 mM MgCl₂, 0.75 µM of each primer (p53exon7 and p53neo) and 1.5 units of Promega Taq DNA polymerase in storage buffer A. The following PCR protocol was used: 12 cycles of 0.35 seconds at 95°C, 45 seconds at 64°C and 45 seconds at 72°C, in which the annealing temperature was ramped down 0.5°C each cycle to reach 58°, followed by 25 cycles of 35 seconds at 95°C, 30 seconds at 58°C and 45 seconds at 72°C, to obtain products of around 600 bp.

ARF wt: A 20 µl reaction mixture contained 1x Takara *Ex Taq* DNA polymerase buffer, 1 µM of each primer (ARF1 and ARF2) and 1.5 units of *Ex Taq* DNA polymerase. The following PCR protocol was used: 35 cycles of 1 minute at 95°C, 1 minute at 66°C and 1 minute at 72°C, to obtain products of around 400 bp.

ARF neo: A 20 µl reaction mixture contained 1x Takara *Ex Taq* DNA polymerase buffer, 1 µM of each primer (ARF2 and ARFNEO2) and 1.5 units of *Ex Taq* DNA polymerase. The following PCR protocol was used: 35 cycles of 1 minutes at 95°C and 2 minutes at 72°C, to obtain products of around 200 bp.

E2F1 wt and E2F1 neo: A 25 µl reaction mixture contained 1x Promega DNA polymerase buffer, 10 mM MgCl₂, 0.5 µM of each primer (E2F1 5', E2F1 3' and PGKB) and 1.5 units of Promega Taq DNA polymerase in storage buffer A. The following PCR protocol was used: 30 cycles of 5 minutes at 95°C, 45 seconds at 92°C, 1 minute at 55°C and 2 minutes at 72°C, to obtain products of 172 bp (wt band) and 227 bp (neo band).

2.7.6 Tumour establishment

Mice were monitored 2 or 3 times per week for behavioral changes (loss in motility, breathing problems, hunched stature, weight loss) and/or the presence of tumour lumps under front/rear legs, throat, neck or in the abdomen, for up to a year. Sick mice were euthanised by CO₂ asphyxiation, followed by cervical dislocation and/or bilateral thoracotomy.

2.7.7 Isolation of primary lymphocytes

Thymi, spleens and lymph nodes were obtained by dissection from mice with lymphomas, from age-matched littermate controls or from 10-week old controls. Tissue was disaggregated by pressing through a 70 µm nylon mesh cell strainer in DMEM medium containing 2% FBS to obtain a single cell suspension. Cells were pelleted by centrifugation at 1500 rpm for 5 minutes. Splenic erythrocytes were eliminated by incubation in ACK lysis

buffer (diluted 2:3 in DMEM/2%FBS; 1 ml medium + 2 ml ACK for cells isolated from a healthy spleen) for 5 minutes at RT, followed by another centrifugation step. Cells were resuspended in 10 ml of medium and counted using a haemocytometer. Where appropriate, cells were cryopreserved in DMEM/10% FBS (90%) and DMSO (10%), as described in section 2.3.2.

2.7.8 Lymphocyte surface antigen staining and analysis

Approximately $2-3 \times 10^6$ splenocytes or lymphocytes were spun in 5 ml FACS tubes for 5 minutes at 1800 rpm. Cellular antigens were blocked by washing cells once with 5 ml of 1% BSA/PBS solution. Cells were next resuspended in 200 μ l of antibody solution (1% BSA/PBS containing a 1:200 dilution of the antibody) and incubated for 20-30 minutes on ice in the dark. Cells were washed once with 5 ml of blocking solution and finally resuspended in 400 μ l of blocking solution and surface antigen expression was determined by flow cytometric analysis.

2.7.9 Centrosome staining of primary lymphocytes

Cryopreserved lymphocytes from tumours and control tissues were thawed, washed with PBS/10% BSA and 1×10^5 cells were taken up in 200 μ l PBS/BSA. These samples were spun on glass slides by centrifugation for 8 minutes at 400 rpm using a cytospin centrifuge. Slides were air-dried and next fixed by incubation for 6 minutes in ice-cold methanol at -20°C . Samples were incubated in PBS and stained with anti γ -tubulin antibody exactly as described in section 2.9.3.2.

2.7.10 Histopathology of tissues

Tissues were fixed by ON incubation in 10% neutral buffered formalin solution (Sigma) at 4°C and next placed in marked OmniSette tissue cassettes (Fisher Scientific). Cassettes were incubated in PBS for 5-10 minutes and tissues were dehydrated by sequential 45 minute washes in 30% Ethanol, 50% Ethanol and 70% Ethanol. Cassettes were handed over to the UCSF Cancer Center mouse pathology core for further processing. Tissues were paraffin embedded, followed by sectioning of tissues (5 micron sections) and haematoxylin and eosin (H&E) stained according to standard procedures. This resulted in the purple staining of the cell nucleus and other acidic structures by the haematoxylin dye, and the pink staining of the cytoplasm and collagen by the eosin dye. Histology images were made using a Zeiss Axioplan 2 imaging microscope and Axiovision software.

2.7.11 Complete blood counts (CBCs) and blood smears

Sick or control mice were anaesthetised by IP injection of 400 μ l of 2.5% Avertin (Sigma). Around 1 ml of blood was collected from the orbital sinus or plexus using a 2 ml natelson blood collecting tube containing 10 μ l of 0.17 M EDTA (Fisher Scientific). The sample was placed in a 500 μ l pediatric EDTA tube and processed the same day for a CBC and a bloodsmear by the UCSF Cancer Center mouse pathology core. Hematology profiles were determined using a HEMAVET[®]850 machine. Bloodsmears were stained with Wrights Giemsa according to standard procedures and were microscopically analysed Zeiss Axioplan 2 imaging microscope and Axiovision software.

2.8 Apoptosis-related techniques

2.8.1 Nuclear microinjection of plasmid DNA

Rat-1 cells were seeded on glass bottom Microwell petri dishes (MatTek Cor. Ashland, OR) and grown for 48 h prior to microinjection. Microinjections were performed at room temperature with a Zeiss automated microinjection system (Zeiss AIS, Thornwood, NY). Needles were pulled from glass capillaries (1.2 mm, Clark Electromedical Instruments, UK) using a Sutter Instruments puller (Model P-97, Reading, UK). About 0.1 picolitre of DNA sample was injected directly into the cell nucleus. DNA samples were typically diluted on the day of injection with filtered milliQ water to a concentration of 100-150 ng/ μ l per plasmid, which is roughly equivalent to 150-250 DNA copies per injection. Co-injections with YFP plasmid (100 ng/ μ l) were performed to track injected cells. Identical standardised conditions of injection pressure (110 hPa) and injection time (0.1 seconds) were used in all experiments. Directly after injections, the medium was replaced with fresh DMEM + 10% FBS. At discrete time points after injection, cells were analysed by fluorescence microscopy and the number of YFP expressing viable (flat, healthy looking) and dead (round, apoptotic looking) cells was counted. The cumulative percentage of dead cells was calculated as the cumulative numbers of dead cells divided by the sum of cumulative blebbing and live cells at each time point. After establishment of K cyclin-induced apoptosis, each following experiment included one dish of cells injected with 2FKpcDNA3 plasmid as a control for injection efficiency and apoptosis induction.

2.8.2 Z-VAD.fmk treatment of cells

Cells were grown in the presence of 100 μ M of the general caspase inhibitor Z-VAD.fmk (Z-Val-Ala-Asp[Ome]-CH₂F, Enzyme Systems Products, Livermore, CA) for 1

hour before microinjection. The medium was changed and Z-VAD.fmk was added 1 hour after microinjection, followed by continued culture of cells in the presence of the caspase inhibitor.

2.8.3 Exposure of cells to various pro-apoptotic stimuli

To establish whether cells were sensitised to undergo apoptosis, cells were grown in medium containing 10% FBS and mouse TNF- α (50 ng/ml, specific activity 1.2×10^7 U/mg, kindly provided by Boehringer-Ingelheim), His-tagged TRAIL (200 ng/ml, R&D Systems, Minneapolis, MN, USA) or etoposide (5 μ M, Sigma). TRAIL ligand was pre-incubated for 30 h at RT with 3 μ g of anti-6x Histidine antibody (R&D systems) to initiate ligand trimerisation.

2.8.4 Propidium Iodide (PI) staining and Fluorescence Activated Cell Sorting (FACS) for Sub-G1 DNA content

To assess the effect of K cyclin expression on DNA content, both cells and the cell culture medium were harvested together. Cells were pelleted in FACS tubes by centrifugation for 10 minutes at 2,000 rpm, after which they were washed twice with PBS. Care was taken when aspirating the supernatants to avoid removal of the less dense apoptotic cells. Cell pellets were resuspended in 1 ml of 70% ethanol by dropwise addition of the ethanol while tubes were continuously vortexed. Cells were fixed during incubation for at least 16 h at 4°C to allow time for the smaller DNA fragments to diffuse out of the cell and thus give a more reproducible separation between the G1 and sub-G1 peak. After this, normal and apoptotic cells were precipitated by centrifugation at 3,000 rpm. Pellets were resuspended in PBS containing 10 μ g/ml propidium iodide (PI), 100 U/ml Ribonuclease A and 0.1% glucose and analysed by flow cytometry around 30-60 minutes later (FACSCalibur, Becton Dickinson). Clumped cells were excluded from the analysis by gating only single cells based on dot plot of the width versus the area of the DNA signal. PI signal was detected in the FL-3 channel and the percentage of sub-G1 events was measured by marking this area in the resulting DNA histogram.

2.8.5 Annexin staining and FACS

Cells and medium supernatants were collected and stained with Annexin V-PE (Pharmingen) according to the protocol of the manufacturer. This method is based on the high affinity of Annexin V for negatively charged phospholipids, such as phosphatidyl serine, in the presence of Ca²⁺ ions. Unfixed cells were washed once with PBS and once with binding buffer (10 mM HEPES/NaOH, pH7.4, 140 mM NaCl, 2.5 mM CaCl₂), followed by incubation

for 15 minutes with 5 μ l Annexin V-PE in the dark. DNA was counterstained with the DNA intercalating dye 7-AAD (Via-ProbeTM, 20 μ l per sample, Pharmingen). The percentage of Annexin V-positive / Via-Probe positive or negative cells was determined by flow cytometric analysis. Debris was excluded from the analysis by gating only the whole cells, based on a dot plot of the forward light scatter versus side light scatter. PE signal was detected in the FL-2 channel and Via-Probe signal in the FL-3 channel. When Annexin V positivity was assessed specifically in a GFP-positive cell population, GFP signal was detected in the FL-1 channel.

2.8.6 “Blue cell assay”

MEFs were plated at 1×10^5 cells per well in a 6-well dish 24 h before transfection. Cells were transfected using Fugene reagent (Roche, Indianapolis, IN, USA) with 1 μ g of cyclin or anti-apoptotic expression plasmids plus 0.4 μ g of pEFLacZ vector plus empty pcDNA3 plasmid to 2.4 μ g total DNA. Cells were grown for 24 h in DMEM medium containing 10% FBS, followed by growth for 24 h in medium containing 0.2% FBS. Cells were then fixed with 0.05% glutaraldehyde and stained using a solution containing 0.05% X-Gal, 0.2 mg/ml spermidine, 10 mg/ml potassium ferrocyanide, 8 mg/ml potassium ferricyanide and 3 mM MgCl₂ (all from Sigma) in phosphate-buffered saline (PBS), at 37°C overnight. The percentage of dead cells was calculated by counting the number of live, flat blue cells and dead, rounded blue cells.

2.9 General Techniques

2.9.1 DNA replication assay by BrdU labelling

To determine the percentage of cells that undergoes DNA replication during a certain time-period, cells were grown in the presence of 50 μ M of 5-bromodeoxyuridine (BrdU, Sigma) to allow incorporation of this thymidine analog in the DNA. After this cells were harvested and fixed. If analysis was by flow cytometry, cells were trypsinised, washed twice in 1% BSA/PBS, fixed with cold 70% ethanol and stored at -20°C . The incorporated BrdU was stained with FITC-conjugated anti-BrdU (Becton Dickinson). Cells (around 1×10^6) were centrifuged for 10 minutes at 2,000 rpm and resuspended in 500 μ l of 2 N HCl/Triton X-100, whilst continuously vortexing the tubes. Cells were incubated for 30 minutes at RT to allow denaturation of the DNA. Cells were next centrifuged and cell pellets resuspended in 500 μ l of 0.1 M Na₂B₄O₇•10 H₂O, pH 8.5 to neutralise the acid. After this, cells were pelleted and taken up in 500 μ l of 0.5% Tween-20/1% BSA/PBS containing 20 μ l of anti-BrdU FITC and

incubated for 30 minutes at RT. Cells were pelleted and resuspended in 1 ml of PBS containing 5 µg/ml of PI followed by flow cytometric analysis.

For microscopic analysis, cells grown on coverslips were fixed with 70 % ethanol for 30 minutes at room temperature. Cells were stored in 70% Ethanol at 4°C until staining with anti-BrdU FITC (up to one week after fixation). Coverslips were air-dried and immersed in 0.07 N NaOH for 2 minutes. Coverslips were washed with PBS to neutralise the base. Coverslips were incubated for 30 minutes at RT on a drop of anti-BrdU antibody solution (20 µl of antibody diluted with 50 µl of 0.5% Tween-20/PBS), and washed with PBS. Next, coverslips were immersed for 1 minute in a solution of 0.04 µg/ml of PI in PBS. Coverslips were washed with water, mounted in MOWIOL (Calbiochem) and analysed by fluorescence microscopy using a Zeiss Axiovert S100 TV microscope (Zeiss, Thornwood, NY, USA).

2.9.2 Luciferase assays

2.9.2.1 NFκB Luciferase assay

Rat-1 cells or 293T cells were split in 6 well dishes to reach 60-80% confluency at the time of transfection (2×10^5 cells or 1×10^5 cells per well respectively). The next day cells were transfected with 0.05 µg of pRL-TK plasmid plus 0.1 µg of pBIIIX-luc NFκB luciferase plasmid together with increasing amounts of v-FLIP plasmid (0.1-2 µg) or 1 µg pcDNA3 negative or 1 µg pGDp65 positive control plasmid. Rat-1 cells were transfected using Fugene 6 reagent according to the manufacturer's protocol (see section 2.6.3). 293T Cells were transfected using SuperFect reagent (Qiagen Inc., Chatsworth, CA, USA), according to the manufacturer's protocol.

Cells were harvested 24 h after transfection and processed according to the protocol of the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The growth medium was removed and cells were washed once with PBS. The cells were actively lysed by addition of 150 µl of 1x passive lysis buffer and harvested immediately by scraping with a cell scraper. Lysates were frozen at -80°C to achieve complete lysis of cells. After thawing, 20 µl of lysate was transferred to a 96-well microplate. Lysates were assayed for firefly and *Renilla* luciferase activities using a microplate luminometer LB96V equipped with two injectors (EG&G Berthold, Bad Bildbad, GER). The injector was programmed to perform the following protocol: priming of the auto-injectors with 2ml of reagent - an automatic dispensation of 100 µl Luciferase Assay Reagent II - measurement of the firefly luciferase activity with a 3-second pre-measurement delay followed by a 12-second measurement period - dispensation of 100 µl Stop&Glo Reagent - measurement of the *Renilla* luciferase activity

with a 3-second pre-measurement delay followed by a 12-second measurement period – 100 wash steps with milliQ water. The values of the luciferase activities were subtracted with the background values of lysates of untransfected cells. Specific luciferase activities were calculated by dividing the firefly luciferase activity by the *Renilla* internal control activity. The specific luciferase value of a lysate transfected with empty pcDNA3 control was set as 1, to which the activities in the various samples were related to obtain the final relative luciferase activity of each sample.

2.9.2.2 p53 Luciferase assay

293T cells were split at 3×10^5 cells/well in 6 well plates. The next day, cells were transiently transfected using Superfect reagent with 0.05 μg of pRL-TK and 0.1 μg of pGL3cyclinG-luc reporter plasmids together with 1.0 μg of pcDNA3 or pCMVp53 and co-transfected with 1.0 μg of LANA or pBpurop53(302-390) (DNp53) plasmid. Alternatively, wt or p53^{-/-} MEFs were plated at 1×10^5 cells per well and transfected the next day using Fugene reagent. Transfection mixtures consisted of 0.05 μg RL-TK and 1 μg of p53luc or mdm2luc reporter plasmid together with 0.5 μg of pcDNA3 or pCMVp53 and co-transfected with 0.5 μg of LANA or DNp53 plasmid. Cell lysates were prepared 24 h after transfection and the firefly and *Renilla* luciferase activities were measured using the Promega dual-luciferase reporter assay system as described in the previous section.

2.9.3 Time-lapse videomicroscopy

MEFs or MRC-5 cells were plated at 1×10^5 cells per well in a 6 well plate. Cells were grown in a humidified system adapted with a CTI-controller (5% CO₂) and a 37-2 digital Tempcontrol (37°C) on an Axiovert S 100 microscope (all systems from Zeiss, Thornwood, NY). About 20-40 cells were followed over the course of 4 days whilst taking photos (magnification 10x) every 10 minutes using an Openlab automator module (Improvision, Coventry, UK). Time-lapse videos were compiled in Openlab and converted to Quicktime movies (0.1 sec. per frame).

2.9.4 Immunocytochemistry of tissue culture and primary cells

2.9.4.1 Staining of Flag-tagged proteins

Cells grown on coverslips were washed twice with PBS and fixed in 3.7% formaldehyde/PBS for 10 minutes at RT. Cells were permeabilised by incubation for 5 minutes in 0.1% Tween-20/PBS, followed by incubation with anti-Flag primary antibody

(1:100) diluted in 0.5% NP-40/10% donkey serum/PBS for 1 h at RT. Coverslips were washed three times with PBS, permeabilised and next incubated for 30 minutes at RT in the dark with FITC-labelled donkey anti mouse secondary antibody diluted in 0.5% NP-40/10% donkey serum/PBS (1:200, Jackson laboratories, PA, USA). Cells were washed three times with PBS and mounted in MOWIOL (Calbiochem, La Jolla, Ca, USA) and analysed by fluorescence microscopy using a Zeiss Axiovert 135TV conversion microscope (Zeiss, Thornwood, NY, USA). All incubations of coverslips with antibodies were performed by turning the coverlip upside down on a drop of antibody solution in a hydrated chamber (petridish with parafilm on top of a piece of whatman paper moistened with PBS).

2.9.4.2 Centrosome staining

MEFs grown on coverslips were fixed in ice-cold methanol for 6 minutes at -20°C . Coverslips were washed with PBS and cells permeabilised in 0.1% Tween-20/PBS, followed by staining for 1 h at room temperature with anti γ -tubulin (1:150) or anti centrin (1:500) primary antibody diluted in 0.5% NP-40/10% goat serum/PBS. After washing and another permeabilisation step, primary antibody binding was detected using Alexa FluorTM 488 goat anti mouse secondary antibody (1:200, Molecular Probes, Oregon, USA). DNA was counterstained with DAPI (4',6-diamidino-2-phenylindole, 0.5 μM , Sigma) and coverslips were mounted in MOWIOL (Calbiochem), followed by a Zeiss Axiovert S100 TV microscope and Openlab imaging system. When indicated, slides were analysed by confocal microscopy using a Zeiss LSM 510 META microscope (see section 2.9.3.7).

2.9.4.3 Cyclin B staining

Cells grown on coverslips were washed twice with PBS and fixed in ice-cold methanol for 6 minutes at -20°C . Cells were stained with primary anti cyclin B1 antibody (1:200), secondary Alexa FluorTM 488 goat anti mouse secondary antibody (1:200) exactly as described in the previous section. DNA was counterstained with DAPI (0.5 μM) and coverslips mounted in MOWIOL were analysed by fluorescence microscopy.

2.9.4.4 Cytochrome C staining

Cells grown on coverslips were washed twice with PBS and placed in ice-cold methanol:acetone (50:50) for 2 minutes, after which the coverslips were air-dried. Coverslips were washed three times with PBS. Next, they were incubated with anti-cytochrome C antibody diluted 1:200 in PBS/3%BSA, for 1 h at RT in a hydrated chamber. The coverslips were washed twice with PBS/BSA, and incubated for 30 minutes at RT with secondary Alexa Fluor 568TM goat anti-mouse IgG antibody (Molecular Probes), diluted 1:200 in PBS/BSA.

Coverslips were washed three times with PBS and then incubated for 1 minute in PBS containing 1 $\mu\text{g/ml}$ Hoechst 33342.

2.9.4.5 DNA quantitation

For quantification of cellular DNA contents, PI was added (3 $\mu\text{g/ml}$, 100 U/ml Ribonuclease A) for 30 min at room temperature. Immunofluorescence microscopy was performed and the DNA content of individual nuclei was measured using the particle analysis tool of Scion Image 1.62c software by integrating the PI signal density of separate particle. Overlapping nuclei or nuclei touching the edges were ignored. Identical threshold limits were used for all analyses to standardise background signals.

2.9.4.6 SA- β gal staining

Cells were washed in PBS, fixed for 3-5 minutes at RT in 2% formaldehyde/0.2% glutaraldehyde, washed, and incubated at 37°C (no CO₂) with fresh senescence-associated β -Galactosidase (SA- β -Gal) staining solution: 1 mg/ml of X-Gal (stock of 50 mg/ml in dimethylformamide), 40 mM citric acid/sodium phosphate pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂. Blue staining was evident after 2-4 h and maximal after 12-16 h incubation. Cells were photographed using a Nikon F70 camera attached to a Nikon Eclipse TE 3000 microscope (Serco, Inc., Livermore CA, USA).

2.9.4.7 Confocal analysis

Immunostained cells were analysed by confocal imaging if this improved visualisation of the intracellular structure. Cells stained with cytochrome C were analysed using a MRC1000 confocal microscope (Bio-Rad Labs, Hercules, CA). Serial z-axis optical sections through the cell were collected at 0.5 μm intervals via a 100x plan-apochromatic oil objective. Z-series of images were projected on a single two-dimensional plane to get final pictures. Cells stained with γ -tubulin and Hoechst were analysed using a LSM 510 META confocal microscope. In this case, single sections that showed the DNA most accurately were imaged.

CHAPTER 3:
K CYCLIN TRIGGERS APOPTOSIS IN FIBROBLASTS

3.1 Introduction

Deregulation of the cellular control mechanisms by K cyclin may create an environment that is conducive to viral replication and thus proliferation. However, many (viral) oncogenes that promote proliferation simultaneously sensitise cells to apoptosis, explained as a cellular fail-safe mechanism against tumourigenesis (Evan and Vousden, 2001). In addition, host cells activate tumour suppressor pathways to eliminate infected cells. The long-term establishment of latently infected cells therefore often relies on the adoption of anti-apoptotic strategies by the virus (Hardwick, 1998). The balance between these pro- and anti-apoptotic signals eventually determines the fate of the cell.

Preliminary experiments in the Jones laboratory showed that it was difficult to derive and maintain cell lines constitutively or inducibly expressing K cyclin (D. Mann and H. Laman personal communication). This led to the hypothesis that K cyclin can trigger apoptosis. The involvement of cyclins in apoptosis had already been suggested. For example, an association of cyclin D1 upregulation with neuronal cell death had been shown (Kranenburg et al., 1996) and there were indications that cyclin D1 could directly trigger apoptosis (Sofer-Levi and Resnitzky, 1996). However, no detailed analysis of cyclin-induced apoptotic pathways had been performed. A study of K cyclin-triggered apoptosis could therefore perhaps serve as a model for cyclin-induced cell death.

The KSHV virus encodes multiple proteins that regulate apoptosis. For example, KSHV ORF16 encodes a viral homologue of the mammalian anti-apoptotic *Bcl-2* gene, called *KSBcl-2*. *KSBcl-2* mRNA is expressed during the lytic viral replication cycle but not in latently infected cells (Cheng et al., 1997b). Although the overall structure of the hydrophobic BH3 domain-binding pocket of *KSBcl-2* is similar to cellular *Bcl-2*, *KSBcl-2* has a shorter unstructured loop region between BH1 and BH2 domains. Importantly, the *KSBcl-2* loop lacks residues involved in the caspase-mediated cleavage of *Bcl-2* to a Bax-like pro-apoptotic protein, suggesting that *KSBcl-2* may evade regulation by caspases (Chang et al., 1997; Cheng et al., 1997a; Clem et al., 1998; Grandgirard et al., 1998; Huang et al., 2002). In addition, recent evidence from the Makela lab has shown that K cyclin/Cdk6 complexes phosphorylate Serines 70 and 87 in this loop region, thereby inactivating cellular *Bcl-2* (Ojala et al., 2000). Again, *KSBcl-2* lacks this region, escaping inactivation upon K cyclin expression. Furthermore, the interaction of *KSBcl-2* with pro-apoptotic *Bcl-2* family members is different. For example, *KSBcl-2* can interact with and antagonise Diva, a pro-apoptotic *Bcl-2* homologue that directly associates with and activates Apaf-1 (Inohara et al., 1998). Together, these intrinsic differences most likely explain why *KSBcl-2* is more effective in inhibiting K cyclin-induced apoptosis compared to cellular *Bcl-2*.

Another putative anti-apoptotic KSHV-encoded protein is v-FLIP, encoded by ORF 71. FLIPs are DEDD containing proteins act as dominant negative inhibitors of FAS/APO-activated apoptosis (Krueger et al., 2001). In addition, FLIPs can activate the NF- κ B survival pathway in *in vitro* transfection studies (Chaudhary et al., 1999; Hu et al., 2000; Kataoka et al., 2000), although it should be noted that NF- κ B activation is not altered in mammalian (cellular) cFLIP^{-/-} mice (Yeh et al., 2000). At the time when the experiments described in this chapter were initiated, no experiments with KSHV v-FLIP had been described, although the viral FLIP from the related γ -herpesvirus EHV-2 had been shown to protect cells from CD95-induced apoptosis (Thome et al., 1997). Furthermore, the Evan lab showed around this time that fibroblasts expressing c-Myc are sensitised to the CD95 death signal and that apoptosis by intracellular c-Myc is inhibited upon death receptor pathway disruption (Hueber et al., 1997). By inference, v-FLIP could therefore also function to indirectly inhibit oncogene-induced apoptosis by altering death receptor signalling

I thus decided to investigate the apoptotic potential of K cyclin and, if K cyclin was pro-apoptotic, the ability of KSBcl-2 and/or v-FLIP expression to inhibit this. A cooperation between K cyclin and KSHV v-FLIP during viral infection was particularly appealing because they are transcribed together on a polycistronic transcript (see Fig. 1.9 and Dittmer et al., 1998). Thus, a study of the apoptotic effects of K cyclin would give more insights into the potential interactions between K cyclin and cellular anti-apoptotic signals that dictate the cellular response to K cyclin expression.

3.2 Microinjection of K cyclin-encoding plasmid in Rat-1 cells induces caspase-dependent apoptosis

To investigate if K cyclin can trigger apoptosis, it was ectopically expressed by microinjection of expression constructs in the nuclei of rodent fibroblasts. This method was

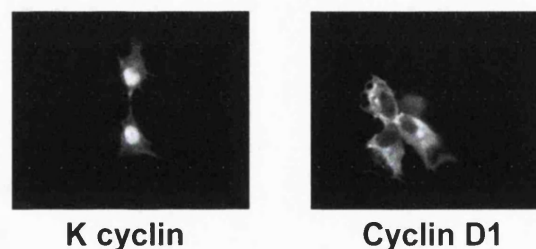


Fig. 3.1 Expression of 2xFlag-tagged cyclins in Rat-1 fibroblasts. Cells growing on coverslips were microinjected with 100 ng/ μ l (~150-200 copies per injection) of pcDNA3 expression vector. 6 Hours later cells were fixed in 3% formaldehyde and coverslips were stained with anti-Flag primary and FITC conjugated anti-mouse IgG secondary antibodies. Immunofluorescent images were taken on a Zeiss axiovert 135 TV conversion microscope.

chosen because it allowed the easy detection of injected cells, the titration of expression constructs and the possibility to co-express combinations of proteins. Functional assays based on DNA microinjection had been applied successfully by A. Alberts, a former ICRF colleague, whose recommendation for DNA concentration was followed (Alberts et al., 1993). Injection of around 150-200 copies of cyclin expression vector resulted in detectable levels of Flag-tagged cyclins by immunocytochemistry (Fig. 3.1). Cells were next co-injected with YFP-encoding vector to identify injected cells and followed for the appearance of dead cells. 24 Hours after injection of K cyclin-encoding plasmid (2FKpcDNA3), cells exhibited typical apoptotic blebbing or rounded morphology, with concomitant DNA fragmentation (Fig. 3.2A).

Dead Rat-1 cells are easily distinguishable from dividing cells by phase microscopy via their loose attachment to the dish and presence of a bright light-reflecting circumference (See Fig. 3.2A lower panel). To quantify cell death, YFP-positive injected cells were therefore microscopically followed for 72 hours and blebbing, dead and live cells were scored. Expression of K cyclin in cells growing in 10% FBS resulted in ~50% cell death

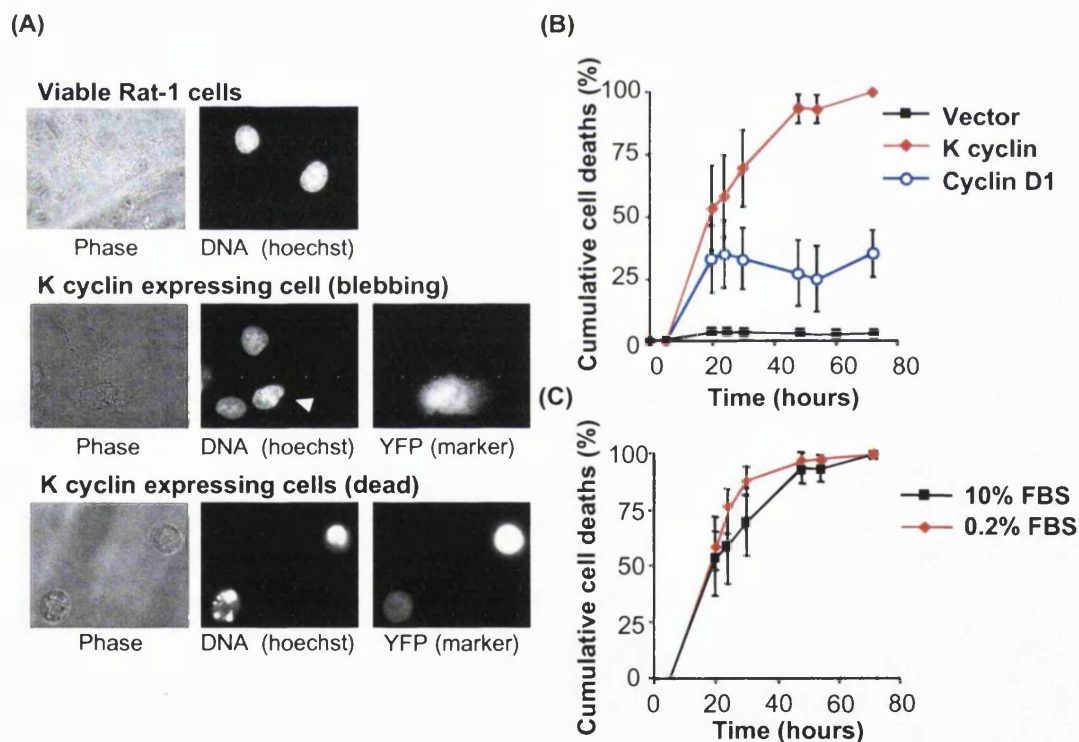


Fig. 3.2 K cyclin triggers cell death upon microinjection of expression plasmid in Rat-1 cells. (A) Morphology of Rat-1 cells growing in 10% FBS 24 h after microinjection with 2FKpcDNA3 (K cyclin) plus YFP plasmid or left untreated. Arrowhead: blebbing cell. (B) Rat-1 cells growing in 10% FBS were microinjected with 2FKpcDNA3, 2FDpcDNA3 (cyclin D1) or empty pcDNA3 plasmid together with YFP. At the indicated time points cells were analysed by fluorescence microscopy and the number of live, blebbing and dead cells was counted. The cumulative percentage of dead cells was calculated as the cumulative number of dead cells divided by the sum of the cumulative blebbing and live cells, and multiplied by 100. Data shown are the means and standard deviations of 4 independent experiments.

within 24 h, increasing to 80-100% after 72 h (Fig. 3.2B). In comparison, injection of an equivalent amount of cyclin D1-encoding vector (2FDpcDNA3) elicited only 35-40% cell death. A QuickTime movie (named Apoptosis Rat-1) of apoptosis after microinjection of Rat-1 cells with K cyclin-encoding plasmid is presented on the supplemented CD in the back cover of this thesis.

Deprivation of cells from growth factors by serum withdrawal can cooperate with certain apoptotic insults in the induction of apoptosis. However, 2FKpcDNA3 microinjections in cells grown in low (0.02%) serum did not significantly increase cell death by K cyclin (Fig. 3.2C). Furthermore, injections were complicated by the increased sensitivity of serum starved cells to apoptosis, thereby increasing the incidence of background killing. It was therefore decided to perform further experiments with cells grown in high (10%) serum.

To confirm that cells died by classical caspase-dependent apoptosis, the effect of the general membrane-permeable general caspase inhibitor Z-VAD.fmk was tested. 2FKpcDNA3 was microinjected in cells grown in medium containing Z-VAD.fmk. As shown in Fig. 3.3A, K cyclin-induced cell death was delayed by 20-30 h upon addition of Z-VAD.fmk. Cells showed typical ongoing membrane blebbing (Fig. 3.3B), visible in some cells at 18 h after injection and continuing until they rounded up. This is consistent with the described ability of Z-VAD.fmk to inhibit characteristic biochemical apoptotic events like the membrane display of phosphatidylserine, but not the onset of apoptosis including membrane blebbing (McCarthy et al., 1997). Interestingly, DNA condensation, normally a late event in the execution of apoptosis, is not inhibited by Z-VAD.fmk in K cyclin-expressing blebbing cells (see Fig. 3.3C for a typical example). Taken together, K cyclin expression in Rat-1 cells

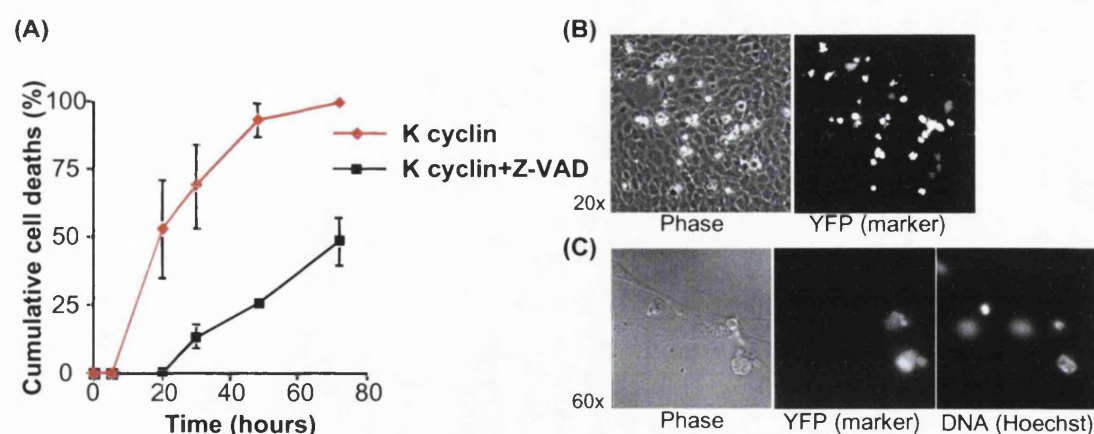


Fig. 3.3 Apoptosis by K cyclin is caspase dependent. (A) Rat-1 cells growing in 10% FBS in the presence or absence of Z-VAD.fmk (100 μ M) were microinjected with K cyclin plus YFP-encoding plasmids (150 ng/ μ l each). Cells were microscopically analysed at the indicated time points. Data shown are the means and standard deviations of 4 (K cyclin) or 3 (K cyclin + Z-VAD) independent experiments. (B) Morphology of Rat-1 cells grown in the presence of Z-VAD 20 h after injection with K cyclin-encoding plasmid (YFP-positive cells). Extensive membrane blebbing is visible in the majority of the injected cells. (C) DNA staining of cells treated as in B) by Hoechst showing DNA condensation in the blebbing cells.

triggers caspase-dependent apoptosis although DNA condensation upon K cyclin expression seems caspase-independent.

3.3 Apoptosis by cyclin D1 is not enhanced by co-expression of cyclin E

Since the activity of K cyclin/Cdk6 complexes resemble the combined activities of G1 and S phase cyclin/Cdk complexes (Ellis et al., 1999; Laman et al., 2001a; Mann et al., 1999), cyclin D1 and E were next co-expressed to see if the extent of K cyclin-induced apoptosis was mimicked. Microinjection of cyclin E-encoding construct alone did not induce a measurable level of apoptosis, which is in agreement with published results (Sofer-Levi and Resnitzky, 1996). Importantly, co-expression of cyclin E did not augment cyclin D-induced cell death (Fig. 3.4A). This implies that K cyclin-induced apoptosis is not merely the result of the ability of K cyclin/Cdk to mimic the activity of multiple cellular complexes that function at the G1/S transition.

One potential complication when comparing the apoptotic potential of different proteins is variable protein expression levels driven by various plasmids. However, the promoters of all the used plasmids were identical (all CMV-driven) and K cyclin and cyclin D1 were expressed at similar levels as measured by anti-Flag immunocytochemistry (Fig. 3.1). To further address the issue of quantitation a titration of cyclin-encoding plasmids was performed. Titration of 2FDpcDNA3 from 100-500 ng/ μ l resulted in a significant increase in cell death to levels comparable to K cyclin-induced apoptosis (Fig. 3.4B). Conversely, apoptosis upon injection of 2FKpcDNA3 at 10 ng/ μ l abrogated apoptosis and was comparable

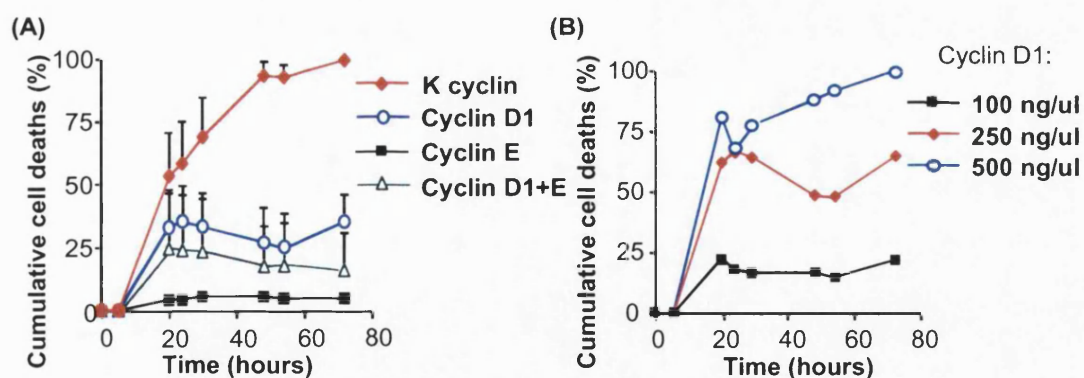


Fig. 3.4 Co-expression of cyclin D1 and E does not mimic K cyclin-induced apoptosis. (A) Rat-1 cells were microinjected and cell deaths were quantified essentially as in Fig. 2. All plasmids were injected at 150 ng/ μ l. Data are means and standard deviations from 4 independent experiments. The K cyclin curve is plotted as a control and is as shown in Fig. 3.2A. (B) Increased amounts of microinjected 2FDpcDNA3 plasmid correlate with enhanced apoptosis. Injections and analysis of apoptosis were as in Fig.3.2. The experiment was performed twice and representative curves are depicted.

to 100 ng/ μ l 2FDpcDNA3 (data not shown), demonstrating that approximately 5- to 10-fold more cyclin D1 needed to be expressed to equalise K cyclin-induced apoptosis.

Other factors such as cyclin stability or localisation may influence the propensity of cyclins to induce apoptosis. Preliminary data suggested indeed that K cyclin protein is more stable than Cyclin D (H. Laman personal communication). However, expression of a stable cyclin D1 mutant (T286A) that is refractory to phosphorylation and ubiquitination (Diehl et al., 1997) did not increase apoptosis compared to wild type cyclin D1 (data not shown). Another difference between K cyclin and cyclin D1 is that K cyclin is exclusively nuclear in the majority of the cells (91%), while cyclin D1 localisation is mainly cytoplasmic (86 %). Of note, Rat-1 cells reportedly do not express p21^{Cip1} (Allan et al., 2000), the so-called assembly factor of cyclin D/Cdk complexes that triggers nuclear accumulation of cyclin D (Cheng et al., 1999; LaBaer et al., 1997). This therefore suggests that K cyclin has most likely adopted a different strategy to ensure its nuclear accumulation.

3.4 Inducible expression of K cyclin triggers apoptosis in Rat-1 fibroblasts

Microinjection studies are not amenable to large-scale biochemical assays. For this reason, and to corroborate the microinjection data, a clonal Rat-1 cell line expressing Flag-tagged K cyclin under the IPTG-inducible promoter was created, called K18. This cell line

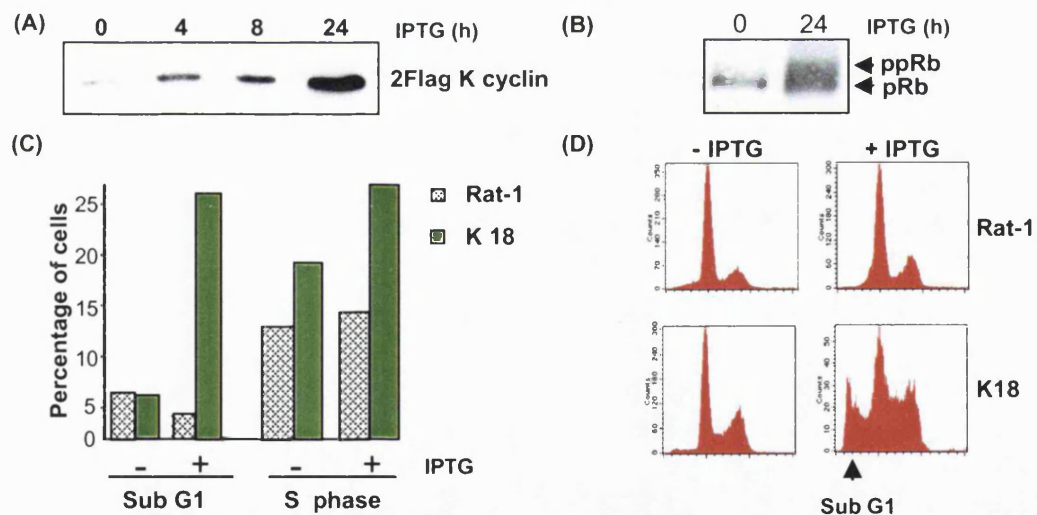


Fig. 3.5 Inducible expression of K cyclin induces apoptosis in serum starved K18 Rat-1 fibroblasts. (A) Immunoblot analysis with anti Flag antibody showing inducible expression of 2x Flag-tagged K cyclin. 0.8×10^5 K18 cells were grown in 10 cm plates in the presence of 10% FBS and 5 mM IPTG for the indicated times. Cells were harvested in Laemmli buffer and equal amounts of protein were loaded on a 15% SDS-PAGE gel. (B) Immunoblot analysis using anti Rb antibody. Lysates were run on a 6% SDS-PAGE gel. (C) K18 or Rat-1 cells grown for 24 h in medium containing 10% FCS with or without 5 mM IPTG, followed by growth for 72 h in medium containing 0.2% FCS with or without IPTG. Cells were harvested, fixed in 70% EtOH, stained with PI and analysed by flow cytometry. Results are the representative of three experiments. (D) Typical flow cytometry histograms of cells treated as in C).

shows optimal expression of K cyclin after 24 hours (Fig. 3.5A), which stayed high until at least 48 h (data not shown). Induction of K cyclin was associated with hyperphosphorylation of the Rb protein (Fig. 3.5B), showing that the expressed K cyclin is functionally active. In contrast to the microinjection experiments, neither inducible expression of K cyclin in cells growing in 10% serum or in 0.2% serum triggered significant apoptosis. However, induction of K cyclin expression for 24 h in cells growing in 10% serum followed by serum deprivation for 72 h resulted in a 16.4-fold increase in the sub-G1 population of apoptotic cells compared to un-induced controls (Fig. 3.5B). Such apoptotic cells exposed phosphatidylserine on the membrane as established by annexin V-FITC binding and flow cytometric analysis (data not shown). These data confirm that K cyclin is pro-apoptotic and emphasise that the sensitisation to apoptosis afforded by K cyclin is dependent on the cellular environment and the assay used.

3.5 K cyclin-induced apoptosis is delayed by KSBcl-2 but not by v-FLIP

Next, the ability of Bcl-2 to inhibit apoptosis induced by K cyclin was investigated. Expression constructs for both human and KSBcl-2 were constructed as described in the materials and methods (hBcl-2pcDNA3 and KSBcl-2pcDNA3). The expression of human Bcl-2 and Flag-tagged KS Bcl-2 proteins from these vectors was confirmed in transiently transfected 293T cells (Fig. 3.6A).

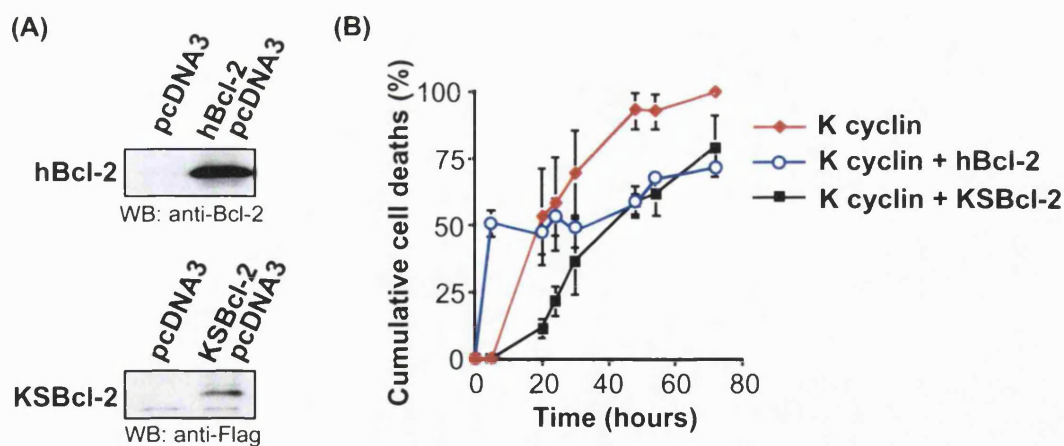


Fig. 3.6 K cyclin-induced apoptosis in Rat-1 cells is delayed upon coexpression of KSBcl-2. (A) A subconfluent culture of 293T cells growing in a 10 cm dish were transiently transfected with 10 μ g of empty pcDNA3, hBcl-2pcDNA3 or KSBcl-2pcDNA3 plasmid using Superfect. Cells were lysed 40 h later in boiling SDS lysis buffer. 20 μ g of protein was resolved on a 12% SDS-PAGE gel, western blotted and probed with anti-human Bcl-2 DHA6 antibody or anti-Flag antibody. Human Bcl-2 migrates around 24 kDa, Flag-tagged KSBcl-2 migrates around 35 kDa. (B) Rat-1 cells growing in 10% FBS were microinjected with K cyclin and YFP-encoding plasmids and the indicated Bcl-2pcDNA3 plasmids (150 ng/ μ l each). Cells were microscopically analysed at the indicated time points. Data shown are the means and standard deviations of 4 independent experiments.

Cells were co-injected with K cyclin and Bcl-2-encoding plasmids and apoptosis was analysed as described in the previous section. Coexpression of KSBcl-2 and K cyclin delayed the initiation of apoptosis by K cyclin for around 20 hours (Fig. 3.6B), consistent with a reported delay of c-Myc-induced apoptosis by human Bcl-2 (McCarthy et al., 1997). In contrast, human Bcl-2 did not significantly delay apoptosis by K cyclin at least during the first 30 hours (Fig. 3.6B). Thus, KSBcl-2 can inhibit K cyclin-induced apoptosis, supporting a model in which apoptosis by K cyclin is via a mitochondrial pathway.

A plasmid encoding v-FLIP (v-FLIP pcDNA3) was constructed as described in the materials and methods to assess whether K cyclin-induced apoptosis is inhibited by v-FLIP. The expression of Flag-tagged FLIP was confirmed in transiently transfected 293T cells and U2OS cells (expression in U2OS cells is shown in Fig. 3.7A). In addition, a bicistronic K cyclin/v-FLIP cDNA was constructed (K/FpcDNA3), as well as a mutant of this vector in which FLIP expression but not K cyclin expression is abrogated (K/FpcDNA3mut). Since no antibodies against v-FLIP were available at the time, the expression of K cyclin and/or v-FLIP from the different expression constructs was confirmed by *in vitro* transcription/translation (Fig. 3.7B).

To confirm that v-FLIP expressed from the v-FLIPpcDNA3 vector has any biological activity, luciferase assays were performed to measure NF- κ B-mediated transcriptional activation, by v-FLIP. Reporter assays were performed in 293T cells and in Rat-1 cells, using expression of the NF- κ B subunit p65 as a positive control. In both cases, expression of v-FLIP resulted in a 4 to 7-fold increase in the relative luciferase activity (see Fig. 3.7C for results in 293T cells), in agreement with the 8-fold increase in the NF- κ B activity by KSHV v-FLIP seen by Chaudhary and colleagues (Chaudhary et al., 1999). This shows that v-FLIP expressed from v-FLIPpcDNA3 is functionally active.

Microinjection experiments in which K cyclin was coexpressed with v-FLIP, either by coinjection of both plasmids or injection of K/FpcDNA3, did not show an inhibitory effect of v-FLIP on K cyclin-induced apoptosis. Furthermore, v-FLIP expression alone did not induce apoptosis (Fig. 3.7D). Since the relative expression level of Flag-tagged v-FLIP compared with K cyclin was relatively low (Fig. 3.7B), titrations of vFLIP plasmid to higher (500 ng/ μ l) and K cyclin to lower (50 ng/ μ l) amounts of injected DNA were performed. Again, no inhibitory effect of v-FLIP on apoptosis by K cyclin was revealed. However, CD95-mediated killing of Swiss 3T3 cells using an anti-mouse CD95 specific antibody was also not inhibited by v-FLIP (Fig. 3.7E). Thus, no definite conclusion regarding the inhibition of K cyclin-induced apoptosis by v-FLIP can be drawn.

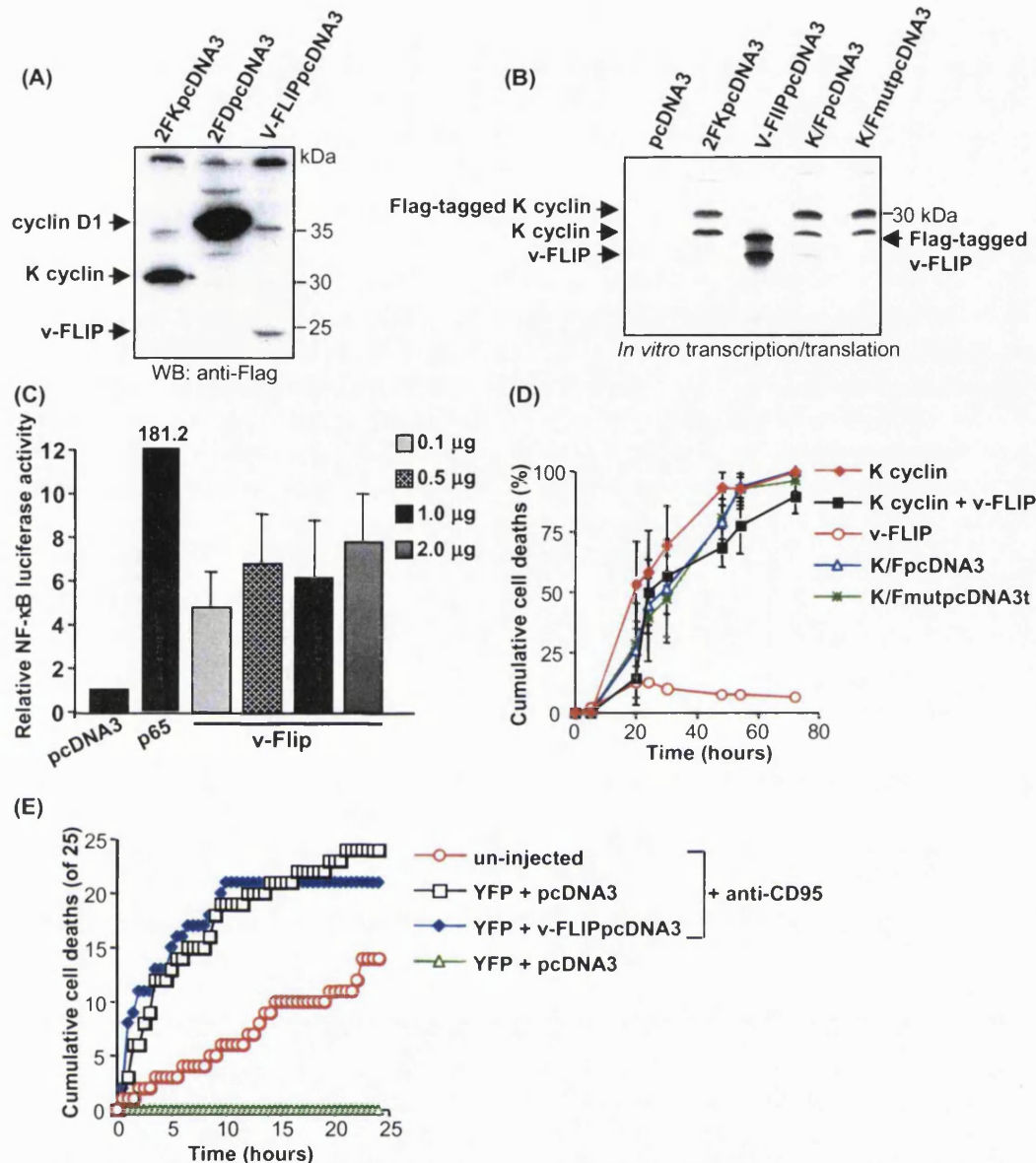


Fig. 3.7 K cyclin-induced apoptosis is not measurably inhibited by v-FLIP. (A) A subconfluent culture of U2OS cells was transfected with 10 μ g of 2FKpcDNA3 or 2FDpcDNA3 or 20 μ g of v-FLIPpcDNA3 plasmid using Calcium Phosphate transfection. 48 h after transfection lysates were prepared in Laemmli buffer and equal amounts of protein were resolved on a 12% SDS-PAGE gel. Protein was immunoblotted using anti-Flag antibody. (B) Proteins expressed from the indicated vector were synthesised by rabbit reticulocyte coupled *in vitro* transcription/translation in the presence of 35 S Methionine and 5 μ l of each reaction was run on a 15% SDS-PAGE gel which was dried and autoradiographed to detect 35 S signal. (C) 293T cells were split at 1×10^5 cells/well in 6 well plates and transfected the next day using Superfect reagent with 0.1 μ g of pRL-TK and 0.05 μ g of pBIIXLuc plasmids together with 1.0 μ g of pcDNA3 or pGDp65 or increasing amounts of v-FLIPpcDNA3 plasmid as indicated. 24 h after transfection firefly luciferase specific activity was measured using the Promega dual-luciferase reporter assay system. (D) Rat-1 cells growing in 10% FBS were microinjected with 2FKpcDNA3, K/FpcDNA3 or K/FmutpcDNA3 plus YFP or co-injected with 2FKpcDNA3, YFP and v-FLIPpcDNA3 (150 ng/ μ l each, empty vector added to 450 ng/ μ l total). Apoptosis was analysed as described in Fig. 3.2. Data shown are the means and standard deviations of 4 independent experiments. (E) Swiss 3T3 cells were microinjected with v-FLIPpcDNA3 or pcDNA3 together with YFP (150 ng/ μ l each). Control un-injected and injected cells were cultured in the presence of 100 ng/ml JO2 anti-CD95 antibody, directly after injections. Time-lapse phase and fluorescent videomicroscopy was started 30 min. later. Pictures were taken every 30 min. for 24 h and a time-lapse movie was compiled using QuickTime. 25 YFP injected or 25 control cells were followed for apoptotic morphology and cumulative cell deaths were plotted.

3.6 Cytochrome C release triggered by K cyclin does not precede nuclear collapse

A crucial event in the activation of effector caspases is the translocation of holocytochrome C from the mitochondria into the cytosol. Such cytochrome C release is prevented by the expression of anti-apoptotic Bcl-2 family members (Green and Reed, 1998). K cyclin-induced apoptosis is inhibited by KSBcl-2 (Fig. 3.6), suggesting that cytochrome C release may be essential for K cyclin-induced apoptosis. Around the time of these experiments, c-Myc was shown to mediate at least part of its pro-apoptotic effect via the early release of cytochrome C, thereby sensitising the cell to apoptosis by CD95 receptor ligation and γ -irradiation (Juin et al., 1999). I therefore asked whether K cyclin triggers the release of cytochrome C into the cytosol and what is the timing with regards to apoptosis.

K18 Rat-1 cells grown on coverslips were induced to express K cyclin for 24 h, followed by growth for 72 h in medium containing 0.02% FBS to trigger apoptosis.

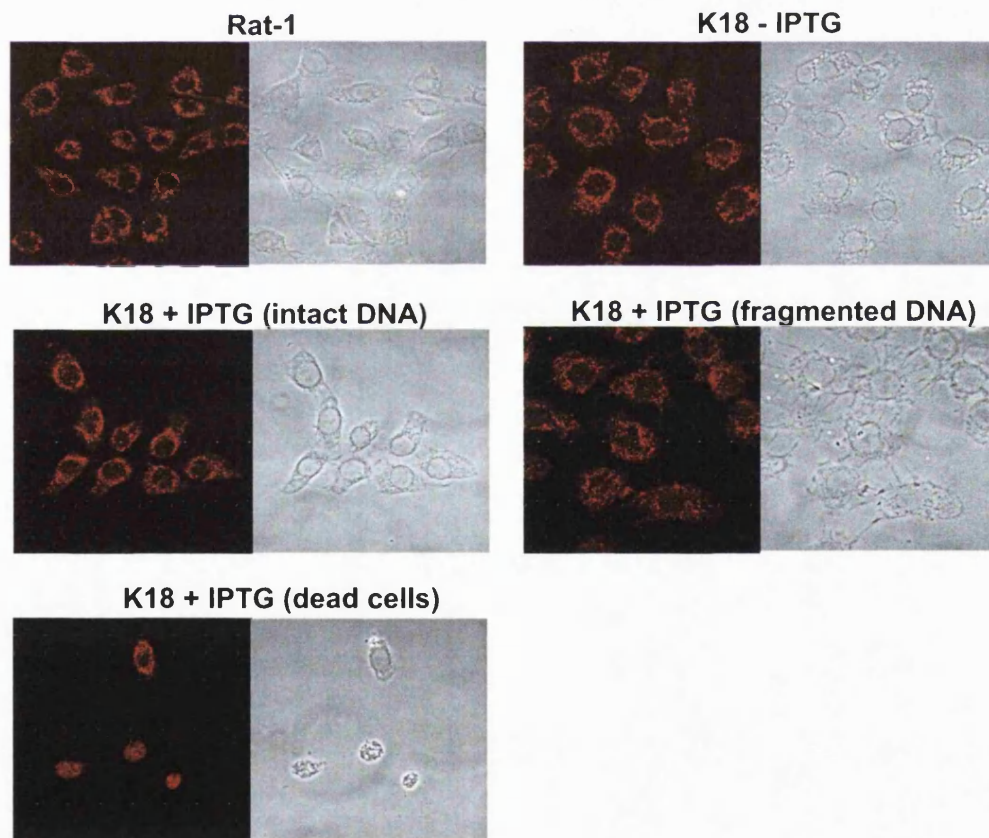


Fig. 3.8 Cytochrome C release upon K cyclin-induced apoptosis is preceded by nuclear DNA condensation. K18 Rat-1 cells growing on coverslips were grown for 24 h in the presence of 5mM IPTG to induce K cyclin expression, followed by growth in 0.02% FBS for 72 h to trigger apoptosis. Cells were fixed in methanol:acetone (50:50), immunostained using anti-cytochrome C antibody and DNA was counterstained with Hoechst. Coverslips were first analysed using a fluorescent microscope to visualise DNA stainings and cytochrome C localisation. Areas with condensed DNA, dead cells or live cells were next analysed using a laser scanning confocal microscope. Images shown are two-dimensional projections of Z-series (0.5 μ m intervals).

Cytochrome C was visualised by immunostaining followed by confocal analysis. As expected, K cyclin expression was associated with DNA condensation and the formation of dead round cells (Fig. 3.8). Many apparently healthy looking cells showed DNA condensation, but a punctate mitochondrial staining for cytochrome C. Cytosolic cytochrome C was only visible in the dead rounded cells (Fig. 3.8). Shorter serum starvation times did not affect this result. Thus, at least in Rat-1 cells, DNA condensation instead of cytochrome C release is the earliest apoptotic event associated with K cyclin expression. This is consistent with the Z-VAD experiment in which DNA condensation was visible even when caspases were inhibited (Fig. 3.4).

3.7 Expression of K cyclin in Rat-1 cells sensitises to apoptosis by various insults

Sublethal apoptotic insults can synergise to promote cell death (Evan et al., 1992; Hueber et al., 1997; Klefstrom et al., 1997). To assess which of these cooperate with K cyclin, K18 Rat-1 cells were exposed various apoptotic triggers. Apoptosis was quantified using PI staining and flow cytometric analysis. Similar to growth factor deprivation, treatment of cells

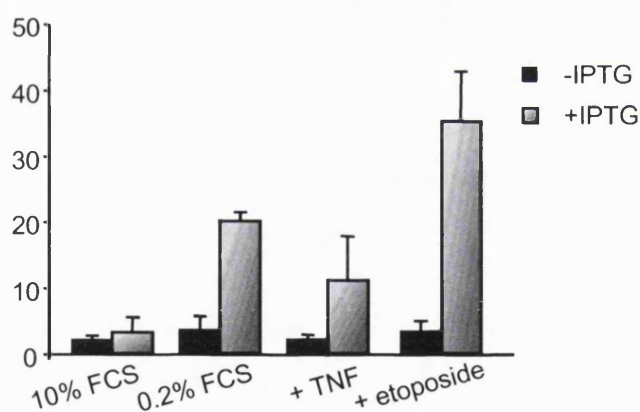


Fig. 3.9 Expression of K cyclin in Rat-1 cells sensitises to various apoptotic stimuli. K18 Rat-1 cells were seeded at 5×10^4 cells per 6 cm dish and stimulated the next day with 5mM IPTG. 24 h later the medium was changed and cells were grown in the presence of 10% or 0.02% FBS, TNF (50ng/ml) or etoposide (0.1 μ M) for another 72 h. Cells were harvested and fixed in 70% EtOH, stained with PI and analysed by flow cytometry. The percentage of cells with a sub G1 content is shown. Results are the mean and standard deviation of 4 experiments.

with TNF sensitised K cyclin-expressing cells to apoptosis. However, the strongest apoptotic response was measured upon treatment of cells with the DNA damage-inducing DNA topoisomerase inhibitor etoposide (Fig. 3.9). The apoptotic potential of K cyclin expression in a particular cell is therefore most likely influenced by survival and/or death signals residing in the microenvironment of the cell.

3.8 Discussion

The results presented in this chapter show that, in addition to its previously established ability to stimulate cell cycle progression, expression of K cyclin in Rat-1 cells also triggers an apoptotic response. This conclusion is based on the typical apoptotic morphology of cells associated with K cyclin expression including DNA condensation and fragmentation, membrane blebbing and phosphatidylserine exposure, together with the observation that caspase inhibition and viral Bcl-2 expression inhibit K cyclin-induced cell death. These results were supported by a subsequent publication by the Makela group, in which they report K cyclin-induced apoptosis in U2OS cells. However, in their experiments transient co-transfection of Cdk6 with K cyclin was required to efficiently trigger apoptosis (Ojala et al., 1999). Perhaps the levels of ectopic cyclin or endogenous Cdks were limiting in their experiments. Indeed, the inducible K cyclin expression did not trigger apoptosis directly, but rather, sensitised cells to various apoptotic. Together, these results therefore emphasise that the outcome of K cyclin expression with regards to cell survival is not only dependent on cell type, and the spectrum of endogenous Cdks, but also on apoptotic stimuli in the cellular environment.

K cyclin-induced apoptosis is caspase-dependent, since Z-VAD delayed the appearance of round apoptotic cells (Fig. 3.3). Interestingly, some form of DNA condensation was still visible in blebbing Z-VAD treated cells. One possible explanation for the residual DNA condensation may be the release of caspase-independent DNA condensation factors such as EndoG, apoptosis inducing factor (AIF) or AIF-homologues such as AMID from the mitochondria (Daugas et al., 2000; Li et al., 2001; Wu et al., 2002). It is not clear how these proteins are exported out of the mitochondria, or what are their nuclear targets. Experiments performed primarily in the Kroemer lab have shown an involvement of the oxidoreductase AIF in a so-called phase I initial nuclear condensation, which is associated with large scale DNA fragmentation (Susin et al., 2000). Of note, the images of phase I DNA condensation are similar to those in Z-VAD treated K cyclin expressing cells (compare Fig. 3.3). Furthermore, AIF is released from the mitochondria prior to cytochrome C and can actually promote cytochrome C release and the subsequent activation of downstream caspases, followed by full oligonucleosomal DNA fragmentation and phase II DNA condensation (Daugas et al., 2000; Susin et al., 1999). Thus, K cyclin expression may promote early nuclear apoptosis via the release of mitochondrial proteins like AIF, followed by late cytochrome C release and full-blown nuclear and cellular apoptosis. This is consistent with the finding that cytochrome C release was only observed in dead cells and was preceded by nuclear collapse (Fig. 3.8).

One pathway by which c-Myc is thought to trigger apoptosis is via the early caspase-independent release of cytochrome C from the mitochondria. This c-Myc mitochondrial pathway differs in several ways from mitochondrial effects associated with K cyclin-induced apoptosis. For example, cytochrome C release is observed in many obviously healthy looking cells with intact nuclear DNA (Juin et al., 1999) (and personal communication with Philippe Juin). Second, caspase inhibition is reported to fully inhibit DNA condensation upon c-Myc-induced apoptosis (McCarthy et al., 1997). Thus, cytochrome C release upon c-Myc expression, rather than the activation of caspase-independent nuclear condensation factors, is a primary mitochondrial effect upon c-Myc expression. This is consistent with the findings that microinjection of holocytochrome C (hcC) recapitulates the sensitisation to apoptosis by c-Myc and does not cooperate with c-Myc-induced apoptosis (Juin et al., 1999). If it is true, therefore, that K cyclin does not primarily act by releasing mitochondrial cytochrome C in the cytosol, one hypothesis is that K cyclin cooperates with hcC microinjection to trigger apoptosis. Attempts to address this premise failed because K18 Rat-1 cells induced to express K cyclin died as soon as they were even mock injected (in collaboration with Philippe Juin). A tempting speculation would be that K cyclin expression promotes the formation of selective mitochondrial channels that release proteins like AIF, while c-Myc promotes formation of cytochrome c specific channel. I have however not pursued experiments to pinpoint these issues. Nonetheless, the above considerations emphasise the absence of one universal but rather the existence of multiple mechanisms for oncogene-induced apoptosis, further warranting studies to assess oncogene-specific apoptotic mechanisms.

Apoptosis induced by K cyclin likely involves a mitochondrial component, as it is inhibited by KSBcl-2, but not by cellular Bcl-2 expression. A similar result was published by the Makela group (Ojala et al., 1999). Both structural modelling and functional data imply that the anti-apoptotic properties of KSBcl-2 differ from cellular Bcl-2. Importantly, KSBcl-2 lacks part of an unstructured loop region that can be cleaved by caspases and as such converts Bcl-2 to a pro-apoptotic protein (see introduction of this chapter). KSBcl-2 may thus evade pro-apoptotic conversion upon caspase cleavage. Moreover, K cyclin/Cdk6 complexes can phosphorylate and thereby inactivate cellular Bcl-2 (Ojala et al., 2000), accounting for the inability of Bcl-2 to inhibit K cyclin-induced apoptosis (Fig. 6B and (Ojala et al., 1999). Together, these intrinsic differences most likely explain why KSBcl-2 is more effective in inhibiting K cyclin-induced apoptosis compared to cellular Bcl-2.

K cyclin-induced apoptosis was more pronounced than apoptosis induced by ectopic cyclin D1, either with or without cyclin E (Fig. 3.2). Quantitative differences in microinjection studies were controlled as much as possible via the use of plasmids with identical backbones and careful determination of plasmid concentrations. However,

quantitative differences may also arise because of intrinsic differences between cyclins that cannot be controlled, such as the robust activation of both G1 and S phase Cdk activities by K cyclin/Cdk complexes (Ellis et al., 1999; Laman et al., 2001a; Mann et al., 1999), together with the fact that K cyclin/Cdk activities are not inhibitable by Cdk inhibitors (Swanton et al., 1997). Other differences are protein localisation or stability, although expression of a stable cyclin D1 mutant did not lead to higher percentages of apoptosis compared to wild type cyclin D (data not shown). Thus, assuming that K cyclin/cdk activity is the sum of uninhibitable G1 and S phase Cdk activities, the potent apoptotic response of which is determined by these quantitative differences, then it remains possible that these findings give insights into a general mechanism for apoptosis induced by deregulated expression of cyclin/Cdks. Furthermore, because comparisons of the biological effects triggered by different cyclins are complicated by the above, subsequent experiments were designed to mainly assess mechanisms of K cyclin-induced apoptosis.

v-FLIP did not measurably inhibit K cyclin-induced apoptosis, although v-FLIP protein was detectably expressed and able to signal NF- κ B transcriptional activation (Fig. 3.7). However, v-FLIP was also unable to prevent CD95 killing in Swiss 3T3 cells, which contrasts with the previously published inhibition of CD95 killing by FADDdn (Hueber et al., 1997). One explanation might be that expression levels of v-FLIP were too low to have a measurable effect. A second reason may be the effect of v-FLIP expression is cell type dependent and relies on high expression levels of the CD95 receptor such as measured in lymphocytes. Since these experiments were done, KS v-FLIP was reported to prevent death receptor-mediated killing in B cells and in this manner promote B cell tumourigenesis (Djerbi et al., 1999). Furthermore, the Makela group reported on a similar lack of a v-FLIP-mediated effect on apoptosis triggered by K cyclin in U2OS cells (Ojala et al., 1999). Instead of continuing studies to establish whether this is a true result, I therefore decided to await results of the K cyclin +/- v-FLIP transgenic mice (see results chapter 6) to see if enhanced tumourigenesis was measured upon v-FLIP expression.

A strong nuclear accumulation of K cyclin after microinjection of expression constructs was detected in Rat-1 cells (Fig. 3.1). The nuclear accumulation of cyclin D/Cdk complexes is facilitated by the p21^{Cip1} and p27^{Kip1} Cdk inhibitors (Cheng et al., 1999; Diehl and Sherr, 1997; LaBaer et al., 1997). However, cyclin D nuclear accumulation still occurs, although less efficiently, in p21^{Cip1}/p27^{Kip1} null MEFs, implying that the p21^{Cip1}/p27^{Kip1} NLS is not strictly required for nuclear import of cyclin D (Cheng et al., 1999). Another model suggests that p21^{Cip1} inhibits cyclin D1 nuclear export (Alt et al., 2002). Presumably, this also requires the binding of p21^{Cip1} to cyclin D1/Cdk complexes. However, K cyclin/Cdk complexes fail to interact with p21^{Cip1} and p27^{Kip1} Cdk inhibitors (Swanton et al., 1997) and

the nuclear localisation of K cyclin is therefore independent from p21^{Cip1}/p27^{Kip1} and is likely to be different from cyclin D. I could not identify an obvious basic NLS sequence in K cyclin, neither a bipartite ((K/R)₂-10spacer-(K/R)_{3/5}) sequence such as in p21^{Cip1}, nor a lysine rich stretch (P(1-3x)(K/R)_{3/4} or (K/R)_{3/4}H/P) such as in SV40 large T or nucleoplasmin (Gerace, 1995). Notably, cyclins E and B also lack an obvious basic NLS, but their nuclear import still occurs via a classical importin α/β -mediated mechanism (Moore et al., 1999). It therefore remains possible that K cyclin nuclear import is mediated by importins. Alternatively, nuclear export of K cyclin may somehow be compromised.

Rat-1 cells lack p21^{Cip1} expression. This highlights a problem with the use of established tissue culture cell lines such as Rat-1, since they usually harbor mutations in their apoptotic or cell cycle machinery. I was interested to investigate the molecular pathway of K cyclin-induced apoptosis, including the involvement of 19^{ARF} (see chapter 4). However, the p19^{ARF} message is also undetectable in Rat-1 cells, as established by Northern blotting with a p19^{ARF} exon 1 specific probe (Lomax and Fried, 2001; Swafford et al., 1997). Thus, to further investigate the molecular mechanism of apoptosis by K cyclin, I initiated experiments using early passage primary mouse embryo fibroblasts (MEFs) that express the full repertoire of cellular and apoptotic proteins.

CHAPTER 4:
ACTIVATION OF P53 UPON K CYCLIN EXPRESSION

4.1 Introduction

In the previous chapter I showed that K cyclin could stimulate apoptosis via a pathway that is caspase-dependent and involves a mitochondrial component. However, studies to address the molecular pathway of apoptosis are difficult in cell lines such as Rat-1. First of all, the specific roles of proteins are difficult to assess, as no “knock-out” cell lines lacking expression of individual proteins of interest exist. Second, no extensive array of anti-rat specific antibodies exists. Third, and most importantly, tissue culture cells grow under continuous stress that is imposed by tissue culture per se (see discussion in Sherr and DePinho, 2000). One of these factors may be DNA damage caused by the exposure to supraphysiological oxygen concentrations. Immortal tissue culture lines have therefore probably been selected for resistance to apoptosis. Indeed many lines such as Rat-1 harbor mutations in their apoptotic or cell cycle machinery, such as p21^{Cip1} promoter methylation and lack of p19^{ARF} expression. I therefore decided to perform further experiments in early passage primary MEFs. These cells are cytogenetically normal and can be derived from mice lacking individual genes, allowing the assessment of their roles in K cyclin-induced proliferation and apoptosis.

Around the time of these experiments, oncoproteins like E2F1, E1A, c-Myc and Ras were shown to trigger apoptosis via the ARF/p53 pathway (Bates et al., 1998; de Stanchina et al., 1998; Palmero et al., 1998; Zindy et al., 1998). p19^{ARF} protein (or human p14^{ARF}) induced by activated oncoproteins associates directly with Mdm2 protein and neutralises its ability to promote the ubiquitination and degradation of p53 (Kamijo et al., 1998; Pomerantz et al., 1998; Zhang et al., 1998). This results in p53 stabilisation and consequent cell cycle arrest and/or apoptosis. Importantly, in human cells, E2F1 is able to directly activate expression of p14^{ARF} by activating transcription from the p14^{ARF} promoter (Bates et al., 1998). Since expression of K cyclin leads to strong phosphorylation of Rb (Fig. 3.5 and Child and Mann, 2001) and subsequent release of E2Fs, I therefore hypothesised that K cyclin-induced apoptosis was via an E2F1/p19^{ARF}/p53 pathway.

Interestingly, KSHV encodes multiple proteins that are known to interfere with the activation of p53. One of these, latency-associated nuclear antigen (LANA) encoded by ORF73, is abundantly expressed in latently infected KS cells and B cell neoplasms (Kellam et al., 1999; Parravicini et al., 2000). Similar to v-FLIP, LANA is transcribed together with K cyclin on the same polycistronic mRNA transcript (Fig. 1.9, chapter 1 and Dittmer et al., 1998; Zhong et al., 1996). LANA has been shown to bind p53 directly, inhibiting its transcriptional activation and thereby preventing apoptosis (Friborg et al., 1999). However, in addition to p53, LANA is also able to bind and regulate the function of other proteins. Among these are binding and inhibition of Rb (Radkov et al., 2000) and binding to KSHV origin of

plasmid replication (oriP) DNA and host Histone H1, which may aid in tethering of the episomal viral DNA to host chromosomes (Ballestas et al., 1999; Cotter and Robertson, 1999). It is unclear which of these functions are important for successful viral infection. Taken together, I decided to use MEFs to investigate the molecular mechanism of K cyclin-induced apoptosis, specifically the involvement of the ARF/p53 pathway, and potential anti-apoptotic effect of LANA expression.

4.2 Transient transfection of MEFs with K cyclin-encoding plasmid triggers apoptosis that involves a mitochondrial component

Initial experiments to determine whether K cyclin expression induces apoptosis in MEFs utilised transient co-transfection assays with β -galactosidase-encoding plasmid as a marker for transfection. This so-called “blue cell assay” relies on ability of β -galactosidase-transfected cells to hydrolyse X-Gal substrate into a blue product, and the visual scoring of round detached cells as dead and flat cells as live. This assay is less laborious than microinjections since many conditions can be tested in a shorter time-frame. Transient expression of K cyclin in wild type (wt) MEFs growing in 10% FBS resulted in the induction of 31% apoptosis compared to 14% with empty vector (Fig. 4.1A). Since serum contains multiple survival factors that could suppress K cyclin-induced cell death, apoptosis was also assessed in MEFs cultured in low (0.2%) FBS. K cyclin-induced apoptosis was only marginally increased to 39%, suggesting that K cyclin killing in this particular assay is largely refractory of serum survival factors.

To explore a potential role of the mitochondrial apoptosis pathway in K cyclin-induced apoptosis in MEFs, the ability of Bcl-2 to inhibit K cyclin-induced cell death was assessed. Expression of human Bcl-2 did not inhibit K cyclin-induced apoptosis (Fig. 4.1B), consistent with its reported functional inactivation upon phosphorylation by K cyclin/Cdk6 complexes (Ojala et al., 2000). In contrast, viral KSBcl-2 partially inhibited K cyclin-induced apoptosis. In contrast, E2F1-induced apoptosis was significantly inhibited not only by KSBcl-2, but also by human Bcl-2 (Fig. 4.1B), showing that both Bcl-2 expression constructs were functional.

Although the inhibition of apoptosis by KSBcl-2 was significant but not very convincing, the involvement of the mitochondrial pathway in K cyclin-induced apoptosis was further assessed using a dominant negative (DN) caspase 9 (Fearnhead et al., 1998). Caspase 9 DN can bind apoptosome cofactors such as Apaf-1 and Cytochrome C but is unable to undergo autocatalytic processing, because it contains a point mutation in the

catalytic site (Cys→Ser²⁸⁷). As a result, caspase 9 DN competes with endogenous caspase for the binding of cofactors, thereby preventing activation. As is shown in Fig. 4.1B, K cyclin-induced apoptosis was around 50% inhibited upon coexpression of caspase 9 DN. Together, these data therefore show that, similar to expression of K cyclin in Rat-1 cells, expression of K cyclin in rodent fibroblasts triggers apoptosis that involves a mitochondrial pathway.

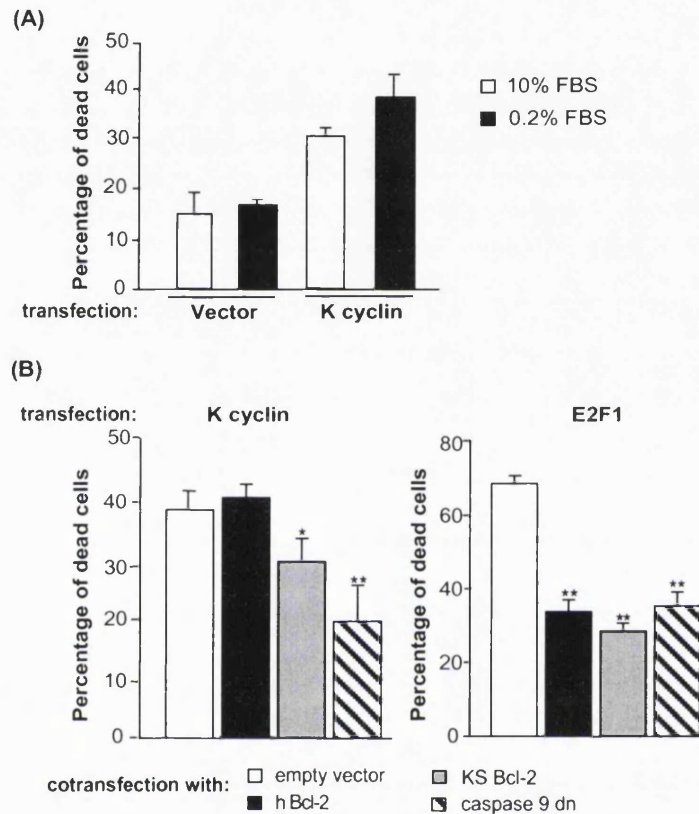


Fig. 4.1 Expression of K cyclin in MEFs triggers an apoptotic response that involves a mitochondrial pathway. (A) Wt C57Bl/6 x 129 MEFs were transfected with 2FKpcDNA3 or empty plasmid plus pEFLacZ reporter plasmid. After transfection, cells were grown for 24 h in medium containing 10% or 0.2% FBS. Cells were stained for β -Galactosidase activity and cell death was scored based on cell morphology. Data shown are the mean percentages of dead cells \pm SEM of three independent experiments. (B) MEFs were co-transfected with K cyclin or E2F1-encoding plasmids and pEFLacZ reporter plasmid plus the indicated vectors encoding anti-apoptotic proteins. After transfection, cells were grown for 24 h in medium containing 0.2% FBS and apoptosis was quantified as described in (A). Data shown are the mean percentages of dead cells \pm SEM of three independent experiments. When indicated, the inhibition of apoptosis was statistically significant as calculated by Student's t-test ($p < 0.02 = **$, $0.02 < p < 0.05 = *$).

4.3 Retroviral expression of K cyclin in MEFs sensitises to apoptosis by various insults

Next, K cyclin was introduced in early passage mouse embryo fibroblasts (MEFs) by retroviral infection, since biochemical assays can be performed with lysates of such cells. K cyclin was expressed from pBMNIresEGFP (K cyclin pBMN) retrovirus vector, that

dicistronically directs the concomitant expression of K cyclin and enhanced green fluorescent protein (GFP) (Fig. 4.2A). Expression of K cyclin from this vector was confirmed by immunoblotting (Fig. 4.2B), using pBabepuro infected cells as a control. Following transduction, around 80% of the cells were GFP-positive, and the apoptotic cells in this population were identified by Annexin V and Via-Probe staining using flow cytometric analysis (see materials and methods for the binding characteristics of these apoptotic markers). The validity of this assay was confirmed by sorting the population of Annexin V+/Via-Probe+ cells, which yielded only blebbing or rounded dead cells, compared to viable cells in the unlabelled population (Fig. 4.2C).

Only a small increase in apoptosis above background was measured in K cyclin-

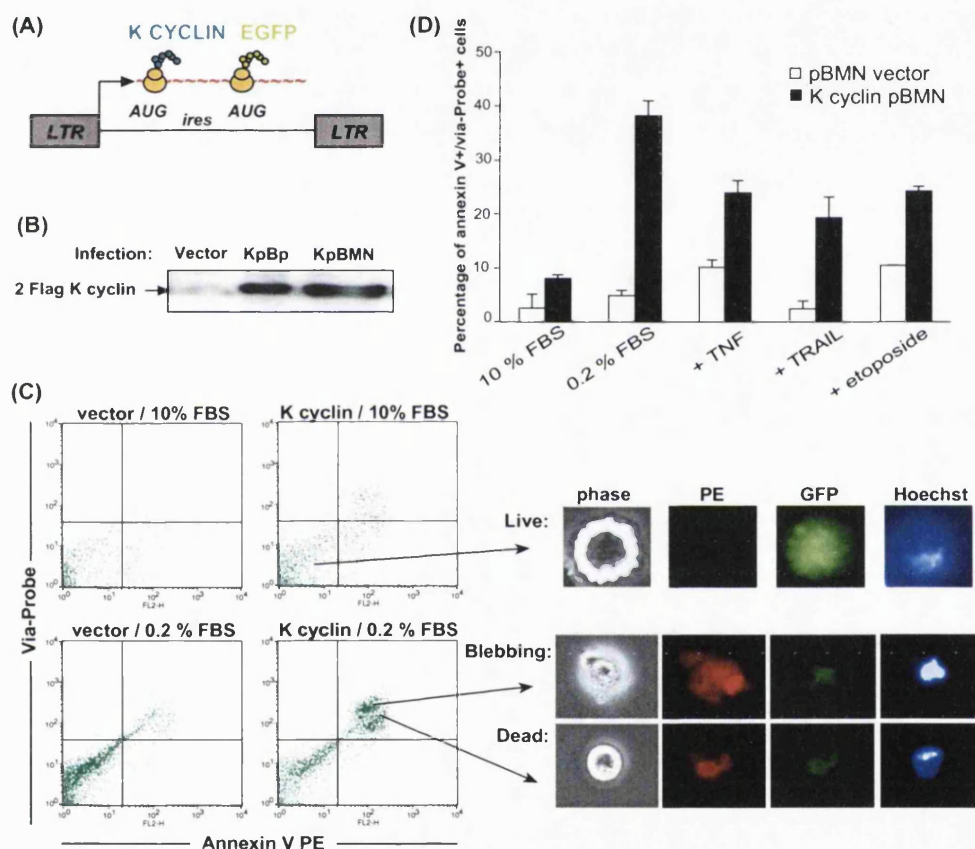


Fig. 4.2 Retroviral expression of K cyclin sensitises MEFs to apoptosis by various triggers. (A) Schematic depicting characteristics of the pBMNIresEGFP retrovirus system. LTR= long terminal repeat; EGFP= enhanced green fluorescent protein; ires= internal ribosomal entry site; AUG= startcodon. (B) Immunoblotting analysis of Flag-tagged K cyclin in MEFs infected with KpBMN, KpBpuro or empty vector control using M2 anti-Flag antibody. Lysates were prepared 48 h after infection and 20 μ g of protein per sample was resolved on a 12% SDS-PAGE gel. (C) MEFs were infected for two subsequent days (8 h each) with KpBMN or empty pBMN vector. After overnight recovery, cells were cultured for 48 h in medium containing 10% FBS or 0.2% FBS. Floating and adherent cells were collected and stained with Annexin V-PE and Via-Probe. Cells were sorted by flow cytometry and analysed by phase or fluorescence microscopy. (D) MEFs were infected for two subsequent days with KpBMN or empty vector and next grown for 48 h in medium containing 10%FBS, 0.2% FBS, etoposide (5.0 μ M), TNF (50 ng/ml) or TRAIL (200 ng/ml). Cells were stained as described in C) and next analysed by flow cytometry. Shown are the mean percentages of dead cells \pm SEM of 3 independent experiments.

expressing cells grown in 10% FBS (around 8%, compared with 3% upon mock infection) (Fig. 4.2D). One reason for this might be a low level of expression upon transduction of K cyclin. However, analogous to IPTG-inducible expression in Rat-1 cells (Fig. 3.5), the presence of sub-lethal levels of K cyclin may still sensitise cells to apoptosis by other apoptotic stimuli. K cyclin-transduced cells were therefore subjected to various pro-apoptotic stimuli for 48 h. While neither the growth of MEFs in 0.2% serum nor treatment of these cells with the DNA damaging agent etoposide, TNF or TRAIL ligand induced any significant cell death (around 5-12% apoptosis), expression of K cyclin together with any of these insults triggered pronounced apoptosis (Fig. 4.2D). The sensitisation to apoptosis by K cyclin was especially manifested in cells grown in 0.2% FBS, in which conditions around 40% of the cells underwent apoptosis. Subsequent experiments addressing K cyclin apoptosis were therefore performed with cells grown in 0.2% FBS. Thus, analogous to the experiments in Rat-1 cells, even when K cyclin expression does not kill MEFs directly, it induces a state of profound sensitisation to induction of apoptosis by DNA damage, death receptor engagement and/or disruption of the mitochondrial integrity.

4.4 K cyclin-induced apoptosis requires p53 but not E2F1 or p19^{ARF}

Expression of K cyclin leads to strong phosphorylation of Rb (Fig. 3.5 and Child and Mann, 2001), which will result in the release of E2Fs. In human cells, expression of E2F1 can trigger the transcriptional upregulation of p19^{ARF} protein (Bates et al., 1998). I therefore asked whether p19^{ARF} protein levels are increased upon K cyclin expression. K cyclin protein was retrovirally expressed in MEFs growing in 10% FBS and 72 h later the cells were assayed for p19^{ARF} protein expression by immunoblotting. A lysate of p53^{-/-} MEFs was run as a positive

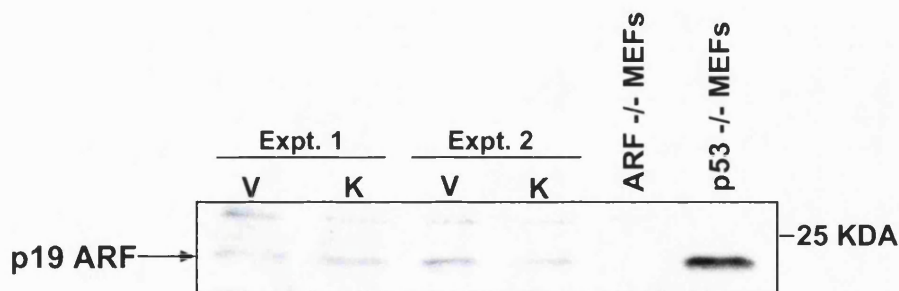


Fig. 4.3 K cyclin expression is not associated with an increase in p19^{ARF} protein levels. Wt C57Bl/6 x 129 MEFs were infected for 24 h with K cyclin-encoding (K) or empty (V) pBabepuro retrovirus. Infected cells were selected by growth in puromycin for two days and grown for a further 72 h in medium containing 10% FBS. SDS whole protein lysates were prepared and equal amounts (20 µg) of protein per sample were resolved on a 15% SDS-PAGE gel and immunoblotted using anti-p19^{ARF} antibody. Lysates from ARF^{-/-} and p53^{-/-} MEFs were run as negative and positive controls, respectively

control, as these cells contain high levels of endogenous p19^{ARF} due to the lack of a poorly defined p53-mediated negative feedback loop (Stott et al., 1998). No upregulation of p19^{ARF} protein upon K cyclin expression was observed (Fig. 4.3). Furthermore, immunohistochemistry using a p19^{ARF} antibody did not reveal a spotted nucleolar staining pattern that is typically seen upon increases in p19^{ARF} protein levels (Quelle et al., 1995; Weber et al., 1999) (data not shown). Thus, K cyclin expression does not trigger an increase in p19^{ARF} protein levels.

The activation of p53 can occur in a p19^{ARF}-independent manner (Kamijo et al., 1997). The finding that p19^{ARF} is not induced upon K cyclin expression does therefore not exclude the possibility that K cyclin activates p53. To examine whether this is the case, I first asked whether p53 protein levels were increased upon K cyclin expression. Retroviral expression of K cyclin in MEFs growing in 10% FBS resulted in an upregulation of p53 protein levels (Fig. 4.4A). However, although an increased stability is a major mechanism by which p53 is activated, post-translational modifications of p53 protein are often required to induce its transcriptional activation (recently reviewed in Vousden, 2002). It was therefore shown that the upregulation of p53 protein levels correlated with an induction of the p53 target gene p21^{Cip1} (Fig. 4.4B), implying an upregulation of transcriptionally active p53 protein upon K cyclin expression. Importantly, K cyclin expression in *E2F1*^{-/-} and *ARF*^{-/-} MEFs also resulted in an increase in active p53 protein levels (Fig. 4.4A and B). Thus, consistent with the finding that K cyclin does not induce p19^{ARF} protein, K cyclin activates p53 via a mechanism that is does not require E2F1 and p19^{ARF}.

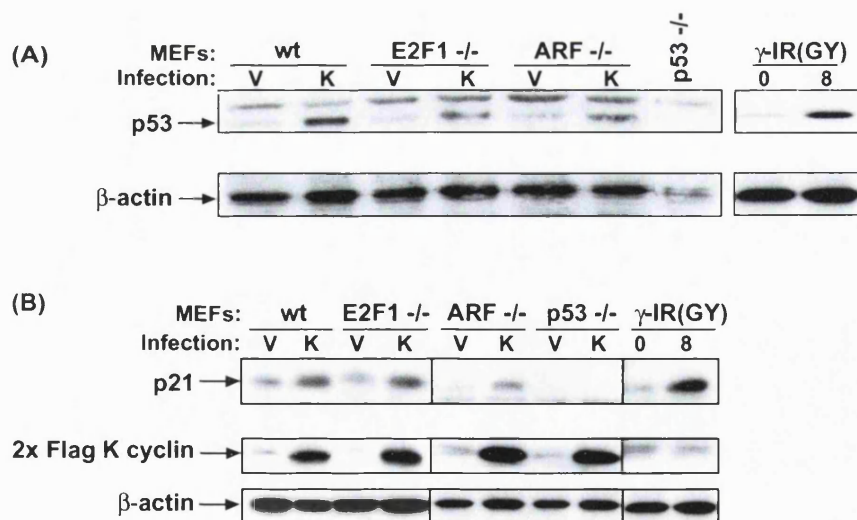


Fig. 4.4 K cyclin expression is associated with elevated p53 and p21^{Cip1} protein levels. MEFs of the indicated genotype were infected with empty (V) or K cyclin-encoding pBabepuro (K) vector for 24 h. Infected cells were selected by puromycin and grown for 72 h in medium containing 10% FBS until cell lysates were prepared. As a control, a subconfluent culture of wt MEFs was exposed to 8 GY of γ -irradiation and grown for a further 24 h. Proteins (20 μ g/lane) were resolved on 15% (p21) or 8% (p53) SDS-PAGE gels and analysed by immunoblotting with (A) CM-5 p53 or β -actin antibody or (B) Flag antibody to detect tagged K cyclin, CM-5 p53, p21 or β -actin antibody.

Next, I determined the requirement of p53 in K cyclin-induced apoptosis. K cyclin was transduced into wt, *E2F1*^{-/-}, *ARF*^{-/-} and *p53*^{-/-} MEFs, followed by culture of cells for 48 h in 0.2% FBS. Apoptosis was analysed by Annexin V and Via-probe staining. Levels of apoptosis induced by K cyclin were similar in wt, *E2F1*^{-/-} and *ARF*^{-/-} MEFs. In contrast, K cyclin induced substantially less apoptosis in *p53*^{-/-} MEFs as compared to wt MEFs (15% in *p53*^{-/-} cells compared to about 38% in other MEFs, Fig. 4.5A). Differences in the percentages of apoptosis as determined by flow cytometry correlated with the appearance of round, GFP-positive apoptotic cells (Fig. 4.5B). These results suggest that p53 is required, but E2F1 and p19^{ARF} are dispensable for K cyclin-induced apoptosis.

The requirement for p53 in K cyclin-induced apoptosis was confirmed by transient transfection experiments. Wild-type and *p53*^{-/-} MEFs were transiently transfected with K cyclin-encoding plasmid and apoptosis was measured using the “blue cell assay”. E2F1-

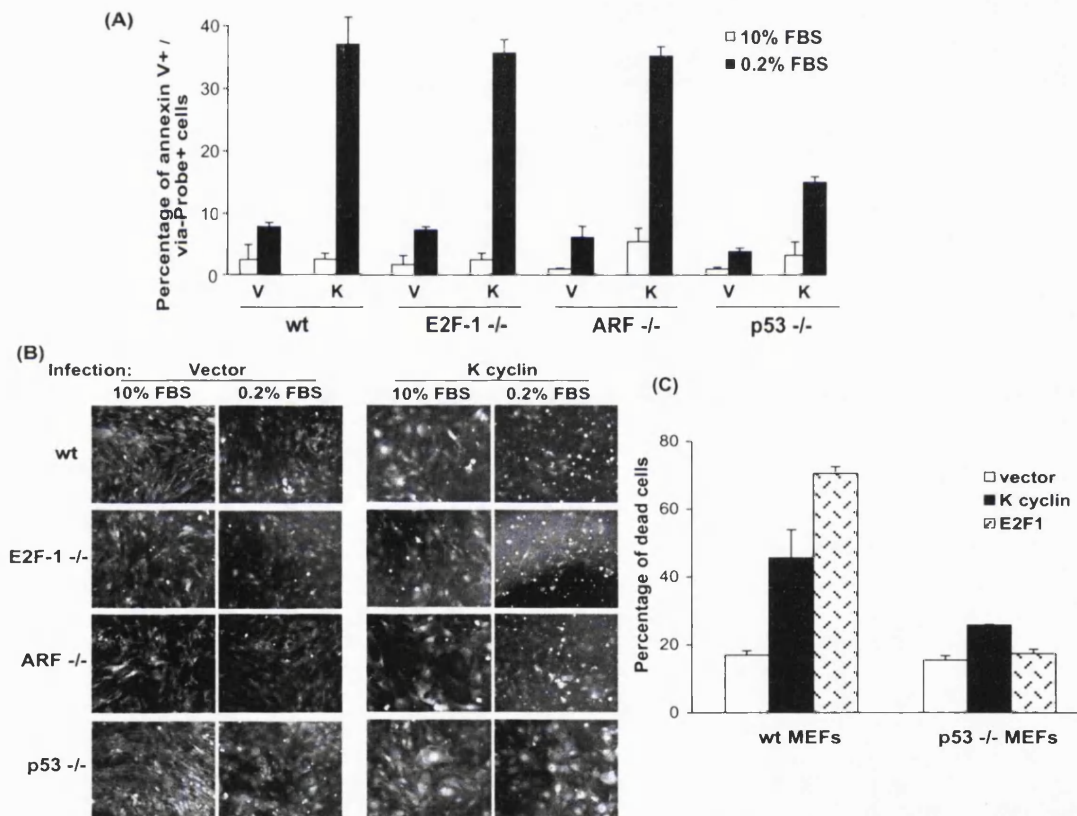


Fig. 4.5 K cyclin-induced apoptosis involves p53 but not E2F1 or p19^{ARF}. (A) Wt, *E2F1*^{-/-}, *p19^{ARF}*^{-/-} and *p53*^{-/-} MEFs were infected for two subsequent days (8 h each) with KpBMN (K) or empty pBMN vector (V). After overnight recovery, cells were cultured for 48 h in 10% FBS or 0.2% FBS-containing medium. Cells were stained with AnnexinV-PE and Via-Probe and analysed by flow cytometry to determine the percentage of apoptotic cells in the GFP-positive cell population. (B) Fluorescent microscopic analysis of GFP-positive MEFs that were infected as described in A). Pictures were taken at a magnification of 20x. (C) Wt C57BL/6 or *p53*^{-/-} MEFs were transfected with K cyclin- or E2F1-encoding plasmid together with LacZ reporter plasmid. After transfection cells were grown for 24 h in medium containing 0.2% FBS. The percentage of apoptotic cells was determined as described in Fig. 4.1A and are the means \pm SEM of three experiments.

induced apoptosis was included as a positive control, since apoptosis by E2F1 had previously been shown to be inhibited in *p53*^{-/-} MEFs, using a similar approach (Irwin et al., 2000). Both E2F1- and K cyclin-induced apoptosis were significantly reduced in *p53*^{-/-} MEFs compared with wt MEFs (Fig. 4.5C). Together, these data show that K cyclin-induced apoptosis does not proceed via a pathway that requires p19^{ARF} and/or E2F1, but involves p53. However, apoptosis upon K cyclin expression was not fully inhibited in *p53*^{-/-} cells, showing some level of p53-independent apoptosis was also likely to occur.

4.5 K cyclin expression triggers a p53-dependent, but p21-independent, growth arrest

During the course of the previously described studies, I noticed that K cyclin expressing cells exhibited a large and flattened phenotype resembling senescence. Oncogenes such as E2F1 and Ras can trigger growth arrest in primary cells (Dimri et al., 2000; Serrano et al., 1997), which prompted me to investigate if K cyclin also induces growth arrest in MEFs.

First, the proliferation capacity of mock and K cyclin-transduced MEF populations were determined. Following infection and selection, mock-infected wt, *E2F1*^{-/-} and *ARF*^{-/-} cells proliferated until they reached confluence (Fig. 4.6A, closed symbols), whereas cells transduced with K cyclin (Fig. 4.6A, open symbols) showed no net propagation. Importantly, K cyclin expressing *p53*^{-/-} MEFs expanded until confluent, although the rate of expansion was somewhat reduced compared with that in mock-infected cells (Figs. 4.6A and B). In contrast, no such inhibition of cell proliferation was observed upon ectopic expression of cellular cyclin D3 in wt MEFs, although protein expression was readily detectable by immunoblotting (Fig.4.6C). These data show that K cyclin expression, but not cyclin D expression, blocks the expansion of MEFs in a p53-dependent manner.

The lack of a net propagation of K cyclin expressing cells may result from an elevated rate of apoptosis relative to proliferation, or from an absence of proliferation due to a block in cell division. To investigate whether K cyclin blocks cell division time-lapse videomicroscopy was performed to follow cultures of infected MEFs. Representative QuickTime movies of these experiments are included on the CD in the back cover of this thesis (named wt pBp, wt K cyclin, *ARF*^{-/-} K cyclin, *p53*^{-/-} pBp and *p53*^{-/-} K cyclin). The quantitations of cell divisions in the movies show that while mock-infected wt MEFs proliferated until confluence, K cyclin expressing wt, *E2F1*^{-/-} and *ARF*^{-/-} and MEFs did not divide at all (See Fig. 4.6E for wt MEFs expressing K cyclin). However, *p53*^{-/-} MEFs expressing K cyclin divided and grew until confluent (Fig. 4.6E). In conclusion, constitutive

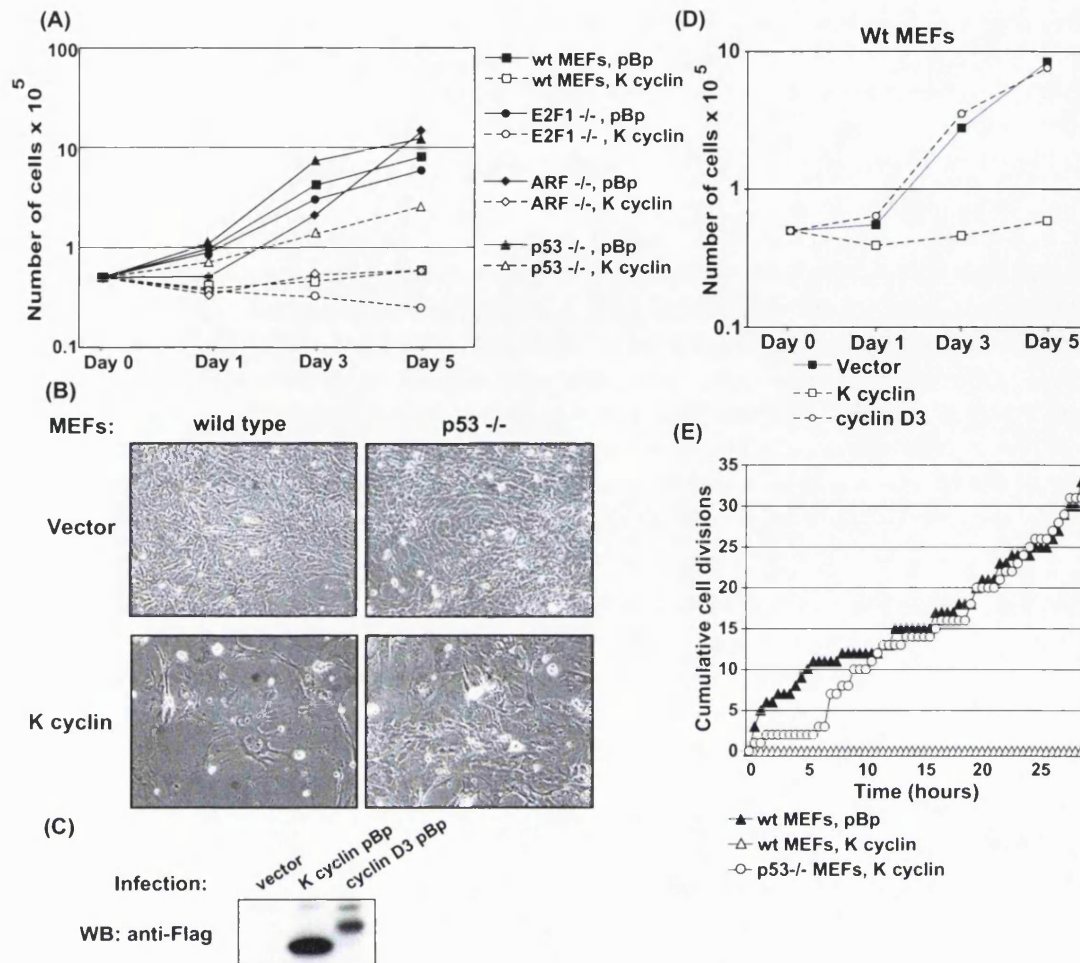


Fig. 4.6 K cyclin expression in MEFs triggers a p53-dependent growth arrest. (A) Growth curves of MEFs constitutively expressing K cyclin. Wt, *E2F1*^{-/-}, *p53*^{-/-} or *p19^{ARF}*^{-/-} MEFs were infected for 24 h with empty (pBp) or K cyclin-encoding pBabepuro retrovirus. Infected cells were selected by puromycin and grown in medium containing 10% FBS. Cell numbers were counted at day 1, day 3 and day 5 after plating. The growth curves represent the average of three independent experiments. (B) Microscopic analysis of cells at day 5 of the growth curve. Phase contrast pictures were taken at a magnification 10x. (C) Wt MEFs were infected with empty or Flag-tagged cyclin-encoding pBabepuro virus. Cell lysates were prepared 48 h after infection and protein lysates (20 μ g/lane) were resolved on a 12% SDS-PAGE gel and immunoblotted with anti-Flag antibody. (D) Growth curves of cells expressing cyclin D3 versus K cyclin or empty vector. Cells were infected and grown as described in A). (E) Cultures of MEFs infected and grown as in A) were followed by time-lapse videomicroscopy (see supplemented CDs in back cover for quicktime movies). Pictures were taken every 10 minutes and cultures were followed for 4 days. Movies were analysed for cell divisions and cumulative cell divisions of wt or *p53*^{-/-} MEFs expressing K cyclin were plotted.

expression of K cyclin induces both p53-dependent apoptosis and growth arrest via a mechanism that does not require E2F1 or p19^{ARF}.

A p53-dependent growth arrest is usually attributed to the Cdk-inhibitory action of the p21^{Cip1} protein (Agarwal et al., 1995; Stewart and Pietsch, 2001). Since p21^{Cip1} accumulates in cells expressing K cyclin (Fig 4.4), the potential role of p21^{Cip1} in growth

arrest was examined by determining whether a K cyclin-mediated arrest was mitigated in *p21*^{-/-} MEFs. A culture of *p21*^{-/-} MEFs expressing K cyclin arrested in a similar fashion compared to wt MEFs (Fig. 4.7A). However, *p21*^{Cip1} is not required for p53-dependent apoptosis (Attardi et al., 1996), and apoptosis by K cyclin was even enhanced in *p21*^{-/-} cells compared to wt MEFs (Fig. 4.7B). The lack of a net propagation of K cyclin expressing *p21*^{-/-} cells might therefore have arisen from an elevated rate of apoptosis relative to proliferation. However, time-lapse videomicroscopic analysis of *p21*^{-/-} cultures expressing K cyclin did not show any cell divisions, similar to wt MEFs (see QuickTime movie on supplementary CD, named *p21*^{-/-} K cyclin). Together, these data show that K cyclin induces a p53-dependent growth arrest that is independent of *p21*^{Cip1}.

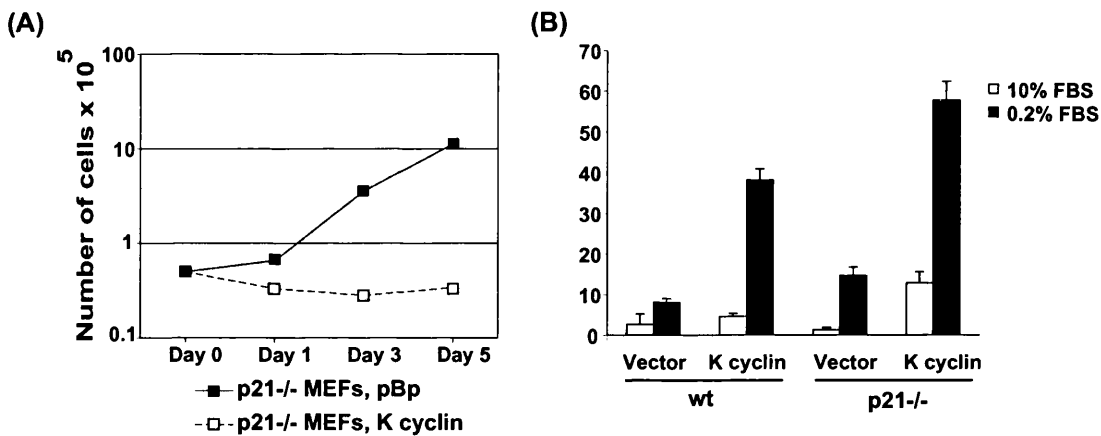


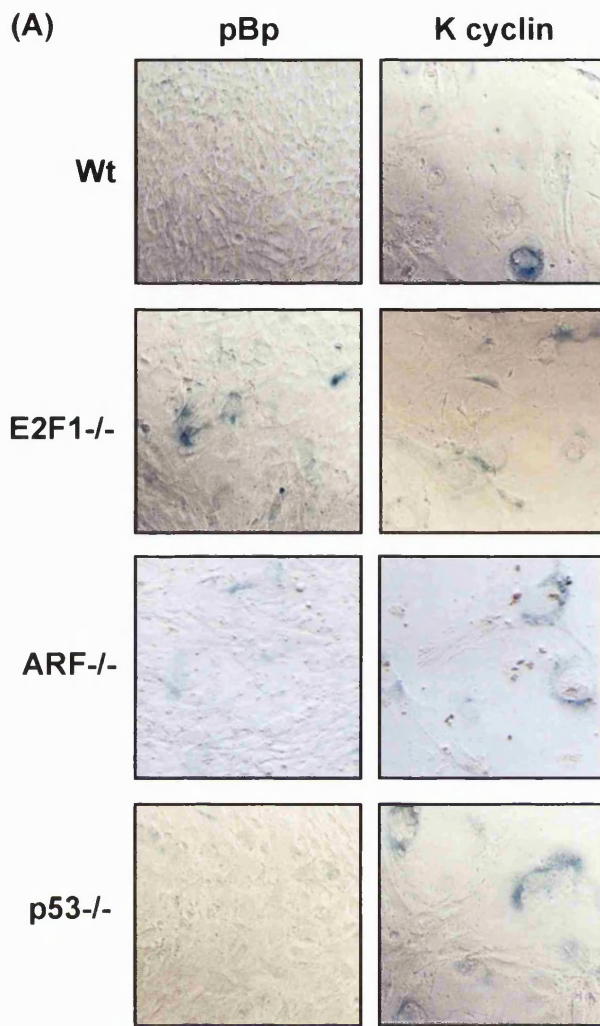
Fig. 4.7 The growth arrest by K cyclin is p21-independent. (A) Growth curves of *p21*^{-/-} MEFs infected with empty or K cyclin-encoding pBabepuro retrovirus and analysed essentially as described in Fig. 15A. (B). Wt and *p21*^{-/-} MEFs were retrovirally infected for two subsequent days (8 h each) with KpBMN (K cyclin) or empty pBMN vector. After overnight recovery, cells were grown for 48 h in 10% FBS- or 0.2% FBS-containing medium. Cells were stained with Annexin V-PE and Via-Probe and analysed by flow cytometry to determine the percentage of apoptotic cells in the GFP-positive cell population.

4.6 Expression of K cyclin in MEFs increases the expression of Senescence Associated β -Galactosidase

K cyclin-expressing growth-arrested MEFs became flat and enlarged. This phenotypic appearance is similar to that described for so-called cellular senescence. Cellular senescence is defined as a state of irreversible growth arrest and can be induced either upon repeated cell division (which in human cells is associated with telomere shortening) or upon “stress” signals, which includes strong mitogenic signalling upon oncogene expression (Campisi, 2001; Serrano and Blasco, 2001). Senescent cells not only show selected changes

in morphology, but also in metabolism. Some of the characteristic senescence-associated changes are increased lysosome biogenesis, secretion of large amounts of matrix metalloproteinases and inflammatory cytokines (Campisi, 2000). However, the most universally used marker for senescence is expression of a β -galactosidase that has a pH optimum of 6 (senescent-associated β -galactosidase, or SA- β -gal) (Dimri et al., 1995). I therefore asked whether SA- β -Gal activity was detected in K cyclin-expressing MEFs.

MEFs were transduced with 2FKpBpuro or control retrovirus and puromycin-resistant cells were cultured for a further two days. Cells were stained for SA- β -Gal activity and the numbers of blue, senescent cells versus unstained cells was determined. K cyclin expression in wt, *E2F1*^{-/-} and *ARF*^{-/-} MEFs led to a dramatic increase in the percentage of SA- β -Gal-positive cells (Fig. 4.8), consistent with the growth arrest induced upon K cyclin expression in such MEFs (see Fig. 4.6). Of note, proliferating mock-infected *E2F1*^{-/-} MEFs showed high background levels of SA- β -Gal activity, suggesting that SA- β -Gal expression does not strictly correlate with growth arrest. Furthermore, a significant proportion of K cyclin-transduced *p53*^{-/-} cells also became SA- β -Gal-positive (Fig. 4.8), which was surprising considering the observation that cultures of K cyclin-expressing *p53*^{-/-} cells did not undergo a growth arrest (Fig. 4.6). In particular, large multinucleated cells (see chapter 5 for detailed analysis of this phenomenon) stained positive for SA- β -Gal. Thus, K cyclin expression triggers expression of a senescence marker in at least a proportion of the cell population.



(B)

	pBp	K
Wt	1%	30%
E2F1-/-	43%	83%
ARF-/-	4%	64%
p53-/-	3%	50%

Fig. 4.8 K cyclin triggers an increased expression of the senescence marker SA- β -Gal.

(A) MEFs of the indicated genotypes were transduced for 24 h with empty pBabepuro or 2FkpBpuro retrovirus. Infected cells were selected by culture for 2 days in puromycin and plated at 1×10^5 cells per well in 6 well plates, followed by culture for another 2 days. Cells were fixed and stained for SA- β -Gal activity and representative photos were taken at a 20x magnification. (B) Quantitation of the experiment described in (A). Around 150-200 cells were scored for senescence marker (blue) or not (unstained) and indicated are the percentages of SA- β -Gal-positive cells.

4.7 K cyclin-induced apoptosis and growth arrest are not convincingly inhibited by LANA or by dnp53

The finding that p53 plays a central role in the execution of apoptosis and growth arrest by K cyclin is somewhat paradoxical, considering that viruses develop diverse strategies to avoid elimination of the infected cells. An attractive hypothesis is therefore that the activation of p53 is counteracted by the action of other viral proteins. KSHV ORF73-encoded LANA protein, which is widely expressed in latently infected cells, and had been reported to inhibit the transcriptional activation of p53, was therefore a likely candidate. I thus decided to investigate whether LANA inhibits the K cyclin-induced cellular effects.

I evaluated two constructs encoding LANA protein, called pcDNA3/73 and pcDNAHis/ORF73. pcDNA3/73 drives expression of full length untagged LANA protein, whereas pcDNAHis/ORF73 drives expression of His-tagged LANA protein together with very low levels of K cyclin protein (see materials and methods). The latter construct, without its His tag, was subcloned into the pBpuro retroviral vector. Western blot analysis of expressed LANA protein revealed a doublet of around 220-235 kDa in pcDNA3/73-transfected 293T cell lysates (Fig. 4.9A), which is in agreement with published results, (Friborg et al., 1999; Rainbow et al., 1997). In addition, three lower molecular weight bands around 160-200 kDa were detected, which presumably represent degradation products. The pcDNAHis/ORF73-encoded LANA protein migrated slightly slower (doublet at around 245-250 kDa) (Fig. 4.9A). Indeed, molecular polymorphism mapped to the number of repeat elements in the central LANA domain (see chapter 1, section 1.6.6) has been reported, probably explaining the difference in mobility between various LANA proteins (Gao et al., 1999). The presence of multiple protein bands is likely to reflect different post-translational modifications (Rainbow et al., 1997).

In experiments aimed to determine the inhibition of p53 by LANA, the C-terminal domain of mouse p53 (aa 302-390, referred to as DNp53) was expressed as a positive control. DNp53 contains the oligomerisation domain and prevents p53-mediated transcriptional activation by inhibiting the binding of full-length p53 to DNA (Shaulian et al., 1992). DNp53 expression was confirmed by immunoblotting (Fig. 4.9B). Importantly, endogenous p53 levels accumulated upon DNp53 expression. This most likely reflects the inhibition of the transcriptional upregulation of the p53 target gene Mdm2, and thus the lack of a p53 negative feedback loop (Kubbutat and Vousden, 1998), showing that DNp53 protein functioned as expected.

Next, luciferase assays using p53-responsive promoters were performed to establish whether the different LANA proteins were able to inhibit p53-mediated transcriptional

activation. Luciferase assays were performed with different cell lines, transfection methods and reporter constructs (Fig. 4.9C). Ectopic expression of p53 in 293T, wt MEFs or *p53*^{-/-} MEFs resulted in a 5.6-fold, 4.9-fold or 40-fold increase in the relative luciferase activity, respectively. Co-transfection of either of the LANA constructs or DNp53 with p53 resulted in a slight, but reproducible inhibition of the p53 luciferase activity (around 20-28% inhibition). These data show that transcriptional activation mediated by ectopic p53 is detectably, but modestly, inhibited by LANA. Unfortunately, expression of K cyclin did not result in an increase of specific luciferase activities, so that the effect of LANA on K cyclin-activated p53 could not be assessed. This may be due to the high background constitutive activation of p53, as γ -irradiation of cells also did not increase specific luciferase activities (data not shown).

To establish whether K cyclin-induced apoptosis was inhibited upon LANA transfection, I first performed co-transfection experiments. Similar to co-expression of KSBcl-2, K cyclin-induced apoptosis was inhibited by around 50% upon LANA co-expression Fig. 4.10A. However, apoptosis upon retroviral expression of K cyclin followed by growth of MEFs in 0.2% FBS was not inhibited by LANA expression (Figs. 4.10B and C). Similarly, retroviral LANA expression did not prevent the growth arrest induced upon K cyclin expression as established by time-lapse analysis (see figure legend Fig. 4.10D and QuickTime movies on the CD in the back cover of this thesis, named pBp+pBp, pBp+K cyclin, pBpDNp53+K cyclin, pBpLANA+K cyclin). The interpretation of these results were complicated by the finding that DNp53 expression was also unable to inhibit apoptosis upon both K cyclin expression and γ -irradiation of MEFs, although DNp53 expression did inhibit growth arrest upon γ -irradiation (data not shown). Together, these results imply that LANA may at least partially inhibit K cyclin-induced apoptosis, although the retrovirus experiments did not support this finding.

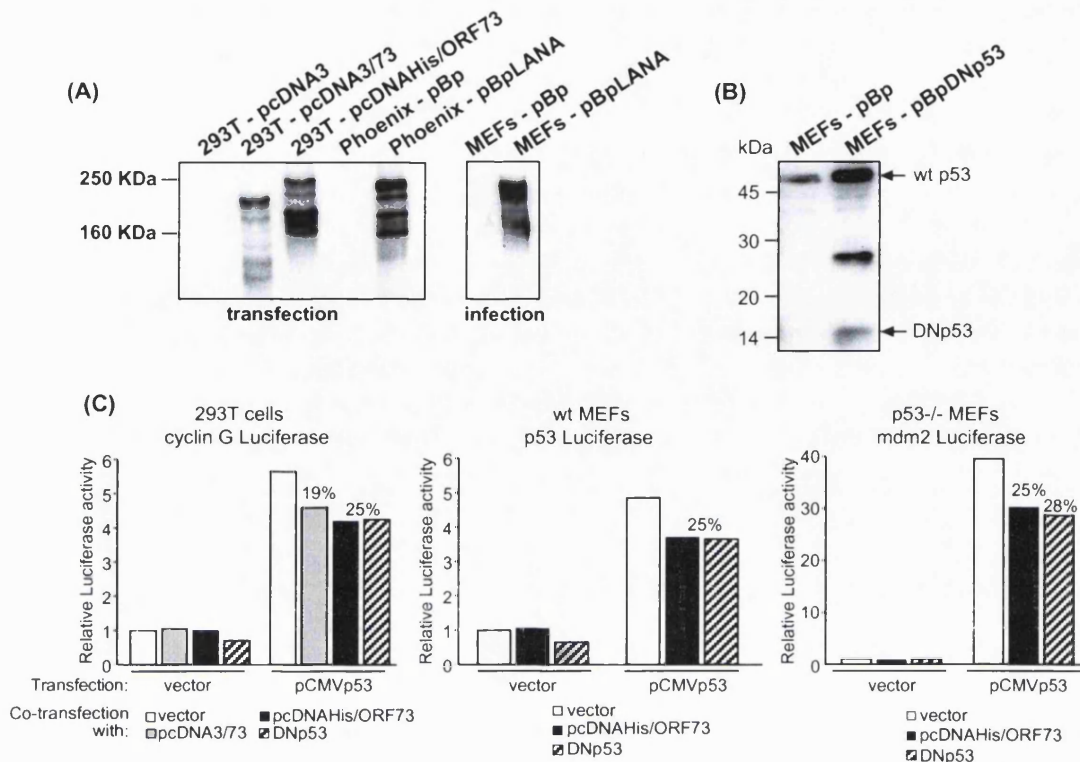


Fig. 4.9 Expression of LANA or DNp53 show a weak inhibitory effect on the transcriptional activation mediated by ectopically expressed p53. (A) 293T cells were transiently transfected with empty pcDNA3 or LANA-encoding pcDNA3/73 or pcDNAHis/ORF73 plasmids using Superfect reagent. SDS lysates were prepared 30 h after transfection. Phoenix retroviral producer cells were transfected with empty pBabepuro or pBpuroLANA vector using Lipofectamine Plus reagent and SDS lysates were prepared four days later. Wt MEFs were infected for 24 h with pBpuro or pBpuroLANA retrovirus and infected cells were selected by growth in puromycin for three days, after which SDS lysates were prepared. Equal amounts (20 μ g) of protein were resolved on a 6% SDS-PAGE gel and proteins were immunoblotted using anti-ORF73 antibody. (B) Wt MEFs were infected for 24 h with pBpuro or pBpurop53(302-390) (DNp53). Infected cells were selected by growth for 2 days in puromycin and cells were cultured for a further 2 days in normal growth medium. SDS lysates (20 μ g per lane) were resolved on a 15% SDS-PAGE gel and proteins were immunoblotted using CM-5 anti-p53 antibody. (C) 293T cells were split at 3×10^5 cells/well in 6 well plates. The next day, cells were transiently transfected using Superfect reagent with 0.05 μ g of pRL-TK and 0.1 μ g of pGL3cyclinG-luc reporter plasmids together with 1.0 μ g of pcDNA3 or pCMVp53 (as indicated) and co-transfected with 1.0 μ g of LANA or pBhydrop53(302-390) (DNp53) plasmid (as indicated). Alternatively, wt or p53^{-/-} MEFs were plated at 1×10^5 cells per well and transfected the next day using Fugene reagent. Transfection mixtures consisted of 0.05 μ g RL-TK and 1 μ g of p53luc or mdm2luc reporter plasmid together with 0.5 μ g of pcDNA3 or pCMVp53 (as indicated) and co-transfected with 0.5 μ g of LANA or DNp53 plasmid (as indicated). Cell lysates were prepared 24 h after transfection and the firefly and *Renilla* luciferase activities were measured using the Promega dual-luciferase reporter assay system, from which the relative luciferase activity was calculated. Results show the averages of two experiments and the percentage by which the luciferase activity is decreased upon LANA or DNp53 expression is indicated.

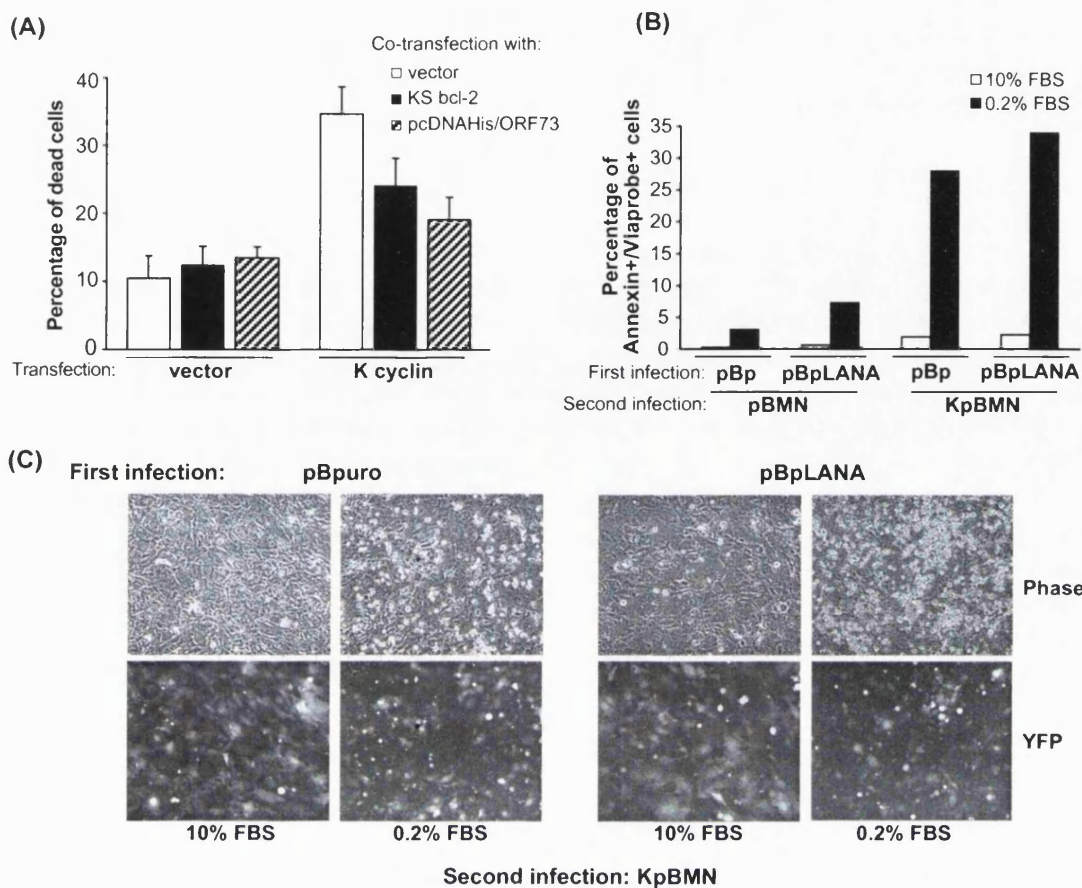


Fig. 4.10 Expression of neither LANA nor DNp53 significantly inhibits K cyclin-induced apoptosis or growth arrest. (A) Wt MEFs were transfected with cyclin-encoding or empty plasmid together with pEFLacZ reporter plasmid and were co-transfected with the indicated plasmids encoding LANA or KSBcl-2. After transfection, cells were grown for 24 h in medium containing 0.2% FBS and the percentage of dead cells was calculated as described in Fig. 10(A). Data shown are the mean percentages of dead cells \pm SEM of 3 (KSBcl-2) or 5 (others) independent experiments. (B) Wt MEFs (4×10^6 cells/10 cm dish) were infected for a first round with pBpuro or pBpuroLANA retrovirus for 24 h, after which infected cells were selected by growth in puromycin for 2 days. Infected cells were plated at 1×10^5 cells per well in a 6-well dishes and infected for a second round with KpBMN or empty pBMN vector for two subsequent days (8 h each). After overnight recovery, cells were cultured for 48 h in medium containing 10% or 0.2% FBS as indicated. Floating and adhered cells were collected and stained with Annexin V-PE and Via-Probe and analysed by flow cytometry to determine the percentage of apoptotic cells. (C) Fluorescent microscopic analysis of GFP-positive MEFs infected with KpBMN as described in (A). Pictures were taken at a magnification of 20x. (D) MEFs (4×10^6 cells/10 cm dish) were infected for 24 h with empty pBpuro, pBpurop53(302-390) or pBpuroLANA and infected cells were selected for 2 days in puromycin. MEFs were plated at 1×10^5 cells per well in a 6-well dishes and infected for a second round with empty pBMN or KpBMN retrovirus for two subsequent days (8 h each). After overnight recovery, the GFP-positive cells were FACS-sorted. Cells were plated at 1×10^5 cells per well in 6 well dishes and followed by time-lapse videomicroscopy. Pictures were taken every 10 minutes and cultures were followed for 4 days. Control cultures all grew until confluent, while K cyclin-expressing MEFs all growth-arrested, independent of the co-expression of LANA or DNp53 (see supplemented QuickTime movies).

Since the retrovirus experiments did not show an effect of LANA on apoptosis induced by K cyclin, I also tested an additional LANA retroviral construct obtained from J. Friberg and G. Nabel, who originally reported on the inhibition of p53 by LANA, called ppGS-orf73/LANA (Friberg et al., 1999). They showed that stable expression of LANA protein in NIH3T3 protected cells against apoptosis upon serum withdrawal, which they claimed to be p53-mediated. However, the NIH3T3 cell line available in our lab did not show significant apoptosis upon serum withdrawal. I therefore made stable cell lines that retrovirally expressed LANA or DNp53 proteins in Swiss 3T3 cells (DNp53 Swiss, ppGS-orf73/LANA Swiss and pBpLANA Swiss). Neither apoptosis upon serum withdrawal on its own (Fig. 4.11A), nor in conjunction with K cyclin expression (Fig. 4.11B and D) was reduced upon expression of either of the LANA proteins (Fig. 4.11B) or DNp53 (Fig. 4.11C). Similar results were seen using pBpLANA (data not shown). Thus, these experiments did not provide convincing evidence for the inhibition of K cyclin-induced apoptosis by either DNp53 or LANA.

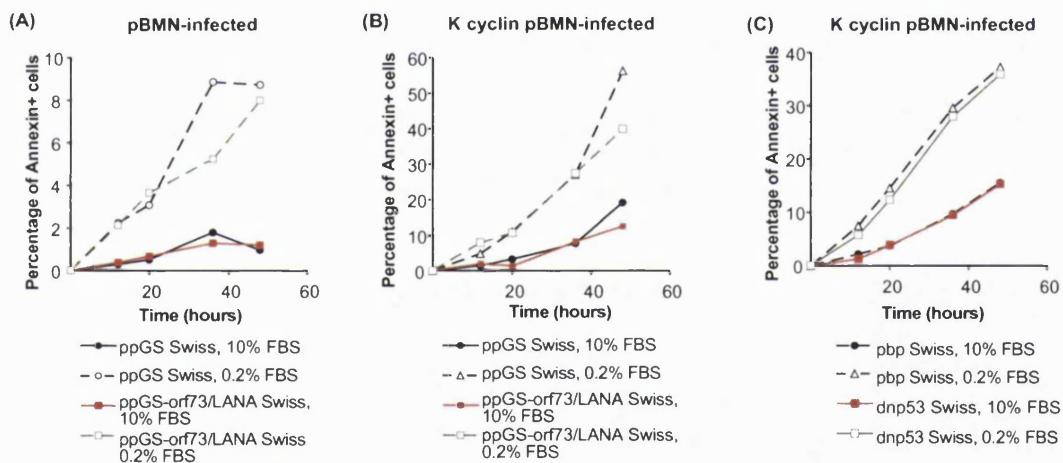


Fig. 4.11 Retroviral expression of LANA or DNp53 in Swiss 3T3 cells does not inhibit K cyclin-induced apoptosis. (A) Swiss 3T3 cells stably expressing LANA or control cells were plated at 1×10^5 cells per well in 6 well dishes. Three days later the medium was replaced with fresh medium containing 10% or 0.2% FBS. Floating and adhered cells were harvested 12, 20, 36 and 48 h later. Cells were stained with Annexin V-PE and Via-Probe and analysed by flow cytometry. (B) Swiss 3T3 cells stably expressing LANA or control cells were plated 1×10^5 cells per well in 6 well dishes and retrovirally infected the two subsequent days with KpBMN retrovirus (8 h each day). After overnight recovery, the medium was replaced with fresh medium containing 10% or 0.2% FBS. Cells were harvested and analysed for apoptosis as described in (A). (C) Swiss 3T3 cells stably expressing DNp53 or control cells were retrovirally infected with KpBMN as described in (B) and analysed for apoptosis as described in (A). Results shown are representative of two experiments.

4.8 Discussion

The results presented in this chapter show that K cyclin expression in early passage primary cells triggers apoptosis, similar to that seen in established tissue culture cell lines described in the previous chapter. Again, dependent on the assay, K cyclin expression either induced apoptosis directly (transient transfection) or strongly sensitised to apoptosis upon stimulation of cells with certain pro-apoptotic insults (retroviral infection). Such differences are presumably due to the differences in K cyclin expression levels. Together, these observations emphasise that the effects of K cyclin expression on cell survival are dependent on the amount of expressed K cyclin, the activity of K cyclin/Cdk complexes determined by the spectrum of endogenous Cdks in a particular cell, and on the presence of apoptotic stimuli in the cellular environment.

The finding that p53 and its target protein p21^{Cip1} were upregulated in wt MEFs, together with the observation that apoptosis was significantly reduced in *p53*^{-/-} MEFs show that p53 is a key mediator of apoptosis induced by K cyclin (Figs 4.4 and 4.5). However, K cyclin also triggers some level of p53-independent apoptosis, since apoptosis was not fully inhibited in *p53*^{-/-} MEFs (Fig. 4.5) or in *p53*^{-/-} SaOS-2 cells (Ojala et al., 1999). One such mechanism might be the transcriptional upregulation of the p53 homologue p73, which was recently shown to mediate E2F1-induced apoptosis (Irwin et al., 2000; Lissy et al., 2000; Stiewe and Putzer, 2000). However, if this were the case, it would be necessary to evoke a redundant activity between E2F family members (discussed below), since E2F1 is dispensable for K cyclin-induced apoptosis. Furthermore, the K cyclin-induced p53 pathway is likely to cooperate with apoptotic pathways that converge either upstream or downstream of p53, as demonstrated by the sensitisation afforded by K cyclin to apoptosis induced by serum depletion, DNA damage or death receptor engagement (Fig. 4.2). One cooperative signal may be the phosphorylation and inactivation of cellular Bcl-2 by K cyclin/Cdk6 complexes (Ojala et al., 2000), priming the mitochondrial apoptotic pathway. Thus, although apoptosis does not necessarily involve one linear pathway, the presented data show that p53 is an important mediator of K cyclin-induced apoptosis.

In addition to apoptosis, K cyclin expression also induced a p53-dependent growth arrest (Fig. 4.6 and time-lapse data). No equivalent growth arrest was observed upon ectopic expression of cyclin D3, supporting the notion that the properties of K cyclin/Cdk complexes differ from those of ectopic G1 cyclins. In most instances of oncoprotein-induced p53 stabilisation, cells undergo a G1 or G2 phase arrest (Agarwal et al., 1995) that is thought to be mediated at least in part by p21^{Cip1} (Brugarolas et al., 1995; Bunz et al., 1998; Waldman et al., 1995). However, *p21*^{-/-} MEFs also arrested upon K cyclin expression (Fig. 4.7 and time-lapse data), showing that the K cyclin-induced growth arrest is independent of p21^{Cip1}. This is

consistent with resistance of K cyclin/Cdk complexes to Cdk inhibitors (Swanton et al., 1997), driving cell cycle progression in the presence of enhanced levels of p21^{Cip1}. No upregulation of p16^{Ink4A} was detected upon K cyclin expression, suggesting that the growth arrest was also independent of p16^{Ink4A} (data not shown). Thus, it remains unclear what downstream effector of p53 mediates the K cyclin-induced growth arrest, which will be further discussed in chapter 5.

The activation of p53, apoptosis and growth arrest upon K cyclin expression all occurred in the absence of E2F1 (Figs 4.4, 4.5 and 4.6A). However, functional redundancy between E2F family members may exist, and a role for E2Fs 2-6 in the activation of p53 by K cyclin can therefore not be excluded. Indeed, in addition to E2F1, E2F3 has recently been shown to play a role in the p53-dependent apoptosis resulting from RB loss (Ziebold et al., 2001). Furthermore, similar to E2F1, inducible expression of E2F2 and E2F3 in Rat-1 cells was shown to induce apoptosis (Vigo et al., 1999). Complicating matters even further, a role for E2F1 in apoptosis could be cell type dependent, as implied by the occurrence of a defined tumor spectrum in *E2F1*^{-/-} mice, which solely show lymphomas, lung tumours and reproductive tract sarcomas (Yamasaki et al., 1996). It therefore remains possible that the activation of p53 by K cyclin involves E2Fs, or even requires E2F1 in cell systems different than MEFs.

In contrast to oncoproteins like c-Myc, Ras and E1A, K cyclin expression did not trigger an increase in p19^{ARF} protein levels (Fig 4.3). In addition, K cyclin-induced activation of p53 and consequent apoptosis and growth arrest did not require p19^{ARF} (Figs 4.4, 4.5 and 4.6A). Thus, the activation of p53 upon expression of K cyclin in MEFs is not via an E2F1/p19^{ARF} pathway, although, as discussed above, E2Fs might still be involved. Although this was initially surprising, several other observations have undermined the assumption that the transcriptional upregulation of p19^{ARF} by E2F1 constitutes a linear and universal pathway by which oncoproteins activate p53. First, although E2F1 expression in human fibroblasts correlates with an increase in p14^{ARF} protein levels (Bates et al., 1998; Dimri et al., 2000), no report documents such a result in MEFs. Second, a series of transcription factors other than E2F1 govern p19^{ARF} expression and have been implied as genetic modifiers of the p19^{ARF}/p53 pathway *in vivo*, including the transcriptional activator DMP1 (Inoue et al., 2000) and the transcriptional repressors Bmi-1, TBX2, TBX3 Twist and JunD (Brummelkamp et al., 2002; Jacobs et al., 2000; Jacobs et al., 1999; Maestro et al., 1999; Weitzman et al., 2000). And third, two groups recently showed that p19^{ARF} is dispensable for E2F1- and p53-dependent apoptosis associated with Rb loss (Tolbert et al., 2002; Tsai et al., 2002), implying that the activation of p53 by E2F1 does not always require p19^{ARF}. Interestingly, p53-dependent cell cycle checkpoints, for example those triggered upon DNA damage, do not require p19^{ARF} and

are intact in *ARF*^{-/-} MEFs (Kamijo et al., 1997). One possibility is, therefore, that K cyclin activates p53 by triggering a checkpoint response. This will be further discussed in chapter 5.

Expression of K cyclin correlated with an increase in the activity of the senescence marker SA- β -Gal in a significant proportion of not only arrested wt, *E2F1*^{-/-} and *ARF*^{-/-} MEFs, but also in the proliferating population of K cyclin expressing *p53*^{-/-} MEFs (Fig. 4.8). In addition, increased SA- β -Gal activity was detected in proliferating mock-infected *E2F1*^{-/-} MEFs. This was surprising, considering the general belief that SA- β -Gal expression correlates with growth arrest and that p53 and its target gene p21^{Cip1} are key mediators of cellular senescence (reviewed in Itahana et al., 2001). For example, growth arrest upon expression of oncogenic proteins of the Ras pathway (Ferbeyre et al., 2002; Lin et al., 1998; Serrano et al., 1997; Sewing et al., 1997; Zhu et al., 1998) or E2F-1 (Dimri et al., 2000) are associated with the induction of p53, p19^{ARF}, p21^{Cip1}, and/or p16^{INK4a} and growth arrest is generally abrogated in the absence of p53. Similar to the p53-independent senescent-like phenotype upon K cyclin expression, human fibroblasts that do not express p53 and p21^{Cip1} (cultured skin fibroblasts from Li-Fraumeni patients (Medcalf et al., 1996) or HPV E6 over-expressing fibroblasts (Dulic et al., 2000) and *p21*^{-/-} MEFs (Dulic et al., 2000) undergo replicative senescence, despite their extended lifespan. Of note, such senescent cells are not able to block DNA synthesis and are multinucleated, a phenotype that resembled the SA- β -gal-positive K cyclin expressing *p53*^{-/-} MEFs.

Another potential explanation for the detection of SA- β -Gal activity in K cyclin-expressing *p53*^{-/-} MEFs is that SA- β -Gal expression does not always mirror growth arrest. Lysosomal β -Gal activity is detected constitutively at pH 4 and it is thought to be the increase in this β -Gal activity in senescent cells that allows SA- β -Gal to be detected at pH 6 (Dimri et al., 1995). However, SA- β -Gal expression on itself does not induce senescence and, as such, is rather a marker of increased lysosome biogenesis and thus of cellular stress (reviewed in Serrano and Blasco, 2001). A manifestation of this is the finding that p21^{Cip1}-induced reactive oxygen species (ROS), rather than inhibition of DNA replication upon p21^{Cip1} expression, is required for p21^{Cip1}-induced senescence (Macip et al., 2002). Thus, the senescence phenotype is heterogeneous and overlaps with cellular stress, and a strict correlation of senescence with SA- β -Gal activity may therefore be assumptive.

K cyclin expression increases SA- β -Gal activity in a significant proportion of both *p53*^{-/-} and wt MEFs. Why then does only a population of K cyclin-expressing *p53*^{-/-} MEFs expand? Perhaps the SA- β -Gal-positive K cyclin-expressing *p53*^{-/-} cells actually proliferate, as increased SA- β -Gal expression in mock-infected *E2F1*^{-/-} MEFs did not result in a reduced growth rate either (Fig. 4.6 and Field et al., 1996). Indeed, time-lapse analysis of such populations showed a large multinucleated *p53*^{-/-} cell undergoing cell division (see

supplemented CD). Alternatively, this may be an issue of kinetics, such that K cyclin-expressing proliferating *p53*^{-/-} MEFs divide at a faster rate compared with K cyclin-expressing wt MEFs. Interestingly, senescent cells have been shown to secrete metalloproteases, inflammatory cytokines and growth factors (Shelton et al., 1999), which can promote the proliferation and tumorigenesis of co-cultured cells, especially if those are pre-neoplastic (Chang et al., 2000; Krtolica et al., 2001). Thus, another potential explanation is that the SA- β -Gal-positive K cyclin-transduced *p53*^{-/-} MEFs actively stimulate the proliferation of the pre-senescent cells in the culture.

Of the KSHV-encoded proteins that have been suggested to inhibit p53, LANA is the most likely candidate to specifically inhibit the activation of p53 by K cyclin. LANA is expressed in all malignancies associated with KSHV expression and is co-expressed with K cyclin on an overlapping transcript. Some inhibition of p53-luciferase activities and K cyclin-induced apoptosis by LANA was detected in transient transfection assays (Fig 4.8 and 4.9A). In contrast, no effect of retrovirally expressed LANA on K cyclin-induced apoptosis or growth arrest was measured. However, DNp53 expression also failed to inhibit K cyclin-induced cellular effects, complicating the interpretation of these results. Of note, two reports describe inhibition of apoptosis by this particular C-terminal DNp53 fragment (Bowman et al., 1996; Gottlieb et al., 1996), although it has been suggested that the absolute levels of DNp53 have to be very high in order to act effectively as dominant negative regulators of wt p53 (Shaulian et al., 1992), and references herein). Thus, in my experiments the expression levels of retrovirally expressed LANA and DNp53 might have too low to achieve detectable anti-apoptotic activity. This might also explain why I was unable to repeat published results from Friborg and colleagues, which showed that LANA expression inhibits apoptosis of rodent fibroblasts upon serum withdrawal (compare Fig. 4.10 and Friborg et al., 1999). Alternatively, p53-mediated apoptosis and/or growth arrest might entail non-transcriptional effects. Supporting this, apoptosis induced upon tetraploidisation of cells treated with mitotic spindle inhibitors is inhibited upon Bcl-xL expression, but not upon expression of a DNp53 mutant (Minn et al., 1996). Interestingly, LANA was recently shown co-localise with p53 in KS cells (Katano et al., 2001), further supporting the hypothesis that LANA may regulate p53 activity *in vivo*. Thus, it remains possible that in the context of high LANA expression in B lymphocytes or endothelial cells, LANA is able to inhibit the activation of p53 by K cyclin.

CHAPTER 5:
K CYCLIN-ASSOCIATED PLOIDY INCREASE AND
CENTROSOME AMPLIFICATION

5.1 Introduction

Expression of K cyclin in primary fibroblasts triggered p53-dependent growth arrest and apoptosis, blocking the expansion of a K cyclin-expressing population of cells (previous chapter). Time-lapse analysis of K cyclin expressing cells showed that cells were not dividing. Noticeably, a significant amount of K cyclin-expressing cells were multinucleated, implying that even though those cells were not undergoing cytokinesis, the nuclear division cycle was ongoing. However, MEFs are heterogeneous and multinucleated cells are visible in untreated control cells as well. In addition, genomic instability can be induced upon tissue culture, which is revealed in the absence of p53 checkpoints (Harvey et al., 1993b). I therefore decided to further define the K cyclin-specific effect on the cellular division cycle.

Accumulating evidence over recent years has shown that the DNA replication and the centrosome replication cycle are tightly co-regulated (Hinchcliffe and Sluder, 2001b). In particular, similar to the initiation and execution of DNA replication, centrosome duplication requires cyclin E/A driven cdk2 activity (Lacey et al., 1999; Matsumoto et al., 1999; Meraldi et al., 1999). Centrosomes constitute the microtubule-organising center (MTOC) of the cell and, as such, assure proper segregation of chromosomes to daughter cells. At the ultrastructural level, they consist of a pair of centrioles surrounded by a cloud of amorphous material called the pericentriolar matrix (PCM). The centrioles themselves are a barrel of nine triplet microtubules ~400 nm in length (Marshall and Rosenbaum, 1999), composed of a large number of proteins, amongst which is centrin (Salisbury, 1995). The function of centrin is unknown. The PCM consists of a fibrous meshwork that binds various proteins, including γ -tubulin ring complexes that nucleate microtubule polymerisation (Wiese and Zheng, 1999) (Gunawardane et al., 2000) (Zimmerman et al., 1999). One known centrosomal protein that is targeted by cyclin E/Cdk2 is nucleophosmin/B23 (NPM/B23) (Okuda et al., 2000) (Tokuyama et al., 2001). Phosphorylation of NPM/B23 by cyclin E/Cdk2 is thought to cause it to dissociate from the centrosomes, allowing the initiation of centrosome duplication (see Fig. 5.1). Additional cell cycle regulatory mechanisms are however likely to exist.

If proliferating cells fail to coordinate centrosome duplication with DNA replication, this will inevitably lead to a change in ploidy. Furthermore, the formation of monopolar or multipolar spindles will lead to aberrant chromosome separation. It was first proposed by Theodor Boveri in the beginning of this century that aberrations in the centrosome duplication cycle may be an important cause of aneuploidy, and perhaps thereby contribute to the development of cancer (reviewed in Salisbury et al., 1999). This view has recently been revived with the finding that many human and mouse tumour cells contain centrosomes that are abnormal in size, shape and/or number, as measured by γ -tubulin or anti-centrin

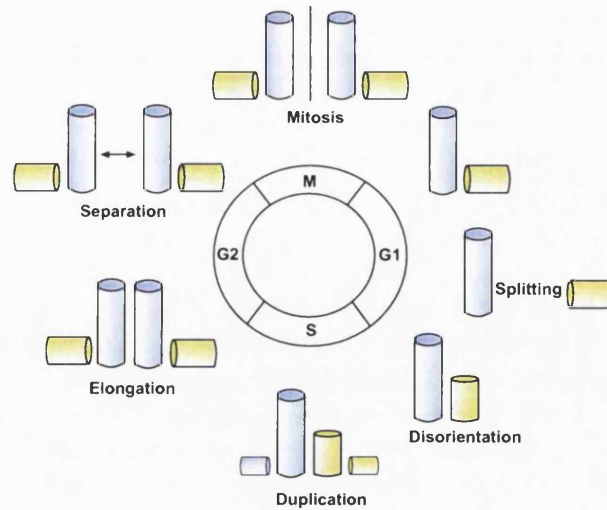


Fig. 5.1 The centrosome reproduction cycle. Each centrosome consists of two centrioles, which are represented as barrels. Blue coloured barrels represent mature mother centrioles and green barrels represent immature daughter centrioles.

immunostainings. As expected, centrosome aberrations always correlated with the occurrence of abnormal mitosis and abnormal chromosome numbers (Carroll et al., 1999; Lingle et al., 2002; Pihan et al., 1998; Pihan et al., 2001). An ongoing debate is whether centrosome aberrations and consequent aneuploidy directly trigger tumorigenesis, or rather are a mere side effect of the loss of checkpoints that is typical of tumour cells (Marx, 2001). At the heart of this issue is the question of how centrosome amplifications exactly arise. A recent elegant study by Meraldi and colleagues has suggested that defects in cell division and concomitant multinucleation, rather than deregulation of the centrosome duplication cycle itself, can give rise to centrosome amplifications (Meraldi et al., 2002). Given the resemblance of K cyclin/Cdk activity to Cdk2 activity, and the potential K cyclin-associated multinucleation, I therefore decided to further characterise the K cyclin-induced nuclear effects and its effect on centrosomes.

5.2 Increase in multinucleation and polyploidy in K cyclin-expressing MEFs

Time-lapse analysis of K cyclin expressing cells indicated that cells and their nuclei enlarged over time. It was also frequently observed that cells rounded up as if they were about to divide, but then flattened again without dividing, yielding cells with multiple nuclei. Such defective cytokinesis was particularly evident in the time-lapse analysis of the LANA experiment described in Fig. 4.10D, in which cells GFP-positive cells were FACS sorted, plated and analysed by time-lapse videomicroscopy soon after K cyclin expression. Cells undergoing abortive cytokinesis remained rounded for longer than cells undergoing normal

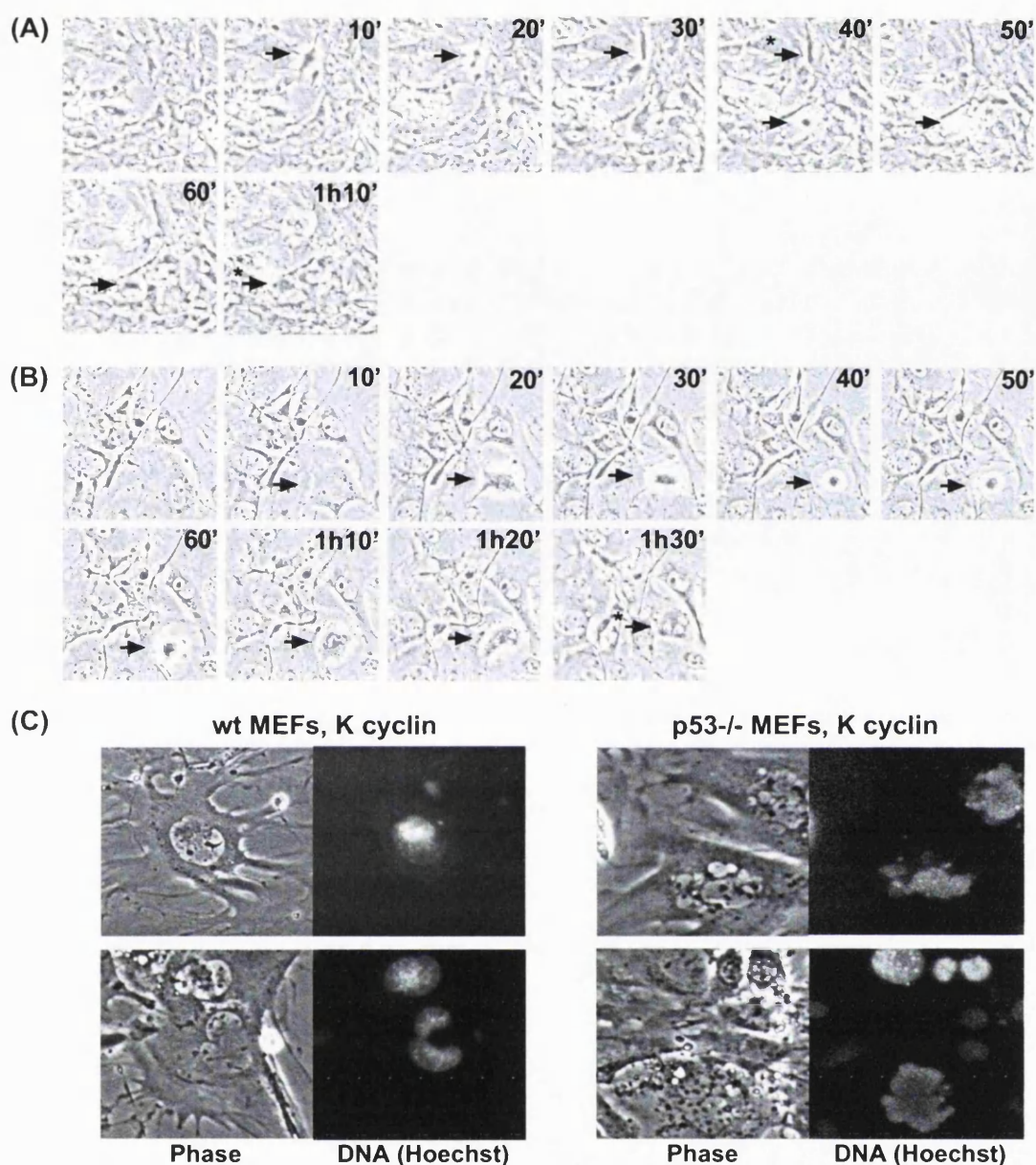


Fig. 5.2 K cyclin expression in MEFs induces the formation of multinucleated cells. Wt MEFs were infected with a first round of pBabepuro and a second round of empty pBMN (A) or K cyclin pBMN virus (B), FACS sorted and cultured as described in Fig. 4.10D. Cells were followed by time-lapse videomicroscopy and photos were taken every 10 minutes. Arrows point at cells undergoing mitosis, starting at the time point when cells first became refractory. The asterisks indicate the time point when daughter cells flatten. Notice the abortive cytokinesis and formation of a daughter cell with three nuclei in K cyclin-transduced MEFs in panel B. (C) Wt or *p53*^{-/-} MEFs were transduced for 24 h with empty 2FKpBabepuro retrovirus. Infected cells were selected by culture for 2 days in puromycin and plated at 1×10^5 cells per well in 6 well plates, followed by culture for another 5 days. DNA was stained with Hoechst and pictures were taken at a magnification of 32x. Representative multinucleated cells are shown.

mitosis (around 1.5 h to 2.5 h in abortive cytokinesis versus 30-40 minutes in normal mitosis, see Fig. 5.2A for an example). Multinucleated cells were seen in both wild type and in *p53*^{-/-} MEFs expressing K cyclin, but was more dramatic in the latter which exhibited large

multilobular nuclei (Fig. 5.2B). This suggested that despite defective cell division in K cyclin expressing cells, the nuclear division cycle was ongoing and DNA replication was still taking place.

To determine whether MEFs arrested upon K cyclin expression maintained DNA synthesis, MEFs of various genotypes were transduced with K cyclin and cultured for three days in the presence of serum. Cells were subsequently labelled for 5 h with BrdU and

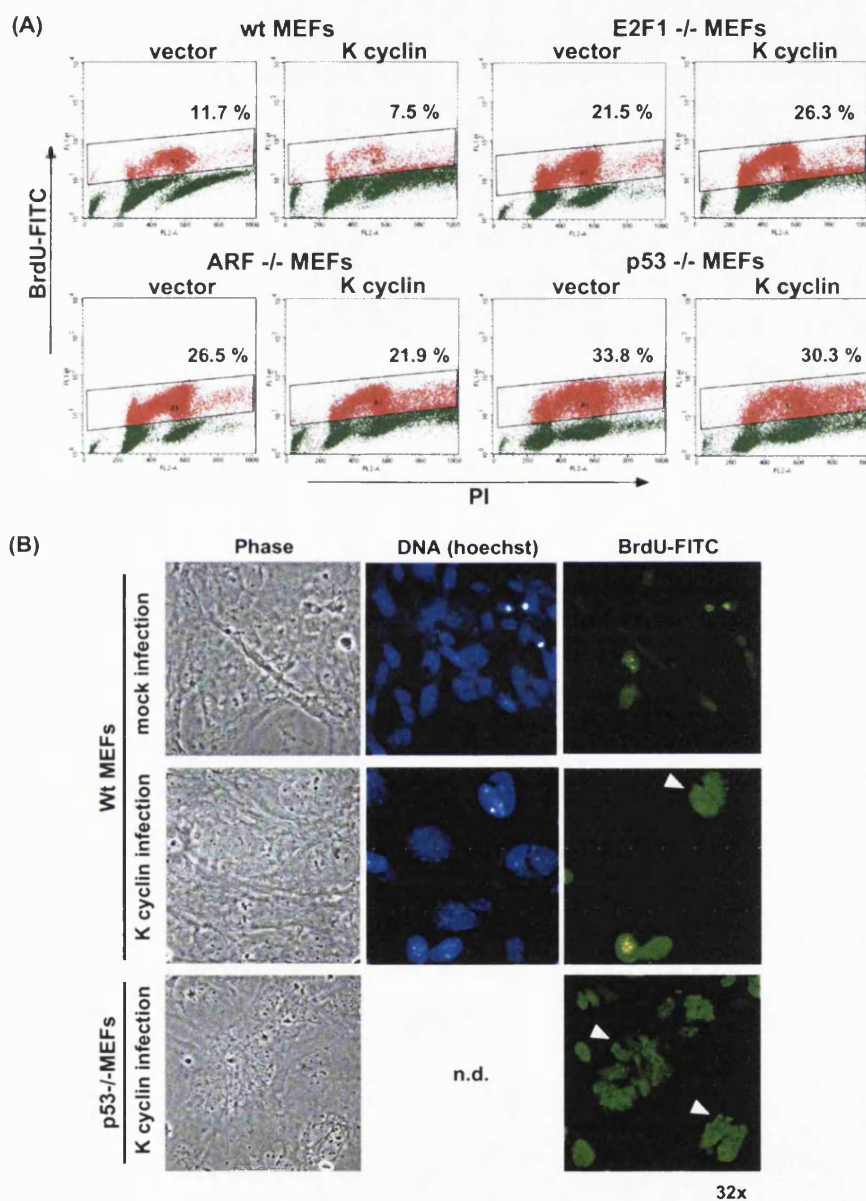


Fig. 5.3 Growth-arrested K cyclin-expressing MEFs synthesise DNA. (A) Wt, *E2F1*^{-/-}, *p19^{ARF}*^{-/-} or *p53*^{-/-} MEFs were transduced for 24 h with empty pBabe puro or 2FkpBabe puro retrovirus. Infected cells were puromycin selection, plated at 0.5×10^5 cells per well and grown for three days in medium containing 10% FBS. BrdU (50 μ M) was added to the medium and the cells were grown for a further 5 hours. Cells were collected and stained with PI and anti-BrdU-FITC conjugate and analysed by flow cytometry. Indicated are the percentages of BrdU positive cells. (B) Wt or *p53*^{-/-} MEFs were infected as in (A) were grown on coverslips for 3 days, followed by growth in medium containing BrdU for 5 h. Cells were fixed in 70% ethanol and stained with anti-BrdU-FITC (green) and Hoechst (blue) to visualise the DNA. Slides were microscopically analysed and pictures were taken at a magnification of 32x. Large, multinucleated BrdU-positive cells are indicated with arrowheads. N.d.= not determined.

analysed by flow cytometry. A significant percentage of cells expressing K cyclin had entered S phase over the course of BrdU labelling (5.3A), with percentages being roughly equivalent to mock-infected cultures. This was evident in cultures of not only *p53*^{-/-}, but also wt, *ARF*^{-/-} and *E2F1*^{-/-} MEFs, showing that cells entered S phase regardless of growth arrest. To confirm that the observed multinuclear cells had incorporated BrdU, we examined the cells by immunofluorescence microscopy using anti-BrdU antibody. BrdU staining was observed in the K cyclin-expressing growth-arrested MEFs with large or multiple nuclei (Fig. 5.3C, arrows), indicating that these cells had synthesised DNA.

Multinucleation and ongoing DNA replication should give rise to polyploid cells, which is defined as a form of aneuploidy in which the complete set of chromosomes is amplified. MEFs comprise a heterogeneous cell population and some multinucleation occurs spontaneously. Polyploidy was therefore quantified by DNA staining and flow cytometric analysis. Mock and K cyclin-transduced MEFs were cultured for 3 days in the presence of

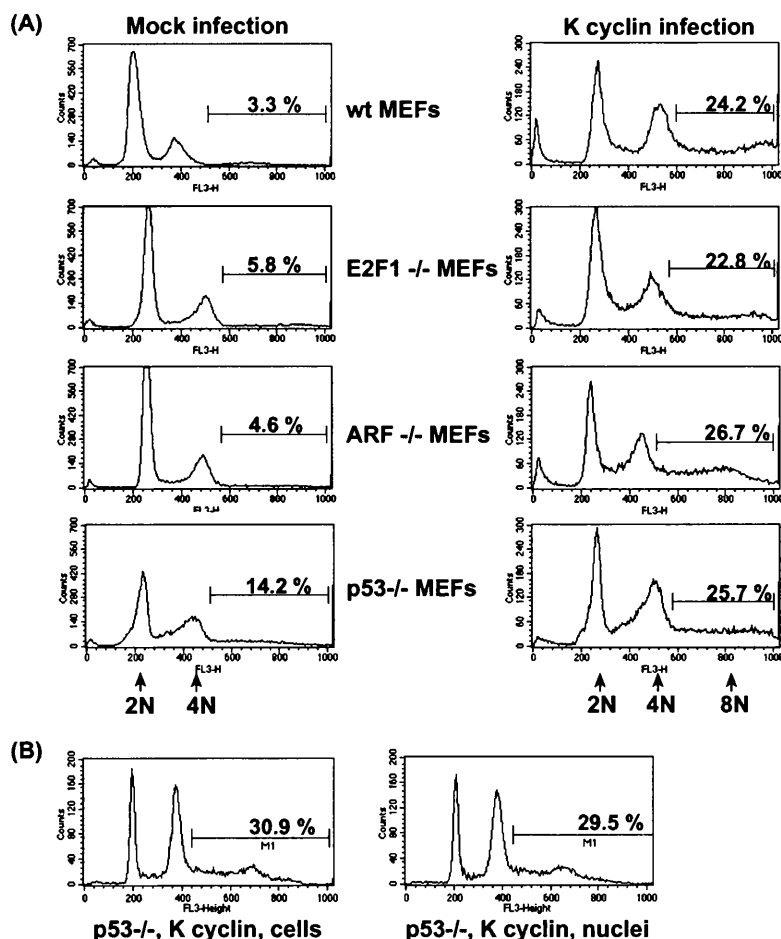


Fig. 5.4 K cyclin-expressing MEFs become polyploid. (A) MEFs infected and grown as described in Fig. 22A were grown for 3 days in medium containing 10% FBS. Adhered cells were harvested, followed by staining of the DNA with PI and flow cytometric analysis to establish cell cycle profiles. (B) *p53*^{-/-} MEFs transduced with 2FKpBabepuro as described in Fig. 22A were grown for 5 days in medium containing 10% FBS. Adhered cells were harvested and, where indicated, nuclei were isolated. The DNA was stained with PI and cells or nuclei were analysed by flow cytometry. Indicated are the percentages of cells with a DNA content greater than 4N.

serum. A significant fraction of wt, *E2F1*^{-/-} and *ARF*^{-/-} MEFs expressing K cyclin showed increased ploidy, reflected by an increase in the fraction of cells with approximately 8N DNA content (from 3-6% in mock infected to around 22% to 27% in K cyclin expressing cultures) (Fig. 5.4). Consistent with published observations (Harvey et al., 1993b), *p53*^{-/-} MEFs showed increased ploidy (~14%) even in control, mock-infected cultures. Importantly, expression of K cyclin also induced accumulation of *p53*^{-/-} MEFs with 8N DNA content (up to the level of 26%). These data show that a significant proportion of the MEFs expressing K cyclin go through multiple rounds of DNA replication without cell division. In addition, since polyploidisation is observed in wt as well as in *p53*^{-/-} MEFs, the induction of polyploidy is independent of the activation of p53.

A polyploid cell could either contain multiple nuclei each with a normal DNA complement, or contain nuclei in which chromosomes are unequally distributed. To see which of these was true, nuclei were isolated from K cyclin and mock-transduced MEFs and subjected to flow cytometric analysis. In case each individual nucleus was diploid, this should result in discrete 2N and 4N peaks and the absence of an 8N DNA peak, while a more irregular profile was expected if nuclei were aneuploid. However, the DNA profile of nuclei was very similar to that of whole cells (see Fig. 5.4B for K cyclin-transduced *p53*^{-/-} MEFs). This shows that nuclei were not physically separated, implying completion of nuclear division does not proceed DNA replication.

5.3 K cyclin expression induces polyploidy in human fibroblasts

To confirm that multinucleation and polyploidy induced by K cyclin are not phenomena specific for MEFs, K cyclin was introduced with amphotropic retroviruses into primary human lung fibroblasts. Similar to MEFs, primary human fibroblasts growth arrested upon K cyclin expression (Fig. 5.5A and B). Some of the K cyclin-arrested MRC-5 cells were multinucleated (see arrows Fig. 5.5B), although this was less evident compared with MEFs. The growth arrest of cells was further confirmed by time-lapse videomicroscopy (see supplementary CD, named MRC-5 pBp and MCR-5 K cyclin). In addition, an increase in the percentage of SA- β -Gal-positive cells from 0.6% in mock-transduced to 83% in K cyclin-transduced cells was measured (Fig. 5.5C). Importantly, flow cytometric analysis of K cyclin expressing cells cultured for three days in the presence of serum showed the formation of a polyploid population of cells (Fig. 5.5D).

Kaposi's sarcoma tumour cells have been shown to exhibit telomerase activity (Chen et al., 2001). To establish whether this immortalisation process affect could affect K cyclin-

induced cellular effects, K cyclin was introduced into MRC-5 cells that constitutively expressed the telomerase catalytic subunit hTERT (Meyerson et al., 1997). No differences between MRC-5 and MRC-5hTERT cells in K cyclin-induced growth arrest, polyploidy and SA- β -Gal activity were measured (see Fig. 5.5A for growth curve). Thus, K cyclin expression leads to the formation of a polyploid population of cells in both human primary fibroblasts, regardless of their immortalisation status.

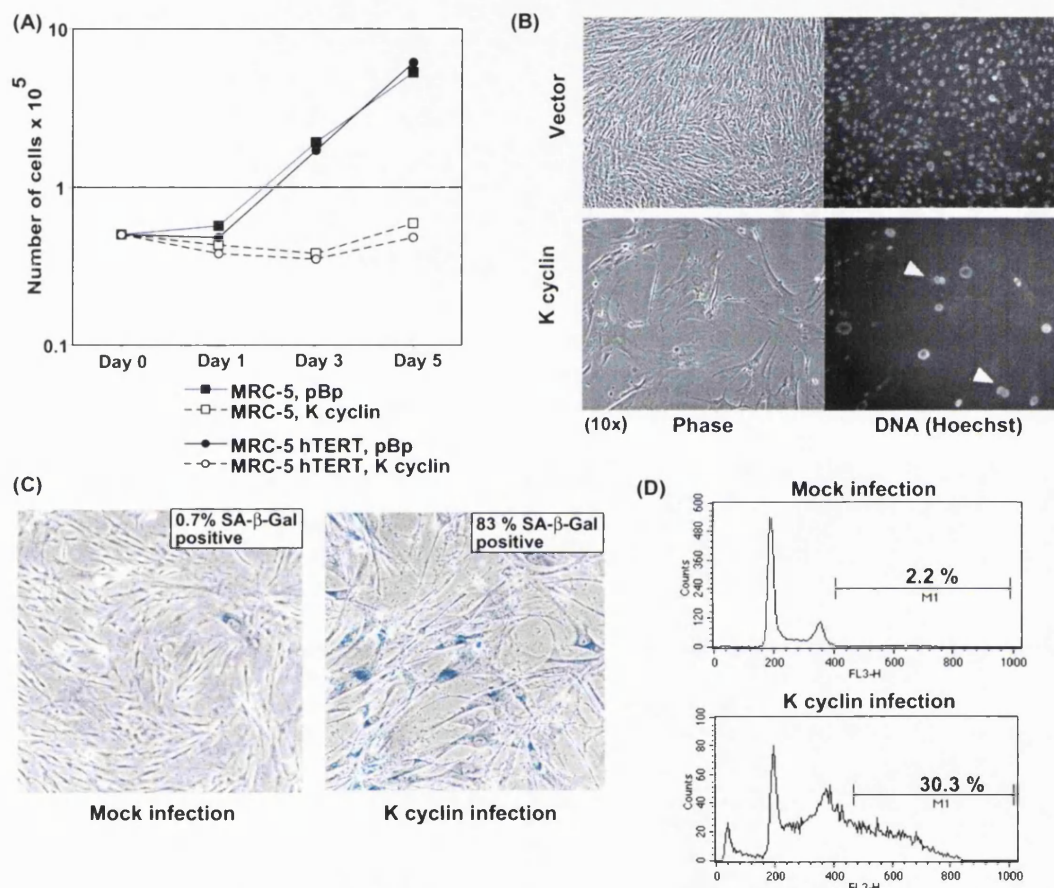


Fig. 5.5 Expression of K cyclin in MRC-5 human fibroblasts triggers growth arrest and polyploidy. (A) MRC-5 or MRC-5/hTERT cells were transduced with amphotropic retrovirus and infected cells were selected by growth in puromycin for three days. Tissue culture supernatants were established to be negative for replicative virus using the Quant RT assay (see materials and methods) and cells were split at 0.5×10^5 cells per well. Cells were grown in medium containing 10% FBS and counted at day 1, 3 and 5 after plating. (B) Cells infected as in A) were grown for four days, fixed in 2% formaldehyde/0.2% glutaraldehyde and DNA was stained with Hoechst. Pictures were taken at 10x magnification. (C) MRC-5 cells infected as in A) were grown for four days and then stained for SA- β -Gal activity. Around 250 cells were scored to be positive (blue) or negative (unstained) for β -gal activity and the percentages of positive cells are indicated. Pictures were taken at a 10x magnification. (D) MRC-5 cells infected as in A) were grown for three days. Adhered cells were harvested and DNA was stained with PI, followed by flow cytometric analysis. Indicated are the percentages of cells with a DNA content greater than 4N.

5.4 Centrosome amplification upon K cyclin expression

It was recently shown that multinucleated cells often contain centrosome amplifications (Meraldi et al., 2002). In addition, the centrosome duplication cycle has been linked with Cdk2-activity (Hinchcliffe and Sluder, 2001a). Since many K cyclin expressing cells are multinucleated and K cyclin/Cdk activity resembles Cdk2 activity, I decided to investigate whether K cyclin-expressing cells exhibit centrosome amplifications by staining of the centrosomal proteins γ -tubulin and centrin. Anti- γ -tubulin staining of MEFs expressing K cyclin showed a marked increase in the average number of centrosomes per cell above the expected number of 1-2 centrosomes (Fig. 5.7). While only 6.5% of mock-infected wt or 15.6% of *p53*^{-/-} MEFs had more than 2 centrosomes, around 46% of wt MEFs expressing K cyclin showed centrosome amplification. Amplification of centrosomes was typically observed in cells with large or multiple nuclei (Fig. 5.7A, middle panel). Loss of p53 exacerbated this phenotype, with around 67.5% of K cyclin expressing *p53*^{-/-} MEFs showing centrosome amplification (Fig. 5.7B). The numbers of centrosomes observed were remarkably high, ranging from 3 to 40 centrosomes per cell. Staining with an anti-centrin antibody showed analogous results, confirming the specific staining of centrosomes (data not shown).

The centrosomes in K cyclin-expressing cells had a typical round shape and were localised in the cytoplasm around the nucleus. This is consistent with previous reports describing centrosome stainings (see for example Okuda et al., 2000). Visual inspection suggested that the size of centrosomes in a normal cell and in cells with amplified centrosomes was similar, which was confirmed by confocal microscopic analysis. The diameter of the centrosomes varied between 0.88 and 1.41 μm , similar to the previously determined centrosomal diameter (Doxsey, 1998).

The DNA and centrosome replication cycles are thought to be co-regulated. To explore a potential correlation between the number of centrosomes and the DNA content (N) per cell, the nuclear DNA content was measured using quantitative immunofluorescence microscopy. The normalised DNA content of mock-infected cultures showed values of N ranging from 2 to 4 (Fig. 5.8A), validating the use of this method. In contrast, a significant proportion of K cyclin-expressing cells had a DNA content of around 8N, increasing in a few cells to around 16N, consistent with the flow cytometric analysis (Fig. 5.4). The DNA content of each individual cell was correlated with the number of centrosomes in the same cell (inset Fig. 5.8A and Fig. 5.8B). Notably, the cells with the highest DNA content contained the highest number of centrosomes. Furthermore, cells with amplified centrosomes were always polyploid (inset Fig. 5.8A), and these cells often contain a single, but very large, nucleus (Fig. 5.8B).

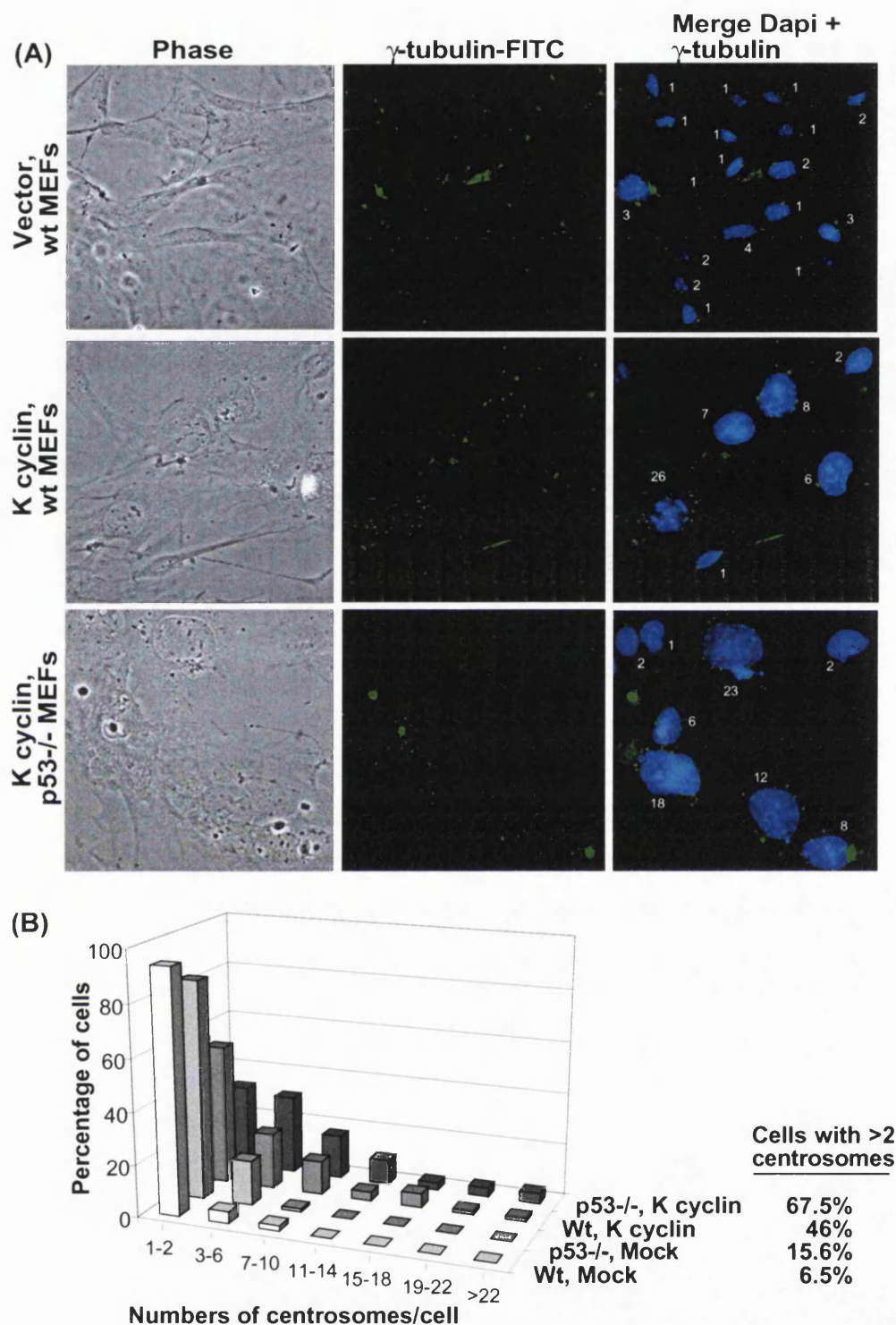


Fig. 5.6 Centrosome amplifications in K cyclin-expressing MEFs. (A) Wt or *p53*^{-/-} MEFs (passage 2-4) were transduced with pBabepuro or 2FKpBabepuro virus for 24 h, followed by growth in puromycin for 48 h. Infected cells were plated at 1×10^5 cells per well on coverslips and stained three days later with anti γ -tubulin antibody for centrosomes (green) and DAPI for DNA (blue). Numbers represent amounts of centrosomes per cell. (B) Quantitation of the centrosome analysis described in A). Shown are the pooled percentages of three experiments of around 70 cells each.

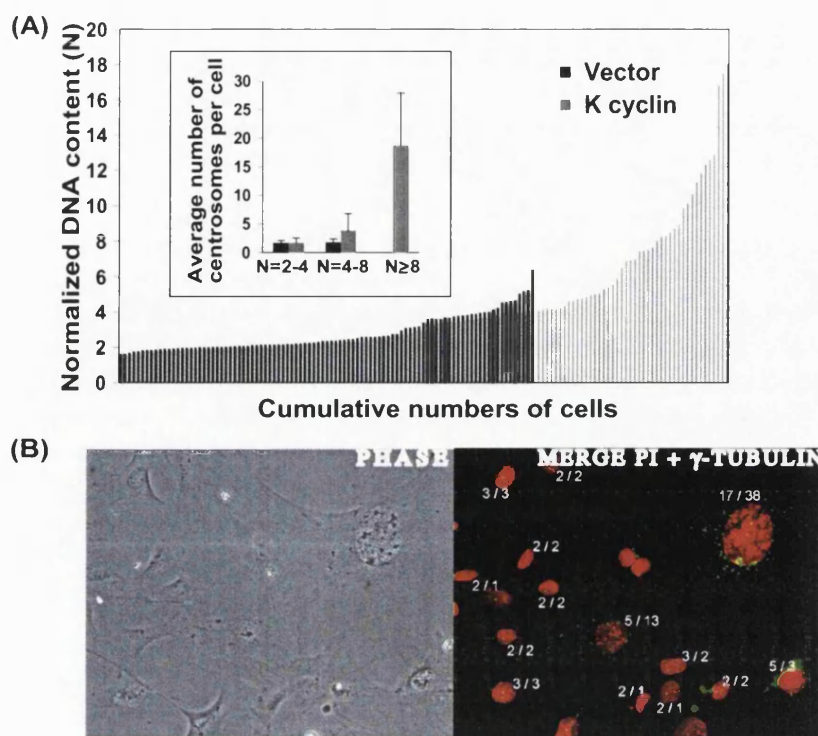


Fig. 5.7 Polyploid cells contain amplified centrosomes. (A) Wt MEFs infected and grown as in Fig. 5.6A were stained with anti- γ -tubulin and PI. The DNA content per nucleus was measured by quantitative immunofluorescence microscopy and normalised to obtain an N=2 population. Around 100 mock infected cells or 150 K cyclin infected cells were analysed. The inset histogram illustrates that average numbers of centrosomes increase with DNA content. (B) Example of the correlation analysis described in A). Shown is a representative field of wt MEFs infected with K cyclin, green = γ -tubulin, red= PI. Numbers before the slash represent DNA contents, numbers after the slash represent centrosome counts.

In addition to MEFs, primary human fibroblasts also exhibited centrosome amplification upon K cyclin expression. Approximately 5-10% of K cyclin-expressing MRC-5 cells contained more than two centrosomes, often as a typical clusters of around 5-7 centrosomes (see upper right panel in Fig. 5.9 for an example). The numbers of centrosomes per cell were remarkably reduced compared with MEFs (maximum of 7 per cell, Fig. 5.9). However, the polyploidy in human fibroblasts was also less pronounced (Fig. 5.6). These results are therefore consistent with the finding that an increase in ploidy correlates with an increase in the number of centrosomes.

The presence of multiple centrosomes in one cell can lead to the formation of multiple spindles and improper chromosome separation. Indeed, confocal studies of K cyclin-expressing MEFs revealed the formation of multipolar spindles and, in *p53*^{-/-} cells, anaphase bridges that were sometimes accompanied by chromosomal fragments (Fig. 5.9). Taken together, these results show that K cyclin expression leads to genomic instability, involving increases in ploidy and centrosome amplifications, a phenotype that is exacerbated upon *p53* loss.

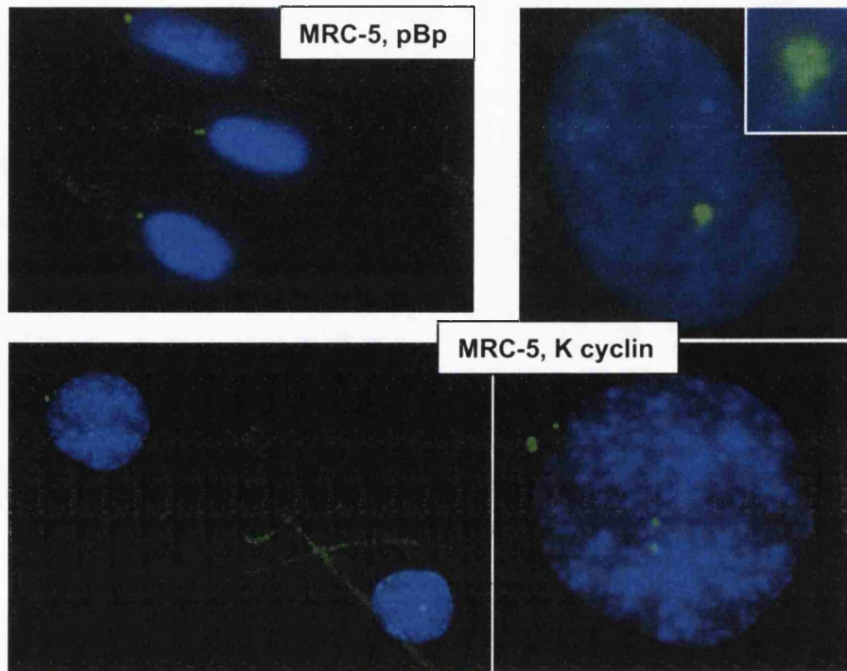


Fig. 5.8 Centrosome amplifications in K cyclin-expressing MRC-5 cells. MRC-5 cells were transduced with pBabepuro (pBp) or 2FKpBabepuro amphotropic virus as described in Fig. 5.6A. Cells were split at 0.5×10^5 cells per well, cultured on coverslips for three days in the presence of 10% FBS and stained with anti- γ -tubulin (green) and DAPI (blue). Cells were analysed by fluorescence microscopy and pictures were taken at a magnification of 32x. Magnified images depicted on the right were obtained using the zoom function in Adobe Photoshop to better visualise the centrosomes.

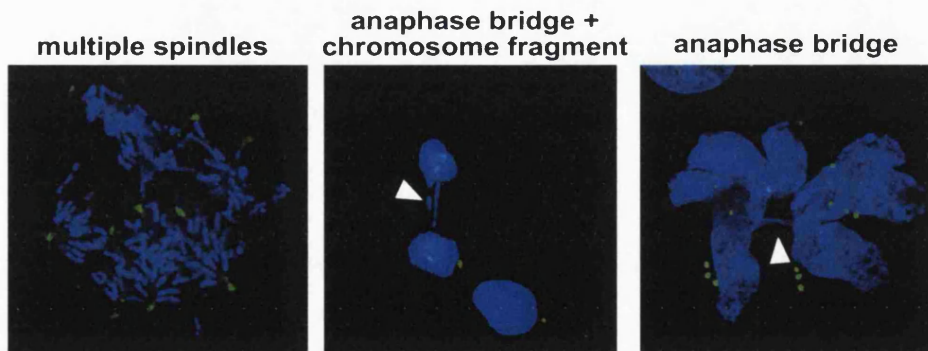


Fig. 5.9 Abnormal chromosome distribution in K cyclin-expressing MEFs. Confocal microscopic analysis of wt (first image) or *p53*^{-/-} MEFs infected with K cyclin retrovirus and grown and stained as in Fig. 5.6A. Pictures are the image layer that best visualised the genomic abnormality, magnification 63x and 2x zoom.

5.5 Enhanced cyclin B nuclear localisation in K cyclin expressing MEFs

Since many K cyclin expressing wt cells exhibited DNA replication without undergoing cytokinesis, it was possible that K cyclin-induced tetraploid cells (4N DNA, 2 nuclei) did not enter mitosis again. Alternatively, K cyclin expression might interfere with endogenous mitotic cyclin B activity, the regulation of which is crucial for the proper execution of mitosis. Cyclin B expression increases around G2/M (Takizawa and Morgan, 2000) and is degraded at the end of mitosis by the anaphase promoting complex (APC) (Morgan, 1999; Peters, 2002), thus constituting a marker for mitotic progression. To investigate whether K cyclin interferes with mitotic progression, MEFs transduced with K cyclin or mock virus were therefore stained with anti-cyclin B antibody.

As expected, control cultures only showed detectable cyclin B levels in dividing rounded cells (around 5-10% of the cells, Fig. 5.10A). No widespread, constitutive upregulation of cyclin B levels was visible in K cyclin-transduced MEFs. Similar to mock-infected cells, around 5-10% of K cyclin-transduced MEFs were rounded and contained

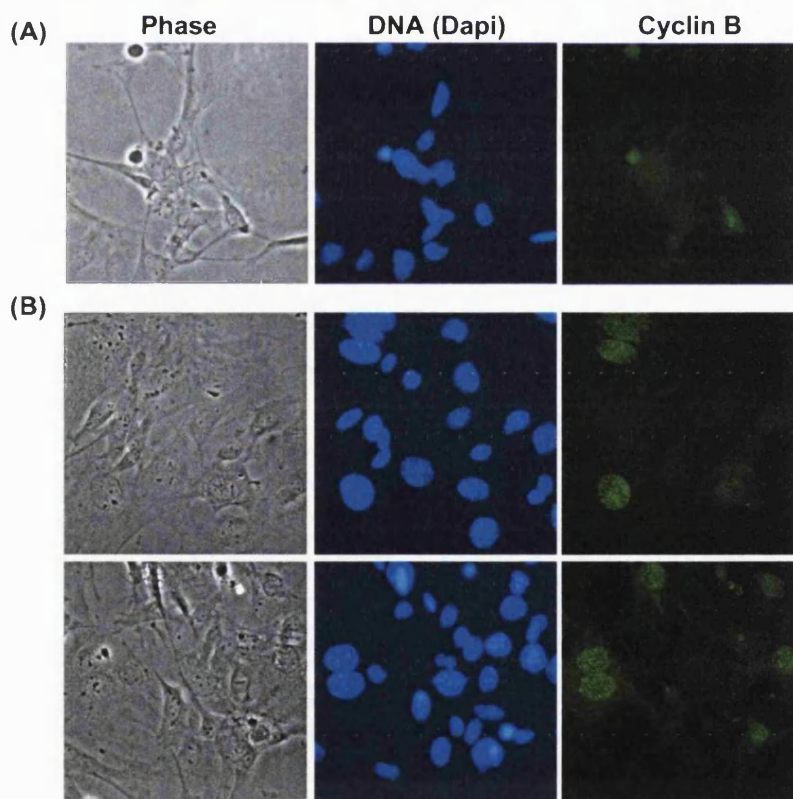


Fig. 5.10 Enhanced nuclear cyclin B in K cyclin-expressing MEFs. (A) Wt MEFs were transduced for 24 h with empty pBabepuro virus, puromycin selected for 48 h and split at 1×10^5 cells per well on coverslips. Cells were cultured for three days and then fixed in -20°C methanol. Coverslips were stained with anti-cyclin B1 antibody. Cells were microscopically analysed and pictures were taken at a magnification of 32x. (B) Wt MEFs were transduced with 2FkpBabepuro virus, cultured and stained as described in A).

increased levels of cyclin B. In addition, a significant proportion of K cyclin-transduced cells with large or multiple nuclei that contained uncondensed DNA showed detectable nuclear cyclin B levels (around 5% of the cells, Fig. 5.10B). This was remarkable, as nuclear cyclin B is normally only seen in rounded, mitotic cells. Thus, arrested K cyclin-expressing cells do enter mitosis, but the enhanced nuclear localisation of cyclin B before chromosome condensation in many cells suggests that mitotic progression is altered in such cells.

5.6 Decreased polyploidy upon prolonged culture of K cyclin-expressing MEFs

The expression of K cyclin triggers the formation of a population of cells with an 8N DNA content. However, if endoreduplication is ongoing, the ploidy of cells might increase over time. Alternatively, at least in the dividing *p53*^{-/-} cells, a certain subpopulation of cells might outgrow the polyploid cells. I thus decided to find out how long-term culture of K cyclin-expressing MEFs affects the DNA content of such cells. While K cyclin-transduced wt MEFs could not be kept in culture, K cyclin-transduced *p53*^{-/-} MEFs were maintained on a 3T9 culture protocol and samples were harvested up to 24 days. As shown in Fig. 5.11, after 12 days of culture, 15% of the MEFs had a DNA content of around N=16 and 40% of the

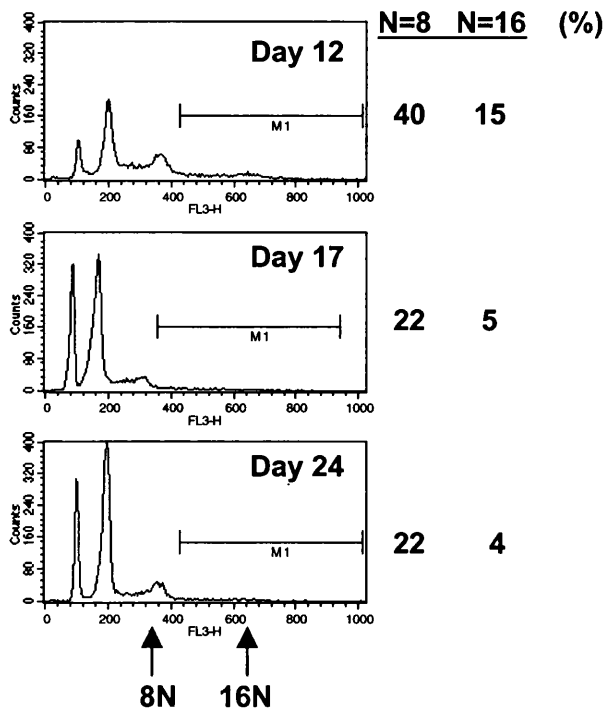


Fig. 5.11 Reduced ploidy upon prolonged culture of K cyclin-expressing *p53*^{-/-} MEFs. MEFs were transduced with 2FKpBabepuro virus for 24 h, selected by growth in puromycin for 48 h and split at 0.5×10^5 cells per well in 6-well plates. Cells were cultured for 5 days, pooled and grown in 10cm plates on a 3T9 protocol for 24 days. Cells were harvested on the indicated days, stained with PI and analysed by flow cytometry. Indicated are the percentages of cells with an 8N or 16N DNA content.

MEFs had a DNA content of around N=8. Surprisingly, at day 17 and 24 the ploidy was decreased, implying that a population of near-diploid *p53*^{-/-} MEFs expressing K cyclin had overgrown the polyploid cells.

5.7 Discussion

The results in the previous chapter showed that K cyclin induced a p53-dependent growth arrest, which was marked by the increased levels of p53 and consequent expression of p21^{Cip1} protein. However, cells containing wild-type p53 did not arrest at the classical G1 and G2 p53-dependent checkpoints (Agarwal et al., 1995), but instead, remained competent to synthesise DNA and became polyploid (Fig. 5.3 and 5.4). This is presumably explained by the insensitivity of K cyclin/Cdk complexes to Cdk inhibitors (Swanton et al., 1997), which are able to drive G1/S progression even in the presence of elevated p21^{Cip1} protein levels. In addition, wt MEFs expressing K cyclin contained amplified numbers of centrosomes (Fig. 5.6), again implying that at least part of the cell cycle-regulated processes were ongoing. Still, K cyclin-expressing cells did not exit the cell cycle and were defective at cytokinesis. I will refer to the ongoing DNA replication cycle in non-dividing cells as endoreduplication, a term that is generally used to describe replication of DNA without the subsequent completion of mitosis and/or cytokinesis, although the mechanism most likely differs from the classical endoreduplication of *Drosophila* larva salivary gland polytene chromosomes (reviewed in Edgar and Orr-Weaver, 2001).

Several questions arise from these observations: 1) why does cytokinesis not occur? 2) how and why is p53 activated 3) what is the sequence of these events, also with regards to centrosome amplification and polyploidisation. The cytokinesis defect appears to be p53-independent, since multinucleation and endoreduplication were also observed, and even enhanced, in *p53*^{-/-} MEFs. This suggests that the cytokinesis defect occurs before, or at least in parallel with, the activation of p53.

What triggers the cytokinesis defect upon K cyclin expression? Although the regulation of cytokinesis in mammalian cells remains largely unknown, the mechanism of cytokinesis mediated by the “mitotic exit network (MEN)” proteins in *S. Cerevisiae* or “SIN (septum initiation network)” in *S. Pombe* has been extensively studied (McCollum and Gould, 2001). MEN and SIN are signalling cascades that control the release of the Cdc14 phosphatase from the nucleolus into the cytoplasm, leading to the dephosphorylation and activation of critical mitotic regulator such as the cyclin/Cdk inhibitor Sic1 and the APC subunit Cdh1 (see introduction chapter 1). Active APC/Cdh1 then promotes mitotic cyclin

degradation and Sic1 activation decreases mitotic cyclin activity, together driving mitotic exit. Thus, although other regulatory mechanisms are likely to exist, it is clear that mitotic cyclin activity has to be shut down in order for cells undergo cytokinesis. Similarly, also in mammalian cells the mitotic cyclins A and B need to be targeted for degradation via ubiquitination by the anaphase-promoting complex (APC) (Morgan, 1999). Furthermore, at least in *Xenopus* egg extracts, the degradation of cyclin B depends on the inactivation of Cdk2 activity (D'Angiolella et al., 2001). So perhaps a premature or constitutive K cyclin-driven Cdk2-like activity deregulates the timing of the mitotic machinery, resulting in defective cytokinesis. Supporting this, recent evidence points to a correlation between the formation of multinucleated cells and deregulated expression of mitotic regulators that either impinge on mitotic spindle formation (Meraldi et al., 2002) or inhibit APC activity (Zhang and Lees, 2001).

Expression of K cyclin has been shown to prolong the duration of S phase (Laman et al., 2001a). Interestingly, a link between the correct timing of DNA replication and the assembly of chromatin that is fully competent to undergo normal chromosome segregation has been shown in various model systems. For example, yeast that lack the Cdk inhibitor Sic1, and therefore show precocious S-phase CDK activation, initiate DNA replication from fewer origins, have an extended S phase and accumulate in early mitosis with partly separated chromatids (Lengronne and Schwob, 2002). Over time, these cells died or resumed division, although cells exhibited double strand breaks and gross chromosomal rearrangements. Second, aberrant replication timing in *Drosophila* ORC2 mutants not only led to the extension of S phases, but also to abnormal chromosome condensation, a delay in mitosis and aberrant execution of mitosis, including chromatid bridges (Loupart et al., 2000). Third, a broad-scale study aimed to identify novel E2F target promoters not only identified the previously known DNA replication and cell cycle targets, but also a large set of genes involved in chromatin assembly/condensation (histones, nucleosomes), chromosome segregation (securin, CENP-E) and the mitotic spindle checkpoint (Bub3, CENP-E, Mad2) (Ren et al., 2002). This was consistent with microarray studies showing the control of mitotic genes by E2Fs (Ishida et al., 2001). Thus, an alternative, or perhaps complementary, explanation for the K cyclin-associated cytokinesis defect is that the K cyclin-induced alteration of the dynamics of S phase indirectly perturbs the execution of mitosis and thereby disrupts cytokinesis.

One manifestation of deregulated mitotic progression upon K cyclin expression is the pronounced nuclear localisation of cyclin B in flat, pre-mitotic cells (Fig. 5.10), indicative of a prophase delay. Cyclin B/Cdk1 complexes are cytoplasmic during interphase and are translocated to the nucleus in late G2 to initiate mitosis (chapter 1, section 1.2.8). Nuclear

localisation is normally only visible in a 10 minute time-window just before nuclear condensation (Hagting et al., 1999). The finding that a significant proportion of K cyclin-expressing cells contain nuclear cyclin B is therefore indicative of a prophase delay. The nuclear translocation of cyclin B is promoted upon its phosphorylation (Izumi and Maller, 1991). K cyclin/Cdk complex activity may therefore drive cyclin B phosphorylation, either directly, or via activation of cyclin B-directed kinases such as cyclin B/Cdk1 itself or polo-like kinase (Plk) (Takizawa and Morgan, 2000) (Peter et al., 2002).

Of note, the G2 DNA damage checkpoint involves the p53-mediated transcriptional upregulation of the 14-3-3- σ protein, which inhibits mitotic entry via binding and sequestration of cyclin B in the cytoplasm (Chan et al., 1999; Hermeking et al., 1997). The finding that nuclear localisation of cyclin B is visible in K cyclin-expressing cells with activated p53 therefore shows again that K cyclin expression evades a p53 checkpoint, through an unknown mechanism in this case. Importantly, cells do not show constitutive upregulation of cyclin B levels and enter S phase at rates comparable with control cultures (Fig. 5.3), implying that although K cyclin expressing wt cells do not divide, they adapt and re-enter the cell cycle machinery inappropriately. This differs from results described for expression of stable cyclin A or cyclin B, which arrests cells in metaphase or late anaphase, respectively (Gallant and Nigg, 1992; Parry and O'Farrell, 2001; Sigrist et al., 1995; Surana et al., 1993). However, in such cases relatively short-term effects were studied and often in synchronised systems. Since I studied the effects of long-term expression any (transient) mitotic arrest might have been overlooked.

The DNA replication licensing system ensures that DNA replication occurs only once per cell cycle. So how can K cyclin-expressing cells endoreduplicate while unable to undergo cytokinesis? Studies in yeast have shown that the inhibition of Cdk activity during the M/G1 transition bypasses the requirement of all other mitotic genes and is sufficient to ensure DNA relicensing (Noton and Diffley, 2000). In particular, high G1/S Cdk activity prevents the tight binding of the pre-RC proteins ORC and Cdc6 to the DNA, but the DNA binding capacity is regained when mitotic cyclin activity drops (see introduction of this thesis and (Blow and Hodgson, 2002; Diffley and Labib, 2002). This implies that component(s) of the pre-RC component(s) is/are phosphorylated and inactivated by Clb/cyclin B mitotic cyclin activity, as well as by G1/S cyclin activity, although the specific targets of mitotic cyclin activity are unknown. An additional regulatory mechanism active in mammalian and *Xenopus* cells is the APC-mediated proteolysis of geminin, an inhibitor of the pre-RC protein Cdt1 (Diffley, 2001). Together, APC activation and mitotic cyclin inhibition thus allow renewed DNA replication licensing. The ongoing nuclear division cycles and presence of mitotic spindles in K cyclin-expressing cells show that such cells, albeit not dividing, progress through all other

mitotic phases and reenter the cell cycle. Thus, K cyclin-expressing cells exit mitosis and presumably exhibit oscillations in APC and mitotic Cdk activities, thereby allowing endoreduplication to occur. Inactivation of Cdc2 in yeast (Correa-Bordes and Nurse, 1995; Dahmann et al., 1995), *Drosophila* (Hayashi, 1996) or human cells (Itzhaki et al., 1997) induces rereplication and bypasses the progression through mitosis, consistent with the notion that Cdc2/Cdk1 prevents untimely replication licensing (Nishitani and Lygerou, 2002). One possibility is therefore that K cyclin expression drives endoreduplication via the untimely inactivation of cyclin B, which releases the block on pre-RC complex formation and causes premature S phase entry.

What triggers the activation of p53 upon K cyclin expression? As mentioned, the finding that *p53*^{-/-} MEFs expressing K cyclin also show increased multinucleation and polyploidy, suggests that the activation of p53 occurs in parallel with or subsequent to the cytokinesis defect. A consequence of abortive cytokinesis is polyploidy. Interestingly, tetraploidy upon the so-called “adaptation” of cells after spindle disruption, in which cells re-enter G1 even though cytokinesis is disrupted, has been shown to trigger the activation of p53 (Andreassen et al., 2001; Lanni and Jacks, 1998; Minn et al., 1996). The activation of p53 was independent of spindle disruption *per se*, since p53 was induced subsequent to exit from mitosis (Minn et al., 1996) and tetraploidy induced upon inhibition of actin assembly also activated p53 (Andreassen et al., 2001). In such cases, the two daughter nuclei are arrested in G1 via the induction of the p21^{Cip} protein and inhibition of G1/S phase cyclin/Cdk activity (Andreassen et al., 2001). However, K cyclin/Cdk complexes are refractory to Cdk inhibitors, including p21^{Cip1} (Swanton et al., 1997). This most likely explains why even wt cells expressing K cyclin remain competent to synthesise DNA and become polyploid, analogous to endoreduplication ascribed to an inability to suppress cyclin E/Cdk2 kinase activity upon spindle disruption in *p53*^{-/-} or *p21*^{Cip1}^{-/-} cells (Di Leonardo et al., 1997; Khan and Wahl, 1998; Lanni and Jacks, 1998; Stewart et al., 1999) or upon γ -irradiation of *p21*^{-/-} cells (Waldman et al., 1996). Thus, tetraploidy upon K cyclin expression may activate p53, as a safeguard mechanism eliminating the survival of a polyploid/aneuploid population of cells.

It is unclear how a cell would detect tetraploidy and how this would result in the activation of p53. Polyploidy upon K cyclin expression correlated with increased numbers of centrosomes (Fig. 5.7). However, an excess number of centrosomes and consequent formation of multiple spindles is unlikely to activate p53, since cells do not have a checkpoint that aborts mitosis in response to extra spindle poles and, instead, divide regardless of abnormal numbers of spindle poles (Sluder et al., 1997). Interestingly, ploidy-specific regulation of gene transcription has been reported in isogenic budding yeast (Galitski et al., 1999). These authors suggested that ploidy affects gene transcription either because transient pairing of

homologous chromosomes directly alters gene expression, or because a presumed reduced nuclear surface area/cell volume ratio of polyploid cells could affect the nuclear import and concentration of transcriptional regulatory proteins. It therefore remains possible that a ploidy-specific transcriptional program leads to the activation of p53. An alternative explanation is that tetraploid cells exhibit changes in chromosomal topology or undergo DNA damage caused by a failure to properly segregate sister chromatids, and thereupon activate an ATM/ATR DNA damage-like checkpoint response. Indeed, anaphase bridges and chromosome fragments were visible in K cyclin-expressing MEFs (Fig. 5.9), suggesting that DSBs might have taken place.

K cyclin-expressing *p53*^{-/-} cells survived and proliferated and at the same time showed increased ploidy compared with mock-infected *p53*^{-/-} cells (Figs 4.6, 5.6 and 5.9). This implies that the K cyclin-induced decoupling of S phase and cell division also took place in the absence of p53, but at least a subpopulation of these cells was able to divide. Furthermore, in the time-lapse analysis of K cyclin-expressing *p53*^{-/-} cells a large cell with multiple nuclear lobules underwent division (see supplementary movie named *p53*^{-/-} K cyclin). The K cyclin-associated cytokinesis defect is therefore subverted in the absence of p53. However, the mechanism of this is unclear. K cyclin expression did not cause a permanent G1 or G2 arrest in wt MEFs (Fig. 5.3) and arrested *p21*^{-/-} MEFs (Fig. 4.7), indicating that p53 activated by K cyclin did not trigger a classical p21-dependent G1/G2 growth arrest (Stewart and Pietsenpol, 2001). Thus, it is unlikely that K cyclin-expressing *p53*^{-/-} cells proceeded through cytokinesis simply because a G1/G2 arrest was absent. Perhaps the prolonged survival of *p53*^{-/-} cells expressing K cyclin eventually releases the cytokinesis block. Alternatively, a negative regulator of cytokinesis may be absent in *p53*^{-/-} cells. This would mean that p53 can trigger a previously unidentified p21-independent cytokinesis arrest, although, as far as I am aware, there is no obvious p53 transcriptional target that would mediate such an arrest.

Both K cyclin-expressing *p53*^{-/-} and wt MEFs showed massive centrosome amplifications to numbers that, as far as I know, have never been detected before (Fig. 5.6). Centrosome amplifications were exacerbated in *p53*^{-/-} MEFs, presumably because the absence of p53 allows the survival of these cells. Recently, defects in cell division and concomitant multinucleation upon overexpression of mitotic regulator such as Aurora A and Polo-like kinase 1 (Plk1) were shown to give rise to centrosome amplifications (Meraldi et al., 2002). This is consistent with the findings in this chapter, since centrosome amplifications measured upon K cyclin expression correlate with ploidy (Fig. 5.7). In the case of K cyclin expression, cells with the highest DNA content contained one large nucleus, adding to the model of Meraldi and colleagues that ploidy, rather than multinucleation per se, correlates with centrosome numbers. However, if centrosomes replicate exactly once per cell cycle, then

a cell with N=16 should contain 8 centrosomes, whereas a significant proportion of the K cyclin expressing MEFs contained more than 8 centrosomes (Fig. 5.6). Thus, even though there is a general correlation between polyploidy and centrosome amplification, the actual timing of the DNA- and centrosome replication cycles may, at least in some cases, be uncoupled. Potential mechanisms may be the phosphorylation of critical inhibitors of the centrosome duplication cycle by K cyclin, including nucleophosmin or the kinase Mps1p (Hinchcliffe and Sluder, 2001a), or the splitting of centrosomes into two centrioles that both form functional spindle poles (for review see Hinchcliffe and Sluder, 2001b). Of note, centrosome amplification upon K cyclin expression in human fibroblasts was much less pronounced (Fig. 5.9), although the increase in ploidy was also diminished compared with MEFs (Fig. 5.6). The reason for the discrepancy in centrosome amplification between mouse and human cells is unclear. Taken together, K cyclin expression promotes chromosome instability at least via the formation of polyploid cells, but may also directly drive centrosome amplifications independent of DNA synthesis.

In summary, the results presented in this and the previous chapter are consistent with a model in K cyclin-expressing cells undergo abortive cytokinesis (the mechanism of which is unclear), adapt from the cytokinesis defect and become tetraploid (Fig. 5.12). Tetraploidy may trigger an E2F1- and p19^{ARF}-independent activation of p53, which elicits concurrent (p21^{Cip1}-independent) growth arrest and sensitisation to apoptosis. This is supported by the time-lapse studies, in which many large, multinucleated cells underwent apoptosis. In addition, DNA damage upon missegregation of chromatids may trigger DNA damage-induced p53 activation, although I have no direct evidence for this. Because K cyclin expressing growth-arrested cells lack functional G1 and G2 checkpoints, this leads to the formation of a polyploid/aneuploid population of cells with amplified centrosomes, which

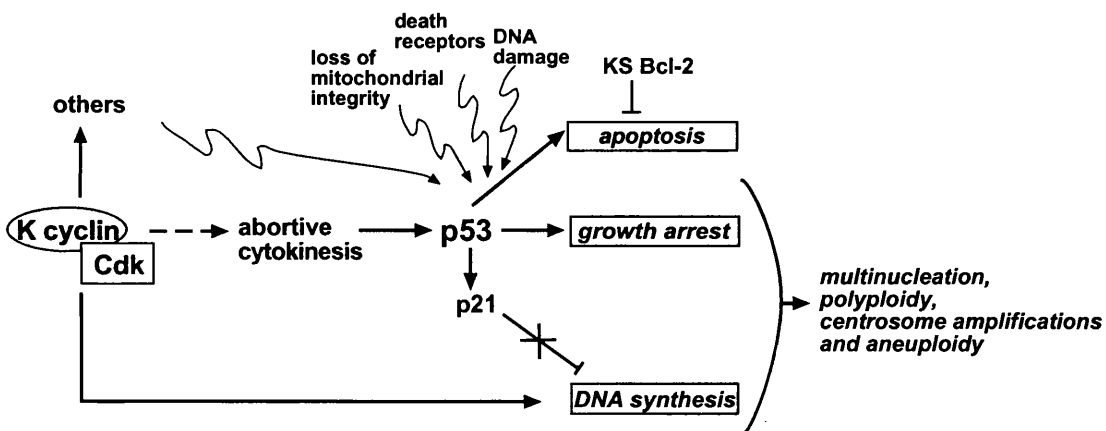


Fig. 5.12 Model showing how expression of K cyclin in MEFs triggers multiple responses. See text for details. Dotted arrow indicates that it is unclear why and how abortive cytokinesis upon K cyclin occurs.

survives and expands in the absence of p53. Additional apoptotic triggers such as the phosphorylation and inactivation of endogenous Bcl-2 are likely to contribute to the final fate of K cyclin-expressing cells.

These cell culture experiments show that K cyclin expression promotes DNA replication and aneuploidy, which in the absence of p53 are able to survive and expand. However, which of these attributes contribute to the tumourigenic potential of K cyclin is unclear. Importantly, long-term culture of K cyclin-expressing *p53*^{-/-} cells resulted in the outgrowth of a near-diploid population of cells (Fig. 5.11). This either suggests that aneuploid cells do not necessarily have a growth advantage, or that there is selection for cells which contain a subset of chromosomes/genes that promotes cell proliferation. Notably, also the prolonged culture of *p53*^{-/-} MEFs in culture suppresses centrosome amplifications present in these cells (Chiba et al., 2000), consistent with the notion that widespread genomic instability may be a transient feature. In the next chapter I attempt to address which of the consequences of K cyclin expression are important for K cyclin-mediated tumourigenesis in an *in vivo* mouse model of cancer.

CHAPTER 6:
CONSTRUCTION AND CHARACTERISATION OF E μ
***K* CYCLIN TRANSGENIC MICE**

6.1 Introduction

The cell culture experiments presented in the previous chapters suggest that K cyclin expression promotes DNA replication and aneuploidy in wt and *p53*^{-/-} cells, which in the absence of p53 are able to survive and expand. In addition, previous experiments performed in the labs of Nic Jones and others have shown that, *in vitro*, K cyclin is a potent activator of Cdk6, triggering the phosphorylation of both cyclin D and E-like targets, and that such complexes are resistant to Cdk inhibitors (Laman et al., 2000). However, although these properties of K cyclin support the hypothesis that it can function as an oncogenic protein, K cyclin expression does not promote fibroblast transformation as measured by soft agar colony forming assays (H. Laman, personal communication). A possible explanation for this is that K cyclin expression arrests cells in a p53-dependent manner, as shown in chapter 4, such that the tumourigenic potential of K cyclin is only detected in the absence of p53. In order to further address these issues and study the K cyclin-mediated tumourigenic properties *in vivo*, I therefore decided to develop a K cyclin transgenic mouse model. I also aimed to generate K cyclin/vFLIP transgenic mice, since v-FLIP is expressed from an IRES 3' of the K cyclin gene (see Fig. 1.9), allowing the cloning of both genes together and the possibility to study a potential effect of v-FLIP on K cyclin-induced tumourigenesis.

The immunoglobulin heavy chain enhancer (IgH enhancer), also called E μ , was chosen to drive K cyclin expression. The E μ enhancer is thought to be required for the opening of chromatin during B lymphocyte immunoglobulin rearrangements (Abbas et al. 1994). Importantly, many leukemias and lymphomas in humans are associated with translocations of the IgH locus on chromosome 14 with genes that regulate cell proliferation and survival, causing deregulated transcriptional activation of such genes. Examples of these are Burkitt's lymphoma, follicular lymphoma and centrocytic B cell lymphoma in which chromosome translocations associated with, *c-myc* t(8;14), *Bcl-2* t(14;18) and cyclin D1 t(11;14) chromosome translocations are observed, respectively (Abbas et al. 1994). Of note, transgenes driven by the E μ enhancer are not only expressed throughout B cell development, but also in T lymphocytes and at low levels in the heart (Bodrug et al., 1994; Grosschedl et al., 1984; Moroy et al., 1990).

There were several reasons for choosing the E μ enhancer to drive lymphocyte-specific K cyclin expression. First, KSHV infection of B lymphocytes predisposes to the development of lymphoproliferative disorders (Cesarman et al., 1995) and E μ K cyclin-driven lymphomagenesis would therefore be a valid model with regards to KSHV pathogenesis. Second, Cdk6, the primary binding partner of K cyclin, is abundantly expressed in lymphocytes (Meyerson and Harlow, 1994). Third, lymphocytes are prone to undergo

apoptosis and roles for E2F1, p19^{ARF}, p53 and/or p73 have been established in apoptosis during thymocyte negative selection and activation-induced apoptosis of peripheral thymocytes (Garcia et al., 2000; Lissy et al., 2000; Zhu et al., 1999). Furthermore, mice that do not express p53, p19^{ARF} or E2F1 are predisposed to the development of lymphomas (Donehower et al., 1992; Kamijo et al., 1999; Yamasaki et al., 1996). Lymphocytes therefore constitute an ideal model to study K cyclin-induced proliferative and apoptotic effects.

Expression of cyclin D1 from the E μ enhancer does not promote lymphomagenesis on its own, but E μ cyclin D1 expression significantly shortens the average latency period of B and T cell lymphomas in E μ *N-*, *L-* or *c-Myc* mice (Bodrug et al., 1994; Lovec et al., 1994). This implies that, in the case of lymphocytic cyclin D1 expression, activation of an additional pathway such as Myc expression is required to drive proliferation. Interestingly, *c-Myc* expression correlates with an increase in cyclin E/Cdk2 activity (Alevizopoulos et al., 1997), explained in part by the *c-Myc*-induced upregulation of cyclin D1 and D2 protein expression, which sequester Cdk inhibitors away from cyclin E/Cdk2 complexes (Bouchard et al., 1999; Perez-Roger et al., 1999). This increase in cyclin E-directed activity could partly explain the ability of Myc to cooperate with cyclin D1 in transformation. Since K cyclin-directed Cdk activity resembles combined cyclin D and E activities, it would therefore be interesting to compare the ability of K cyclin and cyclin D1 to promote lymphomagenesis in E μ transgenic mice.

Taken together, E μ *K cyclin* transgenic mice would be useful to study the effect of K cyclin expression on lymphocyte proliferation and apoptosis, and allow a dissection of the pathway(s) important for K cyclin-induced tumourigenesis. In addition, although *p53*^{-/-} MEFs and lymphocytes are reported to exhibit centrosome amplifications and aneuploidy (Fukasawa et al., 1996; Fukasawa et al., 1997), an ongoing debate is whether centrosome amplification and/or aneuploidy are a requirement or a consequence of tumour formation (Marx, 2001). Considering the finding that K cyclin had pronounced effects on centrosome amplification in MEFs, it would therefore be interesting to establish whether centrosome amplifications and/or aneuploidy are detected in K cyclin-induced tumours *in vivo*.

6.2 Generation of E μ *K cyclin* and E μ *K cyclin/v-FLIP* transgenic mice

Double Flag-tagged *K cyclin* cDNA was subcloned either alone or as a bicistronic construct together with *v-FLIP* cDNA into pHSE3' plasmid, creating K cyclin pHSE3' and K cyclin/*v-FLIP* pHSE3'. These constructs drive transgene expression from the H-2K promoter/IgH enhancer cassette (Fig. 6.1A). Expression of Flag-tagged K cyclin was

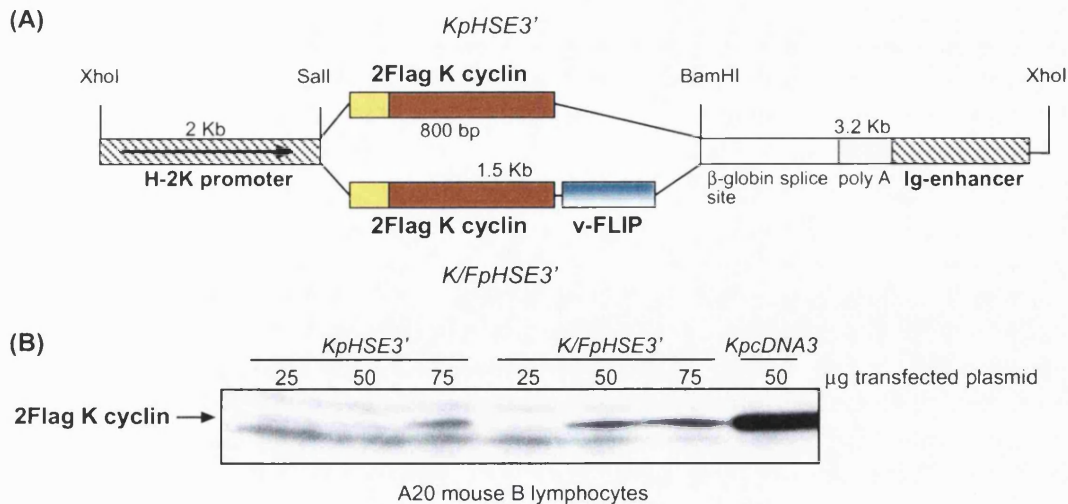


Fig. 6.1 Generation of E μ K cyclin and E μ K cyclin/v-FLIP transgenic constructs. (A) Schematic representation of the E μ K cyclin (*KpHSE3'*) and E μ K cyclin/v-FLIP (*K/FpHSE3'*) transgenic cassettes that were injected into fertilised mouse oocytes. H-2K promoter= Heavy chain promoter, Ig-enhancer= Immunoglobulin enhancer. (B) Expression of double Flag-tagged K cyclin from transgenic constructs. 10×10^6 A20 mouse B lymphocytes per sample were electroporated with varying amounts of the indicated plasmids. Cell lysates were prepared 30 h later using laemmli lysis buffer. All proteins were resolved on a 12% SDS-PAGE gel and immunoblotted using anti-Flag antibody.

confirmed in mouse A20 B cells transiently transfected with K cyclin or K cyclin/v-FLIP *pHSE3'* plasmid (Fig. 6.1B). The co-expression of v-FLIP could not be confirmed by immunoblot analysis, since no antibody against v-FLIP was available at the time. However, v-FLIP expression from the bicistronic construct was confirmed by *in vitro* transcription/translation (Fig. 3.7B).

Linearised, purified plasmids were injected into the fertilised oocytes of CBA x C57BL/6 (F1) founder animals using standard techniques (Brinster et al., 1985). The offspring were screened for the integration of *K cyclin* transgene DNA by PCR analysis and Southern blot analysis of tailsnip-derived DNA. Potential founders were backcrossed to F1 mice, screened for transmission of the transgene DNA and offspring were shown to express Flag-tagged K cyclin by immunoblot analysis of lymphocytes. In this way, two founder lines for *K cyclin* were generated (called E μ K cyclin 1996E-H (6) and E μ K cyclin 2269A.1 (20),

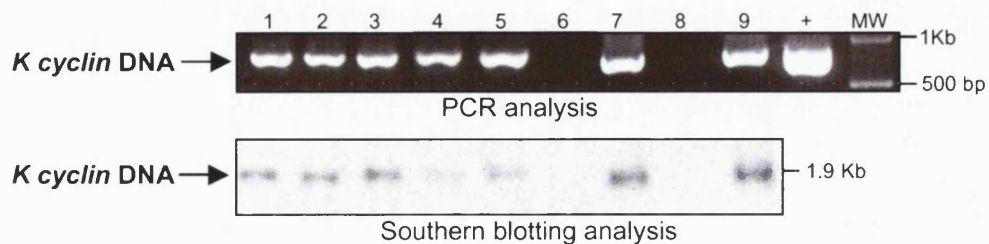


Fig. 6.2 Genotyping of a litter of E μ K cyclin pair 6 mice. DNA was isolated from tailsnips and analysed by PCR (upper panel) or by Southern blotting (lower panel) for the presence of *K cyclin* cDNA. MW= 1Kb molecular weight marker, + = positive control PCR on 2FKpcDNA3 plasmid.

or pair 6 and pair 20), and one founder line for K cyclin/*v-FLIP* was generated (called E μ K cyclin/*v-FLIP* 2284G (14), or pair 14). An example of PCR analysis and Southern analysis of a litter of founder line pair 6 is shown in Figure 6.2.

The copy number of integrated transgene DNA in a particular mouse strain can be estimated by Southern analysis. However, actual protein expression levels are also determined by the transcriptional activity of the region surrounding the site of integration, and possible repeat-induced gene silencing within the integrated transgene array itself (Henikoff, 1998). The expression of transgene DNA in lymphocytes of the various E μ K cyclin lines was therefore compared using IP/Western blotting analysis. E μ K cyclin pair 6 and E μ K cyclin/*v-FLIP* pair 14 showed a similar K cyclin expression pattern, with high levels in the splenocytes and low levels in the thymocytes. In contrast, E μ K cyclin pair 20 only expressed low levels of K cyclin in the thymocytes (Fig. 6.3A).

All lymphocytes originate in the bone marrow. However, T cells mature further in the

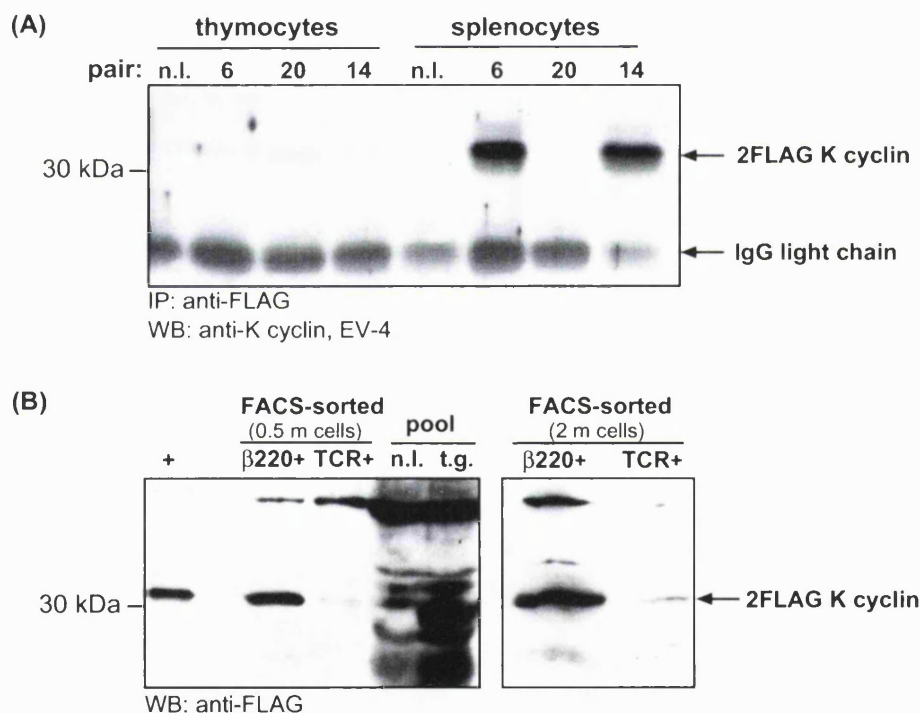


Fig. 6.3 Expression of K cyclin protein in transgenic lines. (A) IP/Western analysis of Flag-tagged K cyclin. Single lymphocyte suspensions were prepared from spleens and thymuses of 8-10 week old mice. Protein lysates were prepared in RIPA buffer, 250 μ g of proteins were pre-cleared with sepharose beads, followed by immunoprecipitation with monoclonal anti-Flag antibody. All immunoprecipitated proteins were separated on a 12% SDS-PAGE gel and immunoblotted using rabbit polyclonal anti-K cyclin EV-4 antibody (see materials and methods for generation of this antibody). (B) Western blot analysis of FACS-sorted splenocytes. Spleens were isolated from two 5 week old mice from pair 6 E μ K cyclin transgenic line. Splenocytes were stained with anti-mouse TCR β chain antibody or anti-mouse CD45R/ β 220 antibody. Splenocytic B and T cells were FACS-sorted and protein lysates of 0.5×10^6 or 2×10^6 cells were resolved on a 12% SDS-PAGE gel. As controls, a sample of K18 IPTG-induced Rat-1 cells (+) and lysates from pooled splenocytes (2.5×10^6 cells per sample) were run. m= million, n.l.= negative littermate, t.g.= transgenic mouse pair 6, WB= western blotting. Expression of K cyclin detected in the TCR-positive splenocytes was not because of protein overflow between wells, as the samples shown in the right hand panel are separated by an empty well.

thymus, whereas B cells leave the bone marrow as mature cells. This is why the bone marrow and thymus are called the primary lymphoid organs. Bone marrow-derived B cells and thymus-derived T cells migrate from these primary organs to the blood and peripheral lymphoid organs, where they proliferate and complete their differentiation. The spleen and lymph nodes are examples of such peripheral lymphoid organs (Abbas et. al. 1994). Splenocytic lymphocytes consists of around 60% B cells and 30% T cells (see for example Fig. 6.9A). To assess whether K cyclin was detected in peripheral B cells as well as T cells, splenocytes stained with cell surface TCR or the pan-B cell marker CD45R (β 220) were subjected to FACS sorting followed by immunoblotting analysis. Both B and T cells isolated from the spleen showed detectable expression of K cyclin protein, showing that both peripheral B cells as well as T cells express the transgene (Fig 6.3B).

6.3 Effect of K cyclin expression on thymocyte maturation

Proliferation and apoptosis are integral to the maturation process of T cells. These maturation events are characterised by specific patterns of surface expression of the T cell receptor (TCR) and the CD4 and CD8 accessory molecules (Fig. 6.4). Specifically, the most immature T cells, CD4⁻ CD8⁻ or double negative (DN) cells, migrate from the bone marrow to the thymus cortex (the peripheral zone of a thymus lobule). Here, they begin to express both CD4 and CD8 accessory molecules (double positive, DP cells), together with low levels of the TCR. As the DP cells migrate from the cortex to the medulla (inner zone of a thymus

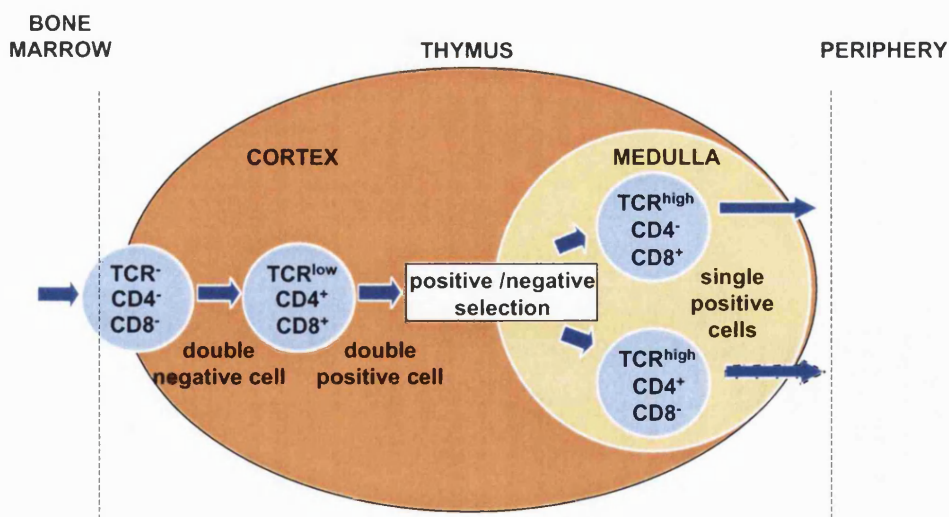


Fig. 6.4 Maturation of T cells in the thymus. CD4⁻ CD8⁻ double negative cells in bone marrow migrate to the thymus cortex, where they express both CD4 and CD8 and low numbers of TCR receptors. TCR expression increases as maturation proceeds. Single positive CD4⁻ or CD8⁻ positive cells are selected from this population by positive and negative selection (see text for details). Adapted from (Abbas et al., 1994).

lobule), their TCRs interact with major histocompatibility complex (MHC) molecules, which contain processed antigenic peptides, expressed on epithelial cells in the cortex. Only T cells containing TCRs that recognise self-MHC appropriately are positively selected and permitted to survive. Negative selection eliminates T cells with TCRs that bind with high affinity to self-peptide antigens via the induction of apoptosis in such T cells. The T cells that survive this selection process bear high levels of functional TCR with appropriate affinity and selectivity and mature to either CD4⁺ CD8⁻ or CD4⁻ CD8⁺ single positive (SP) T cells, which leave the thymus and migrate to peripheral lymphoid tissues (Tough and Sprent, 1995; Zuniga-Pflucker and Lenardo, 1996).

The expression of Eμ K cyclin could affect proliferation and apoptosis of lymphocytes *in vivo*. To establish whether Eμ K cyclin expression alters thymocyte maturation, the ontogeny of thymocytes was determined by T cell surface marker labelling and flow cytometric analysis. K cyclin expression did not affect the total cellularity of the thymus as reflected by the total number of thymocytes (around 70-100 x 10⁶ cells per thymus of 10-week old mice, Fig. 6.5A). In addition, no convincing effect of K cyclin expression on T cell ontogeny was detected, although the number of CD8 SP thymocytes in transgenic thymuses was slightly reduced (4.2 or 4.4 % in controls versus 0.73 or 0.9 % CD8 SP in

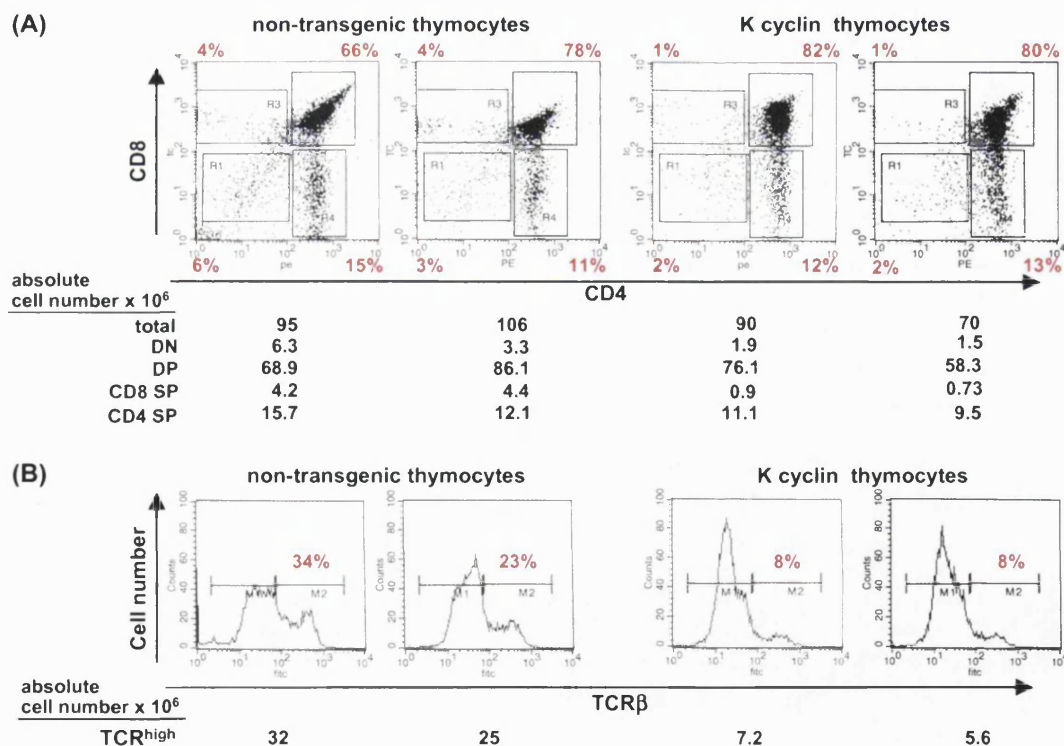


Fig. 6.5 K cyclin expression interferes with thymocyte maturation *in vivo*. (A) Thymocytes from 10-week old littermates were stained with CD4-PE and CD8-TC antibodies and subjected to two-colour flow cytometric analysis. Indicated in red are the percentages of DN (lower left) DP (upper right), CD8 SP (upper left) and CD4 SP (lower right) thymocytes. The absolute numbers of thymocytes of each subpopulation are indicated below the dot-plots. (B) Thymocytes from the same mice as in A) were stained with TCRβ-FITC antibody and analysed by flow cytometry. Percentages of TCRβ^{high} are indicated in red and absolute numbers are shown below the histograms.

transgenics Fig. 6.5A, corresponding to $\sim 4.3 \times 10^6$ SP cells in non-transgenic controls versus $\sim 0.8 \times 10^6$ SP cells in transgenic thymuses). However, K cyclin expression correlated with a pronounced reduction in the percentages and absolute numbers of TCR-expressing cells (23 and 33% in controls versus 8% TCR⁺ in transgenics, corresponding to $\sim 25\text{-}32 \times 10^6$ TCR β^{high} cells in controls versus $\sim 6\text{-}7 \times 10^6$ TCR β^{high} cells in transgenics, Fig. 6.5B). Furthermore, the dot-plot of the DP population of cells in transgenic mice contains less of a “beak shape”, suggesting that K cyclin affects expression levels of CD4 and C8 co-receptors in DP T cells. Taken together, although preliminary, these data suggest that K cyclin expression interferes with the maturation thymocytes from DP to SP stages *in vivo*.

6.4 E μ K cyclin expression predisposes to the development of lymphomas

E μ *K cyclin* (pair 6 and 20) and E μ *K cyclin/v-FLIP* (pair 14) transgenics and non-transgenic control mice were monitored for the development of lymphomas. Two cohorts of mice with slightly different genetic backgrounds were followed: “ICRF mice” on a congenic C57Bl/6 x CBA/Ca (“F1”) background, and the re-derived “UCSF mice” on a mixed C57/Bl6 x CBA/Ca background. Mice were regularly checked for behavioral changes and/or the presence of tumour lumps as described in the materials and methods. None of the control mice (20 mice in ICRF cohort; 25 mice in UCSF cohort) developed lymphomas, in agreement with the described absence of lesions in C57BL/6 mice under 18 months of age (Bronson, T., 1990). In addition, no lymphomas were detected in pair 20 E μ *K cyclin* mice, and only 1 out of 20 of the pair 14 E μ *K cyclin/v-FLIP* mice developed lymphoma at 8 months of age. However, around 15 % of E μ K cyclin pair 6 transgenic mice develop B or T cell lymphomas by around 7 months of age, with no detectable influence of genetic background (Table 6.1). Together, these results suggest that K cyclin expression predisposes to the development of lymphomas, the incidence of which is not enhanced in *K cyclin/v-FLIP* mice.

Table 6.1. Tumour development in E μ K cyclin pair 6 transgenic mice.

Case no.	Sex	Day of detection	Tumour type
"ICRF cohort" (n=21)			
ICRF, 1	F	175	n.d.
ICRF, 2	F	181	Thymoma, CD8+ T cell
ICRF, 3	M	203	Splenomegaly, CD4+ T cell
ICRF, 4	F	238	Splenomegaly, CD4+ T cell
"UCSF cohort" (n=27)			
UCSF, 1	M	181	n.d.
UCSF, 2	M	205	Splenomegaly
UCSF, 3	M	205	Splenomegaly
UCSF, 4	F	270	Thymoma

n.d. = not determined

E μ K cyclin tumours either heavily involved the thymus (thymoma, Fig. 6.6A) or the spleen (splenomegaly) (Table 6.1) and enlarged lymph nodes were detected in all cases, indicating that tumours had disseminated to the lymph. Flag-tagged K cyclin was highly expressed in disseminated lymphomas (Fig. 6.6B), confirming the association of K cyclin expression with tumorigenesis. Three of the 8 lymphomas were analysed by flow cytometry and shown to consist of enlarged (blastoid) CD4 SP or CD8 SP T cells (Fig. 6.6C, D and Table 6.1), implying that these tumours might be clonogenic. However, the finding that some E μ K cyclin mice exhibited splenomegaly without concomitant enlargements of the thymus suggests that K cyclin expression also predisposes to B cell lymphoma.

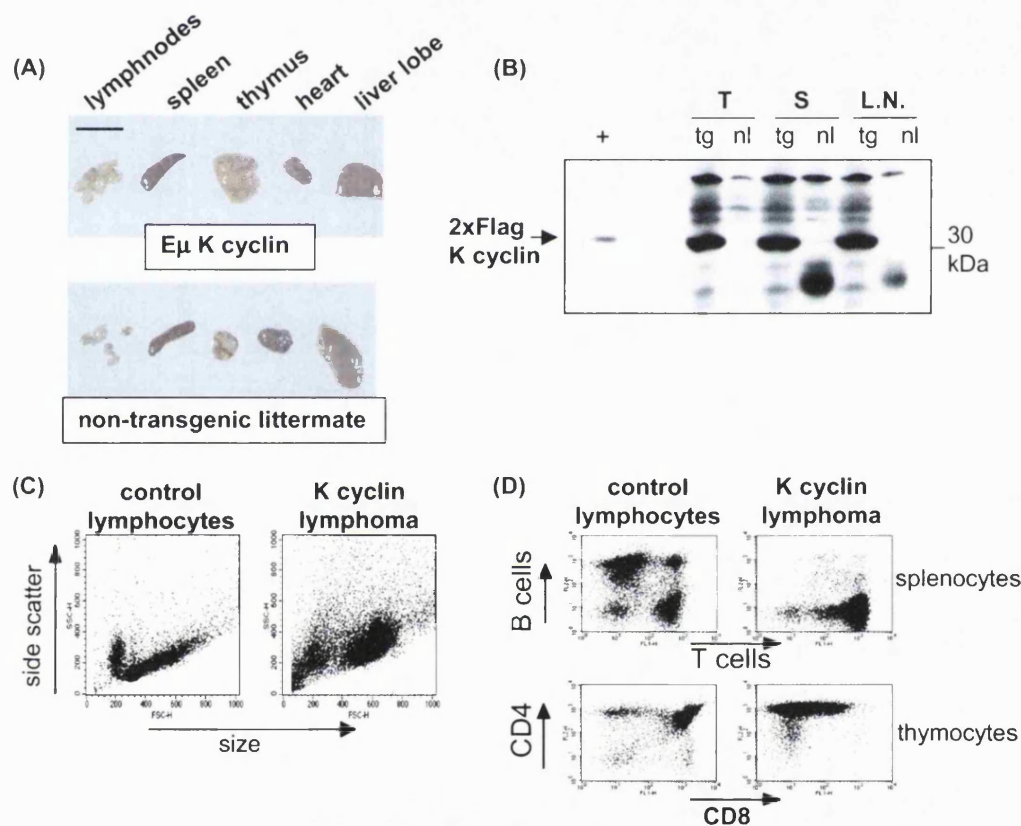


Fig. 6.6 Characterisation of E μ K cyclin tumours. (A) Lymphoid organs (thymus, spleen and axial, inguinal and mesenteric lymph nodes) from a terminally ill E μ K cyclin (ICRF, 2), and a non-transgenic littermate control mouse were dissected and photographed. Heart and liver lobe are shown as a reference. Scale bar: 1 cm. (B) Single cell suspensions of thymocytes (T), splenocytes (S) and lymph node cells (L.N.) were prepared from an E μ K cyclin lymphoma (tg) (ICRF, 3) and cells from a negative littermate control (nl). Lysates from 10 x 10⁶ cells were resolved on a 12% SDS-PAGE gel and immunoblotted with anti-Flag antibody. A lysate of IPTG-induced K18 Rat-1 cells expressing K cyclin was run as a positive control. (C) Single cell suspensions of E μ K cyclin lymphoma cells (ICRF, 3) and control splenocytes were analysed by flow cytometry. Dotplots depict an increased forward and side scatter of lymphoma cells. (D) Single cell suspensions of lymphoma (ICRF, 3) and control lymphocytes were stained with anti-CD4-PE and anti-CD8-FITC or with anti-B220-PE and anti-TCR β -FITC antibodies and analysed by flow cytometry. Dotplots show that this E μ K cyclin lymphoma consists of CD4+ T-cells.

6.5 Disruption of the p53 pathway in E μ K cyclin lymphomas

The finding that E μ K cyclin mice developed lymphomas with a relatively low incidence and late onset suggested that secondary somatic mutations might be required for lymphomas to arise. Based on the *in vitro* experiments described in the previous chapters, mutation of the p53 gene was considered a strong candidate cooperative stimulus. Mutant forms of p53 accumulate to supraphysiological levels because they are transcriptionally inactive and do not induce *Mdm2* to trigger their own destruction (Haupt et al., 1997; Kubbutat et al., 1997). Indeed, detection of high levels by histopathological methods has been used as a diagnostic marker for p53 mutations in tumours (Hall and Lane, 1994; Soussi, 2000) and references herein). Importantly, E μ -myc-induced lymphomas that contained high levels of p53 were shown to contain missense mutations of p53 as established by sequencing of the p53 gene, while p53 was wild-type in randomly selected tumours expressing lower levels of p53 (Eischen et al., 1999). I therefore examined whether p53 levels were upregulated in K cyclin lymphomas.

Immunoblotting analysis demonstrated high levels of p53 in two out of three of the analysed tumours. Furthermore, all three tumours contained increased levels of p19^{ARF} protein compared with non-transgenic control (Fig. 6.7). P19^{ARF} is not detected in mouse cells unless p53 is mutated, explained by the abrogation of a p53-mediated negative feedback loop upon p53 inactivation (Stott et al., 1998). In addition, overexpression of Mdm2 and consequent inactivation of p53 correlates with p19^{ARF} overexpression in lymphomas (Eischen et al., 1999). Thus, the presence of high p53 protein levels in two, and high p19^{ARF} protein levels in all three E μ K cyclin lymphomas, strongly suggests that the p53 pathway is disrupted in the analysed lymphomas.

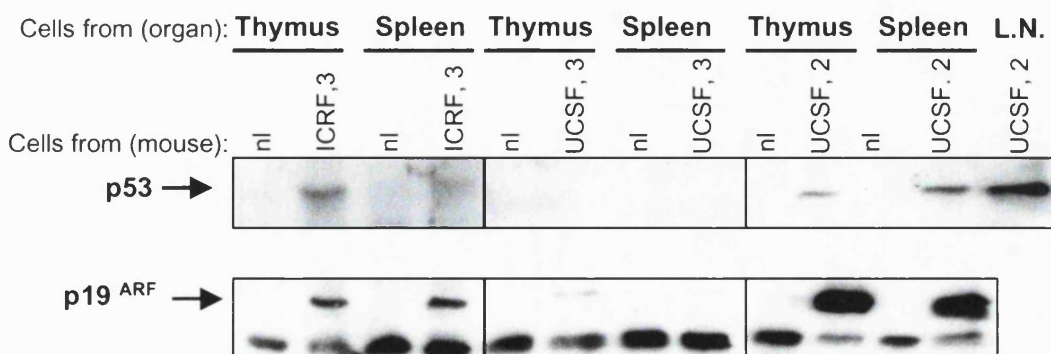


Fig. 6.7 p53 and p19^{ARF} expression in E μ K cyclin lymphomas. Single cells suspensions prepared from tumours or control littermate thymocytes or splenocytes were resolved on 12% SDS-PAGE gels (20 μ g of protein per lane) and immunoblotted with anti-p19^{ARF} or CM5 anti-p53 antibody. nl= negative littermate.

6.6 E μ *K cyclin* cooperates with p53 and p19^{ARF} loss in lymphomagenesis

To determine whether loss of p53 exacerbates the oncogenic action of K cyclin *in vivo*, E μ *K cyclin* pair 6 transgenic mice were bred with *p53*^{-/-} mice and the progeny of different genotypes followed for the development of lymphoma. During a one year follow-up around 15% (3/21) of K cyclin transgenic mice on a wt background developed lymphoma around 7 months (200 days) of age (Fig. 6.8A), similar to the results showed in table 6.1. The absence of one allele of p53 in *K cyclin/p53*^{+/-} mice increased the incidence of lymphomas to 52% (12/23), although the time of tumour onset was not much earlier. The non-transgenic *p53*^{-/-} mice succumbed to a variety of tumours (the majority of which were B or T cell

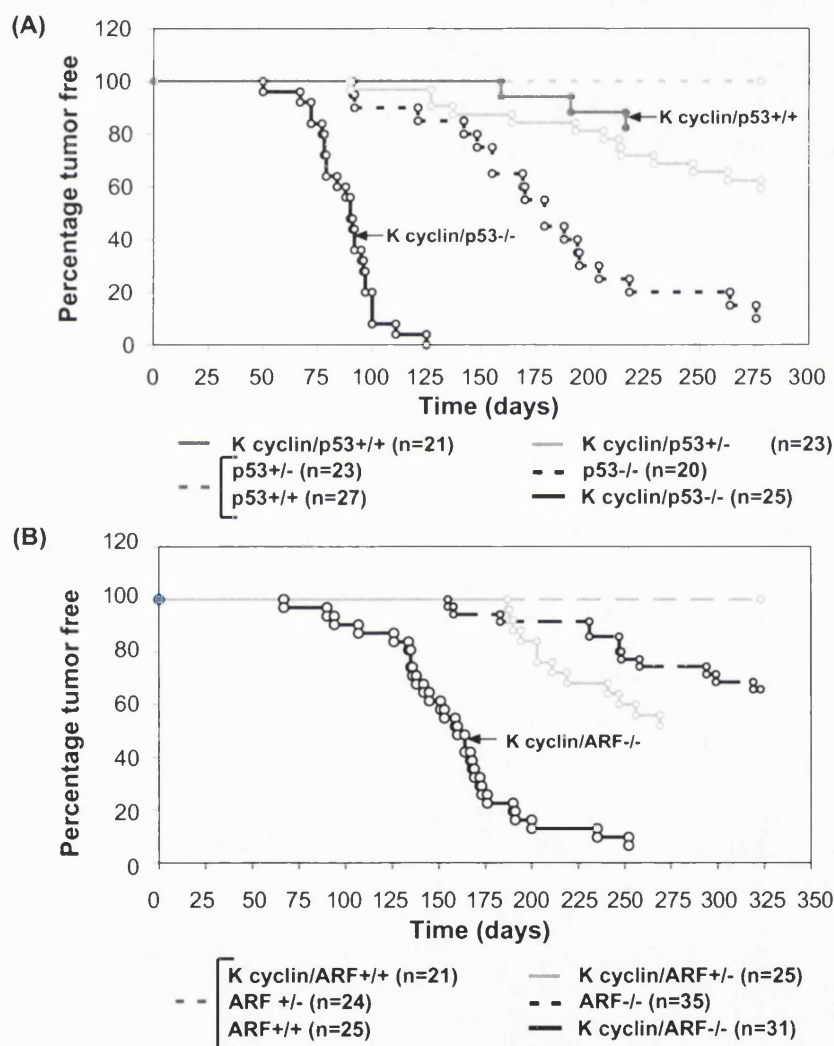


Fig. 6.8 E μ *K cyclin* cooperates with p53 and p19^{ARF} loss in tumourigenesis. (A) E μ *K cyclin* transgenic mice and *p53*^{-/-} mice were bred to generate *K cyclin* transgenic mice on *p53*^{-/-}, *p53*^{+/-} or *p53*^{+/+} backgrounds. Shown are Kaplan Meier survival curves of mice to 9 months of age. The curve of *K cyclin/p53*^{+/+} mice is not finished because some (8) surviving mice are younger than 275 days old. (B) E μ *K cyclin* transgenic mice and *ARF*^{-/-} mice were bred together to generate *K cyclin* transgenic mice on *ARF*^{-/-}, *ARF*^{+/-} or *ARF*^{+/+} backgrounds. Shown are Kaplan Meier survival curves of mice to 11 months of age. Curves of *K cyclin/ARF*^{+/-} and *K cyclin/ARF*^{-/-} mice are unfinished because 4 or 2 mice, respectively, are younger than 325 days old. Mice showing abnormal clinical signs were sacrificed.

lymphomas) around 6-7 months of age, in agreement with previous reports on tumour development in *p53*^{-/-} mice (Donehower et al., 1992; Harvey et al., 1993a). Strikingly, all Eμ *K cyclin* transgenic mice with a *p53*^{-/-} genotype rapidly developed lymphomas with a mean latency of around 2.5-3 months (85-90 days). Thus, the oncogenic potential of *K cyclin* is revealed *in vivo* by loss of *p53*.

Experiments in chapter four showed that the loss of *p19*^{ARF} did not impair apoptosis and growth arrest upon *K cyclin* expression in MEFs. However, the role of *p19*^{ARF} might be cell type dependent. In addition, loss of *p19*^{ARF} has been shown to cooperate with Eμ-*Myc* in the induction of lymphomas (Eischen et al., 1999), indicating that *p19*^{ARF} acts as a tumor suppressor in lymphocytes. To compare the involvement of *p53* and *p19*^{ARF} in *K cyclin* tumorigenesis, Eμ *K cyclin* mice were similarly crossed with *ARF*^{-/-} mice and the resulting mice of various genotypes were monitored for tumour development. As reported, non-transgenic *ARF*^{-/-} mice developed tumours (mainly sarcoma or lymphoma) around 8 months (250 days) of age (Kamijo et al., 1999). Similar to the described cooperation with *p53* loss, *K cyclin* expression increased the incidence and accelerated tumour onset to around 5 months (150 days) of age (Fig. 6.8B). In conclusion, not only *p53*, but also *p19*^{ARF}, are potent antagonists of *K cyclin*-induced oncogenesis in lymphocytes.

Mice showing abnormal behavior or obvious tumour lumps were sacrificed and biopsies were performed. All sick mice developed lymphomas that were often highly dispersed. A summary of the tumour spectrum in these mice is shown in Table 6.2. Most *K cyclin/p53*^{-/-} mice developed thymic T cell lymphoma, in which the thymus had extended throughout the thoracic cavity and thus killed mice by compression of the pericardial cavity and the heart. In striking contrast, *K cyclin/ARF*^{-/-} mice solely developed B cell lymphomas

Table 6.2. Tumour spectrum in Eμ *K cyclin* x *p53*^{-/-} and x *ARF*^{-/-} mice.*

K cyclin / p53^{-/-} (n=25)	K cyclin / ARF^{-/-} (n=29)
12 T cell lymphoma (48%)	0 T cell lymphoma
1 SP, TCRβ high (8%)	
1 DP, TCRβ high (8%)	26 B cell lymphoma (90%)
6 DP, TCRβ low (24%)	
4 n.d.	3 other than lymphoma (10%)
	1 osteosarcoma
4 B cell lymphoma (16%)	2 unknown
9 B and T cell lymphoma (36%)	
1 SP, TCRβ high (4%)	
1 DP, TCRβ high (4%)	
4 DP, TCRβ low (16%)	
3 n.d.	

* No influence of sex on the onset or type of tumour development was detected. In addition, no recurrent difference in age of tumour onset was detected between mice with T and/ or B lymphomas. n.d.= not determined

which was apparent upon dissection by the pronounced splenomegaly. B or T cell tumour cells had often disseminated to the lymph nodes and/or liver. In addition, in some cases T cell infiltrates were found in the spleen and B cell infiltrates in the thymus. A subset of tumours were further characterised by cell surface marker staining and flow cytometric analysis. Of note, all B cell tumours lacked expression of surface IgM, a marker for B cell differentiation, and most T cell tumours consisted of DP cells that expressed low levels of the TCR β chain. These expression patterns are consistent with such lymphomas being precursor B or T cell neoplasms (Morse et al., 2002), implying that K cyclin drives the proliferation of undifferentiated lymphocytes. Examples of the phenotypic analysis of disseminated B cell tumour cells, precursor TCR β^{low} T cell tumour cells isolated from the same mouse and precursor B cell tumour cells from a different mouse are shown in Fig. 6.9. Importantly, the finding that the predominant tumour type differed in mice that lacked p53 or p19^{ARF} expression implies that these tumour suppressors modulate K cyclin-associated tumourigenesis in separate ways.

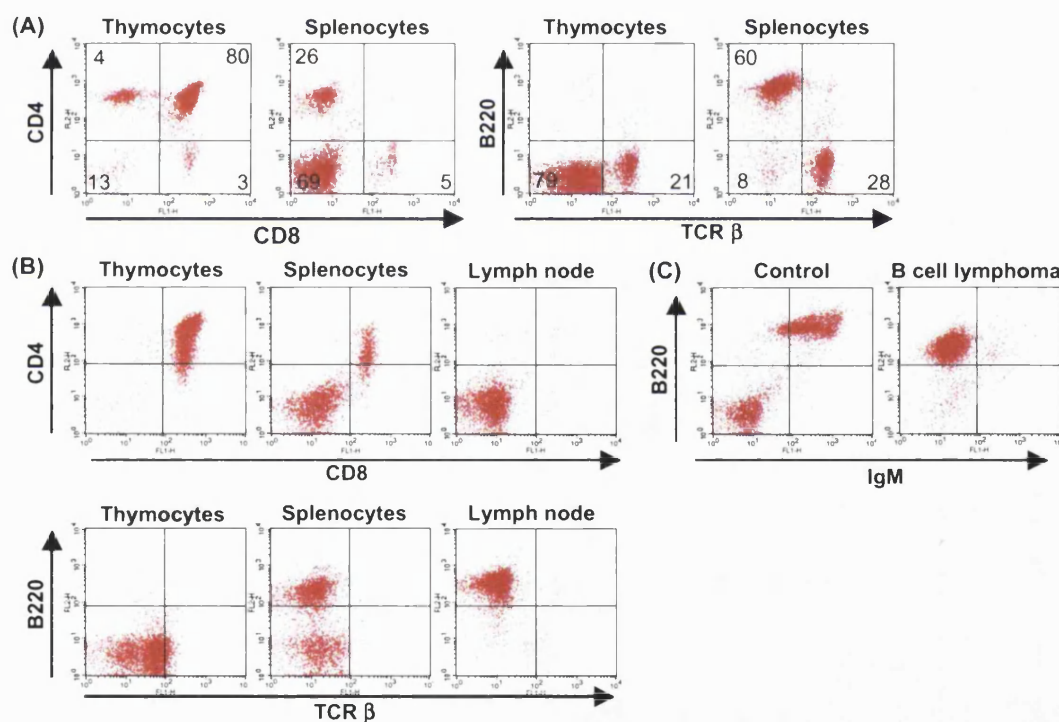
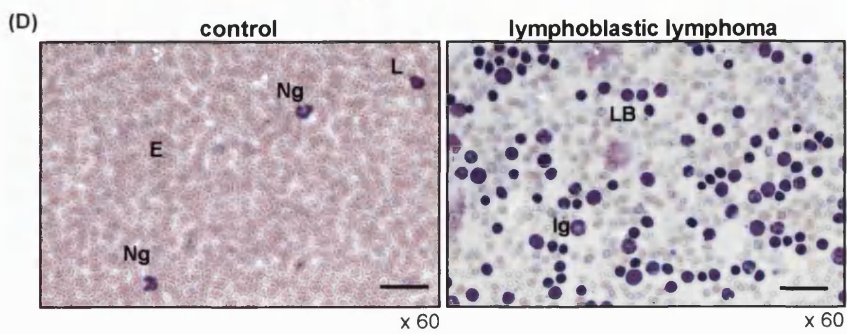
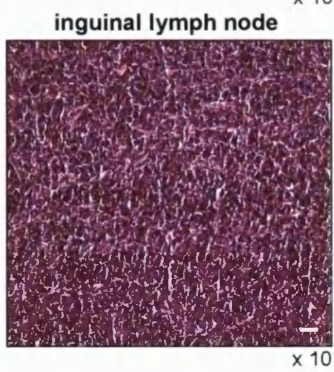
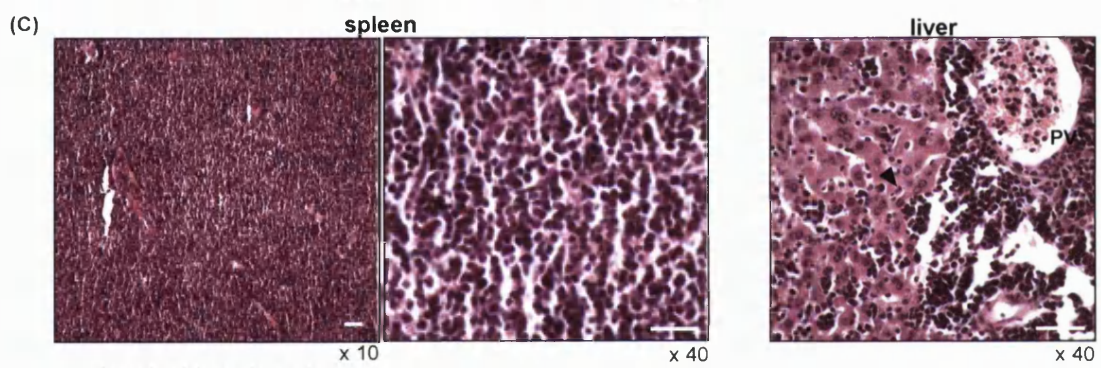
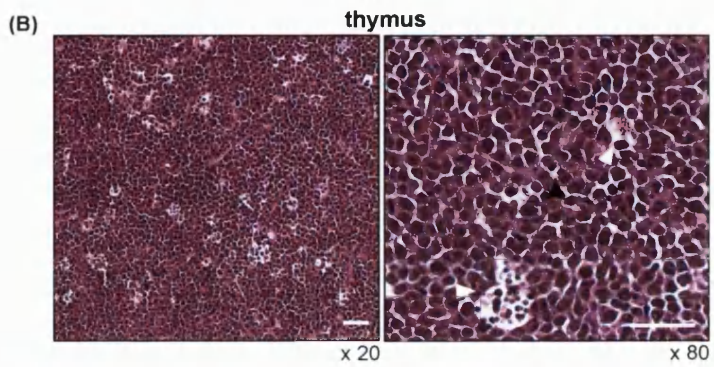
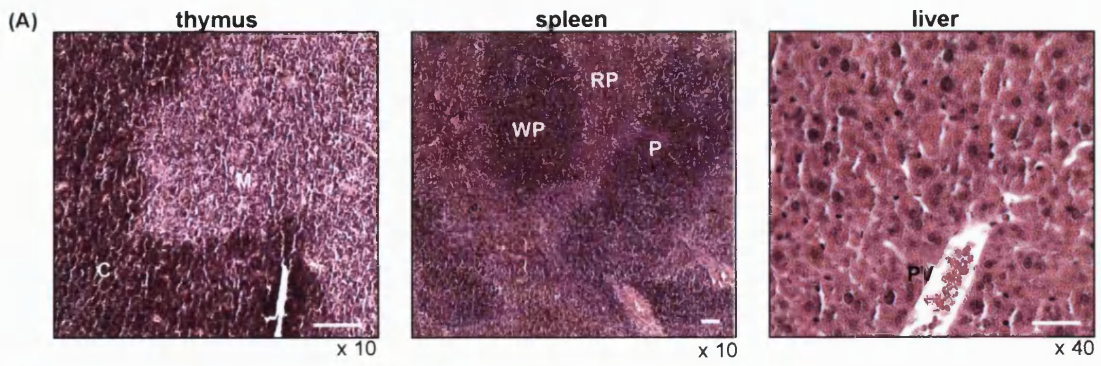


Fig. 6.9 Phenotypic analysis of lymphomas from E μ *K cyclin/p53*^{-/-} mice. (A) Control lymphocyte flow cytometric analysis. Thymocytes and splenocytes isolated from a 10-week old non-transgenic mouse were stained with anti-CD4-PE and anti-CD8-FITC or with anti-B220-PE and anti-TCR β -FITC antibodies and analysed by flow cytometry. Percentages of cells in each quadrant are indicated. (B) Thymocytes, splenocytes and cells from the inguinal lymph nodes from a *K cyclin/p53*^{-/-} mouse with a thymoma were isolated and stained and analysed as described in A). This mouse contained both a T cell lymphoma (note low levels of TCR β expression) and a B cell lymphoma that had infiltrated the lymph nodes. (C) Splenocytes from a non-transgenic 10-week old mouse and a *K cyclin/p53*^{-/-} mouse with a splenic B cell lymphoma were isolated, stained with anti-B220-PE and anti-IgM antibodies and analysed by flow cytometry. Note that tumour cells were surface IgM-negative.

The phenotypic analysis of K cyclin-associated lymphomas indicated that these consisted of precursor lymphocytes. To further define the type of hematopoietic neoplasms, tumours were analysed using histopathological methods. Thymomas in *K cyclin/p53*^{-/-} mice consisted of a uniform population of medium sized T cells with low cytoplasmic to nuclear ratio that had completely replaced the normal thymic structure (compare Fig. 6.10A and B). In addition, a typical “starry sky” pattern of macrophages was visible, caused by an increased amount of apoptosis and consequent phagocytosis of apoptotic cells. Several pyknotic cells were visible throughout the thymus, but nearly all were situated within enlarged macrophages. Furthermore, numerous mitotic figures were detected in thymomas (Fig. 6.10B), indicative of an increased proliferative capacity of the tumour cells. In some cases, thymic tumour cells had taken over the T cell areas in the spleen (periarterial lymphatic sheath, PALS) (data not shown). The blood of mice with thymomas was polycythemic (increased red blood cell count), probably as a secondary response to poor oxygenation of the blood caused by the compression of the lung cavity by the thymus. These characteristics, together with the cell surface marker results, are consistent with these lymphoid neoplasms being precursor T cell lymphoblastic lymphoma (Morse et al., 2002).

Similar to thymomas, splenic tumours in *K cyclin/p53*^{-/-} and *K cyclin/ARF*^{-/-} were typified by a uniform population of medium-sized cells that had disrupted the normal structure of the spleen. B cell lymphoma cells were often found in the liver as periportal vein or parenchymal infiltrates and in the lymph nodes (Fig. 6.10C). In some cases tumour cells had also spread to the kidneys and lungs (data not shown). Numerous lymphoblastic cells were visible in the blood (Fig. 6.10D), which correlated with high white blood cell counts (leukocytosis) as established by hematology profiles. These pathological features, together with the previous observation that B cell neoplasms are surface IgM negative, are typical of precursor B cell lymphoblastic lymphoma (Morse et al., 2002). Thus, K cyclin expression induces precursor B or T cell lymphoblastic lymphoma both in *p53*^{-/-} and *p19^{ARF}*^{-/-} deficient mice.

Fig. 6.10 Histology of Eμ *K cyclin* lymphomas. (A) Tissue sections from a 10-week old non-transgenic wt mouse (H&E). C= cortex, M= medulla, WP= white pulp, RP= red pulp, P= periarterial lymphatic sheath, Pv= portal vein. (B) *K cyclin/p53*^{-/-} precursor T cell lymphoblastic lymphoma (thymoma). H&E stained sections from the thymus. Typical “starry sky” pattern of macrophages is visible in the left panel. Macrophages are indicated with white arrowheads and a mitotic figure is indicated with a black arrowhead. Note that macrophages contain numerous cells with pyknotic nuclei. (C) *K cyclin/ARF*^{-/-} precursor B cell lymphoblastic lymphoma. H&E stained sections of spleen, liver and an inguinal lymph node. Note the infiltrate of dark-stained tumour cells, identical to those visible in the spleen, around the portal vein in the liver (black arrowhead) and in the lymph node. (D) Wrights Giemsa stained bloodsmears of control blood of a non-transgenic mouse and blood from a *K cyclin/ARF*^{-/-} mouse with B cell lymphoblastic lymphoma. Notice the marked increase in the number of lymphoblastic lymphocytes (LB) and intermediate granulocytes (Ig) in mice with lymphoma. Ng= neutrophilic granulocyte, L= lymphocyte, E= erythrocyte. Scale bars: 50 μm.



6.7 Ploidy and centrosome analysis of $E\mu$ *K cyclin* tumour cells

Expression of K cyclin induced marked increases in both ploidy and centrosome numbers in MEFs, irrespective of their genotype (results in chapter 5). These K cyclin-associated effects might contribute to tumourigenesis *in vivo*. I therefore investigated whether $E\mu$ K cyclin lymphomas displayed ploidy changes and/or abnormal centrosome numbers. Cells from *K cyclin/p53*^{-/-} (3 B, 9 T cell) and *p53*^{-/-} lymphomas (6 B, 3 T cell) were stained with PI and subjected to flow cytometric analysis to determine the ploidy. While 3 of the 9 *p53*^{-/-} tumours were clearly aneuploid, only 1 of 11 *K cyclin/p53*^{-/-} lymphomas contained a polyploid population of cells (Fig. 6.11). Thus, at least in the analysed tumours, K cyclin expression was not associated with marked increases in ploidy compared with *p53*^{-/-} controls. Of note, increased amounts of cells with S/G2/M DNA contents were visible in lymphomas compared with control lymphocytes, confirming the high proliferative index of such cells.

Next, cells from the same tumours that were analysed for ploidy were cytospun and stained with anti- γ -tubulin antibody to establish centrosome numbers. Control lymphocytes each contained one or two centrosomes. All *p53*^{-/-} lymphoma cells also contained normal numbers of centrosomes, regardless of whether they expressed K cyclin or not (Fig. 6.12 A and B). Taken together, no evidence for K cyclin-specific increases in ploidy or abnormalities in centrosome numbers were found in late-stage *K cyclin* lymphomas.

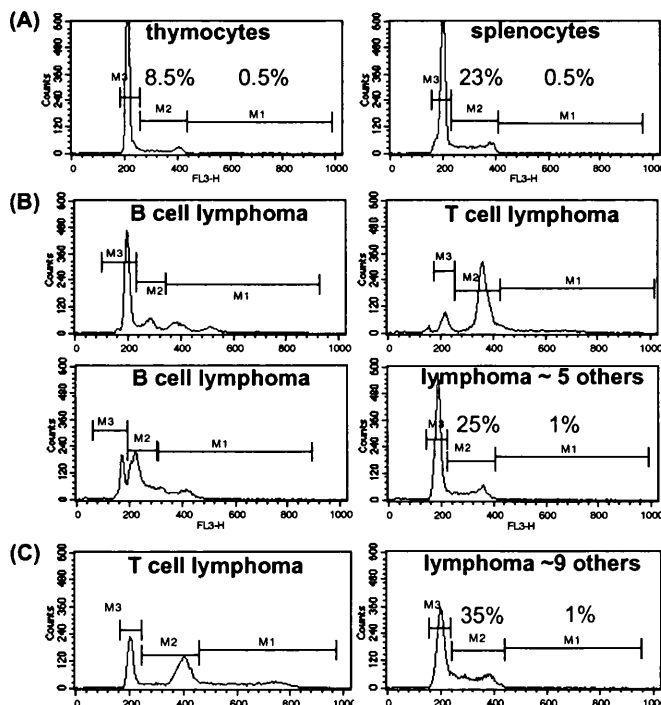


Fig. 6.11 Ploidy of *p53*^{-/-} and $E\mu$ *K cyclin/p53*^{-/-} tumour cells. Control lymphocytes from 10-week old mice (A) or lymphocytes from 9 *p53*^{-/-} (B) or 11 $E\mu$ *K cyclin/p53*^{-/-} lymphomas (C) were stained with PI and analysed by flow cytometry. Indicated percentages reflect cells with S/G2/M or larger than G2 DNA contents

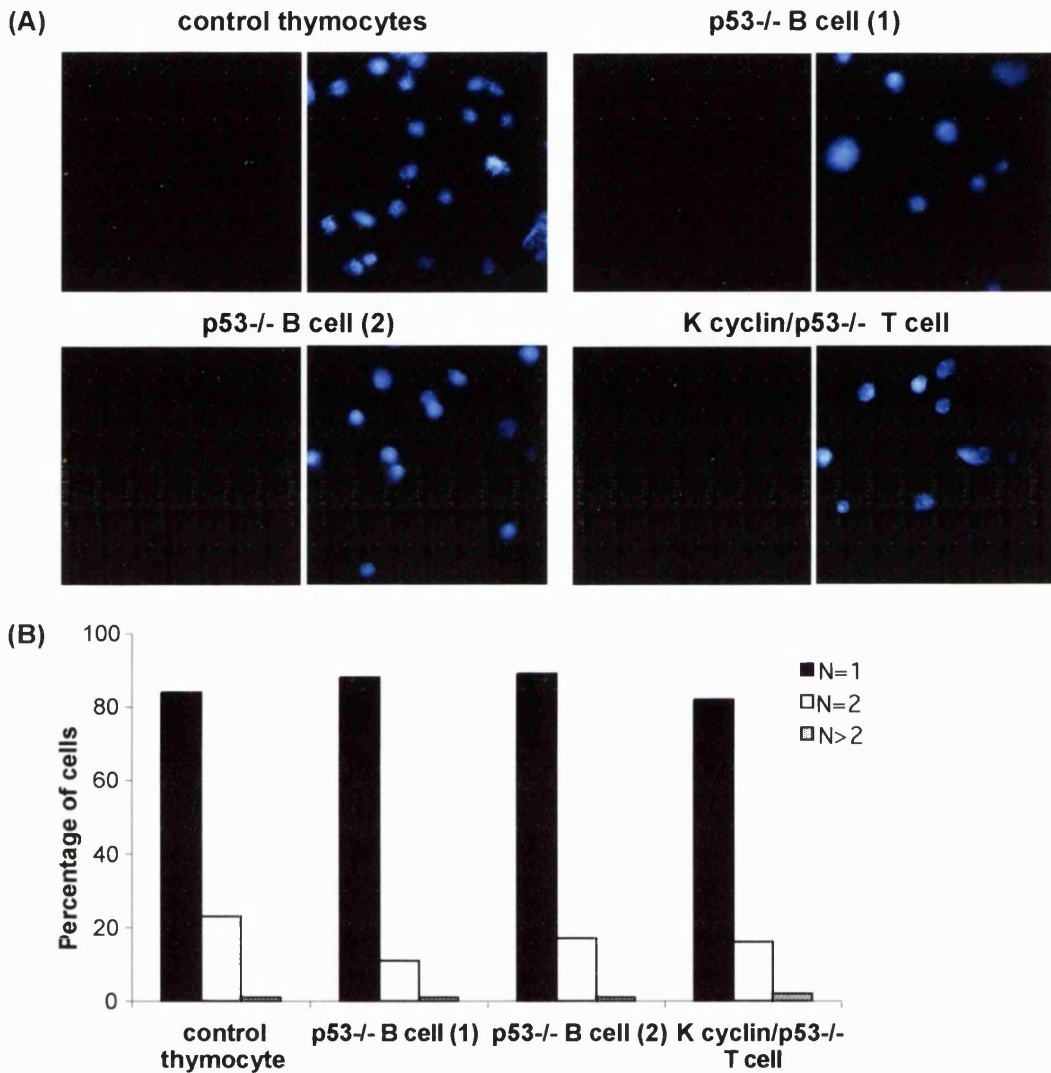


Fig. 6.12 No detectable centrosome amplification in *p53^{-/-}* or *K cyclin/p53^{-/-}* lymphomas. (A) Single cell suspensions from the same lymphomas as analysed in Fig. 42 were cytopun and subsequently stained with anti- γ tubulin antibody (green). DNA was counterstained with DAPI (blue) Slides were microscopically analysed and representative fields of control lymphocytes and three tumours that were aneuploid by FACS are shown (magnification 32x). All other analysed tumour samples showed similar results. (B) Quantitation of centrosome numbers of the samples shown in A). The number of centrosomes of around 150 single cells (judged by DAPI stain) were counted and percentages of cells with 1, 2 or more than 2 centrosomes are shown.

6.8 Discussion

Based on the results in the previous chapters showing that a polyploid population of K cyclin expressing cells is able to survive and expand in the absence of p53, I hypothesised that K cyclin would promote tumourigenesis in a p53-null background. This hypothesis is supported by the E μ *K cyclin* transgenic mouse model. First, K cyclin-induced lymphomas in wt mice were infrequent and, when analysed, contained increased levels of p53 and/or p19^{ARF} protein indicative of disruption of the p53 pathway in such tumours (Fig. 6.7). Second, loss of p53 resulted in a substantial decrease in latency and increase in the incidence of K cyclin-induced lymphomas (Fig. 6.8). Of note, the experiments described in this chapter were performed solely with the E μ *K cyclin* 1996-E-H (6) line, because more than one of the mice of this line got tumours in the initial follow-up period. However, the finding that one E μ *K cyclin*/*v-FLIP* 2284G (14) mouse developed lymphoma at 8 months of age suggests that the E μ *K cyclin*-mediated predisposition to lymphomagenesis is a general phenomenon. Mice of the other two lines have been crossed with *p53*^{-/-} mice and are currently being followed for tumour formation to confirm this.

These data are the first evidence that K cyclin acts as an oncogene *in vivo*, although its tumourigenicity is only exposed in the absence of tumour suppression. The latter finding likely explains why K cyclin expression was unable to promote transformation in soft agar colony forming assays (H. Laman, personal communication). Interestingly, expression of the gammaherpesvirus MHV-68-encoded cyclin D homologue (M cyclin) driven by the proximal *lck* promoter was previously shown to trigger T cell lymphomas in mice between 3 and 12 months of age (van Dyk et al., 1999). This is somewhat surprising, since M cyclin forms complexes mainly with Cdk2 and fails to interact with Cdk6 (Card et al., 2000), an abundant Cdk in T lymphocytes (Meyerson and Harlow, 1994). However, similar to K cyclin/Cdk complexes, M cyclin/Cdk complexes also exhibit reduced sensitivity to Cdk inhibitors. These findings therefore support a concept in which oncogenicity is a general attribute of virally-encoded cyclins. An outstanding question is whether tumour suppression is also disrupted in M cyclin-driven tumours, although the long latency of tumour onset is consistent with such a premise.

No increased incidence of lymphomas was detected in E μ *K cyclin*/*v-FLIP* mice compared with E μ *K cyclin* mice, although the expression pattern and level of Flag-tagged K cyclin were similar in both lines (Fig. 6.3). A potential explanation is that *v-FLIP* expression from the bicistronic transgene construct was too low to have a biological effect. Expression levels of transgenic *v-FLIP* were not measured because no appropriate antibody was available. The expression from this IRES could be intrinsically weak as only low levels of *v-*

FLIP protein were translated *in vitro* from the bicistronic transgene construct (Fig. 3.7). An alternative explanation is that v-FLIP expression acts as a modulator of Fas-mediated apoptosis in peripheral lymphocytes, rather than a tumour-progression factor in immature lymphocytes. The latter possibility is unlikely, since recent studies have uncovered a Fas-ligation-independent ability of constitutively expressed cellular and viral FLIP to augment proliferation of T or B lymphocytes, respectively (Djerbi et al., 1999; Lens et al., 2002), perhaps reflecting the ability of FLIPs to augment survival signalling via ERK and NF- κ B (Chaudhary et al., 1999; Kataoka et al., 2000). Thus, it remains possible that (high levels of) v-FLIP expression could synergise with K cyclin in lymphomagenesis.

All K cyclin-associated B cell lymphomas and most T cell lymphomas consisted of immature lymphocytes, characterised in B cells by the lack of surface IgM expression and the presence of both CD4 and CD8 co-receptors but low level TCR β expression on T cells (Table 6.2 and Fig. 6.9). These markers, together with the characteristic pathological features, classified these lymphoid neoplasms as precursor lymphoblastic lymphomas. Similar types of lymphoma have been reported in other models of transgenic oncogene expression in lymphocytes, such as the E μ -*myc* (Adams et al., 1985; Harris et al., 1988; Strasser et al., 1990), E μ -*ret* (Iwamoto et al., 1991; Wasserman et al., 1998) and *lck-M cyclin* models (van Dyk et al., 1999). In all these cases pronounced proliferation of immature lymphocytes was detected, suggesting that oncogene expression may inhibit the differentiation of lymphocytes through its effects on the cell cycle. In addition, the fact that immature IgM⁺ B cells or DP T lymphoblasts are found in peripheral lymphoid organs and the blood, shows that they have escaped the boundary of their normal somatic environment (bone marrow or thymus, respectively). Whether such “metastasis” is solely dependent on oncogene expression, or whether additional mutations are needed to acquire such a trait, is unclear. Interestingly, the mere inhibition of differentiation in mice lacking E2A proteins, helix-loop-helix proteins that regulate early thymocyte development, predisposes to the early development of thymic lymphoma. Thus, E μ K cyclin tumorigenesis may involve both the induction of proliferation and the inhibition of differentiation.

An unexpected finding was the cooperation of E μ K cyclin with *p19^{ARF}* loss in the induction of lymphomas. This contrasts with the finding that growth arrest and apoptosis upon K cyclin expression in MEFs were not abrogated in the absence of *p19^{ARF}* (results in chapter 4), and may reflect a difference between *in vitro* and *in vivo* experiments and/or cell type. Mutations in *p19^{ARF}* and *p53* have been proposed to exhibit overlapping tumour suppressor functions in the context of E μ -*myc*-induced lymphomagenesis: B cell lymphomas developed with similar kinetics and pathological characteristics whether they were on a *p53*- or *ARF*-null background (Schmitt et al., 1999). However, E μ K cyclin transgenics, which

express the transgene in both B and T cells, mainly developed early onset T cell lymphomas in a *p53*^{-/-} background (~80 days) but, in striking contrast, solely developed B cell lymphomas with a delayed onset in an *ARF*^{-/-} background (~160 days). This discrepancy is unlikely a result of strain variability, as a C57BL/6 background (which was predominant in the *p53*^{-/-} crosses) favored B instead of T lymphoma development in an E μ -*myc* line that expressed c-*myc* in both B and T cells (Yukawa et al., 1989). These results therefore highlight a p19^{ARF}-independent role for p53 in suppression of E μ *K cyclin*-associated T cell lymphomagenesis and emphasise that, in agreement with the cell culture results, the role of these tumour suppressors bifurcate in *K cyclin*-induced tumourigenesis. Of note, the finding that tumourigenesis in *K cyclin/ARF*^{-/-} mice showed a relatively late onset of around 5 months may imply that such tumours have acquired additional tumour-promoting lesions.

Other existing evidence suggests that the tumour suppressor roles of p53 and p19^{ARF} are not strictly overlapping. For example, the onset and spectrum of tumours differs in mice lacking p53 or p19^{ARF}: while *p53*^{-/-} mice mainly develop lymphomas around 18-20 weeks of age (Donehower et al., 1992; Harvey et al., 1993a; Jacks et al., 1994), *ARF*^{-/-} animals mainly develop sarcomas around 38 weeks of age (Kamijo et al., 1999). This is consistent with the idea that p53, but less so p19^{ARF}, is important during lymphoid development. However, non-overlapping roles for these tumour suppressors have also been proposed with regards to E μ -*myc* lymphomagenesis, which is inhibited equally by p53 or p19^{ARF} expression (Schmitt et al., 1999). Specifically, E μ -*myc* B cell lymphomas lacking *Bax* never showed *p53* mutations but still exhibited *ARF* deletions or *Mdm2* overexpression, implying that Myc-induced p53/*Bax* and p19^{ARF} tumour suppressor pathways are genetically separable (Eischen et al., 2001). Taken together, the functions of p53 and p19^{ARF} in tumour surveillance are not on a strict linear pathway but are defined by the type of tissue and oncogenic stimulus; the molecular pathways underlying these differences remain undefined.

One function ascribed to p53 is maintenance of chromosome stability by eliciting cell cycle checkpoints upon DNA damage to allow time for DNA repair (see introduction chapter 1). Importantly, this function does not seem to involve p19^{ARF}, since γ -irradiated *ARF*^{-/-} MEFs upregulate p53 and p21^{CIP1} and undergo G1 arrest with similar efficiencies as wt MEFs (Kamijo et al., 1997). This is further reflected by the characteristic gross aneuploidy as measured by DNA content analysis in *p53* mutant mouse lymphomas, while most *ARF*^{-/-} tumours remained diploid (Schmitt et al., 1999). However, the absence of recurrent aneuploidy in *p53*^{-/-} lymphomas in my experiments (Fig. 6.11) not only questions the frequency of such aneuploidy, but also decreases the likelihood that lack of genome surveillance explains the specific development of T cell lymphomas in E μ *K cyclin/p53*^{-/-} mice, but not in E μ *K cyclin/ARF*^{-/-} mice.

Concurrent with the absence of gross aneuploidy in *p53*^{-/-} or *K cyclin/p53*^{-/-} lymphomas, no abnormalities in centrosome amplification were found (Fig. 6.12). This stands in marked contrast to a previous report describing the presence of abnormally amplified centrosomes in *p53*^{-/-} lymphocytes (Fukasawa et al., 1997). Although the reason for this discrepancy is unclear, it is worth noting that cytopins contain many lymphocyte clusters and the image shown in the report by Fukasawa and colleagues does not convincingly show centrosome amplification in an individual cell. Together, the absence of obvious polyploidy and centrosome amplification in *K cyclin* lymphomas raises the question of whether these *K cyclin* phenomena contribute to tumorigenesis. This question is difficult to answer: if genomic instability is found in tumours this does not necessarily mean that it contributed to tumorigenesis, and if genomic instability is absent in late stage tumours it may still have contributed to early tumour development (Brinkley, 2001).

CHAPTER 7:
CONCLUDING DISCUSSION AND FUTURE DIRECTIONS

This thesis has investigated cellular consequences upon expression of the KSHV-encoded viral cyclin, K cyclin. The main conclusions that can be drawn from the data presented in this thesis is that ectopic expression of K cyclin in fibroblasts induces potential tumour-promoting as well as potential tumour-inhibitory effects, a feature common to many viral oncoproteins. Tumour-inhibitory effects constitute p53-dependent sensitisation to apoptosis and a p53-dependent growth arrest. Tumour-promoting effects constitute centrosome amplification and consequent aneuploidy. Furthermore, the *in vivo* expression of K cyclin in mouse B and T lymphocytes stimulates lymphomagenesis only when the p53 pathway is disrupted, establishing the p53 tumour suppressor pathway as a key restraint to K cyclin-induced tumourigenesis.

However, many questions remain, and even more questions have perhaps been generated. These include the molecular pathways leading to a K cyclin-induced cytokinesis defect and activation of p53, the contribution of various aspects of p53 loss to lymphomagenesis in the E μ K cyclin transgenic mouse model and the contribution of K cyclin-associated cellular effects to KSHV pathogenesis under physiological circumstances. In this chapter I will discuss these questions in relationship to published literature and I will try to define potential experimental ways to further elucidate these issues.

7.1 K cyclin-associated responses as a model for deregulated cellular cyclins?

Results presented in the first two chapters of this thesis show that K cyclin expression in Rat-1 cells and MEFs strongly sensitised cells to undergo p53-dependent apoptosis and growth arrest. Both of these K cyclin-induced responses occurred in the absence of E2F1- and p19^{ARF}. Compared to K cyclin, Cyclin D1-induced apoptosis was less pronounced and was not increased upon co-expression of cyclin E (Fig. 3.5 and Sofer-Levi and Resnitzky, 1996). Furthermore, cyclin D3 expression in MEFs did not induce a growth arrest (Fig. 4.7). These data therefore suggest that cyclin D and E do not mimic K cyclin in its ability to induce apoptosis and growth arrest.

A simple explanation for the above observations could be that K cyclin/Cdk activity also resemble cyclin A/Cdk activity, exemplified by their shared ability to target and phosphorylate Cdc6 and Orc1 and trigger DNA replication (Laman et al., 2001a). This could be addressed by co-expressing cyclins D, E and A and determining the propensity of such cells to die or arrest. However, based on published observations it might be predicted that cyclin A would actually inhibit the activation of p53. In particular, the binding of cyclin A/Cdk2 complexes to E2Fs negatively regulates the DNA binding activity of E2F/DP complexes (Krek et al., 1994; Krek et al., 1995), which would inhibit E2F responses leading to p53 activation. Indeed, inhibition of E2F1 binding to cyclin A dramatically enhances

apoptosis (Krek et al., 1995), a premise that was used to design peptides that selectively kill transformed cells with deregulated E2F activity (Chen et al., 1999). In these studies, apoptosis was dependent on transactivation by E2F, although the role of p53 in apoptosis was not established. In addition, E2F has been shown to directly bind p53 and cooperate with p53 to induce apoptosis, in a manner that is independent of E2F transactivation. Also in this case apoptosis was inhibited by upon binding of cyclin A to E2Fs, which competed with p53 to bind E2Fs (Hsieh et al., 2002). It would therefore be interesting to see whether cyclin A promotes or inhibits K cyclin-induced apoptosis.

Other more subtle differences exist between K cyclin and cellular cyclins. For example, K cyclin lacks a residue homologous to the C-terminal Thr286 residue of cyclin D1 or the Thr380 residue of cyclin E that are involved in the destruction of cyclin molecules (Clurman et al., 1996; Diehl et al., 1997; Won and Reed, 1996). It could therefore be predicted that K cyclin is more stable when expressed *in vivo*. Interestingly, the same cyclin D1 Thr286 residue was recently shown to also govern cyclin D nuclear accumulation, as its phosphorylation by GSK-3 β -triggered a Crm1-dependent nuclear export (Alt et al., 2002). This could imply that K cyclin evades both nuclear export and degradation by its alteration of this residue, and would explain why nuclear accumulation of K cyclin is pronounced (Fig. 3.1 and (Child and Mann, 2001) even though it does not bind p21/p27 CKIs (Swanton et al., 1997). Mutagenesis studies in combination with stability and localisation studies could be performed to address this hypothesis.

Another question concerns the mechanism of nuclear import of K cyclin. Not much is known about the import mechanism of cyclin D and it is unnecessary to propose that cyclin D and K cyclin nuclear import are regulated differently. Interestingly, the mutation of a conserved Thr residue in cyclin D1 to Ala (T156A) disrupts CAK phosphorylation of Cdk4 in complex with mutant cyclin D1 and prevents nuclear import of cyclin D1 T156A/Cdk complexes. Structural analysis shows that the 156 residue is located in a hinge region between the two cyclin folds. K cyclin, as well as other viral cyclins, contain a conserved Leucine residue at this site (Leu150). This residue might therefore be an important determinant for the interaction of K cyclin/Cdk complexes with other proteins, including potential NLS-containing proteins. Differential protein binding to wt versus Leu150 mutant K cyclin/Cdk complexes could be a starting point to investigate potential K cyclin-associated nuclear import factors.

Thus, K cyclin/Cdk complexes differ from cyclin D/Cdk with regards to its localisation, stability, broadened substrate specificity as well as its lack of inhibition by CKIs (Swanton et al., 1997). This complicates direct comparisons between cellular responses to each cyclin. However, thus far no function of K cyclin has been revealed that is not shared by

endogenous cyclin activities. The results therefore reveal the p53 pathway as a likely candidate for tumour suppression when cyclin activity is deregulated. Importantly, it is clear that the functional similarity of K cyclin to E1A/LargeT/E7-type viral oncoproteins in its ability to inactivate Rb is greater than its homology to cellular cyclin D.

7.2 K cyclin-associated senescence?

A significant proportion (30%) of growth-arrested K cyclin-expressing wt MEFs stained positively for the SA- β -Gal marker, implying that K cyclin may induce a form of cellular senescence (Fig. 4.8). Unexpectedly however, around 50% of a proliferating culture of K cyclin-expressing *p53*^{-/-} MEFs and around 43% of mock-infected proliferating *E2F1*^{-/-} MEFs were positive for this senescence marker as well. These results are unlikely due to a staining artifact, as mock-infected wt MEFs did not stain. Many studies however use SA- β -Gal staining as an exclusive marker for senescence (Dimri et al., 1995). These results therefore raise the concern that positive staining may rather be a marker of cellular stress and clearly shows that an increase in the percentage of positive staining does not necessarily correlate with the proliferative capacity of the culture.

An interesting observation was that most SA- β -Gal-positive *p53*^{-/-} MEFs were large multinucleated cells. This implied that such cells were still undergoing DNA synthesis, which was supported by the finding that the percentage of BrdU-positive cells in K cyclin-expressing cultures was similar to wt cultures (Fig 5.3). This is inconsistent with the established definition of cellular senescence, which states that cells undergo an irreversible cell cycle arrest (Campisi, 2000). Are these cells also capable of cell division? This could be investigated by FACS-sorting of the polyploid population of cells and study by time-lapse videomicroscopy whether they regain the ability to divide. Another recent idea is that such SA- β -Gal-positive “senescent” cells secrete growth factors that promote the proliferation of pre-neoplastic cells (Chang et al. 2000; Krtolica et al., 2001). An interesting question therefore is whether the conditioned medium of K cyclin-expressing FACS-sorted polyploid cells would actually promote the outgrowth of, for example, endothelial cells.

7.3 K cyclin-induced cytokinesis defect

The expression of K cyclin in MEFs is associated with a cytokinesis defect. Many questions remain as to what might be the mechanism of this. Accumulating reports in the last year have revealed cases in which misexpression of mitotic regulators correlates with defective mitotic division. Examples are the overexpression of Plk1 and Plk3 (Meraldi et al., 2002; Mundt et al., 1997; Wang et al., 2002), Aurora (Meraldi et al., 2002) and Cdc14A (Kaiser et al., 2002), the small interfering RNA (siRNA) -based ablation of Plk1 (Liu and

Erikson, 2002) or Cdc14A (Mailand et al., 2002), dominant-negative inhibition of Plk1 (Seong et al., 2002) and inhibition of survivin function by antisense or dominant-negative approaches (Li et al., 1999). An unexpected member of this club is the DNA replication protein Orc6: siRNA silencing of Orc6 causes multinucleation and aberrant mitosis (Prasanth et al., 2002). The above proteins have all been shown to localise to mitotic structures such as the centrosomes, mitotic spindle, kinetochores and spindle midzone (see also in introduction), in agreement with their role in mitosis. However, in these cases their association with a correct execution of cytokinesis also remains correlative and no molecular mechanism has been proposed as yet. Putative Plk targets constitute the mitotic kinesin-like proteins MKLP-1 (Lee et al., 1995a) or Pavarotti in *Drosophila* (Adams et al., 1998), which may regulate the formation of the contractile ring.

One hypothesis is therefore that K cyclin expression deregulates mitotic kinase activity and consequently disrupts cytokinesis. Indeed, the pronounced cyclin B localisation in K cyclin-expressing MEFs (Fig. 5.10) is suggestive of increased Plk activity, as phosphorylation by Plk mediates nuclear accumulation of cyclin B (Toyoshima-Morimoto et al., 2001). A kinase that activates Plk has not yet been identified, but it would be interesting to see if K cyclin/Cdk complexes targets Plk and/or regulates its kinase activity. Another possibility is that K cyclin upregulates Plk protein levels, since the *Plk* gene was found to be an E2F target in microarray studies (Ren et al., 2002). Furthermore, preliminary results in the Jackson laboratory have shown that *in vitro*, Cdc14A is an efficient cyclin E/Cdk target (Kaiser et al., 2002). Although the biological consequence of Cdc14 phosphorylation is unknown, it is possible that K cyclin modulates Cdc14 function by targeting its modification.

Another question is why K cyclin-expressing *p53*^{-/-} MEFs are able to overcome the cytokinesis defect and are able to divide. The observation that *p53*^{-/-} MEFs expressing K cyclin exhibited pronounced multinucleation and polyploidy implies that cytokinesis is also improperly regulated in such cells. Do *p53*^{-/-} MEFs lack a specific checkpoint that monitors a disruption of cytokinesis, or does this checkpoint overlap with an existing G1 or G2/M checkpoint? If such a checkpoint exists, it does not involve p21 activation, as *p21*^{-/-} MEFs arrested upon K cyclin expression (Fig. 4.7). Other candidate p53 target genes are the G2/M inhibitors Gadd45 and 14-3-3 σ , which both inhibit the activation of cyclin B/Cdk1 complexes in response to DNA damage (Fig. 1.14). Interestingly, E7/ras-transformed *Gadd45a*^{-/-} MEFs have been reported to become polyploid and contain large lobulated nuclei, while E7/Ras transformed wt MEFs have a normal morphology (Hollander et al., 1999). Furthermore, E7/Ras-transformed *Gadd45a*^{-/-} cells show increased apoptosis, concomitant with a robust outgrowth of cells at a normal plating efficiency. Thus, Gadd45a appears important for the

coordination of mitosis and cytokinesis and constitutes a prime candidate for an oncogene-induced, p53-dependent G2/M/cytokinesis checkpoint.

7.4 Does K cyclin activate a p53-dependent “tetraploidy checkpoint”?

K cyclin triggers the activation of p53 and consequent p53-dependent apoptosis and growth arrest. This, together with the phenotype of multinucleation and increased ploidy, support published observations demonstrating that p53 function is required to impose a G1 arrest in response to tetraploidisation (Andreassen et al., 2001; Di Leonardo et al., 1997; Khan and Wahl, 1998; Lanni and Jacks, 1998; Minn et al., 1996). In these studies, cells treated with the mitotic spindle inhibitors nocodazole undergo a transient arrest at mitosis and, despite failing to undergo cytokinesis, proceed to reenter G1. Importantly, co-staining of cells with p53 and a mitotic marker (MPM-2) revealed that p53 levels increased in the subsequent G1-like phase, instead of in mitotic cells (Minn et al., 1996). In addition, cytokinesis failure upon disruption of actin assembly also induced a p53-dependent arrest of tetraploid cells, showing that spindle disruption *per se* does not activate p53 (Andreassen et al., 2001). Nocodazole-treated cells lacking Rb or p53 undergo endoreduplication, further confirming the activation of a p53/p21-dependent G1 arrest (Di Leonardo et al., 1997; Khan and Wahl, 1998; Lanni and Jacks, 1998).

Does K cyclin similarly trigger p53 upon the induction of tetraploidy? This could be examined by p53 immunohistochemistry and its correlation with DNA content, multinucleation or mitotic marker staining. Of note, the time-lapse analysis shows that multinucleated cells are the ones that die, which would support such a premise. Another question is how p53 would be activated upon such a proposed “tetraploidy checkpoint”. One possibility is that DNA damage during aberrant mitosis induces an ATM/ATR/Chk-type response. This could be addressed by using chemicals to block their activation (e.g. caffeine), by using cells lacking components of this pathway, or by studying the phosphorylation profile of the p53 protein. A recent observation is that syncytia, formed upon fusion between CD4- and HIV-envelope expressing HeLa cells, undergo apoptosis following the nuclear translocation of mTOR, which then phosphorylates Ser15 of p53 (Castedo et al., 2002). The activation of p53 required cyclin B/Cdk1 activity and was only detected in cells undergoing nuclear fusion, suggesting that this pathway may measure the presence of polyploid nuclei. Although mTOR has not yet been established as a common activator of p53 or mediator of apoptosis, it would be interesting to see whether an analogous pathway is activated upon K cyclin-induced tetraploidy.

7.5 Other potential mechanisms of p53 activation by K cyclin

Unless there is unequivocal evidence for the activation of p53 via a “tetraploidy checkpoint”, other possibilities cannot be excluded. One option is that K cyclin/Cdk complexes phosphorylate and activate p53 protein themselves. Phosphorylation of p53 on Ser315 by Cdk2 and Cdk1 has been reported (Price et al., 1995; Wang and Prives, 1995). However, the biological consequences of such phosphorylation are unclear and it is therefore difficult to predict what this would mean in the context of K cyclin activity. Another possibility is that K cyclin expression somehow disrupts a correct timing of DNA replication thereby interfering with the formation of chromatin that is fully competent to undergo normal chromosome segregation (see discussion of chapter 5). This may trigger DNA damage independent of tetraploidy and activate p53 via an ATM/ATR/Chk-type pathway. Related to this is the question of whether transcriptional activation by p53 is required to trigger apoptosis and/or growth arrest, and if so, which transcriptional targets of p53 are involved.

7.6 Effect of LANA on K cyclin-induced p53 activation

Most known DNA tumour viruses disrupt both the Rb and the p53 pathway. KSHV encodes several proteins that inhibit the activation of p53 in *in vitro* studies (Friborg et al., 1999; Park et al., 2000; Rivas et al., 2001). Of these, only LANA is expressed during latent infection both in KS and lymphoproliferative disorders. Although I have not managed to convincingly show that K cyclin-induced apoptosis is inhibited by LANA, this remains an intriguing possibility. Observations that support a role for LANA in inhibiting p53 *in vivo* include the finding that KSHV-associated disorders do not display recurrent p53 mutation (Bergman et al., 1996; Carbone et al., 1998; Katano et al., 2001; Kennedy et al., 1998) and the colocalisation of LANA with p53 in KS samples (Katano et al., 2001). One way to approach this is by co-expressing titrated amounts of K cyclin and LANA in lymphocytes and study the propensity of cells to undergo apoptosis.

7.7 K cyclin and centrosome amplification

A striking result was that K cyclin expression in *p53*^{-/-} as well as wt MEFs triggered a pronounced increase in centrosome numbers (Fig. 5.6). This phenomenon is unlikely to explain the defective cytokinesis, as all wt MEFs arrested, while not all of these cells contained multiple centrosomes. Furthermore, the presence of multiple spindles is not thought to be monitored by checkpoints (Sluder et al., 1997). On the contrary, the disruption of centrosome function is associated with mitotic failure, most dramatically exemplified by the failure of cells to divide upon microsurgical removal of the centrosome (Piel et al., 2001). It could therefore be possible that K cyclin expression interferes with centrosome function,

something that could be further investigated by a more detailed analysis of centrosomes, for example, by electron microscopic imaging of centrosomes or immunohistochemical stainings of various components of the centrosome organelle or the microtubule network.

It was recently proposed by the Nigg laboratory that centrosome amplifications always arise secondary to multinucleation and ploidy increases, a response that is dramatically enhanced in the absence of p53 (Meraldi et al., 2002). Indeed, several other reports describe centrosome amplifications concomitant with cytokinesis defects in cells that lack a functional p53 checkpoint pathway (Chen et al., 2002; Duensing et al., 2000; Hollander et al., 1999). Amplified centrosomes were always detected in polyploid MEFs expressing K cyclin (Fig. 5.7). However, the actual numbers were greater than predicted based on the DNA content of such cells. It would therefore be informative to have a system in which centrosome formation could be followed over time, for example by using cells expressing GFP-tagged γ -tubulin (Khodjakov and Rieder, 1999).

Does constitutive K cyclin-associated kinase activity also stimulate centrosome duplication directly? It should be noted that although cyclin E and A are required for centrosome duplication in S phase (Hinchcliffe et al., 1999; Lacey et al., 1999; Meraldi et al., 1999), cyclin E overexpression stimulates chromosome instability in the absence of centrosome abnormalities (Spruck et al., 1999). Instead, increased Cdk2 activity promotes centrosome splitting – the separation of the two parental centrioles (Meraldi and Nigg, 2001). One reason for the increase in γ -tubulin-positive structures could therefore be an enhanced splitting of centrosomes. Another possibility is that K cyclin expression upregulates E2F targets that control centrosome duplication. One of such candidates identified in E2F array studies is the centrosomal RanBPM protein (Ren et al., 2002), ectopic expression of which causes increases in γ -tubulin-containing microtubule nucleation sites (Nakamura et al., 1998). Alternatively, K cyclin may alter the activation of proteins that regulate centrosome duplication, such as the Mps1p kinase. This kinase is required for centrosome duplication in budding yeast and in mouse cells (Fisk and Winey, 2001), and phosphorylation by Cdk2 triggers its stabilisation. In contrast, human Mps1 is dispensable for centrosome duplication (Stucke et al., 2002). Perhaps this explains why centrosome amplification upon K cyclin expression in human cells is less pronounced compared with mouse cells?

The percentage of polyploid *p53*^{-/-} MEFs expressing K cyclin decreased upon prolonged culture of such cells (Fig. 5.11), and centrosome amplification therefore probably decreased too. Such genomic convergence also occurs upon long-term culture of *p53*^{-/-} cells, explained by the outgrowth of cells that have acquired a chromosome composition optimal for cell growth (Chiba et al., 2000). However, a reduction in centrosome amplification could also be attributed to the fact that successful cell division requires a stable and efficient mitosis,

which presumably favors bipolar over multipolar spindles (Brinkley, 2001). It may even be possible that cells that never exhibited centrosome amplification and always contained bipolar spindles preferentially grow out. These questions could be addressed by following GFP-tagged centrosomes in K cyclin expressing cells, to see whether cells with supernumerary centrosomes lose their extra centrosomes, disappear from the culture or eventually divide and expand.

7.8 Further characterisation of E μ K cyclin mice

Many questions remain with regards to the development of lymphomas in E μ K cyclin transgenic mice. Some of these involve the further characterisation of E μ K cyclin mice, such as the detection of mutations in the p53 sequence in K cyclin-associated tumours or the frequency of LOH in tumours of p53 or ARF heterozygous mice. In addition, it would be informative to establish the apoptotic and proliferative potential of K cyclin-expressing lymphocytes. This is probably best studied with isolated primary lymphocytes, since apoptotic lymphocytes are rapidly cleared by neighboring macrophages which complicates accurate measurement of lymphocyte apoptosis *in vivo* (Surh and Sprent, 1994).

A more challenging subject is which of the effects of p53 loss is required to promote lymphomagenesis: inhibition of apoptosis, inhibition of growth arrest or tolerance to the formation of aneuploid cells. It has recently been suggested that it is the inhibition of apoptosis, rather than the acquisition of aneuploidy, that constitutes the critical tumourigenic effect of p53 loss in the E μ myc model (Gurova et al., 2002; Schmitt et al., 2002a). Several arguments intimate that the promotion of aneuploidy may not be crucial in the E μ K cyclin model either. First, no centrosome amplifications were detected in E μ K cyclin lymphomas. Second, K cyclin transgenic p53^{+/-} mice developed tumours with relatively long latency compared with p53^{-/-} mice, arguing that gross genomic instability does take place. Third, p53^{-/-} lymphocytes are likely to be aneuploid in the absence of K cyclin expression, and it would therefore be more plausible if K cyclin would convey a different tumourigenic stimulus, such as prevention of differentiation and concomitant increase in the proliferative lymphocytic compartment.

It remains however possible that K cyclin-associated polyploidy and centrosome amplification does occur in earlier stages of tumourigenesis. Indeed, abnormal centrosome numbers and genomic instability have been described early during neoplastic progression in epithelial cells expressing E6 and E7 and in pre-invasive HPV-associated genital lesions (Duensing et al., 2000). Similarly, the development of pancreatic cancer in transgenic mice expressing SV40 large T is characterised by supernumerary centrosomes and the sequential appearance of tetraploid and then multiple aneuploid cell populations (Levine et al., 1991).

Apart from studying whether K cyclin-expressing lymphocytes at pre-tumour stages contain abnormal centrosome numbers, it would therefore also be instructive to establish the clonality of lymphomas. Surface marker staining by FACS implies that tumours are clonal, but this should be confirmed by for example VDJ heavy chain rearrangement patterns. In addition, comparative genome hybridisations could reveal whether recurrent gains or losses of whole chromosomes or large parts of chromosomes are detected, indicative of genomic instability. Preliminary data in collaboration with Graeme Hodgson and Joe Gray show that this may indeed be the case. Further analysis is required to establish whether certain gains or losses are preferentially detected, which may hint at a cooperative event.

7.9 Do K cyclin-associated cellular responses affect KSHV pathogenesis?

Of course the ultimate goal is to translate all above findings or predictions into a physiological role for K cyclin in KSHV-associated pathogenesis. This is not easy, first and foremost due to the lack of permissive systems and animal models for pathogenesis. Ideally, the infectivity or tumourigenic potential of a virus lacking K cyclin expression should be investigated. Mutant viruses lacking the expression of the MHV68 viral cyclin show normal latent infection patterns, but inefficient reactivation from latency (Hoge et al., 2000; van Dyk et al., 2000). However, this system addresses acute infection with a different virus, and no deductions for persistent HHV-8 infection in a physiological context can therefore be made.

Kaposi's sarcoma endothelial cells or PEL-derived cells are described as grossly normal looking judged by microscopic analysis and do not display apparent ploidy changes as measured by flow cytometry (see for example Bisceglia et al., 1992; Dictor et al., 1991; Kaaya et al., 1992). Still, KS and PEL are thought to begin as polyclonal hyperplasias that develop into monoclonal tumours (Gill et al., 1998; Judde et al., 2000). Does K cyclin expression promote the proliferation of such tumour cells?

7.10 Conclusion

This thesis shows that the combination of K cyclin expression with lack of p53 expression forms a potent tumourigenic stimulus, supplying the first evidence of a previously suspected oncogenic potential for K cyclin. The data highlight that deregulation of G1/S control can lead to mitotic abnormalities and give rise to genomic instability in the absence of appropriate checkpoint controls. Whether this information impacts on our understanding of KSHV pathogenesis remains to be seen. Still, the finding that the mitogenic effects of K cyclin are antagonised by p53 may provide an explanation for the presence of a set of p53-inhibiting proteins in the KSHV genome and suggests that pharmacological disruption of this inhibition may be a potential means of anti-KSHV therapy.

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