

The Cell Cycle Regulators p18^{Ink4c} and p19^{Ink4d}

***In vivo* studies of their roles in tumorigenesis and development**

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Now this is not the end.
It is not even the beginning of the end.
But it is, perhaps, the end of the beginning.

Sir Winston Churchill

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ABSTRACT

Progression through the G1, S, G2 and M phases of the cell cycle is controlled by cyclin-dependent kinases (Cdks) and cyclins. These proteins form active Cdk:cyclin complexes that phosphorylate specific substrates. The Cdk:cyclin complexes of the G1/S transition regulate the progression of cells into the S phase by phosphorylating the retinoblastoma protein (Rb). This prevents Rb from sequestering E2F, a transcription factor that induces expression of genes required for DNA synthesis. This process is in part regulated by a family of Cdk inhibitors (CKIs) called the Ink4 family (Inhibitors of Cdk4). The Ink4 family of CKIs consists of four members; p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d}, and they bind specifically to Cdk4 and Cdk6, thereby negatively regulating their kinase activities and cell cycle progression. Because of its cell cycle inhibitory role, p16^{Ink4a} is frequently mutated or deleted in human cancer, whereas the other *Ink4* genes are only occasionally altered in cancer. The overall aim of this thesis was to study the roles of p18^{Ink4c} and p19^{Ink4d} using *in vivo* models of cancer and embryonic development. In paper I, we analyzed the tumor spectrum in mice lacking *p53*, *Ink4c* and *Ink4d*. *p53* is a tumor suppressor and one of the most frequently mutated genes in human cancer. Mice carrying mutated *p53* alleles are highly tumor-prone but develop predominantly lymphomas. However, the combined loss of *p53* and *Ink4c* (but not *Ink4d*) caused a shift in the tumor spectrum to increased incidences of hemangiomas and hemangiosarcomas, as well as appearance of medulloblastomas, a tumor of the cerebellum. These data, revealed in the absence of *p53*, suggest a cell-type specific tumor suppressing role for p18^{Ink4c}. In paper II, loss of *Ink4c* was evaluated in another tumor-prone mouse model; the Eμ-*Myc* mouse. This is a transgenic mouse overexpressing c-Myc in B cells causing clonal B cell lymphomas. Surprisingly, precancerous B cells and lymphomas from Eμ-*Myc* mice exhibited elevated levels of p18^{Ink4c} mRNA and protein despite high rates of proliferation. Moreover, loss of *Ink4c* in this model did not affect the rate of cell proliferation or the onset of tumor development. We conclude from these studies that *Ink4c* is not an important tumor suppressor of Myc-induced lymphomas. To gain insight into the role of *Ink4* genes in early vertebrate development, the African clawed frog, *Xenopus laevis*, was analyzed for the presence of *Ink4* homologs. Paper III describes the cloning and characterization of a gene homologous to *Ink4d*, *Xl-Ink4d*. This CKI is expressed throughout frog embryo development, making *Xl-Ink4d* the only CKI present during the cleavage stages of *X. laevis*. Antisense morpholino oligonucleotides directed against *Xl-Ink4d* were used to knock down the protein level of *Xl-Ink4d* during development. This resulted in defects in head tissues and reduced expression of *Twist*, a gene important for neural crest cell migration. We therefore propose that *Xl-Ink4d* is important for proper neural crest differentiation in the frog.

LIST OF PAPERS

This thesis is based on the following papers, which will be referred to in the thesis by their Roman numerals:

- Paper I.** Nilsson LM*, Zindy F*, Nguyen L, Meunier C, Smeyne RJ, Rehg JE, Eberhart C, Sherr CJ, Roussel MF. (2003) Hemangiosarcomas, medulloblastomas, and other tumors in Ink4c/p53-null mice. *Cancer Res.* 63: 5420-5427.
- Paper II.** Nilsson LM, Keller U, Yang CY, Nilsson JA, Cleveland JL, Roussel MF. Ink4c is dispensable for tumor suppression in Myc-induced B-cell lymphomagenesis (2007). *Oncogene* 26: 2833-2839.
- Paper III.** Doherty J*, Nilsson LM*, Kuliyeve E, Cleveland JL, Roussel MF, Mead P. The *Xenopus* cyclin-dependent kinase inhibitor Ink4d is functionally conserved with murine p19Ink4d and is required for neural crest development. Submitted.

* These authors contributed equally to the work.

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ADDITIONAL PUBLICATIONS NOT PART OF THIS THESIS:

Nilsson JA, Nilsson LM, Keller U, Yokota Y, Boyd K, Cleveland JL. (2004) Id2 is dispensable for Myc-induced lymphomagenesis. *Cancer Res* 64: 7296-7301.

Nilsson JA, Keller UB, Baudino TA, Yang CY, Norton S, Old JA, Nilsson LM, Neale G, Kramer DL, Porter CW and Cleveland JL. (2005) Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. *Cancer Cell* 7: 433-444.

Keller UB, Old JA, Nilsson JA, Yang CY, Nilsson L, Maclean, KH, Nakayama KI, Reed SI and Cleveland JL. (2007) Myc targets Cks1 to provoke the suppression of p27^{Kip1}, proliferation and lymphomagenesis. *EMBO J* 26: 2562-2574.

ABBREVIATIONS

A	Alanine, amino acid
ATP	Adenosine 5'-triphosphate
CAK	Cdk-activating kinase
Cdk	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitor
E11.5	Embryonic day 11.5
E	Glutamate, amino acid
EGL	External granule layer
GCP	Granule cell precursor
HPV	Human papilloma virus
I	Isoleucine, amino acid
LOH	Loss of heterozygosity
MBT	Mid-blastula transition
MEF	Mouse embryo fibroblast
NC	Neural crest
P	Proline, amino acid
R	Arginine, amino acid
Rb	Retinoblastoma protein
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser (S)	Serine, amino acid
Thr (T)	Threonine, amino acid
Tyr	Tyrosine, amino acid

INTRODUCTION

In all eukaryotes, ranging from yeast cells to mammalian cells, there is a set series of events that take place as a cell divides into two cells. The components differ to some extent, but the process remains the same; the cell has to duplicate all its cellular material and split into two exact parts, leading to the formation of two identical units. This is the process of cell division, and the carefully controlled chain of events leading to cell division is referred to as the cell cycle [reviewed elsewhere (Morgan, 2007)]. Cells exist in an environment together with other cells forming tissues and organs. It is of great importance for the organism that the cells are controlled and behave in an ordered way; they live and die and proliferate in a way suitable to their surroundings. This includes obeying signals from the environment, mediated by a variety of growth factors and mitogens. In order to respond to extracellular signals the cells are equipped with receptors in the cell membrane that relay signals through the cell membrane via the cytoplasm and in to the nucleus, causing changes in gene expression and protein activity.

THE CELL CYCLE

The cell cycle is the controlled chain of events when one cell becomes two, and it is divided into four separate phases according to the events taking place at each specific stage. The progression from one phase to another is governed by checkpoints at which the status of the cell is carefully monitored; a cell that is damaged or not behaving the way it should is not allowed to proceed to the next phase, and will thus not generate daughter cells. This system serves as a control where only healthy intact cells are allowed to divide. The four phases of the cell cycle are G1 (gap phase 1), S phase (DNA replication), G2 (gap phase 2) and finally M phase (mitosis) and division (Figure 1). On any given time point, the vast majority of cells in the body will not be actively dividing, instead they will be in a resting state called G0. When stimulated by signals from growth factors and mitogens, cells will enter the G1 phase and end up at the restriction point (Pardee, 1974). If the mitogenic signals are strong enough the cells will progress into the S phase. It is thus at the restriction point where the decision is made to continue the cell cycle or not and after passing the restriction point, the cell will complete the process of dividing even in the absence of further mitogenic stimuli.

Cells that pass the restriction point are further monitored in the checkpoints present in the S, G2 and M phases, ensuring proper completion of each phase. During the S phase, the

entire genome of the cell is replicated rendering the cell with double amount of genetic material. In diploid cells (two copies of each autosome, referred to as $2n$), this means that their genome should carry four copies ($4n$) of each autosome by the end of the S phase in order for the cell to progress into the G2 phase. The G2 checkpoint, also referred to as the G2/M checkpoint, dedicates its efforts into revealing any mistakes done while replicating the genome (Lukas et al., 2004). Finally, there is also a checkpoint dedicated to control that the cell does not progress into anaphase from metaphase unless all chromosomes are assembled on the mitotic spindle (Musacchio and Salmon, 2007). This checkpoint will assure equal distribution of genetic material into the two daughter cells.

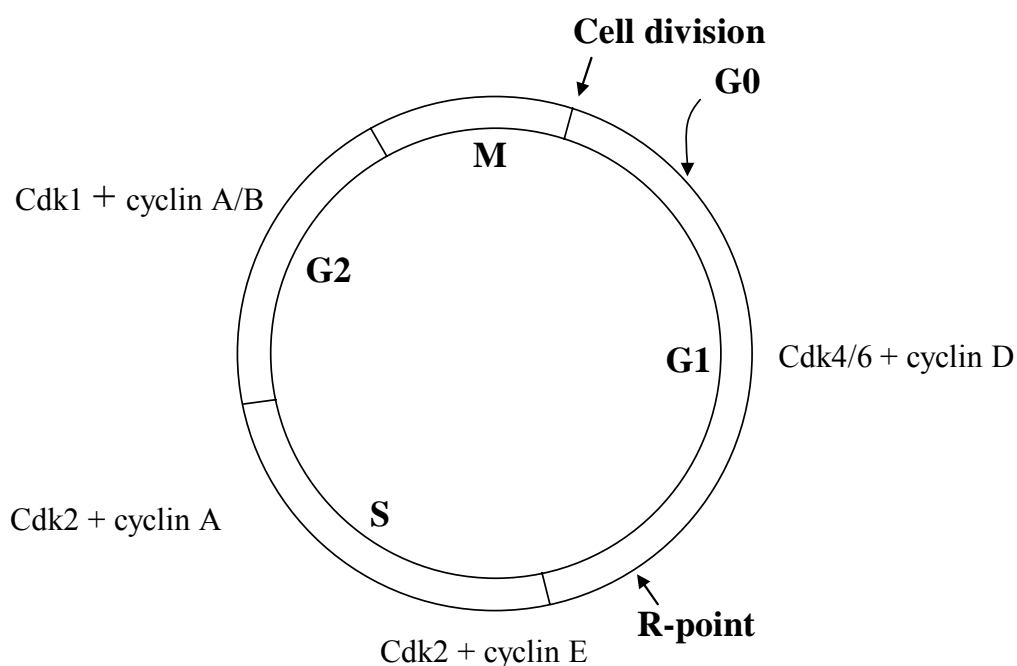


Figure 1. The different phases of the cell cycle and the cyclin:Cdk complexes controlling each phase.

Each phase of the cell cycle is carefully orchestrated by the actions of proteins called cyclins and cyclin-dependent kinases (Cdks). To this date, there are 13 Cdks and numerous cyclins identified in mammals (Table 1). A subset of these has been firmly connected to cell cycle regulation in various organisms (bold faced in Table 1). The Cdks and cyclins form heterodimers, resulting in active Cdk:cyclin complexes, and each phase of the cell cycle is characterized by a specific set of complexes being active (Figure 1). The active kinase complex will exert its effect by phosphorylating specific target substrates (Table 1). Throughout the cell cycle, the Cdks are ubiquitously expressed, whereas the protein levels of the cyclins oscillate dramatically, as a result of increased transcription and translation

Table 1. The Cdks identified so far in mammalian cells, their cyclin partners and substrates. The bold faced Cdks are directly connected to cell cycle regulation. A selection of substrates are denoted, for a more complete list and review of the topic, see elsewhere (Malumbres and Barbacid, 2005).

Cdk	Cyclin partner(s)	Phosphorylation targets
Cdk1	A, B	Cdc20, Cdc25, Cdk7, Dynein, Lamins, MAP4, MCM2 and 4, Npm, Nucleolin, Op18, pRb
Cdk2	A, E	Cdc6, MCM2 and 4, Npm, p107, p21 ^{Cip1} , p27 ^{Kip1} , p53, pRb
Cdk3	A, C, E	pRb, Cables 1
Cdk4	D1, D2, D3	pRb, p107, p130, Smad3
Cdk5	p35, p39	Disabled 1, p53, Stat3, Synapsin
Cdk6	D1, D2, D3	pRb, p107, p130
Cdk7	H	Cdk1-6, p53, RNA pol II
Cdk8	C	Cyclin H, RNA pol II
Cdk9	K, T	pRb, RNA pol II
Cdk10	Unknown	Unknown (function in transcription)
Cdk11	L	Cyclin L
Cdk12	L	Unknown (function in alternative splicing)
Cdk13	L	Unknown (function in alternative splicing)

followed by rapid destruction. Since cyclins are required for Cdk activity, the oscillations result in different Cdks being active in different phases of the cell cycle (Figure 2).

Following mitogen stimulation in the early G1, the expression of the D-type cyclins is induced leading to formation of active Cdk4/6:cyclin D complexes. These will phosphorylate their target, the Rb protein, before the restriction point. As the cells approach the end of the G1 phase, active Cdk2:cyclin E complexes will phosphorylate Rb on additional residues, rendering Rb hyperphosphorylated and inactivated. The Cdk:cyclin complexes that are active during the remaining phases are important for regulating the replication of DNA during the S phase and chromosome condensation and separation during mitosis. An overview of the major Cdk:cyclin complexes together with the known phosphorylation targets are summarized in Table 1.

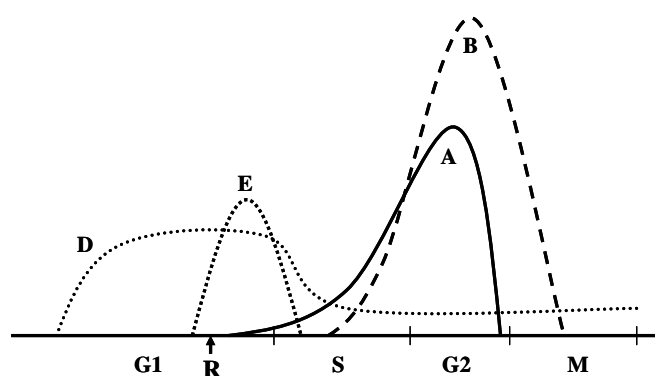


Figure 2. Cyclins and corresponding kinase activities fluctuate during the cell cycle, where Cdk4/6:cyclin D complexes are activated in response to mitogens during the early G1 phase, followed by Cdk2:cyclin E at the restriction point (R). Cdk2:cyclin A and Cdk1:cyclin B are activated during the S, G2 and M phases, regulating replication and subsequent cell division. Based on Sherr, 1996.

Ever since their discovery it has been clear that the cyclin D proteins do not behave like the cyclins A and B, which are both rapidly degraded after completing their functions. Cyclin D proteins are induced in response to mitogens, but will be detectable throughout the cell cycle and are not cycling as dramatically as cyclins A and B. The tight regulation of cyclins A and B serves a crucial function; they are active only once every cell cycle making it impossible for a cell to halt and go backwards. The focus of this thesis concerns the regulation of the G1/S transition and the following chapter will go into further details of how the Rb tumor suppressor is regulating these events.

THE G1/S TRANSITION

The G1 phase of the cell cycle is the phase in which a quiescent cell will re-enter the cell cycle when receiving the proper signal to divide and it is the phase in which an actively proliferating cell will end up in upon completion of mitosis. As mentioned earlier, it is during this phase that the cell will decide whether to continue through another round of cell division or to stop and exit the cell cycle and become quiescent. The early-to-mid G1 phase of the mammalian cell cycle is governed by three D-type cyclins (D1-3) and the kinases Cdk4 and 6, whereas the later part of the G1 phase also includes the cyclins E1-2 and their partner Cdk2 (Sherr and Roberts, 1999). These proteins will form dimers consisting of one Cdk and one cyclin subunit that together form an active Cdk:cyclin complex. The unifying action of these kinase complexes is the combined regulation of the common phosphorylation target; the Rb protein. Rb is the protein encoded from the *Rb* gene locus, and this was the first tumor suppressor identified in humans, where mutations in the gene were identified in retinoblastoma, a malignant tumor developing in the retina in children. Rb, together with its family members p107 and p130, make up the pocket protein family, where the pocket is the conserved domain present in all three proteins to which several DNA tumor virus oncoproteins bind, for example adenovirus E1A, human papillomavirus E7 and SV40 large T antigen (DeCaprio et al., 1988; Munger et al., 1989; Whyte et al., 1988). The binding of the viral proteins leads to inactivation of the pocket proteins and subsequent cell proliferation, but of these three pocket proteins, only Rb has been firmly connected to an essential role in tumorigenesis (Goodrich, 2006).

Rb function is controlled by a series of phosphorylations, and a simplified schematic of this regulation is summarized in Figure 3. At the start of the G1 phase Rb is unphosphorylated since it has been dephosphorylated by the protein phosphatase type 1 (PP1) at the end of mitosis (Ludlow et al., 1993). Unphosphorylated Rb binds the E2F proteins on promoters in complexes together with histone deacetylases (HDACs) and other chromatin remodelling proteins, keeping E2F transcriptional activity repressed (Classon and Harlow, 2002). In the early-to-mid G1 phase, Cdk4 and Cdk6 together with their D-type cyclins phosphorylate Rb on specific amino acids (Zarkowska and Mittnacht, 1997). Upon phosphorylation of Rb by Cdk4/6:cyclin D, the HDACs are released from the complexes whereby E2F can transcribe the *cyclin E* gene. The initial phosphorylation carried out by Cdk4/6:cyclin D sets the stage for subsequent hyperphosphorylation by Cdk2:cyclin E complexes at the restriction point and it is not until the phosphorylation of Rb is complete that

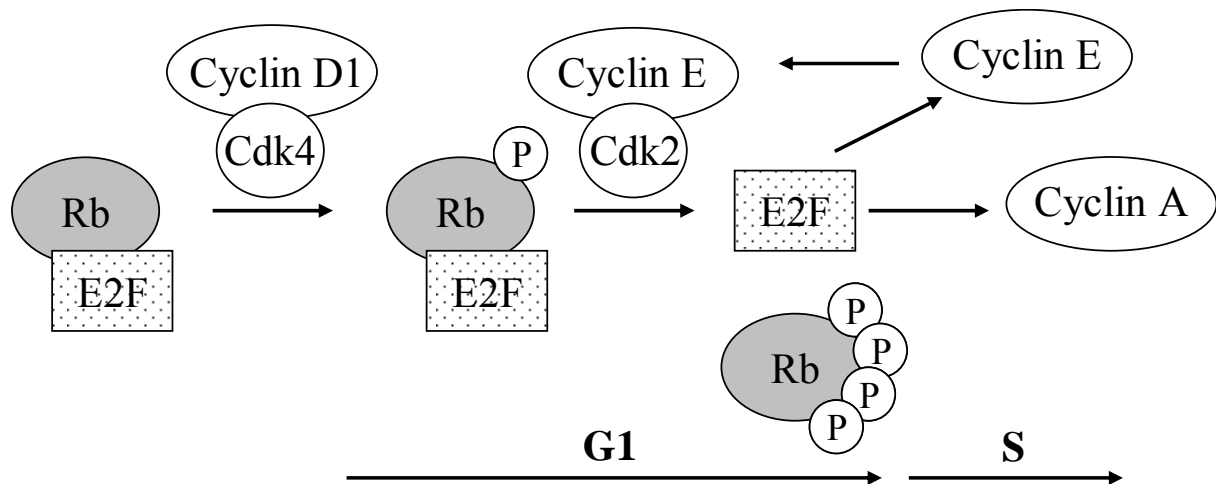


Figure 3. The phosphorylation of Rb regulates E2F transcriptional activity and progression into the S phase. Based on Obaya and Sedivy, 2002.

transcription of *cyclin A* starts. The fact that cyclin E is part of the complex regulating E2F and the fact that the *cyclin E* gene is an E2F target leads to an intensification of the Cdk2:cyclin E activity and further increase of Rb phosphorylation, which ultimately results in cells entering the S phase. In this way, Rb is controlling the transcriptional activity of E2F proteins, which in turn regulate S phase entry and progression.

REGULATION OF CDK ACTIVITY

The activities of the Cdk proteins of the cell cycle can be regulated in many different ways. This is an area of intense research, since if one can regulate the Cdks that phosphorylate Rb, it is possible to control E2F transcriptional activity and thereby proliferation. Regulation of

Cdks in the cell occurs in different ways; there is regulation of kinase activity by phosphorylation and there is regulation by binding of inhibitory proteins called cyclin-dependent kinase inhibitors (CKIs).

The most basal activation of Cdk activity occurs upon cyclin binding. All cyclin proteins share a domain referred to as the cyclin box, which is the part of the cyclin molecule that interacts with the PSTAIRE (the amino acid sequence using the single letter code) domain on the Cdk proteins (Morgan, 1996). The PSTAIRE domain is a conserved domain found in the various Cdks, representing one of the contact points between the cyclin and the Cdk. The Cdk is built up by two lobes; the N lobe and the C lobe, where the N lobe is rich in β -sheets and the C lobe consists primarily of α -helices (Pavletich, 1999). The catalytic cleft is located between the two lobes, and this is where the ATP molecule as well as the substrate will be brought for catalysis, i.e. where the γ -phosphate is transferred from ATP to a specific Thr/Ser on the substrate. The monomeric Cdk lacks kinase activity, due to the fact that the catalytic cleft is not properly formed and since the cleft is blocked by a structure called the T-loop (see below). When a cyclin binds, a conformational shift is imposed on the Cdk. This reshapes the catalytic cleft by altering the angle between the two lobes and by moving the PSTAIRE domain into the cleft, making the cleft suitable for ATP catalysis.

Inactivating modifications of Cdks include phosphorylations and cyclin-dependent kinase inhibitor (CKI) binding. All these modifications are summarized in Figure 4. In order to become fully active, the newly formed Cdk:cyclin complexes need to be phosphorylated by

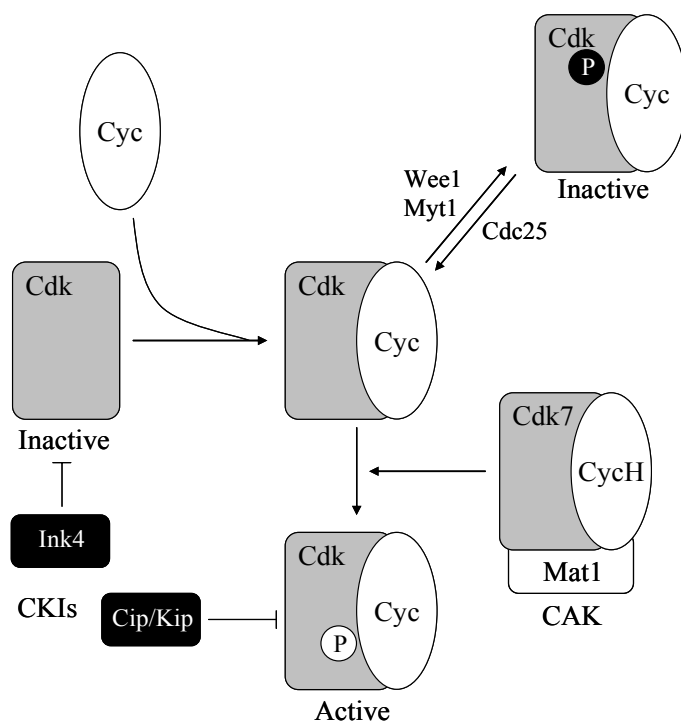


Figure 4. Cdk activity is regulated by several mechanisms. The first activating modification is the cyclin binding to the Cdk. The Cdk:cyclin complexes are further regulated by phosphorylations or binding of CKIs. Inhibiting modifications (black in the figure) are phosphorylations by Wee1 and Myt1 on Thr residues on the Cdk, whereas activating modifications are dephosphorylations by Cdc25 phosphatases and phosphorylations by CAK. CKIs from the Ink4 family bind and inactivate primarily monomeric Cdks (Cdk4 and Cdk6). CKIs from the Cip/Kip family bind to active Cdk:cyclin complexes thereby forming inactive trimers. Modified from a review (Malumbres and Barbacid, 2005).

the Cdk-activating kinase (CAK) (Kaldis, 1999). This kinase complex consists of Cdk7 and cyclin H, and the assembly factor Mat1. CAK phosphorylates Cdk1, Cdk2, Cdk4 and Cdk6, and these events occur in the nucleus. In early studies it was shown that Cdk4 with the CAK-modified threonine residue (Thr172) mutated to prohibit phosphorylation, can still bind and form complexes with cyclin Ds (Kato et al., 1994), but the phosphorylation will further increase the activity around 100 times. As mentioned earlier, the catalytic cleft of monomeric Cdks is blocked by a structure called the T loop. Upon binding of the cyclin subunit, the T loop will shift to open up the cleft, and this shift is completed upon phosphorylation of the threonine in the T loop by CAK. Binding of the CKIs, discussed in more detail in the following section, will block the CAK phosphorylation of Cdks (Jeffrey et al., 2000).

Phosphorylation of Cdks can also be carried out on additional residues. The Myt1 and Wee1 kinases phosphorylate Cdk1 and Cdk2 on tyrosine 15 and threonine 14, and these are inhibiting modifications leading to a stop in cell cycle progression (McGowan and Russell, 1993; Mueller et al., 1995), and occur in response to for example DNA damage (Rhind et al., 1997). The inhibiting phosphorylations of Cdks can be counteracted by a family of phosphatases; the Cdc25 proteins (Malumbres and Barbacid, 2001). These phosphatases dephosphorylate specifically Tyr15 and Thr14, rendering the Cdk accessible for further activation by cyclin binding and CAK-activation. The residue equivalent to Tyr15 on Cdk4 is Tyr17, and there are reports suggesting that not only Cdk1 and Cdk2 but also Cdk4 function can be inhibited in this manner, although the functional relevance and responsible kinase is currently not known (Terada et al., 1995).

Apart from modifications including phosphorylations, Cdk activities are also regulated by binding of CKIs (Sherr and Roberts, 1999). There are two families of CKIs in mammals, and these two families display specific characteristics when it comes to target recognition. The Cip/Kip family consists of three members; p21^{Cip1}, p27^{Kip1} and p57^{Kip2}. This family has the ability to bind to a broad spectrum of Cdk:cyclin complexes; they can bind all known Cdk:cyclin complexes. They inhibit Cdk2 and Cdk1 activities, and have been implicated in proper formation of active Cdk4/6:cyclin D complexes (Cheng et al., 1999), although this is still debatable since cells lacking p21 and p27 show Cdk4/6:cyclinD activity (Bagui et al., 2000; Sugimoto et al., 2002). The Cip/Kip family of inhibitors interacts with both the Cdk and the cyclin subunit. This is different from the second group of CKIs in cells, referred to as the Ink4 family; the name being an abbreviation of Inhibitor of Cdk4. In addition to Cdk4, these proteins also inhibit the activity of Cdk6, and the result of this inhibition is to stop the Cdk4 and Cdk6 from phosphorylating their substrate Rb, thereby causing a cell cycle arrest. The

Ink4 family consists of four members; p16^{Ink4a}, p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d}. The founding member of this family is p16^{Ink4a}, where p16 refers to its size when separated by SDS-PAGE. p16^{Ink4a} was identified as a protein associated with Cdk4 in certain immortalized cells (Serrano et al., 1993). Shortly after that initial observation, p16^{Ink4a} was shown to be the protein encoded from the *MTS1/CDKN2A* gene which is often found mutated in human melanoma (Kamb et al., 1994; Ruas and Peters, 1998). p16^{Ink4a} is firmly established to be a tumor suppressor in human cancers, where it is often deleted or mutated (Ruas and Peters, 1998). During the years that followed the initial discovery of p16^{Ink4a}, three more *Ink4* genes were discovered in both humans and mice; p15^{Ink4b}, p18^{Ink4c} and p19^{Ink4d} (Chan et al., 1995; Guan et al., 1994; Hannon and Beach, 1994; Hirai et al., 1995). The proteins have a high sequence similarity and consist of ankyrin repeats throughout the entire length of the protein. Ankyrin repeats are composed of several consecutive blocks of ~33 amino acid stretches rendering an L-shaped structure with a β -hairpin loop followed by two α -helices. The α -helices form the core of the protein and the β -hairpins are exposed generating the surface for interactions. Ankyrin repeat-containing proteins exist in different classes of proteins in both prokaryotes and eukaryotes and this structure is believed to promote protein-protein interactions.

REDUNDANCY AMONG GENES OF THE G1/S TRANSITION

In attempts to increase our knowledge about how the cell cycle is governed, a large number of genetically modified mice have been generated over the last decades. To remove a gene by homologous recombination in embryonic stem cells is a useful method to elucidate the function of the gene product by studying what happens when it is absent. During the last decades, all the major players of the G1/S transition have been knocked out in the mouse and these strains have meant a lot for our current understanding of the physiological cell cycle regulation. The most important strains and corresponding phenotypes are summarized in Table 2.

Table 2. Mice lacking Cdks and cyclins important for G1/S progression.

Gene(s)	Survival	Major phenotype(s)	References
Cdk2	Viable	Reduced body size, female and male infertility. MEFs show delayed S phase entry, but can be transformed but to a lower degree compared to wildtypes.	(Berthet et al., 2003; Ortega et al., 2003)
Cdk4	Viable	Reduced body size, female and male infertility, diabetes. MEFs can be cultured but resist transformation.	(Rane et al., 1999; Tsutsui et al., 1999; Zou et al., 2002)
Cdk6	Viable	Thymic and splenic hypoplasia, mild defects in hematopoiesis	(Malumbres et al., 2004)
Cdk4/Cdk6	Lethal from E14.5 and on	Defective hematopoiesis (reduced number precursors) with severe megaloblastic leukaemia. MEFs proliferate slower with reduced S-phase and resist transformation.	(Malumbres et al., 2004)
Cdk2/Cdk6	Viable	Same phenotype as reported for the single KOs.	(Malumbres et al., 2004)
Cdk2/Cdk4	Lethal ~E15	Reduced body size, heart defects, defective hematopoiesis. Progressive loss of Rb phosphorylation and E2F target gene expression. MEFs proliferate slower, which is partially rescued by HPV-E7.	(Berthet et al., 2006)
Cdk2/Cdk4/Cdk6	Lethal E12.5	Severe haematological defects and heart defects. MEFs proliferate but slower, Cdk1 associates with all cyclins, Rb is phosphorylated at all residues believed to be interphase-specific.	(Santamaria et al., 2007)
Cyclin D1	Viable	Reduced body size, neurological defects, impaired proliferation of mammary epithelium	(Fantl et al., 1995; Sicinski et al., 1995)
Cyclin D2	Viable	Female sterility, hypoplastic testes in males, cerebellar defects, impaired proliferation of peripheral B lymphocytes.	(Huard et al., 1999; Lam et al., 2000; Sicinski et al., 1996)
Cyclin D3	Viable	Hypoplastic thymus	(Sicinska et al., 2003)
Cyclin D2/D3	Lethal ~E18.5	Severe megaloblastic anemia	(Ciemerych et al., 2002)
Cyclin D1/D3	Lethal P1	Neurological defects	(Ciemerych et al., 2002)
Cyclin D1/D2	Viable ~3 weeks	Reduced body size, hypoplastic cerebella	(Ciemerych et al., 2002)
Cyclin D1/D2/D3	Lethal E16.5	Severe megaloblastic anemia, hematopoietic failure, heart defects. MEFs can be cultured but show reduced susceptibility to transformation	(Kozar et al., 2004)
Cyclin E1	Viable	No reported abnormalities	(Geng et al., 2003; Parisi et al., 2003)
Cyclin E2	Viable	Decreased male fertility	(Geng et al., 2003; Parisi et al., 2003)
Cyclin E1/E2	Lethal E11.5	Placental defects (tetraploid complementation prevented lethality), heart defects. MEFs proliferate slower and resist transformation, but cannot exit quiescence due to failure of loading MCM proteins onto prereplicative origins.	(Geng et al., 2003; Parisi et al., 2003)

Mice lacking single *cyclin D* genes have been generated, and these mice are all viable but display unique phenotypes (Fantl et al., 1995; Lam et al., 2000; Sicinska et al., 2003; Sicinski et al., 1996; Sicinski et al., 1995). Interestingly, when deleting all three *cyclin D* genes in the mouse, the embryos still develop to embryonic day (E) 16.5, indicating that cells can divide in the absence of cyclin D proteins (Kozar et al., 2004). However, fibroblasts lacking all three cyclins are resistant to transformation by various oncogenes (Kozar et al., 2004), indicating that there is a different need for cyclins in the process of transformation compared to embryo development. ‘Single-cyclin’ mice have also been generated. In these

mice, two of three *cyclin D* genes have been deleted, leading to expression of only one *cyclin D* (Ciemerych et al., 2002). These embryos develop relatively normally, and both *D2*-only and *D3*-only pups are born but they die after birth. The *D1*-only mice die at the very last stages of embryonic development, around E17.5-18.5. Mouse embryo fibroblasts (MEFs) generated from ‘single-cyclin’ mice have been used in proliferation assays, where the overexpression of the oncogenes *ras* (plus dominant-negative p53) or *c-myc* normally leads to an increased rate of cellular proliferation. Despite the presence of only one *cyclin D* gene in these MEFs, the cells were capable of responding to oncogene-induced hyperproliferation (Yu et al., 2005).

The knockouts of the individual *cyclin E1* and *E2* genes are viable, but mice lacking both *cyclin E1* and *E2* die at E11.5 (Geng et al., 2003; Parisi et al., 2003), the major problem being an inability of the trophoblast giant cells of the placenta to undergo the required endoreplication. The lethality of the double knockout embryos could be rescued with tetraploid complementation.

Mice lacking *Cdk4*, *Cdk6*, or both have been generated. *Cdk4* knockouts are small, sterile and develop diabetes due to defects in β -islet cells in the pancreas (Moons et al., 2002; Rane et al., 1999; Tsutsui et al., 1999; Zou et al., 2002). *Cdk6* knockouts, on the other hand, show only mild defects in hematopoiesis and hypoplastic thymus and spleen (Malumbres et al., 2004). Mice doubly deficient in *Cdk4* and *Cdk6* display embryonic lethality from E14.5 and on, with major defects in hematopoiesis (Malumbres et al., 2004). Fibroblasts from *Cdk4/Cdk6* double mutant embryos can proliferate but in absence of these kinases, cyclin D1 can interact with Cdk2 and form complexes that harbour Rb kinase activity. Therefore, when knocking down the expression of Cdk2 in the *Cdk4/Cdk6* double knockout cells using shRNA, there was an inhibition in proliferation which was not seen in wildtype cells (Malumbres et al., 2004). *Cdk2* has also been knocked out in mice and, to the surprise of many, these mice are fine apart from effects on oogenesis and spermatogenesis leading to both male and female sterility (Berthet et al., 2003; Ortega et al., 2003). Removing *Cdk4* in these mice resulted in an embryonic lethal phenotype due to a heart defect (Berthet et al., 2006). *Cdk2/Cdk4* double knockout MEFs proliferate slower than wildtype cells, which is associated with a lack of E2F transcriptional activity. Several E2F target genes, for example *Cdk1*, *cyclin E* and *cyclin A*, show reduced expression which can be partially restored by co-expression of the Rb inhibitory protein HPV E7. Finally, during the writing of this thesis, the *Cdk1*-only mouse was published (Santamaria et al., 2007) where all the Cdks of the interphase, i.e. *Cdk2*, *Cdk4* and *Cdk6* (*Cdk3* is lacking in most lab strains of mice due to a

point mutation rendering a truncated protein) were deleted in the germ line. Extraordinarily, the cells were still able to proliferate in culture, implying that the basic cell cycle machinery in mice is similar to the situation in yeast where one Cdk governs the entire cell cycle (Morgan, 2007). Supporting its important role, Cdk1 is essential for early embryogenesis (Santamaria et al., 2007).

To summarize this section, the results from mice lacking Cdks and cyclins of the G1/S transition alone or in combinations, indicate that these genes are to a large extent dispensable for normal cell division. However, the cell cycles in cancer are not normal and many of the genes regulating the G1/S transition are altered during development of tumors.

ALTERATIONS OF GENES IN CANCER

The results in the previous section describe the development of an organism in the absence of genes regulating the G1/S transition, but development of an embryo is very different from development of tumors. Since the major focus of this thesis is tumor biology and tumor development, the following sections aim to shed some more light on this topic by describing in more detail the genetic alterations involved in tumorigenesis.

The Hallmarks of Cancer

In the field of tumor biology it is well established that for a tumor to develop, certain genetic alterations need to occur. These alterations do not occur in a random manner and by studying them we can learn how the process of tumorigenesis occurs. Tumors do not solely reflect an increase in cell proliferation, but a broad set of events that take place before tumors appear as a final step in a multistep process. The six hallmarks for malignant growth, as once proposed, are; 1) self-sufficiency of growth signals, 2) insensitivity to anti-growth signals, 3) evading apoptosis, 4) limitless replicative potential, 5) sustained angiogenesis, and 6) tissue invasion and metastasis (Hanahan and Weinberg, 2000), and these characteristics are shared by most malignant tumors.

Tumor Suppressor Genes (TSGs) and Oncogenes

Cancer is a genetic disease and research on the genes causing tumors has established that there are two types of genes involved in cancer development: TSGs serving as proliferative brakes, and oncogenes that promote growth and cellular proliferation. In tumors, the functions of TSGs are lost whereas oncogenes are overexpressed or constantly activated.

The most studied TSG in human cancer is *TP53*, which is mutated in at least 50% of all human cancers (Hollstein et al., 1991). Germline mutations in *TP53* also cause the Li-Fraumeni syndrome where affected people inherit a mutated *TP53* gene and develop a large variety of cancers at a young age (Malkin et al., 1990). *TP53* encodes the protein p53, a transcription factor that is induced by several cellular stress signals such as genotoxic, non-genotoxic and oncogenic stress (Giaccia and Kastan, 1998; Levine, 1997; Sherr and McCormick, 2002). p53 exerts its function by regulating transcriptional target genes that encode proteins involved in cell cycle control (e.g. p21^{Cip1} and 14-3-3 σ), apoptosis (e.g. Puma and Noxa) and control of its own stability (Mdm2 or Hdm2 in humans) (Vogelstein and Kinzler, 2004). p53 therefore functions to protect the organism against the appearance of damaged cells that may become cancer cells. For cancer to develop, mutations in *TP53* are a near pre-requisite for malignant transformation. These mutations are most often point mutations clustered in the DNA binding domain, cancelling p53's function as a transcription factor. Since mutated p53 no longer can induce Hdm2 and its own degradation, tumors accumulate the mutant protein to a high level.

Loss of p53 function has been modelled in mice for almost twenty years. The first mice created were simple transgenics where the p53 mutations identified in tumors were expressed in whole animals (Lavigne et al., 1989), and even though we consider this crude today, it was the first *in vivo* support for mutated *p53* causing tumors. Later, mice carrying a deletion (Donehower et al., 1992; Jacks et al., 1994) or point mutation (Lang et al., 2004; Olive et al., 2004) in *p53* were shown to be highly prone to develop a broad spectrum of tumors. Lastly and most recently, mice were created that lack p53 function, but the targeting was designed with an 'on-off' switch, allowing tissue-specific and temporal reactivation of p53 function (Christophorou et al., 2005; Ventura et al., 2007).

Since levels of p53 are kept low in a cell by its own transcriptional target Mdm2, the interaction between p53 and Mdm2 has to be disrupted for p53 levels to accumulate in response to stress signals (Sherr, 2001). This can be accomplished in two conceptually different ways: phosphorylation of p53 and Mdm2 by stress kinases (e.g. ATM) or induction of the Arf tumor suppressor by oncogenes such as Myc and E1A. Arf is encoded from the *Ink4a* locus using a different reading frame and first exon compared to p16^{Ink4a} (Quelle et al., 1995). Arf activates p53 by binding, inactivating and translocating Mdm2 into the nucleolus (Honda and Yasuda, 1999; Weber et al., 1999). These protein interactions make up the Arf-p53 pathway depicted in Figure 5A.

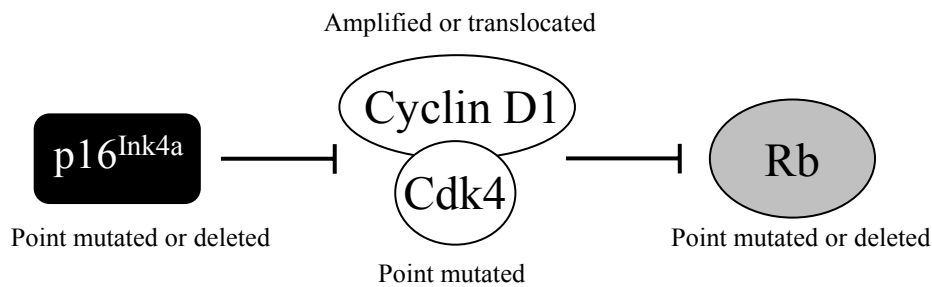
A**B**

Figure 5. Schematic of the Arf-p53 and Rb pathways with the type of alterations seen in cancer depicted. **A)** Arf causes p53 activation by binding and inhibiting Mdm2, the E3 ubiquitin ligase for p53. This causes stabilization and activation of p53. **B)** p16 blocks the kinase activity of CDK4/6 thereby preventing inactivation of Rb.

The RB pathway (Figure 5B) is also altered in many tumors, where the most frequent alterations include *cyclin D1* translocations and amplifications, *RB* mutations and *INK4A* deletions, mutations and silencing (Sellers and Kaelin, 1997). The alterations of these genes occur to a high degree in a tumor-specific manner, where certain tumor types carry specific mutations. For instance, mantle cell lymphomas are almost exclusively caused by overexpression of cyclin D1 due to a translocated *cyclin D1* gene, whereas pancreatic cancers often display loss of *INK4A* (Sherr and McCormick, 2002). The mutations in the RB pathway are most often affecting only one member, implying that this pathway is linear. The frequent alterations in this pathway have motivated further studies of its importance for controlling the events leading to cancer.

Common to classical TSGs, is that both copies of the gene need to be lost in order for a tumor to form. The tumors developing in the retinas of people carrying a mutation in *RB* always display loss of heterozygosity (LOH) in the *RB* locus. The loss of TSGs by this two-step mechanism is referred to as the Knudson's two hit model (Knudson, 1971), where the second allele is believed to be lost by different genetic events including mitotic recombination and gene conversion (Tischfield, 1997). However, not all genes showing tumor suppressive functions follow these rules; the CKI p27^{Kip1} has been shown to be haploinsufficient for tumor development in mice (Fero et al., 1998), where tumors develop despite retention of a wildtype allele. Haploinsufficient tumor suppressors are not completely understood, but most likely

there is a threshold effect where one remaining copy of a gene is not enough to suppress tumor formation. p27^{Kip1} is for example very rarely mutated in human cancers, but lower expression of p27^{Kip1} protein correlates with reduced survival time of patients suffering from various cancers (Sgambato et al., 2000; Slingerland and Pagano, 2000).

At variance with TSGs, oncogenes promote cellular proliferation. Numerous oncogenes have been identified to date, exemplified here by *c-Myc*. The *Myc* genes (*c-Myc*, *N-Myc* and *L-Myc*) are very frequently deregulated in cancer; an estimation of around 70% of all cancers show increased expression of these genes (Nilsson and Cleveland, 2003). The gene was originally identified in the MC29 myelocytomatosis virus where expression of *v-myc* gave rise to tumors in chickens (Sheiness et al., 1978). The origin of *v-myc* was the cellular gene *c-myc* present in the chicken genome (Sheiness and Bishop, 1979), and this is how many of the known oncogenes were once identified; by finding the cellular homologs of genes expressed in tumor-causing retroviruses (Weinberg, 2006). Myc proteins carry out their functions as transcription factors by binding to their obligate partner Max, and these heterodimers will bind specific DNA sequences called E-boxes (CACGTG) in the regulatory regions of target genes and activate transcription (Blackwell et al., 1993; Blackwell et al., 1990; Blackwood and Eisenman, 1991; Blackwood et al., 1992). Max can also dimerize with other proteins, such as Mad1-4 or Mnt, whereby the formed heterodimers serve repressing functions together with repressor complexes including Sin3 and HDACs (Ayer et al., 1993; Ayer et al., 1996; Ayer et al., 1995; Hurlin et al., 1997; Hurlin et al., 1995). Myc does not only induce proliferation but overexpression of Myc also promotes apoptosis (Nilsson and Cleveland, 2003). Studies on Myc function have provided us with important findings supporting the notion that in order for tumors to form, the apoptosis caused by Myc and other oncogenes needs to be evaded.

There are many mouse models available for studies of the tumor forming process caused by overexpressed Myc, one being the E μ -Myc mouse where the consequence of the translocation t(8;14) of human Burkitt lymphoma has been mimicked. In this model *c-Myc* has been inserted downstream of a promoter regulated by the Immunoglobulin heavy chain enhancer E μ . This results in Myc overexpression in B cells and mice positive for the transgene develop pre-B cell lymphomas early in life (Adams et al., 1985). This model has been utilized for *in vivo* studies to determine the roles of collaborating lesions for development of lymphomas. Analyses of tumors arising in the E μ -Myc mouse have shown that in the vast majority, the apoptotic pathway governed by Arf-Mdm2-p53 is inactivated (Eischen et al., 1999; Zindy et al., 1998). Another way for tumor cells to escape the apoptosis

induced by Myc is by induction of anti-apoptotic proteins such as Bcl-2 and Bcl-X_L (Eischen et al., 2001).

Apart from Myc's apoptosis-inducing abilities, genes influencing proliferation have also been tested for collaboration with Myc overexpression in the Eμ-Myc mouse model. The CKI p27^{Kip1} negatively regulates Cdk2:cyclin E and Cdk2:cyclin A complexes in the G1/S phase of the cell cycle and both the p27 mRNA and protein are downregulated in response to Myc overexpression (Vlach et al., 1996). The *in vivo* support of p27^{Kip1} being important for suppressing Myc-induced proliferation came from Eμ-Myc;p27^{-/-} mice where an acceleration of disease was observed (Martins and Berns, 2002). On this note, very recent studies indicate that the reduced levels of p27^{Kip1} in response to Myc rely in part on Myc's ability to upregulate the degradation machinery for p27^{Kip1}. Thus, Eμ-Myc mice null for *Cks1*, a protein involved in degrading p27^{Kip1}, display a delayed onset of lymphomagenesis concomitant with increased levels of p27^{Kip1} (Keller et al., 2007).

Ink4 Proteins and Cancer

Since p16^{Ink4a} is an important tumor suppressor in human cancer, the other Ink4 family members have also been tested for similar functions. A lot of work has gone into screening *Ink4* genes for mutations and expression levels in different tumors and the results point towards a unique role for *Ink4a* in its high frequency of mutation and deletion in human tumors (Ruas and Peters, 1998). *Ink4b* is deleted to some extent, but this often occurs simultaneous with *Ink4a* deletions, making it hard to define the individual contribution. For p18^{Ink4c} and p19^{Ink4d} the situation is different, where mutations in *Ink4c* have been identified in some tumors and cell lines, whereas *Ink4d* is practically never altered (Ruas and Peters, 1998). An explanation for the observed difference in the mutation frequency of different *Ink4* genes in cancer is that the Ink4 proteins perform separate functions in the cell. Indeed, p16^{Ink4a} and p15^{Ink4b} respond to external growth inhibitory signals, oncogenic stress and cellular senescence (programmed ageing) (Lowe and Sherr, 2003; Ortega et al., 2002), whereas p18^{Ink4c} and p19^{Ink4d} are involved in physiological withdrawal from the cell cycle during terminal differentiation (Chen et al., 2003; Franklin and Xiong, 1996; Morse et al., 1997; Phelps et al., 1998; Tourigny et al., 2002). The genomic locations of these genes are interesting, where both *Ink4a* and *Ink4b* reside on chromosome 9 in humans and on chromosome 4 in mice, together with *Arf* (Figure 6). Hence, the *Ink4a* locus encodes two tumor suppressors that together regulate the two most important pathways (p53 and Rb) in human cancer. In mice, three out of the four *Ink4* genes reside on chromosome 4 with *Ink4c*

located 20 million bases away from the *Ink4b-Arf-Ink4a* loci (Figure 6). *Ink4d* is in both

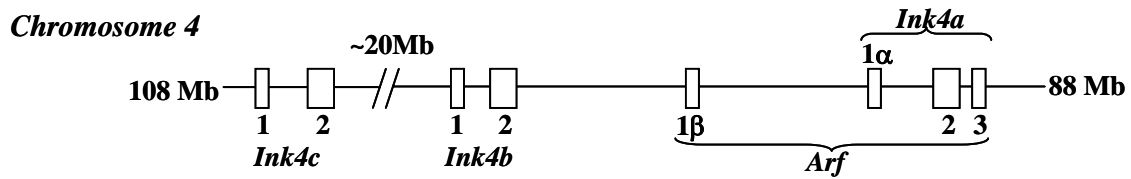


Figure 6. *Ink4a*, *Ink4b* and *Ink4c* all reside on chromosome 4 in the mouse genome together with *Arf*.

human and mouse located on a different chromosome than the rest of the *Ink4* genes (chromosome 19 in humans and chromosome 9 in mice).

Mice lacking one or two *Ink4* genes have been generated (see Table 3). To the disappointment of many, the loss of *Ink4* genes is not strongly predisposing mice to develop spontaneous tumors. Mice lacking *Ink4a* or *Ink4b* develop tumors with low incidences and relatively late in life, *Ink4c* null animals develop pituitary adenoma at a high frequency as well as testicular tumors and lymphomas with lower incidences, whereas loss of *Ink4d* has not been connected to any tumor phenotype at all. Interestingly, even though the Ink4 proteins display identical biochemical properties when assayed *in vitro*, the phenotypes in mice lacking the individual *Ink4* genes are all unique. Nevertheless, mice expressing a melanoma-

Table 3. Phenotypes of mice lacking one or two *Ink4* genes or expressing Ink4-insensitive Cdk4.

<i>Gene(s)</i>	<i>Survival</i>	<i>Phenotypes</i>	<i>References</i>
<i>Ink4a</i>	Viable	Thymic hyperplasia, increased incidence of spontaneous and carcinogen-induced tumors (lymphomas and sarcomas).	(Krimpenfort et al., 2001; Sharpless et al., 2001)
<i>Ink4b</i>	Viable	Extramedullary hematopoiesis, lymphoproliferative disorders, MEFs are susceptible to transformation by <i>ras</i> .	(Latres et al., 2000)
<i>Ink4c</i>	Viable	Pituitary adenomas with high incidence, lymphoproliferative disorders.	(Franklin et al., 1998; Latres et al., 2000)
<i>Ink4d</i>	Viable	Testicular atrophy and progressive hearing loss.	(Chen et al., 2003; Zindy et al., 2000)
<i>Ink4a;Ink4c</i>	Viable	Aggressive pituitary tumors as well as lymphomas and sarcomas), enhanced proliferation of cells in pancreatic islets, pituitary and B lymphocytes.	(Ramsey et al., 2007)
<i>Ink4b;Ink4c</i>	Viable	MEFs show increased susceptibility to transformation by <i>ras</i> , compared to <i>Ink4b</i> -null.	(Latres et al., 2000)
<i>Ink4c;Ink4d</i>	Viable	Male sterility.	(Zindy et al., 2001)
<i>Ink4a;Ink4b</i>	Viable	Increased tumor susceptibility compared to <i>Ink4a</i> -null.	(Krimpenfort et al., 2007)
<i>Cdk4^{R24C/R24C}</i>	Viable	Develop broad spectrum of tumors, MEFs do not senesce in culture.	(Sotillo et al., 2001)

derived Ink4-insensitive Cdk4 mutant (*Cdk4*^{R24C}; equivalent to *Ink4abcd*-null) are highly tumor-prone (Sotillo et al., 2001), indicating that the combined loss of Ink4 function has a profound effect on cell cycle control. Furthermore, it is a known fact that *Ink4* genes have the ability to compensate for each other, most recently described in cells lacking p18^{Ink4c} where the levels of p16^{Ink4a} were increased (Ramsey et al., 2007). This redundancy may be the reason why deletion of the individual *Ink4* genes not always generates tumor-prone mice.

AIMS

The overall aim of this thesis was to further evaluate the roles of the Cdk4/Cdk6-inhibiting proteins p18^{Ink4c} and p19^{Ink4d} using *in vivo* models of cancer and embryonic development.

More specifically, the aims were:

- 1) To evaluate how loss of these CKIs influence the development of tumors in the tumor-prone *p53* knockout mouse.
- 2) To study how loss of *Ink4c* affects lymphomagenesis in the E μ -*Myc* mouse.
- 3) To search for *Ink4* genes in *Xenopus laevis* for the purpose of studying the role of Ink4 proteins in early vertebrate development.

RESULTS AND DISCUSSION

Lack of *Ink4c* alters the tumor spectrum in *p53*-deficient mice (Paper I)

To further evaluate the roles of $p18^{Ink4c}$ and $p19^{Ink4d}$ in cancer, mice doubly deficient in *Ink4c* and *Ink4d* were bred to the *p53* knockout mouse. Mice lacking *p53* alone are highly tumor-prone and develop a wide range of tumors, predominantly T-cell lymphomas (Donehower et al., 1992; Jacks et al., 1994). The original reason for the cross was to investigate whether blocking apoptosis of spermatocytes in mice doubly deficient in *Ink4c* and *Ink4d* (Zindy et al., 2001) by removing *p53* would cause an increase in the incidence of testicular tumors. However, the loss of *Ink4d* did not affect the incidence of tumors at all. Mice doubly deficient in *p53* and *Ink4d* displayed the same phenotype as mice deficient in *p53* alone. On the other hand, when mice doubly deficient in *Ink4c* and *p53* started to develop tumors it became clear that instead of testicular tumors, these mice developed blood vessel tumors to a higher degree than previously reported. Additional to frank tumors, the mice from this cross also displayed high incidences of various vascular dysplasias including (1) endothelial hyperplasias, where there is an increased proliferation of endothelial cells; (2) angiectasia, defined as a marked dilation of the blood vessel; (3) hemangiomas, which are benign blood vessel tumors, and (4) hemangiosarcomas which are the malignant counterparts. These tumors develop in animals lacking only *p53* but to a lower degree (Jacks et al., 1994). In our cohorts of mice, lacking *p53* and at least one copy of *Ink4c*, we observed an increased vascular tumor incidence but the onset of disease is unaffected compared to the mice lacking *p53* alone, where 50% of the cohort had succumbed to disease by 15 weeks of age. Apart from the increase in vascular tumor incidence, the mice developed the tumors of the parental strains with unaltered incidences. It is worth noticing that the incidence of the vascular dysplasias was higher in our *p53* knockout control group compared to the incidences reported in the literature for *p53* knockout mice (Jacks et al., 1994) and this may reflect an effect on the tumor spectrum depending on background. There are often changes in tumor spectrum of knockout mice depending on genetic background, and so also in the *p53* knockout mouse, where the incidence of hemangiosarcomas increased from 8% in the pure 129SvJ mouse to 23% in the mixed C57BL/6 x 129 SvJ mouse (Harvey et al., 1993). There is certainly a possibility that the background also affected our results, since our mice were on a mixed C57BL/6 x 129SvJ background. To define this effect of genetic background the cross would need to be done on a pure background.

Some of the hemangiosarcomas were successfully transplanted in mice, but due to their composition no detailed analyses have been possible. The tumors consist of a mixture of different cell types; endothelial cells, pericytes, stroma cells, and added to that all the cells present in blood. Immunohistochemical analyses reveal that cells present in the tumors express markers typical for this tumor type (CD31, CD34, VEGF-R2), but any further genetic analyses and gene expression studies have not been possible.

There are examples in the literature of increased incidences of hemangiosarcomas in mouse models relevant to ours. In a similar study as ours, *Ink4c/p53* double knockout mice were generated and the authors reported increased incidences of several tumor types, including hemangiosarcomas and hepatocellular carcinoma (Damo et al., 2005). Another model is the *Cdk4*^{R24C} mutant mouse (Sotillo et al., 2001), where arginine at position 24 has been mutated to a cysteine, rendering Cdk4 insensitive to the inhibitory effects of Ink4 proteins (Wolfel et al., 1995; Zuo et al., 1996). This mouse developed a wide range of tumors and 56% of the mice developed hemangiosarcomas (Sotillo et al., 2001). Similar incidences of hemangiosarcomas were seen in mice doubly deficient for *p53* and *Ink4a* (Sharpless et al., 2002). These results would argue that the Rb pathway is involved in the development of this particular tumor type, as is the p53 pathway. As a matter of fact, when the *Cdk4*^{R24C} mouse was bred to the *p53* knockout mouse there was a shift in tumor spectrum with increased rates of sarcomas and a concomitant decrease in lymphomas (Sotillo et al., 2001). This finding is in agreement with what we observed in the cohorts of mice lacking *p53* and one or both *Ink4c* alleles. In contrast to our results, Sotillo and co-workers reported a shift to shorter survival in *Cdk4*^{R24C/R24C}; *p53*^{-/-} mice (3.3 months) compared to *Cdk4*^{+/+}; *p53*^{-/-} littermates (4.7 months). In our studies we instead observed that mice lacking *p53* and one or both copies of *Ink4c* generally lived longer. When mice, either wildtype, heterozygous or null for *Ink4c*, were given the mutagenic compound dimethylnitrosamine (DMN) in drinking water, there was an increased rate of tumorigenesis in general and of hemangiosarcomas of the liver in particular (Bai et al., 2003). The authors concluded that *Ink4c* is a haploinsufficient tumor suppressor for chemically induced tumors.

Lack of *Ink4c* increases the incidence of medulloblastomas in *p53*-null mice (Paper I)

Another striking observation from our crosses was that mice lacking *p53* and at least one allele of *Ink4c*, but retaining one copy of *Ink4d*, developed medulloblastomas to an incidence of 8-25%. Interestingly, this tumor is not seen in any of the parental strains. Medulloblastoma is a tumor of the cerebellum affecting children, and there are several mouse models of this particular tumor (Lee et al., 2003). One reason for the large interest in models of this disease is that the children suffering from these tumors have to go through surgery and radiation therapy which often are detrimental to the health and development of these patients. Therefore this and other mouse models of medulloblastomas have been followed up in a series of papers (Lee et al., 2003; Uziel et al., 2005; Zindy et al., 2007).

Due to the increased incidences of medulloblastomas in mice lacking *Ink4c* and *p53*, we analyzed the RNA expression of the *Ink4c* and *Ink4d* genes by *in situ* hybridization in the developing cerebellum from embryonic day (E) 11.5 to adult animal. These studies showed that *Ink4c* and *Ink4d* exhibited different mRNA expression patterns. In brief, *Ink4d* was expressed throughout the developing cerebellum, but became restricted to the Purkinje cell layer and to the deeper regions of the cerebellum with time. *Ink4c*, on the other hand, was detected in the pial membrane at all stages analyzed, as well as in the external granule layer (EGL) of newborn mice. The expression of *Ink4c* in the EGL is interesting because this is the region that undergoes an enormous expansion during the first two weeks after birth, and the granule cell precursors (GCPs) populating the region are believed to give rise to medulloblastomas. Recently, continued work on *Ink4c* and medulloblastoma have indicated that p18^{Ink4c} controls proliferation of the GCPs and plays a tumor suppressive role in medulloblastomas developing in mice, and that p18Ink4c expression is lost in ~20% of medulloblastomas developing in humans (Uziel et al., 2005).

Loss of *Ink4c* does not alter the onset of disease in the E μ -Myc mouse model of B cell lymphoma (Paper II)

Several studies indicate a role for *Ink4c* in differentiation of the B cell lineage. Upon careful characterization of the immune responses in mice null for *Ink4c* it became clear that they had a defect in plasma cell function (Tourigny et al., 2002). The plasma cell, which is the antibody-secreting cell, differentiates in a multistep manner including class switching and hypermutation in germinal centers, and as a last step they terminally differentiate and exit the cell cycle. It is at the last stage that they become fully active and antibody-secreting. In the

Ink4c-deleted mice, the plasma cells display problems to exit the cell cycle in the very last step of differentiation and do not terminally differentiate.

Even though mutations in *Ink4c* are uncommon, there is an overrepresentation of deletions, mutations and loss-of-expression in tumors arising in cells of B-cell origin, as for instance multiple myeloma, mantle cell lymphoma and Hodgkin's lymphoma (Kulkarni et al., 2002; Sanchez-Aguilera et al., 2004; Williams et al., 1997). To investigate further the role of *Ink4c* as a tumor suppressor in mice specifically in B cells, we generated E μ -*Myc* mice lacking one or both copies of *Ink4c*, as well as wildtype controls. The E μ -*Myc* mouse was created by Adams et al. to mimick the genetic alteration seen in human Burkitt lymphoma, a tumor of B-cell origin. Mice positive for the transgene all develop clonal pre-B-cell lymphomas, with 50% of the animals affected at 100 days after birth (Adams et al., 1985; Nilsson et al., 2005).

Despite the interesting connections between B-cell malignancies, plasma cell defects and *Ink4c*, we could conclude that loss of *Ink4c* did not alter the onset of disease in E μ -*Myc* mouse. In addition, there was no difference in location or in type of tumors; the mice developed tumors characteristic of the E μ -*Myc* mouse, namely palpable clonal pre-B-cell lymphomas locating to the lymph nodes. If *Ink4c* would have been an important tumor suppressor in this model we would have expected an acceleration of disease in mice null for *Ink4c*, as is the case for other tumor suppressors that have been tested in this model (Eischen et al., 1999; Martins and Berns, 2002; Schmitt et al., 1999). A subset of mice wildtype for *Ink4c* seemed to live longer than mice in the other two groups, but this difference was not statistically significant.

p18^{Ink4c} expression is higher in Myc-overexpressing cells and tumors in mouse and human (Paper II)

A surprising finding of ours was that p18^{Ink4c} RNA and protein exhibited elevated levels in Myc-expressing cells and tumors compared to normal B cells, in both mice and in human tumors. Upregulation of p18^{Ink4c} was in contrast to results on other tumor suppressors, for instance p27^{Kip1}, which is downregulated following Myc overexpression in this mouse model (Keller et al., 2007). However, we could not detect any changes in proliferation or apoptosis in B cells from pre-cancerous E μ -*Myc* animals with or without *Ink4c*. These results, in conjunction with high levels of *Ink4c* in tumors, indicated that p18^{Ink4c} was not efficient as a cell cycle inhibitor in this scenario. This is in agreement with previous studies of the E μ -*Myc* model, where loss of *Ink4a* or heterozygosity of *Rb* do not accelerate onset of disease

(Krimpenfort et al., 2001). One factor that might explain these results is the ability of c-Myc to override the growth-suppressive effects of Ink4 proteins (Alevizopoulos et al., 1997). If this is true also in B lymphocytes, there would be no further advantage for these cells to lose p18^{Ink4c} protein expression.

The transcription of both *Ink4c* and *Ink4d* are regulated by the E2F proteins, whereas *Ink4a* and *Ink4b* are not, separating the Ink4 family into two groups (Blais et al., 2002; DeGregori et al., 1997). E2F levels are increased following Myc overexpression (Leone et al., 1997), and this could explain the increased levels of p18^{Ink4c} mRNA and protein, at least in this cellular setting. To determine if the high levels of p18^{Ink4c} are simply a response to high levels of E2F, analyses of p18^{Ink4c} levels in Eμ-*Myc* tumors lacking *E2f1* could have been performed. These mice, on the other hand, develop tumors later than controls due to high levels of p27^{Kip1} (Baudino et al., 2003), and there may be compensatory increases of other E2F family members. Nevertheless, the question if E2F regulates p18^{Ink4c} in Eμ-*Myc* mice is of an academic nature since neither high nor low expression levels of p18^{Ink4c} impacts lymphomagenesis.

Loss of heterozygosity (LOH) at the *Ink4c* locus in B cell lymphomas of the Eμ-*Myc* mice (Paper II)

The tumors that developed in Eμ-*Myc* mice either *Ink4c*^{+/+}, *Ink4c*^{+/-} or *Ink4c*^{-/-} were all analyzed by Southern blot and Western blot. These analyses were carried out to evaluate eventual LOH in the *Ink4c* locus in tumors arising in Eμ-*Myc*; *Ink4c*^{+/-} animals, but also to study secondary genetic events that take place during tumorigenesis. When performing Southern blots for *Ink4c*, we observed LOH in 42% of the heterozygote tumors analyzed (10/24). Of ten tumors, three had become wildtype and the remaining seven had become null for *Ink4c*. This was an interesting and unexpected result since in three of ten tumors the wildtype locus had been re-established, i.e. not at all in line with how TSGs are supposed to behave. There was no significant change in survival between the mice becoming wildtype for *Ink4c* and the ones becoming null, but a larger number of mice would be needed to be sure. If *Ink4c* would behave like a haploinsufficient tumor suppressor in these tumors, the remaining wildtype allele would not be lost at all, which was the result in 50% of the tumors. One explanation for the genetic events observed lies in the genomic location of *Ink4c* in the mouse genome where it is located on chromosome 4 together with *Ink4a* and *Ink4b* and, most importantly *Arf* (Figure 6). Even though we did not observe a complete correlation between

LOH at the *Ink4c* locus and *Arf* deletions, it is likely that the gene conversion event affecting *Arf* spreads to the *Ink4c* locus.

When analyzing the secondary mutations in the tumors arising in the different cohorts of Eμ-*Myc* mice, we observed a dramatic drop in the frequency of *Arf* deletions in tumors arising in Eμ-*Myc*;*Ink4c*^{-/-} animals. This was surprising since it implicated that loss of *Arf* can be bypassed in the absence of *Ink4c*. This suggests that *Ink4c* has tumor suppressing capabilities in this mouse model after all, but that it normally is masked by the rapid onset of *Arf* deletions occurring in Eμ-*Myc* mice. Changes in the frequency *Arf* deletions have previously been reported by us in tumors arising in Eμ-*Myc* mice lacking one allele of *Odc* (Nilsson et al., 2005). However, in this setting the absence of *Arf* deletions were associated with a delay in tumorigenesis and may therefore just reflect the importance of *Arf* deletions in this tumor model.

The fact that loss of *Ink4c* did not alter the onset of disease in the Eμ-*Myc* mouse may be a function of the B cell developmental stage, where previous studies suggest a tumor suppressive role for p18^{Ink4c} in a more mature B cell compared to the pre-B-cell lymphomas in Eμ-*Myc* mice (Kulkarni et al., 2002; Sanchez-Aguilera et al., 2004; Williams et al., 1997). It would be interesting to evaluate the loss of *Ink4c* in a model where *Ink4c* has been shown to play a role for tumorigenesis.

***Xenopus laevis Ink4d* is the predominant Cdk inhibitor expressed during early embryogenesis (Paper III)**

In order to study the functions of Ink4 proteins in early vertebrate development, I turned to a model where development occurs externally. The model system used was the African clawed frog, *Xenopus laevis*. Previously two CKIs belonging to the Cip/Kip family had been reported (Shou and Dunphy, 1996; Su et al., 1995), but not any Ink4 proteins. Fortunately, degenerate oligonucleotides designed to recognize a conserved region of *Ink4*-like genes had been described (Gilley and Fried, 2001) and these were used to amplify a fragment of an exon that turned out to be from the frog homolog of *Ink4d*. This fragment was used to clone the full length cDNA, hereafter referred to as *Xl-Ink4d*. Expression of *Xl-Ink4d* was detected throughout embryo development at RNA and protein levels, as early as in the unfertilized egg. Whole-mount in situ hybridization on more developed embryos (stage 33-45) showed the strongest expression in the developing nervous system including the eyes, the spinal cord and the brain (data not shown), similar to the expression pattern of the developing mouse embryo (Zindy et al., 1997).

We successfully knocked down the expression of *Xl-Ink4d* protein by injecting antisense morpholino oligonucleotides into the early frog embryo. When injected into either one cell of 2-cell embryos or one dorsal cell of 4-cell embryos, we observed severe defects on morphology and development. Reduced *Xl-Ink4d* protein levels interfered with differentiation of cranial neural crest-derived tissues. The neural crest (NC) is a region formed from the ectoderm in the dorsal region of the neural tube, and the cells in the NC migrate and differentiate into a large number of cell types and tissues. The cranial NC specifically contributes to cartilage, bone and connective tissue of the face as well as thymic cells, odontoblasts of the tooth and cartilage of the inner ear and jaw (Le Douarin et al., 2004). In the injected embryos, we also observed reduced mRNA levels of *Twist*, a gene important for proper NC migration (Hopwood et al., 1989). The observation that *Xl-Ink4d* may be involved in NC development is not the first; increased levels of p19^{Ink4d} in NC-derived cells were present in the developing tooth of the *Msx-1* knockout mouse, and this had effects on proliferation (Han et al., 2003).

The consequences of reducing the level of *Xl-Ink4d* protein during early development would argue that it is required for development in the frog. This is contrary to studies on mice where *Ink4d* was deleted in the germ line causing only mild defects (Zindy et al., 2000). We do not know why reduction of *Xl-Ink4d* levels causes such severe effects in frog compared to mice. To try to control for specificity of the antisense morpholino oligonucleotides, a second one was designed to recognize a different part of the mRNA. The two morpholinos caused identical effects when injected, supporting their specificity.

From previous work on CKIs in the frog it is known that the expression of *Xic1* and *Kix1* is initiated at the midblastula transition (MBT) (Shou and Dunphy, 1996; Su et al., 1995), which is the time point when the embryos start zygotic transcription (Stage 8/9). During our work, we also identified the frog homolog of *Ink4b*, but this gene is not expressed until later in development, and always to a much lower level than *Xl-Ink4d*. Thus, in early development *Xl-Ink4d* is the predominant CKI, being present as a maternal transcript in the pre-MBT embryo and throughout development.

The only known function of the Ink4 proteins is to inhibit Cdk4/6:cyclin D kinase activity during the G1 phase, thereby regulating Rb phosphorylation and E2F transcriptional activity. During the cleavage stages, the majority of E2F is found free, and is not associated with Rb until later stages of development (Philpott and Friend, 1994). Accordingly, Rb has been shown to exist in its hyperphosphorylated inactive form throughout the early stages of frog development and also in mouse embryonic stem cells (Cosgrove and Philpott, 2007;

Savatier et al., 1994). The first divisions of the frog embryo are referred to as the cleavage stages, and these are characterized by only S and M phases, interchangeably. Since there is no growth of the cells, they become smaller with every cell division. The G1 and G2 phases do not appear until after MBT. It is therefore interesting that *Xl-Ink4d* is present throughout development, even at time points where there are no G1 phases. Since Rb already resides in its inactive form it is unlikely that the phenotype associated with loss of *Xl-Ink4d* is due to an increased activity of Cdk4/6 on Rb. Recently, Smad3, a key component of the TGF β signalling pathway, was identified as the first non-pocket protein substrate of Cdk4:cyclin D complexes (Matsuura et al., 2004). The inhibitory actions of the Ink4 proteins will most likely regulate Smad3 phosphorylation as well as Rb phosphorylation, even though the experiments confirming this are yet to be published. TGF β signalling via the highly homologous Smad2 is important for NC differentiation (Chai et al., 2003), and it would be interesting to find out if Cdk4:cyclin D is capable of phosphorylating both Smad2 and Smad3.

An evolutionary interest in the Ink4 proteins and the Arf tumor suppressor has the last years generated several publications describing the arrangement of this locus in lower vertebrates. Several interesting aspects of the evolution of the *Ink4a/Arf* locus have been revealed. In fish, as represented by the puffer fish *Fugu rubripes*, there are two *Ink4* genes (*CDKN2A/B* and *CDKN2D*) but no *Arf* (Gilley and Fried, 2001). In birds, represented by the hen *Gallus gallus*, there is only one complete *Ink4* gene, *Ink4b*, and an exon 2 of *Ink4a*. However, exon 1 β of *Arf* is present in this locus (Kim et al., 2003). Exon 1 β of chicken *Arf* harbours some of the known functions of *Arf*, in agreement with studies in mouse showing that all its functions reside within exon 1 β (Quelle et al., 1997). In our studies we have utilized two frog species as representative for amphibians; the pseudotetraploid *X. laevis* and the diploid relative *X. tropicalis*. The genome of *X. tropicalis* is currently being sequenced and the results are accessible to the public via www.ensembl.org. Sequence analysis in this database reveal three *Ink4* genes in this species; *Ink4b*, *Ink4c* and *Ink4d*. In the chromosomal region of *Ink4b*, we have only identified one *Ink4* gene and so far no *Arf*.

GENERAL DISCUSSION AND CONCLUDING REMARKS

The papers in this thesis are all based on *in vivo* models to further study the roles of the Ink4 proteins p18^{Ink4c} and p19^{Ink4d} in tumorigenesis and during development. The Ink4 proteins are CKIs that have the ability to stop cell proliferation by inhibiting phosphorylation of Rb. However, there are clear differences within this group as to which *Ink4* genes are mutated or deleted in human cancer; *Ink4a* being the gene most often affected. The Ink4 proteins studied here, p18^{Ink4c} and p19^{Ink4d}, are very rarely altered in human cancer. Nevertheless, the *Ink4c* knockout mice develop tumors, primarily pituitary adenomas but also testicular tumors and lymphomas (Franklin et al., 1998; Latres et al., 2000).

In mice lacking both *p53* and *Ink4c* we observed increased incidences of hemangiomas and hemangiosarcomas, as well as the appearance of medulloblastomas, indicating a tumor suppressive function of *Ink4c* in the cell of origin of these tumors. Surprisingly, in the Eμ-*Myc* model, loss of *Ink4c* did not alter the onset of disease, despite bypassing the need for *Arf* deletions, arguing that in B cells overexpressing *Myc*, p18^{Ink4c} is not active as a tumor suppressor. Nevertheless, recent publications support tumor suppressive functions of *Ink4c* in certain cell types in both mice and humans. These include diseases like medulloblastoma (Uziel et al., 2005), multiple myeloma (Kulkarni et al., 2002) and multiple endocrine neoplasia (MEN) syndrome (Bai et al., 2007). These results validate further studies of *Ink4c* and its involvement in tumor development.

Our identification of the *XI-Ink4d* is novel and exciting. We conclude that *XI-Ink4d* is the only CKI present during the early stages of frog development. All other identified CKIs in frog are first visible at mid-blastula transition (MBT), the time point when frog embryos start zygotic transcription and when the G1 and G2 phases first appear in the cell cycle. We also found that depleting the levels of *XI-Ink4d* protein during development causes defects in neural crest-derived tissues. This effect is intriguing since Ink4 proteins are only known to inhibit Cdk4 and Cdk6 from phosphorylating and thereby inactivating Rb. In the early frog development, Rb is already fully phosphorylated (Cosgrove and Philpott, 2007), and the defects observed from knocking down *XI-Ink4d* may reflect an Rb-independent function.

In summary, the conclusions of this thesis are:

1. Loss of p18^{Ink4c}, but not p19^{Ink4d}, alters the tumor spectrum in p53 knockout mice, arguing for a tumor suppressive role of Ink4c in cells causing hemangiosarcomas and medulloblastomas.
2. Loss of p18^{Ink4c} does not affect onset of B cell lymphoma development in the E μ -*Myc* transgenic mouse, but alters the secondary mutations occurring during tumorigenesis.
3. The frog homolog of p19^{Ink4d}, *XI-Ink4d*, is the only CKI expressed during early frog development. Depletion of *XI-Ink4d* using antisense morpholino oligonucleotides causes defects in neural crest-derived tissues as well as reduced expression of *Twist*, a gene important for neural crest migration.

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REFERENCES

- Adams, J. M., Harris, A. W., Pinkert, C. A., Corcoran, L. M., Alexander, W. S., Cory, S., Palmiter, R. D., and Brinster, R. L. (1985). The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature* 318, 533-538.
- Alevizopoulos, K., Vlach, J., Hennecke, S., and Amati, B. (1997). Cyclin E and c-Myc promote cell proliferation in the presence of p16INK4a and of hypophosphorylated retinoblastoma family proteins. *Embo J* 16, 5322-5333.
- Ayer, D. E., Kretzner, L., and Eisenman, R. N. (1993). Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. *Cell* 72, 211-222.
- Ayer, D. E., Laherty, C. D., Lawrence, Q. A., Armstrong, A. P., and Eisenman, R. N. (1996). Mad proteins contain a dominant transcription repression domain. *Mol Cell Biol* 16, 5772-5781.
- Ayer, D. E., Lawrence, Q. A., and Eisenman, R. N. (1995). Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. *Cell* 80, 767-776.
- Bagui, T. K., Jackson, R. J., Agrawal, D., and Pledger, W. J. (2000). Analysis of cyclin D3-cdk4 complexes in fibroblasts expressing and lacking p27(kip1) and p21(cip1). *Mol Cell Biol* 20, 8748-8757.
- Bai, F., Pei, X. H., Godfrey, V. L., and Xiong, Y. (2003). Haploinsufficiency of p18INK4c sensitizes mice to carcinogen-induced tumorigenesis. *Mol Cell Biol* 23, 1269-1277.
- Bai, F., Pei, X. H., Nishikawa, T., Smith, M. D., and Xiong, Y. (2007). p18Ink4c, but not p27Kip1, collaborates with Men1 to suppress neuroendocrine organ tumors. *Mol Cell Biol* 27, 1495-1504.
- Baudino, T. A., Maclean, K. H., Brennan, J., Parganas, E., Yang, C., Aslanian, A., Lees, J. A., Sherr, C. J., Roussel, M. F., and Cleveland, J. L. (2003). Myc-mediated proliferation and lymphomagenesis, but not apoptosis, are compromised by E2f1 loss. *Mol Cell* 11, 905-914.
- Berthet, C., Aleem, E., Coppola, V., Tessarollo, L., and Kaldis, P. (2003). Cdk2 knockout mice are viable. *Curr Biol* 13, 1775-1785.
- Berthet, C., Klarmann, K. D., Hilton, M. B., Suh, H. C., Keller, J. R., Kiyokawa, H., and Kaldis, P. (2006). Combined loss of Cdk2 and Cdk4 results in embryonic lethality and Rb hypophosphorylation. *Dev Cell* 10, 563-573.
- Blackwell, T. K., Huang, J., Ma, A., Kretzner, L., Alt, F. W., Eisenman, R. N., and Weintraub, H. (1993). Binding of myc proteins to canonical and noncanonical DNA sequences. *Mol Cell Biol* 13, 5216-5224.
- Blackwell, T. K., Kretzner, L., Blackwood, E. M., Eisenman, R. N., and Weintraub, H. (1990). Sequence-specific DNA binding by the c-Myc protein. *Science* 250, 1149-1151.
- Blackwood, E. M., and Eisenman, R. N. (1991). Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science* 251, 1211-1217.
- Blackwood, E. M., Luscher, B., and Eisenman, R. N. (1992). Myc and Max associate in vivo. *Genes Dev* 6, 71-80.
- Blais, A., Monte, D., Pouliot, F., and Labrie, C. (2002). Regulation of the human cyclin-dependent kinase inhibitor p18INK4c by the transcription factors E2F1 and Sp1. *J Biol Chem* 277, 31679-31693.
- Chai, Y., Ito, Y., and Han, J. (2003). TGF-beta signaling and its functional significance in regulating the fate of cranial neural crest cells. *Crit Rev Oral Biol Med* 14, 78-88.

- Chan, F. K., Zhang, J., Cheng, L., Shapiro, D. N., and Winoto, A. (1995). Identification of human and mouse p19, a novel CDK4 and CDK6 inhibitor with homology to p16ink4. *Mol Cell Biol* 15, 2682-2688.
- Chen, P., Zindy, F., Abdala, C., Liu, F., Li, X., Roussel, M. F., and Segil, N. (2003). Progressive hearing loss in mice lacking the cyclin-dependent kinase inhibitor Ink4d. *Nat Cell Biol* 5, 422-426.
- Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. (1999). The p21(Cip1) and p27(Kip1) CDK 'inhibitors' are essential activators of cyclin D-dependent kinases in murine fibroblasts. *Embo J* 18, 1571-1583.
- Christophorou, M. A., Martin-Zanca, D., Soucek, L., Lawlor, E. R., Brown-Swigart, L., Verschuren, E. W., and Evan, G. I. (2005). Temporal dissection of p53 function in vitro and in vivo. *Nat Genet* 37, 718-726.
- Ciemerych, M. A., Kenney, A. M., Sicinska, E., Kalaszczynska, I., Bronson, R. T., Rowitch, D. H., Gardner, H., and Sicinski, P. (2002). Development of mice expressing a single D-type cyclin. *Genes Dev* 16, 3277-3289.
- Classon, M., and Harlow, E. (2002). The retinoblastoma tumour suppressor in development and cancer. *Nat Rev Cancer* 2, 910-917.
- Cosgrove, R. A., and Philpott, A. (2007). Cell cycling and differentiation do not require the retinoblastoma protein during early *Xenopus* development. *Dev Biol* 303, 311-324.
- Damo, L. A., Snyder, P. W., and Franklin, D. S. (2005). Tumorigenesis in p27/p53- and p18/p53-double null mice: functional collaboration between the pRb and p53 pathways. *Mol Carcinog* 42, 109-120.
- DeCaprio, J. A., Ludlow, J. W., Figge, J., Shew, J. Y., Huang, C. M., Lee, W. H., Marsilio, E., Paucha, E., and Livingston, D. M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* 54, 275-283.
- DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc Natl Acad Sci U S A* 94, 7245-7250.
- Donehower, L. A., Harvey, M., Slagle, B. L., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., and Bradley, A. (1992). Mice deficient for p53 are developmentally normal but susceptible to spontaneous tumours. *Nature* 356, 215-221.
- Eischen, C. M., Weber, J. D., Roussel, M. F., Sherr, C. J., and Cleveland, J. L. (1999). Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev* 13, 2658-2669.
- Eischen, C. M., Woo, D., Roussel, M. F., and Cleveland, J. L. (2001). Apoptosis triggered by Myc-induced suppression of Bcl-X(L) or Bcl-2 is bypassed during lymphomagenesis. *Mol Cell Biol* 21, 5063-5070.
- Fantl, V., Stamp, G., Andrews, A., Rosewell, I., and Dickson, C. (1995). Mice lacking cyclin D1 are small and show defects in eye and mammary gland development. *Genes Dev* 9, 2364-2372.
- Fero, M. L., Randel, E., Gurley, K. E., Roberts, J. M., and Kemp, C. J. (1998). The murine gene p27Kip1 is haplo-insufficient for tumour suppression. *Nature* 396, 177-180.
- Franklin, D. S., Godfrey, V. L., Lee, H., Kovalev, G. I., Schoonhoven, R., Chen-Kiang, S., Su, L., and Xiong, Y. (1998). CDK inhibitors p18(INK4c) and p27(Kip1) mediate two separate pathways to collaboratively suppress pituitary tumorigenesis. *Genes Dev* 12, 2899-2911.
- Franklin, D. S., and Xiong, Y. (1996). Induction of p18INK4c and its predominant association with CDK4 and CDK6 during myogenic differentiation. *Mol Biol Cell* 7, 1587-1599.

- Geng, Y., Yu, Q., Sicinska, E., Das, M., Schneider, J. E., Bhattacharya, S., Rideout, W. M., Bronson, R. T., Gardner, H., and Sicinski, P. (2003). Cyclin E ablation in the mouse. *Cell* 114, 431-443.
- Giaccia, A. J., and Kastan, M. B. (1998). The complexity of p53 modulation: emerging patterns from divergent signals. *Genes Dev* 12, 2973-2983.
- Gilley, J., and Fried, M. (2001). One INK4 gene and no ARF at the Fugu equivalent of the human INK4A/ARF/INK4B tumour suppressor locus. *Oncogene* 20, 7447-7452.
- Goodrich, D. W. (2006). The retinoblastoma tumor-suppressor gene, the exception that proves the rule. *Oncogene* 25, 5233-5243.
- Guan, K. L., Jenkins, C. W., Li, Y., Nichols, M. A., Wu, X., O'Keefe, C. L., Matera, A. G., and Xiong, Y. (1994). Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes Dev* 8, 2939-2952.
- Han, J., Ito, Y., Yeo, J. Y., Sucov, H. M., Maas, R., and Chai, Y. (2003). Cranial neural crest-derived mesenchymal proliferation is regulated by Msx1-mediated p19(INK4d) expression during odontogenesis. *Dev Biol* 261, 183-196.
- Hanahan, D., and Weinberg, R. A. (2000). The hallmarks of cancer. *Cell* 100, 57-70.
- Hannon, G. J., and Beach, D. (1994). p15INK4B is a potential effector of TGF-beta-induced cell cycle arrest. *Nature* 371, 257-261.
- Harvey, M., McArthur, M. J., Montgomery, C. A., Jr., Bradley, A., and Donehower, L. A. (1993). Genetic background alters the spectrum of tumors that develop in p53-deficient mice. *Faseb J* 7, 938-943.
- Hirai, H., Roussel, M. F., Kato, J. Y., Ashmun, R. A., and Sherr, C. J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Mol Cell Biol* 15, 2672-2681.
- Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991). p53 mutations in human cancers. *Science* 253, 49-53.
- Honda, R., and Yasuda, H. (1999). Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *Embo J* 18, 22-27.
- Hopwood, N. D., Pluck, A., and Gurdon, J. B. (1989). A *Xenopus* mRNA related to *Drosophila* twist is expressed in response to induction in the mesoderm and the neural crest. *Cell* 59, 893-903.
- Huard, J. M., Forster, C. C., Carter, M. L., Sicinski, P., and Ross, M. E. (1999). Cerebellar histogenesis is disturbed in mice lacking cyclin D2. *Development* 126, 1927-1935.
- Hurlin, P. J., Queva, C., and Eisenman, R. N. (1997). Mnt, a novel Max-interacting protein is coexpressed with Myc in proliferating cells and mediates repression at Myc binding sites. *Genes Dev* 11, 44-58.
- Hurlin, P. J., Queva, C., Koskinen, P. J., Steingrimsson, E., Ayer, D. E., Copeland, N. G., Jenkins, N. A., and Eisenman, R. N. (1995). Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. *Embo J* 14, 5646-5659.
- Jacks, T., Remington, L., Williams, B. O., Schmitt, E. M., Halachmi, S., Bronson, R. T., and Weinberg, R. A. (1994). Tumor spectrum analysis in p53-mutant mice. *Curr Biol* 4, 1-7.
- Jeffrey, P. D., Tong, L., and Pavletich, N. P. (2000). Structural basis of inhibition of CDK-cyclin complexes by INK4 inhibitors. *Genes Dev* 14, 3115-3125.
- Kaldis, P. (1999). The cdk-activating kinase (CAK): from yeast to mammals. *Cell Mol Life Sci* 55, 284-296.
- Kamb, A., Shattuck-Eidens, D., Eeles, R., Liu, Q., Gruis, N. A., Ding, W., Hussey, C., Tran, T., Miki, Y., Weaver-Feldhaus, J., and et al. (1994). Analysis of the p16 gene

- (CDKN2) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nat Genet* 8, 23-26.
- Kato, J. Y., Matsuoka, M., Strom, D. K., and Sherr, C. J. (1994). Regulation of cyclin D-dependent kinase 4 (cdk4) by cdk4-activating kinase. *Mol Cell Biol* 14, 2713-2721.
- Keller, U. B., Old, J. B., Dorsey, F. C., Nilsson, J. A., Nilsson, L., MacLean, K. H., Chung, L., Yang, C., Spruck, C., Boyd, K., *et al.* (2007). Myc targets Cks1 to provoke the suppression of p27Kip1, proliferation and lymphomagenesis. *Embo J* 26, 2562-2574.
- Kim, S. H., Mitchell, M., Fujii, H., Llanos, S., and Peters, G. (2003). Absence of p16INK4a and truncation of ARF tumor suppressors in chickens. *Proc Natl Acad Sci U S A* 100, 211-216.
- Knudson, A. G., Jr. (1971). Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci U S A* 68, 820-823.
- Kozar, K., Ciemerych, M. A., Rebel, V. I., Shigematsu, H., Zagozdzon, A., Sicinska, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R. T., *et al.* (2004). Mouse development and cell proliferation in the absence of D-cyclins. *Cell* 118, 477-491.
- Krimpenfort, P., Ijpenberg, A., Song, J. Y., van der Valk, M., Nawijn, M., Zevenhoven, J., and Berns, A. (2007). p15(Ink4b) is a critical tumour suppressor in the absence of p16(Ink4a). *Nature* 448, 943-946.
- Krimpenfort, P., Quon, K. C., Mooi, W. J., Loonstra, A., and Berns, A. (2001). Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. *Nature* 413, 83-86.
- Kulkarni, M. S., Daggett, J. L., Bender, T. P., Kuehl, W. M., Bergsagel, P. L., and Williams, M. E. (2002). Frequent inactivation of the cyclin-dependent kinase inhibitor p18 by homozygous deletion in multiple myeloma cell lines: ectopic p18 expression inhibits growth and induces apoptosis. *Leukemia* 16, 127-134.
- Lam, E. W., Glassford, J., Banerji, L., Thomas, N. S., Sicinski, P., and Klaus, G. G. (2000). Cyclin D3 compensates for loss of cyclin D2 in mouse B-lymphocytes activated via the antigen receptor and CD40. *J Biol Chem* 275, 3479-3484.
- Lang, G. A., Iwakuma, T., Suh, Y. A., Liu, G., Rao, V. A., Parant, J. M., Valentin-Vega, Y. A., Terzian, T., Caldwell, L. C., Strong, L. C., *et al.* (2004). Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell* 119, 861-872.
- Latres, E., Malumbres, M., Sotillo, R., Martin, J., Ortega, S., Martin-Caballero, J., Flores, J. M., Cordon-Cardo, C., and Barbacid, M. (2000). Limited overlapping roles of P15(INK4b) and P18(INK4c) cell cycle inhibitors in proliferation and tumorigenesis. *Embo J* 19, 3496-3506.
- Lavigne, A., Maltby, V., Mock, D., Rossant, J., Pawson, T., and Bernstein, A. (1989). High incidence of lung, bone, and lymphoid tumors in transgenic mice overexpressing mutant alleles of the p53 oncogene. *Mol Cell Biol* 9, 3982-3991.
- Le Douarin, N. M., Creuzet, S., Couly, G., and Dupin, E. (2004). Neural crest cell plasticity and its limits. *Development* 131, 4637-4650.
- Lee, Y., Miller, H. L., Jensen, P., Hernan, R., Connelly, M., Wetmore, C., Zindy, F., Roussel, M. F., Curran, T., Gilbertson, R. J., and McKinnon, P. J. (2003). A molecular fingerprint for medulloblastoma. *Cancer Res* 63, 5428-5437.
- Leone, G., DeGregori, J., Sears, R., Jakoi, L., and Nevins, J. R. (1997). Myc and Ras collaborate in inducing accumulation of active cyclin E/Cdk2 and E2F. *Nature* 387, 422-426.
- Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* 88, 323-331.
- Lowe, S. W., and Sherr, C. J. (2003). Tumor suppression by Ink4a-Arf: progress and puzzles. *Curr Opin Genet Dev* 13, 77-83.

- Ludlow, J. W., Glendening, C. L., Livingston, D. M., and DeCarprio, J. A. (1993). Specific enzymatic dephosphorylation of the retinoblastoma protein. *Mol Cell Biol* 13, 367-372.
- Lukas, J., Lukas, C., and Bartek, J. (2004). Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time. *DNA Repair (Amst)* 3, 997-1007.
- Malkin, D., Li, F. P., Strong, L. C., Fraumeni, J. F., Jr., Nelson, C. E., Kim, D. H., Kassel, J., Gryka, M. A., Bischoff, F. Z., Tainsky, M. A., and et al. (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. *Science* 250, 1233-1238.
- Malumbres, M., and Barbacid, M. (2001). To cycle or not to cycle: a critical decision in cancer. *Nat Rev Cancer* 1, 222-231.
- Malumbres, M., and Barbacid, M. (2005). Mammalian cyclin-dependent kinases. *Trends Biochem Sci* 30, 630-641.
- Malumbres, M., Sotillo, R., Santamaria, D., Galan, J., Cerezo, A., Ortega, S., Dubus, P., and Barbacid, M. (2004). Mammalian cells cycle without the D-type cyclin-dependent kinases Cdk4 and Cdk6. *Cell* 118, 493-504.
- Martins, C. P., and Berns, A. (2002). Loss of p27(Kip1) but not p21(Cip1) decreases survival and synergizes with MYC in murine lymphomagenesis. *Embo J* 21, 3739-3748.
- Matsuura, I., Denissova, N. G., Wang, G., He, D., Long, J., and Liu, F. (2004). Cyclin-dependent kinases regulate the antiproliferative function of Smads. *Nature* 430, 226-231.
- McGowan, C. H., and Russell, P. (1993). Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. *Embo J* 12, 75-85.
- Moons, D. S., Jirawatnotai, S., Tsutsui, T., Franks, R., Parlow, A. F., Hales, D. B., Gibori, G., Fazleabas, A. T., and Kiyokawa, H. (2002). Intact follicular maturation and defective luteal function in mice deficient for cyclin- dependent kinase-4. *Endocrinology* 143, 647-654.
- Morgan, D. O. (1996). The dynamics of cyclin dependent kinase structure. *Curr Opin Cell Biol* 8, 767-772.
- Morgan, D. O. (2007). *The cell cycle, principles of control*: New Science Press.
- Morse, L., Chen, D., Franklin, D., Xiong, Y., and Chen-Kiang, S. (1997). Induction of cell cycle arrest and B cell terminal differentiation by CDK inhibitor p18(INK4c) and IL-6. *Immunity* 6, 47-56.
- Mueller, P. R., Coleman, T. R., Kumagai, A., and Dunphy, W. G. (1995). Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. *Science* 270, 86-90.
- Munger, K., Werness, B. A., Dyson, N., Phelps, W. C., Harlow, E., and Howley, P. M. (1989). Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *Embo J* 8, 4099-4105.
- Musacchio, A., and Salmon, E. D. (2007). The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* 8, 379-393.
- Nilsson, J. A., and Cleveland, J. L. (2003). Myc pathways provoking cell suicide and cancer. *Oncogene* 22, 9007-9021.
- Nilsson, J. A., Keller, U. B., Baudino, T. A., Yang, C., Norton, S., Old, J. A., Nilsson, L. M., Neale, G., Kramer, D. L., Porter, C. W., and Cleveland, J. L. (2005). Targeting ornithine decarboxylase in Myc-induced lymphomagenesis prevents tumor formation. *Cancer Cell* 7, 433-444.
- Obaya, A. J., and Sedivy, J. M. (2002). Regulation of cyclin-Cdk activity in mammalian cells. *Cell Mol Life Sci* 59, 126-142.

- Olive, K. P., Tuveson, D. A., Ruhe, Z. C., Yin, B., Willis, N. A., Bronson, R. T., Crowley, D., and Jacks, T. (2004). Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* 119, 847-860.
- Ortega, S., Malumbres, M., and Barbacid, M. (2002). Cyclin D-dependent kinases, INK4 inhibitors and cancer. *Biochim Biophys Acta* 1602, 73-87.
- Ortega, S., Prieto, I., Odajima, J., Martin, A., Dubus, P., Sotillo, R., Barbero, J. L., Malumbres, M., and Barbacid, M. (2003). Cyclin-dependent kinase 2 is essential for meiosis but not for mitotic cell division in mice. *Nat Genet* 35, 25-31.
- Pardee, A. B. (1974). A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci U S A* 71, 1286-1290.
- Parisi, T., Beck, A. R., Rougier, N., McNeil, T., Lucian, L., Werb, Z., and Amati, B. (2003). Cyclins E1 and E2 are required for endoreplication in placental trophoblast giant cells. *Embo J* 22, 4794-4803.
- Pavletich, N. P. (1999). Mechanisms of cyclin-dependent kinase regulation: structures of Cdk, their cyclin activators, and Cip and INK4 inhibitors. *J Mol Biol* 287, 821-828.
- Phelps, D. E., Hsiao, K. M., Li, Y., Hu, N., Franklin, D. S., Westphal, E., Lee, E. Y., and Xiong, Y. (1998). Coupled transcriptional and translational control of cyclin-dependent kinase inhibitor p18INK4c expression during myogenesis. *Mol Cell Biol* 18, 2334-2343.
- Philpott, A., and Friend, S. H. (1994). E2F and its developmental regulation in *Xenopus laevis*. *Mol Cell Biol* 14, 5000-5009.
- Quelle, D. E., Cheng, M., Ashmun, R. A., and Sherr, C. J. (1997). Cancer-associated mutations at the INK4a locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. *Proc Natl Acad Sci U S A* 94, 669-673.
- Quelle, D. E., Zindy, F., Ashmun, R. A., and Sherr, C. J. (1995). Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 83, 993-1000.
- Ramsey, M. R., Krishnamurthy, J., Pei, X. H., Torrice, C., Lin, W., Carrasco, D. R., Ligon, K. L., Xiong, Y., and Sharpless, N. E. (2007). Expression of p16Ink4a compensates for p18Ink4c loss in cyclin-dependent kinase 4/6-dependent tumors and tissues. *Cancer Res* 67, 4732-4741.
- Rane, S. G., Dubus, P., Mettus, R. V., Galbreath, E. J., Boden, G., Reddy, E. P., and Barbacid, M. (1999). Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. *Nat Genet* 22, 44-52.
- Rhind, N., Furnari, B., and Russell, P. (1997). Cdc2 tyrosine phosphorylation is required for the DNA damage checkpoint in fission yeast. *Genes Dev* 11, 504-511.
- Ruas, M., and Peters, G. (1998). The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1378, F115-177.
- Sanchez-Aguilera, A., Delgado, J., Camacho, F. I., Sanchez-Beato, M., Sanchez, L., Montalban, C., Fresno, M. F., Martin, C., Piris, M. A., and Garcia, J. F. (2004). Silencing of the p18INK4c gene by promoter hypermethylation in Reed-Sternberg cells in Hodgkin lymphomas. *Blood* 103, 2351-2357.
- Santamaria, D., Barriere, C., Cerqueira, A., Hunt, S., Tardy, C., Newton, K., Caceres, J. F., Dubus, P., Malumbres, M., and Barbacid, M. (2007). Cdk1 is sufficient to drive the mammalian cell cycle. *Nature* 448, 811-815.
- Savatier, P., Huang, S., Szekely, L., Wiman, K. G., and Samarut, J. (1994). Contrasting patterns of retinoblastoma protein expression in mouse embryonic stem cells and embryonic fibroblasts. *Oncogene* 9, 809-818.

- Schmitt, C. A., McCurrach, M. E., de Stanchina, E., Wallace-Brodeur, R. R., and Lowe, S. W. (1999). INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev* 13, 2670-2677.
- Sellers, W. R., and Kaelin, W. G., Jr. (1997). Role of the retinoblastoma protein in the pathogenesis of human cancer. *J Clin Oncol* 15, 3301-3312.
- Serrano, M., Hannon, G. J., and Beach, D. (1993). A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 366, 704-707.
- Sgambato, A., Cittadini, A., Faraglia, B., and Weinstein, I. B. (2000). Multiple functions of p27(Kip1) and its alterations in tumor cells: a review. *J Cell Physiol* 183, 18-27.
- Sharpless, N. E., Alson, S., Chan, S., Silver, D. P., Castrillon, D. H., and DePinho, R. A. (2002). p16(INK4a) and p53 deficiency cooperate in tumorigenesis. *Cancer Res* 62, 2761-2765.
- Sharpless, N. E., Bardeesy, N., Lee, K. H., Carrasco, D., Castrillon, D. H., Aguirre, A. J., Wu, E. A., Horner, J. W., and DePinho, R. A. (2001). Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature* 413, 86-91.
- Sheiness, D., and Bishop, J. M. (1979). DNA and RNA from uninfected vertebrate cells contain nucleotide sequences related to the putative transforming gene of avian myelocytomatosis virus. *J Virol* 31, 514-521.
- Sheiness, D., Fanshier, L., and Bishop, J. M. (1978). Identification of nucleotide sequences which may encode the oncogenic capacity of avian retrovirus MC29. *J Virol* 28, 600-610.
- Sherr, C. J. (1996). Cancer cell cycles. *Science* 274, 1672-1677.
- Sherr, C. J. (2001). The INK4a/ARF network in tumour suppression. *Nat Rev Mol Cell Biol* 2, 731-737.
- Sherr, C. J., and McCormick, F. (2002). The RB and p53 pathways in cancer. *Cancer Cell* 2, 103-112.
- Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev* 13, 1501-1512.
- Shou, W., and Dunphy, W. G. (1996). Cell cycle control by Xenopus p28Kix1, a developmentally regulated inhibitor of cyclin-dependent kinases. *Mol Biol Cell* 7, 457-469.
- Sicinska, E., Aifantis, I., Le Cam, L., Swat, W., Borowski, C., Yu, Q., Ferrando, A. A., Levin, S. D., Geng, Y., von Boehmer, H., and Sicinski, P. (2003). Requirement for cyclin D3 in lymphocyte development and T cell leukemias. *Cancer Cell* 4, 451-461.
- Sicinski, P., Donaher, J. L., Geng, Y., Parker, S. B., Gardner, H., Park, M. Y., Robker, R. L., Richards, J. S., McGinnis, L. K., Biggers, J. D., *et al.* (1996). Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 384, 470-474.
- Sicinski, P., Donaher, J. L., Parker, S. B., Li, T., Fazeli, A., Gardner, H., Haslam, S. Z., Bronson, R. T., Elledge, S. J., and Weinberg, R. A. (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* 82, 621-630.
- Slingerland, J., and Pagano, M. (2000). Regulation of the cdk inhibitor p27 and its deregulation in cancer. *J Cell Physiol* 183, 10-17.
- Sotillo, R., Dubus, P., Martin, J., de la Cueva, E., Ortega, S., Malumbres, M., and Barbacid, M. (2001). Wide spectrum of tumors in knock-in mice carrying a Cdk4 protein insensitive to INK4 inhibitors. *Embo J* 20, 6637-6647.
- Su, J. Y., Rempel, R. E., Erikson, E., and Maller, J. L. (1995). Cloning and characterization of the Xenopus cyclin-dependent kinase inhibitor p27XIC1. *Proc Natl Acad Sci U S A* 92, 10187-10191.

- Sugimoto, M., Martin, N., Wilks, D. P., Tamai, K., Huot, T. J., Pantoja, C., Okumura, K., Serrano, M., and Hara, E. (2002). Activation of cyclin D1-kinase in murine fibroblasts lacking both p21(Cip1) and p27(Kip1). *Oncogene* 21, 8067-8074.
- Terada, Y., Tatsuka, M., Jinno, S., and Okayama, H. (1995). Requirement for tyrosine phosphorylation of Cdk4 in G1 arrest induced by ultraviolet irradiation. *Nature* 376, 358-362.
- Tischfield, J. A. (1997). Loss of heterozygosity or: how I learned to stop worrying and love mitotic recombination. *Am J Hum Genet* 61, 995-999.
- Tourigny, M. R., Ursini-Siegel, J., Lee, H., Toellner, K. M., Cunningham, A. F., Franklin, D. S., Ely, S., Chen, M., Qin, X. F., Xiong, Y., *et al.* (2002). CDK inhibitor p18(INK4c) is required for the generation of functional plasma cells. *Immunity* 17, 179-189.
- Tsutsui, T., Hesabi, B., Moons, D. S., Pandolfi, P. P., Hansel, K. S., Koff, A., and Kiyokawa, H. (1999). Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. *Mol Cell Biol* 19, 7011-7019.
- Uziel, T., Zindy, F., Xie, S., Lee, Y., Forget, A., Magdaleno, S., Rehg, J. E., Calabrese, C., Solecki, D., Eberhart, C. G., *et al.* (2005). The tumor suppressors Ink4c and p53 collaborate independently with Patched to suppress medulloblastoma formation. *Genes Dev* 19, 2656-2667.
- Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. *Nat Cell Biol* 1, 20-26.
- Weinberg, R. A. (2006). *The biology of cancer*: Garland Science.
- Ventura, A., Kirsch, D. G., McLaughlin, M. E., Tuveson, D. A., Grimm, J., Lintault, L., Newman, J., Reczek, E. E., Weissleder, R., and Jacks, T. (2007). Restoration of p53 function leads to tumour regression in vivo. *Nature* 445, 661-665.
- Whyte, P., Buchkovich, K. J., Horowitz, J. M., Friend, S. H., Raybuck, M., Weinberg, R. A., and Harlow, E. (1988). Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* 334, 124-129.
- Williams, M. E., Whitefield, M., and Swerdlow, S. H. (1997). Analysis of the cyclin-dependent kinase inhibitors p18 and p19 in mantle-cell lymphoma and chronic lymphocytic leukemia. *Ann Oncol* 8 Suppl 2, 71-73.
- Vlach, J., Hennecke, S., Alevizopoulos, K., Conti, D., and Amati, B. (1996). Growth arrest by the cyclin-dependent kinase inhibitor p27Kip1 is abrogated by c-Myc. *Embo J* 15, 6595-6604.
- Vogelstein, B., and Kinzler, K. W. (2004). Cancer genes and the pathways they control. *Nat Med* 10, 789-799.
- Wolfel, T., Hauer, M., Schneider, J., Serrano, M., Wolfel, C., Klehmann-Hieb, E., De Plaen, E., Hankeln, T., Meyer zum Buschenfelde, K. H., and Beach, D. (1995). A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. *Science* 269, 1281-1284.
- Yu, Q., Ciernerych, M. A., and Sicinski, P. (2005). Ras and Myc can drive oncogenic cell proliferation through individual D-cyclins. *Oncogene* 24, 7114-7119.
- Zarkowska, T., and Mitnacht, S. (1997). Differential phosphorylation of the retinoblastoma protein by G1/S cyclin-dependent kinases. *J Biol Chem* 272, 12738-12746.
- Zindy, F., den Besten, W., Chen, B., Rehg, J. E., Latres, E., Barbacid, M., Pollard, J. W., Sherr, C. J., Cohen, P. E., and Roussel, M. F. (2001). Control of spermatogenesis in mice by the cyclin D-dependent kinase inhibitors p18(Ink4c) and p19(Ink4d). *Mol Cell Biol* 21, 3244-3255.

- Zindy, F., Eischen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J., and Roussel, M. F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev* 12, 2424-2433.
- Zindy, F., Soares, H., Herzog, K. H., Morgan, J., Sherr, C. J., and Roussel, M. F. (1997). Expression of INK4 inhibitors of cyclin D-dependent kinases during mouse brain development. *Cell Growth Differ* 8, 1139-1150.
- Zindy, F., Uziel, T., Ayrault, O., Calabrese, C., Valentine, M., Rehg, J. E., Gilbertson, R. J., Sherr, C. J., and Roussel, M. F. (2007). Genetic alterations in mouse medulloblastomas and generation of tumors de novo from primary cerebellar granule neuron precursors. *Cancer Res* 67, 2676-2684.
- Zindy, F., van Deursen, J., Grosveld, G., Sherr, C. J., and Roussel, M. F. (2000). INK4d-deficient mice are fertile despite testicular atrophy. *Mol Cell Biol* 20, 372-378.
- Zou, X., Ray, D., Aziyu, A., Christov, K., Boiko, A. D., Gudkov, A. V., and Kiyokawa, H. (2002). Cdk4 disruption renders primary mouse cells resistant to oncogenic transformation, leading to Arf/p53-independent senescence. *Genes Dev* 16, 2923-2934.
- Zuo, L., Weger, J., Yang, Q., Goldstein, A. M., Tucker, M. A., Walker, G. J., Hayward, N., and Dracopoli, N. C. (1996). Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nat Genet* 12, 97-99.

APPENDIX

PAPERS I-III