CELL CYCLE AND CANCER: THE ROLE OF CYCLIN DEPENDENT KINASES IN TUMOURIGENESIS

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By

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1.Abstract

Most human cancers carry mutations in cell cycle regulators that result in deregulated Cdk activity, which can either be amplification of the *cyclins*, elimination of the Cdk inhibitors or mutations in the *Cdks*. These modifications have a high prognostic value. Cdk2 activity has been shown to be upregulated in different kind of tumours (mammary and prostate carcinomas, and lymphomas) due to the mutation of its regulators, $p27^{Kip1}$ and *cyclin E*; and this alteration has a high prognostic value. Moreover, an insensible INK4 point mutation in *Cdk4* has been described in human melanomas. To evaluate the importance of Cdks in neoplastic development, the loci encoding *Cdk4, Cdk6* and *Cdk2* were ablated to study the effect of Cdk deficiency in tumour development. To this end, the corresponding *Cdk* knock out mice were crossed with the $K\text{-}Ras^{+/LSLG12V};RERT^{ert/ert}$ strain that carries an endogenous K-Ras oncogene whose expression is dependent on Cre-mediated recombination. Postnatal expression of this oncogene leads to the development of lung adenomas and adenocarcinomas. Primary MEFs derived from $K\text{-}Ras^{+/LSLG12V};RERT^{ert/ert}$ embryos lacking either Cdk4, Cdk6 or Cdk2 displayed decreased proliferation in culture and prevented the growth in low serum condition. However, no obvious differences were detected in immortal MEFs regardless of the missing Cdk. In vivo, $Cdk6^{-/-};K\text{-}ras^{+/LSLG12V};RERT^{ert/ert}$ and $Cdk2^{-/-};K\text{-}ras^{+/LSLG12V};RERT^{ert/ert}$ developed the same number of tumours and with the same latency as the parental strain carrying the full complement of Cdk activity. In contrast, $Cdk4^{-/-};K\text{-}ras^{+/LSLG12V};RERT^{ert/ert}$ mice displayed a significant decrease in the number of adenomas as well as a delay in their progression

into adenocarcinomas. In addition, the elimination of *Cdk4* after tumour formation results in an important decrease of the tumours. These results, along with those observed in other tumour systems, suggest that Cdk4, but not Cdk2 and Cdk6, may be a target of potential therapeutic interest, at least in K-Ras^{G12V} driven lung adenocarcinomas.

To Borja and my parents

2.Acknowledgments

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6.Abbreviations

4-OHT	4-Hydroxy Tamoxifen
A	Aliphatic amino acid
AT-2	Alveolar type -2
ATP	Adenosine triphosphate
BASCs	Bronchi Alveolar Stem Cells
BRCA	Breast Cancer
C	Carbon
CAK	CDK Activating Kinase complex
CC10	Clara cell-specific marker
CCSP	Calra cell secretory protein
Cdk	Cyclin dependent kinase
C-terminal	Carboxi-terminal
cyc	Cyclin
Cys	Cystein
D	Aspartic acid
DBACR	Debenz(c,h)acridine
DKO	Double Knock out
dl	Decilitre
DMBA	Dimetil-benzantrocene
E	Embryonic day
ER	Estrogen Receptor
ERK	Extracellular signal regulated kinases
ERT2	Estrogen receptor transgene modified 2
ES	Embryonic stem cells
EtOH	Ethanol
G	Glycine
g	Gram
G0 phase	Quiescence
G1 phase	Gap 1 phase
G2 phase	Gap 2 phase
GDP	Guanosine biphosphate
GF	Growth factors
GFP	Green Flourecent Protein
GSK-3b	Glycogen synthase kianse 3b
GTP	Guanosine triphosphate
HDAC	Histonadeacetilases
HOAAF	N-hidroxi-2-acetil-aminofluoride
HODE	1-hidroxi-2-3-dihidroestragol
H-ras	Harvey-ras
HSC	Haematopoietic Stem Cell
Icmt	Isoprenylcysteine carboxy methyltransferase
IRES	Internal Ribosome Entry
iRNA	interference RNA
Kb	kilobases
KD	Kinase Dead
KDa	Kilodaltons
KI	Knock in

KO	Knock out
K-ras	Kirsten-ras
LOH	Loss of Heterozygosity
LTR	Long Terminal Repeat
M phase	Mitosis phase
MCA	3-metil-colantrene
MCM	Mini chromosome maintenance
MEFs	Mouse Embryonic Fibroblasts
MEK	MAP-ERK kinase
Met	Methionine
mg	Milligram
min	Minutes
mm	Millimetres
MMTV	Mouse mammary tumour virus
MNU	Nitroso-methylurea
moi	Multiplicity of infection
NfkB	Nuclear factor kB
NFR	Nuclear Fast Red
NNK	4-(metilnitrosamine)-1-(3piridil)-1-butanone
N-ras	Neuroblastoma-ras
NSCLC	Non-small cell lung cancer
N-terminal	Amino-terminal
o.n.	Over night
OCT	Optimum cutting temperature
OIS	Oncogene induce senesence
PCR	Polymerase Chain reaction
PCR	Polymerase Chain Reaction
PICe	Phospholipase Ce
pfu	Plaque-forming units
pRb	Phosphorylated retinoblastoma
R point	Restriction point
Raf	Rapidly growing fibrosarcomas
Ras	Rat sarcoma oncogene
Ras-GAP	Ras-GTPase Activity Proteins
Ras-GEF	Ras-Guanine nucleotide Exchange Factors
Rb	Retinoblastoma
rpm	Revolutions per minute
RSV	Rous Sarcoma Virus
RT	Room Temperatura
S phase	Synthesis phase
S. cerevisiae	Schizosaccharomyces cerevisiae
S. pombe	Schizosaccharomyces pombe
SA-b-gal	Senecence Asociated b -gal
Ser	Serine
shRNA	Short hairpin RNA
SP-C	Prosurfactant apoprotein C
TB	Terminal bronchiole
Thr	Threonine
TKO	Triple Knock out

TKR	Thyrosine-kinase receptors
TNM	Tetranitrometane
TPA	12-o-tetradecanoil, forbol-13-acetato
V	Valine
WT	Wildtype
Xgal	5-bromo-4-chloro-3-indolil-β-D-galactopiranoside
Y	Tyrosine

7.Associated publications/presentations

Presentation: *Validation of cell cycle Cdks as potential targets for therapeutic intervention.* M.Puyol, A. Martin, D.Santamaria, A. Seddon G.Khan and M. Barbacid. **Postgraduate day : Kingston University June 2006. Obtained Second Prize.**

Poster: *Validation of cell cycle Cdks as potential targets for therapeutic intervention.* M.Puyol, A. Martin, D.Santamaria, A. Seddon G.Khan and M. Barbacid. **Keystone Symposium March 2007: Molecular Targets for Cancer/Mouse Models at the Frontier of Cancer Discovery.**

Poster: *Validation of cell cycle Cdks as potential targets for therapeutic intervention.* M.Puyol, A. Martin, D.Santamaria, A. Seddon G.Khan and M. Barbacid. **Cold Spring Harbour Laboratory Congress August 2008 : Mechanisms & Models of Cancer.**

8.Introduction

8.1 Cell Cycle

8.1.1 Cell cycle Overview

The cell cycle brings together a whole set of molecular processes that lead to the division of the cell into two daughter cells, genetically identical to one another and equal to the cell from which they come from. The progression through the different cell cycle stages culminates in cell division (Norbury and Nurse, 1992). The main objective pursued in cell division is the exact replication of its genetic material and the subsequent distribution of it between the two daughter cells that are going to be formed. These two facts, define the two major phases of the cell cycle: S & M phases. Along the S phase or synthesis phase the replication of genetic material takes place, the DNA replication occurs only once during each cycle. Later, at the M stage, or mitosis phase, the replicated chromosomal material is divided between the two daughter cells through a process that culminates in the cytoplasmic division or cytokinesis. Accordingly, each of the newly generated daughter cells will receive a single copy of chromosome material. Between these two events are some key preparation stages known as G1 (Gap 1) and G2 phase (Gap 2). More specifically, the G1 phase precedes S and the G2 phase is between S and M. The objectives of the Gap phases are: 1) increasing the cell size, 2) observe that the status and conditions of the cell environment are appropriate for the cycle progression and 3) ensure that events that have occurred until then have been correctly performed (Hartwell and Weinert, 1989; Losick and Shapiro, 1993) and thus, the cell is able to proceed to the next phase of the cycle. In

higher eukaryotic organisms, most of its cells are not in the division process, rather they are in a latent state, called quiescence state, from which they will only escape when they receive specific molecular signals. The state of quiescence is also called G0 phase (Figure 1).

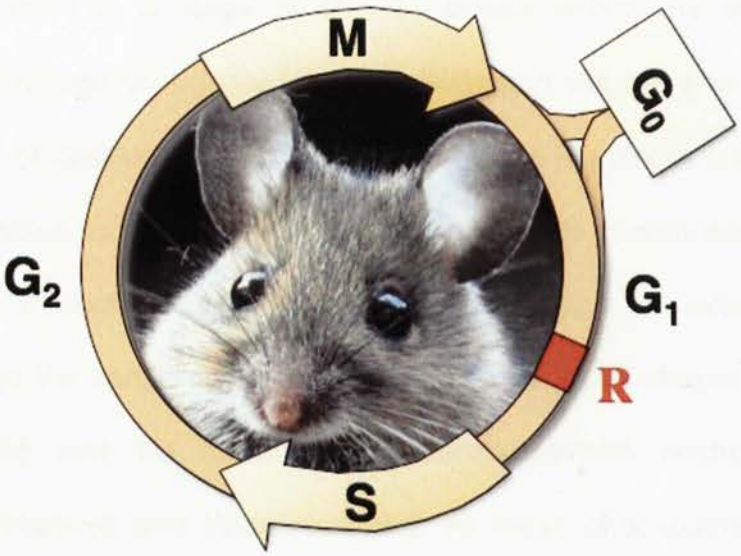


Figure 1: The cell cycle distribution in eukaryotic cells consists of four different phases: G1, S, G2 and M. When the cells are in a quiecence phase, or G0, they require mitogenic signals to enter into a round of cell division.

One of the major decisions that any cell in quiescence has to take when mitogenic signals start to arrive is whether or not to enter into cell cycle. Cells in G0 phase have this capability. Only those cells that have reached a terminal differentiation state will not be able to re-entrer the cycle, unless they undergo an undifferentiation process, such as the neoplastic process. There are several factors that the cell has to take into account before "taking a decision" in one way or another. The extracellular conditions, such as the availability of nutrients, the presence of growth factors and other mitogenic signals are

essential in determining the cell fate in the G1 phase (Malumbres and Barbacid, 2001).

An analysis of the dependence on external cell growth factors during the earliest stages of the G1 phase led to the concept of "restriction point or R point". The R point is a stage at the G1 phase where the cell becomes independent of mitogenic signals for its division and will have to complete at least one cycle of division (Pardee, 1989). Once the R point is surpassed, the cells are committed to enter into S phase, doubling its chromosomal material and continuing through G2 and M phases, irrespective of external factors. However, due to the sensitivity of the whole process, in all stages of cell cycle (G1, intraS, G2 and M) there are scattered several control points or "checkpoints" (Hartwell and Weinert, 1989). At these checkpoints, if the cell detects a failure in one of the molecular processes carried out so far, it can arrest the advance across the cycle and decide whether it may continue to progress towards the next phase of the cycle. If the defect cannot be repaired and proves to be fatal to the cell viability, it can choose to enter into a process of programmed cell death or apoptosis or alternatively senescence.

8.1.2 Molecular control of cell cycle in mammalian cells

From the facts mentioned above it is clear the need for eukaryotic organisms to have a series of molecular mechanisms that control the cell cycle stage and the transition from one phase to another, to ensure the success of this whole process.

The orderly progression through successive phases of the cycle is driven by a family of kinases, the serine threonine-cyclin dependent kinases (Cdks). The Cdks are the catalytic subunit of the heterodimeric complex (Sherr and Roberts, 2004; Malumbres and Barbacid, 2005) and the regulatory subunit is constituted by a family of proteins whose expression levels fluctuate throughout the various stages of the cycle and therefore have been called Cyclin (Sherr and Roberts, 2004; Malumbres and Barbacid, 2005). Due to variations in levels of expression of the cyclins, there is a sequential activation of each of the cyclin-CDK heterodimeric complexes. Those complexes are the fundamental basis for cell cycle control.

Recent studies of human and mouse genome have identified the presence of 11 genes that code for Cdk proteins (Cdk1-11) and 9 more genes encoding proteins similar to Cdks (PCTAIRE, 1, 2 and 3, PFTAIRE 1 and 2, PITSLRE, CCRK, Chedid, CRK7) (Malumbres and Barbacid, 2005). In addition, 29 different genes that code for the cyclin proteins have been identified in the human genome (Cyclin A1 and A2, B1, B2 and B3, D1, D2 and D3, E1, E2, C, F, G1 and G2, H, I, J, K, L1 and L2, M1, M2, M3, M4, O, T1 and T2, Cables 1 and 2) (Malumbres and Barbacid, 2005). Despite the high number of Cdks and cyclins found, only a small number of these complexes have been demonstrated to have a role in controlling the cell cycle.

8.1.2.1 Cyclin-dependent Kinases (Cdks)

The first member identified in the Cdk family was Cdk1. This protein was found in a genetic screening tests using *Schizosaccharomyces pombe* (*S.pombe*) and

Saccharomyces cerevisiae (*S.cerevisiae*). The protein identified was called Cdc2 in the case of *S. pombe* and Cdc28 in the case of *S. cerevisiae* (Lee and Nurse, 1987; Draetta *et al.,* 1987). A few years later, in 1991 during the Cell Cycle symposium at Cold Spring Harbor Laboratories the CDK nomenclature was established, and both Cdc2 and Cdc28 were renamed Cdk1. From the list set out in the above paragraph of the existing Cdks only Cdk1, 2, 3, 4 and 6 have been described to have a specific role in controlling the cell cycle (Figure 2).

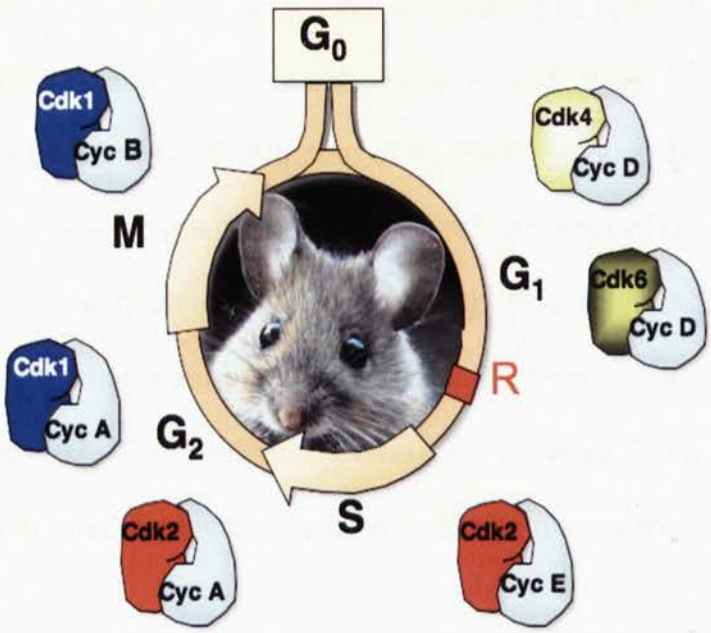


Figure 2: The sequential activation of the different Cdks, by the association to its appropriate cyclin, are able to regulate the different stages of the cycle. According to the classical model, Cdk4 and Cdk6, together with cyclin D, will be the first Cdks responding to the mitogenic stimuli and promoting the cell cycle initiation. Cdk2 will then associate with cyclin E and later to cyclin A. Cyclin A will also bind to Cdk1 at the G2/ M transition. During mitosis Cdk1 will associate with cyclin B.

The Cdks, together with its corresponding cyclin, act as the molecular cell cycle "engines". Additionally, some Cdks act as intracellular sensors, being responsible for the integration of signalling coming from the external

environment. Thus, Cdk4 and Cdk6 coupled with cyclin D1, D2 and D3 (Figure 2) will be the link between the mitogenic stimuli and the cell cycle machinery during the early stages of G1. It's important to point out that while Cdk4 expression is more ubiquitous, Cdk6 expression is mainly restricted to the haematopoietic compartments (Malumbres and Barbacid, 2005). Then, the complex Cdk2/cyclin E1 or E2, is activated during the late G1 stages and at the G1/S transition. Once reached to the S stage, there will be a rapid degradation of cyclin E, and Cdk2 will then be able to associate with cyclin A1 and A2. Cdk1 will be responsible for the passage through G2 and M phases, by firstly associating with cyclin A and, later by binding to cyclin B1 and B2. Cyclin B3 acts as an intermediate between cyclin A and cyclin B (Nieduszynski *et al.*, 2002) and binds to Cdk2 (Nguyen *et al*, 2002) (Figure 3). Finally, it seems that Cdk3 intervenes, coupled with cyclin C, in the output of G0 for cell cycle re-entry in human cells (Ren and Rollins, 2004). However, most murine laboratory strains carry an spontaneous mutation that introduces a premature stop codon at position 187. This mutation creates a truncated Cdk3 protein that lacks at least one third of the kinase domain and thus makes the protein inactive (Ye *et al*, 2001).

The rest of Cdks described in this paragraph (Cdk5, 7, 8, 9, 10 and 11) are not entirely characterized in detail and its possible role as direct regulators of the cell cycle has yet to be established. Cdk5 is activated by binding to p35 and p39, and its role has been widely described in brain (Kesavapany *et al*, 2004; Cruz and Tsai, 2004) although its function could be extended to other tissues such as pancreas, heart and gonads (Rosales and Lee, 2006).

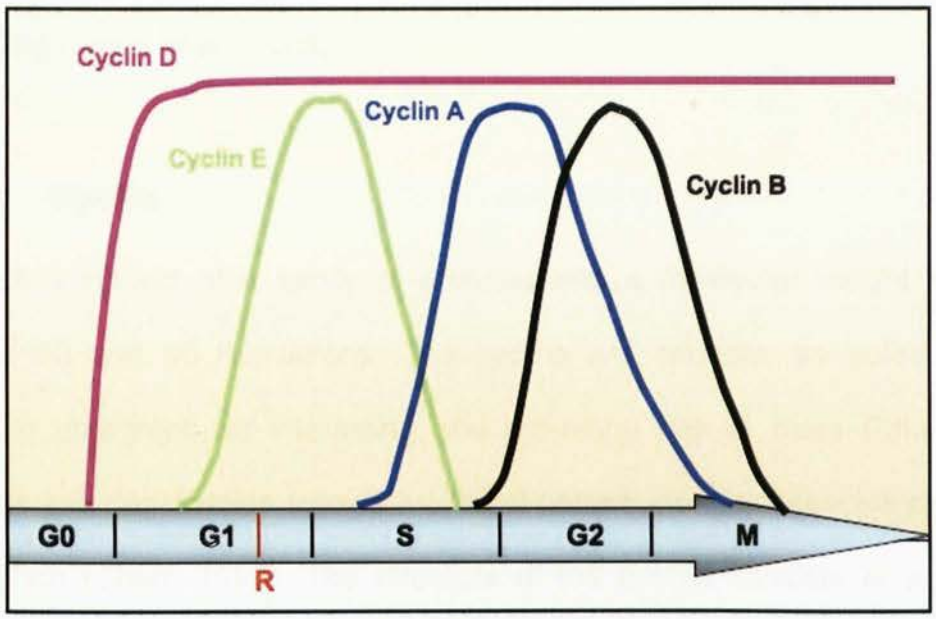


Figure 3: Although Cdk protein levels are maintained during the different cell cycle phases, the cyclin levels vary. The fluctuation in the different cyclin levels will allow the binding of the Cdks their appropriate cyclin partner. The expression of the cyclins is restricted to the stages were they are playing a role.

Moreover, it seems that Cdk5 governs various aspects of brain activity, such as neural function and synaptic transmission. Cdk7 bound to and activated by the cyclin H, together with Mat1 will form the Cdk Activating Kinase complex (CAK). It has been described how the CAK acts as a positive regulator of the cell cycle implicated Cdks. The complex Cdk8/cyclinC and Cdk9/CyclinT are also involved in regulating transcription by phosphorylation of the C-terminal domain of the largest RNA polymerase II subunit (Akoulitchev *et al.*, 2000). Cdk8/cyclinC also regulates the inhibitory activity of the CAK complex by phosphorylation. Additionally, it has been observed that Cdk9 can phosphorylate pRB, although the relevance of this phosphorylation is yet to be studied in greater detail. Although no cyclin partner has been identified for Cdk10, it has been reported a possible role for Cdk10 in the Cdk1 regulation. In addition, Cdk11 bound to

cyclin L is involved in the production of RNA transcripts and its subsequent processing (Loyer *et al.*, 2005).

8.1.2.2 Cyclin

The cyclins consist of a family of proteins with a molecular weight ranging between 30 and 90 Kilodaltons. The cyclins are capable, as noted in the preceding paragraph, of interacting and activating one or more Cdks. Their synthesis and degradation follows a cyclical pattern along successive cycles of cell division (Sherr, 1996). The structure of the cyclins consists of a pair of central domains the cyclin box, with 5-α helixes each, flanked at the N- and C-terminal regions by a short helical structure. While the cyclin box domain is conserved among various cyclins, the structure and position of the flanking regions vary between the different family members. The cyclins also play an essential role as they confer substrate specificity to the Cdks.

8.1.3 Regulating the activities of Cdks

As we have mentioned, activation of Cdks is obtained by the association to the cyclins. However, this is not the only mechanism of Cdk regulation. There are four major groups of mechanisms regulating Cdk activity: 1) protein-protein interactions (association to the cyclins and the binding to inhibitory proteins) 2) post-translational modifications (activating and inhibitory phosphorylations) and 3) subcellular localization 4) Interaction with inhibitory molecules. One of the main mechanisms regulating the Cdk/cyclin complexes activity is conferred by the cyclin. Thus, the synthesis and degradation of cyclin are of vital importance when monitoring the Cdk enzyme activity (Figure 4).

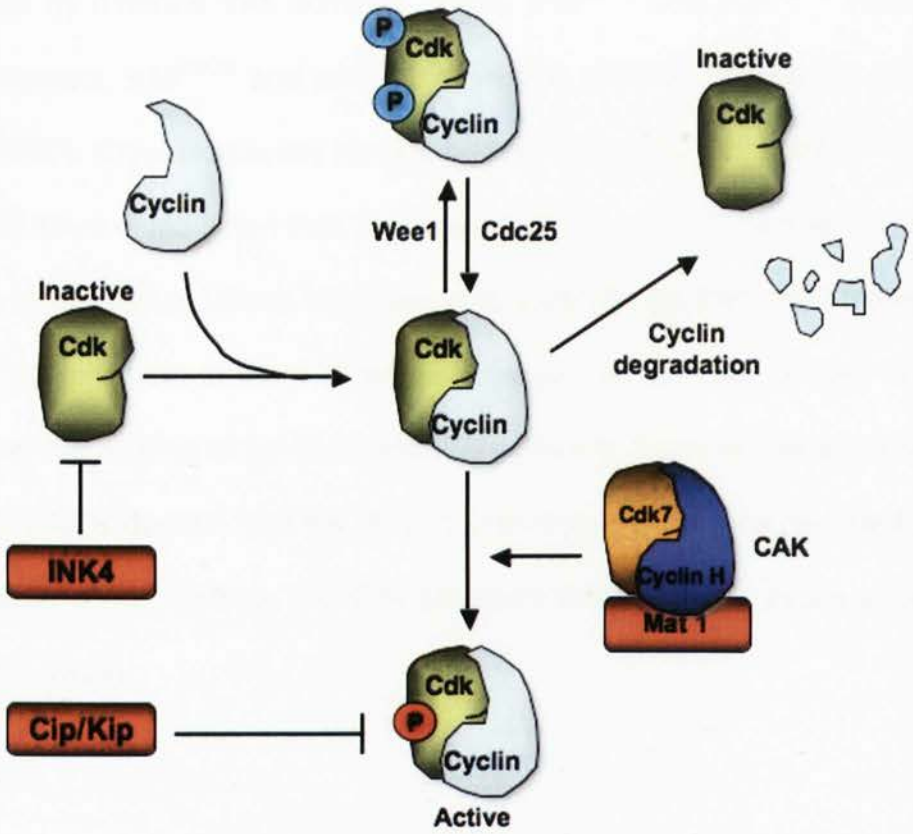


Figure 4: Main mechanisms of Cdk/cyclin activity regulation. The inhibition of the Cdk/ cyclin complex can be obtained by the binding of inhibitory molecules to the complex, or the Cdk alone. But also, the inhibition of Cdk activity can be achieved by phosphorylation of the complex or degradation of the cyclin.

However, the interactions with inhibitory molecules provides the Cdks an additional mechanism of control:

There are two families of inhibitory Cdk proteins: 1) INK4 inhibitors, which consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$, $p19^{INK4d}$ proteins, and 2) the inhibitor family Cip/Kip, composed by $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$.

INK4 family inhibitors are specific Cdk4 and Cdk6 inhibitors, they bind to the N-terminal region of the Cdks and act as competitive inhibitors for cyclin D (Figure 5). The four INK4 proteins share the structural ankyrin repeats motive, which consists of pairs of α antiparallel helixes bound by the terminal ends and

connected by different fork domains. While $p16^{INK4a}$ and $p15^{INK4b}$ contain four ankyrin repeats, $p18^{INK4c}$ and $p19^{INK4d}$ have five of these repetitions (Ruas and Peters, 1998). Crystallographic studies performed on the Cdk6/$p16^{INK4a}$ complex (Figure 5) have established that the binding of the $p16^{INK4a}$ occurs in a position opposite to the region where Cdk6 binds to cyclin D. So that, the binding of the inhibitor will promote several allosteric changes in the Cdk protein structure, preventing the binding of cyclin D. More specifically, there will be a reorientation of the PSTAIRE domain and the N- and C-terminal ends. The resultant change will produce a 15° shift on the Cdk structure impeding the cyclin association (Pavletich, 1999).

Figure 5: Three-dimensional structure of the Cdk6/ $p16^{Ink4a}$ complex. The inhibitor bind to the Cdk and so, the kinase is not able to associate with the cyclin. (Adapted from Pavletich, 1999).

Regarding the Cip/Kip family, its inhibitory action is obtained by binding to the heterodimeric Cdk/cyclin complexes. It seems quite clear that its association to the Cdk2/cyclin E complex will produce an inhibition in their kinase activity (Figure 6) (Sherr and Roberts, 1999). More controversial is the effect exerted on the Cdk4 or 6/cyclin D complexes. Some studies suggest that the Cip/Kip proteins also have an inhibitory activity over them (Sherr and Roberts, 1999). In contrast, other publications seem to indicate that they have a positive effect on the activity of the Cdk4 or 6/cyclin D complexes (Bagui *et al*, 2003). Indeed, there are studies that indicate that the association between the Cdk4-6/cyclin D complex, and at least $p21^{Cip1}$ or $p27^{Kip1}$ contribute to the stabilization of the complex, and to strengthen its activity (Sherr and Roberts, 1999). In fact, it has been postulated that the progression through the G1 phase is provided in some extent by the titration of the Cip/Kip to the Cdk4-6/cyclin D complex and thus the prevention from binding to the Cdk2/cylin E complexes, contributing, to the activation of the second Cdk/cyclin complexes (Sherr and Roberts, 1999). It has been shown that the growth arrest imposed by the overexpression of $p16^{INK4a}$ or $p21^{Cip1}$ can be overcome by the overexpression of an inactive Cdk4 mutant. This Cdk4 isoform conserves intact its ability to interact with inhibitors Cip/Kip (Sherr and Roberts, 2004).

The solving of the Cdk2/cyclin A/$p27^{Kip1}$ ternary complex crystal structure has helped to understand the mechanism by which the Cip/Kip inhibitors regulate the Cdk/cyclin complexes (Russo *et al*, 1996). The binding of $p27^{Kip1}$ to the Cdk will take place at the C-terminal region, inserting in the catalytic subunit of Cdk2 a small α helix, which would inhibit the ATP binding. So that, the presence of

ATP in the catalytic centre of the Cdk will interfere with the action of molecules Cip/Kip but it would not prevent the binding of $p27^{Kip1}$ to the complex, through the regulatory subunit. This could explain the existence of functional heterotrimeric Cdk/cyclin/Cip-Kip complexes (LaBaer *et al.*, 1997; Zhang *et al.*, 1994, Harper *et al.*, 1995, Soos *et al.*, 1996). Recently it has been discovered a novel function for $p27^{Kip1}$ when associated to the Cdk4/cyclin D complex. The phosphorylation of the $p27^{Kip1}$ tyrosines (Y) at position Y 88 and Y 89, generates a molecular switch that turns $p27^{Kip1}$ from a non-inhibitor to an inhibitor state (Blain, 2008). The $p27^{Kip1}$ Y-phosphorylation provides an additional pathway of cell cycle control.

Figure 6: Three-dimensional structure of the Cdk2/ partial cyclin A/ $p27^{Kip1}$ ternary complex. The inhibitor binds to the Cdk/ cyclin complex and prevents ATP binding, and thus its activity (Adapted from Pavletich, 1999).

8.1.4 Molecular events during the cell cycle

8.1.4.1 Regulation of cell cycle re-entry and progression across the G1 stages

As a consequence of an adequate mitogenic stimulation, and once the cell has taken the "decision" to leave the molecular state of quiescence, the cell will activate various signalling pathways that promote the activation of the complex Cdk4-6/cyclin D in the different levels of regulation: cyclin D transcription, translation and stabilization, binding with their respective Cdks and eventually accumulating in the nucleus. At the nucleus the heterodimers will carry out the phosphorylation of their corresponding substrates (Sherr and Roberts, 1999). The activation of Ras and consequently the Ras/Raf/Mek/Erk mitogenic pathway, raises, among others, the levels of cyclin D1 mRNA (Lavoie, 1996; Balmanno and Cook, 1999; Peeper *et al.,* 1997; Kerkhoff and Rapp, 1997: Aktas *et al.,* 1997). Moreover, the simultaneous activation of the PI3K/Akt route will extend the half-life of cyclin D1 by inhibiting its phosphorylation in the residue Thr286 by glycogen synthase kinase 3β (GSK- 3β kinase) (Diehl and Sherr, 1997; Cross *et al.,* 1995; Alt *et al.,* 2000). This phosphorylation promotes the association of cyclin D1 with the exportine CRM1 and subsequent translocation to the cytoplasm where it would be ubiquitinated and degraded by the proteosome.

8.1.4.2 G1 / S transition.

High levels of cyclin D will be able to assemble and form the Cdk4-6/cyclin D complexes. The complex will acquire kinase activity and will be able to phosphorylate several substrates. The main target of these heterodimic

complexes is the retinoblastoma family of protein, or Rb, which consists of pRB, p107 and p130 (Ewen *et al.*, 1993; Kato *et al.*, 1993) (Figure 7). The Rb protein family regulates the activity of a number of transcription factors, being E2F the main factor (Dyson, 1998). The E2F factors, associated with the DP proteins, will promote the expression of essential genes necessary for the progression through the G1/ S phases.

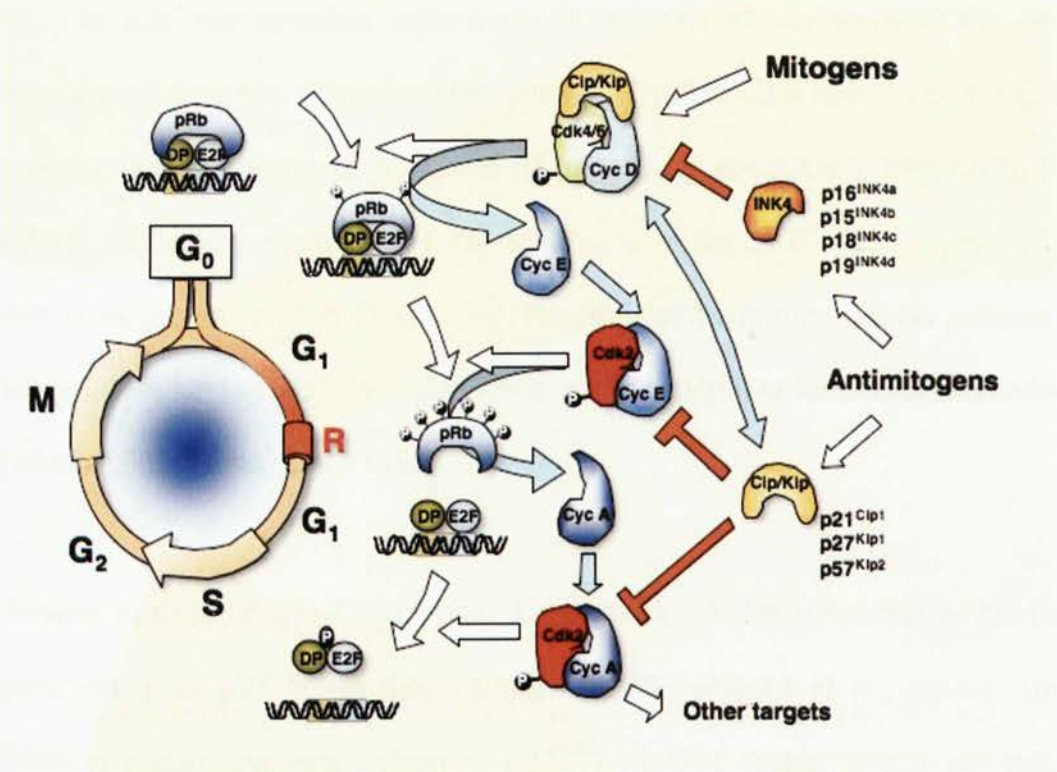


Figure 7: Main molecular events that regulate the progression through the different cell cycle phases. The Rb inactivation at the G1/ S transition will trigger a cascade of events that will lead to the expression of genes responsible for the progression through the S phase and will guide the cells to the G2 and M phases.

When Rb is hypophosphorylated, and thus active, it will interact with the transcription factor E2F and blocks its activity. At the same time, in an Rb-dependent manner the histon deacetilases or HDAC, the Sw1/SNF chromatin remodelling complexes, together with other proteins are recruited to the

chromatin (Brehm *et al*, 1998; Luo *et al.,* 1998; Zhang *et al.*, 2000). Those protein complexes will bind to the promoter regions of the genes whose expression is dependent on the E2F factor.

The Cdk4-6/cyclin D complex carries out the first rounds of Rb phosphorylation. The partial phosphorylation of Rb results in an incomplete decoupling of inhibitory macrocomplex described above and thereby loss of the HDAC activity. The E2F transcription factors would lower its affinity towards Rb, and will be released from the complex (Harbour *et al.,* 1999). The release of the E2F will promote the synthesis of a subset of genes, by specifically binding to its promoters, inducing among others the synthesis of cyclin E. Once cyclin E is coupled to its corresponding Cdk, Cdk2, the resultant complex will be activated and will contain kinase activity, generating various positive feedback cascades (Koff *et al.*, 1992; Dulic *et al.,* 1992).

The kinase activity of the Cdk2/cyclin E complex will be responsible for the phosphorylation of $p27^{Kip1}$ on the residue Thr 187 (Aleem *et al.*, 2005). This modification allows the recognition of $p27^{Kip1}$ by SCF Skp2, which will poly-ubiquitinate the protein and promotes its degradation by the proteasome (Bloom and Pagano, 2003). The decrease on $p27^{Kip1}$ will cause an exponential increase in the kinase activity of Cdk2. Moreover, it has also been described that in the absence of Cdk2, Cdk1 is also capable of phosphorylating $p27^{Kip1}$ (Aleem *et al.,* 2005). In addition, the Cdk4-6/cyclin D complex seems to titrate $p27^{Kip1}$ preventing the binding of $p27^{Kip1}$ from the Cdk2/cyclin E complex. The Cdk2/cyclin E complex will be also responsible for the completion of Rb

phosphorylation, and thus inactivation. pRb inactivation is essential for progression through the R point, a stage that marks out the commitment of the cell to initiate DNA synthesis (Malumbres & Barbacid, 2005). The passage through the G1/S transition results in degradation of the E-type cyclins by the proteasome.

8.1.4.3 The S phase of the cycle, the G2 / M transition and mitosis

The replication of chromosomal material takes place during the S phase, along with the initiation of the centriole duplication and these processes will run through G2. There are several studies indicating that Cdk2 is involved in these processes regardless of its role as direct regulator of Rb activity (Tsai *et al.*, 1993; Jackson *et al.*, 1995; Hua *et al.*, 1997; Krud *et al.,* 1997; Duronio *et al.*, 1998; Krude, 2000; Futstenthal, 2001; Coverly, 2002). It has been described that Cdk2, coupled with its corresponding cyclin, is capable of phosphorylating a large number of substrate (Malumbres and Barbacid, 2005), independently of Rb; these substrates include CBP/p300, Cdc6, Cdc7, Cdt1, C/EBP β, DP1, MCM2, MCM4, thymidine kinase, BRCA1 and BRCA2. The target substrates are involved in various processes such as the onset of DNA replication, elongation and repair. From a biochemical point of view, the decline of cyclin E by its degradation is important to prevent chromosomal instability. The chromosomal instability will occur when the average life of the cyclin stretches more than it should. The decrease in cyclin E levels is due to its proteolytic degradation that is originated via two different pathways, both associated with the ubiquitin-proteasome system.

When the levels of cyclin E start to decline, cyclin A levels would increase, forming an heterotrimeic complex firstly with Cdk2 and, secondly, with Cdk1 (Girard *et al.*, 1991). Previous studies showed that Cdk2 along with the appropriate cyclin, has not only an important role in cell cycle progression, but also the active heterodimer has additional functions in other relevant processes such as chromosome and centrosome duplication, histone synthesis and chromatin assembly.

Cyclin B is formed by three family members: cyclin B1, B2 and B3. While cyclin B1 and B2 only interact with Cdk1, cyclin B3 can also associate with Cdk2. Although during the S and G2 phases cyclin B levels can be detected, it is not until the G2/M transition when the Cdk1/cyclin B complex is activated (Pines and Hunter, 1990). The Cdk1/cyclin B complex is able to translocate into the nucleous due to the loss of inhibitory phosphorylation (Izumi *et al.,* 1992; Kumagai and Dunphy, 1992; Hoffman *et al.,* 1993). At this location, Cdk1/ cyclin B will be able to phosphorylatate a wide range of substrates, that participate in a variety of processes (Malumbres and Barbacid, 2005). Cdk1 will regulate proteins such as Cdc25A and C, laminin A, B and C, B lamin recetor, nucleolin, separase, survivine, among others. The list extends to more than 70 potential substrates. Those Cdk1/cyclin B substrates intervene in the regulation of processes such as chromosome condensation, fragmentation of the Golgi apparatus, breaking the nuclear membrane, etc. Finally, the activation of the APC in the metaphase-anaphase transition promotes the decline of cyclin B levels and thus, the progressive decrease of its associated kinase activity. In fact, the inactivation of the Cdk1/cyclin B complex is necessary for mitosis

conclusion (King *et al.*, 1996; Harper *et al.,* 2002). When the cell cycle has been completed, the divided cells will face again the decision of entering into a new round of mitotic division or entering into the state of quiescence or G0. The decision is taken based on the extra-cellular conditions.

8.1.5 Genetic analysis of the cell cycle

Most of the initial data obtained from the inhibition of cell cycle proteins was provided from *in vitro* studies on cell lines. However, the development of analytical techniques and genetic modification in the mouse genome has helped to generate a more precise picture of the events that occur at every stage of the cell cycle, as well as reporting the physiological role played by some cell cycle regulators. We will now review those models that have a more direct involvement with the work described in this thesis:

8.1.5.1 *Cdk4* KO Model

The generation of a knock out (KO) mice model that has been obtained through homologous recombination techniques, provides us the opportunity to study the *in vivo* role of Cdk4. The germline *Cdk4* KO model (Tsutsui *et al.*, 1999) that have been used in the experiments reported later, a resistance neomycin cassette has been inserted in the genomic *Cdk4* locus, which replaces the region enclosed between exons 1 and 5, eliminating, among others, the ATG sequence and the protein catalytic centre encoduing region. The result is the inhibition of the Cdk4 expression.

The *Cdk4* KO animals are viable, demonstrating that Cdk4 is dispensable for

the cell cycle, despite what might have been expected (Table 1). However, the size of the animals was smaller than their wildtype littermates. In addition, the absence of Cdk4 causes a defect in the post-natal proliferation of pancreatic β cells (resulting in the development of type I diabetes in those mice) and in the adenohypophysis cells (causing partial sterility in males and full sterility in females). Primary MEFs isolated from these animals were able to divide without major deficiencies (only a slight delay of 3-6 hours at the S phase entry was described) (Tsutsui *et al.*, 1999).

TARGETED GENE	FUNCTIONAL EFFECT	LIFESPAN	MAJOR PHENOTYPE	REFERENCE
Cdk1	Knock-out	Embryonic lethality at E3.5		Santamaria et al. 2007
Cdk2	Knock-out	Viable. Normal life span	Spermatocytes die in pachytene. Oocites die in diplotene. 100% esterility in males in females. Strain dependent reduced body size.	Ortega et al., 2003 Berthet et al., 2003
Cdk3	Knock-out	Viable	Most laboratory mouse strains carry this mutation. Therefore all other models are also null for Cdk3.	Ye et al., 2001
Cdk4	Knock-out	Viable. Reduced life span due to diabetes	Pancreatic beta cell proliferation impaired: diabletes. Anterior pituitary cell proliferation impaired, particulary lacotrophs. Leydig cell number reduced. Defective spermatogenesis. Reduced body size.	Rane et al., 1999 Tsutsui et al,. 1999
Cdk6	Knock-out	Viable. Normal life span	Hypoplastic thymus and spleen. Reduced erythrocytes and megakaryocyte numbers. Reduced body size only in females.	Malumbres, et al. 2004
Cdk4 and Cdk6	Double knock-out	Embryonic lethality at E14,5 - E18,5	Defective fetal hematopoiesis. Severe anemia.	Malumbres, et al. 2004
Cdk2 and Cdk6	Double knock-out	Viable. Normal life span.	Addition of individual knock-out phenotypes.	Malumbres, et al. 2004
Cdk2 and Cdk4	Double knock-out	Die at birth	Cardiac defect.	Barriere, et al. 2007
Cdk2, Cdk4 and Cdk6	Double knock-out	Embryonic lethality at E13,5	Defective fetal hematopoiesis. Severe anemia.	Santamaria et al. 2007

Table 1: Summary of the *Cdk* knock out mice models and the main phenotypes of the different strains present. Adapted from Santamaría and Ortega, 2006.

8.1.5.2 Model of *Cdk6* KO

As in the case of *Cdk4*, the *Cdk6* KO mice model (Malumbres *et al.*, 2004) is

viable. In order to ablate the *Cdk6* locus, the first coding exon was replaced with a *PGK-neo* cassette. This first exon encodes the ATG initiation codon and the PLSTIRE domain essential for cyclin binding. The resultant modification of the gene prevents the transcription of Cdk6. The *Cdk6* KO mice phenotype is not very strong, females have a smaller size than their wildtype littermates and approximately 30% of them are sterile. The main defect of these animals occurs in the haematopoietic system (Table 1). While the levels of haematopoietic precursors are normal, there is a decrease in levels of several cell lines (such as red blood cells, megakaryocyte, etc.). The lymphocytes isolated from the thymus of the KO mice reached its S phase peak with a 24 hour of delay when compared with lymphocytes isolated from the thymus of wildtype littermates. However, the *Cdk6* KO MEFs grow normally and with similar kinetics to those of wildtype MEFs.

8.1.5.3 Models of *Cdk2* KO

The *Cdk2* KO mouse model used in this thesis was obtained by the removal of exons 2 and 3 of the *Cdk2* locus (Ortega *et al.*, 2003). Those two exons contain the PSTAIRE domain sequence and its excision generates frameshift in the coding sequence of the gene, transcribing a non related Cdk2 mRNA. The *Cdk2* KO animals were viable and have an average life similar to their wildtype littermates. Surprising, KO animals (both males and females) are sterile due to defects in chromosome pairing during the first prophase of meiosis, indicating an essential role for Cdk2 in meiosis (Table 1). The KO MEFs derived from these animals proliferate and can be immortalized in the same manner as the wildtype MEFs.

8.1.5.4 Model of *Cdk1* KO

Cdk1 has demonstrated to be the only cell cycle Cdk absolutely essential for cell division. Thus, the ablation of *Cdk1* results in a very early embryonic lethality (embryonic day 3.5 or E3.5), reinforcing the importance of this protein in embryonic development. The disruption of the Cdk1 expression was performed by introduction of a β-geo trap cassette in the intron 2 of the *Cdk1* locus. The resultant protein generated is a Cdk1 β-geo fusion protein that only contains the 12 amino-terminal residues of Cdk1 and is unable to act as Cdk1 protein.

8.1.5.5 Functional redundancy among the cell cycle regulators

The combination of different *Cdk* KO mice models as well as *cyclin* KO have illustrated the functional redundancy that exists between these proteins. Cdk1 seems to be the only cell cycle regulator protein, whose activity cannot be compensated by other family members.

As the deficiency of a single Cdk does not result in a dramatic phenotype, with exception of diabetic phenotype in the *Cdk4*-null mice, one of the hypothesis was that the different components of the Cdk or cyclin families can compensate for the lack of a member. In consequence, the next step was the generation of mice carrying deleterious mutations for various combination of the protein members.

Absence of *Cdk4* and *Cdk6* results in embryonic lethality at E14.5-18 due to a defect in the erythroid lineage proliferation, provoking a severe anemia.

However, organogenesis of these animals was almost normal, which leads to the conclusion that Cdk4 and Cdk6 are dispensable for the proliferation and differentiation of most cell types in the organism. *Cdk4* and *Cdk6* double mutant MEFs can proliferate normally, with kinetics similar to wildtype MEFs, although with less efficiency than the latter, and are immortalized normally.

Importantly, *cyclin D1, D2* and *D3* triple KO (TKO) as well as the double combinations have complementary phenotypes to that observed in the simple KO models. Thus, for example, *cyclin D1* KO animals developed some neurological abnormalities and the females had mammary glands atrophic (Fantl *et al.,* 1995; Sicinski *et al.,* 1995). The *cyclin D2* KO females were sterile due to defects in proliferative cells of the ovaries, while the males were fertile but had testicular hypertrophy (Sicinski *et al.,* 1996). Moreover, in the absence of this cyclin, the development of the cerebellum (Huard, 1999), adult neurogenesis (Kowalczyk *et al.*, 2004), B-lymphocytes proliferation (Lam *et al.,* 2000; Solvason *et al.,* 2000) and β cell proliferation, were all affected (Georgia and Bhushan, 2004). The *cyclin D3* KO mice have proliferative defects in T lymphocytes (Sicinska *et al.,* 2003). Double KO (DKO) animals of the different *cyclin D* combinations, show an accumulation of the single KO phenotypes (Ciemerych *et al.*, 2002). In addition, the triple *cyclin D* KO mice (Kozar *et al.,* 2004) managed to reach intermediate stages of embryonic development, dying at day E17.5. The cause of death is a failure of proliferative myocardiocytes and haematopoietic stem cells (HSCs). However, as in the *Cdk4; Cdk6* deficient mice, the organogenesis of the rest of tissues remained relatively unchanged. The earlier lethality of the TKO *cyclin D* animals when compared to the DKO

Cdk4; Cdk6 could be due to the ability of cyclin D (at least D1 and D2) to bind to Cdk2 in order to compensate for the absence of the G1 Cdks (Malumbres *et al.,* 2004).

The deficiency of obvious phenotypic defects in the *Cdk2* and *Cdk6* KO mice led to the generation of a model for DKO model (Malumbres *et al.,* 2004) to determine the potential compensatory roles between the two kinases. However, these animals show only the addition of defects present in the single KOs. Thus, they are completely sterile due to a defect in meiosis, and in the maturation of immature germ cells. In addition they have the same haematopoietic defects in the maturation of certain haematopoietic cell lines (blood cells, megacariocytes, among others).

Cyclin E1 or *E2* DKO mice (Geng *et al.,* 2003; Parisi *et al.,* 2003) are also viable and show no remarkable phenotypes, except for testicular abnormalities in the *cyclin E2* KO males. However, when comparing the results obtained with the model *Cdk2* KO to the combined absence of *cyclin E1 and E2* (Geng *et al.,* 2003), cyclin E deficient mice have embryonic lethality at E11.5 of the embryonic development (Table 2). This notable difference among the two mice models demonstrates the redundant role of the cyclins E during mouse embryonic development. The cause of death of the DKO mice is a defect in the trophoblast proliferation, which are components of the cellular placenta. Thus, in the absence of cyclin E, giant trophoblast cells cannot complete their cycles of endo-replication.

TARGETED GENE	FUNCTIONAL EFFECT	LIFESPAN	MAJOR PHENOTYPE	REFERENCE
Cyclin D1	Knock-out	Viable	Neurological abnormalities. Impaired mmamary epthelial proliferation during pregnancy. Retinal hypoplasia. Reduced body size.	Fantl et al., 1995 Sicinski et al., 1995 Ciemerych et al., 2005 Atanasoski et al., 2001
Cyclin D2	Knock-out	Viable	Impaired pancreatic beta cell proliferation. Impaired granuloss cell proliferation in response to FSH. Females sterility. Hypoplastic testes, decreased sperm counts. Impaired proliferation of B-lymphocytes. Impaired cerebellar cell development.	Sicinski et al., 1996 Georgia et al., 2004 Huard et al., 1999 Solvason et al., 2000 Lam et al., 2000 Kowalczyk et al., 2004
Cyclin D3	Knock-out	Viable	Hypoplastic thymus.	Sicinska et al., 2003
Cyclin D1 and D2	Double knock-out	Viable. Die in the first three weeks	Hyperplastic cerebellum. Reduced body size.	Ciemerych et al., 2002
Cyclin D1 and D3	Double knock-out	Neonatal death. Some survive up to two months.	Neurological defects. Respiratory failure.	Ciemerych et al., 2002
Cyclin D2 and D3	Double knock-out	Embryonic lethality at E17.5- E18.5	Megaloblastice anemia.	Ciemerych et al., 2002
Cyclin D1, D2 and D3	Triple knock-out	Embryonic lethality at E16.5	Megaloblastice anemia. Defective fetal hematopoiesis.	Kozar et al., 2004
Cyclin E in cyclin D1	Knock-in. Gene replacement.	Viable	Rescue of the cyclin D1 knock-out phenotype	Geng et al., 1999
Cyclin D2 in cyclin D1	Knock-in. Gene replacement.	Viable	Rescue of the cyclin D1 knock-out phenotype in breast	Carthon et al., 2005
Cyclin E1	Knock-out	Viable	No detctable abnormalities.	Geng et al., 2003 Paisi et al., 2003
Cyclin E2	Knock-out	Viable	Reduced male fertility.	Geng et al., 2003 Paisi et al., 2003
Cyclin E1 and E2	Double knock-out	Embryonic lethality at E11.5	Lack of trophoblast-derived polyploid giant cells of the placenta: plancental failure. Tetraploid rescue: normal embryo development. Perinatal death: cardiac abnormalities.	Geng et al., 2003 Paisi et al., 2003
Cyclin A1	Knock-out	Viable	Male sterility. Meiotic arrest at the prophase-metaphase transition in spermatocytes.	Liu et al., 1998 Liu et al, 2000
Cyclin A2	Knock-out	Embryonic lethal at implantation	?	Murphy et al., 1997
Cyclin B	Knock-out	Embryonic lethality at E10.5	?	Brandeis et al., 1998
Cyclin B2	Knock-out	Viable	No detectable abnormalities.	Brandeis et al., 1998

Table 2: Summary of the *cyclin* knock out out mice models and the main phenotype of the different strains present. Adapted from Santamaría and Ortega, 2006.

Additionally, the MEFs extracted from these mice, when growing asynchronously, behave almost identical to the wildtype MEFs grown under the same conditions. However, DKO MEFs for *cyclin E1* and *E2,* which are synchronized in G0, are unable to re-enter into cycle due to a defect in the load of the MCM protein to the origins of replication, and thus activation of replication.

Mice lacking *Cdk4* and *Cdk2* in the germ line, complete embryonic development and are born alive, but die soon thereafter, possibly due to limited numbers of cardiomyocytes. The rest of the tissues display normal levels of cell proliferation (Barriere *et al.,* 2007).

Embryos deficient in the three G1/ S Cdks (*Cdk2, Cdk4* and *Cdk6*) can undergo organogenesis and develop to mid-gestation, dying at E13.5 to E15.5. Those TKO mice show reduced cellularity in the livers as well as decrease proliferation of the cardyomyocytes (Santamaría *et al.,* 2007).

Although it has been demonstrated that there exists a functional compensation among the Cdks, the compensation is not universal. Some cell types require specific Cdks to drive their cycle progression, such as Cdk4 in pancreatic β cells. This specific necessity of Cdk proteins provides the opportunity for the inhibition of Cdk activity in cancer therapy. The therapeutic ablation of the Cdk in a tissue specific manner would allow the control of tumour growth without secondary effects in non targeted tissues. For example, in this respect, indeed it has been shown that the ablation of Cdk4 in wildtype keratinocytes has no

effect in cell proliferation, however Cdk4 has shown to be essential for tumour promotion in keratinocytes (Rodriguez-Puebla *et al.,* 2002). In contrast, it is necessary to take into account special requirements that the wildtype tissues could have from the targeted Cdk.

8.1.6 Alterations in the transition and G1 / S in cancer

The importance of a precise control in advancing through the G1 to S phase is evidenced by the fact that most of the molecules involved in controlling the different cell cycle phases are mutated in several types of tumours. Thus, the Cdk4-6/cyclin D/INK4/Rb pathway is one of the most frequently mutated cascades in human tumours (Ruas and Peters, 1998; Malumbres and Barbacid, 2001) (Figure 8).

The *Cdk4* gene is located in the chromosomal region 12q13, which suffers amplifications in several types of tumours: gliomas (Schmidt *et al.*, 1994; He *et al.,* 1994; Sonoda *et al.*, 1995), osteosarcomas (Khatib *et al.*, 1993; Wunder *et al.*, 1999; Wei *et al.*, 1999), rhabdomyosarcomas (Khatib *et al.*, 1993) and breast tumours (An *et al.*, 1999). In addition, melanomas have been described to be associated to a point mutation in this kinase, $Cdk4^{R24C}$, which prevents the protein from bindining to the INK4 inhibitory family, and thus not repressing Cdk activity (Zuo *et al.*, 1996; Sotillo *et al.,* 2001).

On the other hand, Cdk6 has also been found overexpressed in T-cell lymphomas (Chilosi *et al.*, 1998) and gliomas (Costello *et al.*, 1997). A mutation similar to the R24C in CDK4 has been described for Cdk6, $Cdk6^{R31C}$, in neuroblastoma cell lines (Easton *et al.*, 1998).

Figure 8: The mutation frequency of the different G1/ S regulators in different tumours. Only represented are the mutations that occur in at least 10% of primary tumours. The percentage represents the total number of tumours where at least one mutation in the shown molecules has been reported. In green are shown the molecules for which no mechanism has been found to explain the relationship between the mutation and the tumour appearance. The asterisk represents the mutations that contain a prognostic value (adapted from Malumbres and Barbacid, 2001).

It is also very common to find mutations in the *INK4* inhibitor family of genes in different types of tumours. The loss of heterozygosity in the *INK4a* locus (which encodes for the genes $p16^{INK4a}$ and $p19^{INK4d}$) or in the $p15^{INK4b}$ locus occurs in a high percentage of bladder cancer and melanoma patients. In addition, the total deletion of the *INK4a* locus has been described in leukaemia, bladder cancer, gliomas, melanomas, nasopharyngeal and pancreatic carcinomas (Herman *et al.*, 1996; Ruas and Peters, 1998). Moreover, mutations affecting $p16^{INK4a}$, without disrupting $p19^{INK4d}$ expression, have been described in pancreatic carcinoma and melanoma patients (Ruas and Peters, 1998). Moreover, INK4

inhibitor genes are also frequently altered in the neoplastic process by alterations in the methylation patterns of the gene promoters, encouraging the silencing of their transcription. Thus, the *p16*^{INK4a} promoter is hypermethylated in bladder tumours, nasopharyngeal carcinoma, gliomas, myelomas, melanomas and colon cancer (Ruas and Peters, 1998). In the case of *p15*^{INK4b}, it has been described hypermethylation of its promoter region in leukaemias, lymphomas and gliomas (Herman *et al.*, 1996; Malumbres *et al.*, 1997). Mutations of *p18*^{INK4c} and *p19*^{INK4d} genes are not as frequent, although they have been reported in leukaemia, myeloma and osteosarcomas (Ruas and Peters, 1998).

The amplification and overexpression of cyclin D is also a common event in tumour development. In cases of breast, oesophagus and lung cancer cyclin D1 has been shown to be overexpressed. In addition, chromosomal reorganizations involving cyclin D have been described in different types of lymphomas and the parathyroid adenomas (Weinberg, 1995; Sherr, 1996; Donnellan and Chetty, 1998). In addition, Cyclin D2 overexpression has been reported in ovarian and testicular tumours (Sicinski *et al.*, 1996).

In the case of Cdk2, and despite the fact that its activity is altered in a high number of cancer patients, alterations in the levels of expression of the proteins have been found in rare cases. Even so, there are few cases of Cdk2 overexpression; specially found in certain types of oral cancer, ovarian tumours and colon-rectal carcinomas correlated with low survival rates (Marone *et al.*, 1998; Li *et al.*, 2001; Mihara *et al.*, 2001).

Cyclin E-type overexpression has been observed in lymphomas, leukaemias, sarcomas and breast, ovarian, lung and cervix cancinomas, among others (Hwang and Clurman, 2005). In fact, cyclin E has an important prognostic value, especially in breast tumours, and different studies show a correlation between high levels cyclin E and a lower survival rate (Keyomarsi *et al.*, 1994; Porter *et al.*, 1997; Span *et al.*, 2003). Some studies have suggested that the cyclin E oncogenic capacity is conferred by the generation of a truncated protein isoform (Keyomarsi, 2002) in tumour cells and this isoform is resistant to the action of Cip/Kip type inhibitors. Other researchers suggest that the oncogenic ability of cyclin E arises not as a consequence of its cell cycle role, but due to the chromosomal instability associated with high levels of cyclin E expression (Spruk *et al.*, 1999; Minella *et al.*, 2002). Those high protein levels will produce an incomplete cellular replication or an aberrant chromosomal distribution (Kawamura *et al.*, 2004).

There is no clinical evidence on the role the Cip/Kip family of inhibitors in the tumour processes. No *p27^{Kip1}*mutations have been described in human tumours, however in certain types of cancers the levels of p27^{Kip1} are used as a prognostic factor (Lloyd *et al.*, 1999; Tsihlias *et al.*, 1999). In contrast, the loss of p57^{Kip2} expression has been described associated with certain types of lung carcinomas (Kondo *et al.*, 1996).

Finally, loss or inactivation of *Rb* and, less frequently, of *p130*, is one of the most frequent alterations found in human neoplastic processes (lung, breast,

prostate, bladder, pancreatic carcinoma, nasopharyngeal, endometrial, liver tumours ovarian, testicular, leukaemias, lymphomas,...) (Paggi and Giordano, 2001).

In sum, increased Cdk activity is observed in a wide spectrum of tumours, occuring either as direct mutation of the genes or, more commonly, by deregulation of their regulators. These findings suggest that the Cdks are a key target for cancer treatment.

8.1.7 The Cdks as targets for therapeutic intervention

As a consequence of the frequent alteration of the Cdk activity levels in tumours, the Cdks have been targets of major pharmaceutical studies. Therefore, a wide variety of inhibitors have been developed against those kinases. However, none of them have reached commercialisation. One of the main problems that Cdk inhibitors have faced is that the drugs have been generated against the ATP binding site of the kinase proteins. However, because this domain is highly conserved among all the kinases, the Cdk inhibitor compounds have a problem of selectivity towards their target protein.

The generation of genetically modified mice strains give the opportunity to observe the behaviour of a hypothetically specific Cdk inhibitor in different tumour context. Moreover, previous biochemical studies have suggested that although the lack of a Cdk can be compensated by the other cell cycle kinases (Malumbres *et al.,* 2004, Santamaria *et al.,* 2007), this compensation only takes place in particular tissues and context.

Although some studies have examined the potential of Cdk2 and Cdk4 as therapeutic targets, no study has described the effect of *Cdk6* ablation in a tumour context. Very early *in vitro* studies using a *Cdk2 Kinase Dead (KD)* isoform (van der Heuvel and Harlow, 1993) suggested the importance of Cdk2 inhibition in highly proliferative cells, as human tumour cell lines infected with the *Cdk2* isoform stopped cell cycle in the G1 phase. Several studies have extended these observations in tumour scenarios, looking into the role of Cdk2 in the neoplasic environment. The target validation of the kinase followed different strategies, giving insights of Cdk2 in various scenarios. An early study analyzed the importance of Cdk2 in the growth arrest in colon cancer cell lines. The inhibition of the protein was carried out by different techniques, resulting in the same conclusion; colon cancer cell lines grow despite the inhibition of Cdk2 protein (Tetsui and McCormick, 2003). Moreover, previous studies carried out in our laboratory looked into the role of Cdk2 in a $p27^{Kip1}$ null background. In this tumour prone scenario, the ablation of Cdk2 does not revert the organomegalia and pituitary tumours resultant from the depletion of $p27^{Kip1}$ (Martin *et al.*, 2005). Both studies concluded that Cdk2 is non essential for proliferation in tumours from different origins.

In addition, a *Cdk4* KI stain that expressed the Cdk4 oncogenic mutation R24C was generated in our laboratory. These animals, although they do not develop R24C driven tumours, they are highly susceptible to DBA/TPA induced melanomas (Sotillo *et al.*, 2001). Importantly, $Cdk4^{R24C}$ mutation made the mice insensitive to the INK4 inhibition (Sotillo *et al.*, 2001).

On the other hand, several models suggest Cdk4 as a therapeutic target, as the lack of *Cdk4* has been shown to abolish the hyperproliferative phenotype of the pituitary gland associated with $p18^{INK4c}$ loss (Pei *et al.*, 2004). Moreover, the loss of *Cdk4* confers resistance to ErbB-2 driven breast tumours (Yu *et al.*, 2006) and Cdk4 deficiency results in a significant reduction in the number of tumours generated after DMBA/TPA treatment (Rodriguez-Puebla *et al.*, 2002). Additionally, it was also necessary to look into Cdk4 in a more therapeutic manner, and study the effect of Cdk4 ablation once the tumour has already been formed.

8.2 Ras oncogene

8.2.1 The origin of the genetic study of cancer: Discovery of the ras proteins

Cancer genetic studies started in the mid twentieth Century in the virology field. In 1909, Peyton Rous, managed to transmit between chickens a disease that caused them to develop tumours and which further led to metastasis. He did this by inoculating sarcoma subcellular extracts from ill animals into the plectoral muscle of healthy chickens. These experiments led to the discovery of the first virus that could cause tumours: the Rous Sarcoma Virus, RSV (Rous, 1911).

In 1970, experiments carried out simultaneously in various laboratories by Steve Martin, Peter H. Duesberg and Peter K. Vogt demonstrated the existence of a gene responsible for cell transformation (Duebserg and Vogt, 1970; Martin 1970). The *v-src* (viral gene responsible for the sarcoma) was the first oncogene, or gene implicated in cancer. Finally, in 1976 Bishop and Varmus, in collaboration with Vogt, Stehelin purified the DNA corresponding to the genes responsible for the cell transformation by DNA hybridisation of RSV transformed and non-transformed strains. The comparison, by hybridisation experiments, with chicken DNA demonstrated the existence of an homologous gene to v-src in the wildtype chicken cells (Stehelin *et al.*, 1976). These experiments confirmed that the genome contained genes that could lead to cancer.

Similar experiments allowed the discovery of new oncogenes. The oncogene ras was discovered in 1964 when Harvey managed to reproduce sarcomas in

healthy rats by the inoculation of subcellular extracts derived from rats suffering from leukaemia (Harvey, 1964). The virus responsible for this effect was the oncogene Harvey Rat Sarcoma (*v-H-ras* viral). Following this experiment three other retroviral lines where shown to contain the ras oncogene: Kirsten Mouse Sarcoma Virus (Kirsten-MSV) that was carrying the oncogene viral Kirsten Rat Sarcoma (*v-K-ras*) (Kirsten, 1967); BALB-MSV (Peters, 1974) and the Rasheed-MSV (Rasheed *et al.*, 1978). In 1973, Scolnick confirmed that the retrovirus Ha-MSV and Ki-MSV were hybrid retrovirus carrying genes of the wildtype rat genome (Scolnick *et al.*, 1973).

Nowadays, it is well known that although viruses are still an important cause of tumour formation, in humans they are only responsible for a 5-10% of the total number of tumours as the majority of human cancers have their origin in mutations of their own cellular genome. The development of techniques for cellular transfection in the 1970's, in other words, the possibility of introducing *in vitro* exogenous DNA into mammalian cells and this gave the opportunity to analyze the oncogenic potential of different cellular genes. In 1971 Hill and Hillova managed to transform chicken embryo fibroblasts grown *in vitro* by the direct transfected of RSV DNA. In a similar way, the laboratories of Weinberg and Wigler (Perucho *et al.*, 1981; Shih *et al.*, 1981; Shih *et al.*, 1979) transformed NIH 3T3 cells by transfecting DNA, which had been isolated from tumour cells. The conclusive experiment to define cancer as a consequence of an alteration of human genes was carried out by Weinberg, Wigler and Barbacid. In 1982 these investigators cloned from a cell line derived from human bladder carcinoma (T24 and EJ) a human gene with transformant

qualities. This was the first time a human activated oncogene gene was *H-ras*, and the sequence modification that conferred the gene an oncogenic potential was the point mutation G-T (Goldfarb *et al.*, 1982; Pulciani *et al.*, 1982; Shih and Weingberg, 1982). This mutation resulted in a modification in the amino acid sequence of the protein, changing a Glycine (G) to a Valine (V) in the position 12 of the protein (Reddy *et al.*, 1982). *The H-ras* human oncogene was homologous to the viral oncogene *v-H-ras*. Following these experiments, the human *K-ras* oncogene was cloned from a cell line derived from human lung carcinomas (LX-1), and was shown to be homologous to the retroviral *v-K-ras* (Der *et al.*, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982). Finally, in 1993 Wigler and Wiess discovered a new gene belonging to the ras family with transforming abilities, *N-ras* (Hall *et al.*, 1993; Shimizu *et al.*, 1983).

Since the first experiments in the molecular oncology field, the three ras protein members (*H-ras, K-ras* and *N-ras*) have always been of prime concern for research in this field. This early discovery in the cancer genetic study era, led to the investigation by the scientific community of the role of these proteins in oncogenesis.

8.2.2 Ras Proteins

8.2.2.1 *Ras* genes: chromosomal localisation and genomic structure

As already mentioned above three ras genes have been identified: *H-ras, N-ras* and *K-ras* have been identified in the mammalian genome. Moreover, there are also a number of pseudogenes. The chromosomal localisation of the ras genes

has been perfectly characterised in humans and in mice. In humans, *H-ras* is located on the small arm of chromosome 11 while *K-ras* and *N-ras* are located on the small arm of chromosome 12 and 1, respectively. In mice, *H-ras* is located on chromosome 7 and *K-ras* and *N-ras* on chromosome 6 and 3 respectively (Barbacid, 1987).

The genomic structure of the three ras genes is highly conserved. Their coding sequence is distributed along four exons. In the case of *K-ras*, it contains two alternative exons 4 (4A and 4B) which will result in the synthesis of two K-Ras proteins (K-ras4A and K-ras4B). For simplicity and due to the major translation frequency in mammals, the protein K-ras4B has been designated as K-ras throughout this thesis. The fact that the genomic structure of the *ras* genes is so well conserved suggests that they derive from a unique common ancestor. However, the intronic structure is very different in these genes, and it can vary from 4,5 kilobases (kb) as *H-ras* or 50kb as *K-ras* (Barbacid, 1987).

8.2.2.2 Ras proteins form a highly conserved protein family

The amino acid sequence of the three Ras proteins are also highly conserved (Hancock, 2003). Regarding the conservation between the proteins, its sequence has been classically divided into two: the conserved domain and the hypervariable region (Figure 9). The conserved domain (1-165 amino acid) extends from the amino terminal region to approximately three quarters of the protein, and it is 90-100% identical between the three proteins. Moreover, the first 85 amino acids are identical in the three human Ras proteins and with the mice ras amino acid sequences. The 80 following amino acids form a domain

that slightly differs between the proteins, being 85% identical. The conserved domain contains the majority of the sequences responsible for binding to GDP/GTP as with the effectors domain.

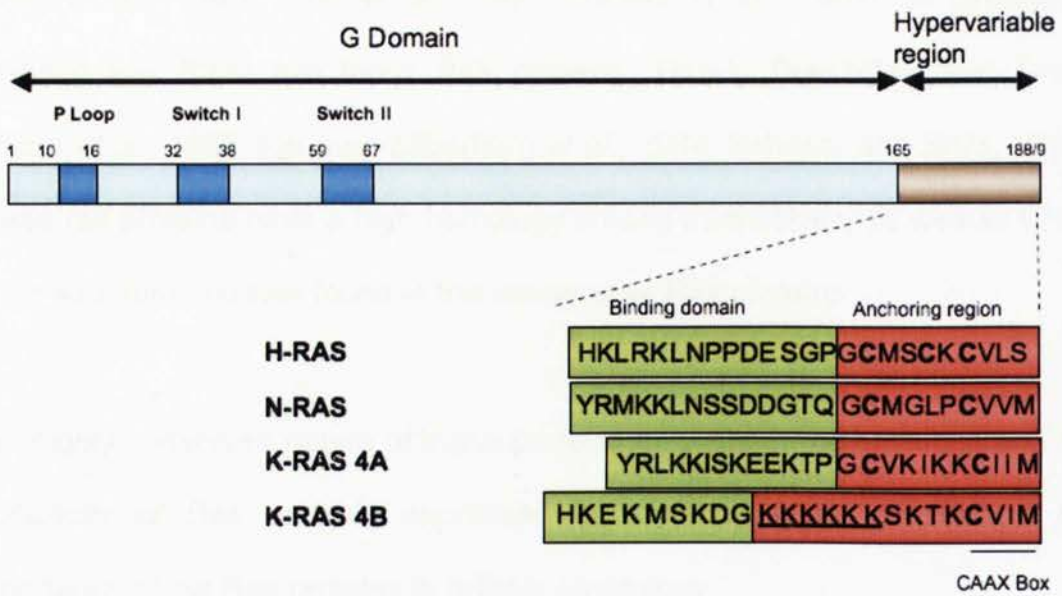


Figure 9: Analysis of the primary sequences of the Ras proteins. Structural domains conserved among the different Ras proteins.

The hypervariable region (amino acid 166-188/189) corresponds to the carboxyl terminal region. In this region the differences in the sequence of the major amino acid are observed, except for the last four amino acids where all the Ras proteins contain a structure C186-A-A-X. C is a cystein (Cys), A aliphatic amino acid and X will be a serine amino acid in the case of H-ras, and a methionine (Met) in N-ras and K-ras. These last four amino acids are responsible for ras anchoring to the membrane.

Phylogenetic studies have also shown a high conservation of the Ras proteins not only among mammals, but also with all the eukaryotic species, so that there has been conservation of the Ras family of proteins during evolution. In *S.cerevisiae* two Ras proteins have been defined: Ras1 and Ras2 (DeFeo-Jones *et al.*, 1983; Dhar *et al.*, 1984; Powers *et al.*, 1984). In *Drosophila melanogaster* there are three Ras proteins: Dras1, Dras2/64B and Dras3 (Mozer *et al.*, 1985, Neuman-Silberberg *et al.*, 1984; Schejter and Shilo, 1985). These ras proteins have a high homology among themselves, as well as to the same structural motives found in the mammalian Ras proteins.

The highly conserved nature of these proteins throughout the evolution and the multiplicity of Ras proteins expressed in each organisim emphasise the importance of the Ras proteins in cellular physiology.

8.2.2.3 Functions of the Ras protein: its role in signal transduction

Ras proteins are small molecules (21 Kilodaldons, KDa) that participate in signal transduction from the exterior to the interior of the cell. The transduction of proliferative signals induced by the binding of growth factors (GF) to thyrosine-kinase receptors (TKR) localised in the plasma membrane will be dependent on Ras. The same happens with the signal coming from the binding of hormones to receptors associated with the heterotrimeric protein G (G proteins) or the cascade induced by the non-receptor thyrosine- kinases, adhesion molecules or second messengers (Figure 10). Ras proteins therefore,

act as mediators of the signal transduction by binding to different receptors or non-receptors and allowing the binding of cytoplasmic second messengers.

Ras proteins intervene in many signalling pathways. Their involvement in different signal transduction cascades shows their importance as regulators of signal transduction, proliferation, survival and cell differentiation.

8.2.2.4 Biochemical properties of the Ras proteins

8.2.2.4.1 The GTPase activity of the Ras proteins

Biochemically, the modulation of Ras protein signal integration is conferred by the ability of these proteins to bind GTP and GDP molecules as well as for their intrinsic GTPase activity (Lenzen *et al.*, 1998) (Figure 10).

In a quiescent cell, Ras is found in an inactive state, bound to a molecule of GDP. Ras activation will imply an exchange of a molecule of GDP for a molecule of GTP. The exchange is catalyzed by nucleotide exchange factors or Ras-GEF (Ras-Guanine nucleotide Exchange Factors) (Figure 10). It has been reported that various Ras-GEFs: Sos1, Sos2, RasGRF or RasGRPs play a key role in Ras activation. When Ras binds to GTP transducers the signal is transferred to other molecules, known as Ras effectors. When the signal has been transferred, Ras protein is then able to hydrolyse the molecule of GTP to a molecule of GDP due to its intrinsic GTPase activity, becoming inactive. There also exist some factors that potentiate the Ras inactivation, the Ras-GAP (Ras-GTPase Activity Proteins). Various Ras inactivators have also been described: Ras-GAP, Nf1, GPA1m, GAP III or CAPRI (Figure 10). All of them negatively

modulate Ras activity. Ras proteins act as a switch blocking or allowing the flow of signal transduction (Downward, 2003; Malumbres and Barbacid, 2003).

Figure 10: Ras activation diagram. The Ras proteins are activated through different stimuli mediated by tyrosine kinase receptors, G proteins, adhesion molecules and second messengers. Those stimuli activate the guanine exchange nucleotides (GEF, green). Different GTPase activator proteins (GAP, orange) are involved in the negative regulation of Ras. The GAP catalyses the GTP hydrolisis. DAG, diacetilglicerol. PLC, phospholipase C. Adapted from Malumbres and Barbacid, 2003.

8.2.2.4.2 Anchoring of Ras proteins to the plasma membrane

The biological activity of Ras proteins implies their localisation in the cytoplasmic side of the plasma membrane. Ras proteins are initially synthesised as cytosolic proteins, and thus inactive. For their localization at the plasma membrane Ras proteins undergo different post-translational modifications at the

carboxy terminal (Figure 11). The farnesiltransferease enzyme, catalyses the first reaction, adding a farnesyl group (derived form 15 atoms of carbon isoprene) at the residue Cys 186 from the CAAX box of the protein (Casey *et al.*, 1989). Then, the enzyme Ras converting enzyme1 (Rce1) is responsible for the catalytic inhibition of the protein, freeing the last three amino acids (-AAX) (Hancock *et al.*, 1991). Finally, the carboxymethylation of the new Farnesyl-Cys residue of the protein takes place (Stephenson and Clarke, 1990). This last reaction is catalysed by the enzyme Isoprenylcysteine carboxy methyltransferase (Icmt). The hydrophobicity conferred by this hydrocarbonated chain allows the anchoring of Ras to the plasma membrane, where these proteins can perform their function by the interaction with different substrates and effectors. The farnesilation of these proteins is essential for their association with the membrane and so its activity.

Additional post-translational modifications stabilise the anchoring of Ras to the plasma membrane. Thus, H-ras suffers two palmitoylations in the cysteines adjacent to the farnesylation position, at the residues Cys 181 and Cys 184. N-ras lack Cys 184, so then it is palmitoylated only at Cys 181 (Hancock *et al.*, 1989). On the other hand, K-ras is not palmitoylated,s but its interaction with the plasma membrane is promoted by a group of consecutive lysines, near to the carboxy terminal end, that establish ionic interactions with the lipids negative charges, that form the membrane (Hancock *et al.*, 1990) (Figure 9 and 11).

Figure 11: The anchoring of the Ras proteins to the plasma membrane depends on a series of post-translational modifications, being the Farnesyltrasnferase protein the starting modification. Modified from Bivona and Philips, 2003.

These posttranslational modifications make the difference between the three Ras proteins, defining the difference upon the cellular trafficking among the proteins (Figure 11). The farnesylation of the Ras proteins is located in the cellular cytoplasm. The hydrophobicity conferred by the isoprene derivative will favour Ras integration into the endoplasmic reticulum membrane. At this location will take place the excision of the -AAX tripeptide and the final methylation. H and N-ras, after being palimitoylated, are integrated into the classic secretion pathway. They will go though the Golgi apparatus to their final destinations, the plasma membrane. On the other hand, K-ras will abandon the endoplasmic reticulum, and by an unidentified Golgi apparatus independent

pathway, K-ras will get to the plasma membrane (Apolloni *et al.*, 2000). Finally, the differences at the carboxy terminal and among Ras proteins, will determine the differential insertion of the proteins into the membrane.

8.2.3 Ras protein effectors

We define Ras effectors as any proteins that are activated in a Ras-dependent manner, as a consequence of simulation by either GF or hormones. The effectors are localised at the plasma membrane, where they will preferentially bind to the Ras active form, Ras-GTP (Figure 12) (Reviewed in Downward, 2003; Malumbres and Barbacid, 2003).

The first Ras effector described was the serine/ theronine kinase, Raf. The Raf family consists of three proteins, C-raf, B-raf and A-raf. When Raf proteins are active they will have the capability of phosphorylating MAPK and extracellular signal-regulated kinase (Erk) kinase 1-2 (Mek1 and Mek2) (Wellbrock *et al.*, 2004). The activation of these two proteins will led to the phosphorylation, and thus activation of Erk1 and Erk2. Once Erk is activated, it would be translocated to the nucleolus where it would bind to a wide range of transcription factors. An example of Erk1 regulated transcription factor is Elk, this protein regulate the expression of genes such as Fos or c-Jun. The consequence of the stimulation of the transcription factors activation will be that specific cell cycle regulatory proteins will be synthesised. Cyclin D, a crucial protein in cell cycle initiation and progression though G1 phase will be synthesised via this pathway. Moreover, Ras will also induce other pathways that will promote cyclin D stabilization and the assembly into the Cdk/cyclin complexes (Marshall, 1999).

Figure 12: Diagram of the main Ras effectors. Ras activation induces a wide variety of cellular processes such as, transcription, translation, cell cycle progression, apoptosis or cell survival. In blue these are represented as adaptor complexes. Adapted from Malumbres and Barbacid, 2003.

The activation of Raf promotes cell cycle progression due to the activation of the Raf/Mek/Erk pathway, commonly known as the mitogenic or Ras proliferative pathway.

A second cascade activated by Ras is the phosphatidylinositol 3-kinases (PI3Ks). The activation of these kinases will produce a lipid-based second messengers, such as phosphatidylinositol 3,4,5 triphosphate. These lipid factors can then activate a wide number of enzymes such as Pdk1. Pdk1 is

important for the activation of the AGC family of kinase proteins, such as Akt, PKCs and p70S6K. These last two proteins are specially implicated in signal transduction. Akt, also denominated Pkb, has a key anti-apoptotic role, and it is this feature, which has led the PI3K/Pdk1/Akt pathway to be called the Ras cell survival pathway. Akt is also involved in cell mobility by activating cytoskeletal proteins, such as RhoB. The PI3K proteins also lead to the activation of Rac, a member of the Rho family that not only has a role in cytoskeleton regulation but also in the stimulation of specific transcription factors such as Nf-kB (Nuclear factor-kB) (Krasilnikov, 2000).

The Ral-GEFs are a third class of Ras effectors. The Ral-GEFs are nucleotide-exchanging factors of the Ral proteins (Ral family, are small size GTPase proteins). Ral drives the inactivation of Cdc42 and Rad, two proteins that work together in the cytoskeleton regulation. On the other hand, Ral activation inhibits the transcription factor Forhead, of the FoxO family, leading to the expression of the cell cycle inhibitor, $p27^{kip1}$ (Essers *et al.,* 2004).

Ras proteins play a universal role in cellular signalling transduction due to the wide range of effectors they bind to; having an effect in most cellular biological responses (Malumbres and Barbacid, 2003).

8.2.4 The use of murine models for the study of Ras proteins

Mechanistically, the three Ras proteins are capable of activating any of the effectors described above. However, in wildtype cells, H-ras and K-ras seem to activate Raf-1, PI3K or Rac1 with different efficiencies (Walsh and Bar-Sagi

2001; Yan *et al.,* 1998): K-ras activates more potently Raf-1 or Rac1, while H-ras has higher effect over PI3K. These biochemical differences among the Ras proteins lead to different responses to the same stimuli.

Although Ras proteins expression is ubiquous, the three proteins have differences on their mRNA levels in different mouse adult tissues (Table 3) as well as in the different embryonic developmental stages (Leon *et al.,* 1987). K-ras and N-ras expression is higher during the first half of the embryonic development (E10), decreasing during the last gestation period. In contrast, H-ras levels are constant during all the embryonic developmental stages.

The possibility of altering the expression of specific genes, using gene targeting techniques, in embryonic stem cells (ES) has allowed the generation of mice strains lacking *H-, N-* and *K-ras* (KO mice). Phenotypic studies of these mice models have provided clear evidence of the differential functions of the Ras family of proteins in mammals. The ablation of *K-ras* results in embryonic lethality at day 12 to 15 of severe anaemia, due to an increase in foetal liver apoptosis, as well as an increase in neural and cardiac cell death. As K-ras does not seem to have a predominant expression at these embryonic developmental stages (Leon *et al.,* 1987), it can be concluded that the embryonic death of these mice comes from a K-ras specific function in embryonic tissues.

Tissue	mRNA levels		
	K-ras	H-ras	N-ras
Bone and bone marrow	++	++	+++
Brain	++	++++	++
Heart	+	+++	++
Kidney	++	+++	+
Liver	+	+	+
Lung	++	++	+++
Ovary	+	+	++
Skeletal muscle	+	++++	++
Skin	+	++++	++
Spleen	++	++	+++
Stomach	++	+++	+++
Testicles	++	+++	++++
Thymus	+++	++	++++

Table 3: Differential expression of the three Ras mRNAs. Adapted from Leon *et al.*, 1987.

On the other hand, mice lacking *N-* or *H-ras* either individually or combined are perfectly viable. In the case of *N-ras* KO mice, a defect in the T-lymphocyte mediated immune response has been described. *H-ras* KO mice do not have any phenotypic alteration. Mouse embryonic fibroblasts (MEFs) lacking *N-ras* have a higher susceptibility to Fas and TNF induced apoptosis. This phenotype can be rescued by the ectopic expression of N-ras but not K-ras, concluding that N-ras has a specific role in cell survival (Pérez de Castro *et al.*, 2003).

The higher homology among the different Ras proteins led to the belief in a redundant function of the three proteins. However, it is becoming clearer that they have differential biological functions that not only depend on the differences in affinity to the regulators and effectors (Raf, PI3K, Mekk, PKC) (Figure 12) but also to the subcellular localisation in the different cellular

compartments (Golgi apparatus, endoplasmic membrane, cytoplasm or plasma membrane) (Figure 11) or to the localisation at the different plasma membrane microdomains.

8.2.5 Ras Oncoprotein

Due to the high implication of Ras in numerous cellular pathways, the functional alteration of Ras proteins leads to the deregulation of different cascades implicated in cellular homeostasis.

8.2.5.1 Point mutations that disregulate Ras protein function

In mammals, *ras* genes acquire transformant properties by a point mutation in specific bases of their sequence. A change in a unique base in specific locations of the sequence is sufficient to confer the oncogenic capability. Most tumours have mutated *ras* genes, which are mainly localised at exons 12 and 13. These positions correspond to GDP/GTP binding regions, so that the mutations will increase the GDP/GTP exchange, saturating the intrinsic GTPase activity of the Ras proteins or even producing a conformational change that will no longer require the guanine nucleotide binding. These mechanisms will lead to a higher stabilisation of the active protein (Ras-GTP) and will result in a continued flow of signal transduction and so an uncontrolled signal transduction (Manne *et al.*, 1985).

The constitutive activation of the transduction pathways in which Ras is implicated will affect numerous cellular processes, including, proliferation, survival and cell differentiation. This alteration will confer the cell tumour

properties (Figure 12). Ras produces an increase in proliferation as a consequence of induction of cell cycle regulators, such as cyclin D1. This activation will lead to the inactivation of the Rb pathway and will inhibit the expression of cell cycle inhibitors such as $p27^{kip1}$ (Marshall, 1999). On the other hand, cells become insensitive to apoptosis or programmed cell death mechanisms via the PI3K/Akt pathway or Raf. Ras will also induce angiogenesis processes, especially by Erk mediated activation of angiogenesis factors. Moreover, Ras activation contributes to the invasive properties of transformed cells by the activation of the expression of cell matrix metaloproteases by Erk and Rac (Denhardt, 1996). In conclusion, Ras and its effectors have a key role in different phenotypic aspects characteristic of tumour cells.

8.2.5.2 Frequency and distribution of Ras mutations in human tumours

Approximately 20-30% of human tumours have point activating mutations in the *ras* genes. *K-ras*, is the *ras* gene more frequently mutated in human tumours (approximately 85%). More precisely, up to 90% in pancreatic tumours, 35% in lung adenomas and 50% of colon tumours carry mutated *K-ras* oncogenes (Barbacid, 1987; Bos, 1989) (Table 3). *N-ras* mutations are found in 15% of tumours. Finally, *H-ras* modifications are rarely identified in tumours, being mutated only in less than 1% of the Ras driven tumours (Downward, 2003. Table 4).

AFFECTED TISSUE*	K-RAS	H-RAS	N-RAS
Bladder	4%	11&	3%
Breast	4%	-	-
Colon	32%	-	3%
Digestive Track	33%	-	1%
Endometrium	15%	1%	-
Kidney	1%	-	-
Liver	8%	-	10%
Lung	17%	1%	1%
Melanoma	2%	6%	18%
Mieloid Leukaemia	5%	-	14%
Ovary	17%	-	4%
Pancreas	60%	-	2%
Thyroid	4%	5%	7%
Uterous	9%	9%	1%

Table 4: Frequency of *Ras* mutation in the different tissues. Obtained from the Somatic Mutation Catalog, from the Sanger Institute (COSMIC-Sanger). * The frequency of mutations listed, make reference to the total frequency observed in tumours in an specific tissue, without distinction between cell types affected or type of tumour. So that, for example, the *K-ras* mutation frequency is increased if we concentrate in the pancreatic ductal adenocarcinoma (90%), in the colon carcinoma (35%) or in the non small cell lung adenoma or adenocarcinoma (45%).

8.2.6 Transgenic and carcinogenic murine models for the study of *ras* oncogenes

Ras importance in tumourigenesis has been demonstrated by a high variety of models or systems where ras proteins have been involved. During the 60s-70s, four retroviral lines containing *ras* oncogenes (Harvey-MSV, Kirsten-MSV, BALB-MSV and Rasheed-MSV) were isolated from mouse specimens. This fact set the trend during the decade and thus cancer research was mainly based on the virology field.

In addition, *ras* oncogenes are mutated in a reproducible manner in different physical and chemical induced tumour models. During 1983, experiments done in rats showed that *H-ras* was mutated in 86% of the NMU (Nitroso-methylurea) induced breast carcinomas (Sukumar *et al.,* 1983; Zarbl *et al.,* 1985) or in 23% of the DMBA induced breast carcinomas (dimethylbenzanthracene) (Zarbl *et al.,* 1985). In mice, *H-ras* is mutated in 90% of the DMBA or DBACR (dibenz(c,h)acridine) induced and TPA (12-O-Tetradecanoylphorbol-13-acetate) promoted papillomas and skin carcinomas (Balmain and Pragnell 1983; Quintanilla *et al.*, 1986; Bizub *et al.,* 1986). The X-ray exposure of the mice or the NMU teatment lead to the development of lymphoma, in which most (70%) have activated forms of K-ras, N-ras of H-ras (Guerrero *et al.,* 1984). K-ras is also activated in adenomas and adenocarcinomas induced by TNM (tetranitromethane) treatment or by MNK (4-(methylnitrosamino)-1-(3pyridyl)-1-butanone) (Stowers *et al.,* 1987; Lantry *et al.,* 2000). K-ras is also mutated in 80% of the thymus tumours and 50% of the MCA (3-metil-colantrene) induced fibrosarcomas (Eva and Trimmer, 1986). Finally, *H-ras* is mutated in 100% HOAAF (N-hydroxiy2-acetylaminofluoride), VC (vinil carbamate), HODE (1-hydroxy-2-3-dihydroestragol) (Wiseman *et al.,* 1986) or Furfural induced hepatocarcinomas (Reynolds *et al.,* 1987). The frequency and reproducibility of Ras activation in carcinogenic induced tumours has made it an important model for the study of Ras in tumourigenesis. Cancer is a disease that can be divided in various stages, which results in the accumulation of different genetic alterations as well as epigenetic modifications. Hence, the majority of aberrations in tumour cells do not appear during the initial stages of tumour development, but during the malignant tumour development. This is why the

doubt of whether the high level of Ras disregulation in tumours is necessary during tumour initiation or is a consequence of the tumour process.

The use of transgenic *ras* animals has been the final proof on the oncogenic properties of these genes *in vivo*, and their implication in different tumour stages. The expression of different *ras* oncogenes under tissue specific promoters (transgenic mice) has led to tumour development. An example is the MMTV LTR (mouse mammary tumour virus, long terminal repeat) promoter. *MMTV-v-H-ras* (Sinn *et al.,* 1987; Kohl *et al.,* 1995) or *MMTV-v-K-ras* mice (Omer *et al,* 2000) develop mammary and salivary gland carcinomas, Harder gland hyperplasias and moderate retina degeneration. *MMTV-v-N-ras* mice develop mammary gland carcinomas as well as splenic lymphomas (Mangues et al., 1998). When the promoter used is the pancreas specific Elastase I, the *Elastase-I-H-ras* mice rapidly develop foetal exocrine pancreatic neoplasias (Quaife *et al.,* 1987).

The development of *ras* inducible transgenic mice (Table 5; and references therein) shows the importance of the oncogenic expression of Ras for the tumour maintenance and progression. Thus, the *Tyr (Tet)-H-ras* mice, where the oncogenic *H-ras* expression is driven by a specific tyrosine promoter, is inducible by tetracycline. The developed melanomas disappear as a consequence of a high apoptotic response when the activating treatment by tetracycline is removed (Chin *et al.,* 1999). In *CCSP (Tet)- K-ras* ^{G12D} mice, where a lung clara cell specific promoter drives oncogenic K-ras expression, is

also inducible by tetracycline. The lung adenocarcinomas also vanish when tetracycline is removed (Fisher *et al.*, 2001).

Model	Description	Phenotype	Reference
MMTV-v- *H-ras*	Expression of the H-ras oncogene under the MMTV promoter.	Breast and salivary carcinoma. Harderian gland hyperplasia and retina atrophy.	Sinn et al., 1987 Kohl et al., 1995
MMTV-v- *N-ras*	Expression of the N-ras oncogene under the MMTV promoter.	Breast Carcinoma and laeukemia.	Mangues et al., 1998
MMTV-v- *K-ras*	Expression of the K-ras oncogene under the MMTV promoter.	Breast and salivary carcinoma. Retina atrophy.	Omer et al., 2000
Elastase-I- *H-ras*	Expression of the H-ras oncogene under the Elastase I promoter.	Faetal exocrine pancreas neoplasia.	Quaifer et al., 1987
Tyr (tet)- *H-ras*	Expression of the H-ras oncogene under the tetracycline inducible tyrosine promoter.	Melanoma.	Chin et al., 1999
CCSP (Tet)- *K-ras*^{G12D}	Expression of the K-ras oncogene under the tetracycline inducible clara cell promoter.	Lung adenocarcinoma.	Fisher et al., 2001
LSL- *K-ras*^{G12D}	Expression of the K-ras oncogene under the endogenous promoter inducible by the Cre recombinase.	Lung adenoma and adenocarcinoma.	Jackson et al., 2001
LSL-*K-ras*^{G12V}	Expression of the K-ras oncogene under the endogenous promoter inducible by the Cre recombinase.	Lung adenoma and adenocarcinoma.	Guerra et al., 2003
H-ras^{G12V}	Expression of the H-ras oncogene under the endogenous promoter inducible by the Cre recombinase.	Facial dismorphia. Cardiomyopathies. Mammary gland hyperplasia.	Schuhmacher et al., 2008

Table 5: Different mouse models generated to study the effect of Ras oncoproteins. Adapted from references listed

The development of gene targeting models, based in the modification of the endogenous *K-ras* locus, resembles the physiological conditions of the oncogene, as its own promoter regulates its expression. In the case of the knock in model, the oncogenic *K-ras* expression is inducible in a conditional manner and leads to the development of lung adenomas/adenocarcinomas.

H-ras knock in oncogenic mice models surprisingly do not have a tumour phenotype, only females present with mammary hyperplasias and they rarely progress to anaplastic carcinomas (Shuhmacher *et al.*, 2008).

Due to the high frequency of oncogenic *ras* mutations found in human tumours, as well as the importance of Ras in tumour mantainance and progression, *ras* oncogenes are an important antitumour therapeutic target.

8.2.7 Antitumoural techniques based on ras oncogenes

Antitumour therapies based on Ras as molecular target has two strategies: to impede Ras localization at the plasma membrane, where it has to be located to interact with the receptors and substrates, and the direct inhibition of Ras expression (Downward, 2003).

8.2.7.1 Ras expression inhibition as antitumour therapy

Ras expression inhibition was first achieved by the design of antisense oligonucleotides. This technology involves the use of oligonucleotides that bind specifically, by sequence homology, to ras mRNA. These experiments were done using retrovirus infection in nude mice. It showed a 70% reduction in the number of lung tumours developed by human tumour lung cells in where ras expression was altered by the use of antisense oligonucleotides (Georges *et al.*, 1993). The main problem of antisense oligonucleotide use is the short half life, as they are rapidly degraded by intracellular nucleases. Finally, sequence independent cytotoxic effects have been described associated to the use of antisense oligonulceotides that give rise to serious doubts about the benefit of this strategy as antitumoural therapy (Mani *et al.,* 1999).

Specific antibodies have been developed against the Ras protein mutant forms, in an attempt to block the anti-tumour activity of the oncoproteins. Adenoviruses

capable of expressing anti-Ras antibodies have been tested in different tumour cell lines. The adenovirus diffusion by the hepatic artery in rats showed an important regression in hepatic tumours (Van Etten *et al.,* 2002). In other tested administration systems (intravenous, hepatic perfusion or antitumoural injection) did not show antibody diffusion or expression, so there is a big doubt about its use (Van Etten *et al.,* 2002).

In addition, the development of interference RNA (iRNA) technology has opened the possibilities of obtaining Ras inhibition of expression. This technology is based on the posttranscriptional silencing by the creation of a double stranded RNA molecule. In this way we obtain a more specific inhibition and less toxic. So, the tumourigenic capability of the pancreatic derived tumour cells (CAPAN-1) inoculated in athymic mice is abolished by previously treating the cells with specific K-ras iRNA (Brummelkamp *et al.,* 2002).

The main problem of the therapies that try to inhibit ras expression (genetic therapy) is still the vehicle used *in vivo* to reach to the target cells. Pharmaceutical companies are doing a big effort towards the design of such vehicle.

8.2.7.2 Disruption of Ras plasma membrane localization as an antitumoural therapy strategy

Ras proteins are synthesised as cytoplasmic precursors that suffer different post-translational modifications in order to reach to its plasma membrane location, and so its biological activity. The first and most important modification

is the farnesyl group covalently binding to Cys 186. This Cys is conserved among the Ras family. The lipid modification is catalysed by the farnesyltransferase (Zhang and Casey, 1996). By the farnesyl modification, Ras proteins will acquire a hydrophobic residue, which is necessary for Ras anchoring at the membrane, being responsible for the signalling transduction of Ras proteins. In our laboratory, a strain deficient on farnesyltransferase was generated in order to observe the therapeutic value of the protein. The study demonstrated that the farnesyltansferase intervention is not required for Ras activation (Mijimolle *et al.*, 2005). In addition, different inhibitors have been generated against the farnesyltransferase protein, showing no clinical response.

8.2.7.3 Inhibiting downstream ras effectors

Although many attempts have been made in order to inhibit Ras protein activity, none of them have given good results. Fortunately, Ras signalling pathways, mainly those primarily involved in tumourigenesis, contain proteins as well as phospholipid kinases. All these proteins are amenable to the development of selective inhibitors. Indeed, a significant percentage of Ras downstream effectors have been found to be mutated in certain human tumours, thus providing valuable information as to which Ras signalling pathways are relevant to tumourigenesis. These pathways are the Raf/Mek/Erk and the PI3K/PTEN/Akt pathways. All these molecules, with the exception of the PTEN tumour suppressor, are kinases (García-Echeverria and Sellers, 2008). Currently, the pharmaceutical industry is actively pursuing the development of specific inhibitors for the related C-raf and B-raf kinases as well as for the Mek kinases (Sebolt-Leopold and Herrera, 2004). Indeed some of these inhibitors

have already been tested in clinical trials and a C-raf inhibitor, Sorafenib, has already been approved for the treatment of renal cell carcinoma (Sebolt-Leopold, 2008). Likewise, another promising set of therapeutic targets are the Cdks, which are also Ras downstream effectors.

8.2.8 *K-ras* oncogene mouse model

Advances in technology of genetic manipulation of mice, have recently enabled the design of models that reproduce the best physiological characteristics of human tumours. Those models mimic the nature of the mutations responsible for the initiation of tumourigenesis. In the early nineties, a new technological revolution was launched with the introduction of recombinases that recognize specific sites. These recombinases have been isolated from various viruses, phage and yeast. These enzymes recognize specific sequences (or sites) in the DNA and perform a recombination reaction that results, depending on their orientation, in the elimination of DNA between both sites. The recombinase system most commonly used is the Cre-LoxP system (including both, the Cre recombinase and DNA sites known as: "LoxP", of 34 bp).

Thus, advances in the field of mouse embryonic stem cells homologous recombination, has provided the development of conditional KI models. In those conditional models, the expression of the endogenous modified gene will be controlled by the recombinase system. The first mouse model generated by modifying the endogenous locus of *K-ras* (Johnson *et al.,* 2001) was obtained by introducing one or two copies of a mutated exon 1 (G12D). These mice developed multiple adenomas and lung adenocarcinomas. The activation of the

endogenous oncogene *K-ras*, in this model, is dependent on the rate of spontaneous intragenic recombination for each type of the somatic cell. This model, therefore, does not have a control system of activation.

In order to control the expression of *K-ras* oncogene, laboratories of Dr. Jacks and Dr. Barbacid developed, independently, a strain of mice carrying a mutated endogenous allele (mutations G12D and G12V, respectively), which remains silenced thanks to a transcriptional stop cassette flanked by two LoxP sites (LSL, LoxP - STOP-LoxP) that can be eliminated by the recombinase Cre (Jackson *et al.,* 2001; Guerra *et al.,* 2003).

In the model developed in the laboratory of Dr. Barbacid, an additional modification that allows the identification of the cells that express the oncogene but do not show morphological alterations was added. Thus, in the 3' non-coding region of the *K-ras* locus a cassette composed of β-geo chimeric gene, the fusion gene of bacterial β-galactosidase (LacZ), and the neomycine antibiotic resistance gene, preceded by a Internal Ribosome Entry Site (IRES) sequence is present. This modification allows the bicistronic expression (Mountford *et al.,* 1994) along with the K-ras ^{G12V} oncoprotein. For detection of β-galactosidase enzyme activity, the substrate Xgal (5-bromo-4-chloro-3-indolil-β-D-galactopiranoside) forms a blue precipitate, which allows us to identify cells that express the oncogene *K-ras*^{G12V}.

In the group of Dr. Barbacid, the *K-ras* KI model was crossed with a lineage, RERTn, developed in the laboratory by Dr. Campuzano (Guerra *et al.*, 2003).

The RERTn is expressed under the promoter of the RNA polymerase II, thus providing ubiquitous Cre recombinase expression (CreERT2, Brocard *et al.*, 1997). Cre recombinase is merged to the binding domain of the steroid hormone oestrogen receptor (ER), previously modified (ERT2) in a way that prevents their binding to endogenous steroids. The addition of certain synthetic steroid, 4-OH-tamoxifen (4-OHT), provokes the dissociation of the ERT2 to HSP40 at the cytoplasm, resulting in Cre recombinase to translocate to the nucleus. The cDNA of this Cre-ERT2 recombinase was introduced by homologous recombination in ES cells, preceded by the IRES sequence at the 3´-end of the gene that encodes the largest subunit of the *RNA polymerase II*, the enzyme responsible for the synthesis of messenger RNA. The addition of 4-OHT results in the postnatal expression of the *K-ras* ^{G12V} endogenous oncogene leads in all animals to the development of multiple adenomas and lung adenocarcinomas, the mice dying of respiratory problems. However, most of the different cell types tolerate the expression of endogenous physiological levels of oncogene *K-Ras* ^{G12V} without developing neoplastic lesions (Guerra *et al.*, 2003).

In the model of Dr. Tyler Jacks, the postnatal expression of the *K-ras*^{G12D} oncogene, activated in lung through intranasal instillation of a Cre encoding adenovirus, leads to the development of adenomas and carcinomas in the mice lungs (Jackson *et al.*, 2001).

8.3 Target validation of the G1/S Cdks

As we have shown above, the activation of Ras by growth factors promotes cell cycle progression by the synthesis of cyclin D. Studies have shown that Ras activation will remain through G1 phase in order to stimulate cells to enter into DNA synthesis. Ras acts in two different stages, a first stage immediately after addition of the mitogenic stimuli, and a second stage during mid G1 (Marshall, 1999).

The relationship between Ras activation and cell cycle progression is very tight and complex. It has been shown that Rb deficient cells have a lower proliferation rate, however this cell growth is no longer dependent on Ras signalling. Moreover, the block of Ras results in an inhibition of cell proliferation. Both observations show the importance of Ras in cell proliferation. But Ras activation is important in several stages of the cell cycle. First, Ras will activate the Erk/MAPK pathways, which is responsible for the synthesis of cyclin D, by the activation of the transcription factor complex AP1. Moreover, in order to maintain high levels of cyclin D, the activation of a second Ras dependent cascade is required (Malumbres and Barbacid, 2003). The PI3K is a post-transcriptional regulator of cyclin D. Cyclin D is degraded by the phosphorylation at Thr 286 by the GSK-3β protein, the inactivation of the GSK-3β by PKB provides a PI3 kinase and Ras- dependent way to stabilize cyclin D. In addition, PI3K together with mTor regulates the translation of the cyclin D mRNA (Marshall, 1999).

The use of a Ras dominant negative isoform has demonstrated that there also

exist a relationship between Ras activation and $p27^{Kip1}$ regulation. These studies showed that the downregulation of $p27^{Kip1}$ at the late G1 phase is Ras dependent. Moreover, studies with the *K-ras* KI generated in the laboratory of Dr. Jacks show an increase in Cdk2 protein levels in the embryonic fibroblasts derived from these mice and raised kinase levels for Cdk2 and Cdk4 (Tuveson *et al.*, 2004).

In conclusion, there are high expectations on the possibility to study the effect of the ablation of the different G1/S implicated Cdks in a tumour prone scenario. In addition, the validation of the Cdks as potential targets for human cancers in a $K\text{-}ras^{G12V}$ driven tumour context will be of high value due to the relationship between the different cascades.

9.Aims

The main aim of this thesis has been to determine the role placed by Cdk2, Cdk4 and Cdk6 as targets for lung cancer treatment. In order to achieve these aims we have used a *K-ras* KI mouse model that promotes the generation of lung adenomas and adenocarcinomas. To reach our goals we aimed to address the following objectives:

1. Assess the individual role of the G1/S Cdks in proliferation, immortalisation and transformation *in vitro* in a K-ras^{G12V} expressing background.

2. Determine the importance of Cdk2, Cdk4 and Cdk6 in the formation and development of K-ras^{G12V} induced lung tumours; and assess the impact of the G1/S Cdks lack in mice survival.

3. Understand the mechanism underlying the possible inhibition of tumour formation or development, obtained by the ablation of Cdk4.

4. Study the impact of abrogating Cdk4 in K-ras^{G12V} driven lung adenomas, mimicking a therapeutic approach.

5. Try to transfer the results obtained to humans by ablating the G1/S Cdks in human NSCLC cell lines.

10.Materials and Methods

10.1 in vitro Assays

10.1.1 Cell lines

MEFs	Mouse embryonic fibroblasts (Guerra *et al.*, 2003)
Phoenix E	Derived human embryonic kidney cells (DiCiommoze *et al.*, 2004)
293 T	Human embryonic kidney cells (Pear *et al.*, 1993)
293 A	Human embryonic kidney cells (Pear *et al.*, 1993)
A549	Human adenocarcinoma cell line (Phillips *et al.*, 2005)
H23	Human adenocarcinoma cell line (Phillips *et al.*, 2005)

10.1.2 Preparation of embryonic fibroblasts and cell culture conditions

Most of the *in vitro* experiments in this thesis are based on the use of MEFs (Mouse embryonic fibroblast) obtained from embryos at E13.5.

Obtaining MEFs

1. Pregnant mouse were sacrified using CO_2.
2. The embryos were extracted from the mother's uterus and put it into PBS+ P/E (GibcoBRL).
3. The embryos were separated from the yolk sac.
4. The embryo´s head were removed and placed it into an eppendorf tube, for further genotyping.
5. The liver was removed from the embryo.
6. The rest of the embryo was subjected to, a first mechanical breakdown with the help of a scalpel
7. Secondly to an enzymatic breakdown by treating each embryo with 3 ml of trypsin-EDTA (GibcoBRL) for 10 minutes at 37°C.

8. The cell suspension from each embryo was placed in a plate of 150 mm of diameter.
9. When it reaches to a 70% of confluence (2-3 days) cells were collected and diluted 1:2 in freezing medium (40%medium, 40%serum, 10% DMSO) and placed in cryotubes, which would be stored in frozen N_2.

Cells seeded in culture after thawing will be consider passage 1.

The MEF cultures were maintained in DMEM with 4500mg/l glucose (GibcoBRL) supplemented with antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin, GibcoBRL) and 10% foetal bovine serum (foetal bovine serum, FBS, Sigma).

For expression of the *K-ras*$^{+/LSLG12V}$ allele and/or complete ablation of allele conditional *Cdk2*$^{lox/lox}$ MEFs were treated with 4-OHT (600nM) from the moment they were set into culture and until the day they were frozen. Although the activation of oncogenic *K-ras* and the complete inactivation of *Cdk2* occurred 6 days after starting the 4-OHT treatment, we decided to continue the culture in 4-OHT presence to ensure that the generated cell lines were free from any K-ras^{G12V} expressing cells.

10.1.3 Immortalization assays

Immortalization tests were carried out in accordance with the protocol described by Todaro and Green in 1963 based on the serial culture of MEFs.

1. The MEFs of interest were removed from the liquid nitrogen storage.

2. The cells were thawed.
3. MEFs were tested for mycoplasma negativity.
4. In passage 2, 10^6 cells were seeded per 10 cm plate in DMEM with 4500 mg/l glucose (GibcoBRL) supplemented with antibiotics (penicillin and streptomycin) and 10% foetal bovine serum (foetal bovine serum, FBS), with or without 4OHT (600nM).
5. The cells were maintained in culture for 3-4 days.
6. The number of cells in the plate is counted, and 10^6 cells were replated into a new plate.
7. This process is repeated many times as it was necessary for cells to overcome the crisis period and acquire the ability to proliferate exponentially.
8. When cells have acquired these properties it is considered that the cells have sucessfully become immortal.
9. Cells were collected and diluted 1:2 in freezing medium (40%medium, 40%serum, 10% DMSO) and placed in criotubes, which would be stored in frozen N_2.

The graphs displayed in the result section represents on a logarithmic scale. The number of cells in each passage is quotient obtained by dividing the number of cells counted in a given passage between the number of cells seeded in the previous passage.

10.1.4 Growth curves

1. The MEFs of interest were removed from the liquid nitrogen storage.
2. The cells were thawed.

3. MEFs were tested for mycoplasma negativity
4. In passage 2, 10^6 cells were plated per 10 cm plate in DMEM with 4500 mg/l glucose (GibcoBRL) supplemented with antibiotics (penicillin and streptomycin) and 10% FBS, with or without 4OHT (600nM).
5. After 5 days 3 x 10^4 cells (primary or immortal, depending on the circumstances) were seeded per 6-well plates in duplicates (for each point and clone).
6. Cells were counted every three days from day 0 to day 21 in the case of the MEFs. The NSCLC human cell lines are counted every two days from day 0 to day 8.
7. Throughout this time the culture medium was changed twice a week to maintain the best possible proliferation conditions.

10.1.5 DNA transfection and viral infection

10.1.5.1 DNA transfection for retroviral production

1. Phoenix E cells were removed from the liquid nitrogen storage.
2. The cells were thawed.
3. Phoenix E cells were tested for mycoplasma negativity.
4. The human cells were amplified.
5. Phoenix E cells were seeded $8x10^6$ in DMEM-FBSi10% medium without Penicillin/Streptomycin.
6. The transfection of the virus to the Phoenix E cells was obtained by mixing 12 µg of the retroviral vector of interest (pBabe H-ras, pBabe K-ras, pBabe E1a, pBabe Cdk4, pBabe Cdk2) together with 10 µg of pCLEco and 30µg of lipofectamine in 1.5ml of DMEM.

7. The mixture was preserved for 20 min at RT.
8. 1.5 ml of mixture were added to the Phoenix E plate drop by drop.
10. The plates were incubated at 37ºC over night (o.n.)
11. The plates were changed to the 32ºC cell culture incubator and leave them at this temperature for 48 hours.
12. The supernatant was removed and centrifuged at 1500 rpm 5 min.

10.1.5.2 DNA Transfection for lentiviral production

1. 293T cells were removed from the liquid nitrogen storage.
2. The cells were thawed.
3. 293T cells were tested for mycoplasma negativity.
4. The human cells were amplified
5. 293T cells were seeded $8x10^6$ in DMEM-FBSi10% medium without Penicillin/Streptomycin.
6. The transfection of the virus to the 293T cells was obtained by mixing 10 µg of the lentivector of interest (pLKO.1 shRNACdk2, pLKO.1 shRNACdk4, pLKO.1 shRNACdk6) together with 3.9 µg of pLP1, 2.6 µg of pLP2, 3.3 µg of pLPVsVg and 30µg of lipofectamine in 1.5ml of DMEM.
7. The mixture was preserved for 20 min at RT.
8. 1.5ml of mixture were added to the 293T plate drop by drop.
9. The plates were incubated at 37ºC o.n.
10. The plates were changed to the 32ºC incubator and leave them at this temperature for 48 hours.
11. The supernatant was removed and centrifuged at 1500 rpm 5 min.

10.1.5.3 Cell Infection with retrovirus and lentivirus

1. 5ml were added of the virus supernatant to the appropriate plates cell along with polibrene (8 µg/ml-12 µg/ml).
2. The cells were incubated at 32°C for 12-16 hours.
3. The culture medium of the plates was changed and the cells were placed in 37°C cell culture incubators.
4. Within 36-48 hours depending on the antibiotic used, the selection process started.

Thus, in the presence of puromycin (2 µg/ml) (Sigma), 2 days were enough to be able to work with a cell population highly enriched in infected cells. Virus using hygromycin (50 µg/ml) (Calbiochem) or neomycin (400 µg/ml) (Calbiochem) for the selection, the duration of the selection was increased to nearly 5 days.

Finally cells were seeded for further cell culture tests or collected for submission to various biochemical tests.

10.1.6 Foci formation assay

1. The MEFs of interest were removed from the liquid nitrogen storage.
2. The cells were thawed.
3. MEFs were tested for mycoplasma negativity.
4. In passage 2, 10^6 cells were seeded per 10 cm plate in DMEM with 4500

mg/l glucose (GibcoBRL) supplemented with antibiotics (penicillin and streptomycin) and 10% FBS, with or without 4OHT (600nM).

5. After 5 days 1 x 10^5 cells (primary or immortal, depending on the circumstances) were seeded per 10 cm plates in duplicates (for each infection and clone).
6. 5ml of the viral supernatant containing the oncogenic combination formed by the tandem *H-ras*^{G12V} and *E1a* were added to the MEFs along with polibrene (8 µg/ml-12 µg /ml).
7. The day after transfection, the cell culture was divided 1:2 and maintained in culture over 3-4 weeks.
8. Those MEFs were left to grow exponentially in 10 cm plates.
9. After this time, small foci started to arise in the plates.
10. From each clone we had quadruplets and the four plates were stained with methylene blue.
11. The resultant colonies were quantified.

10.1.7 Protein extraction

1. Either tissues or cells were resuspendend in lysis buffer: 50 mM Tris-HCl (pH 7.4) solution, containing 150 mM NaCl, NP-40 0.5% and protease (PMSF 100mM, Roche) and phosphatase inhibitors (100mM vanadate sodium, Roche and 1 mM NaF, Sigma).
2. For tissue protein extraction we used the homogeniser to mechanically breakdown the tissue samples and maximise the efficiency of the lysis.
3. The protein extracts were sonicated for 5 to 10 seconds at 10% amplitude.
4. The samples were incubated on ice for 15 min.

5. Extracts were centrifuged for 12 min at 13000 rpm to remove the undigested extracts.

6. The supernatant was transferred to a new tube.

7. In order to quantify the amount of protein obtained, we used the Bradford method. (1μl of the protein extract was added to 1ml of Bradford reagent and the absorbance is read on a spectrophotometer).

8. Samples were store at -80ºC.

10.1.8 Western Blot

10.1.8.1 Gel Run

1. The amounts of total protein extracts needed to load the same concentration of whole cell extract was estimated (40-60 μg).

2. 5 μl of 4X Loading Buffer were added.

3. The samples were boiled for 1 min.

4. The samples were placed on ice for 2min.

5. A polyacrylamide electrophoresis gel was prepared (SDS-Page). The SDS-Page consists of two different gels:

Resolving gel:

- 2.5 ml of milli-q H_2O
- 3.75 ml Tris HCl 1M pH 8.8
- 3.1 ml 40% Acrylamide-Bis (BioRad)
- 100 μl of 10% SDS
- 500 μl of 10% APS (Sigma)
- 5 μl of TEMED (BioRad).

Stacking gel:

- 2.8 ml of milli-q H_2O
- 1.25 ml Tris HCl 1M pH 8.8
- 625 µl 40% Acrylamide-Bis (BioRad)
- 50 µl of 10% SDS
- 250 µl of 10% APS (Sigma)
- 4 µl of TEMED (BioRad).

6. The glass plates and spacer were assembled.
7. The resolving gel solution was poured.
8. The gel was sealed 1 ml H_2O-saturated 1-butanol.
9. When gel was set, the buthanol was poured off and rinsed with milli-q H_2O.
10. The stacking gel was poured and the comb was inserted immediately.
11. When the stacking gel was set, the comb was taken out and placed it into the western blot running cassette.
12. The running buffer was added into the cassette.
13. The samples were centrifuged and loaded them into the gel.
14. Also 10 µl of the molecular weight colour marker was added.
15. The gel was run with a constant voltage of 121 V for usually 1.5 hours.

10.1.8.2 Transfering the denatured proteins to a membrane

1. A piece of nitrocellulose transfer membrane was cutted as well as four pieces of Watmann paper.
2. The membrane and the papers were wet on transfer buffer.
3. The gel was also wet in the transfer buffer.
4. The transfer "sandwich" was assembled in a semi-dry blotter.

- Two Whatman papers- membrane- gel- two Whatman papers.

5. 1 gel was tranfer for 30 min at constant 0.35 A.
6. When the transfer was finished, we checked the amount of protein transfer by staining the membrane with Ponceau S solution (Sigma)

10.1.8.3 Blocking and Antibodies

1. The membrane was inmerse in the blocking solution (5% milk in 1X TBS-T) for 1 hour at RT.
2. The membrane was incubated with the primary antibody diluted in the blocking solution o.n. at 4ºC: Cdk1, Cdk2, CDK4, Cdk6, cyclin D2, cyclin A, cyclin E, p53, p21, p27, GADPH and actin.
3. The membrane was washed twice with 1X TBS-T.
4. The membrane was incubated with the appropriate secondary antibody diluted in the blocking solution for 1 hour at RT.
5. The membrane was washed twice with 1X TBS-T.
6. Protein visualization was carried out with ECL (Amersham) using different exposure times depending on the antibody.

10.1.9 Immunoprecipitation and kinase assay

1. Equal amounts of whole cell extracts were estimated.
2. The corresponding antibody was added in a 1:200 concentration (anti-Cdk1 (polyclonal, Santa Cruz), anti-Cdk2 (polyclonal, in-house), anti-Cdk4 (polyclonal, in-house), anti-Cdk6 (polyclonal, in-house), anti-Flag (monoclonal, Sigma)
3. Made up to 200 µl with the protein lysis buffer.

4. The mixture was set on a rotating wheel at 4°C o.n.

5. 120 µl of pre-washed protein A was added.

6. 2-3 hours of incubation on a rotating wheel at 4°C.

7. The samples were centifuged 13000 rpm for 1 min.

8. The supernatant was discarded.

9. 1ml was added of protein lysis buffer.

10. The samples were centrifuged and discarded the supernatant.

11. The washes were repeated twice more.

For immunoprecipitation assays:

a) 30 µl of 4X loading buffer (BioRad) was added.

b) Boiled 5 minutes

c) Centrifuged at 6000 rpm for 5 min.

d) The supernatant was loaded into a polyacrylamide electrophoresis gel (SDS-Page).

For kinase assays:

a) The last three washes were done in the Kinase Buffer (20mm Tris HCl pH 8, 10mM MgCl2, 1 mM EGTA, 1mM DTT).

b) The pellet was resuspended with:

- 0.5 µl of GST-Rb
- 1 µl 1mM cold ATP
- 17.5 µl Kinase buffer.
- 1 µl γ^{32} pATP.

c) The samples were incubated at 30 °C for 30 min.

d) 10 µl of 4X loading buffer (BioRad) was added.

e) The samples were boiled 5 minutes.

f) The samples were centrifuged at 6000 rpm for 5 min.

g) The supernatant was loaded into a polyacrylamide electrophoresis gel (SDS-Page).

h) The gel was dismatled from the western blot cassette and placed it in a PhosphorImager Cassette.

The time of exposure should vary from 4 hours to various days, depending on the antibody efficiency and the amount of protein present.

The results from the radioactive tests were analysed in the PhosphorImager scan.

10.1.10 Generation of the Cdk4 Kinase Dead (KD) isoform

(Using the QuickChange Site-Directed Mutagenesis Kit from Stratagene)

1. Primers containing the *Cdk4* kinase dead mutation were designed positioning the modification bases in the middle of the primers, with around 10 to 15 correct nucleotides at both sides:
2. The "mutagenic" reaction was prepare including:
   - 5-50ng of the original plasmid containing the WT *Cdk4*.
   - 5 μl of the reaction buffer.
   - 125ng of each primer.
   - 1μl of dNTPs mix.
   - 1 μl of PfuTurbo DNA polymerase.
   - up to 50 μl of milli-q H_2O
3. An additional control reaction was preparewith the provided pWhitescript plasmid.

4. Both reactions were place in the PCR machine.
5. The reaction was placed for 2 minutes on ice.
6. 1 μl of the restriction enzyme DPN I was added to the reaction, in order to digest the methylated (non mutated) parental DNA template.

10.1.10.1 Transformation

1. The supercompetent cells were thawn on ice.
2. 1 μl of the template DNA was added.
3. The reaction was incubated 30 minutes on ice.
4. Heat pulse the transformation reaction for 45 seconds at 42ºC, and then placed the reaction 2 minutes on ice.
5. 500 μl of LB medium was added and the reaction was incubated for 1 hour at 37ºC.
6. 100 μl of the final reaction was plate on agar plates containing the appropriate antibiotic for the plasmid vector.
7. The transformation plates were incubated at 37ºC o.n.
8. Colonies were picked and sequenced to identify the clones of interest.

10.2 *in vivo* experiments

10.2.1 Maintenance and genotyping of mice

All animals used in this project were been maintained at the animal facility of the CNIO (Spanish National Cancer Centre) according to the FELASA (Federation of European Laboratory Animal Science Association) recommendations and following the European Union legislation. All the experiments described in this thesis had been previously approved by the

Bioethics and Animal Welfare Committee of the Institute for Health Care Carlos III. At the CNIO animal facility the daytime cycle comes from fluorescent lamps that emit a white light (TLD 36W/840 and TLD 58W/840).

In all the colonies of mice that are described throughout this thesis, consisted of two groups of prominent individuals. A first group denominated aging, these mice are designed to study the survival of the colony and are comprised by an equal number of males and females, and a second group, composed of young breeding animals, responsible for the maintenance and/or expansion of the colony as well as producing embryos, which will be used for MEFs extraction.

10.2.1.1 Necropsy

1. The mouse was sacrified in CO_2 chamber.
2. Various tissue samples were collected (muscle, skin, liver, pancreas, spleen, kidney, adrenal gland, colon, stomach, gonads, lung, heart, thymus, white fat, brain, pituitary, eyes and bone samples are most commonly collected).
3. The samples were fixed them in formalin.
4. Samples were processed, cutted and stained with Hematoxylin and Eosin (H&E) by the comparative pathology unit.

Colon, stomach, pancreas and lung would also be stained with Xgal, and so that they were also fixed with the adequate solution. (see section 9.2.6.1)

A lung lobule was also frozen in OCT, and stored at -80ºC, for future cryostat processing.

10.2.1.2 Genotyping

The genotyping of mice were routinely made by extracting DNA from the mice tails.

1. 490μl of lysis buffer was added (20mM Tris/HCl pH8.0, 100mM NaCl, 0.5% SDS, 10mM EDTA pH8.0 and milli-q H_2O) and 10μl of proteinase K to each tail, and left o.n. at 55ºC.
2. 300μl of saturated NaCl was added.
3. The solutions was mixed and left at 4ºC 10 min
4. The sample was centrifuged at 13000rpm for 10 min.
5. The supernatant was place in a new eppendorf.
6. 800μl of isopropanol was added and mixed vigorously.
7. The mixture was left at -20ºC for at least 1 hour.
8. The sample was centrifuged at 13000 rpm for 10 min.
9. The supernatant was discarded.
10. 400 μl of 70% ethanol (EtOH) was added
11. The sample was centrifuged at 13000 rpm for 10 min.
12. The supernatant was discard.
13. The pelletwas left to dry.
14. The pellet was resuspended in 100 μl of milli-q H_2O

Once the DNA was isolated we can perform the genotyping of the mice using polymerase chain reaction (PCR) technology. Adding for each reaction:

- 1 μl $MgCl_2$
- 2 μl of Buffer Solution.

- 1.5 μl of dNTPs.
- 1 μl of each of the primers.
- 1 μl of DNA
- Fill up to 20 μl with milli-q H_2O.

The genotyping of the *K-ras*^{G12V} KI allele was done by the amplification of sequence using the primers:

Kras2F_16B5: 5´ CGTCCAGCGTGTCCTAGACTTTA 3'

Kras2r_15B9: 5´ ACTATTTCATACTGGGTCTGCCTT 3'

NeoF_2B1: 5´ TGACCGCTTCCTCGTGCTT 3'

The *K-ras*^{G12V} KI allele will be amplified by the use of the primers Kras2r_15B9 and NeoF_2B1, obtaining a 390bp fragment. The wildtype allele will be amplified by the primers Kras2F_16B5 and Kras2r_15B9, obtaining a 240bp amplified sequence.

For genotyping of *K-ras*^{lox} KO alleles the oligonucleotides used were:

Kras2F_8B2: 5' CCACAGGGTATAGCGTACTATGCAG 3'

3'Ex1: 5' CTCAGTCATTTTCAGCAGGC 3'

These combination of the primers will amplify the wildtype allele, 550bp fragment, and the conditional KO allele, 350bp sequence.

The *Cdk2* germline null locus and the conditional *Cdk2*^{lox} KO allele, were both amplified by the use of the oligonucleotides:

Cdk2F_3B9: 5' GAAGACCCTCCAGGTGAATGAA 3';

Cdk2R_3B8: 5' CAAGTTGACGGGAGAAGTTGTG 3' and

IN3R3: 5' GCGATAAGCTTCGAGGGACC 3'.

The combination of the Cdk2F_3B9 and the Cdk2R_3B8 primers will amplify the wildtype allele, resulting in a 560 bp fragment. The Cdk2-/- allele is amplified by the use of Cdk2R_3B8 and IN3R3, resulting in a 360bp sequence. Finally, the Cdk2 lox allele is amplified by the use of the Cdk2F_3B9 and the Cdk2R_3B8 oligonucleotides and provides a 660bp fragment.

The genotyping of *Cdk6* null allele was done by the use of the primers:

Cdk6r_4B1: 5' ATTGGCAAACACATTCTTACACATT 3'

Cdk6F_4B3: 5' CGCGAGTGCGACTCCCC 3'

PGKr_2B2: 5' GCCTACCCGCTTCCATTGCT 3'

The wildtype allele will be identified by the amplification of 700bp fragment by the use of the primers Cdk6r_4B1 and Cdk6F_4B3. The combination of the Cdk6r_4B1 and the PGKr_2B2 oligonucleotides will result in the amplification of the KO allele, resulting in a 530bp fragment.

The genotyping of *Cdk4* null mice was preformed by use of the oligonucleotides:

Cdk4f_8B9: 5' GTAGCTGTGGGCAGGCTGTAGT 3'

NeoF_2B1: 5' TGACCGCTTCCTCGTGCTT 3'

MM 266: 5' CGGAAGGCAGAGATTCGCTTAT 3'

The *Cdk4*$^{-}$ allele was amplified by the primers NeoF_2B1 and MM 266, giving a 270bp fragment. The wildtype *Cdk4* allele was amplified by the primers Cdk4f_8B9 and MM 266, resulting in the amplification of a 180bp fragment.

In addition, the *Cdk4*^{frt} conditional KO allele was genotyped by the use of the primers: For the wildtype allele reaction, we will use the pDC1 and the pDCWC primers.

pDC1: 5' TCTTTGTGCCTAGTGCGATG 3'

pDCWC: 5' ATGATCACCAGCTAGTCGTC 3

pDCC4: 5' TCTAGAGGATCATAATCAGCC 3'

The combination of these oligonucleotides will result in a 630bp amplification product. The conditional Cdk4 KO allele will be genotyped using the primers pDC1 and pDCC4. The resultant amplified fragment will be of 350bp.

For genotyping the *RERT* KI allele, the oligonucleotides used were:

Polr2aR_10B6: 5' CCTCTCTGAGCCTCAATTAAGCAG 3'

ESRF1f_10B7: 5' TGAGTAACAAAGGCATGGAGCA 3'

Polr2aF_14B5: 5' CCAGATGACAGCGATGAGGA 3'

The combination of the primers Polr2aR_10B6 and Polr2aF_14B5 results in the amplification of the wildtype fragment (480bp) while the oligonucleotides Polr2aR_10B6 and ESRF1f_10B7 amplifies the KI allele, resulting in a 390bp sequence.

10.2.2 Tamoxifen (4-OHT) treatment

The 4OHT has a steroid type chemical structure, so it is only soluble in organic solvents. These solvents can never be used as a vehicle in the intraperitoneal administration due to the significant toxic effects that they trigger. For the administration of 4-OHT, the steroid is dissolved in corn oil.

1. 50mg of 4-OHT (Sigma) were dissolved in 10ml of corn oil (Sigma).

2. The mixture was sonicated until the 4-OHT has been dissolved. (2 min at 42% amplitude)

3. 200µl of the dissolved 4-OHT were injected via intraperitoneal to each mouse.

The duration of the treatment varied depending on the mouse strain. The *Cdk2* conditional KO mice were subjected to a 4 month treatment, injecting twice a week. The *Cdk2*, *Cdk4* and *Cdk6* germline KO animals were treated for two weeks with two injections per week. While the *Cdk4* conditional KO mice were injected three times in two weeks.

10.2.3 Adenovirus amplification and purification

10.2.3.1 Amplification

1. The 293A were removed from the liquid nitrogen storage
2. The cells were thawed.
3. 293A cells were tested for mycoplasma negativity.
4. The cells were amplified.
5. 30 p150 plates with 293A cells were infected with 30 µl of AdenoFlp/GFP with a MOI: $12x10^{10}$.
6. Infected cells were incubated in specific virus incubators at 37ºC.
7. 3 days after the infection all the human 293A cells were generating the virus (the 293A infected cells loose adherence to the plate). The medium was collected and centrifuged at 1500 rpm for 5 minutes.
8. The supernatant was discarded and the pellet is frozen at -80ºC.

10.2.3.2 Purification

1. The pellet was thawed.
2. Pellet was resuspened in 15ml 0.1M Tris/HCl pH 8.0
3. 1.5ml of 5% Na-Deoxycholate were added.
4. The saple was mixed and incubated it 30 min at room temperature (RT)
5. 150 μl of 2M $MgCl_2$ and 75 μl of DNaseI solution were added.
6. The saple was mixed and incubated 30-60 min at 37ºC.
7. The saple was centrifuged at 4000 rpm 15 min at 5ºC.
8. The gradient was prepared in the Beckman tubes.
    - 0.5ml CsCl (Roche) 1.5 density solution.
    - 3ml CsCl (Roche) 1.35 density solution.
    - 3ml CsCl (Roche) 1.25 density solution.
9. 5ml of the viral supernatant was added to each gradient tube.
10. The saple was centrifuged in the ultracentrifuge with the rotor SW40 Ti, for 1 hour at 35000 rpm 10ºC.
11. The sample was carefully left the gradient flow and only retain the viral band (white) in a new Beckman tube.
12. The tubes containing the virus were filled with CsCl 1.35 density solution.
13. The saple was centrifuged in the ultracentrifuge, using rotor SW40Ti, o.n. at 35000rpm 10ºC.
14. The sample was carefully left the gradient flow and only retain the viral band (white) in a new tube.
15. The virus was injected in a dialysis cassette.

16. The sample was dialysed for 24 hours in 10mM Tris/HCl pH 8.0.

17. The virus was taken out from the dialysis cassette and added glycerol to the virus to get a final concentration of 10% of glycerol.

18. The virus was stored at -80ºC.

10.2.4 Adenovirus delivery

Mice were infected with Ad-FLP and Ad-GFP (80 μl MOI: $3x10^{10}$) into the caudal vein in the mice tail. Seven days post Ad-FLP infection, the excision of the conditional allele was optimal, and this level of excision was maintained during the lifespan of the mice.

10.2.5 Diabetes Inducement protocol

1. The Sodium Citrate 0.9% NaCl pH:4.5 solution was prepared. This solution can be stored at 4ºC
2. Steptozotocine was dissolve in Sodium Citrate 0.9% NaCl pH:4.5 to a final concentration of 10 mg/ml.
3. The streptozotocine was injected via intraperitoneal during 5 consecutive days, injecting 0.5mg of streptozotocine (Sigma) for each 10 gr of weight. Control animals were treated with only the vehicle solution.
4. The diabetes development was monitored by Blood glucose analysis:
    - The tail of the mouse was cutted.
    - Few μl of blood obtained were introduced into the GlucoMed Vision Sensor.
    - The blood glucose was measured in mg of glucose/ dl of blood.
    - We considered a mouse diabetic when the value exceeded

190mg/dl.

- Animals were also weighed each month during the experiment using a top-loading balance.

10.2.6 Xgal staining

10.2.6.1 Whole Mount Staining

1. During the necropsy of the mice, colon, stomach, pancreas and lungs were obtained.
2. The tissues were fixed at RT for 30-90min in:
   - 0.2% glutaraldehyde (Sigma).
   -1.5% formalin solution (Sigma).
   - 2mM $MgCl_2$.
   - 5mM EGTA.
   -100mM sodium phosphate pH7.5
   - milli-q H_2O
3. The tissues were washed 3 times in the washing solution:
   - 0.2% NP-40.
   - 0.1% sodium deoxycolate (Sigma).
   - 2mM $MgCl_2$.
   -100mM sodium phosphate pH7.3 solution
   - milli-q H_2O

   For 20 min at RT.
4. Following the washes the tissues were stained for 48 hours at 37ºC in the staining solution:
   - 0.2% NP-40.

- 0.1% sodium deoxycolate (Sigma).
- 2mM $MgCl_2$.
-100mM sodium phosphate pH7.3 solution
- 5mM $K_3Fe(CN)_6$ (ProLab).
- 5mM $K_4Fe(CN)_6$ (ProLab).
- 1mg/ml Xgal (solved in dimethylformamide)
- milli-q H_2O

5. The tissues were washed 3 times and for 10 min with the above mentioned wash solution.
6. The samples were postfixed o.n. in phosphate buffered 4% formaldehyde.
7. The fixed and stained tissues werewashed twice with 1X PBS for 5 min.
8. The tissues were washed twice with 50% EtOH, and twice with 70% EtOH.
9. At this point the tissues were processed by the comparative pathology unit, which produce the standard 4μm sections on a 3-aminopropyltrethoxylaan coated slides and counter stained with Nuclear Fast Red.

10.2.6.2 Cryostat section staining

10.2.6.2.1 Cryostat sections

1. The frozen samples were take out from -80ºC.
2. The samples were cutted in the cryostat at a measure of 10 microns.
3. The cut samples were placed in a slide.
4. Slides should be stored at 4ºC o.n. for further processing.

10.2.6.2.2 Xgal section staining

1. Sections were washed with PBS 1X

2. Sections were fixed for 10 min at RT in the solution:

- 0.2% glutaraldehyde (Sigma)
- 5mM EGTA.
- 2mM $MgCl_2$
- 0.1M phosphate buffer pH7.3
- milli-q H_2O

3. The sections were washed three times with:

- 2mM $MgCl_2$
- 0.1M phosphate buffer pH7.3
- 0.02% NP-40
- 0.01% sodium deoxycolate (Sigma)
- milli-q H_2O

4. When the washes were finished the sections are stained o.n. at 37ºC with:

- 2mM $MgCl_2$
- 0.1M phosphate buffer pH7.3
- 0.02% NP-40
- 0.01% sodium deoxycolate (Sigma)
- 1mg/ml Xgal
- 5mM $K_3Fe(CN)_6$ (ProLab).
- 5mM $K_4Fe(CN)_6$ (ProLab).
- milli-q H_2O

5. The sections were washed 3 times with the washing solution previously described.

6. The counter-staining of cryostat sections was performed with nuclear fast red (NFR) protocol.

10.2.6.2.3 Nuclear Fast Red

1. The NFR solution was prepared:
    - 25gr. Aluminium sulphate (Sigma)
    - 0.5gr NFR (Sigma)
    - 500 ml miliqH_2O.
    - Boil the solution for 2-3 min.
    - Filter the solution.
2. The samples were washed with H_2O
3. The sections were stained with NFR for 1.30 min.
4. The sections were wash with H_2O
5. 2 washes of 2 min with 70% EtOH.
6. 2 washes of 2 min with 100% EtOH.
7. The sections were rinsed with Xilol (Merck) for 1 min.
8. The sections were mounted with the coverslip.

10.2.6.2.4 Senescence associated β-gal staining

1. The 10μm sections were left at 4ºC o.n. before we can start the senescence associated β-gal staining protocol.
2. The senescence associated β-gal staining was observed by following the Cell Signaling senescence β-galactosidase staining kit.
    - The slides were washed once with 1X PBS
    - The cells were fixed with 2 ml of 1X Fixative solution for 10-15 minutes at RT.

- While the slides were in the Fixative solution, prepare the Staining solution.

a) 1860 ml of 1X Staining solution

b) 10 ml of 10mg/ml of $K_3Fe(CN)_6$.

c) 10 ml of 10mg/ml of $K_4Fe(CN)_6$.

d) 50 ml of 20mg/ml of Xgal in DMF.

- The slides were washed twice with 2ml of 1X PBS.

- 2ml of the Staining solution were added to the slides. Incubate o.n. at 37ºC.

- The slides were washed with 2ml of 1X PBS.

- The cells were checked under a microscope for development of blue colour.

3. The sections were counter stained with Nuclear Fast Red.

10.2.7 Immunofluorecence

1. The sections were deparaffinized.

2. The deparaffinized sections were then submitted to the antigen retrieval protocol:

- the sections were treated with sodium citrate 10mM (pH 6) at 95ºC for 20 minutes.

- the samples were submitted to a 15 minutes treatment at RT with 3% hydrogen peroxide diluted in methanol to eliminate the endogenous peroxidase activity of the tissue.

3. The samples were permeabilised for 30 min at RT with:

- 0.4% Triton

- 0.04% SDS

- up to 15 ml of 1X PBS.

4. The slides were washed twice with 1X PBS.
5. The slides were blocked for 30- 45 min in 5% BSA 45 min at 37ºC.
6. The primary antibodies was diluted in 5% BSA (SP-C 1:1500 Neomarkers, CC10 1:1500 Santa Cruz) and leave it on the sample 1 hour at 37ºC.
7. The slides were washed twice with 1X PBS.
8. The secondary antibodies was diluted in 5% BSA. (Alexa Fluor 555 donkey anti-goat 1:200, Alexa Fluor 488 goat anti-rabbit 1:200) at 37ºC for 1 hour.
9. The first wash was done with diluted DAPI 1:1000 in 1X PBS.
10. Two additional washes were performed with 1X PBS.
11. The slides were mounted in mowiol and the coverslip.
12. The slides were ready to be analysed by the confocal microscope SP5-MP.

10.2.8 Southern blot

10.2.8.1 DNA extraction

1. 490μl of lysis buffer was added (20mM Tris/HCl pH8.0, 100mM NaCl, 0.5% SDS, 10mM EDTA pH8.0 and milli-q H_2O) and 10μl of proteinase K (20mg/ml) to each tail, and incubate it o.n. at 55ºC.
2. 500 μl of Phenol
3. 20 min at RT on a rotating wheel.
4. The sample was centrifuged 13000 rpm for 5 minutes.
5. The supernatant was placed into a new eppendorf tube.
6. 500 μl of isoamylic phenol/chloroform was added.
7. 20 min at RT on a rotating wheel.

8. The supernatant was placed into a new eppendorf tube.
9. 500 μl of chloroform was added.
10. The sample was centrifuged 13000 rpm for 5 minutes.
11. The supernatant was placed into a new eppendorf tube.
12. 30 μl of Sodium Acetate (Sigma) was added.
13. 1ml of cold 100% EtOH was added.
14. The sample was left at -80ºC 4 hours.
15. The sample was centrifuged 13000 rpm for 5 minutes.
16. The supernatant was discarded.
17. The pellet was left to dry.
18. The pellet was resuspended in 50 μl of miliqH_2O

10.2.8.2 Digestion

1. 1 μl of the DNA was added into 500 μl of miliqH_2O
2. The DNA concentration was measured in a spectrophotometer at a wavelength of 595nm.
3. The volume of total DNA sample needed to have 20 μg of DNA for each sample was estimated.
4. Were added:
    - 3 μl of the restriction enzyme (BamHI, New England, for the Cdk2 southern blots and SSPI, New England, for the Cdk4 Souther Blots).
    - 1 μl of RNase, DNase-free (Roche).
    - 4 μl of spermidine (Sigma).
    - 4 μl of the appropriate restriction enzyme buffer.

Up to 40 μl of milli-q H_2O.

5. The digestion was left at 37ºC o.n.

10.2.8.3 The Gel

1. A gel 1% of agarose was made in 1X TAE.
2. The samples and the molecular weight marker, λ-HindIII were loaded in the gel.
3. The gel was run at 100V.
4. When the run was finished the gel was putted in a 0.25N HCl solution for 10 min.
5. The gel was washed with miliqH_2O.
6. The denaturing solution (1.5 M NaCl/0.5 N NaOH) was added to the gel and left it for 20 min.
7. The gel was washed with milli-q H_2O.
8. The neutralising solution (1.5 M NaCl/0.5 M Tris-HCl pH 7.4) was added to the gel and left it for 20 min.
9. The gel was washed with milli-q H_2O.

10.2.8.4 Setting the Blot

1. A double layer of Whatman paper (fits size of the gel) was laid onto the transfer tray, it should reach the buffer reservoir on both sides.
2. The gel was placed on the Whatman paper. Parafilm was stretch across three sides of tray surrounding the gel to prevent paper from wicking anywhere else but through the gel.
3. A nylon membrane was put on the gel.

4. 2 layers of Whatman paper were placed on top of it.
5. ~1 L of 10X SSC buffer was poured into the tray.
6. ~10-20 cm layers or 2/3 of a pack of paper towels were added (the white ones are the better soakers).
7. A weight was placed on top.
8. The transfer time should be 12-24 hours (o.n.).

10.2.8.5 Crosslinking

1. The membrane was rinsed with 2X SSC.
2. UV cross-linked the membrane.
3. The membrane was inserted into a hybridisation tube.
4. 14ml of hybridisation solution was added.
5. The tube was inserted into the oven at 42°C and leave the membrane to pre-hybridise for 4 hours.

10.2.8.6 Probe

1. 50ng of the probe and of the λ-HindIII marker were added, and taken it up to 45 μl of miliqH_2O.
2. The samples were boiled for 3 min.
4. The samples were put in ice for 5 min.
5. The probes were resuspended in a ReadyPrime (Amersham) tube.
6. 5 μl of α-p^{32}-dCTP was added.
7. The probe mixtures were putted at 37°C for 30 min.
8. The probe was added to column.
9. The sample was centrifuged 1500 rpm 2 min.

10. The supernatant 5 μl of NaOH 5N was addded.
11. The probe mixture was added to the hybridisation buffer that was on the membrane tube.
12. The hybridisation time should be 12-24 hours.

10.2.8.7 Washes and exposure

1. The hybridisation buffer was discarded with the probe and washed the membrane firstly with 2X SSC/ 0.1%SDS for 20 min at 42ºC.
2. A second wash was perfomed with 1X SSC/ 0.1%SDS for 20 min at 42ºC.
3. A third wash with 0.1X SSC/ 0.1%SDS for 20 min at 42ºC.
4. The membrane was taken out from the hybridisation tube and placed it in an PhosphorImager Cassette.
5. The time of exposure should go from 4 hours to various days, depending on the probe signal.
6. The results from the readioactive tests were analysed in the PhosphorImager scan.

The *Cdk2* probe was located between exons 4 and 5, and will generate up to a total of 3 fragments with sizes of 15, 3.9 kb and 2.6 kb, corresponding to the wildtype alleles, conditional and deleterious respectively.

The *Cdk4* probe was localised between exons 2 and 3 and will potentially also generate 3 fragments of 4.3, 4.7 and 3.5 kb. The sizes of the detected fragments correspond to the wildtype, conditional and deleterious allele, respectively.

10.2.9 Histopathology and immunohistochemistry

Tissues including, heart, intestine (small and large), kidney, liver, lung, mammary gland, ovary, pancreas, pituitary, prostate, skeletal muscle, skin, spleen, stomach, thymus, testis, urinary bladder and uterus are dissected, were fixed in 10%-buffered formalin (Sigma) and embedded in paraffin. Three- or five-micrometer-thick sections are cut and stained with hematoxylin and eosin (H&E). Antibodies used for immunohistochemistry included mouse monoclonal anti-Xgal (1:200 dilution, Neomarkers), rabbit polyclonal anti-Cdk4 (1:25 dilution, Santa Cruz); mouse monoclonal anti-Cyclin E (1:25 dilution, Neomarkers); rabbit polyclonal anti-Cdk6 (1:25 dilution, Santa Cruz); rabbit polyclonal anti-p53 (1:25 dilution, Santa Cruz) and rabbit polyclonal anti-p21 (1:25 dilution, Santa Cruz). For detection of β-galactosidase activity in adult tissues, samples were included in O.C.T.^{TM} compound (Sakura) and frozen. Xgal staining of 10 μm thick cryosections was performed as described (9.2.6.2).

10.2.10 Tumour Quantification

In order to estimate the number of tumours in the mice lung we did serial sections of two lobules of the mouse and stained with the β-Gal associated with the K-ras^{G12V} expression. We observed that there was a high correlation between the number of tumours estimated by section with the complete serial section and the number obtained by counting the first (section 1) the middle (around section 50) and the final (around section 100). So that, we performed serial sections of the lungs and analysed the tumours (number and aggressiveness) in the three sections. The pathologist counted the number of

lesions and the aggressiveness of them, observing form hyperplasias to adenocarcinomas.

10.2.11 K-ras^{G12V} expression and senescence quantification

The quantification of the stained sections to see both oncogenic K-ras expression or senescent cells was performed by counting the total amount of cells in the section over the number of either SA β-gal positive or K-ras^{G12V} expressing cells respectively. From the data obtained we performed a percentage of stained cells over the total number of cells in the lung section.

11.Results

11.1 *In vitro* studies

11.1.1 Proliferative advantage of *K-ras*^{G12V} MEFs is eliminated in the absence of individual interphase Cdks

In order to evaluate the contribution of the interphase Cdks to the various phenotypes triggered by oncogenic *K-ras* we took advantage of an inducible *K-ras*^{G12V} knock-in strain (Guerra *et al*, 2003). It carries an oncogenic point mutation in the endogenous *K-ras* locus that is maintained silenced by a Lox-Stop-Lox cassette that inhibits the transcription of the *K-ras* gene when there is no recombination, and thus designated as *K-ras*^{LSLG12V}. In addition, it carries a colour marker in the form of an *IRES-β-geo* sequence inserted at the 3´untranslated region of the targeted *K-ras* allele. Upon Cre-recombinase-mediated excision of the Stop cassette, the oncogene is expressed together with the colour marker from a single bicistronic transcript. The allele resulting from this activation event will be referred to as *K-ras*^{LG12V}. In order to mediate the cleavage of the Stop cassette all compound strains carry an inducible Cre-ERT2 recombinase under the control of the locus encoding the large subunit of the RNA polymerase II (Guerra *et al*, 2003). We then established independent colonies by crossing *K-ras*$^{+/LSLG12V}$;*RERT*$^{ert/ert}$ animals with mouse strains deficient for Cdk2, Cdk4 or Cdk6 (Ortega *et al*, 2003; Malumbres *et al*, 2004; Barriere *et al* 2007).

11.1.1.1 Growth in limiting serum conditions

Our first test was to examine whether the single ablation of the G1/ S Cdks could have any effect on the proliferation properties conferred by the expression of the oncogenic *K-ras*^{G12V}. We took advantage of the primary mouse embryonic fibroblasts (MEFs) coming from the different Cdk deficient strains and expressing the conditional *K-ras*^{G12V} oncogene were seeded in low serum concentrations. It has been published, that although a primary wildtype culture is unable to grow under limiting serum conditions, cells expressing endogenous levels of oncogenic *K-ras* show enhanced proliferative properties and are able to grow in 2% serum (Tuveson *et al*, 2004). *K-ras*$^{+/LSLG12V}$; *RERT*$^{ert/ert}$ MEFs lacking a single G1/ S implicated Cdk (with the appropriate control) were grown for five days in the presence of 4-OHT in order to activate the oncogene and subsequently seeded in 2% serum. As expected, *K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ MEFs were able to grow under low serum conditions (Figure 13 blue symbols). Importantly, this growth advantage was suppressed in the absence of any of the three interphase Cdks (Figure 13 orange symbols). These results clearly show that the elimination of a single Cdk from an oncogenic K-ras context will eliminate the proliferative advantage conferred by the oncogene expression. This was the first evidence we observed on the important role that the Cdks may have in an oncogenic *K-ras* expressing context. If the *K-ras*^{G12V} cells lacking any Cdk could not undergo the first stages of tumour formation we could be dealing with a model which could help us to attack *K-ras*^{G12V} tumours by ablating the interphase Cdk proteins. Thus, these data highlight the importance of G1/S Cdks in driving cell cycle in oncogenic K-ras^{G12V} activated cells.

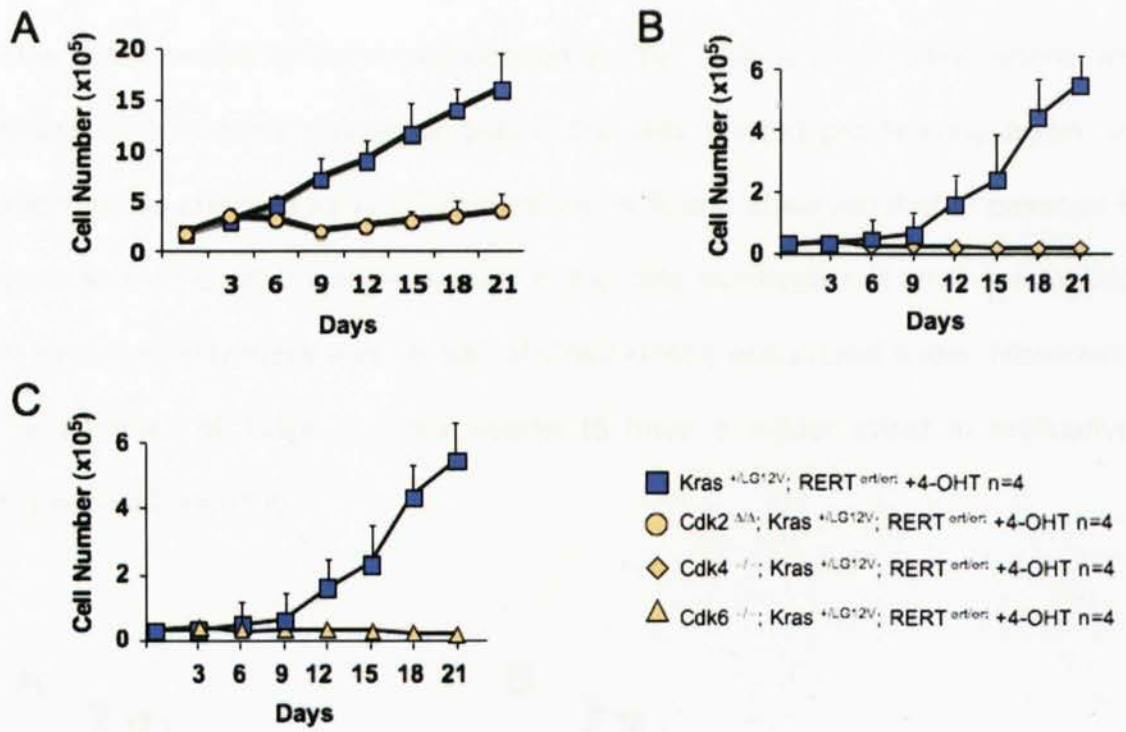


Figure 13: Growth of primary MEFs in limiting serum conditions. In A we can see how the elimination of Cdk2 has a very drastic effect in the ability of oncogenic K-ras expressing cells to grow in 2% foetal serum. In B and C we observed the same effect in the absence of Cdk4 and Cdk6, respectively (n=4 for each genotype).

11.1.1.2 Immortalisation

The proliferative advantage conferred by the expression of K-ras ^{G12V} mutation was further evaluated by submitting the E13.5 isolated MEFs to the classical 3T3 immortalization protocol (Todaro and Green, 1963). In 10% foetal serum cell culture medium, wildtype MEFs undergo a proliferative crisis, known as replicative senescence, that takes place after few passages in culture. In contrast, cells expressing endogenous levels of the activated oncogenic *K-ras* allele, proliferate continuously as immortal cells bypassing the crisis period (Guerra *et al*, 2003; Tuveson *et al*, 2004). This effect of continous proliferation has also been observed in MEFs lacking p53 (Finlay, 1992). Interestingly, the absence of individual interphase Cdks (Figure 14 blue symbols) *Cdk2, Cdk4* or *Cdk6*, reinstates the crisis period, which was bypassed by the expression of

oncogenic *K-ras* in MEFs with wildtype Cdk activity (Figure 14 orange symbols). The crisis period is more pronounced in the cells lacking Cdk2 where we observed four crisis passages before the cells started proliferating again. In addition, we checked for Cdk2 deletion by PCR and observed that at passage 6 there was no *Cdk2* lox allele present in the cells genotype and when we looked at kinase activity there was no sign of Cdk2 kinase associated levels. Moreover, the absence of *Cdk6* or *Cdk4* seems to have a milder effect in replicative senescence induction.

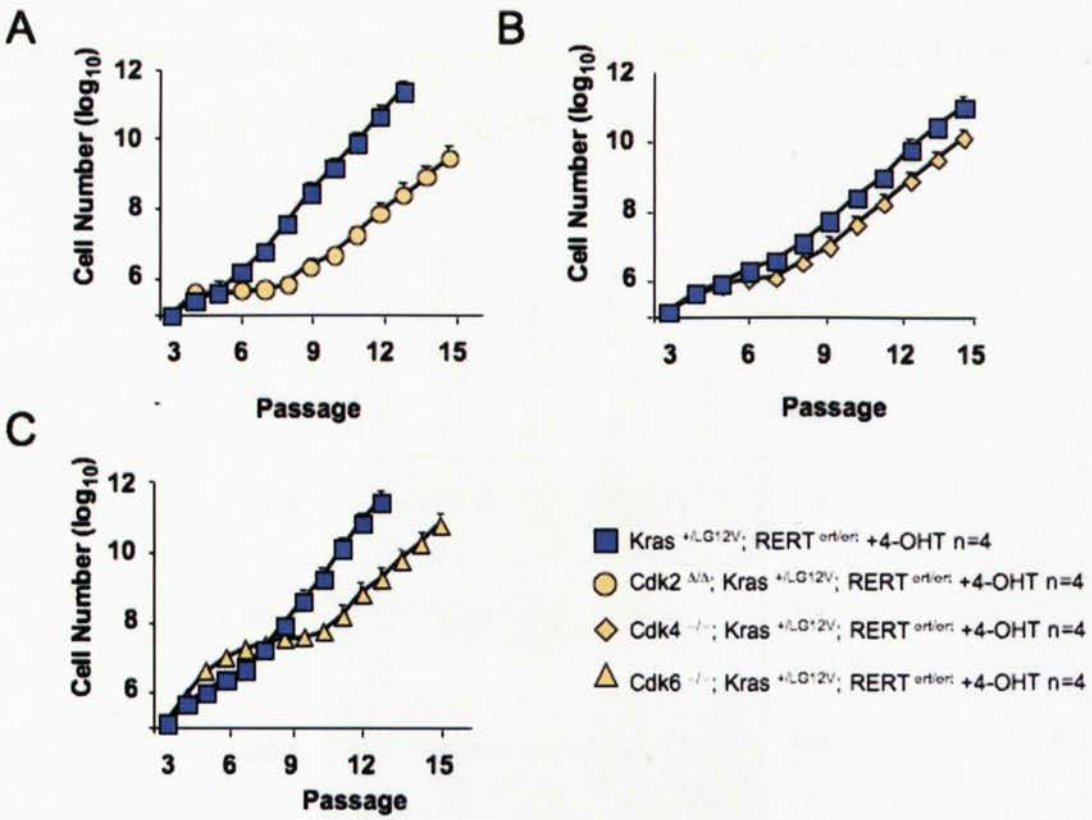


Figure 14: Immortalisation. The depletion of Cdk2 has a pronounced effect on the immortalisation properties of the K-ras ^{G12V} expressing MEFs, by restoring the crisis period and decreasing their proliferation rate (A). When MEFs expressing the oncogenic mutation are defective on Cdk4 we observe a mild crisis period (B). Cdk6 null MEFs expressing the oncogenic K-ras protein also restore the crisis period, although it is observed in later passages (C) (n=4 for each genotype).

These results show the importance of the absence of a single interphase Cdk in continuously proliferating cells. Thus, the ablation of *Cdk2*, *Cdk4* or *Cdk6* reinstalls the senescence crisis that should have been abolished by the expression of the endogenous K-ras oncogene.

11.1.1.3 Kinase Assays and Western Blots

The *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ cells had the most pronounced effect in decreasing the proliferation capabilities conferred by the *K-ras* oncogene. In order to look deeply into the compensation role that could take place when Cdk2 was absent, we analysed if any cell cycle Cdks was compensating for the lack of *Cdk2*. For this purpose, we analysed the kinase levels of Cdk1, Cdk4 and Cdk6 in *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ cells.

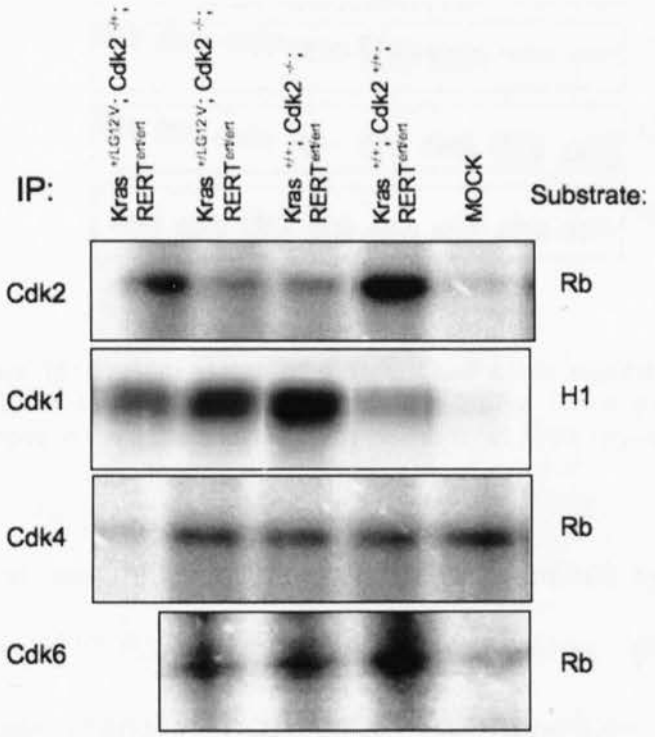


Figure 15: Kinase assays in Cdk2 lacking MEFs. We observe how the elimination of Cdk2 drastically reduces the levels of Cdk2 activity, and more interestingly, cells lacking Cdk2 in a K-ras oncogenic background would have increase levels of Cdk1 activity. There are no differences in the protein levels of Cdk4 and Cdk6.

We observed that neither Cdk4 nor Cdk6 kinase activity levels vary in the absence of Cdk2. However, Cdk1 kinase activity is increased in immortal MEFs lacking Cdk2 (Figure 15).

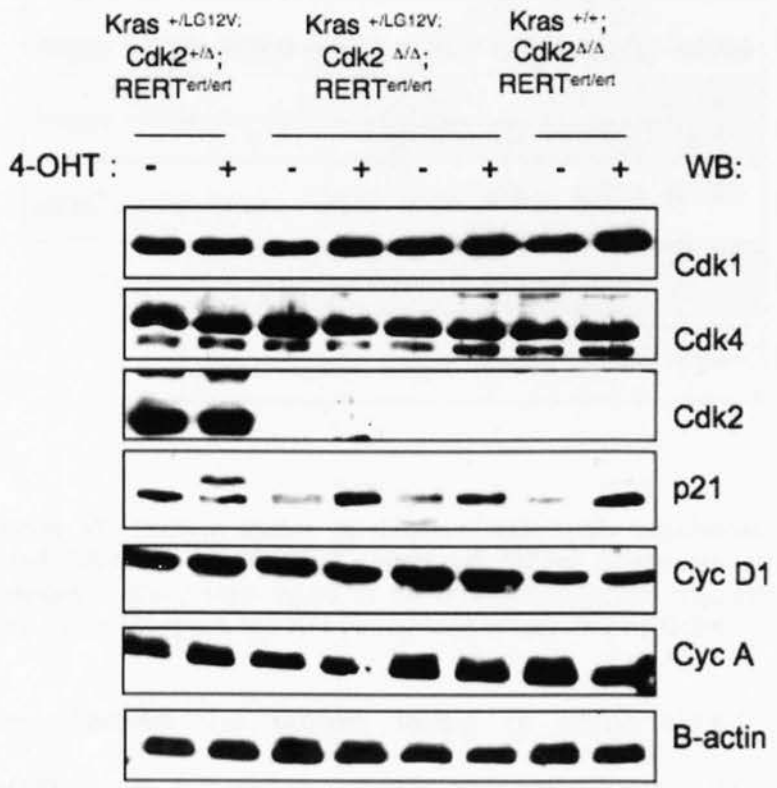


Figure 16: Protein status of different cell cycle regulators in the *Cdk2$^{\Delta/\Delta}$;K-ras$^{+/LG12V}$;RERT$^{ert/ert}$* immortal MEFs. There are no major changes in the protein levels of the different cell cycle regulators.

On the other hand, we analysed the status of different cell cycle regulators in the *Cdk2$^{\Delta/\Delta}$;K-ras$^{+/LG12V}$;RERT$^{ert/ert}$* immortalised clones (Figure 16). We observed no major changes in the pattern of expression of the observed molecules. Importantly Cdk1 protein levels do not seem to be altered in the *Cdk2$^{\Delta/\Delta}$;K-ras$^{+/LG12V}$;RERT$^{ert/ert}$* cells, while its kinase activity is increased.

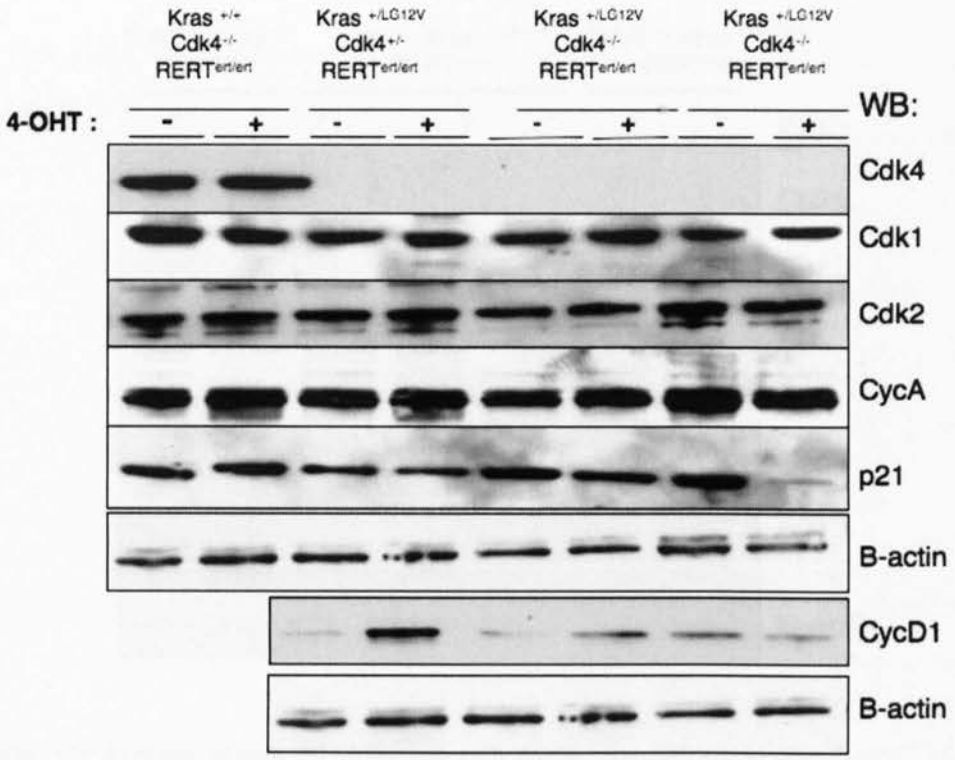


Figure 17: Protein status of different cell cycle regulators in the *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ immortal MEFs. There are no major changes in the protein levels of the different cell cycle regulators, and only Cyclin D1 levels seem to be reduced in cells lacking Cdk4.

Additionally we checked the protein levels of immortalized *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ and *Cdk6*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ MEFs. No differences were observed in the pattern of expression of the different cell cycle regulators (Figure 17 and 18). Only cyclin D1 levels seem to be increased when oncogenic *K-ras*^{G12V} is expressed. This increase can be explained by the high proliferation rate of these cells (Figure 17 and 18).

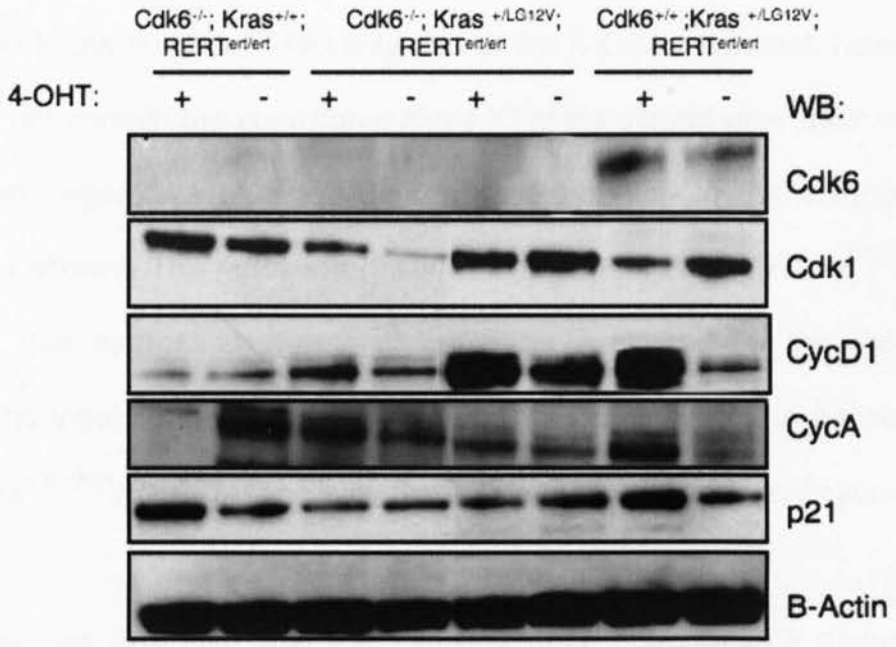


Figure 18: Protein status of different cell cycle regulators in the *Cdk6*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ immortal MEFs. No major differences were observed in the protein levels of the different cell cycle regulators examined.

11.1.1.4 Effect of the absence of the G1/ S Cdks on transformation

Cell transformation has been defined as alterations of morphological appearance reflected by changes in shape and growth patterns. The transformation assay is a gold standard in *in vitro* tumourigenic studies and provides us a clue of the tumourigenic potential of the cell population tested. In order to analyse the impact of the lack of an interphase Cdk in cell transformation, we transfected the *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$, *Cdk4*$^{-/-}$; *K-ras*$^{+/LG12V}$; *RERT*$^{ert/ert}$ and *Cdk6*$^{-/-}$; *K-ras*$^{+/LG12V}$; *RERT*$^{ert/ert}$ primary MEFs with both *H-ras*^{G12V} and *E1a* oncogenes . The transfected cells were maintained for three weeks, until the foci started to arise in the plates, were then stained and counted. Surprisingly, we detected a decrease in the number of foci when *K-ras* ^{LG12V} endogenous expression was activated compared to cells that were not

treated with 4-OHT (Figure 19 blue symbols). We checked to see if the reduction in the number of foci was due to the 4-OHT treatment, however cells that did not contain the conditional *K-ras* KI allele did not alter their rate of foci formation, regardless of the 4-OHT supplementation in the culture medium (data not shown). The reduced transformation capabilities by *H-ras*^{G12V} and *E1a* in cells that expressed *K-ras*^{LG12V} could be explained by the induction of oncogenic induced senescence (OIS) that was presumably to be activated in the *K-ras* $^{LG12V/+}$;*RERT*$^{ert/ert}$ cells by the accumulation of oncogenic stimuli.

Moreover, we observed that the elimination of a single *Cdk* decreased the number of foci obtained by the transformation with *H-ras*^{G12V} and *E1a* (Figure 19). Indeed, the reduction in the appearance of foci was even more dramatic when the endogenous *K-ras* oncogene was expressed, although this effect is less obvious when Cdk2 is abrogated.

These results suggest that the elimination of a single *Cdks* not only has an important role in reducing the growth and immortalisation capabilities in oncogenic *K-ras* expressing cells but also reduces the transformation potential in response to *H-ras*^{G12V} and *E1a*.

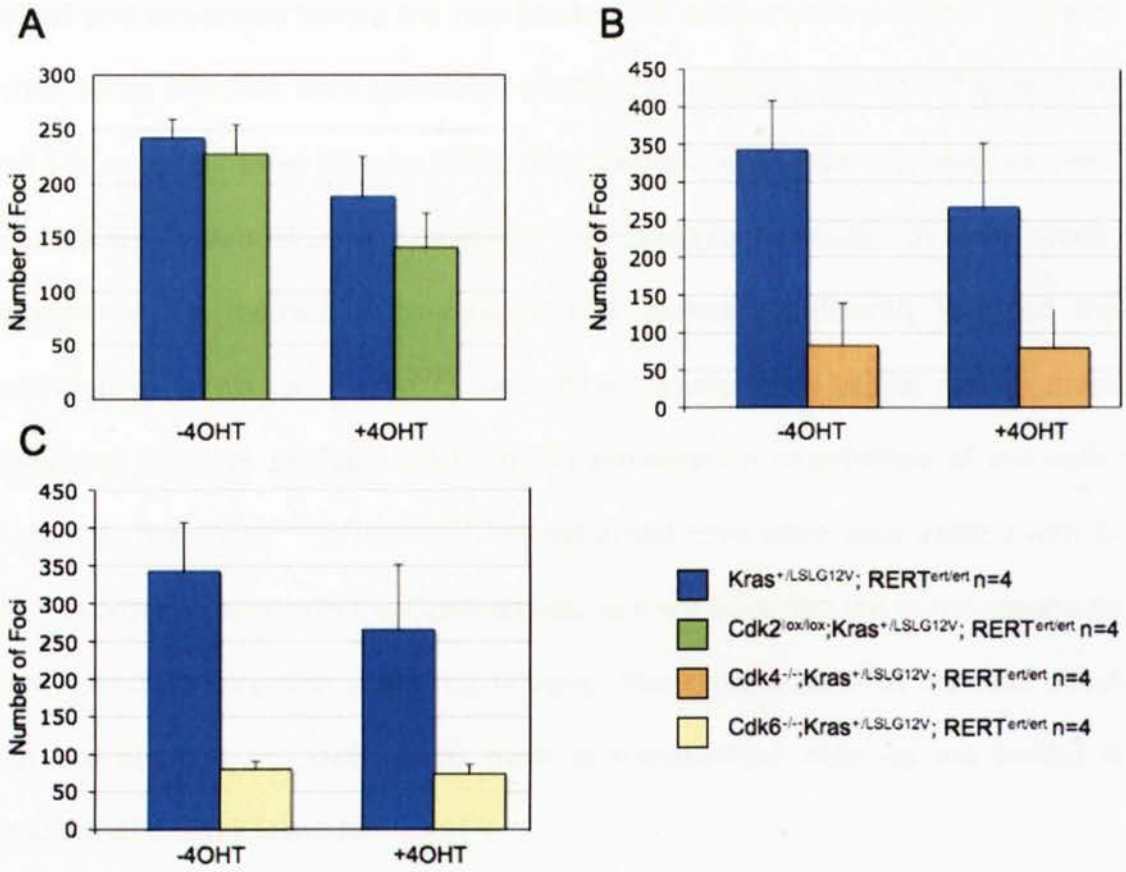


Figure 19: Foci formation after *H-ras*^{G12V} and *E1a* infection. The Infection of *Cdk2* null cells with *H-ras*^{G12V} and *E1a* results in a slight decrease in the number of foci obtained. This difference is more obvious when the *Cdk2* lack is combined with the expression of the endogenous *K-ras*^{G12V} oncogene (A). The number of foci obtained after the double oncogenic infection in a *Cdk4* null background is highly reduced, and this reduction is conserved when there is expression of the endogenous *K-ras* oncogene (B). *Cdk6* deficient MEFs significantly reduce the number of foci obtained after *H-ras*^{G12V} and *E1a* infection, and this difference is also observed in a *K-ras*^{G12V} expressing background (C) (n=4 for each genotype).

11.1.1.5 Effect of the elimination of the kinases after transformation

We next wanted to look further into the therapeutic capabilities the lack of *Cdk2* or *Cdk4* could provide in patient tumours. For this purpose, conditional Cdk2 and Cdk4 primary MEFs were subjected to transformation. To this end, we transfected conditional *Cdk2*$^{lox/lox}$ (Ortega *et al*, 2003) and *Cdk4*$^{frt/frt}$ (Barriere *et al*, 2007) MEFs with the *H-ras*^{G12V} and *E1a* oncogenes. Individual foci were

picked and expanded before the conditional *Cdk* alleles were excised. This was achieved by infection with adenoviral particles expressing the Cre (Figure 20A) and Flp recombinases (Figure 20B). As a control, both MEF populations were infected with adenoviruses expressing GFP (empty symbols). In both cases, ablation of the respective conditional *Cdk* alleles significantly reduced the proliferation levels of *H-ras*^{G12V} and *E1a* transformed MEFs, being more significant the lack of *Cdk2* allele in the proliferation capabilities of the cells. Moreover, the *Cdk2*$^{lox/lox}$;*RERT* $^{ert/ert}$ transformed cells were also treated with 4-OHT in order to eliminate the *Cdk2* alleles, and we obtained the same results as with AdenoCre infection (data not shown). We were unable to test the effect that the ablation of Cdk6 would have in transformed cells as we lacked a conditional *Cdk6* KO model.

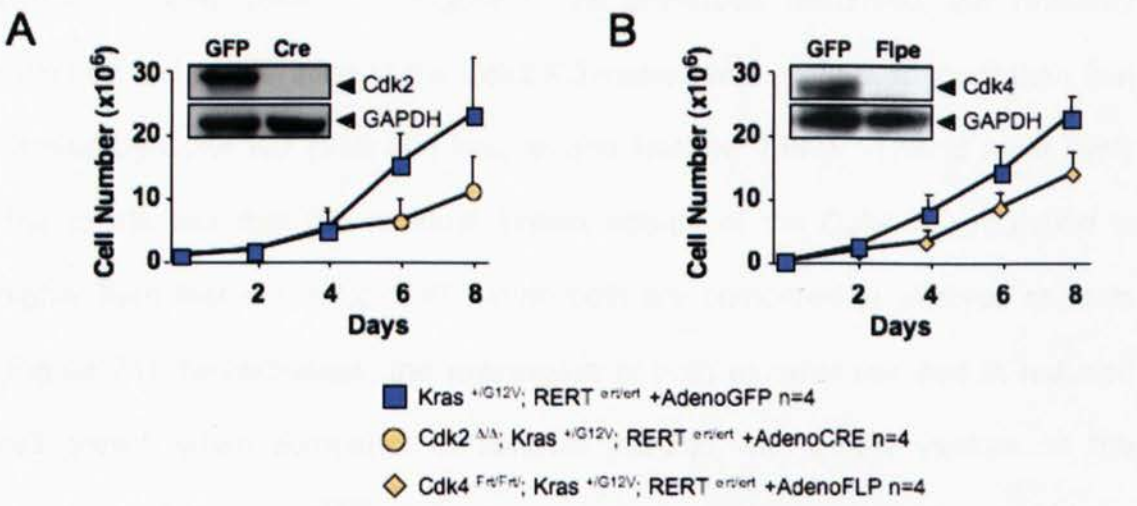


Figure 20: Effect of the elimination of *Cdk2* or *Cdk4* in *H-ras*^{G12V} and *E1a* transformed MEFs. The acute elimination of *Cdk2* in transformed foci has a severe effect on the cell growth of the cells. We can observe a clear elimination of Cdk2 protein after AdenoCre infection (A). Moreover, when *Cdk4* is depleted in foci, the growth of the cells is also reduced Cdk4 is totally eliminated after AdenoFLP cell infection (B) (n=4 for each genotype).

In conclusion, these results suggest that the proliferative advantage conferred by the transformation by *H-ras*^{G12V} and *E1a* is reduced when the transformed

cells acutely eliminate *Cdk2* or *Cdk4.* Those results reinforce the idea of the necessity of transformed cells for an intact Cdk capabilities.

11.1.2 Mimicking an *in vitro* therapeutic approach

The ultimate purpose of the project was to analysis if the Cdks were appropriate targets for drug design. However, when tumours are treated with inhibitors against specific targets, the target proteins remain in the cell binding to their substrates, although they are catalytically inactive. To this end we generated point mutations in specific residues that have been described to be essential in the phospho-transfer reaction, thus functioning as dominant negative forms (Van den Heuvel and Harlow, 1993). Specifically, we infected primary MEFs with retroviral vectors expressing the Cdk2 or Cdk4 Kinase dead (KD) mutants ($Cdk2^{D145N}$ and $Cdk4^{D158N}$) (Figure 2). As previously described, the inhibitory effect on cell proliferation of the Cdk2 KD mutant was more prominent than that caused by Cdk4 KD (Van den Heuvel and Harlow, 1993). This is most likely due to the fact that the residual kinase activity of the Cdk4 KD mutation is higher than that of the Cdk2 KD when both are compared to wildtype controls (Figure 21). Nevertheless, the expression of both mutants resulted in reduced cell growth when compared to controls infected with empty vectors. In the presence of the $K\text{-}ras^{G12V}$ allele, the expression of these mutants reduced the proliferation rate to wild type levels suggesting that the *K-ras* oncogene requires both Cdk2 and Cdk4 activity to convey a fully oncogenic activity (Figure 22).

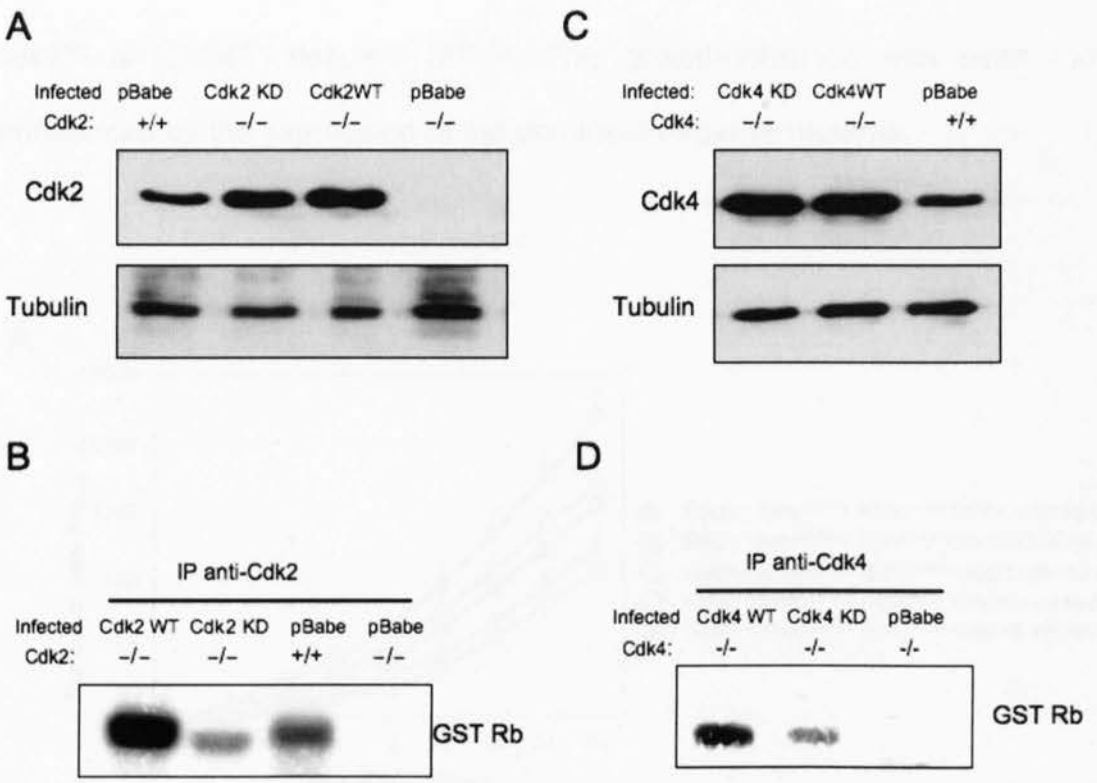


Figure 21: Checking the expression levels of the infected KD isoforms and the kinase activity of the mutants. We can observe that upon *Cdk2* KD or WT isoforms transfection, their expression is enhanced (A). When analysing the kinase levels of the Cdk2 KD isoform (B) we observe a significant reduction in their activity levels when compared to wildtype endogenous or overexpressed Cdk2 activity. When both Cdk4 isoforms are transfected into MEFs, they were overexpressed (C). Cells containing the *Cdk4 KD* isoform, also show a remarkable reduction in kinase activity when compared to wildtype endogenous levels of Cdk4 activity (D).

We next wanted to compare to what extent the proliferative advantage conferred *in vitro* by oncogenic *ras* was more effectively counteracted by germline deletion of the Cdk alleles or by the expression of their dominant-negative mutants. To this end *K-ras*$^{+/LSLG12V}$;*RERT*$^{ert/ert}$ MEFs that were either *Cdk2*$^{-/-}$, *Cdk4*$^{-/-}$ as well as their respective wild type controls were all treated with 4-OHT in order to activate the expression of the endogenous *K-ras*^{G12V} allele. Subsequently, the cells were infected with retroviruses expressing Cdk2 or Cdk4 KD mutants as appropriate and their proliferation capacity was monitored following a 3T3 protocol. As shown in Figure 22, the increased cell

growth associated to the expression of the oncogene was partially attenuated in *Cdk2*$^{-/-}$ or *Cdk4*$^{-/-}$ deficient MEFs. This growth-inhibition was even more pronounced by the expression of the dominant-negative mutants.

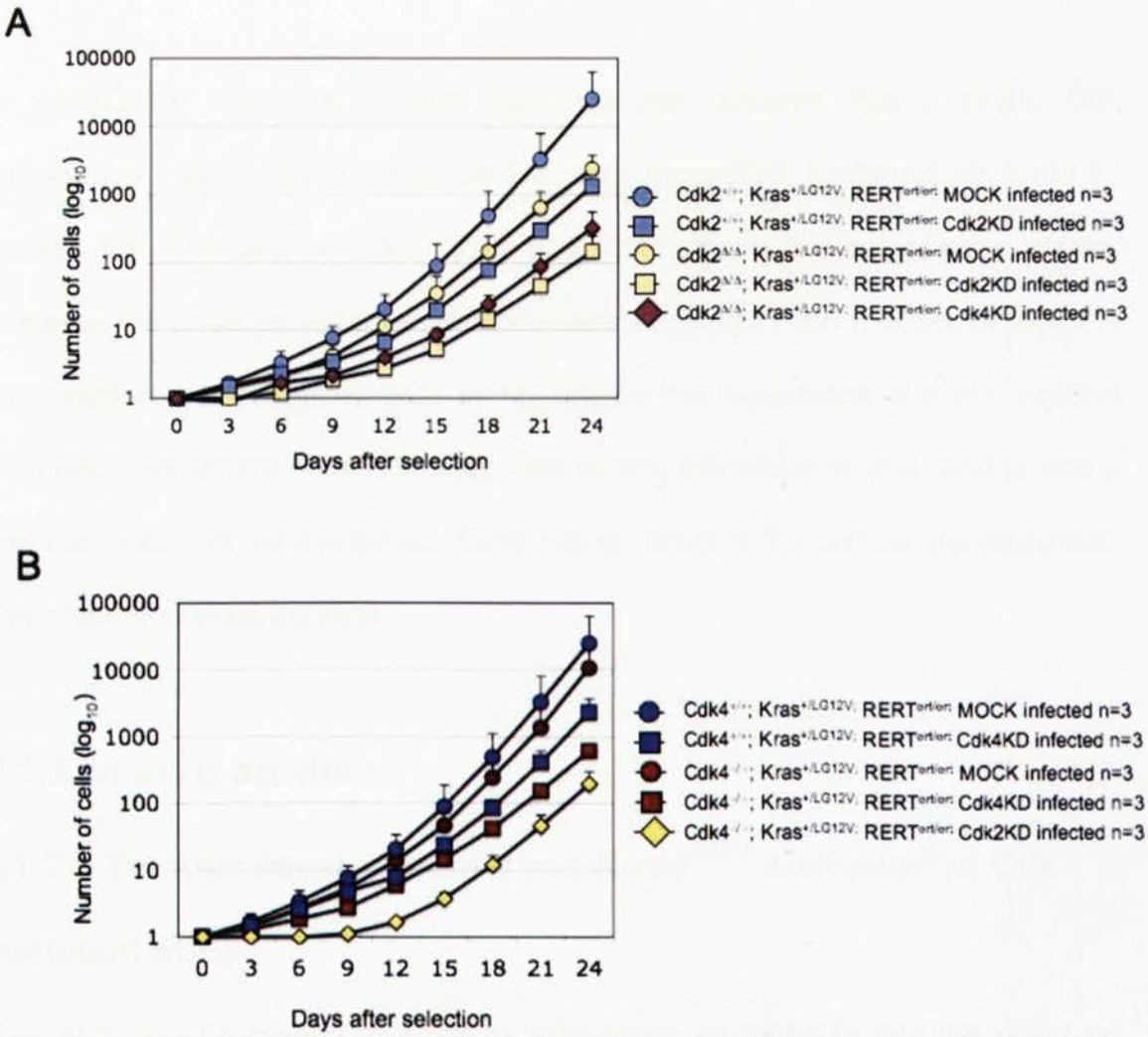


Figure 22: Effect of the expression of the Cdk2 and Cdk4 KD in cell growth. The expression of Cdk2 KD in *Cdk2*$^{\Delta/\Delta}$*;K-ras*$^{+/LG12V}$*;RERT*$^{ert/ert}$ and *Cdk2*$^{+/+}$*;K-ras*$^{+/LG12V}$*;RERT*$^{ert/ert}$ results in a remarkable decrease in proliferation. Moreover when the Cdk4 KD is expressed in a Cdk2 lacking background, there is a reduction in proliferation, however this reduction is less obvious than with the Cdk2 KD isoform expression (A). When the Cdk4 KD isoform is expressed in a *Cdk4*$^{-/-}$*;K-ras*$^{+/LG12V}$*;RERT*$^{ert/ert}$ and *Cdk4*$^{+/+}$*;K-ras*$^{+/LG12V}$*;RERT*$^{ert/ert}$ there is also a decrease in the proliferation which is more acute when the Cdk2 KD isoform is expressed in a Cdk4 null background (B) (n=3 for each genotype).

Finally, combining the expression of catalytically-dead Cdks in germline knock-out MEFs resulted in a synergistic effect reaching approximately 50% reduction in the growth rate (Figure 22). In summary, the data presented here demonstrate that interphase Cdks are key mediators downstream the *K-ras*^{G12V} allele and, at least *in vitro*, their inhibition can partially neutralize its effects.

In conclusion from the *in vitro* work we can observe that a single Cdk elimination has a severe effect on the main properties conferred by *K-ras* KI expression. The lack of *Cdk2, Cdk4* and *Cdk6* affects the cell tumour growth, restores the crisis period upon immortalisation, reduces the number of foci, the proliferation of transformed cells and highlights the importance of these proteins for cancer treatment. The next step was to test this effect *in vivo*, and to see if the elimination of the interphase *Cdks* has an effect in the tumour development, formation and mice survival.

11.2 *In vivo* studies

11.2.1 Tumour development upon *K-ras*^{G12V} activation in Cdk deficient mice

The encouraging results obtained *in vitro* made us move to see the effect on tumour development of the absence of the G1/S Cdks following activation of the endogenous *K-ras*^{G12V} allele. To this end we used strains with *Cdk2* $^{lox/lox}$, *Cdk2*$^{-/-}$, *Cdk4*$^{-/-}$ or *Cdk6*$^{-/-}$ alleles in a *K-ras*$^{+/LSLG12V}$;*RERT*$^{ert/ert}$ background. The activation of the endogenous expression of the *K-ras*^{G12V} allele in adult somatic cells was performed when the litters were weaned (P21) by intraperitoneal administration of 4-OHT (1 mg twice a week for 2 weeks for the germline KO

mice and 1mg twice a week for 4 months in the *Cdk 2* $^{lox/lox}$;*K-ras*$^{+/LSLG12V}$;*RERT*$^{ert/ert}$ model).

11.2.1.1 Effect of the ablation of the kinases on mice survival

The animals were then left unperturbed and observed for tumour incidence. *K-ras*$^{+/LSLG12V}$;*RERT*$^{ert/ert}$ mice under this 4-OHT regime developed lung adenomas and adenocarcinomas and eventually died due to respiratory failure at about eight months of age (Guerra *et al.*, 2003) (Figure 23 empty symbols).

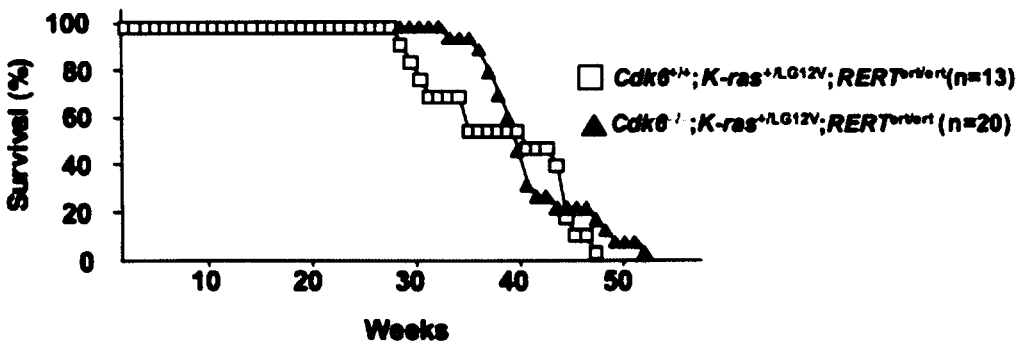


Figure 23: Survival of the *Cdk6*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice. Cdk6 deficiency has no impact on the mice lifespan upon activation of the oncogene expression.

In contrast to the results obtained *in vitro*, *Cdk6* deficient mice in which *K-ras*^{G12V} expression was postnatally activated, did not present any difference in terms of mice survival (Figure 23 solid symbols).

Additionally, the contribution of Cdk2 lack to the expansion of mice survival in K-ras^{G12V} expressing mice was confirmed with the *Cdk2*$^{lox/lox}$ animals suggesting that Cdk2 could have a role partial therapeutic role in these kinds of tumours (Figure 24A).

Moreover, in the *Cdk2*$^{-/-}$ model that was subjected to *K-ras*^{G12V} activation, we observed a slight increases in the mice lifespan. In these mice we also observe an increase in the mice lifespan in mice associated with Cdk2 abrogation (Figure 24B).

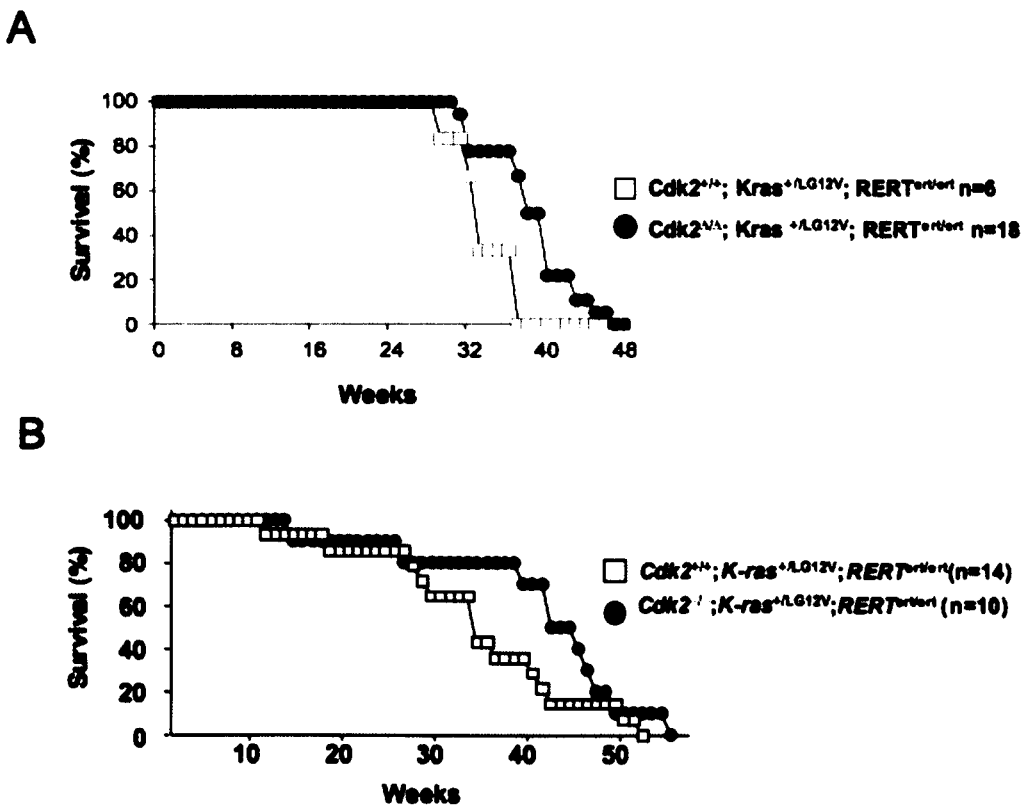


Figure 24: Survival of the *Cdk2*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ and *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice. The elimination of Cdk2 and expression of K-ras ^{G12V} results in a significant increase in the mice lifespan (A).Moreover, the germline Cdk2 knockout elongates the animal average lifespan expressing the K-ras oncogene (B).

The *Cdk4* null mice develop diabetes, a phenotype that masks the potential extension of the mice lifespan. The diabetes occurs as a result of defects in the proliferation of the β-cells of the pancreatic islets, in the majority of the homozygous animals and it results in the death of the mice at five to eight months of age (Rane *et al*, 1999; Tsutsui *et al*, 1999). The diabetes is not overcome by the expression of *K-ras*^{G12V} and this phenotype prevented us from

studying the impact of the *Cdk4*$^{-/-}$ background on the survival of *K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice as the animals died due to diabetes before we could see any sign of respiratory failure (Figure 25).

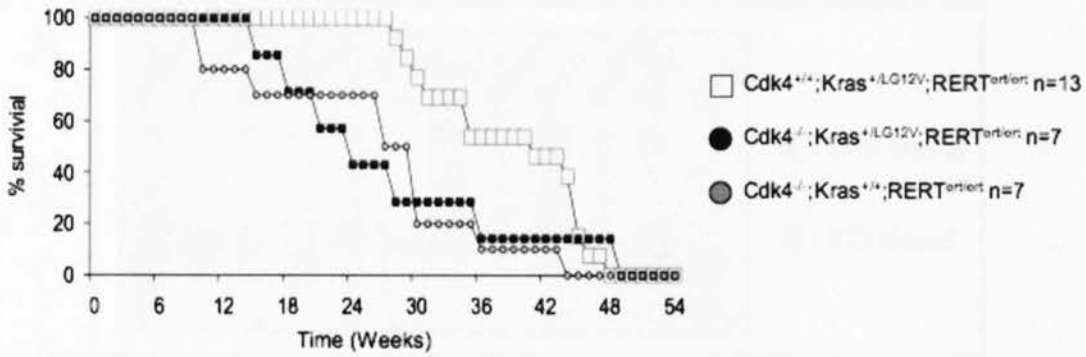


Figure 25: Survival of the *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice. The Cdk4-/- mice die due to the development of a diabetic phenotype regardless of the expression of the K-ras oncogene. We are unable to observe a potential lifespan in the *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ due to the diabetes.

11.2.1.2 *Cdk2* conditional KO mice expressing oncogenic K-ras

Cdk2$^{lox/lox}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice were subjected, as we have mentioned above, to a 4 months continuous 4-OHT administration protocol in order to maximise the excision of the *Cdk2* conditional allele in the mice. After the treatment we analysed the excision of the conditional allele in a wide variety of mouse tissues, and we observed high levels of excision in most of the tissues, being especially remarkable in spleen, pancreas, kidney, colon and thymus (Figure 26A). More importantly we observed over 85% of excision in the lungs, implying that the vast majority of lung cells were deficient for Cdk2 protein. However, no excision was observed in brain where the 4-OHT is less accessible. Moreover we checked *Cdk2* status in the adenomas of 4-OHT treated mice and observed that all the examined adenomas had a 100% excision of *Cdk2* (Figure 26B). These data shows that the *Cdk2* gene is efficiently eliminated in the same cells where the K-ras^{G12V} oncogene

expression is activated. Those results indicate that Cdk2 is dispensable for tumour formation as the lung adenomas are able to grow in spite of lacking Cdk2.

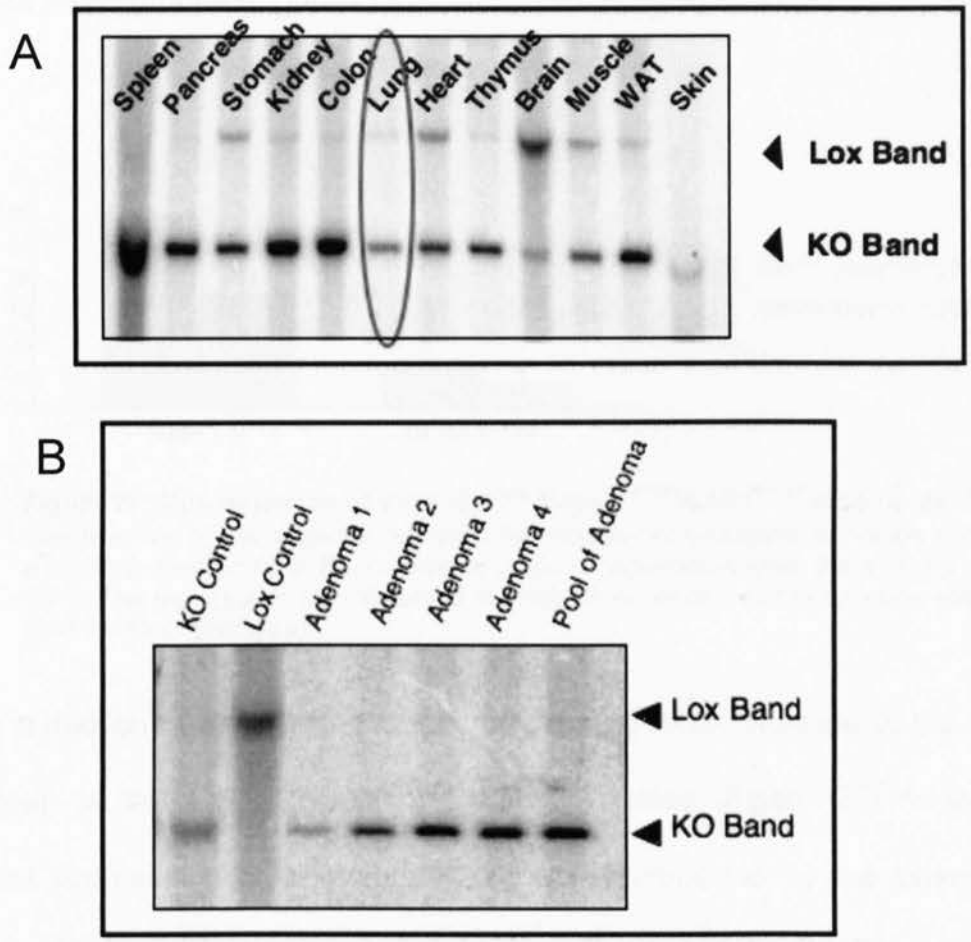


Figure 26: Checking the excision of the *Cdk2* locus. After 4 month 4-OHT treatment we maximised the excision levels in the different tissues (A). We obtain 100% excision in spleen, pancreas and skin. The elimination of the Lox allele is also high in stomach, kidney, colon, lung, thymus, muscle and WAT. The heart and brain being the most resistant tissues to the 4-OHT treatment. Adenomas isolated from the $Cdk2^{lox/lox}$; $K\text{-}ras^{+/LG12V}$;$RERT^{ert/ert}$ mice were examined for *Cdk2* excision (B) and we observe that all the tumours had 100% excision of the *Cdk2* lox allele.

Pathologists histopathologically evaluated the lesions and the tumours were assigned different grades according to the severity of the phenotype (Jackson *et al*, 2005). Grade I and II corresponds to premalignant lesion, grade III and IV to an established adenocarcinoma and Grade 5 to an invasive carcinoma. Interestingly, when we quantified the number of lesions at 7 month of age, and

thus six months after initiation of the 4-OHT treatment we observed a significant decrease in the number of grade I-II lesions and also in the number of adenocarcinomas of grade III –IV. No invasive carcinomas were identified in any of these groups (Figure 27).

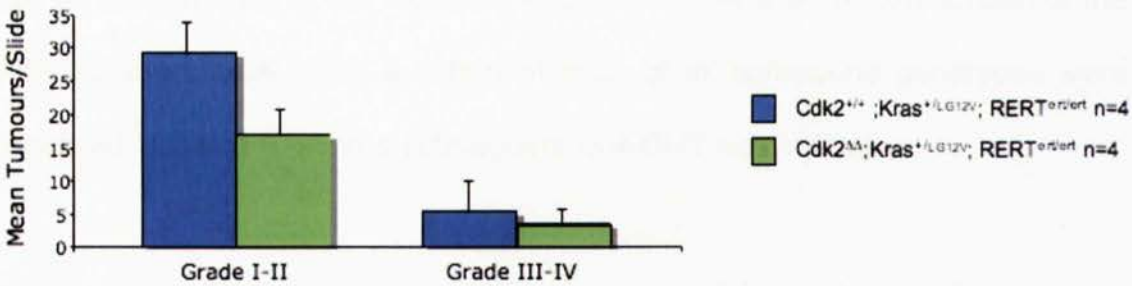


Figure 27: Quantification of the *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice lungs. The quantification of the lungs six months after initiation of oncogene activation shows a significant reduction in the number of grade I-II adenomas when the animals lack *Cdk2*. The reduction is also observed in grade III–IV lesions, but to a smaller extent (n=4 for each genotype).

The reduction in the number of tumours results in an increase in the average lifespan of the *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice (Figure 27) supporting a partial suppression of the tumourigenic ability conferred by the expression of the endogenous K-ras oncogenic mutation. The use of the *Cdk2* conditional KO mice provides a scenario in where Cdk2 levels are normal until K-ras^{G12V} is expressed. The tumour reduction observed in the *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice can be a consequence of Cdk2 itself or the result of the acute elimination of the *Cdk2* gene in lung. In order to distinguish between both possibilities we generated the *Cdk2* germline KO model expressing the *K-ras*^{G12V} oncogene.

These results suggest that the elimination of Cdk2 has only a partial therapeutic effect by reducing the number of tumours and slightly increasing the mouse life-span.

11.2.1.3 Quantification of the tumours in the KO strains

As mentioned above, a post-mortem examination of the lungs upon *K-ras*^{G12V} activation revealed a broad spectrum of lesions with characteristic bronchio-alveolar hyperplasias, adenomas and adenocarcinomas (Guerra *et al*, 2003) as well as hyperplasias in the stomach. In order to evaluate the contribution of the different interphase Cdks, a cohort of mice of all compound genotypes were sacrificed at 3 and 6 months subsequent to 4-OHT administration.

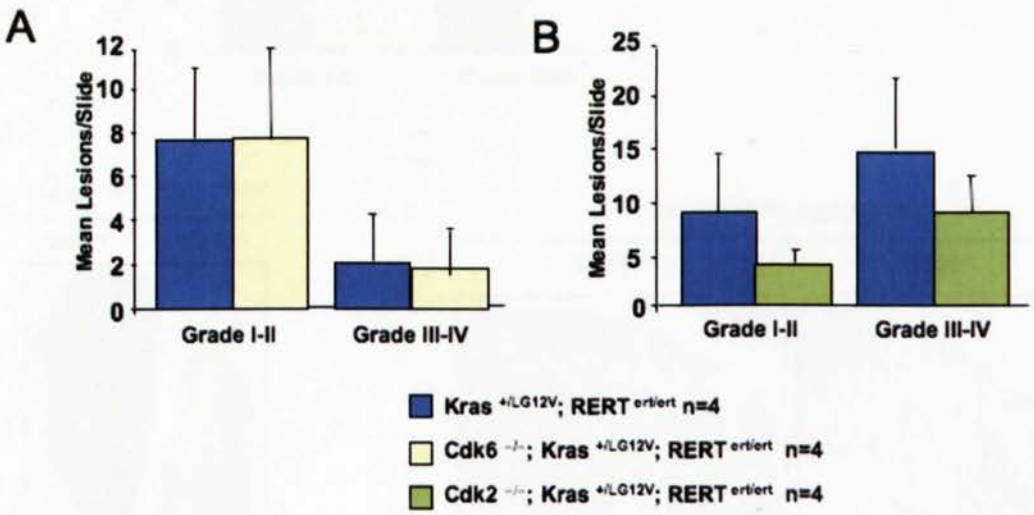


Figure 28: Quantification of the *Cdk2*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ and *Cdk6*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice lungs. The presence or absence of Cdk6 does not have an effect in the number of lesions obtained 6 months after 4-OHT treatment (A). As it has been previously shown, the absence of Cdk2 has an effect in the number of lesions obtained of either grade I-II or grade III-IV (B) (n=4 for each genotype).

As shown in Figure 28A, the distribution of tumours did not show any quantitative nor qualitative variation in the absence of *Cdk6* by 6 months after 4-OHT treatment. However, in the case of *Cdk2*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice we observed a 45% decrease in the incidence of grade I-II lesions and a comparable reduction in adenocarcinoma burden when compared to *Cdk2*$^{+/+}$;*K-*

ras$^{+/LG12V}$;*RERT*$^{ert/ert}$ littermates (Figure 28B) at 6 months after K-ras^{G12V} activation. To what extent this variation may account for the minimal lifespan extension observed remains to be determined (see Figure 24). Moreover, the result of this model is comparable to the Cdk2^{lox} KO model.

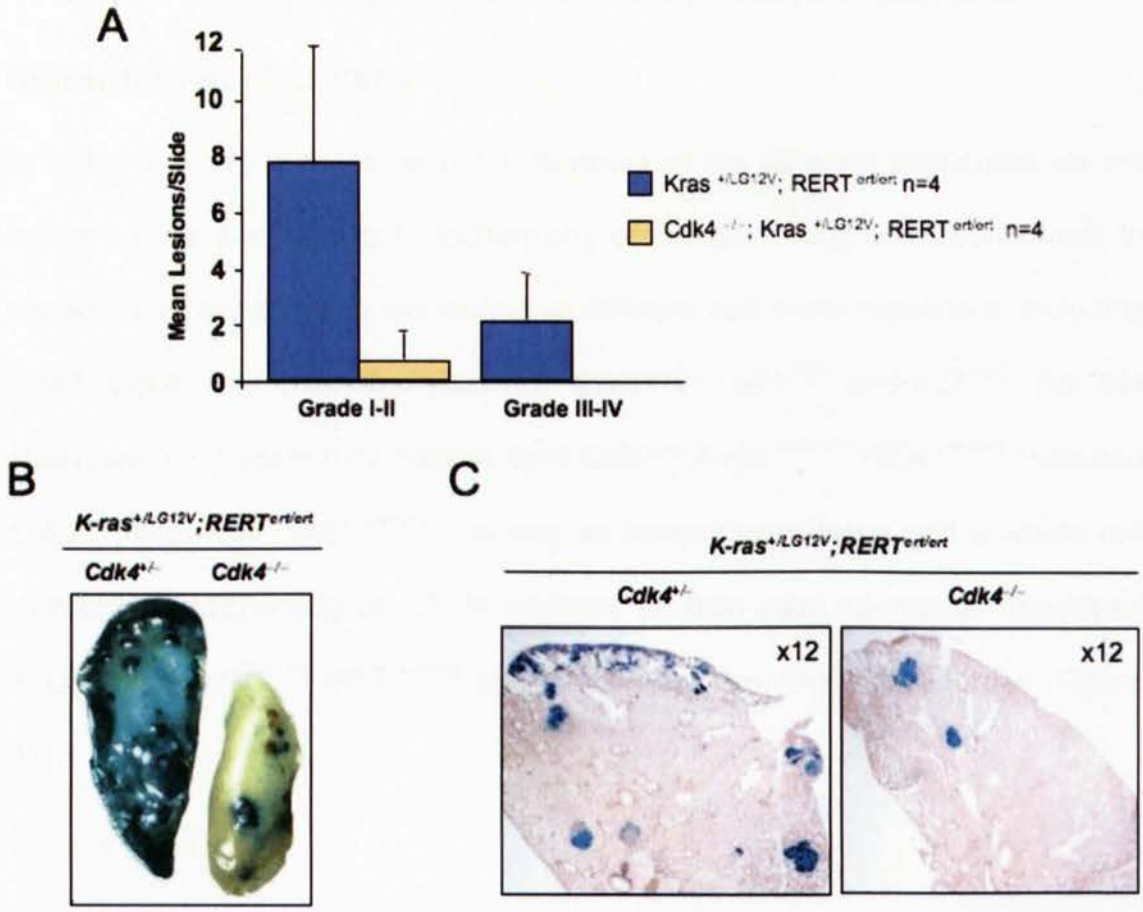


Figure 29: Lesions of the *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice lungs 6 months after oncogenic K-ras activation. There is a 80% reduction in the number of grade I-II adenomas found in animals that lack *Cdk4* and no grade III-IV lesions at this time (A). Macroscopically we can observe the *Cdk4*$^{+/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice have an increase in the size of the lobules due to inflammation and the size and number of tumours (n=4 for each genotype). (B), while *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ do not suffer this increase and have a significant lower number of *K-ras* expressing cells (Blue cells). When observing the sections of both genotypes, we observe that *Cdk4*$^{+/+}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice have not only high number of tumours, but also hyperplatic areas in the lung edges, that are not found in the *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ lungs (C).

Remarkably, the impact of the endogenous *K-ras*^{G12V} was nearly neutralized in a *Cdk4*$^{-/-}$ background. Histopathological analysis of the pulmonary lesions showed that there were no tumours nor hyperplasias at 3 months after 4-OHT

treatment and by 6 months after the K-ras oncogene activation we can observe a 80% reduction in the incidence of grade I-II lesions with none progressing into more aggressive stages (Figure 29).

11.2.1.4 Protein status in adenomas by Western Blot and Immunohistochemistry

In order to study in more detail the tumours of the different genotypes we did western blots and immunohistochemistry of the 3mm lung adenocarciomas. In western blot experiments we looked at different cell cycle regulators, including Cdk2, Cdk4, Cdk1, Cdk6, Cyclin D1, Cyclin E1, $p21^{Cip1}$ and $p27^{Kip1}$. For this study, we used adenomas coming from *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice and *Cdk2*$^{+/+}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ as well as normal lung tissue and a whole cell extract from MEFs (Figure 30). In addition, we also used adenomas developed in *Cdk6*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ and their respective control adenomas (Figure 31).

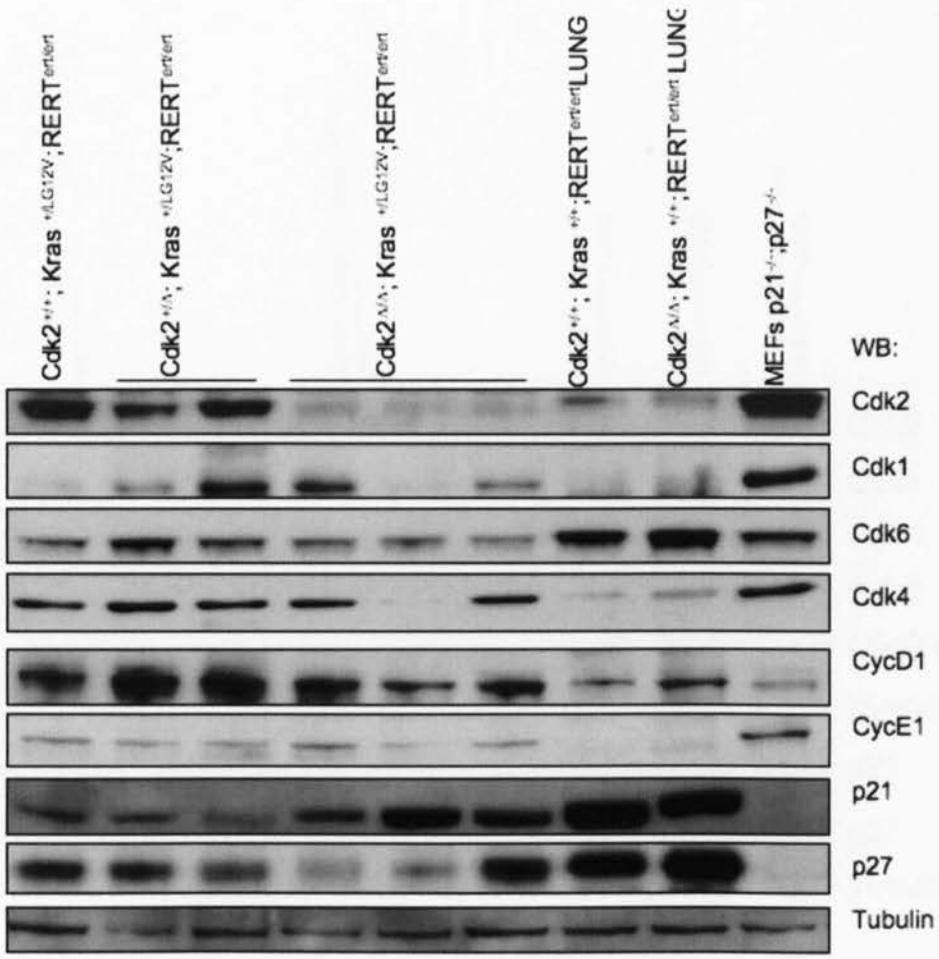


Figure 30: Status of different cell cycle regulators in the adenomas derived from the *Cdk2*$^{\Delta/\Delta}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice. Cdk2 is eliminated in those adenomas that were homozygous for the conditional allele. Cdk1 levels are increased in the tumours. Cdk6 is downregulated in the tumours, when compared to normal lung expression. Cdk4 is overexpressed as well as Cyclin D1 and Cyclin E1. Consequently, p21^{Cip1} and p27^{Kip1} levels are decreased in the adenomas.

In both experiments we observe the same behaviour, Cdk1, Cdk4, Cyclin D1 and Cyclin E levels were upregulated in 3mm long adenocarcinomas, while Cdk6, p21^{Cip1} and p27^{Kip1} expression was decreased. We were unable to observe the protein levels of the different cell cycle regulators from the *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice as we never obtained adenomas big enough to be isolated.

In the immunohistochemistry studies, we looked at the levels of expression of Cdk4, Cdk6, cyclin D1, p27^{Kip1}, Ki67 and performed a TUNNEL assay.

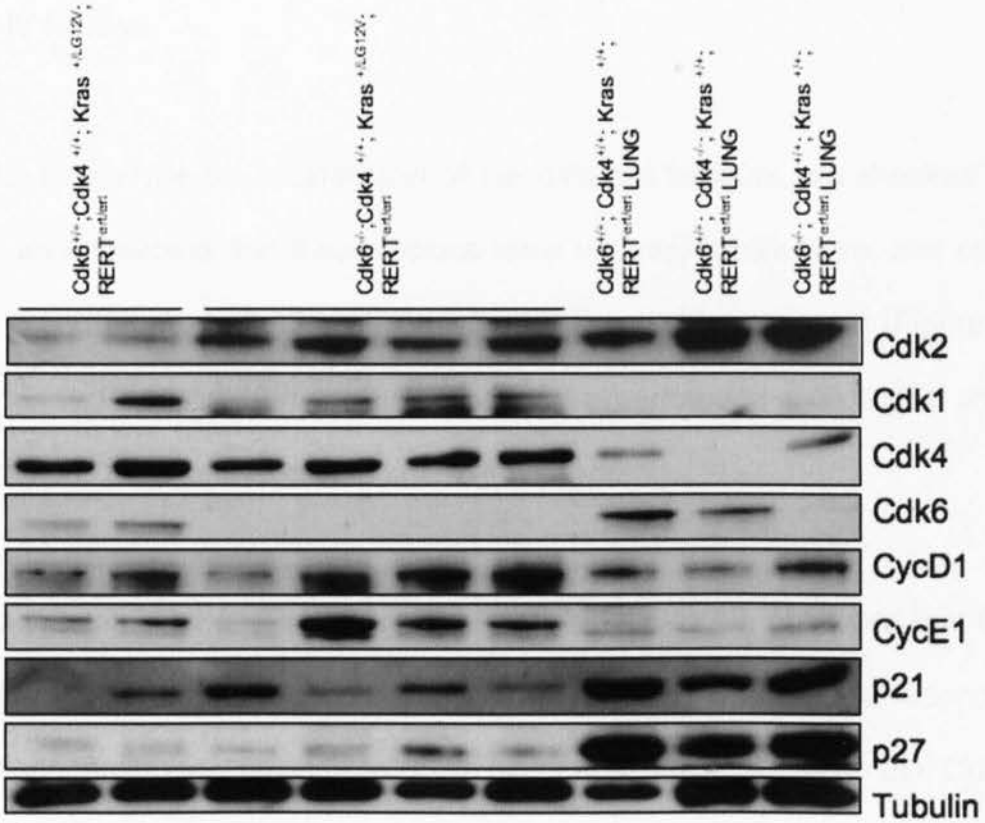


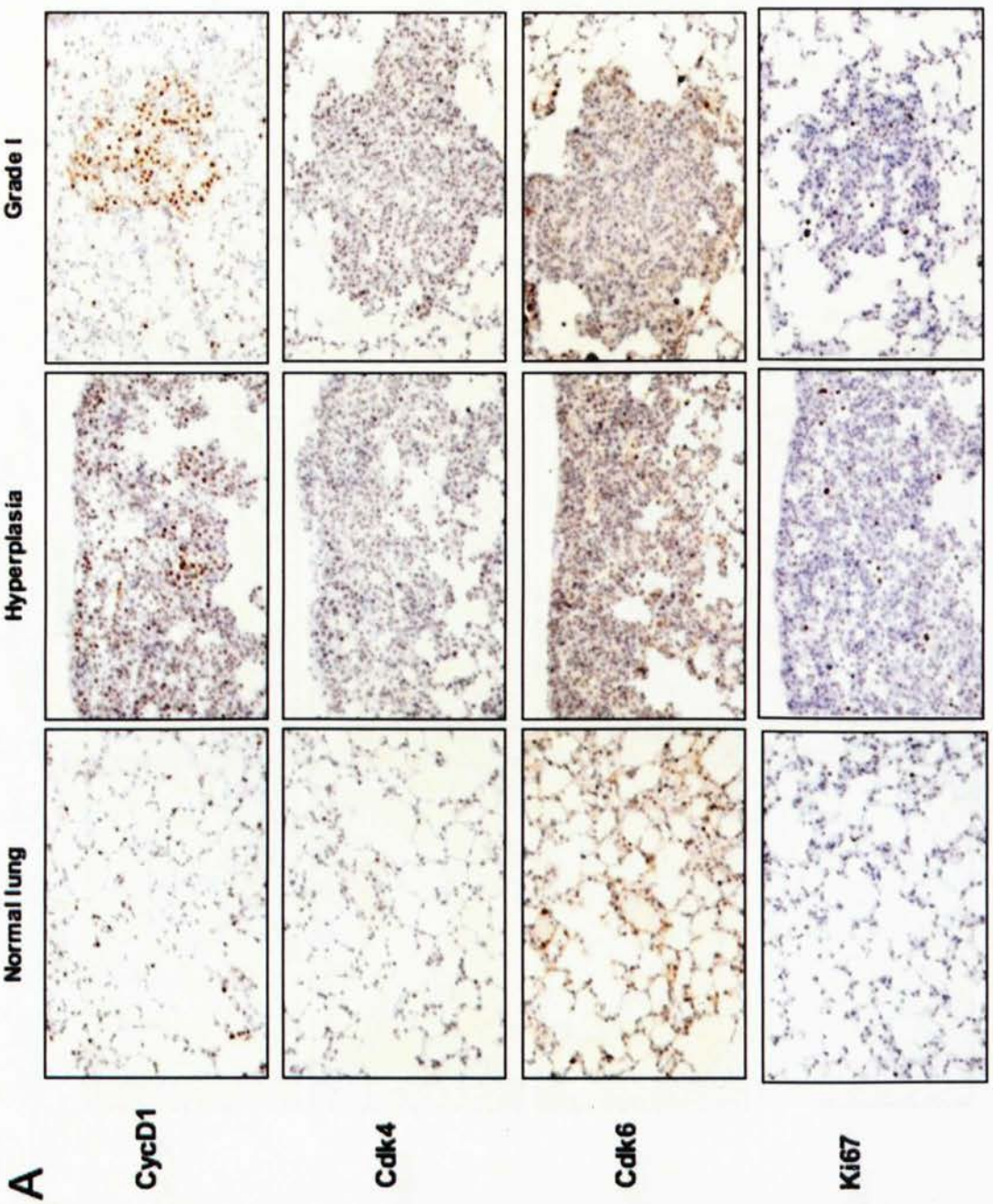
Figure 31: Status of different cell cycle regulators in the adenomas derived form the *Cdk6*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice. Cdk2 is normally expressed in the adenomas. Cdk1 levels are raised in the tumours. Cdk6 is, of course, eliminated in the tumours of the *Cdk6*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice. Cdk4 is overexpressed as well as Cyclin D1 and Cyclin E1. Consequently, p21^{Cip1} and p27^{Kip1} levels are decreased in the adenomas.

The immunohistochemistry allowed us to study the behaviour of each of the proteins at different tumour stages. We observed how Cdk4 and Cyclin D1 levels were increased from hyperplasias to grade IV adenomas. Importantly the increased protein levels correlated with the malignancy of the tumours. In contrast, by immunohistochemical analysis we were unable to detect Cdk6 in the lesions. Hence, the Cdk6 levels appear to be absent in K-ras driven lung adenomas (Figure 32). p27^{Kip1} levels seem to be expressed in hyperplasias

and in premalignant tumours, however, their levels are sharply decreased in grade IV lesions.

In order to analyse the proliferation of the different tumours, we checked Ki67 levels, and observed that these lesions were not very proliferative, and only in grade II and higher tumours, we observed a low proliferation rate (Figure 32). We analysed apoptosis in the lung tissues by performing a TUNNEL assay, nevertheless none of the lesions seem to be apoptotic.

In both western blot and immunohistochemical studies we observed that Cdk6 expression is undetectable or extremely low in lung hyperplasias, adenomas and adenocarcinomas regardless of the histopathological grade, so that Cdk6 is not able to upregulate its expression in the absence of Cdk4 or Cdk2 and thus has no role in compensation; and more importantly, the lung cells that give rise to the K-ras^{G12V} driven adenomas do not express Cdk6.


A
Normal lung
Hyperplasia
Grade I
CycD1
Cdk4
Cdk6
Ki67

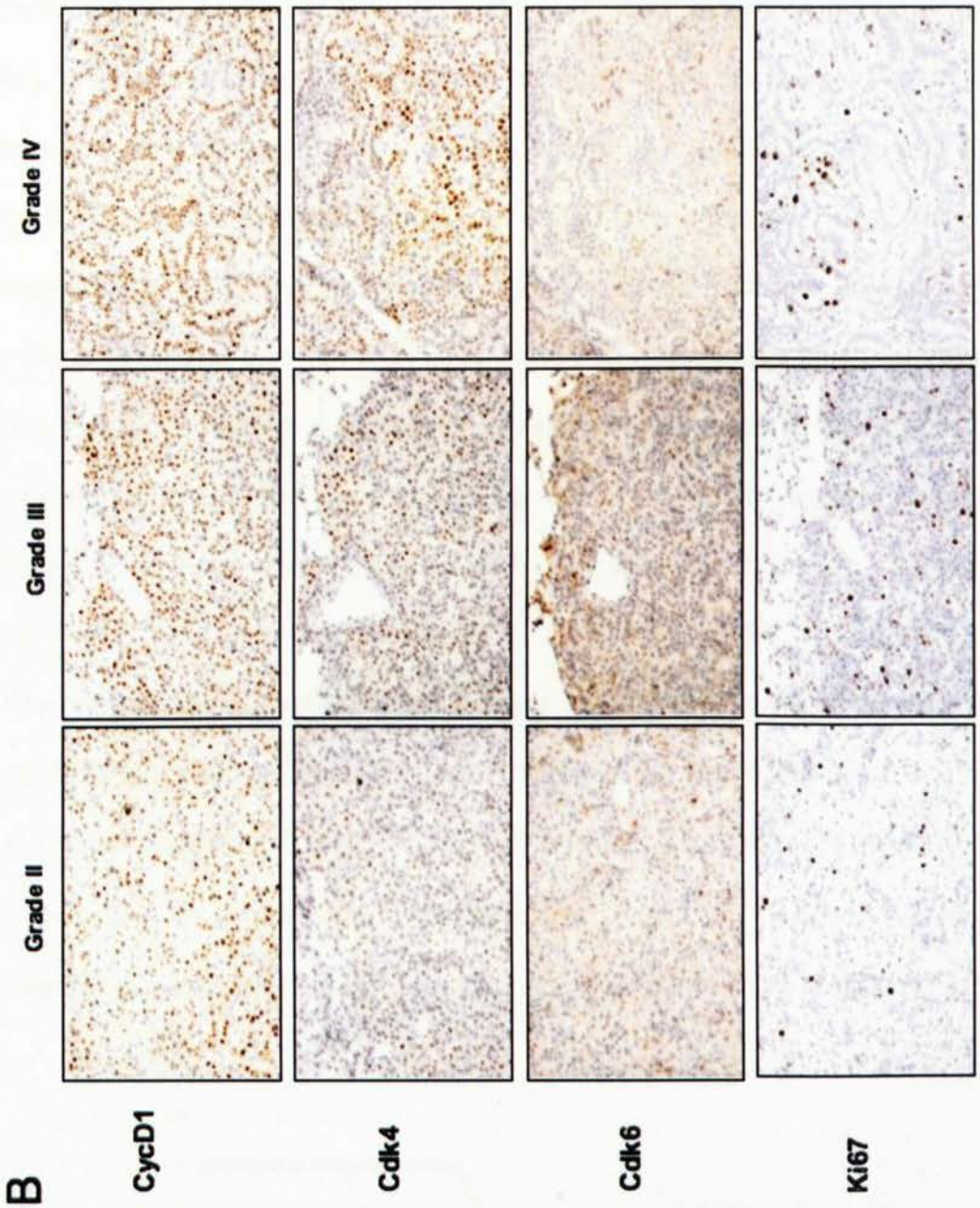


Figure 32: Immumohistochemical analysis of the *K-ras*^{G12V} induced lesions. Cyclin D1, Cdk4, Cdk6 and Ki67 status in the different grade lesions of the K-ras^{LG12V} ; RERT$^{ert/ert}$ mice (200X). We observed that the increase in cyclin D1 and Cdk4 accumulation goes together with the severity of the lesions. Cdk6 is not detected by immunohistochemical analysis. Proliferation of the tumours (detected by Ki67 expression) increases with the severity of the lesion.

11.2.2 Requirement for Cdk4 in lung tumourigenesis

In order to further characterize the implication of *Cdk4* in *K-ras*-driven lung adenocarcinomas we decided to use a more aggressive tumour model. In this respect, it has been previously reported that the murine *K-ras* wild type allele displays tumour suppressor properties and as a consequence is frequently lost during lung tumourigenesis (Zhang *et al*, 2001). Indeed, this loss of heterozygosity (LOH) can be detected in all human lung cancer samples that carry *K-ras* activating mutations (Li *et al*, 2003). In order to mimic spontaneous LOH we took advantage of a conditional *K-ras* allele developed in the laboratory (Guerra, unpublished). We went on to generate a strain carrying this wild type conditional knock-out allele together with the inducible mutation described above. These mice were designated as *K-ras*$^{lox/LSLG12V}$;*RERT*$^{ert/ert}$. Induction of the Cre-recombinase results in the deletion of the wild type copy and the concomitant induction of the oncogenic mutation. We have designated these simultaneous events as *K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$. The elimination of the wildtype *K-ras* allele and the expression of the oncogenic *K-ras* ^{G12V} mutation will result in an earlier onset of tumours and thus a reduction in the mice lifespan (Figure 33).

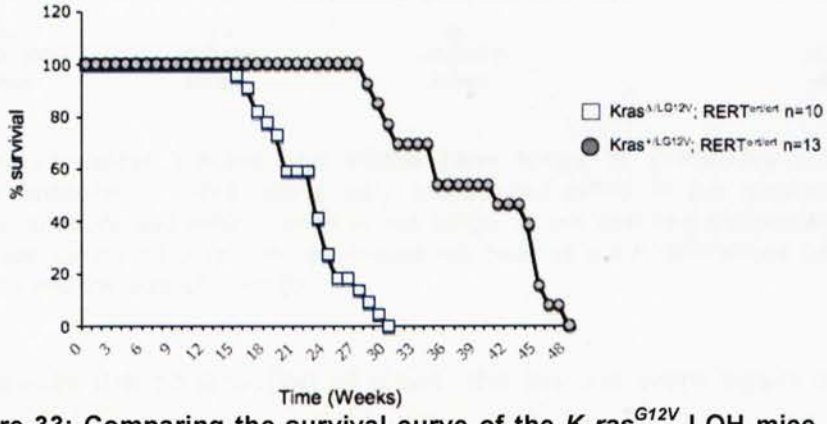


Figure 33: Comparing the survival curve of the *K-ras*^{G12V} LOH mice versus non LOH *K-ras*^{G12V} expressing mice. As we can observe, the lack of a wildtype copy of the *K-ras* allele results in an earlier mice death due to a rapid tumour development.

11.2.2.1 Tumour quantification in the LOH model

The imitation of the LOH event by this genetic tool resulted in reduced life span (Figure 33) ensuing from elevated number of tumours with exacerbated malignancy. Figure 34 depicts the aspect of whole lung lobules 6 months after the administration of the 4-OHT. The augmented tumour burden as a result of the LOH caused a significant enlargement of lung lobules. Remarkably, the impact of *Cdk4* deficiency was fairly evident even at the macroscopical level since it nullified the phenotypic changes observed in wild type littermate controls. Indeed, histopathological analysis of lung specimens revealed a dramatic reduction in the number of lesions in samples from a $Cdk4^{-/-}$ background (Figure 35).

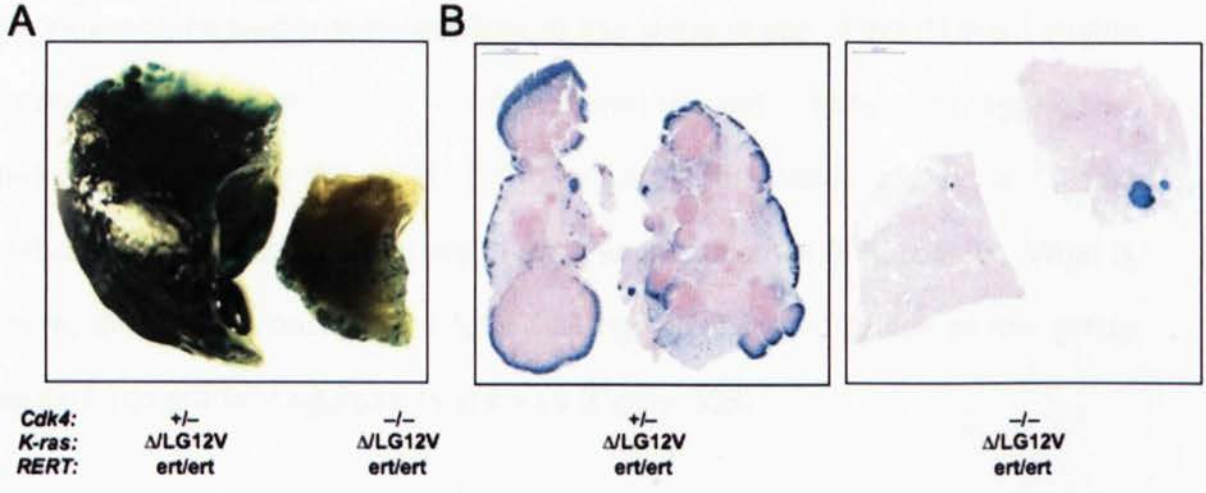


Figure 34: Macroscopical lobules and slides form lungs at 6 months after 4-OHT treatment. The absence of Cdk4 has a very pronounced effect in the number of K-ras expressing cells, tumours and inflammation of the lungs, as we can see macroscopically (A). When sections are examined from both genotypes we observe great differences between the number of lesions and the size of them (B).

In order to assess the contribution of Cdk4, the lesions were again quantified and classified depending to the severity of the phenotype (Jackson *et al*, 2005).

In the *K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice, lung lesions detected 3 months following 4-OHT treatment were scarce and mainly described as benign hyperplasias. Interestingly, lung inspection of *K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$ mice at this early stage already showed a significant number of lung adenomas, indicative of a more aggressive phenotype characteristic of tumours with LOH (Figure 35A). Remarkably, when the LOH model was tested on a *Cdk4*$^{-/-}$ background, the early onset of these aggressive tumours was abolished (Figures 35). Moreover, the effect of the Cdk4 deficiency was even greater when assessed 6 months after 4-OHT administration. As mentioned above, tumour burden in lungs from *K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$ mice not only expanded in number but also showed increased malignancy. Indeed we found lesions with stroma desmoplasia surrounding the nest of tumour cells, indicative of aggressive grade V carcinomas (Jackson *et al*, 2005). These lesions were never found in the presence of the wild type *K-ras* allele at this stage in any of the different strains analysed (see Figures 26, 27 and 28). Moreover, and in spite of the aggressive nature conferred by the LOH, Cdk4 deficiency practically caused a 10 fold reduction in the number of grade III-IV adenocarcinomas (Figure 35B). What is more, the *Cdk4*$^{-/-}$ background totally abrogated the progression of low grade lesions into grade V aggressive tumours (Figure 35B).

In sum, we conclude that Cdk4 deficiency results an important restraint for *K-ras*-driven tumour progression even in a genetic background mimicking a naturally occurring LOH event.

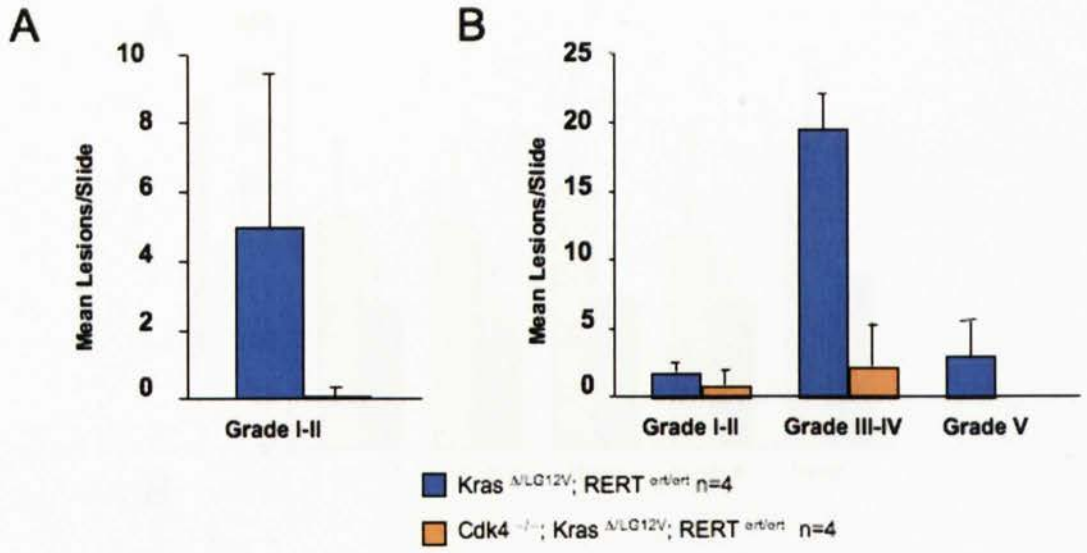


Figure 35: Quantification of the lesions at 3 and 6 months after 4-OHT treatment. The number of lesions at 3 months LOH activation, the number of grade I-II adenomas is reduced by 80% (A). When observed at 6 months we detected a decrease in the number of lesions, and a 75% reduction in the number of grade III-IV adenomas (B). More importantly, in the animals lacking *Cdk4*, the lesions never progressed to adenocarcinomas (n=4 for each genotype).

The appearance of a diabetic phenotype in the *Cdk4*$^{-/-}$;*K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$ mice prevented us again from obtaining a survival curve in these animals for lung cancer. However, we studied the diabetes as well as the weight of these mice over the control. We observed that *Cdk4* null mice start to show increased levels of blood glucose since it is first tested (at three months of age) reaching a peak at 4-6 months post 4-OHT (Figure 36A). Analysing the mice weight, we observed a previously reported decrease in animal weight in the *Cdk4* deficient mice that is not reverted by the expression of *K-ras*^{G12V} and it is maintained during the mice lifespan (Figure 36B).

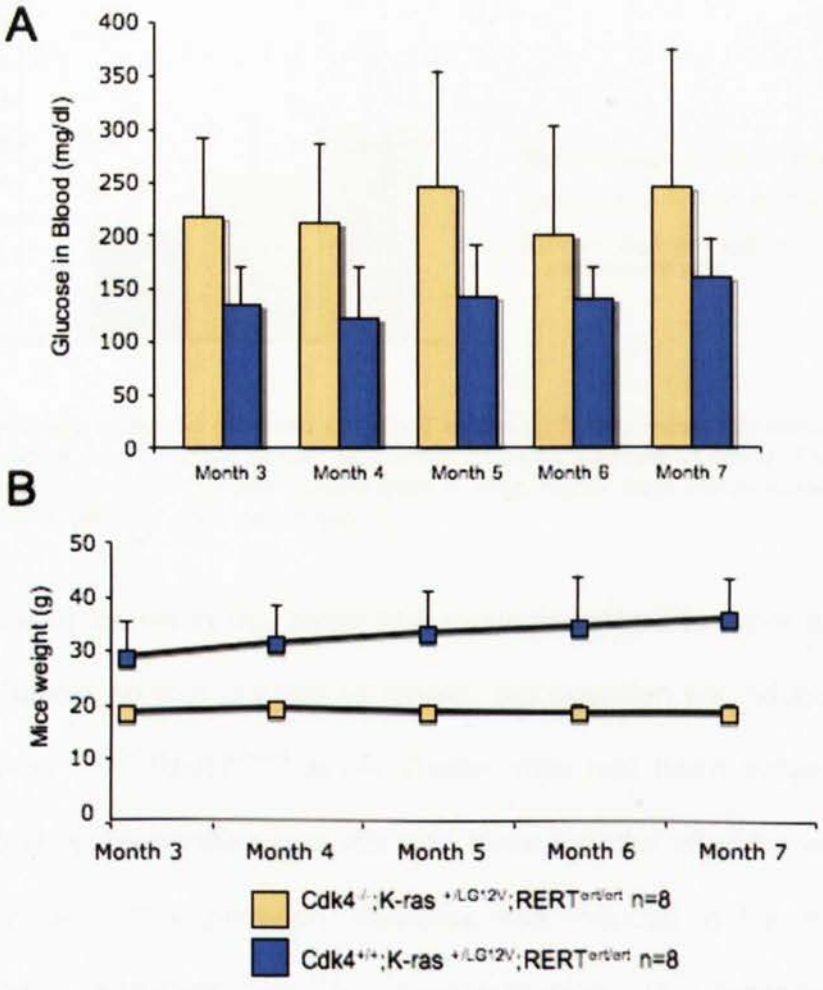


Figure 36: Diabetes and animal weight in the *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice. We tested the glucose in blood of the *Cdk4*$^{-/-}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ animals from the third month after birth to the seventh month, when they were sacrified (A). We observed how the glucose in blood of the *Cdk4* KO mice is much higher than their WT littermates. We considered a mice to be diabetic when its blood glucose is higher than 200 mg/dl. The *Cdk4* –null mice are smaller and thus have a reduced weight when compare to their controls (B). (n=8 for each genotype).

11.2.3 Effect of the diabetes in tumour progression

As we have already mentioned above, the main phenotype of the *Cdk4* null mice is diabetes. We wanted to study whether the tumour suppression effect that we were observing was a cause of the diabetes or of the lack of Cdk4 protein in the lung.

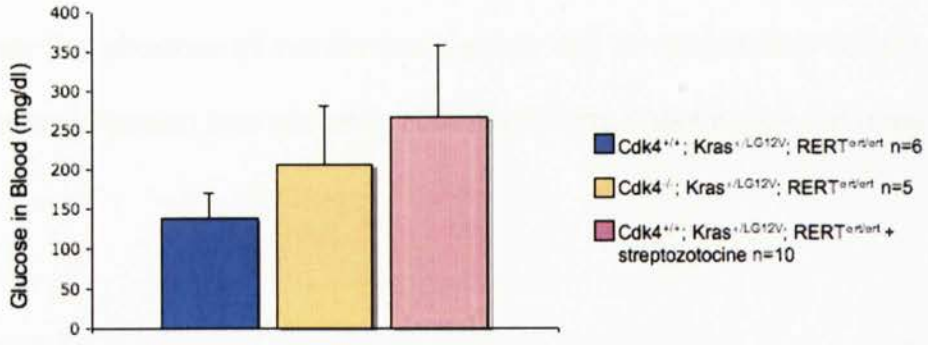


Figure 37: Average of blood glucose detected in the different mice. We observe that after the streptozotocine treatment we obtain high levels of blood glucose in blood. The increase in blood glucose in the streptozotocine treated mice is even higher than the increase observed in the *Cdk4* null mice (n=5 for each genotype).

The absence of insulin in the blood has been described to have an effect on tumour formation, so that in order to answer our question we induced diabetes type-I to *K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice. These mice had been subjected to the normal 4-OHT administration protocol and three months after the endogenous oncogenic *K-ras* ^{G12V} expression, diabetes was induced in the mice by the intrapreritoneal administration of streptozotocine (5 consecutive days intraperitoneal injection of 50ml/gr animal weight with streptozotocine dissolved in 0.9%NaCl pH4.5). The mice were monitored over the following 3 months by weighing them, measuring their blood glucose levels. They were sacrificed when they reached to 6 months post-4-OHT administration.

We observed that the mice subjected to the diabetes protocol showed high levels of glucose in blood at this time point (levels over 200mg/dl) when compared to the control treated (60-150 mg/dl) (Figure 37). In addition we observed that the tumour development is reduced in the diabetic mice (Figure 38) obtaining a 50% reduction of both grade I-II and grade III-IV tumours, however the effect of *Cdk4* ablation is more dramatic. Thus, we can conclude

that limiting amount of insulin in blood has an effect in tumour development however the absence of insulin could solely not be responsible for the massive tumour suppression that we have observed in the *Cdk4* knock out mice.

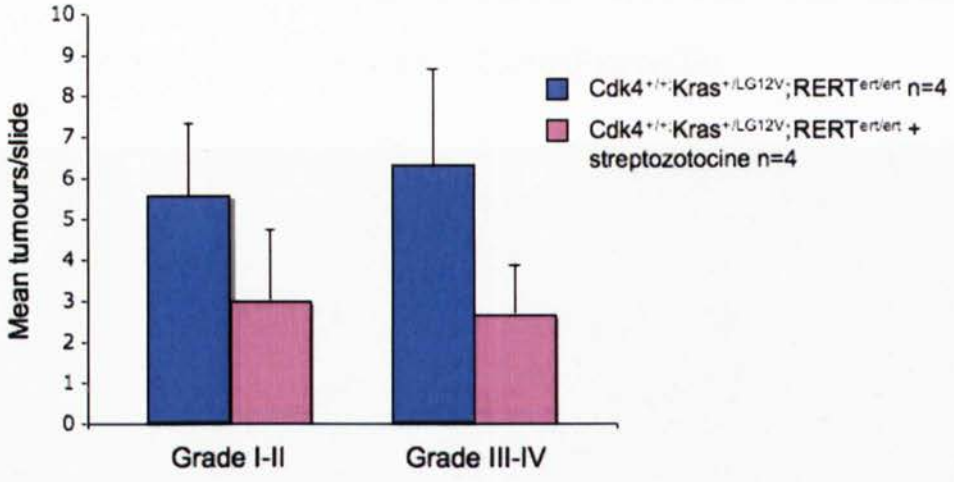


Figure 38: Involvement of diabetes in tumour formation. The quantification of the lesions three months after diabetes inducement and six months post oncogene activation results in a decrease in the number of lesions of either grade I-II and grade III-IV. The diabetic phenotype has a slight impact in the number of lesions and in the malignancy of them (n=4 for each genotype).

11.2.4 Looking for a mechanism

We have shown that there is a clear relationship between *Cdk4* ablation and a tumour suppression role. However, to better understand the mechanism that underlies this tumour decrease that we are observing, we treated *Cdk4*$^{-/-}$;*K-ras*$^{lox/LSLG12V}$;*RERT*$^{ert/ert}$ mice with a single dose of 4-OHT at weaning (P21) and followed the fate of K-ras^{G12V} expressing cells in a Cdk4 deficient background.

11.2.4.1 Is essential Cdk4 for amplification of BASCs?

It has been proposed that lung adenocarcinomas arise as a consequence of deregulated proliferation of a putative stem cell population known as Bronchio

Alveolar Stem Cells, BASCs (Kim *et al*, 2005). Those stem cells localize to the epithelial junction between terminal bronchiole (TBs) and alveoli, typically as isolated cells. However, six weeks subsequent to the activation of oncogenic *K-ras*, single BASCs expand to form groups of up to 8 cells retaining their characteristic double labelling of prosurfactant apoprotein-C (SP-C) and Clara cell-specific marker (CC10) (Kim *et al*, 2005) (Figure 39).

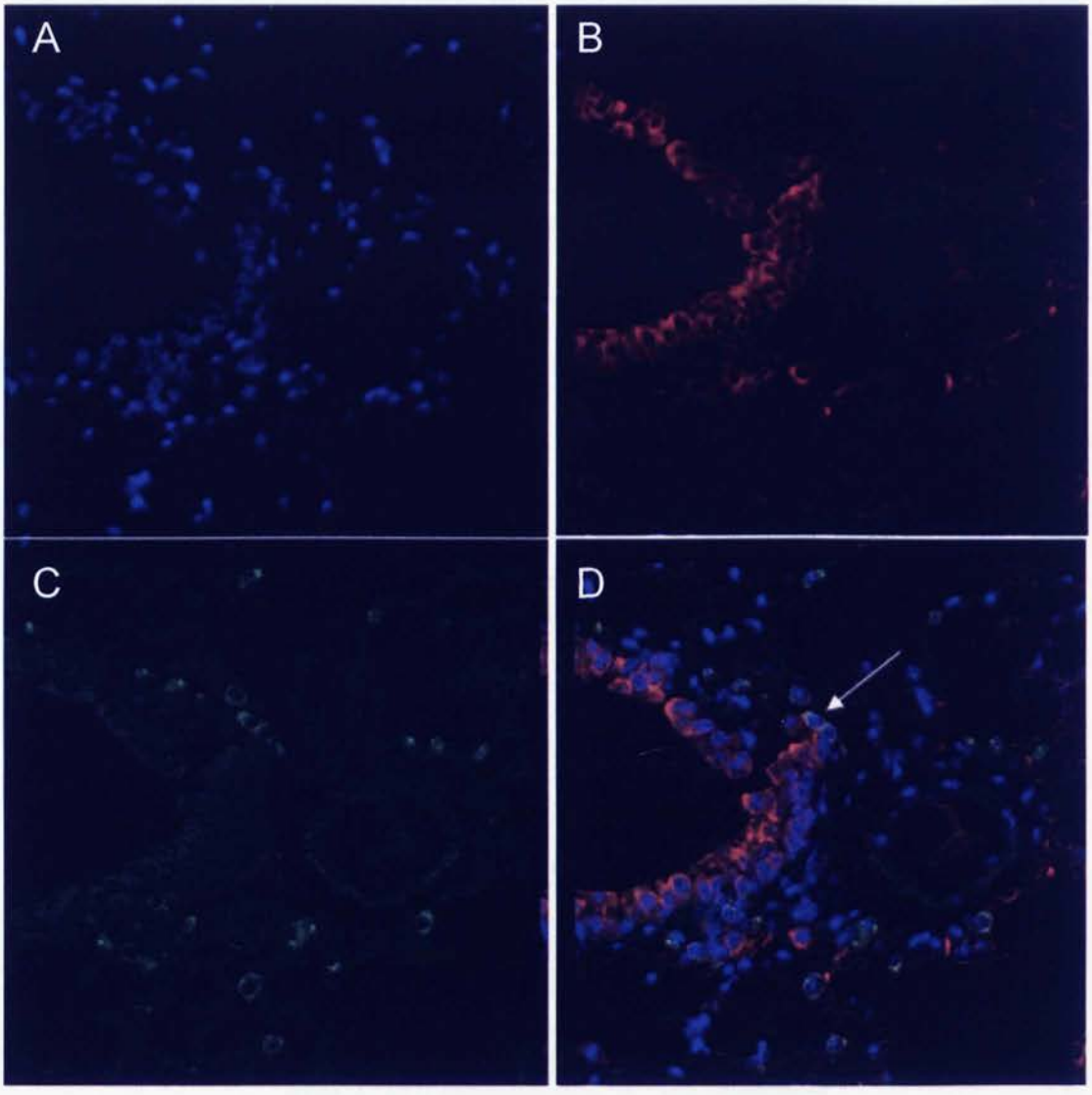


Figure 39: Identification of BASCs by immunofluorescence. In A we can observe the DAPI staining that will mark all the cell nuclei. In B we obtain the CC10 staining that will allow us to identify the clara cells. In C we observe the SP-C staining that is specific for the alveolar type-2 cells. In D we observe a merge of all the images, showing the existence of double positive SP-C/CC10 cells that are the potential lung cancer stem cells.

We wondered whether the observed differences in tumour development described above might originate from the inability of BASCs to proliferate in the absence of Cdk4. Incidentally, Cdk4 has recently been reported to be essential for the proliferation of skin stem cells (Horsley *et al*, 2008). We have performed double inmunofluorecence with both CC10 and SP-C antibodies in either *Cdk4*$^{-/-}$;*K-ras*$^{\Delta LG12V}$;*RERT*$^{ert/ert}$ and the control *Cdk4*$^{+/+}$;*K-ras*$^{\Delta LG12V}$;*RERT*$^{ert/ert}$ lung sections of mice sacrificed 30 days after oncogenic activation.

In *Cdk4*$^{+/+}$;*K-ras*$^{\Delta LG12V}$;*RERT*$^{ert/ert}$ sections we have observed high number of double positive cells, and interestingly we have observed some BASCs mislocalised (Figure 40). The different localisation of the amplified BASCs population could be due to the different way of activation of the oncogene, as we perform intraperitoeneal injections of 4OHT while in the study of Kim *et al*, 2005 the activation is by intranasal AdenoCRE infection.

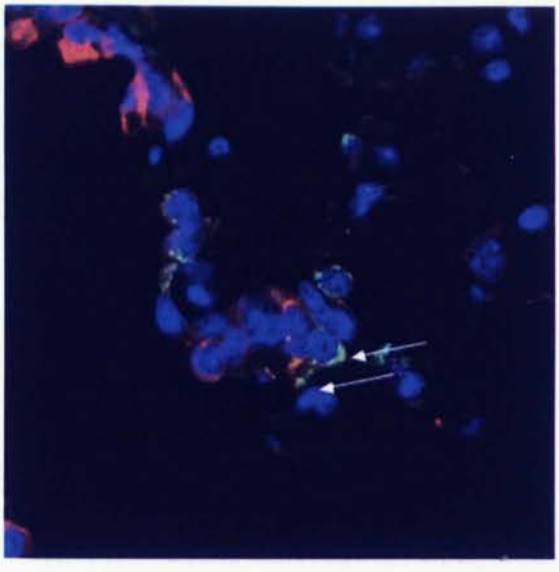


Figure 40: Identification of BASCs mislocalised. In this merge image we can observe a group of double SP-C/CC10 cells in a non terminal bronchioalveolar localisation.

When scoring the number of double positive cells of both genotypes we have not only observed a significant decrease in the number of lung stem cells in *Cdk4*$^{-/-}$;*K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$ over the control lungs but also a reduction in the ability of these cells to expand (Figure 41). The quantification of the BASCs was performed in relationship to the area of the sections.

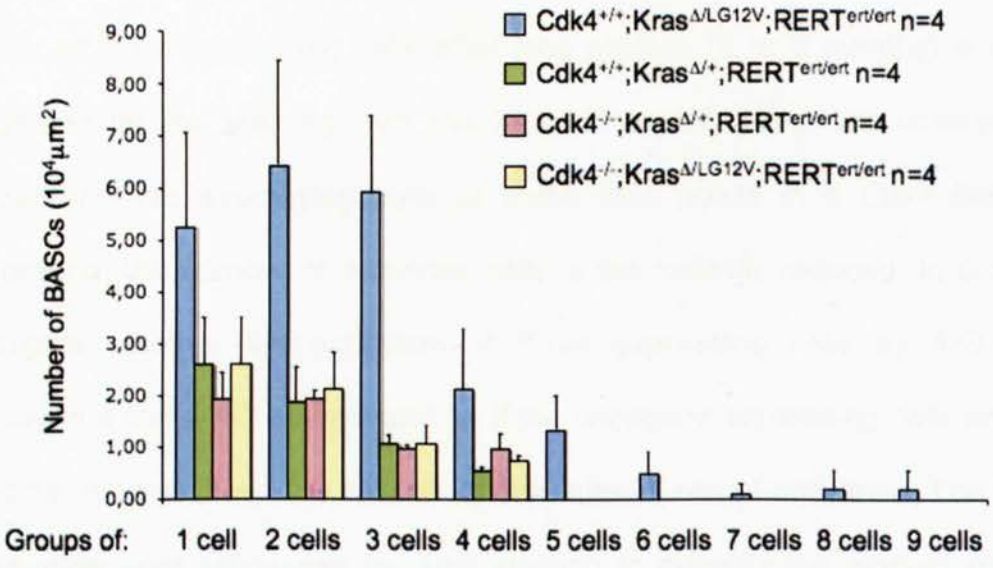


Figure 41: Quantification of the BASC amplification upon K-ras^{G12V} activation. The activation of the oncogene K-ras^{G12V} results in the amplification in the number of groups of BASCs but also in the number of cells forming the group. When Cdk4 in not present, the BASC amplification is impaired (n=4 for each genotype).

It has been shown that the number of BASCs are amplified upon endogenous oncogenic *K-ras* expression, and this expansion is inhibited when Cdk4 is not present. However, it still remains uncertain whether the BASCs are the sole cellular type that proliferate upon oncogenic *K-ras* expression.

11.2.4.2 Quantification of activated cells at short periods of time

During our study we have observed how *K-ras* activation in a *Cdk4* wild type background promotes the amplification of these cells, as the expression of the oncogenic mutation provides the cell with a proliferative advantage. The high number of *K-ras* expressing cells after long periods (3 or 6 months) is easily recognised by the staining with the Xgal. However, when we observe the number of *K-ras* expressing cells at these time points in a *Cdk4* deficient background, the number of activated cells is dramatically reduced. In order to investigate whether the activation of *K-ras* expressing cells by 4-OHT is reduced in a *Cdk4* KO background, or if the oncogene expressing cells are lost with time, we sacrificed the animals shortly after *K-ras* KI activation. The lungs of the mice were processed for Xgal staining to quantify the amount of cells expressing the oncogene. Two weeks after administration of 4-OHT, we saw an equal amount of oncogenic *K-ras* expressing cells in $Cdk4^{+/+}$;$K\text{-}ras^{\Delta/LG12V}$;$RERT^{ert/ert}$ and $Cdk4^{-/-}$;$K\text{-}ras^{\Delta/LG12V}$;$RERT^{ert/ert}$ mice lungs (Figure 42).

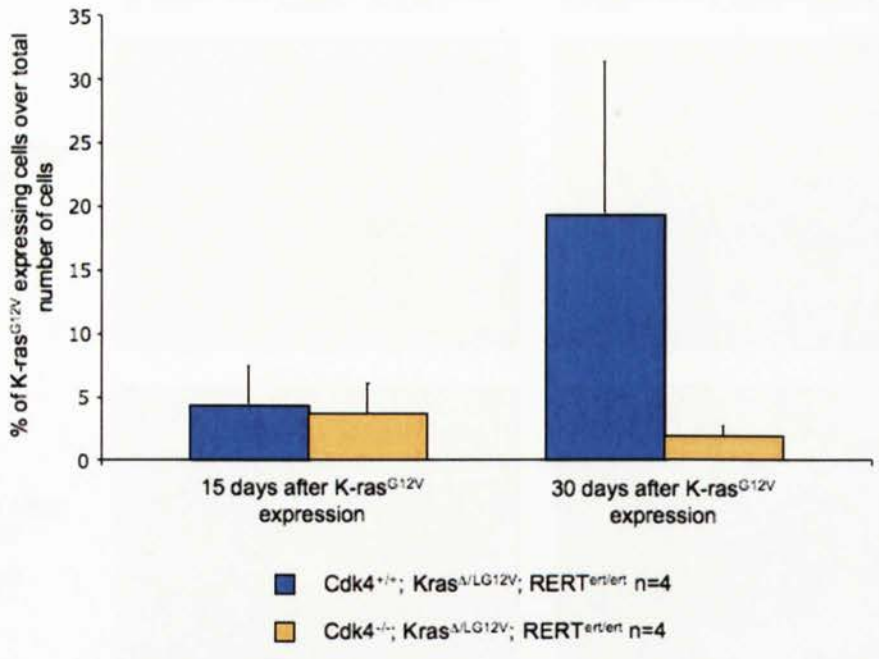


Figure 42: Quantification of the K-ras^{G12V} expressing cells 15 and 30 days after induction of the oncogene expression. 15 days after the activation of the endogenous *K-ras* KI, the number of cells expressing the oncogene is the same. However, 30 days after the oncogene activation there is an important increase in the lungs that retain Cdk4 activity, while in the *Cdk4* KO lungs the oncogene expressing cells are unable to proliferate (n=4 for each genotype).

This result suggested that 4-OHT is able to activate the same number of *K-ras^{G12V}* expressing cells independently of Cdk4 presence. However, when we analysed the lungs of these mice one month after 4-OHT administration we saw an important increase in the number of *K-ras* expressing cells in the *Cdk4$^{+/+}$;K-ras$^{\Delta/LG12V}$;RERT$^{ert/ert}$* while the *Cdk4$^{-/-}$;K-ras$^{\Delta/LG12V}$;RERT$^{ert/ert}$* mice lungs had a significantly lower number of activated blue cells (Figure 42 and 43). Moreover, in mice sacrificed two moths after 4-OHT treatment, the difference between both genotypes was more evident. These results show that although *K-ras ^{G12V}* activation is similar in both genotypes, only in mice carrying wild type *Cdk4* alleles are the activated cells able to expand.

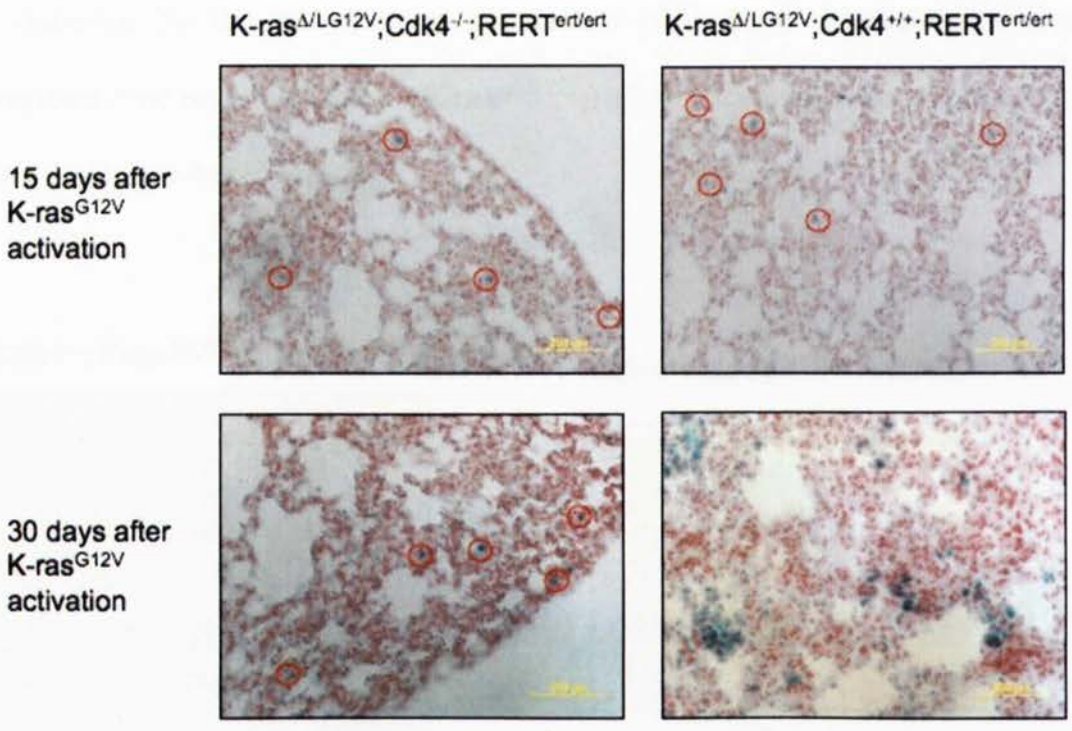


Figure 43: Number of cells expressing K-ras^{G12V} at 15 and 30 days after 4-OHT administration. The activation of the K-ras oncogene is similar in both genotypes at 15 days post 4-OHT treatment. However, 30 days after 4-OHT administration, only few cells expressing K-ras^{G12V} are detected on the *Cdk4* null background, while in a *Cdk4* WT context there is a very significant increase in the number of cells expressing the K-ras^{G12V}.

11.2.4.3 Senescence and Apoptosis

Since we have observed that K-ras^{G12V} expressing cells are not able to expand in a *Cdk4* deficient background, we next wanted to identify the mechanism underlying the lack of proliferation of these cells. It has been described (Collado *et al.* 2005) how *K-ras* induced adenomas are senescent and only when they are converted into adenocarcinomas the senescence status is abolished. However, in hyperplastic lesions or in early stages of *K-ras^{G12V}* activation, there is little or no senescence in the lung cells. To study the possible mechanism we used animals treated at weaning with one injection of 4-OHT and sacrificed again at 15 days and 1 month after 4-OHT administration. We measured the

senescence by the senescence associated (SA) β-gal expression and we observed how only the *Cdk4*$^{-/-}$;*K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$ mice lungs express such marker (Figure 44).

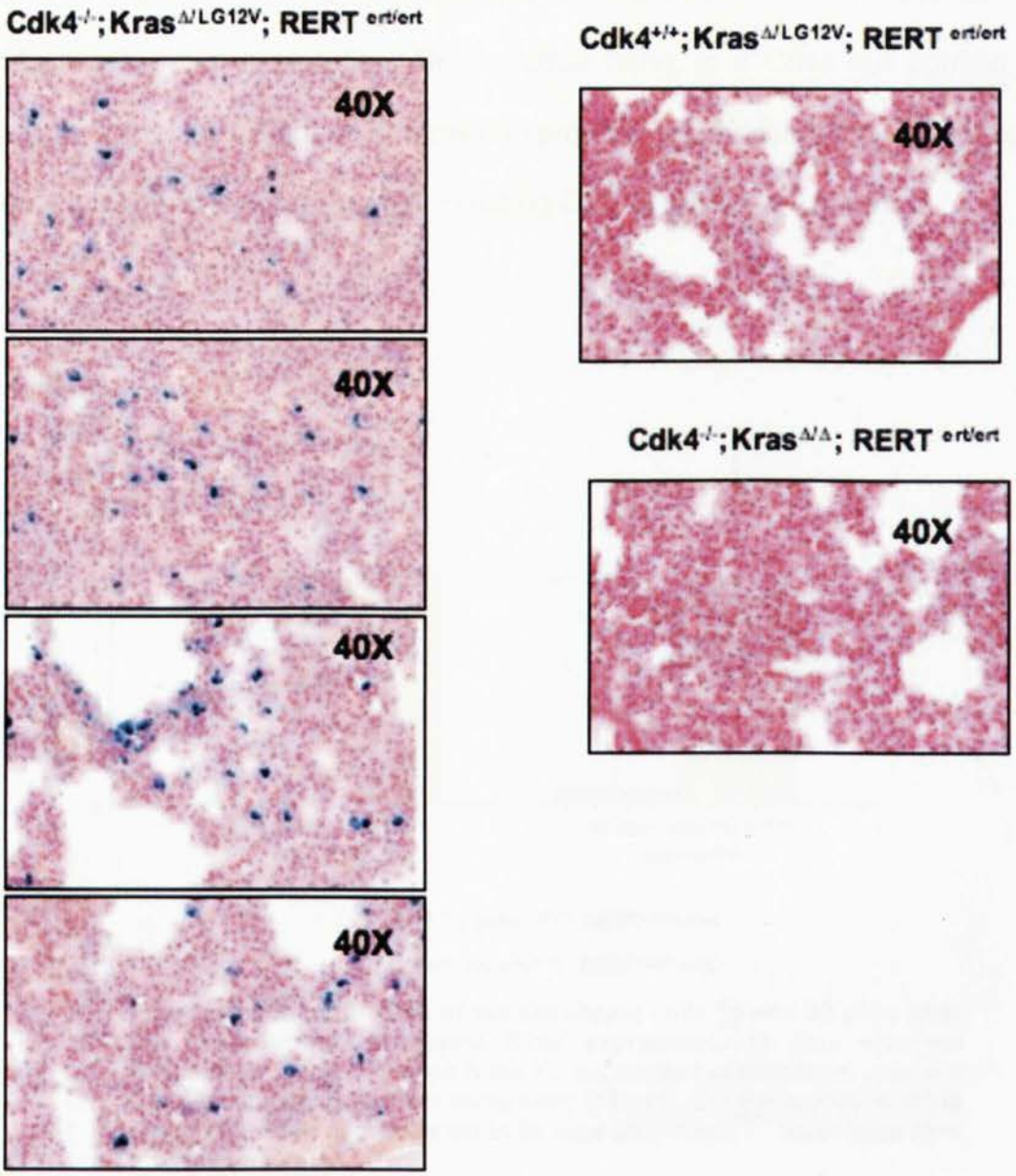


Figure 44: Senescence analysis shortly after oncogene activation. The senescence associated β-gal staining is highly present only in lungs that have suffered the oncogene activation in a *Cdk4* null background. Showing that the remarkable decrease in the tumour incidence must be by the activation of a senescence status in the cells, impeding the oncogenic expressing cells to proliferate.

Moreover, high levels of SA-β-gal staining can be detected at 1 month after 4-OHT administration, however the number of senescent cells seem to decline with the time (Figure 45). This finding supports the idea that the activation of the oncogenic K-ras^{G12V} depends on Cdk4 to regulate the cell cycle. Only when Cdk4 is present the mitogenic cascade activated by K-ras^{G12V} expression will induce cell cycle proliferation. On the other hand, in a *Cdk4* null context, the mitogenic stimuli will not be sufficient to promote cell proliferation and K-ras^{G12V} could accumulate in the lung cell inducing OIS, and thus cell arrest.

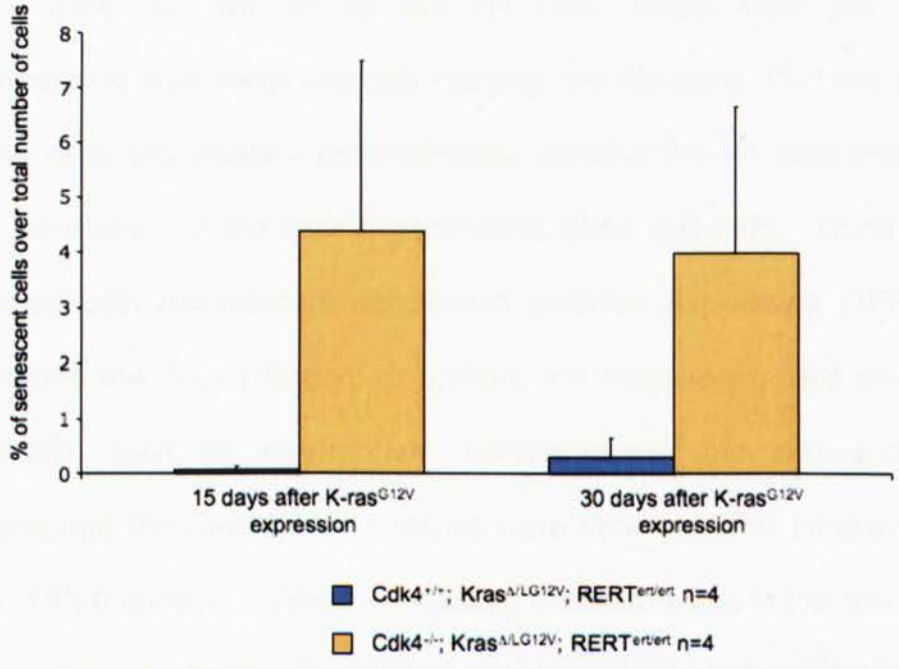


Figure 45: Quantification of the senescent cells 15 and 30 days after the induction of oncogenic K-ras expression. 15 days after the activation of the endogenous *K-ras* KI, the number of senescent cells in a Cdk4 lacking background is remarkably induced, and the activation of the senescent status is maintained at 30 days after *K-ras^{G12V}* expression (n=4 for each genotype).

In addition, we looked at the different short intervals for the presence of apoptosis using active caspase 3 antibody. We observed no positive staining for the apoptotic protein at any of the time points examined. However, as

apoptosis is a very fast cell mechanism we are unable to exclude the possibility that apoptosis might have a role in the tumour reduction of the *Cdk4* null mice at this early stage.

11.2.5 Conditional *Cdk4* KO mice

Cdk4 ablation has an obvious impact on tumour formation or development. In order to study in detail whether Cdk4 is essential for the formation or the development of K-ras^{G12V} driven tumours we used a conditional *Cdk4* mice generated in our laboratory (Barriere, *et al.* 2007). The exons 2 to 4 of the *Cdk4* allele are flanked by two Frt sites. When mice are infected with recombinant adenoviral particles carrying the *Flp* gene, FLP will be expressed in the cells and create a recombination between the Frt sites that will result in the elimination of the exons, generating *Cdk4* null cells. Control mice were infected with recombinant adenovirus particles expressing GFP. Firstly, we optimised the virus infection; to achieve this purpose we tried several infection methods, such as intratracheal, intraperitoneal and intravenous infection. Surprisingly the best excision results were obtained after intravenous infection (pfu: 10^9) (Figure 46). When we injected the adenovirus in the caudal vein in the mouse tail we observed a 50% of excision in the lung, however this infection cannot be repeated to increase the excision at the Frt sites, because the mice rapidly generate an immune response against the virus.

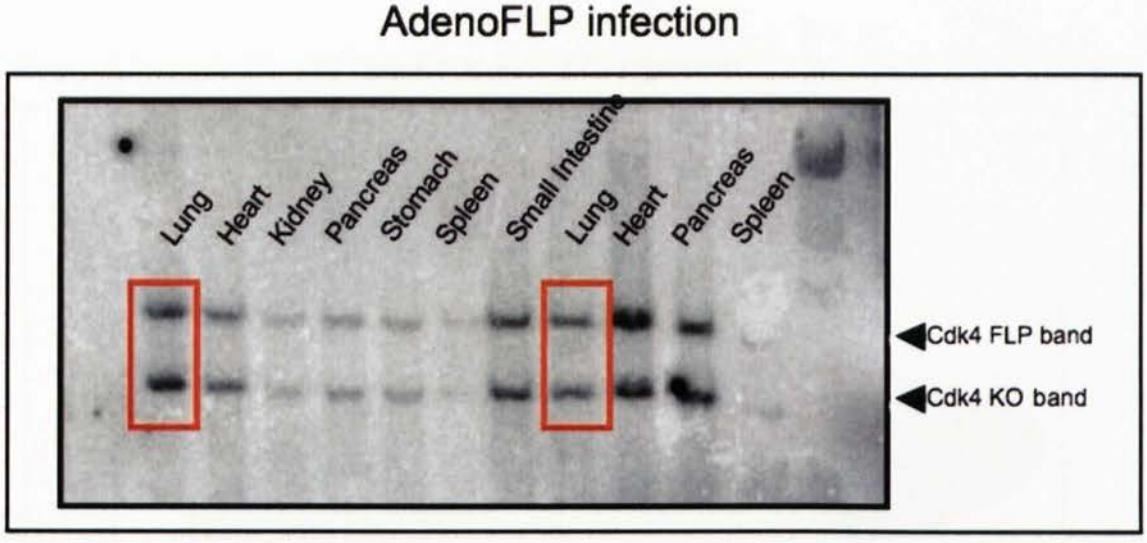


Figure 46: Excision of the *Cdk4* Frt allele upon intravenous AdenoFLP infection. The intravenous infection of AdenoFLP in *Cdk4*$^{-/frt}$; *K-ras*$^{+/LSLG12V}$; *RERT*$^{ert/ert}$ results in a 60% percent of excision in all the examined tissues of the conditional allele.

Due to the levels of *Cdk4*^{frt} excision obtained in lung from the AdenoFLP infection (50-60%), and since the appearance and progression rate of the pulmonary lesions in *Cdk4*$^{+/-}$ mice is indistinguishable from those in *Cdk4*$^{+/+}$ littermates; we decided to carry the conditional *Cdk4* allele together with the *Cdk4 null* allele. We therefore generated *Cdk4*$^{-/frt}$; *K-ras*$^{+/LSLG12V}$; *RERT*$^{ert/ert}$. The oncogene was induced, following the protocol previously described, by intraperitoneal injection of 4-OHT (1mg twice a week during two 2 weeks), and 3 months post oncogene expression activation, the mice were infected with the adenovirus particles. Importantly, at three months after 4-OHT treatment *Cdk4*$^{-/frt}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice showed low grade adenomas, demonstrating that at the point when Cdk4 is excised the lung lesions were already obvious (Figure 47).

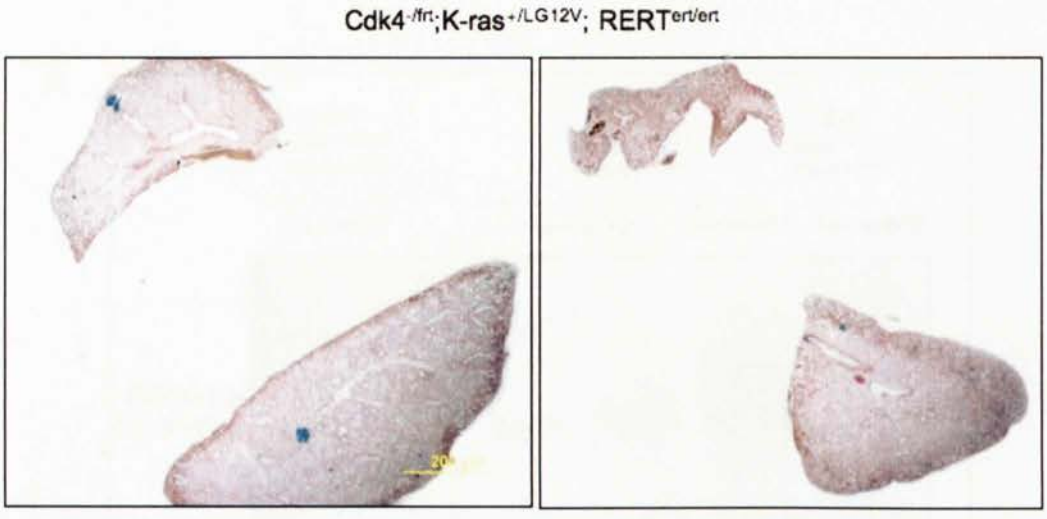


Figure 47: Lesions of the *Cdk4*$^{-/frt}$; *K-ras*$^{+/LSLG12V}$; *RERT*$^{ert/ert}$ lungs at three months after K-ras^{G12V} expression. At the point when Cdk4 is excised by AdenoFLP infection there are already lesions in the mice subjected to the treatment.

Three months later (6 months after 4-OHT administration) the infected mice were sacrificed, as they showed a diabetic phenotype. At this time point, we looked at the excision of the *Cdk4* conditional allele and observed that mice which had been infected with AdenoFLP had 60% of recombination between the *Frt* sites in the mice lungs, this means that 60% of the lung cells were *Cdk4* null cells (Figure 48A), when compared to *Cdk4*$^{-/frt}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice infected with AdenoGFP particles.

In addition, when we looked at the Cdk4 protein expression levels we observed that the protein was reduced to a similar extent in the *Cdk4*$^{-/frt}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ AdenoFLP infected lungs (Figure 48B) over control mice.

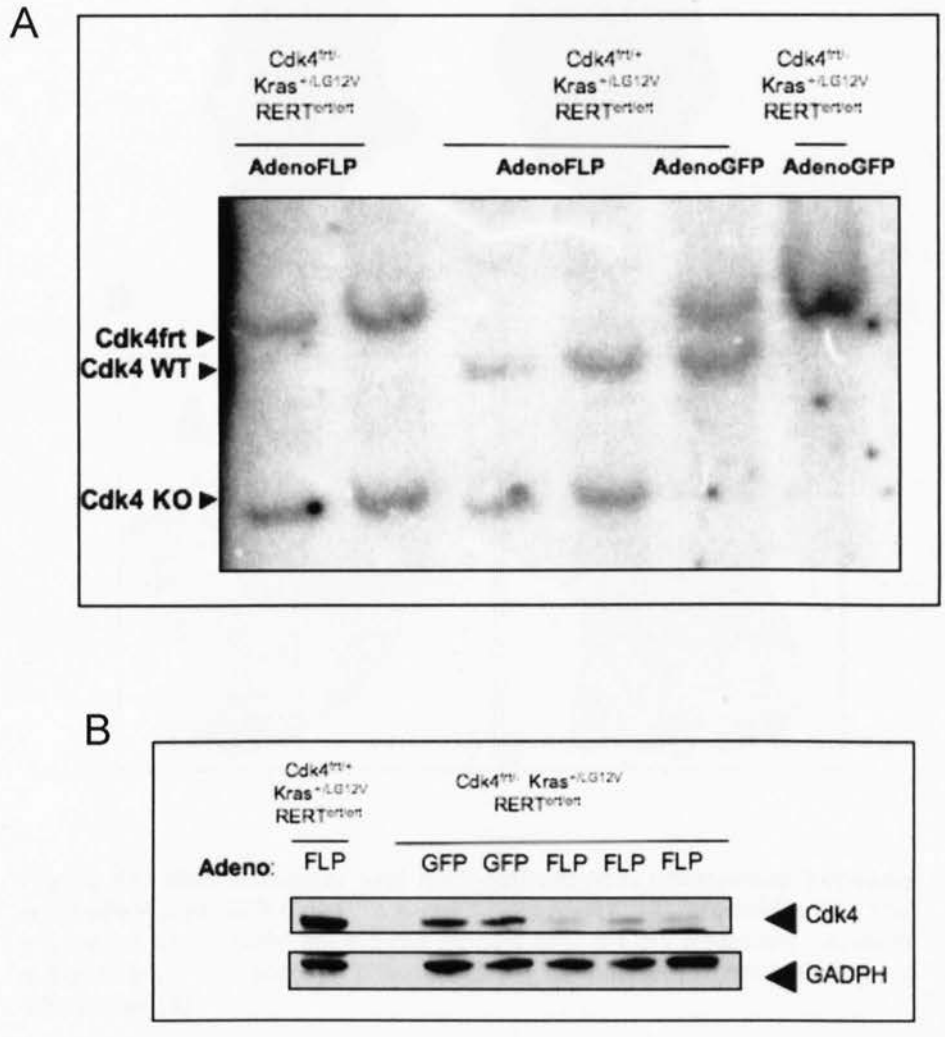


Figure 48: *Cdk4* allele excision and protein levels three months after AdenoFLP/GFP infection. The infection with AdenoFLP results in a 60% excision of the Cdk4 allele in the lung that remains three months after the infection (A). The Cdk4 protein levels are nearly undetectable in the mice lungs that were subjected to the AdenoFLP infection (B).

Interestingly, when we quantified the lesion at 6 months after 4-OHT treatment, and three months after AdenoFLP infection, we observed macroscopically a high reduction in the number of blue cells and no detectable lesions (Figure 49A).

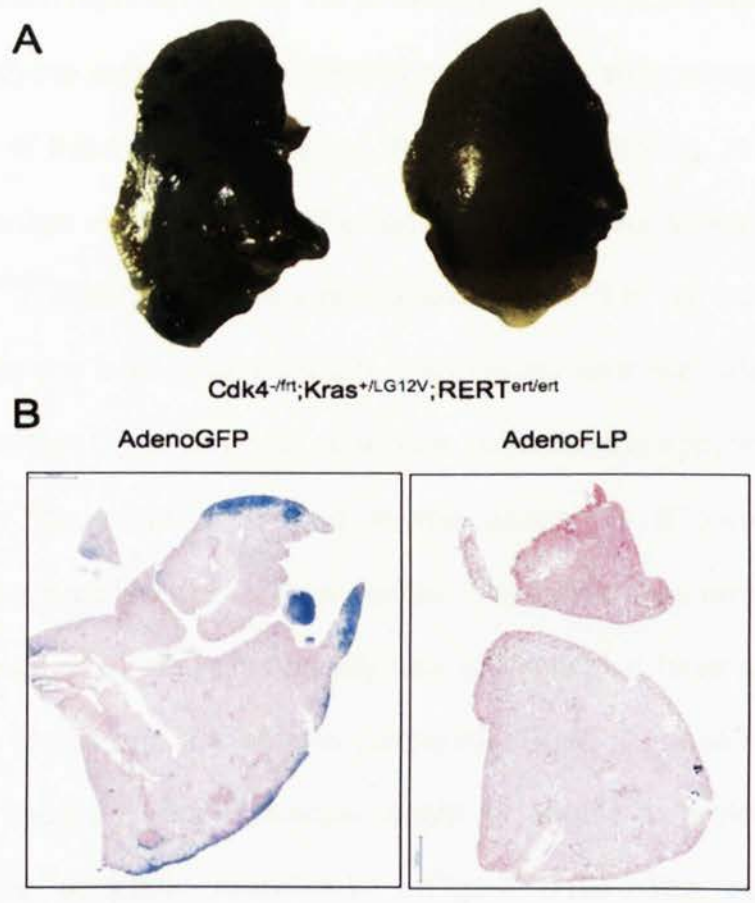


Figure 49: Macroscopical and histopathological differences between AdenoFLP and GFP *Cdk4*$^{-/frt}$; *K-ras*$^{+/LG12V}$; *RERT*$^{ert/ert}$ infected mice. The elimination of the *Cdk4* allele three months after 4-OHT treatment results in a significant macroscopical differences (A) as well as in histopathological differences (B).

Moreover, when the lesion of the *Cdk4*$^{-/frt}$; *K-ras*$^{+/LG12V}$; *RERT*$^{ert/ert}$ AdenoFLP and the *Cdk4*$^{-/frt}$; *K-ras*$^{+/LG12V}$; *RERT*$^{ert/ert}$ AdenoGFP lungs were quantified by the pathologist we observed that the *Cdk4* ablation results in a 80% reduction in the number of grade I-II adenomas and no grade III- IV tumours were obtained (Figure 49B and 50). Hence, at three months of age, when low grade lesions have already been formed, the abrogation of *Cdk4* eliminates tumour cells and consequently results in a decrease in the number of lesions.

As it has been reported that all the premalignant lesions are senescent (Collado *et al.*, 2005) the activation of a different mechanism, such as apoptosis, must be the cause of the tumour regression that we are observing. In order to identify the mechanism responsible for the reduction of tumour incidence in the *Cdk4*$^{-/frt}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice infected with AdenoFLP, we sacrificed the mice shortly after the adenoviral infection. Two weeks after the AdenoFLP infection we observe that the few tumour cells have activated the apoptotic pathway, and importantly this is only detected in the adenomas (Figure 51). As these adenomas are senescent at three months, the proliferation rate in these lesions is low (Figure 51A), and presumably only the cells that have escaped from the senescent status and are able to proliferate. Upon *Cdk4* elimination the cells that have escaped from senescent would be unable to proliferate and would activate the apoptotic machinery (Figure 51B). The activation of this mechanism would explain the observed regression of the lesions.

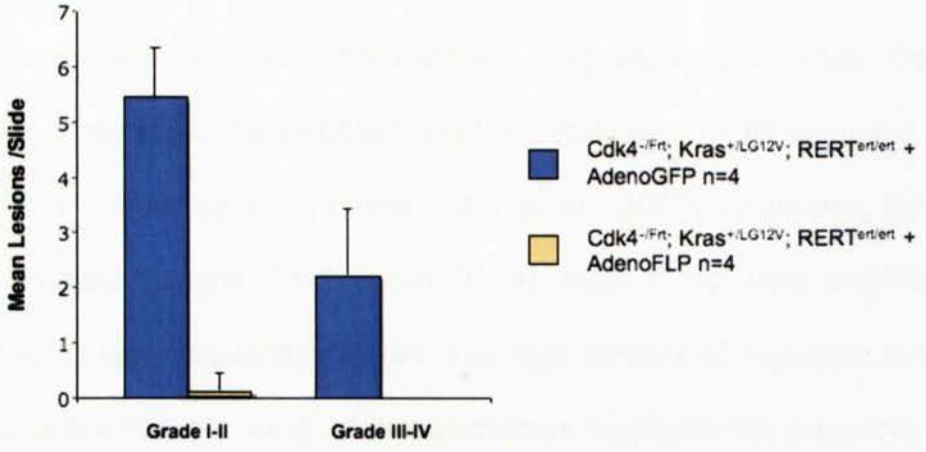


Figure 50: Quantification of the *Cdk4*$^{-/frt}$; *K-ras*$^{+/LSLG12V}$; *RERT*$^{ert/ert}$ mice lungs. We observe a very significant reduction in the number of adenomas found in the *Cdk4*$^{-/frt}$; *K-ras*$^{+/LSLG12V}$; *RERT*$^{ert/ert}$ infected with AdenoFLP three months after oncogene activation (n=4 for each genotype).

These results indicate that Cdk4 is not only necessary for tumour initiation or formation, but Cdk4 also plays an essential role in the tumour development of

the *K-ras*^{G12V} driven lesions. These results open a wide window for the use of Cdk4 inhibitors in oncogenic K-ras^{G12V} derived tumours.

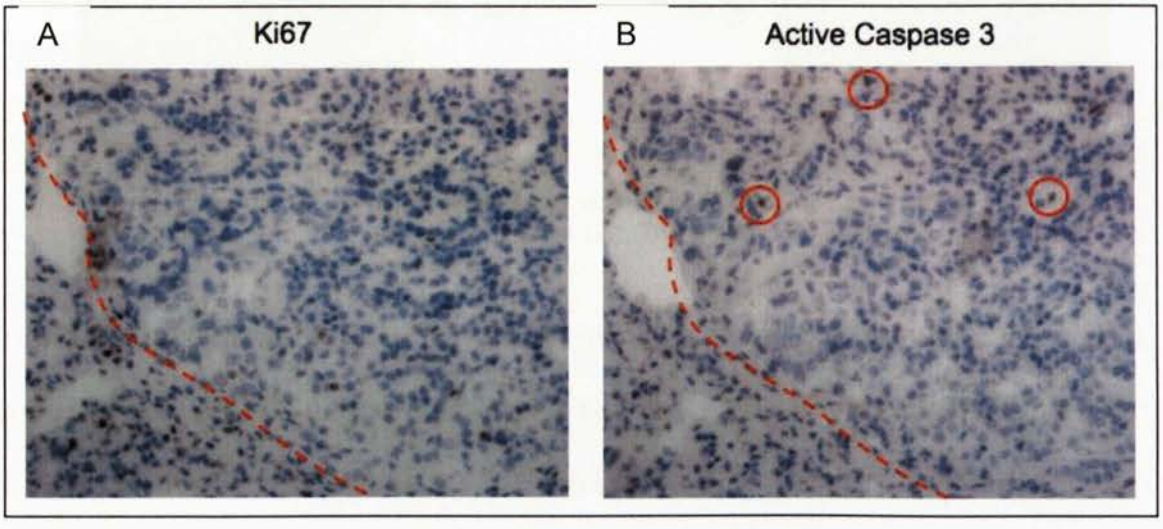


Figure 51: Looking at proliferation and apoptosis two weeks after *Cdk4* excision by AdenoFLP infection in serial sections. By active caspase 3 inmunohistochemistry it can be observed that the *Cdk4*$^{-/frt}$; *K-ras*$^{+/LSLG12V}$; *RERT*$^{ert/ert}$ tumours have activated apoptosis upon is Cdk4 excision. Upon proliferation of the tumours is very low (Ki67).

11.3 From mice to humans

We have established that Cdk4 has a very important role in lung tumourigenesis in mice cells. In human primary lung adenocarcinomas, *Cdk4* locus has been observed to be amplified, implying that Cdk4 might also play an important role in human tumourigenesis (Weir *et al.*, 2007). Moreover, these studies also showed that the *Cdk6*, *cyclin D* and *cyclin E* loci were amplified, while *p16*^{INK4} locus was frequently deleted. The high amount of mutations in G1 cell cycle regulators found in lung adenocarcinomas highlights the necessity of a correct control of cell cycle entry in wildtype cells.

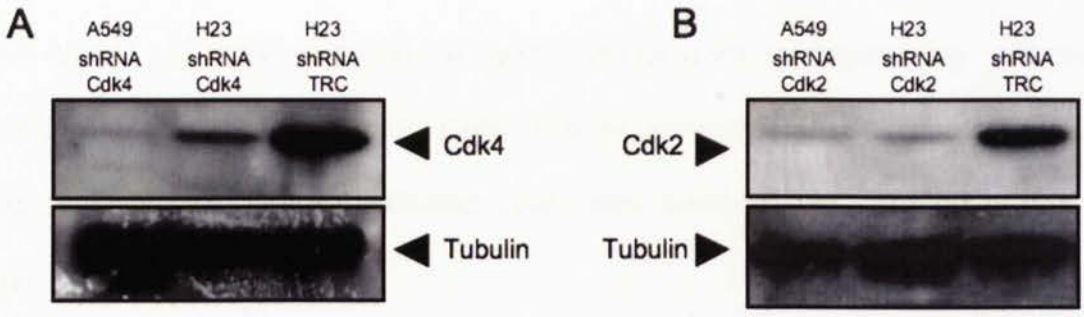


Figure 52: Verification of the efficiency of the shRNAs. The shRNA against Cdk4 highly reduces the protein content of Cdk4 (A). The Cdk2 shRNA is also higly efficient in both NSCLC human cell lines (B).

In order to examine the role that Cdk4 might be playing in human lung cancer we used two NSCLC tumour cell lines, A549 and H23. A549 contains mutations in *K-ras* at the codon 12 and in *Lkb1*, while H23 cells express an oncogenic *K-ras* mutation as well as additional mutations in *Lkb1* and *p53*.

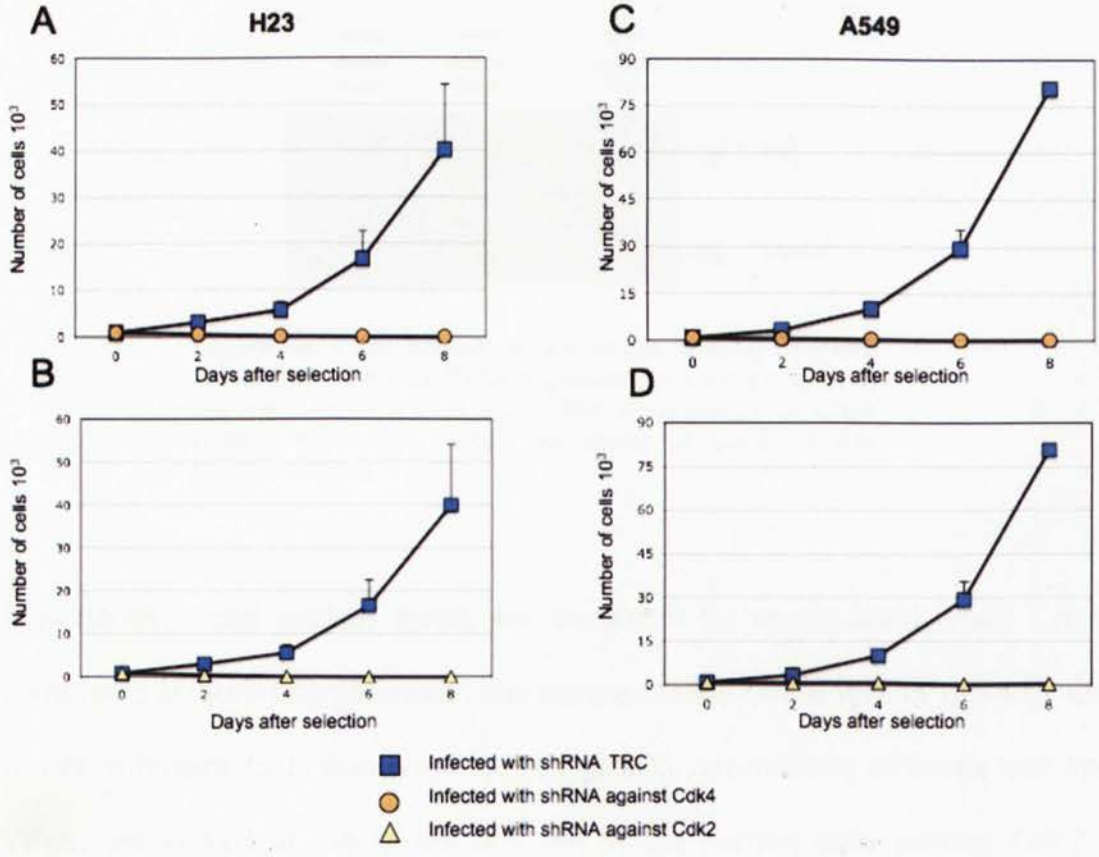


Figure 53: Effect of the elimination of *Cdk2* and *Cdk4* in NSCLC human cell lines. The elimination of *Cdk4* by shRNA induces cell arrest in human lung cancer cells (A and C). The depletion of *Cdk2* also results in inhibiting the NSCLC cells to proliferate (B and D).

Both NSLC cell lines were infected with lentiviral particles expressing effective shRNAs against human *Cdk2*, *Cdk4* and an scramble shRNA control (TRC) (Figure 52). The different infected cells were seeded and counted during 8 days.

We observed that the cells infected with TRC grow continuously, as transformed cells. However, when the cells are infected with shRNA for *Cdk4*, the exponential growth of the cells is impaired (Figure 53). Moreover, when both cell lines are infected with the shRNA of *Cdk2* the same effect was achieved.

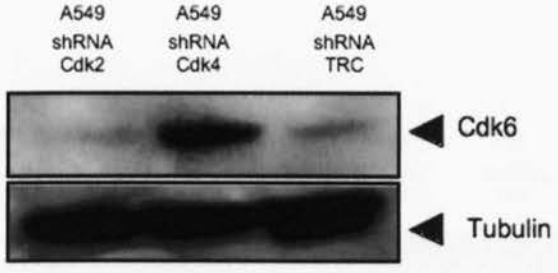


Figure 54: Cdk6 protein levels in the NSCLC cell line A549 after Cdk2 or Cdk4 depletion. In contrast to what we have observed in mice, the suppression of Cdk4 protein expression raises the levels of Cdk6, in this tumour cell line.

Importantly, Cdk6 protein levels are shown to be upregulated when Cdk4 is eliminated (Figure 54). However, the compensation of the lack of Cdk4 by Cdk6 is not sufficient to restore normal cell growth capabilities of these cell lines. When we looked at the levels of Cdk4 in the human cells lacking Cdk2 we obtain similar protein levels. On the other hand, when Cdk2 levels are examined in human NSCLC cells lacking Cdk4, Cdk2 levels seem to be

decreased (Figure 55). This downregulation in the levels of Cdk2 can be due to the low rate of proliferation that these cells suffer from.

We tried to look at the mechanism behind the inhibition of proliferation in human lung tumour cells lacking either *Cdk2* or *Cdk4* and observed that there was no induction of senescence and that apoptosis must be playing a key role in the decreased cell number observed.

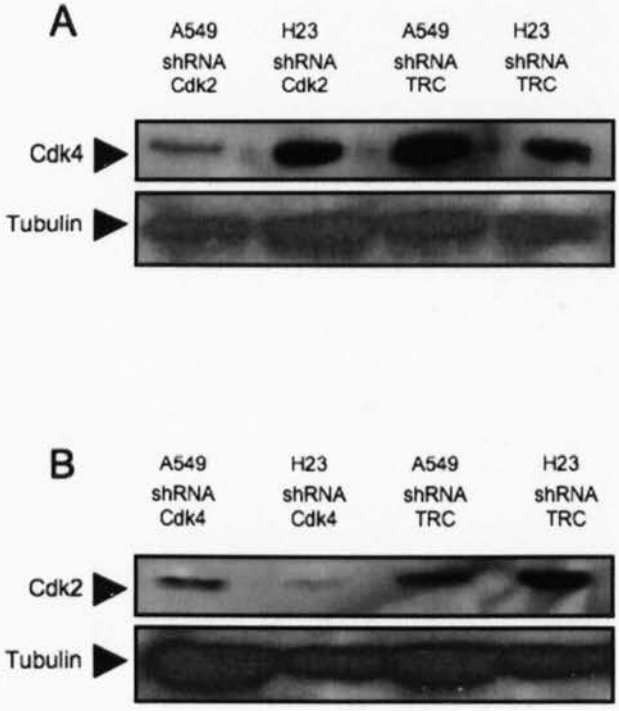


Figure 55: Protein levels of Cdk2 and Cdk4. The elimination of *Cdk2* by the use of shRNA results in a sightly decrease in the levels of Cdk4 protein levels (A). When Cdk4 protein expression is inhibited by shRNA, the levels of Cdk2 are reduced (B).

Moreover, $2x10^6$ cells from the A549 cell line infected either with the shRNA for Cdk2, Cdk4 or TRC were introduced into nude mice to study the tumourigenic potential of these cells. One month after the subcutaneous injection of the NSCLC cells into the mice we observed that the A549 cells infected with either the shRNA against Cdk2 or TRC were able to form tumours in the mice, while

the cells lacking Cdk4 were unable to do so. These results, once again, highlight the importance of Cdk4 in NSCLC tumours. And shows that Cdk4 not only has an important role in the formation and development of mice lung adenomas, but may also have a key role in the control of human lung adenocarcinomas.

12.Discussion

12.1 The *K-ras*^{G12V} oncogene KI model

The generation of a KI mouse strain that expresses the oncogenic *K-ras* isoform under the control of its own promoter has provided us the opportunity to investigate the relationship between the activation of the mutation and cancer development. First studies showed that although *K-ras* is mutated in 60% of the pancreatic tumours and 32% of the colon tumours (Table 4), *K-ras*^{G12V} only promotes tumour formation in lungs (Guerra *et al.,* 2003, Tuveson *et al.,* 2004), and animals die from respiratory failure due to the high number of adenomas and adenocarcinomas. Importantly, only sporadic lesions have been found in the pancreas of mice expressing the oncogenic K-ras^{G12V} mutation and the pancreatic tumour incidence is highly increased when the activation of the oncogene is performed in an inflammatory context (Guerra *et al.,* 2007) induced by a continuous treatment with cerulein.

The activation of Ras by mitogenic stimuli initiates a wide range of cascades, including the Erk/MAPK and the PI3K pathways. The activation of Erk/MAPK cascade promotes the synthesis of cyclin D, while the PI3K pathway stabilises cyclin D. The coordinated action of both pathways promotes cell cycle entry. As there is a direct relationship between the activation of Ras, and the cell cycle promotion, in this thesis we have tried to dissect the implications of the different G1/S Cdks in *K-ras*^{G12V} driven lung tumours. For this purpose we crossed *K-ras*^{G12V} KI model with the KO models for *Cdk2, Cdk4* and *Cdk6.*

12.2 Discussion of the *in vitro* results

The generation of MEFs from the different compound genotypes gave us the opportunity to test *in vitro* the possible effect that the individual Cdk ablation would have in an oncogenic *K-ras* context.

Primary cells expressing the K-ras^{G12V} oncoprotein have been reported to have the ability to grow under limiting serum conditions (Tuveson *et al.*, 2004) however, we have observed that this growth capacity is only obtained when the cells expressing the *K-ras*^{G12V} oncogene have an intact set of the interphase Cdk proteins. Thus, cells lacking *Cdk2, Cdk4* or *Cdk6* are unable to proliferate in such low serum conditions (please see figure 13, page 112). These limiting serum conditions may resemble the situation found in the initial stages of tumour formation, where only cells having special tumourigenic potential are able to proliferate over the majority of cells that are unable to do so. These results suggest a possible necessity of the cells expressing *K-ras*^{G12V} to retain key cell cycle regulators.

When endogenous *K-ras*^{G12V} is expressed in primary MEFs, these cells immortalise without entering in any senescence-like period, and grow continuously. However, when the expression of the oncogene is combined with the ablation of a single G1/S *Cdk*, the crisis period upon immortalisation is restored. So that, the intact set of the Cdks is necessary for maintaining the immortalisation properties conferred by the expression of K-ras^{G12V} (please see figure 14, page 113). These results together with the previous discussed data, support the necessity of primary MEFs expressing *K-ras*^{G12V} to have intact G1/S

Cdk capabilities, in order to maintain the hyperproliferative phenotype specific of the oncogene expression.

In addition, studying the protein levels, we have analysed the protein status of the main cell cycle regulators and no major changes have been observed, only a predictable increase in cyclin D1 in cells expressing the oncogene (please see figures 16 to18, pages 115 to117). So that, on one hand the expression of the activated K-ras^{G12V} does not produce major changes in cell cycle protein levels, and on the other hand, the ablation of the individual Cdks does not alter the expression profile of the other kinases. Thus, the effect that we are observing must come from the elimination of the specific Cdk kinase activity.

In order to analyse in depth the effect of the G1/S Cdks ablation in growth of transfomed cells expressing *K-ras^{G12V}* we firstly tested the effect of the *K-ras^{G12V}* expression together with the ablation of the G1/S Cdks in foci formation by transfection with the oncogenic tandem *H-ras^{G12V}* and *E1a.* The absence of the studied Cdks has also shown to have an important tumour suppressor role by reducing the number of foci obtained after cell transformation with H-ras^{G12V} and E1a. The absence of a single Cdk reduces the number of foci obtained in presence or absence or the endogenous K-ras^{G12V} expression (please see figure 19, page 119). Importantly, we have also observed that the number of foci obtained after oncogenic *H-ras^{G12V}* and *E1a* transfection is reduced when cells are expressing the endogenous *K-ras^{G12V}* oncogenic mutation. The reduction in the number of foci might be due to the activation of oncogene induce senescence in cells, as a result of expression of both exogenous (K-

ras^{G12V}) and endogenous (H-ras^{G12V} and E1a) oncogenic signalling. Those cells that have activated the OIS due to accumulation of oncogenic stimuli will presumably undergo cell cycle arrest.

Secondly, we tested the conditional removal of either *Cdk2* or *Cdk4* in H-ras and E1a transformed cells. The transformed cells showed a decrease in proliferation rate after *Cdk2* or *Cdk4* abrogation (please see figure 20, page 120). These data supports the notion that Cdk2 and Cdk4 play a role not only in early stages of tumour formation but also in later stages, where the tumour cell is fully transformed. From both experiments we observe how the absence of the G1/S *Cdks* has a great impact on transformation, showing the importance of the Cdks in proliferation of the transformed MEFs.

To further evaluate the importance of Cdk2 and Cdk4 in MEFs expressing K-ras^{G12V}, the cells expressing the K-ras oncogene were transfected with catalytically inactive versions for *Cdk2* and *Cdk4*, the KD isoforms. The transfection with these KD isoforms resembles the treatment with a Cdk2 or Cdk4 specific inhibitors, where the Cdk protein is present in the cell and binds to its substrates but its kinase activity is blocked. The KD Cdk will then be unable to dissociate from its bound partners. The difference between a KO or a KD is that in a KO context we lack the protein altogether, and this absence could be compensated by other protein, while the KD retains the protein and only its activity is blocked. When the cells expressing the oncogenic K-ras^{G12V} are transfected with the *Cdk2* or *Cdk4 KD* isoforms we obtain a significant reduction in the cell growth regardless of having wildtype endogenous copies of Cdk2 or

Cdk4 in the tested cells (please see figure 22, page 123). Importantly we observe a higher reduction in the proliferation rate of the Cdk2 KD transfected MEFs. The Cdk2 KD might be titrating cyclin A and thus preventing its association to Cdk1, leading to a lower proliferation profile. The remarkable decrease in proliferation highlights the importance that a specific treatment against *Cdk2* or *Cdk4* could have in *K-ras*^{G12V} driven tumour therapy.

From all the *in vitro* results presented above, we can conclude that the ablation of a single G1/ S *Cdk* results in the abrogation of essential properties conferred to the cell by the expression of the oncogenic K-ras. Having an intact set of the *Cdk2, Cdk4* and *Cdk6* genes seems to be essential for the oncogenic expressing cell to grow in limiting serum conditions, immortalise and have a high proliferation rate after cell transformation. Moreover, the cell growth decrease after *Cdk2* and *Cdk4 KD* transfection allowed us to hypothesise that the proliferation of the K-ras^{G12V} driven cells could be decreased by the use of specific inhibitors against the kinases. To further examine the relationship between the G1/S Cdks and oncogenic *K-ras*^{G12V} we designed mouse strains that would explain the role of Cdk2, Cdk4 and Cdk6 in *K-ras*^{G12V} tumour formation and development *in vivo*.

12.3 Discussion of the *in vivo* results

The results that we had obtained *in vitro* led us to postulate that Cdks could be possible key targets in K-ras induced tumour therapy. For this purpose, in order to evaluate the possible importance of the Cdks in patient treatment we generated different mouse models combining the lack of any of the three G1/S

Cdks with the oncogenic *K-ras* KI model. We induced *K-ras*^{G12V} activation by 4-OHT administration and observed the tumour development and the impact of the tumour burden on the mice life span. As we have shown in the result section, the presence or lack *Cdk6* has no effect in lung tumour development nor impact in mice survival (please see figures 23 and 28, pages 125 and 130). However, the cause of this lack of effect must be the near absence of Cdk6 protein expression in wildtype tumours (please see figure 32, pages 136-137). All lung tumours driven by K-ras^{G12V} are positive for alveolar type 2 (AT-2) markers, being Cdk4 the main substrate for cyclin D association. More importantly, there is no upregulation of Cdk6 protein levels when Cdk4 or Cdk2 are absent (please see figures 30-31, pages 133 -134). The lack of an increase in the protein levels suggests that in this particular cell type Cdk6 is unable to compensate for the lack of any tested Cdk and to drive the cell cycle.

As to study the potential therapeutic role of Cdk2 in K-ras^{G12V} driven lung adenomas we generated two different mice models. In the first Cdk2 mouse model generated, we used the *Cdk2* conditional KO allele. The 4-OHT treatment of these mice was extended in order to obtain high levels of *Cdk2* excision since the ablation of the *Cdk2* allele is also dependent on the Cre-Lox system (please see figure 26, page 128). When we quantified tumour number at six months after initiation by 4-OHT administration we observed a decrease in the number of tumours, as well as an increase in the mouse lifespan (please see figures 24 and 27, pages 126 and 129). Importantly all the examined tumours were *Cdk2* deficient, showing that Cdk2 activity is dispensable for tumour formation and development. The use of the *Cdk2* conditional KO mice

allowed us to observe the effect of the elimination of *Cdk2* at the same time of the oncogene expression (please see figures 24 and 28, pages 126 and 130). Although in the *K-ras* KI model, the *Cdk2* conditional KO does not resemble a therapeutic approach, it is important to highlight that Cdk2 will be normally expressed until the expression of *K-ras*^{G12V} is activated. Thus, the decrease in tumour development could be associated to the lack of Cdk2 itself or to a possible initial disruption of the cells when the *K-ras*^{G12V} oncogene is expressed and at the same time Cdk2 expression is impaired. In order to clarify the pathway responsible for the partial tumour suppression obtained in the *Cdk2* conditional model, we generated a second *Cdk2* KO mice model. The second model contained the *Cdk2* germline KO allele. In these mice we induced K-ras^{G12V} expression as we did in *Cdk6* KO animals, and waited to observe the effect of Cdk2 deficiency on tumour formation and the life span of the mice. *Cdk2* deficiency had the highest effect *in vitro* by decreasing K-ras oncogenic potential in MEFs, restoring a four-passage crisis upon immortalisation and significantly reducing the cell proliferation when *Cdk2 KD* isoform was expressed. Moreover it had been reported how Cdk2 levels were increased in K-ras^{G12V} tumours (Tuveson *et al.,* 2004 and our own results). In contrast to what we had hypothesised, the lack of Cdk2 has a significant effect on the tumour incidence and impact on mice survival. These results suggest, that Cdk2 might have a key role in regulating cell proliferation and transformation in *in vitro* fibroblasts, however this suppression effect could be cell type specific. As a consequence, Cdk2 protein expression does not seem to be essential in the adenomas nor adenocarcinomas, and Cdk2 itself seems to be the responsible

for the reduction in the number of tumours and in the extension of the mice life-span.

Moreover, it has been demonstrated in a number of tumour models that the elimination of *Cdk2* has no effect in tumour suppression, as it is unable to revert the organomegalia or the pituitary tumours arising from *p27* deficiency (Martin *et al.,* 2005), and it has no effect on reducing the number of papillomas induced by the two-step skin carcinogenic treatment of DMBA-TPA (Martin *et al.,* unpublished). In contrast, we observed a significant decrease in the number of tumours of grade I and II as well as in grade III and IV lesions six months after 4-OHT treatment, and a slight increase in mouse survival. *Cdk2* lack seems to decrease tumour development in the K-ras^{G12V} tumour context, however those results were much less important to what we had hypothesised from the *in vitro* data. The possible explanation for the mild results obtained for the evaluation of Cdk2 as a target for therapeutic intervention could be the compensation by Cdk1. Thus, in the *Cdk2* null mice we observe compensation from the beginning, while in the conditional KO this compensation has to be obtained after the *Cdk2* conditional alleles are excised. However, we do not observe this compensation *in vitro* where Cdk2 has a significant effect on cell proliferation. The differences between the *in vitro* and *in vivo* observations could be explained by the cell nature and context.

The *in vitro* results had also shown that Cdk4 may also have a therapeutic role in tumour development. However this Cdk had the mildest effect *in vitro* among the tested G1/S Cdks. In contrast to those *in vitro* results, Cdk4 and Cdk2

protein and kinase levels had been reported to be increased in MEFs expressing the K-ras^{G12D} oncogene (Tuveson *et al.,* 2004) and we have observed how Cdk2 and Cdk4 protein levels are rised in K-ras^{G12V} driven lung adenomas. To examine the role of Cdk4 in tumour formation we generated a mouse model that combined the *K-ras^{G12V}* KI allele with the *Cdk4* germline KO and examined tumour progression at different time points. When mice were sacrified three months post 4-OHT administration, the *Cdk4* null mice containing the oncogenic mutation showed no sign of tumours, nor hyperplasias. Moreover, when we looked at a later time point (six months after treatment initiation) we observed a clear reduction in the number of premalignant lesions (grade I and grade II) and no malignant lesions (grade III and IV) (please see figure 29, page 131).

However, mice lacking *Cdk4* develop a diabetic phenotype that included polyuria and polydypsia with impaired locomotion, staggering and hyperactivity (Rane *et al.,* 1999). They develop this phenotype as a consequence of having a defect in the development of the β-cells and therefore insuline production. The diabetic phenotype prevented us from obtaining a mice survival curve where we could test the effect of the lack of *Cdk4* in a K-ras^{G12V} expressing mice and its impact on the mice lifespan (please see figure 25, page 127).

As the relationship between cancer and diabetes have always been uncertain (Hall *et al,* 2005), we wanted to test whether the effects that we were observing with the *Cdk4* null mice were due to the absence of the *Cdk* of interest or due to the diabetic phenotype. We subjected oncogenic K-ras^{G12V} expressing mice in a

Cdk4 wildtype background to a diabetic treatment, inducing this phenotype by the injection of streptozotocin at three months after 4-OHT administration. When quantifying the tumour development at three months after the diabetic induction, and thus six months after 4-OHT administration and tumour initiation, we observed a reduction (50% reduction in the grade I and II, and 60% decrease in grade III and IV) in the number of lesions of both grade I and II, and grade III and IV (please see figure 38, page 144). Those results suggest that diabetes may play an inhibitory role in tumour progression, however the diabetes is not solely responsible for the results obtained with the *Cdk4* deficient mice. In addition, the number of grade I-II tumours shown in the diabetes induced animals are three times more than in the *Cdk4* KO mice. Moreover, mice that underwent the diabetic treatment do have grade III and IV while *Cdk4* null mice lack these kind of lesions.

We have shown the importance of Cdk4 in K-ras^{G12V} driven lung adenomas. However, in order to study in greater detail the role of Cdk4 in tumour development we used the K-ras LOH model generated in the laboratory. The LOH model was a more challenging tumour background. In fact, this LOH can be detected in all human lung cancer lesions that carry oncogenic *K-ras* mutations (Li *et al*, 2003). In the K-ras LOH model we were able to express the oncogenic form of K-ras at the same time that we inactivated the wildtype copy of the *K-ras* gene. These LOH mice showed a faster onset of tumours and thus died much earlier (at around six months after 4-OHT treatment) (please see figure 33, page 138). Interestingly, the tumours resulting from the LOH *K-ras* model were more aggressive and by 6 months after 4-OHT treatment we even

observed invasive carcinomas (grade V lesions). When we crossed the LOH model with the *Cdk4* null mice and activated the tumour model, we observed 7 fold reduction in the number of tumours when Cdk4 was no longer present (please see figures 34-35, pages 139-141). Importantly, we observed how *Cdk4* null adenomas never progress to grade V lesions, while in Cdk4 expressing mice we mainly observed adenocarcinomas. However, in this tumour background we were again unable to study the incidence of *Cdk4* ablation in mice survival because of the sudden death of the animals due to the diabetic phenotype.

Nevertheless, other studies have provided strong evidence showing the role of *Cdk4* deficiency in decreasing tumour formation. Cdk4 protein expression levels have been shown to be essential for the development of pituitary hyperplasias in *p18* $^{-/-}$ mice (Pei *et al.,* 2004). Moreover, *Cdk4* deficiency suppresses ErbB-2 driven mammary oncogenesis in mice; importantly *Cdk4*$^{-/-}$ females have a defect in breast development, which could be the responsible for the tumour suppression (Reddy *et al*, 2005). However in *Cdk4*$^{-/-}$ mice, the lungs have a normal development, and thus cannot be the cause of the effect that we are observing. Additionally, it was also shown how the inactivation of Cdk4 by shRNA in primary mammary tumour cultures, when reintroduced into nude mice result in an inability to produce tumours. Yu *et al.,* 2006 also demonstrated how Cdk4 kinase activity is the responsible for the tumour decrease, as the transfection of murine breast cancer cells with a *Cdk4 KD* isoform results in an inhibition of the ability to form tumours when the infected cells are inoculated into mammary fat pads (Yu *et al.,* 2006).

In addition, Rodriguez de la Puebla performed a skin carcinogenic treatment using DMBA/TPA in Cdk4 lacking mice and observed a 77% reduction in the number of papillomas resulting from the treatment. Importantly, they observed how *H-ras* is not mutated in the resultant papillomas, this result suggests that skin cells lacking Cdk4 that are able to form lesions do so through a Ras independent pathway (Rodriguez-Puebla *et al.,* 2002).

12.4 Mechanism underlying the tumour suppression

We have shown through this thesis how Cdk4 plays a key role in the formation and/or development of K-ras^{G12V} induced tumours. Moreover there is a clear connection between Ras activation and the initiation of the cell cycle by the synthesis and stabilisation of cyclin D. There have been other studies that have shown the importance of Cdk4 for tumour development (Rodriguez-Puebla *et al.* 2002, Pei *et al*, 2004, Yu *et al*, 2006) in different backgrounds, however no one has shown the mechanism underlying this evidence. For this purpose we used the LOH model combined with the *Cdk4* deficiency, and activated the *K-ras* LOH system by a single injection of 4-OHT and observed the effect at different time points. First we analysed the number of oncogenic K-ras expressing cells at 15 and 30 days after 4-OHT treatment. We observed that 15 days post oncogenic activation, the number of cells expressing K-ras^{G12V} was equal in both control and *Cdk4* deficient lungs. However, by 30 days post infection the difference in K-ras^{G12V} expressing cells was very significant, 5 fold, between both groups (please see figures 42-43, pages 149-150). So that, in control animals we observed how the number of K-ras^{G12V} expressing cells is

increased as time goes by, while in *Cdk4* null context, the oncogenic expressing cells are probably not able to proliferate adequately and so their levels are reduced.

From these results we observed that the reduction in the number of tumours must be due to the impaired capacity of cells expressing K-ras^{G12V} to proliferate in *Cdk4*$^{-/-}$ lungs. However, what is the mechanism underlying the difference in the number of cells that are expressing the K-ras^{G12V} oncogene? We first tested if there was an increase in apoptosis at early time points, as we observed a difference between the number of K-ras expressing cells, but there were no active caspase 3 stained cells regardless of *Cdk4* status. The second mechanism that could be playing a role in reducing the number of cells expressing K-ras^{G12V} was the activation of senescence. Cell senescence is an reversible proliferative arrest that can be activated by various mechanisms: telomere deficiency, cytotoxic agents, DNA damage, activation of the INK4a locus or oncogene activation. Senescencent cells acquire an irreversible statuts in the chromatin that prevents its division. The cell senescence appears as a consequence to the activation of the ARF-p53 or the p16^{INK4a}-Rb pathways. *In vivo*, it has been observed that the sencecent cells disappear with time (Kay, 1975), so that sometimes when a tumour is treated with chemotherapeutic agents the cells do not undergo apoptosis but senescence. The activation of senescence in the tumourigenic cells leads to the regression of the lesions due to the activation of the immune system involving phagocytosis of senescent cells (Gewirtz *et al.,* 2008). Due to the lack of good antibodies for other senescence markers, we tested senescence status, by looking at the

senescence associated β-gal expression. Interestingly we observe how lungs lacking *Cdk4* but expressing the oncogene have activated senescence pathways. The activation of growth arrest is obtained shortly after K-ras^{G12V} induction (15 days after 4-OHT administration) and maintained for 15 and 45 days more (please see figures 44-45, pages 151-152). It is important to state that premalignant lung lesions have been reported to be in a senescent status (grade I and II tumours) while hyperplasias, malignant lesions (grade III–IV) or invasive carcinomas are negative to growth arrest (Collado *et al.,* 2005). So, the senescence that we are observing is specific of *Cdk4* ablation in an oncogenic K-ras expressing context, and can be the responsible for the tumour growth inhibition that we are observing.

Although by one month after activation of the K-ras^{G12V} oncogene expression in a *Cdk4* null background the number of oncogene expressing cells is reduced, by three and six months after the 4-OHT treatment we do observe K-ras^{G12V} driven tumours lesions. The tumours obtained in a *Cdk4* deficient context could be the result of the accumulation of additional mutations that allow the cells to evade from senescence, thus indicating that K-ras^{G12V} is not able to promote tumour formation by its own in a *Cdk4* deficient background. It is also interesting to point out the fact that all the lesions that we have obtained in a *Cdk4* null background are of low grades. There could be two arguments supporting the low grade lesions obtained in our *Cdk4* deficient mice containing the oncogenic K-ras^{G12V} mutation. Firstly, the lesions arise as grade I lesions and only by accumulation of additional mutations the tumours progress into a carcinoma as pancreatic lesions (Hruban *et al.,* 2006) and colon tumours do (Shibata and

Aaltonen, 2001). On the other hand, it could be argued that depending on the cell of origin, some tumours would be genetically design to progress into grade V (carcinomas) while others would only be able to become benign adenomas. Whatever the situation, *Cdk4* null lesions are not able to progress to invasive carcinomas, at least during its lifetime. The most probable mechanism responsible for the tumour reduction and absence of high grade lesions is the activation of senescence after the activation of K-ras^{G12V} expression.

Moreover, it has been established by the laboratory of Dr. Jacks (Kim *et al.,* 2005) that the cells responsible for the tumour formation in the endogenous *K-ras^{G12V}* KI model are the bronchoalveolar stem cells (BASCs). These cells stain for markers of both Clara and AT-2 cell and thus are easily recognised. It has been proposed that as a consequence of oncogenic *K-ras^{G12V}* mutation, there is an amplification of the number of BASCs at the terminal bronchiols (TB), and this expansion in the number of BASCs cells is responsible for the tumour formation (Kim *et al.,* 2005). Importantly, a p27 mouse model containing point mutation that makes the protein unable to bind to the Cdks, and thus results in a continuous input of Cdk activity, when combined to the K-ras oncogene KI model substantially increases the BASC amplification (Bessons *et al.,* 2007). In addition, in a second tumour model where the oncogenic mutation of K-ras is enhanced by the lack of *p38α*, the number of BASCs is also expanded (Ventura *et al.,* 2006).

Remarkably, it had been published that Cdk4 is repressed in skin stem cells to retain these cells in quiescence. A microarray analysis identified the

transcription factor, nuclear factor of activated T cells c1 (NFATc1) to be upregulated in the skin stem cells. NFATc1 is able to block the expression of Cdk4 and thus prevent the stem cells from entering into the cell cycle, while not affecting Cdk6 expression. Only when NFATc1 levels decrease, Cdk4 levels would rise and allow the stem cell to cycle (Horsley *et al.,* 2008). These interesting results show the importance of Cdk4 activity in a stem cell population and its key role in the proliferation. According to this data, the ablation of Cdk4 might inhibit the proliferation abilities of the skin stem cell population.

Since the expression of the K-ras^{G12V} oncogene induces amplification of the putative lung stem cells (Kim *et al.,* 2005, Ventura *et al.,* 2006, Besson *et al.,* 2007) does the lack of Cdk4 prevent the BASCs proliferation? In order to evaluate the importance of *Cdk4* lack in the number of putative cancer stem cells amplified by K-ras^{G12V} expression we quantified the amount of cancer stem cells at an early time point after K-ras^{G12V} expression (30 days after a single doses of 4-OHT). One month after K-ras^{G12V} activation, the number of BASCs should have increased, as Kim *et al.,* 2005 reports.

When we quantified the number of BASCs cells in either a *Cdk4* null or wildtype background, we observed in *Cdk4* wildtype lungs, which expressed the K-ras oncogenic mutation, the number of BASC cells had been amplified upon K-rasG12V expression. In contrast, the number of BASCs is not able to expand when the K-ras oncogenic mutation is activated in a *Cdk4* deficient context (please see figure 41, page 147). The inability of the *Cdk4* null mice to amplify the lung stem cell population accentuates the importance of Cdk4 in this

specific cell-type. Moreover, as it has been postulated that the BASCs are the cells responsible for the K-ras^{G12D} driven tumours (Kim *et al.*, 2005) the lack of BASC amplification could result in a reduction in the number of tumours.

12.5 Assessing the therapeutic potential of Cdk4 inhibition

In order to best examine the potential therapeutic role of Cdk4, new generation of genetically modified mice that still express the proteins of interest but in a catalytically inactive state need to be created. The Cdk4 KD isoform, containing the mutation K35M, is being generated in our laboratory, this mice strain will allows us to see the effect of a theoretical target specific inhibitor for Cdk4. The Cdk4^{K35M} isoform will express a kinase dead mutation that would prevent Cdk4 from having phosporylation capabilities. The isoform will be expressed at normal levels and be able to associate to its natural substrates, however the formed complexes will be unable to dissolve. Crossing the *K-ras^{G12V}* KI mouse model with the *Cdk4 KD* strain will provide the opportunity to see the therapeutic role that a specific Cdk4 kinase activity inhibitor might have. However, this work is still ongoing.

We have established that Cdk4 is a key player on the oncogenic K-ras tumourigenesis and we have identified a possible mechanism that could be underlying the Cdk4 antitumour potential. However there is still a question that remains unanswered. Does Cdk4 have a role in tumour formation, development or in both processes? In order to respond to this question, we took advantage of the conditional *Cdk4* KO mice generated our laboratory by Dr. Santamaria and

Dr. Barriere (Barriere *et al.,* 2007) under the Frt/FLP system, and crossed it with the *K-ras*^{G12V} KI strain. Since we have observed that at three months post oncogenic K-ras expression, the mice start to have grade I and II lesions in the lungs, (please see figure 47, page 155) we decided to inject at this point the mice with AdenoFLP or AdenoGFP. As the levels of excision that we obtained using various infection methods were not very high, and due to the rapid immune response that the mice developed against the introduced virus, the injections could not be repeated. In order to improve the Cdk4 excision, we decided to take the conditional *Cdk4* frt allele in a heterozygous background together with the germline *Cdk4* KO allele. The genomic excision levels that we obtained from the conditional allele in lungs were of around 60% (please see figure 48, page 156), meaning that 60% of all the lung cells were *Cdk4* KO three months after treatment and that only 20% of Cdk4 has remaining in the *Cdk4* conditional lungs. Moreover, it has been described how the adenovirus has a special tropism towards the lung epithelial cells, that include AT-2 and Clara cells (Strayer *et al.,* 1998), being the majority of the cells responsible for tumour formation deficient for *Cdk4*. In addition, we analysed the levels of Cdk4 protein expression at six months after the 4-OHT treatment (3 months after AdenoFLP infection) and observed that Cdk4 protein levels were highly decreased (90% reduction) (please see figure 50, page 157). As control mice for this experiment we used animals with the same genotype that had been infected with AdenoGFP, to discard the possibility that the adenoviral infection could be having a tumour suppressor role on its own. As an additional control we also treated with AdenoFLP *Cdk4*$^{+/frt}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice in order to rule out the effect that *Cdk4* cleavage could have.

We have clearly showed how *Cdk4* elimination has an important role in tumour development, as we see a 82% reduction in the number of grade I and II lesions, while no grade III and IV tumours appear, in animals that have been subjected to *Cdk4* depletion three months after tumour initiation. Interestingly, at three months after treatment initiation, the *Cdk4*$^{-/frt}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice lungs have lesions that are unable to progress. We have observed the effect of the *Cdk4* excision shortly after AdenoFLP infection (two weeks after the viral infection) and we try to find the mechanism that it is behind the possible tumour regression. The lesions that are found on the lungs of the *Cdk4*$^{-/frt}$;*K-ras*$^{+/LG12V}$;*RERT*$^{ert/ert}$ mice at the time of the adenoviral infection are premalignant tumours. Collado *et al.,* 2005 demonstrated that these premalignant tumours (grade I-II) are senescent. So that the induction of a senescent status in the tumour cells in which *Cdk4* is excised cannot be the cause of the tumour growth inhibition. Moreover, as these adenomas are not very proliferative, the mechanism underlying the tumour regression should not be very drastic if it is proliferative-dependent. By detecting active caspase 3 we identified that only in the tumours in which *Cdk4* was excised we got single cells positive for apoptosis (please see figure 51, page 159). These finding supports the possibility that in premalignant lesions, cells are in senescence and when they are able to exit from this non proliferative status and enter into cell cycle, the elimination of Cdk4 may promote these tumour cells to enter into an apoptotic cascade. In conclusion, we have demonstrated how Cdk4 plays a key role both in tumour development and in formation. Besides, our results suggest

that Cdk4 is the key G1/S Cdk to be targeted in K-ras driven neoplasias, and these are the first *in vivo* evidences of the Cdk4 therapeutic potential.

The elimination of *Cdk4* after the tumour onset and its surprising effect shows the importance of Cdk4 in lung cancer treatment. In order to better characterise the effect of Cdk4 in tumour progression we have used the LOH model, as it is a more aggressive and faster tumour context. In the first experiment, $Cdk4^{-/frt}$; $K\text{-}ras^{Lox/LSLG12V}$;$RERT^{ert/ert}$ and $Cdk4^{+/frt}$;$K\text{-}ras^{Lox/LG12V}$;$RERT^{ert/ert}$ mice were treated with 4OHT at p21, and 1.5 month later, when small adenomas started to appear in the $K\text{-}ras^{Lox/LG12V}$;$RERT^{ert/ert}$ mice, the animals were infected with AdenoFLP/GFP. Taking advantage of the computed tomography (CT) technology, we are monitoring tumour growth every month after Cdk4 excision by adenoviral infection. By three months after adenovirus infection, all the control mice: $Cdk4^{+/frt}$;$K\text{-}ras^{\Delta/LG12V}$;$RERT^{ert/ert}$ infected with AdenoFLP and $Cdk4^{-/frt}$;$K\text{-}ras^{\Delta/G12V}$;$RERT^{ert/ert}$ infected with AdenoGFP are tumour positive by the CT scan, while the $Cdk4^{-/frt}$;$K\text{-}ras^{\Delta/G12V}$;$RERT^{ert/ert}$ mice infected with AdenoFLP were CT negative. Those results reinforce the importance of *Cdk4* elimination in $K\text{-}ras^{G12V}$ driven tumours. In the second experiment, we are waiting until the $Cdk4^{+/frt}$;$K\text{-}ras^{\Delta/G12V}$;$RERT^{ert/ert}$ or $Cdk4^{-/frt}$;$K\text{-}ras^{\Delta/G12V}$;$RERT^{ert/ert}$ mice are positive by CT to infect them with the AdenoFLP/GFP and then monitor them every month by CT to be able to detect a possible tumour regression. These experiments, are still ongoing, and we hope they will shed light on the mechanism underlying the effects observed after *Cdk4* ablation in tumours.

During cell transformation a series of different genetic alterations occur, among them the loss of control over replication mechanisms, DNA repair and segregation of the genetic material. The cell cycle is under a tight genetic control necessary to maintain the homeostatic equilibrium between cell growth, differentiation, survival and cell death. The appropriate control of the cell proliferation is based in the cell capacity to detect mitogenic and antimitogenic signals, and acting in consequence by inducing or arresting cell division. So that, the regulation of the cell cycle by the Cdk/cyclin complexes, as well as by inhibitors of the Cdk/cyclin complexes (INK or Cip/Kip) or negative regulators such as Rb and p53 is essential.

The drug design towards cell cyle regulators has been based in different strategies, however the Cdks inhibitor design has been a major target. Moreover, most human cancers carry mutations in cell cycle regulators that result in deregulated Cdk activity. These alterations can either be upregulation of the cyclins, elimination of the Cdk inhibitors or mutations in the Cdks. These modifications have a high prognostic value. Cdk2 activity has been shown to be upregulated in different kind of tumours (mammary and prostate carcinomas, and lymphomas) due to the alterations of its regulators $p27^{Kip1}$ and overexpression of *cyclin E;* and this alteration has a high prognostic value. In addition, and insensitive INK4 point mutation in *Cdk4* has been described in human melanomas. Design of inhibitors for the Cdks has been based on drugs that prevent its kinase activity. Small molecules, analog to the ATP, that are synthesised and introduced into the cell easily. Those inhibitors bind to the active site of the Cdk and compete for the ATP binding, turning the Cdk into a

non functional protein. However, these drugs have a problem of selectivity towards the Cdks as the ATP binding site of the majority of the kinases is highly conserved. So that, those drugs will inhibit a wide panel of kinases. Due to the problems in toxicity during the clinical trials, no Cdk inhibitor has been approved for commercial use.

A second generation of selective Cdk inhibitors has been design and some of the compounds are in clinical trials. Remarkably, Pfizer has generated a specific Cdk4 and Cdk6 inhibitor that has obtained successful results *in vitro* (PD-0332991) (Toogood *et al,* 2005). Tumour cell lines treated with PD-0332991 have a lower proliferation profile, and when these cell lines are introduced into nude mice, tumour regression is observed. We are starting to use the specific Cdk4 inhibitor in our mice in order to see if the inhibition of the kinase activity of the protein will be able to block tumour growth or even promote tumour regression. In order to test the effects of PD-0332991, we have created two groups of mice. Animals CT positive, with 1 or 2 tumours of 1-2mm form the first group. In the second cohort we have those mice that are CT positive with 2 or more lung tumours of more than 2mm. Mice are treated for a month with the inhibitor by gavage using a dose of 150mg/Kg diairy, while the control mice are treated with the vehicle (Sodium Lactate 50mmols pH: 4). When the one-month treatment is over, a new CT will be done to the animals in order to monitor the growth of the tumours over the month. Moreover, these mice will be monitored by CTs until their time of death, so that expansions in the lifespan of the mice will be correlated with the tumour burden.

We have observed a very consistent effect of the ablation of Cdk4 in tumour progression in mice. However, our aim is to identify a good target for the treatment of human lung cancer. *Cdk4, cyclin D* and *cyclin E* have been found to be commonly amplified in human lung carcinomas, suggesting that the regulation of the G1/S has also a critical role in tumour progression in humans. In order to test the effect of the G1/S Cdks in human cells we used two different human NSCLC cell lines, A549 and H23, which contained mutations in the *K-ras* locus. Additionally H23 also contained a mutation in the *p53* gene. Both cell lines were infected with lentivirus expressing shRNA against Cdk4, Cdk2 and a control scramble shRNA (TRC). By the use of a specific shRNAs we were able to destroy the mRNA of our target gene when the complementation is complete, destroying thus the target Cdk mRNA. The infected cells were used to test their proliferation capabilities as well as to observe the tumour properties of the infected cells. Interestingly we observed how cells lacking *Cdk4* or *Cdk2 in vitro* are not able to grow (please see figure 53, page 160). From these experiments we can conclude that the elimination of either *Cdk2* or *Cdk4* in human lung tumour cells results in a cell proliferation impairment, however we still have to test whether this effect is specific of NSCLC cell lines and dependent on K-ras mutation. Additionally, we observed that NSCLC cells lacking *Cdk4* may try to overcome its ablation by increasing Cdk6 protein levels (please see figure 54, page 160), however, the rise of Cdk6 is not able to compensate the deficiency of *Cdk4* and these cells are not able to grow. Moreover, when A549 infected cells are injected subcutaneously into nude mice *Cdk4* knock down cells were unable to form tumours, while *Cdk2* lacking and TRC infected cell were able to form lesions with the same kinetics. The difference between Cdk2 and Cdk4 in

in vitro cell growth and *ex vivo* tumour formation could be due to the different mechanisms that are activated and the different proliferation necessities of the cells. In conclusion, these experiments with human NSCLC cell lines emphasize the importance of the G1/S Cdks in tumour progression and accentuate Cdk4 as a key target for the human lung cancer treatment.

For our results, the difference among the two *K-ras*^{G12V} KI models do not interfere, as we have shown how the deficiency of Cdk4 has an effect not only in the classical *K-ras*^{G12V} KI model but also in a more aggressive oncogenic background. In this more aggressive K-ras tumour model we will have the expression of the oncogene together with the ablation of the wildtype allele, obtaining a LOH *K-ras* model.

In conclusion, Cdk4 is a main target for lung cancer treatment. The deficiency on *Cdk4* decreases the transformant properties conferred by the expression of K-ras^{G12V} such as BASC amplification and increased proliferation of the cells expressing the oncogene at early time points. In addition, cell lungs lacking *Cdk4* and expressing the K-ras^{G12V} mutation enter in a senescent status in the initial stages of the oncogene expression. Cdk4 has also an important role in tumour development, as low grade adenomas are wiped out by inhibition of Cdk4 expression, activating the apoptotic machinery. Moreover, in human NSCLC cell lines, the ablation of *Cdk2* or *Cdk4* results not only in inhibition of cell proliferation but in cell death. Importantly, Cdk4 activity in mice is only essential for the proper development of the pancreatic β-cells, so that a putative selective Cdk4 inhibitor would then prevent K-ras^{G12V} driven tumours from

progressing while having only minor secondary effects on the wildtype cell homeostasis.

13.Future Work

As it has been mentioned in the discussion section, the confirmation of an interesting target for lung cancer treatment and identification of the mechanism underlying tumour suppression has opened new questions, which are now under study.

One of the most interesting aspects that we have observed is the arrest in proliferation of the K-ras^{G12V} cells in *Cdk4* deficient lungs, and the activation of senescence in this tissue. We are now looking into the specificity of this effect in lungs, and whether it occurs in all *Cdk4* null tissues that express the endogenous K-ras^{G12V} oncogene. In addition, we are trying to identify the mechanism through which the senescence is activated in the *Cdk4*$^{-/-}$ lungs. We have detected that 15 days after 4-OHT administration, most K-ras^{G12V} expressing cells express similar levels of cyclin D1, regardless of the Cdk4 status. In contrast, when we observe cyclin D1 status 30 days after *K-ras* oncogenic activation, most cells expressing K-ras^{G12V} in *Cdk4* deficient lung no longer express cyclin D1. This result confirms that in a *Cdk4* null background K-ras^{G12V} cells, at early time points after 4-OHT administration, are not able to proliferate. This data is still under discussion and still needs more work but we could postulate that expression of endogenous levels of *K-ras*^{G12V} in *Cdk4*$^{-/-}$ lung cells, although sufficient to promote the synthesis of Cyclin D1 fails to result in cell proliferation inducing instead a senescence response.

A second line of research has been opened due to the possibility of monitoring *in vivo* tumour development. As it has already been discussed, several experiments have been initiated using the CT technology. In a first cohort of *Cdk4*$^{-/frt}$;*K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$ mice the expression of the K-ras^{G12V} oncogene is activated at weaning, and 1.5 months later (when the mice already show small premalignant lesions, still not visible by CT) the animals are infected with the AdenoFLP virus. The viral infection results in the excision of the *Cdk4* conditional locus in most tissues. Since *Cdk4* elimination we are performing monthly CTs in order to monitor tumour development. Preliminary results show that only when *Cdk4* is abrogated, tumours are undetectable by CT, while all AdenoGFP infected have CT positive tumours.

In a second cohort of mice the *Cdk4*$^{-/frt}$;*K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$ animals are also treated with 4-OHT at weaning, but the AdenoFLP infection is not performed until the lung tumours are detectable by CT. Following *Cdk4* excision, monthly CTs are being performed. A third cohort of *K-ras*$^{\Delta/LG12V}$;*RERT*$^{ert/ert}$ mice was used to observe the effect of the selective Cdk4/6 inhibitor, PD-0332991. CT positive animals are divided into two groups depending in the number and size of the tumours. Both groups are treated daily with the inhibitor, and tumour growth is monitored by monthly CT scans. Due to initial problems of toxicity, this experiment is still ongoing.

The oncogenic mutation of *K-ras* gives rise to mainly benign lesions, so in order to test if *Cdk4* ablation has also a tumour protective effect in more aggressive tumour backgrounds we are generating different strains containing additional

deleterious mutations in *p16*^{INK4a} and in *p53*. In addition, we are planning to use the *Cdk4 KD* mice, to observe the effect of having a catalytically inactive Cdk4 protein in K-ras^{G12V} driven lung adenomas.

Finally, the surprising result observed after Cdk2 and Cdk4 elimination in human NSCLC cell lines raised the question of whether the effect was only restricted to tumour cell lines which were mutant for *ras*, and if the result was specific of NSCLC. In order to address both questions, we are extending the study, using a wide panel of human cell lines derived from lung adenocarcinoma, pancreatic and colorectal tumours. Those cell lines contain or not oncogenic mutations in the *ras* genes. We only have preliminary results from these experiments, but the elimination of the interphase Cdks seems to only have an effect on proliferation in *ras* mutated NSCLC cell lines.

14.Conclusions

1. The elimination of the G1/S Cdks in MEFs inhibits the proliferative advantage conferred by the expression of the oncogenic K-ras^{G12V}.

2. In contrast to what is observed *in vitro*, Cdk6 activity is not essential for the formation and development of K-ras^{G12V} associated lung tumours, and mice lifespan.

3. The suppression of Cdk2 activity has a partial effect on the tumour formation, and increases the average lifespan of the mice lacking the protein in a K-ras^{G12V} tumour context.

4. The elimination of *Cdk4* has an impact on the K-ras^{G12V} driven tumour formation and development. Moreover, in a more aggressive model, where we obtain activation of the *K-ras* oncogene together with elimination of the *K-ras* wildtype copy, *Cdk4* ablation continues to have a very significant effect in the suppression of tumour formation.

5. Although the activation of oncogenic K-ras^{G12V} is equal in a Cdk4 wildtype and deficient background, only in lungs where Cdk4 is present the oncogenic expressing cells proliferate at early time points. The mechanism underlying the inhibition of proliferation of the K-ras ^{G12V} expressing cells is the induction of a senescent response on those *Cdk4* -null lung cells.

6. The amplification of the lung cancer stem cells (BASCs) upon K-ras^{G12V} activation is dependent on the expression of Cdk4.

7. Cdk4 activity is necessary for K-ras^{G12V} driven tumour development, as the conditional elimination of *Cdk4* in adenomas results in a reduction in the number of lesions. The mechanism underlying the tumour decrease seems to be the activation of apoptosis.

8. The elimination of *Cdk2* or *Cdk4* from established human NSCLC cell lines results, in the inhibition of cell proliferation.

To sum up all the conclusions, Cdk4 seems a very good candidate for therapeutic intervention in patients that have oncogenic K-ras^{G12V} driven tumours.

15.Bibliography

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