The role of CKS proteins in cancer development

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Declaration

I declare that this thesis contains no material, which has been submitted in any form for another degree or diploma at any university or other institute. Except where specific reference is made, the work presented in this thesis has been carried out by myself.

Abstract

The mammalian CKS family consists of two highly conserved proteins, CKS1 and CKS2. Both are able to bind to cyclin dependent kinases 1 and 2 (CDK1 and CDK2) with high affinity and were suggested to modulate CDK activity and thus cell cycle control. CKS proteins have also been reported to facilitate the ubiquitination of cyclin A by the anaphase promoting complex (APC). Additionally CKS1 has been shown to have an important role in the recognition of the cyclin dependent kinase inhibitor $p27^{Kip1}$ by the SCF^{Skp2} (Skp1-Cul1-F-box-protein) ubiquitin ligase. Elevated expression of both CKS proteins is often found in a variety of tumours and is correlated with poor prognosis. This might indicate an involvement of CKS proteins in the process of transformation from normal to cancer cells. Previously it was shown that down-regulation of CKS proteins in cancer derived cell lines results in a loss of their high proliferative capacity. I surmise, that a possible oncogenic effect of CKS proteins might be due to the deregulation of replication.

To study the role of CKS proteins as oncogenes, different stages of cancer development were modelled in the human diploid fibroblast cell line, IMR90. Over-expression of CKS proteins was combined with defined genetic events known to be associated with transformation such as activation of telomerase (hTERT) and blockage of the p53 and RB pathways. Over-expression of *CKS* variants in IMR90 cells led to checkpoint activation and growth arrest reminiscent of oncogenic stress. Down-regulation of the checkpoint proteins p53 and RB however could alleviate the growth arrest and led to a slight growth advantage of the *CKS* over-expressing fibroblasts. Analyses to understand the molecular mechanisms behind the checkpoint activation in primary IMR90 were performed addressing, in particular, the involvement of an altered CDK activity.

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Abbreviations

4OHT	4-hydroxytamoxifen
ABL	Abelson murine leukaemia viral oncogene
ADP	adenosine diphosphate
APC	adenomatous polyposis coli
APC/C	anaphase promoting complex/cyclosome
ARF	alternative reading frame
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3 related
BCL-2	B-cell lymphoma 2
BCR	breakpoint cluster region
BUBR1	budding uninhibited by benimidazoles 1 homologue beta
BUB3	budding uninhibited by benimidazoles 3 homologue
BrdU	5-bromo-2'-deoxyuridine
BSA	bovine serum albumin
CAK	cyclin-dependent-activating kinase
CDC	cell division cycle
CDE	cell cycle dependent element
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
CHR	cell cycle genes homology region
CKI	CDK inhibitor
CKS	cyclin-dependent kinase subunit
CKSAM	CKS anion mutant
CMV	cytomegalovirus
CUL1	Cullin1
DAPI	4,6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
DNA	desoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
Dox	doxycycline
E. coli	Escherichia coli

EDTA	ethylenediaminetetraacetic acid
EMT	epithelial-mesenchymal transition
ER	estrogen receptor
FAP	familial adenomatous polyposis
Gag	group-specific antigen
G0-phase	gap 0-phase
G1-phase	gap 1-phase
G2-phase	gap 2-phase
GLB1	galactosidase beta 1
H1	histone 1
H2AX	histone 2, variant X
HDM2	human homologue of murine double minute 2
HEK	human embryonic kidney
HNPCC	hereditary nonpolyposis colorectal cancer
HP1	heterochromatin protein 1
HRP	horseradish peroxidase
HSF	heat shock factor
HU	hydroxyurea
hTERT	human telomerase reverse transcriptase
IF	immunofluorescence
IGFBP7	insulin-like growth factor–binding protein 7
IL	interleukin
INK4	inhibitor of CDK4
IP	immunoprecipitation
JMJD3	Jumonji domain-containing protein 3
KRAB	Krüppel associated box
LB	lysogeny broth
LTR	long terminal repeats
MAD2	mitotic arrest deficient 2
MCM	mini-chromosome maintenance
MMP3	matrix metalloproteinase-3
MPF	maturation-promoting factor
M-phase	mitotic phase
MRE11	meiotic recombination 11
MRN	MRE11, RAD50, NBS1
mRNA	messenger RNA

MYT1	membrane associated tyrosine/threonine1 protein kinase
NBS1	Nijmegen breakage syndrome 1
OD	optical density
ORC	origin recognition complex
PAL-1	plasminogen activator inhibitor 1
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PVDF	polyvinylidene fluoride
PEI	polyethylenimine
PFA	paraformaldehyde
Pol	polymerase
Pre-RC	pre-replication complex
RB	retinoblastoma
RBX1	RING-box protein 1
Rev	regulator of virion expression protein
RNA	ribonucleotide acid
RPA	replication protein A
rTetR	reverse tetracycline repressor
rtTA	reverse tetracycline-controlled transactivator
ROS	reactive oxygen species
SA-β-gal	senescence-associated β-galactosidase
SAHF	senescence associated heterochromatic foci
SASP	senescence associated secretory phenotype
SCF E3 ligase	SKP-Cullin-F-box E3 ligase
SDS	sodium dodecyl sulfate
SIN	self-inactivating
SKP	S-phase kinase associated protein
S-phase	synthesis phase
SV40 TAg	simian virus 40 large tumour antigen
SWI/SNF	switch/sucrose nonfermentable
tetO	tetracycline operator
TetR	tetracycline repressor
TRAMP	transgenic adenocarcinomas of the mouse prostate
TRE	tetracyclin responsive promoter element
TGF-β	transforming growth factor beta

Tyr	tyrosine
VEGF	vascular endothelial growth factor
VSV-G	vesicular stomatitis virus glycoprotein
WB	Western blotting
wt	wild-type

Chapter 1 - Introduction

1.1 Intrinsic barriers to cancer progression

1.1.1 Models describing cancer development

As early as 1890, David von Hansemann studied carcinoma cells microscopically and described the presence of aberrant mitosis with asymmetric division of the cell. He saw that during these divisions, cells with altered chromatin content derive (Dell, 2006; Marte, 2006; von Hansemann, 1890; Wunderlich, 2011).

In 1914, Theodor Boveri built on these observations and developed his cancer theory based on studies using sea urchin eggs (Marte, 2006). Via double fertilization of these eggs, he could induce multipolar mitosis which led to abnormal chromosome constitutions and cells with a cancer-like phenotype (Boveri, 1914; Weinberg, 2008). He assumed that one chromosome bears several hereditary units and postulated that a perturbed combination of chromosomes leads to cancer development (Balmain, 2001; Boveri, 1914). Boveri also introduced the terms 'proliferation inhibiting chromosomes', which are lost in growing tumours and 'proliferation assisting chromosomes', which are amplified during tumour development (Balmain, 2001; Boveri, 1914; Dell, 2006; Marte, 2006). Today these 'proliferation inhibiting and assisting chromosomes' are synonymous with tumour-suppressor genes and oncogenes respectively. Tumours are believed to develop from one cell and tumour progression happens via sequential changes with increasing activity of growth-stimulatory chromosomes and loss of growth-inhibitory chromosomes. Boveri suggested that there is a predisposition for cancer by inheriting chromosomes with reduced ability to suppress cancer. When an individual is homozygous for such a 'weak chromosome', there is a high penetrance of cancer (Balmain, 2001; Boveri, 1914). He even imagined that certain agents as well as radiation or pathogens lead to cancer development by their influence on mitosis or other chromosome altering processes (Marte, 2006).

The term 'somatic mutation' with respect to a tumour was first used by Ernest Tyzzer in 1916 who described a tumour as 'manifestation of somatic mutation' (Knudson, 2001; Tyzzer, 1916).

Hermann Muller postulated later on that several of these mutations in one cell would be necessary to transform a cell into a cancer cell. He also found X rays to be a cause for these mutations. The delay of the onset of the disease after exposure to radiation suggested, that 'a row of mutations' were required (Knudson, 2001; Muller, 1927, 1955).

Later on, inherited cancer syndromes were discovered. The development of skin cancer in Xeroderma Pigmentosum for example could be explained by a defect in an enzymatic mechanism, which is now known as excision repair (Knudson, 2001). Due to this defect, DNA damage caused by ultraviolet light cannot be repaired and an accumulation of mutations occurs which finally leads to the development of carcinomas (Cleaver, 1968; Dell, 2006; Knudson, 2001). But alterations in gene expression and function are not only due to mutations within the gene itself. In the case of chronic myelogenous leukaemia for example, onset of disease results from a reciprocal translocation between chromosomes 9 and 22 (Knudson, 2001; Rowley, 1973). The newly formed chromosome 22 is shorter and was named Philadelphia Chromosome. As a result of the translocation, the Abelson murine leukaemia viral oncogene (*ABL*) gene, which was located on chromosome 9, is now fused to the breakpoint cluster region (*BCR*) gene on chromosome 22. This resulting *BCR-ABL* fusion gene encodes for a constitutively active tyrosine kinase, which leads to a higher rate of cell proliferation (Knudson, 2001; Shtivelman et al., 1985; Stam et al., 1985). Other genes acting like *ABL*, which promote tumour development upon excessive activation were discovered and called proto-oncogens (Stehelin et al., 1976). Later on genes suppressing tumour development were analysed in more detail (Balmain, 2001). One of the first of these tumour-suppressor genes identified was the retinoblastoma (*RB*) gene (Friend et al., 1986; Knudson, 2001) which controls progression through the cell cycle (Weinberg, 1995). Deletion of the *RB* gene often leads to the development of cancer of the eye called retinoblastoma. In 1971, Knudson analysed onset, severity and development of retinoblastoma in a group of patients. He set up his 'two-hit hypothesis' in which he points out that not only one but both copies of the tumour suppressor gene must be inactivated in one cell before a tumour develops. Thus retinoblastoma occurs with a higher frequency and in younger age in patients, which already inherited an inactive gene from one of their parents (Knudson, 2001, 1971).

Later on, Vogelstein named tumour suppressor genes like the *RB* gene 'gatekeepers'. They directly regulate the growth of tumours by inhibiting growth or promoting cell death (Kinzler and Vogelstein, 1997). During his analyses of sporadic and inherited colorectal cancer, he pointed out that the adenomatous polyposis coli (*APC)* gene, which is mutated in patients with familial adenomatous polyposis (FAP), is another of these gatekeeper genes (Kinzler and Vogelstein, 1996). APC interacts with β-catenin, which in turn is responsible for the regulation of various processes like cell proliferation, differentiation, motility, adhesion and apoptosis (Fodde et al., 2001). In contrast to FAP, hereditary nonpolyposis colorectal cancer (HNPCC) is based on defects

in enzymes, which are necessary for the mismatch repair of DNA. Genes encoding for such repair enzymes are called 'caretakers' (Kinzler and Vogelstein, 1997). The initiation of cancer development is much more likely via the mutation of a gatekeeper like *APC*. Once both copies of the gatekeeper loose their proper function, the loss of caretakers subsequently leads to genomic instability and facilitates tumour progression (Kinzler and Vogelstein, 1996).

Today, we know that the process of cell transformation is a multi-step process and involves several genetic alterations in the genome of a cell. These alterations include both, mutations in oncogenes leading to a gain of function and mutations in tumour suppressor genes leading to a loss of function of the respective proteins. Because they provide gain of function, oncogenes are typically dominant (Cooper, 1990). Tumour suppressor genes are recessive (Hansen and Cavenee, 1988). The action of an active oncogene (gain of function) leads to the activation of tumour suppressor genes. These tumour suppressor genes first need to be bypassed (loss of function) before the effect of the oncogene becomes visible (Boehm and Hahn, 2005). Genetic alterations also contribute to the essential changes in cell physiology necessary for the progression of tumour formation, including: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000). To sustain proliferative signalling and thus chronic proliferation, cancer cells might acquire the ability to produce their own growth factor ligands. Alternatively, they might get other cells within the tumour-associated stroma to release more growth factors. There could be an up-regulation of growth factor receptors on the cancer cell surface, firing via cell surface receptors could be facilitated or other components of the pathway could be constitutively active. Another way of tumour cells to enhance proliferative signalling is to disrupt negative feedback mechanisms. To evade growth suppression and/or cell death, tumour cells must circumvent pathways, which are regulated by tumour suppressor genes like *RB* and *p53* (Hanahan and Weinberg, 2000, 2011). Furthermore, cells achieve immortality by sustaining the protective ends of DNA, called telomeres (Belgiovine et al., 2008). In order to achieve a good supply of nutrients and oxygen, tumour tissue must be infiltrated by new vessels and thus angiogenesis must be induced (Hanahan and Weinberg, 2011). This is possible by vascular endothelial growth factor (VEGF) signalling which might be up-regulated by hypoxia or oncogenes. To be finally able to leave their own tissue and metastasise, cancer cells undergo a program called epithelial-mesenchymal transition (EMT). EMT is regulated by a change in the expression of transcription factors, which are responsible for the expression of matrix

degrading enzymes, increased motility, heightened resistance to apoptosis and loss of adherent junctions (Hanahan and Weinberg, 2011).

1.1.2 Tumour suppressor genes

In 1969, Henry Harris made the observation that upon fusion of a malignant cell with a normal somatic cell, tumour growth could be suppressed. After elimination of certain chromosomes from the hybrid, these suppressing characteristics are lost again (Dell, 2006; Harris et al., 1969). The first gene, which could be identified of having tumour suppressing abilities was retinoblastoma (*RB*) (Burkhart and Sage, 2008; Knudson, 2001). After *RB*, many other tumour suppressor genes have been identified and analysed. To prevent tumour development, tumour suppressors, like the well studied RB and p53 proteins, inhibit enhanced cell proliferation, regulate an apoptotic response and initiate the DNA damage response upon the activation of oncogenic stress. In later stages of carcinogenesis, tumour suppressor genes also block loss of contact inhibition and metastasis (Sherr, 2004).

1.1.2.1 RB

The RB family of proteins consists of RB (p105), p130 and p107. The best-characterised tumour suppressor function of RB is its ability to arrest cells in G1. To achieve this arrest, different members of the RB family bind in their hypophosphorylated form to members of the E2F family of transcription factors and either antagonise or potentiate their function. RB itself binds to the transcriptional activators E2F1, 2 and 3a and prevents them from binding to the promoter of genes required for S-phase entry. p130 and p107 on the other hand bind to repressors like E2F4 and 5. These complexes then bind to promoters of S-phase genes and recruit chromatin remodelling factors to silence the genes. Phosphorylation of the RB proteins is performed by the cyclin D-cyclin-dependent kinase (CDK) 4/CDK6 and cyclin E-CDK2 complexes and leads to its inactivation (Macaluso et al., 2006; Sun et al., 2007; Woo et al., 1997).

In addition to their ability to bind E2F 4 and 5, p107 and p130 were also reported to form stable complexes with cyclin A-CDK2 and cyclin E-CDK2 complexes. They contain a cyclin binding domain as well as a CDK inhibitor domain, which has been shown to inhibit CDK2 activity (De Luca et al., 1997; Macaluso et al., 2006; Woo et al., 1997)

Due to mutations in *RB* itself or in transforming growth factor beta (*TGF β*), one of the anti-proliferative factor sensing receptors upstream of RB, the RB pathway is often disrupted in tumour cells. RB is also down-regulated by the E7 protein of human

papillomavirus. This disruption in the RB pathway makes the cells insensitive to growth-inhibitory signals and allows the progression of the cells through cell cycle (Burkhart and Sage, 2008; Sun et al., 2007).

Apart from cell cycle arrest, more functions of RB in tumour suppression emerged. These include genomic stability, apoptosis and differentiation (Burkhart and Sage, 2008).

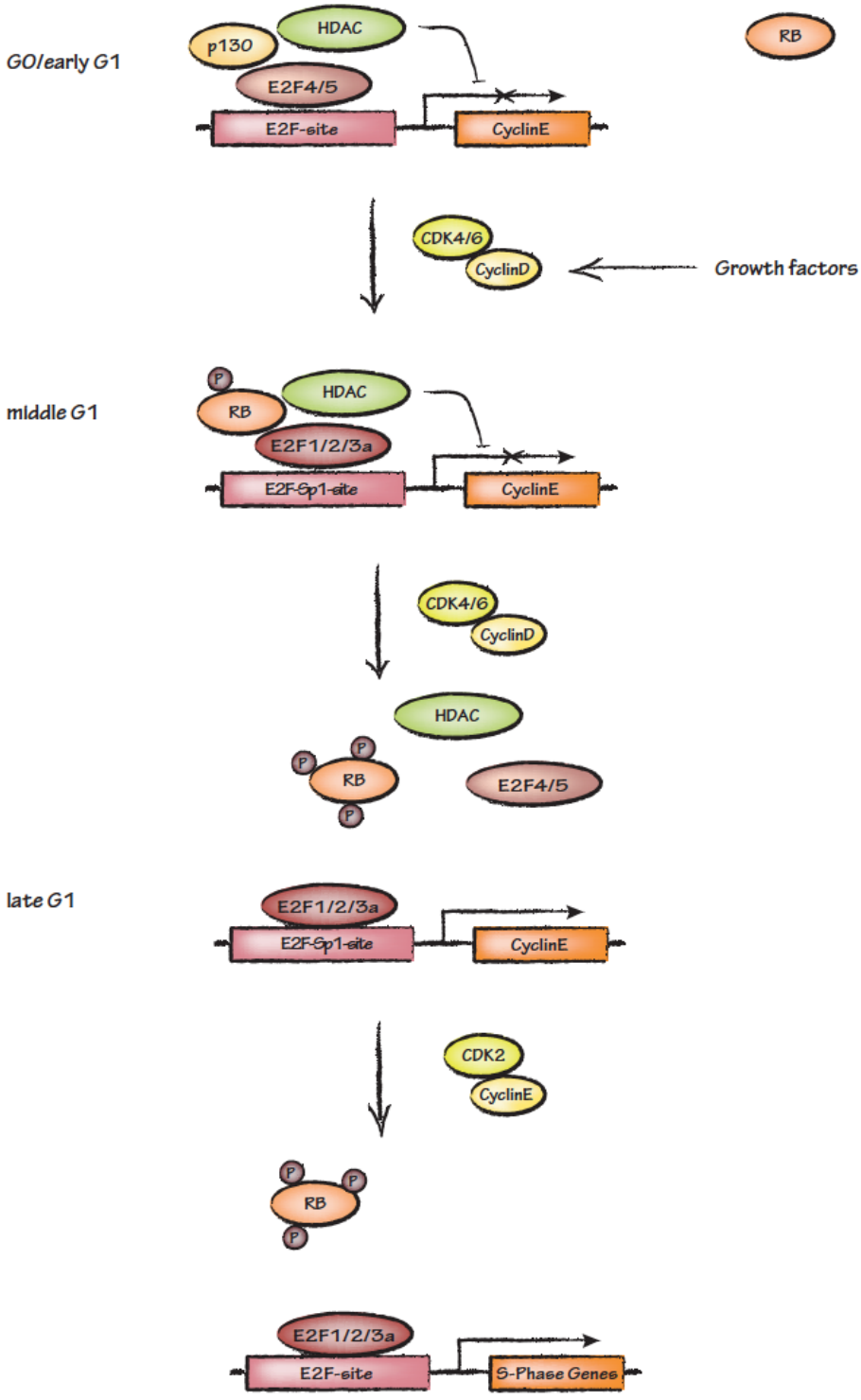


Figure 1.1 **Function of the RB tumour suppressor family.** Proteins of the RB family regulate the transcription of E2F responsive S-phase genes by targeting members of the E2F family of transcription factors. p130 is the member of the RB family, which is predominantly expressed in quiescent cells in G0 (Sun et al., 2007). It binds to the transcriptional repressors E2F4 and E2F5, which lack a nuclear localisation sequence and transports them to E2F responsive promoters in the nucleus. RB proteins like p130 mediate the repression of E2F target genes by recruitment of chromatin remodelling factors such as switch/sucrose nonfermentable (SWI/SNF) and the hisone deacetylase HDAC (Macaluso et al., 2006). In middle G1 p130 levels begin to decrease (Sun et al., 2007). Additionally initial growth factor signalling leads to transcription of cyclin D, subsequent activation of CDK4/6 and hypophosphorylation of RB (Haberichter et al., 2007). In its hypophosphorylated form RB, which is expressed during the whole cell cycle, binds to the transcriptional activators E2F1, E2F2 and E2F3a. This binding prevents the E2F activators from stimulating transcriptional activity of the promoters (Macaluso et al., 2006). By further phosphorylation of RB by the cyclin D CDK4/6 complex and due to increasing levels of the transcriptional activators, RB proteins are not able to supress certain genes like cyclin E anymore (Cobrinik, 2005; Macaluso et al., 2006). Cyclin E expression leads to CDK2 activation and further phosphorylation of the RB proteins. Now genes for the further progression through the cell cycle are transcribed (Blomen and Boonstra, 2007; Haberichter et al., 2007).

1.1.2.2 p53

p53 is crucial in preventing transformation of cells. p53 itself or one of the members of the pathways upstream or downstream of p53 is inactivated in almost every cancer (Junttila and Evan, 2009). Like RB, p53 is a target of viral oncoproteins like E6 from the human papillomavirus (Scheffner et al., 1990).

p53 is well known for its function as transcription factor. It possesses a functional transactivation domain and activates nearby genes after binding to specific DNA sequences (Levine and Oren, 2009). Target genes of p53 are involved in metabolic homeostasis, anti-oxidant defence, DNA repair, Growth arrest and senescence and apoptosis.

More recently, p53 was shown to regulate the transcription of various microRNAs as well (He et al., 2007). Besides transcriptional activation, p53 has other functions including the interaction with members of the B-cell lymphoma 2 (BCL-2) family. This leads to mitochondrial outer membrane permeabilisation, release of cytochrome c and apoptosis (Levine and Oren, 2009).

1.1.3 The cell cycle

A regulated transition of the cell through cell cycle is important to allow the precise replication of the cell's genome and division of the cell with the correct partition of the chromosomes onto the resulting daughter cells (Vermeulen et al., 2003).

Cells divide during mitosis (M-phase). Before cells are able to replicate their genome again in S-phase, proteins, which are required for entry and completion of the replication process, are synthesised in G1-phase (gap 1-phase). After successful replication, cells prepare for mitosis by producing proteins such as microtubules in G2 (gap 2-phase). Cells in G1 which are not ready for DNA replication enter into a state called G0 (gap 0-phase) (Vermeulen et al., 2003).

1.1.3.1 Cell cycle transition

Transition through the cell cycle is regulated by cyclin dependent kinases (CDK). CDK are serine threonine protein kinases and are activated during specific stages of the cell cycle. CDK4 and 6 are active during early G1. Later CDK2 is activated, which is responsible for a proper DNA replication in S-phase and CDK1 is active during M-phase. For CDK to be active the binding of a specific cyclin to the CDK is necessary. Whereas CDK are stably expressed during the cell cycle, the levels of cyclins oscillate. Cyclin D activates CDK4/6 early in G1. CDK2 interacts with cyclin E in late G1 and with cyclin A predominantly in S-phase. Cyclin B is present in the nucleus in M-phase to interact with CDK1 (Vermeulen et al., 2003).

Upon completion of one cell cycle, cyclin levels are low and cells would exit the cell cycle and enter the G0 state, which is called quiescence. Only when cells receive the right stimulation they will progress further through the cell cycle. This stimulation can arise from growth factor signalling via the mitogen-activated kinase (MAPK) pathway. This pathway results in the transcription of cyclin D in G1. Now cyclin D is able to bind and activate CDK 4 or 6 (Blomen and Boonstra, 2007). Active CDK complexes phosphorylate target proteins on CDK consensus sites (Vermeulen et al., 2003). The CDK4/6-cyclin D complex phosphorylates the proteins of the RB family in G1. As mentioned above this initial phosphorylation is important for the release of E2F transcription factors and the transcription of cyclin E. The CDK2-cyclin E complex then helps to further phosphorylate RB leading to further transcription of proteins necessary for S-phase entry. When the phosphorylation of RB has reached a certain threshold, cells are independent of mitogenic signalling and commit to the progression from G1- to S-phase and through the whole cell cycle (Blomen and Boonstra, 2007).

The CDK2-cyclin E complex further promotes S-phase entry by the phosphorylation of its inhibitors $p21^{Cip1}$ and $p27^{Kip1}$, which are degraded by the proteasome later on. Another target protein of CDK2-cyclin E is the histone 1 (H1). At lower levels this phosphorylation was suggested to help decondensation of chromosomes and thus enables DNA replication (Happel and Doenecke, 2009; Vermeulen et al., 2003).

For DNA replication to occur, origin licensing has to take place already in late M-phase and early G1 when CDK are still inactive (Depamphilis et al., 2012). Here the pre-replication complex (pre-RC) is loaded onto the origins of DNA replication. The pre-RC consists of the DNA helicase loader, which is made up of origin recognition complex (ORC) 1-6, cell division cycle (CDC) 6 and DNA replication factor Cdt1 and the mini-chromosome maintenance (MCM) helicase, which is important for the unwinding of the DNA double helix. For activation of the helicase as well as for further loading of proteins essential for replication in late G1 and S-Phase, phosphorylation events are necessary, which are conducted by kinases, including CDK2-cyclin E (Depamphilis et al., 2012).

Targets of CDK2-cyclin A also include CDC 6 and MCM proteins. Phosphorylation of these replication proteins in S-phase by CDK2-cyclin A leads to their relocalisation to the cytoplasm and thus prevention of re-replication (Depamphilis et al., 2012; Ishimi et al., 2000; Paolinelli et al., 2009; Petersen et al., 1999).

Cyclin A also binds to CDK1. The complex further prevents pre-RC assembly by phosphorylation of CDC6 (Depamphilis et al., 2012).

The complex made up of CDK1 and cyclin B is called maturation-promoting factor (MPF). MPF promotes the condensation of chromosomes probably via phosphorylation of condensins and further phosphorylation of H1 (Hagstrom et al., 2002; Happel and Doenecke, 2009; Kimura et al., 1998; Vermeulen et al., 2003). MPF also phosphorylates lamins, which induces nuclear lamin disassembly and nuclear envelope breakdown during mitosis (Ward and Kirschner, 1990). Other targets of the MPF are for example microtubule-associated proteins, which are important for the formation of the mitotic spindle (Shiina et al., 1992; Tsukahara et al., 2010).

The CDK activating cyclins themselves are degraded via the proteasome. G1 cyclins are ubiquitinated by the S-phase kinase-associated protein (SKP), Cullin, F-box (SCF) E3 ligase. Mitotic cyclins are ubiquitinated by the anaphase promoting complex (APC). APC is not activated until Anaphase. Activation is regulated by CDC20 which in turn needs to be activated by CDK1 (Murray, 2004).

In addition to cyclin binding, CDK also need to be phosphorylated on conserved threonin and tyrosine residues to be active. The cyclin-dependent-activating kinase (CAK), which is the complex between CDK7 and cyclin H, performs these

phosphorylations. They lead to a conformational change of CDK, which allows enhanced cyclin binding (Clarke, 1995; Solomon et al., 1992; Vermeulen et al., 2003).

Inhibitory phosphorylations of CDK1 performed by WEE1 and the membrane associated tyrosine/threonine1 protein kinase (MYT1) need to be eliminated by the phosphatase CDC25 (Coleman and Dunphy, 1994; Fattaey and Booher, 1997; Vermeulen et al., 2003).

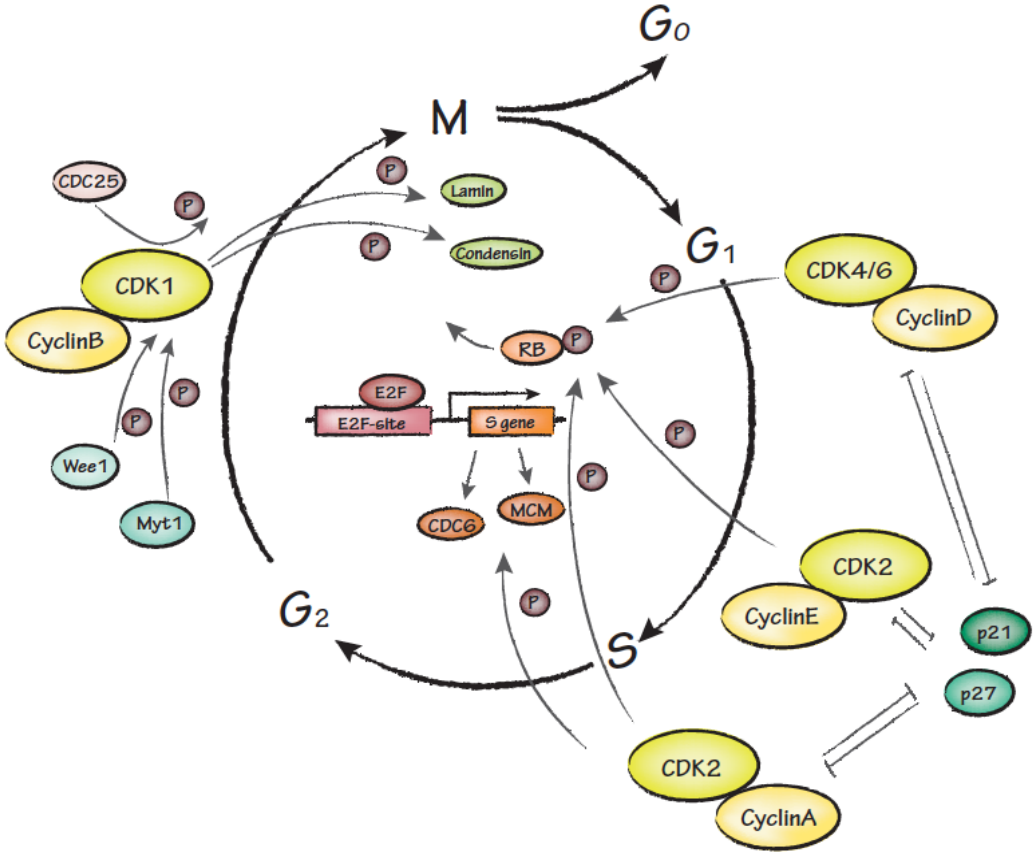


Figure 1.2 **Cell cycle regulation.** In proliferating cells the cell cycle consists of four phases: G1, S-phase, in which the chromosomes of the cells are duplicated, G2 and M-phase, in which the replicated chromosomes are divided onto daughter cells (Vermeulen et al., 2003). Transition through the phases of the cell cycle is regulated by CDK. CDK in turn are regulated by the presence of their appropriate cyclin, phosphorylation status as well as CDK inhibitors. Upon activation of the CDK4- and CDK6-cyclin D complex by the CAK, these complexes phosphorylate RB proteins. Phosphorylation of RB is continued by the CDK2-cyclin E complex. Phosphorylation of RB proteins results in the release of the E2F transcription factors and subsequently the transcription of genes coding for proteins essential during replication. During S-phase, cyclin E gets degraded and is exchanged for cyclin A. The CDK2-cyclin A complex maintains RB phosphorylation and regulates S-phase proteins. CDK4, 6 and 2 are inhibited by $p21^{Cip1}$ as well as $p27^{Kip1}$ upon checkpoint activation. Another, less well studied, CDK inhibitor, $p57^{Kip2}$ is especially known for its function in embryogenesis (Borriello et al., 2011). In G2 a premature entry into Mitosis is prevented by inhibitory phosphorylations of CDK1 by WEE1 and MYT1. The phosphatase CDC25 reverses these phosphorylations. The active CDK1-cyclin B complex phosphorylates proteins like condensin and lamin, which leads to chromatin condensation and nuclear envelope break down (Vermeulen et al., 2003).

1.1.3.2 Cell cycle checkpoints

To ensure that cells replicate and divide in a correct way, cell cycle checkpoints exist which stall the transition from one phase of the cell cycle to another (Elledge, 1996). The checkpoints which are most studied are DNA damage checkpoints and the spindle checkpoint. DNA damage leads to arrest of the cell cycle either in transition from G1 to S or in S to G2 to allow DNA repair to take place (O'Connor, 1997; Taylor and Stark, 2001). Arrest induced at the G1/S transition is p53-dependent (Vermeulen et al., 2003). There are several ways in which aberrant replication structures or DNA damage is sensed. Single stranded DNA for example, which might be a result of nicks during replication or of stalled replication forks, is coated by replication protein A (RPA) (Jones and Petermann, 2012). The RPA coated single stranded DNA acts as trigger for ataxia telangiectasia and Rad3 related (ATR) activation via binding of the ATR interacting protein/ATR complex. ATR activation leads further downstream to the activation of checkpoint kinase 1 (CHK1). CHK1 in turn inhibits the CDK activating phosphatase CDC25 and activates p53 (Branzei and Foiani, 2008; Jones and Petermann, 2012; Lopez-Contreras and Fernandez-Capetillo, 2010). Double-strand breaks, which occur for example after replication fork collapse, are sensed by the MRN (meiotic recombination 11 (MRE11), RAD50, Nijmegen breakage syndrome 1 (NBS1)) complex (Lamarche et al., 2010). Ataxia telangiectasia mutated (ATM) is recruited to the double strand break and activated by autophosphorylation. ATM then phosphorylates several downstream proteins including histone H2A (H2AX) and checkpoint kinase 2 (CHK2). CHK2 leads to the phosphorylation of CDC25A, which is then degraded. CHK2 also activates p53 (Derheimer and Kastan, 2010; Jones and Petermann, 2012). p53 in turn activates for example the transcription of the CDK inhibitor (CKI) $p21^{Cip1}$, which leads to cell cycle arrest (el-Deiry et al., 1993).

CDK inhibitors are one way of down-regulating CDK activity. In mammalian cells these are divided into members of the INK4 family and members of the Cip/Kip family of CDK inhibitors. Members of the INK4 family include $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$ (Besson et al., 2008). The INK4 protein family members specifically inhibit CDK4 and CDK6 activity by interacting directly with these kinases. In this way the association of the kinases with their activating D-type cyclins is impaired (Besson et al., 2008; Canepa et al., 2007).

Members of the Cip/Kip family are $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. $p57^{Kip2}$ plays an important role in cell cycle regulation during embryonic development. As mentioned above, $p21^{Cip1}$ transcription is initiated by p53 upon DNA damage signalling (Besson et al., 2008). $p27^{Kip1}$ is activated via different stimuli like mitogen starvation, cell density,

differentiation signals, loss of adhesion to the extracelluar matrix or TGF ß signalling (Chu et al., 2008). Cip/Kip proteins are known for their inhibitory effect on all cyclin-CDK. As they interact directly with the cyclin and CDK subunit, Cip/Kip proteins were also reported to facilitate the formation of the cyclin D-CDK4/6 complex. In the presence of high INK4 protein levels however, INK4 proteins will displace the Cip/Kip proteins from CDK4/6 and the newly available Cip/Kip proteins will intensify the inhibition of the cyclin E-CDK2 complex. Similarly the up-regulation of Cip/Kip proteins themselves can have the same effect (Blomen and Boonstra, 2007).

The spindle checkpoint ensures during mitosis that all duplicated sister chromatids are attached to the mitotic spindle before the onset of anaphase. The kinetochore of a chromatid, which is not captured by microtubules of the mitotic spindle, is recognised by mitotic arrest deficient 2 (MAD2). Together with budding uninhibited by benzimidazoles 1 homologue beta (BUBR1) and budding uninhibited by benimidazoles 3 homologue (BUB3) it forms the mitotic checkpoint complex, which binds CDC20. CDC20 is the activator of the ubiquitin ligase APC. Thus APC stays inactive and can not promote transition of the cell into anaphase via ubiquitination of securin and cyclin B (Ciliberto and Shah, 2009).

1.1.3.3 Telomere shortening

Although these cell cycle checkpoints might be interrupted in a certain population of cells, which can now proliferate independently from their surroundings, this population is still limited in its number of successive divisions. After a certain number of population doublings, untransformed human cells would enter a senescent state and stop growing due to telomere shortening (Collado et al., 2007; Zakian, 1995). Telomeres, which are located at the end of chromosomes, consist of repetitive G-rich DNA, which is bound by various telomere proteins (Stewart et al., 2012). Telomeres protect the end of the chromosomes from destruction. During each cell cycle however several base pairs of telomeric DNA are lost due to an insufficiency to complete replication of 5' ends of chromosomal DNA during S-phase. Only cells, which obtain a telomerase function and are able to maintain the length of their telomeres are able to survive and expand (Zakian, 1995). The main components of human telomerase, which are important for its catalytic activity are the Telomerase Reverse Transcriptase (hTERT), the RNA molecule which serves as template for the synthesis of the telomeric repeats and the RNA binding protein dyskerin (Belgiovine et al., 2008). The hTERT expression is down-regulated early in human development and introduction of the cDNA for hTERT is sufficient to restore telomerase activity in cultured human cell lines (Belgiovine et al., 2008; Harley,

2002). In contrast to humans, mice constitutively express telomerase in many tissues (Smith and Kipling, 2004).

1.1.4 Senescence as a mechanism to suppress tumour development

Cellular senescence was first observed in cultured human fibroblasts (Hayflick, 1965). It is characterised by an irreversible growth arrest. There are two types of senescence. Replicative senescence is induced in aging human cells mainly due to telomere shortening (Collado et al., 2007). Senescence is also induced by checkpoint pathways in response to oncogene activation (Serrano et al., 1997). This so-called oncogene-induced senescence is, like apoptosis, seen as one of the strategies to prevent cancer development. Whether the cell undergoes apoptosis or enters senescence upon oncogenic stress is dependent on cell type and type of oncogenic stress (Erol, 2011).

1.1.4.1 Characteristics

In contrast to the non-proliferative state of quiescent cells (G0), which is responsive to mitogenic stimulation, the growth arrest of senescent cells is irreversible. Senescence is well studied in fibroblasts, which arrest in G1, but depending on cell type and activated oncogene, cells can also arrest in other phases of the cell cycle (Campisi and d'Adda di Fagagna, 2007; Di Leonardo et al., 1994; Wada et al., 2004). Proliferating cells are able to incorporate the uridine derivate 5-bromo-2'-deoxyuridine (BrdU) into the newly replicated DNA strand during S-Phase. Since senescent cells are unable to proliferate and thus do not take up BrdU, a decrease in BrdU incorporation indicates a senescent cell population (Campisi and d'Adda di Fagagna, 2007).

Senescent cells also undergo morphological changes. A reorganisation in the cytoskeleton leads to a flattened and enlarged appearance. The reorganisation is thought to be due to a down-regulation of actin and an overproduction of vimentin (Nishio et al., 2001).

Another marker, which is more specific for the identification of senescent cells, is an elevated beta-galactosidase (ß-gal) activity detectable at a pH of 6 (Campisi and d'Adda di Fagagna, 2007; Dimri et al., 1995). SA-ß-gal activity is due to an increase in galactosidase beta 1 (*GLB1*) expression, the gene encoding lysosomal ß-D-galactosidase (Lee et al., 2006). As a lysosomal enzyme, it reaches its maximal activity at a pH of 4.0 - 4.5. In senescent cells lysosomal biogenisis is increased. Therefor a ß-gal activity can be detected even at a suboptimal pH of 6 (Kurz et al., 2000). For the detection, X-gal can be used as a chromogenic substrate for the galactosidase (Dimri et al., 1995). As ß-gal activity at a pH of 6 is a general marker for increased lysosome number or activity it is

not exclusively observed in senescent cells (Kurz et al., 2000; Lee et al., 2006; Severino et al., 2000).

Often, a reorganisation of chromatin is observed upon the activation of oncogenic stress. These heterochromatic structures that are formed are called senescence associated heterochromatic foci (SAHF) (Campisi and d'Adda di Fagagna, 2007). Histone modifications found in euchromatin, like acetylated lysine 9 or tri-methylated lysine 4 on histone 3 (H3K9Ac and H3K4me3), are excluded from SAHF. Instead heterochromatin-associated proteins like heterochromatin protein 1 (HP1) and Lys9 tri-methyl on histone H3 (H3K9me3) are enriched (Kuilman et al., 2010). These proteins as well as the RB tumour suppressor proteins are thought to accumulate on E2F-responsive promoters, which results in a stable repression of E2F target genes (Narita et al., 2003). Genes like cyclin A, cyclin B and proliferating cell nuclear antigen (*PCNA*) are down-regulated (Pang and Chen, 1994; Stein et al., 1991). Therefore the formation of SAHF is thought to be one of the ways, how senescent cells alter their profile of gene expression. It is of note that SAHF formation is a common feature of senescence, which is a result of oncogene induced DNA replication stress. Oncogenic events in proliferating transformed cells and cancer cells *in vivo* still lead to SAHF resembling heterochromatin formation. In contrast the appearance of SAHF seems to be cell type dependent in replicative senescence and other forms of stress induced senescence (Di Micco et al., 2011; Kosar et al., 2011).

Senescent cells also express secreted factors. This phenomenon is described as senescence associated secretory phenotype (SASP). Secreted factors include interleukins, chemokines, growth factors and proteases (Rodier and Campisi, 2011). The secretory proteins are thought to regulate the secreting senescent cells themselves as well as neighbouring cells and extracellular environment (Acosta et al., 2008; Campisi and d'Adda di Fagagna, 2007). Several implications for the formation of SASP were reported. Thus factors such as PAI-1, IGFBP7, IL6 and IL8, which are secreted by senescent cells, were shown to reinforce cell cycle arrest and senescence especially in primary cells (Acosta et al., 2008; Rodier and Campisi, 2011; Young and Narita, 2009). On the other hand IL6 and IL8 for example are known for their positive effect on angiogenesis and epithelial-mesenchymal transition of premalignant cells. Using a Boyden chamber it was shown that both cytokines enhance the invasion ability of premalignant epithelial cells (Coppe et al., 2008; Rodier and Campisi, 2011; Young and Narita, 2009). Secretion of other factors which can facilitate tumour cell invasiveness like matrix metalloproteinases or VEGF have been reported (Rodier and Campisi, 2011).

By the secretion of inflammatory cytokines, SASP formation can also help to promote the clearance of senescent cells (Young and Narita, 2009).

1.1.4.2 Pathways to senescence

The main reason for replicative senescence in primary human cells is the shortening of telomeres. The ends of short telomeres are recognised as double strand breaks. This leads to a generation of a persistent DNA damage response (DDR) and ultimately to the activation of p53 and consequently RB (Belgiovine et al., 2008; Rodier and Campisi, 2011). Active p53 induces transcription of $p21^{Cip1}$. $p21^{Cip1}$ mainly acts as cell cycle inhibitor via its interaction with CDK2 although its interaction with CDK4/6-cyclin D complex is also known. Inactive CDK are unable to phosphorylate RB proteins. Thus RB proteins can sequester E2F transcription factors and cell cycle progression is inhibited (Campisi and d'Adda di Fagagna, 2007; Sherr and Roberts, 1999).

To avoid telomere shortening and the associated DNA damage response in cultured human cells, telomerase activity is often restored by introducing hTERT into the cells. Telomerase reactivation is often sufficient to prolong the cellular life span of human fibroblasts indefinitely (Belgiovine et al., 2008). In some immortalised fibroblast cell lines the loss of $p16^{INK4a}$ expression was found. For other cells like keratinocytes, down-regulation of $p16^{INK4a}$ expression is obligatory for immortalisation. It was suggested that $p16^{INK4a}$ activation in these cells is due to cell culture conditions, which lead to stress, the induction of $p16^{INK4a}$ and cell cycle arrest (Belgiovine et al., 2008).

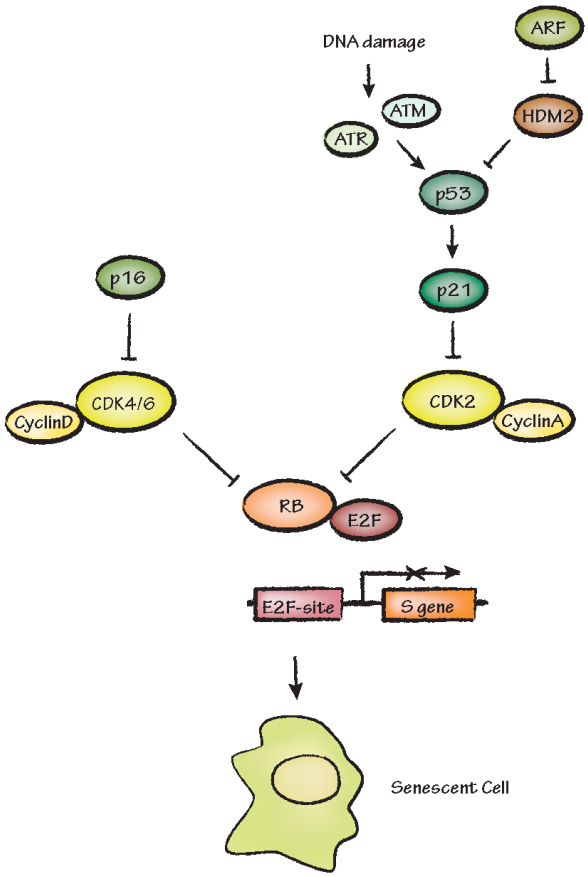
Although murine embryonic fibroblasts (MEF) obtain telomerase activity and do not suffer from telomere shortening, they senesce after a short period of time in culture (vom Brocke et al., 2006). This is due to a high susceptibility of MEFs for stress caused by reactive oxygen species (ROS), which result from the unphysiologically high oxygen levels (ca. 20%), cells are frequently cultured in. ROS leads to DNA modifications and subsequently to the activation of the p53 pathway. Therefor senescence can be bypassed in these cells by a spontaneous acquisition of a p53 mutation. It was also reported that MEFs with intact p53 pathways, which are cultured at physiological oxygen levels (3%) do not senesce (vom Brocke et al., 2006).

Premature senescence, which is a result of the activation of oncogenic stress, is also often triggered by DNA damage pathways. As mentioned above, DNA damage like double strand breaks as well as single-strand breaks induce a signalling cascade, which leads to the activation of p53.

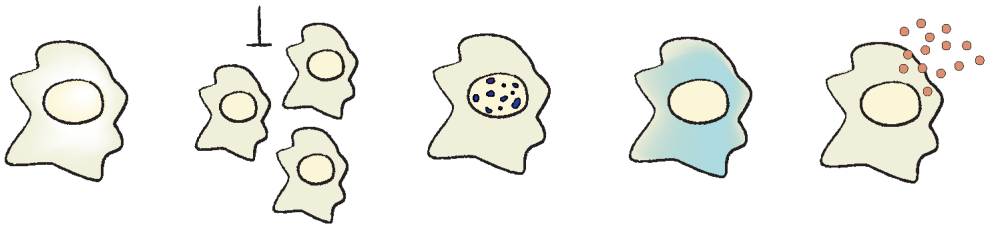
Another consequence of oncogenic signalling is the activation of the *INK4b/ARF/INK4a* locus. In cancers, this locus is often altered for example by point mutations or epigenetic changes. The *INK4b/ARF/INK4a* locus encodes for the CDK inhibitors $p15^{INK4b}$ and $p16^{INK4a}$ as well as ARF (Canepa et al., 2007). $P15^{INK4b}$ and $p16^{INK4a}$ activate RB via inhibition of CDK. ARF inhibits the p53 inhibitor HDM2. When HDM2 is active it can either bind to p53 and sterically block its transactivation domain or function as p53 specific ubiquitin ligase (Levine and Oren, 2009). For HDM2 inhibition, ARF binds to HDM2, which promotes its rapid degradation (Zhang et al., 1998).

Usually polycomb repressive complexes repress the transcription of the *INK4b/ARF/INK4a* locus (Bracken et al., 2007; Gil et al., 2004). Induction of the locus upon oncogene activation might be possible due to decreased expression of polycomb proteins or dissociation of the complexes from the locus. Polycomb proteins bind to histone H3 trimethylated on Lysine 27 (H3K27me3). The *RAS* oncogene for example was reported to increase expression of the H3K27me3 demethylase JMJD3, which led to an increase in $p16^{INK4a}$ expression (Barradas et al., 2009).

In a variety of cancer cells the p53 and RB pathways are inhibited (Burkhart and Sage, 2008; Levine and Oren, 2009). Thus oncogene activation in these cells does not lead to cell cycle arrest and senescence.



Characteristics of senescent cells



Flattened and enlarged cell morphology

Growth Arrest

Heterochromatin Foci (SAHF)

Senescence Associated β-Galactosidase

Secreted Factors (SASP)

Figure 1.3 **Induction of oncogene-induced senescence.** In unperturbed cells CDK activity leads to hyperphosphorylation of RB proteins. Hyperphosphorylated RB proteins release the E2F transcription factors and E2F responsive genes can be described. Transcription of these genes, which are essential for S-phase, results in entry and progression through S-phase. Senescence-inducing signals lead to the inhibition of E2F regulated transcription. Therefore one of two tumour suppressor pathways are activated, the p53 or $p16^{INK4a}$-RB pathways. The p53-pathway is activated as a result of DNA-damage signalling. Down-regulation of the p53 inhibitor HDM2 by ARF also results in increased p53 activity. Active p53 establishes senescence in part by inducing the expression of $p21^{Cip1}$. $p21^{Cip1}$ inhibits CDK activity and thus perturbs RB hyperphosphorylation. $p16^{INK4a}$ directly inhibits CDK4/6, which also results in inhibition of RB phosphorylation. Hypophosphorylated RB is therefore able to interact with E2F and thereby suppresses expression of genes required for cell cycle progression. Cells arrest in G1 and enter senescence (Campisi and d'Adda di Fagagna, 2007). Apart from the inability to proliferate, senescent cells display a typical phenotype. Characteristics include a flattened and enlarged cell morphology, chromatin remodelling, which is visible as senescence-associated heterochromatic foci, a higher β-galactosidase activity and the secretion of senescence-associated secretory phenotype factors (Campisi and d'Adda di Fagagna, 2007).

1.2 CKS (Cyclin-dependent kinase subunit)

The human CKS1 and 2 are members of the Suc1/Cks family of proteins. The members of this family are conserved in all eukaryotes (Harper, 2001) and were first discovered in fission yeast as Suc1 (Hayles et al., 1986; Pines, 1996) and budding yeast as Cks1 (Hadwiger et al., 1989). Since its discovery, this family of proteins has been studied in several model organisms such as fission and budding yeast, *Xenopus*, *C. elegans* and mice, as well as in human-derived cultured cells. Whereas in yeast and *Xenopus* only one Cks protein has been identified, two homologues have been found in *C. elegans*, mice and humans.

The human CKS1 and 2 consist of a four-stranded ß-sheet with two α-helices on top of one side (Seeliger et al., 2002). The Cks proteins in yeast contain additionally two large insertions of a long α-helix at the N-terminus and a large loop between the two other α-helices (Endicott et al., 1995).

1.2.1 Non-mammalian CKS proteins

CKS proteins (Suc1 in fission yeast) were first identified to be a binding partner of Cdc2, which is the fission yeast homologue of CDK1. The function of CKS proteins within this complex is still not well defined although some progress has been made using *in vitro* systems. For example, in *Xenopus* interphase egg extracts, the CKS homologue xe-p9 has been shown to prevent the dephosphorylation of the *Xenopus* CDK1 homologue Cdc2 on Tyrosine 15. Thus the kinase stays inactive and cells arrest in G2 (Patra and Dunphy,

1996). It is thought that this effect is due to phosphorylation of Cdc25 by the Cdc2/xe-p9 complex. The Cdc25 phosphatase is then no longer able to dephosphorylate Cdc2 on Tyrosine 15 resulting in an inactive kinase, which is not able to promote important mitotic events like spindle formation.

In addition to cell cycle inhibitory effects, many cell cycle promoting functions for the CKS binding to CDK have been postulated as well. In *Xenopus* egg extracts for example xe-p9 enhances the hyperphosphorylation of an APC component by recombinant Cdc2-cyclin B complex. This hyperphosphorylation is important for the activation of the APC. The activated APC can then act as ubiquitin ligase and target proteins inhibiting progression through mitosis (Patra and Dunphy, 1998).

A potential role for CKS proteins in the activation of the APC has not only been observed in mitosis. Studies in *C. elegans, Drosophila* and murine germ cells revealed problems in spindle formation and progression past Metaphase when CKS is missing (Pearson et al., 2005; Polinko and Strome, 2000; Spruck et al., 2003). Besides these studies in multicellular eukaryotes, showing cell cycle promoting functions for CKS, which are likely to be associated with higher CDK activity, this was also shown in *Saccharomyces cerevisiae* where Cks is important for the kinase activity of several G1 Cyclins-Cdc28 (CDK1) complexes (Reynard et al., 2000).

Assuming that CKS proteins promote CDK activity in a direct way, CKS proteins need to be able to interact with the kinase itself. CKS proteins are able to interact to a certain extent with CDK themselves. A study in *Xenopus* egg extracts however showed, that cyclin binding and subsequent phosphorylation of the *Xenopus* CDK homologue, Cdc2, on Threonine 161 is required for CKS binding (Egan and Solomon, 1998). This kind of cyclin stimulated binding is also believed to be the case for the human CDK2-cyclin A-CKS complex. A structural analysis revealed that cyclins and CKS1 bind to opposite sides of CDK2 (Bourne et al., 1996). It was presumed that cyclin binding to CDK2 induces conformational changes, which facilitate the phosphorylation of CDK2 on Thr-160 by CAK and subsequent CKS binding. The study also showed, that CKS is positioned in a way that it extends the site of interaction for a substrate of the kinase. Additionally, the adenosine tri-phosphate (ATP) binding site of the kinase is on the same site as a positively charged region on the CKS protein. In a crystal structure analysis of CKS1, this region bound the phosphate analogue, vanadate (Arvai et al., 1995). These structural analyses would support a role of CKS proteins in targeting the cyclin-CDK complex to phospho-proteins and facilitating their phosphorylation in this way. In structural analysis, CKS proteins were also found to form dimers (Endicott et al.,

1995; Parge et al., 1993; Watson et al., 1996). Dimer formation of excess CKS might counteract its function within the cyclin-CDK complex.

Apart from regulating the cell cycle via direct interactions with cell cycle proteins, an effect of CKS proteins on transcription was observed in yeast. It recruits CDK and the proteasome to the promoters of various genes and thus helps to remodel the chromatin to facilitate transcription. Genes reported to be regulated in this manner include *GAL1* and the APC activator *CDC20* (Chaves et al., 2010; Morris et al., 2003; Yu et al., 2005).

1.2.2 Mammalian CKS proteins

In mammals, CKS binds to three CDK, CDK1, CDK2 and CDK3 (Harper, 2001). CDK3 was suggested to be important in the exit from G0 (Ren and Rollins, 2004) but further functions remain poorly understood. In contrast CDK1 and 2 have been extensively studied and play important roles within the cell cycle. Besides their still not well-defined function within the CDK-cyclin complex, the mammalian CKS proteins were shown to take part in the ubiquitination of certain cell cycle regulators.

In 2001, Spruck et al., as well as Ganoth et al., reported a function for CKS1 within the SCF^{Skp2} ubiquitin ligase (Ganoth et al., 2001; Spruck et al., 2001). The ubiquitination of the CDK2 inhibitor $p27^{Kip1}$ could be reconstituted by human CKS1 in a purified system (Ganoth et al., 2001; Spruck et al., 2001) and was impaired in *Cks1*$^{-/-}$ mice (Spruck et al., 2001). SCF^{Skp2} is an E3 ubiquitin ligase, which consists of SKP1, Cullin1 (CUL1), RING-box protein (RBX) 1 and SKP2. RBX1 and SKP1 are placed on CUL1, which serves as scaffold. RBX1 binds the ubiquitin transferase (E2) and SKP1 binds SKP2 (Deshaies, 1999; Jackson and Eldridge, 2002). SKP2 is the F box protein, which binds the substrate. For the ubiquitination of $p27^{Kip1}$, CKS1 interacts with SKP2. CKS1 recognises phosphorylated $p27^{Kip1}$. $p27^{Kip1}$ then binds to both CKS1 and SKP2 to be ubiquitinated. CDK2-cyclin A complexes stimulate this process possibly by the ability of CDK2 to bind CKS1 and the ability of cyclin A to bind SKP2 (Hao et al., 2005). Ubiquitination of $p21^{Cip1}$ by the SCF^{Skp2} ubiquitin ligase was also enhanced in the presence of CKS1 (Bornstein et al., 2003). Further targets of the SCF^{Skp2} ubiquitin ligase, which might require CKS1 as linker protein are $p57^{Kip2}$ and p130 (Bornstein et al., 2003; Kamura et al., 2003; Tedesco et al., 2002). p130 is the pocket protein and CDK inhibitor, which is active primarily in G0 and degraded thereafter (see chapter 1.1.2.1). $p57^{Kip2}$ has been shown to be essential during embryogenesis (Borriello et al., 2011). It shares CDK inhibitory functions with $p21^{Cip1}$ and $p27^{Kip1}$ and levels decrease at the end of G1.

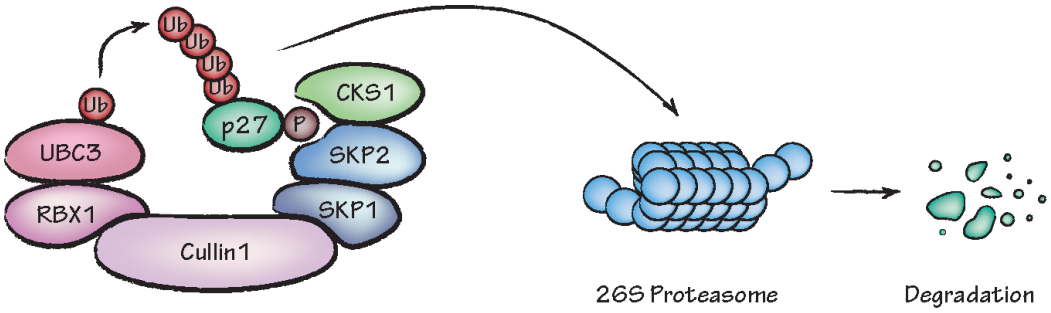


Figure 1.4 **p27^{Kip1} ubiquitination by the SCF^{Skp2} ubiquitin ligase.** The SCF^{Skp2} ubiquitin ligase is composed of the subunits SKP1, Cullin1, RBX1 and the F-box protein SKP2 (Jackson and Eldridge, 2002). The scaffold protein Cullin1 forms the backbone of the ligase and separates the substrate binding and catalytic components. It binds SKP1 at its N-terminal and RBX1 at its C-terminal end. The adaptor protein SKP1 binds SKP2, which functions as a substrate specificity factor. It recognises and binds phosphorylated p27^{Kip1}. p27^{Kip1} binding is enhanced by CKS1 (Spruck et al., 2001). The E2 ubiquitin-conjugating enzyme UBC3 is recruited to the Cullin1 backbone via RBX1. UBC3 can now ubiquitinate the substrate p27^{Kip1}. Polyubiquitinated p27^{Kip1} is then degraded by the 26S proteasome. (Adapted from (Wang et al., 2012))

Later it was discovered that CKS proteins are also involved in the function of another ubiquitin ligase, which plays a crucial role in cell cycle progression, the APC/C. It was shown that CKS facilitates the binding of cyclin A to the APC/C. Here cyclin A, which is in complex with CDK2 and CKS, binds to the APC/C cofactor CDC20. Cyclin A is then recruited to the APC/C through the CKS interaction with phosphorylated APC/C (Di Fiore and Pines, 2010).

Expression of CKS proteins in mammals is regulated by transcription as well as protein degradation. mRNA levels for *CKS1* are low in G0, increase at the end of G1 and peak in S/G2. The responsible region in the promoter of *CKS1*, which is leading to the repression in G0 and early G1 was identified as a cell cycle dependent element (CDE) and cell cycle genes homology region (CHR) tandem site (Rother et al., 2007b). The transcription of both, CKS1 and 2, is down-regulated by p53 (Rother et al., 2007a; Rother et al., 2007b).

CKS1 protein levels were observed to decrease in G1 and again in M phase. Down-regulation in G1 was shown to be dependent on the APC/C^{Cdh1} ubiquitin ligase (Bashir et al., 2004). Low levels of CKS1 in G1 lead to the stabilisation of the CDK inhibitor p27^{Kip1} preventing premature entry into S-phase. It was suggested that CKS down-regulation in M-phase is also dependent on the ubiquitin proteasome pathway (Hattori et al., 2003).

1.2.3 *CKS* as oncogenes

Both proteins, CKS1 and 2, are over-expressed in various types of cancer and are often associated with aggressive forms characterised by high proliferation and a tendency to metastasise. For example, *CKS1* was found to be over-expressed in multiple myeloma (Chang et al., 2010; Fonseca et al., 2006; Zhan et al., 2007), renal cancer (Liu et al., 2008), gastric cancer (Masuda et al., 2003), colorectal cancer (Shapira et al., 2005), lung cancer (Inui et al., 2003), breast cancer (Slotky et al., 2005; Wang et al., 2009; Westbrook et al., 2009), prostate cancer (Lan et al., 2008) and hepatocellular cancer (Calvisi et al., 2009; Lee et al., 2011; Shen et al., 2010). *CKS1* over-expression was also observed in cancer cell lines and animal models (Kitajima et al., 2004; Lan et al., 2008; Lee et al., 2011; Wang et al., 2009). In cell cultures derived from carcinomas of these animal models or patients, the role of *CKS* as an oncogene has been studied by down-regulation of *CKS*. The loss of *CKS* in these cells, which otherwise show high levels of CKS, led to a decrease in proliferation and tumorigenicity. *CKS1* knock-down is frequently accompanied by the accumulation of $p27^{Kip1}$. Furthermore high levels of CKS1 are often correlated to low levels of $p27^{Kip1}$ in cancer tissue samples (Kitajima et al., 2004; Lee et al., 2011; Wang et al., 2009). Thus the function of CKS1 as part of the SCF^{Skp2} ubiquitin ligase in $p27^{Kip1}$ degradation is believed to promote tumorigenesis. High levels of CKS1 are not always associated with a reduction in $p27^{Kip1}$ (Tsai et al., 2005; Westbrook et al., 2009). Additional SKP2 and $p27^{Kip1}$ independent mechanisms such as inhibition of apoptosis, G2/M transition, up-regulation of interleukin 8 and promoting anchorage independent growth and migration activities were associated with *CKS1* expression in cancer cell lines as well (Lan et al., 2008; Lee et al., 2011; Tsai et al., 2005; Wang et al., 2009).

A possible tumour promoting function of *CKS2*, which is also over-expressed in various cancer types like breast cancer (van 't Veer et al., 2002), colon cancer (Li et al., 2004), skin cancer (de Wit et al., 2005), cervical cancer (Lyng et al., 2006), prostate cancer (Lan et al., 2008), liver cancer (Chen et al., 2011) and bladder cancer (Chen et al., 2011) is not well studied.

More recently Liberal et al. proposed, that *CKS* over-expression in cancer might cause override of the intra S-phase checkpoint (Liberal et al., 2011). To demonstrate this point they treated cancer cell lines as well as immortalised human mammary epithelial cells with thymidine, which would typically lead to S-phase arrest induced by replication stress. *CKS1*- and *2* over-expressing cells could not be fully arrested. By using *CKS2* over-expressing cells over *CKS1* over-expressing cells for subsequent experiments they ensured that an observed effect is not due to the well-studied function of CKS1

within the SCF^{Skp2} ubiquitin ligase. The authors showed that the intra S-phase checkpoint in these cells was working properly by monitoring CHK1 phosphorylation status, the amount of CDC25A and CDK phosphorylation on Tyr15. They claimed that no difference in the CHK1 pathway was visible in control and *CKS2* over-expressing cells. Thus they concluded, that the ability of *CKS* over-expressing cells to override the intra S-phase checkpoint is due to an unknown function of CKS. CKS proteins might be able to maintain Tyr15-phosphorylated CDK2 in an active state. To confirm their hypothesis they showed that immunoprecipitated CDK2 from *CKS2* over-expressing HEK293A cells had an increased kinase activity towards RB. In an assay with recombinant proteins, addition of wtCKS1 enhanced CDK2 activity as well. A mutant form of CKS1, which shows a reduced ability to bind CDK (CKS1E63Q), does not have a positive influence on CDK2 activity. In contrast to this, addition of a CKS1 mutant, which is unable to bind phospho-proteins (CKS1AM), leads as well to a higher CDK2 activity albeit not to the same degree as the wild-type protein. These results would support the idea that CKS keeps CDK2 in an active conformation regardless of the inhibitory phosphorylation on Tyr15. This CKS function would not require the ability of CKS proteins to bind to phospho-proteins (Liberal et al., 2011).

1.3 Aim of this study

Both CKS proteins have been shown to be over-expressed in a variety of advanced metastatic cancers. Studies on animal models (Lan et al., 2008; Westbrook et al., 2009) and cell culture have further suggested a role of CKS proteins as oncogenes. These studies showed an impaired proliferative capacity of cancer cells upon down-regulation of CKS proteins (Lee 2011, Wang 2009, Lan 2008, Kitajima 2004). Whether *CKS* acts as a real oncogene and has the ability to promote the transformation process of primary cells to cancer cells remains unclear. Known oncogenes lead to checkpoint activation upon their over-expression in primary cells. Therefore the response of primary cells to *CKS* over-expression should be studied. In case of checkpoint activation upon *CKS* over-expression the nature of this activation should be analysed. Furthermore a tumour promoting function of CKS in checkpoint inhibited cells should be investigated.

Chapter 2 – Material and Methods

2.1 Cell lines and tissue culture methods

IMR90 human foetal lung fibroblasts and human embroyonic kidney (HEK) 293T cells were obtained from the American Type Culture Collection (ATCC). The packaging cell line Takara (GP2-293) was obtained from Takara Bio. Unless otherwise stated cells were maintained in Dulbecco's modified Eagle's medium (DMEM, PAA) with 10% (v/v) foetal bovine serum (PAA), 1% (v/v) non essential amino acids (PAA), 1% (v/v) L-glutamine and 1% (v/v) Penicillin/Streptomycin (PAA), 50 µg/ml Normocin (Invivogen) and 1% (v/v) antibiotic-antimycotic (Gibco). Cells were grown at 37˚C with 5% CO_2.

2.2 Retrovirus production and transduction of target cells

Amphotropic retroviral stocks were prepared by transient transfection of the Takara packaging cell line. Confluent Takara cells were split one to four and plated in 10 cm culture dishes one day prior transfection. A solution consisting of 1 ml DMEM, 75 µl of the transfection reagent polyethylenimine (PEI, Sigma Aldrich), 2 µg of a VSV-G viral coding helper plasmid and 14 µg of the desired expression plasmid was prepared, incubated for 20 minutes and added drop wise to each plate of Takara cells. On the day following transfection, culture medium was replaced with 5 to 7 ml of fresh culture medium and the fibroblasts, to be infected the following day were plated at 40% confluence in a 10 cm plate. Two days after transfection, the virus-containing supernatants from the Takara cells were collected and filtered through 0.45 µm pore sized acetate filters (Anachem). Polybrene (Sigma Aldrich) was added at a 1 to 2 µg/ml concentration to boost the interactions between cells and viral particles. After 24 hours, cells were aspirated and incubated in regular culture media with selection in medium containing the appropriate amount of the required antibiotic initiated three days post-infection (Table 1).

For the infection with the lentiviral vector TetO-FUW ZEOCIN, 293T cells were used as packaging cell line. DMEM and PEI were mixed with 20 µg of TetO-FUW, 5 µg of pRSV-Rev (a vector expressing the regulator of virion expression protein (Rev) for transporting the viral mRNA from the nucleus to the cytoplasm), 10 µg of pMDLg (a vector expressing the group-specific antigen (Gag) and polymerase (Pol) for the assembly of the retroviral particles and the synthesis of viral DNA and integration of the

DNA into the host genome) and 6 µg of pMD2.G (a vector expressing VSV-G for the interaction with target cells). Transfection of 293T cells was then performed as described for the transfection of Takara cells with retroviral vectors.

Table 1 **Retroviral constructs used and selection conditions**

Vector	cDNA	Antibiotic resistance	Concentration of antibiotics	Period of selection
pMaRX	c-MYC	puromycin	0.5 to 1 µg per ml	7 days
pBABE	HRAS G12V			
pBABE	CKS1 wt			
	CKS1 E63Q			
	CKS1 K11E S51E K71A			
	CKS2 wt			
	CKS2 E63Q			
	CKS2 K11E S51E K71A			
pBABE-sin-tet	CKS1 wt			
	CKS1 E63Q			
	CKS1 K11E S51E K71A			
	CKS2 wt			
	CKS2 E63Q			
	CKS2 K11E S51E K71A			
pWZL	hTERT	blasticidin	2 µg per ml	2 weeks
TetO-FUW	CKS1 wt	zeocin	25 µg per ml 10 µg per ml for further culturing	2 weeks
	CKS2 wt			
MSCV	M2rtTA	hygromycin	20 µg per ml	2 weeks
pLV	tTRKRAB	neomycin	400 µg per ml	3 weeks
LXSN	E6/E7			

2.3 Growth assays

2.3.1 Colony formation assay and growth curves

For colony formation assays 5 x 10^4 cells were seeded in duplicates in 6 cm dishes. Medium was replaced every 3 to 4 days. After approximately 14 days cells were washed once with 1 x phosphate buffered saline (PBS) fixed for 10 minutes with 0.5% glutaraldehyde (Sigma Aldrich) and washed twice with PBS. Fixed cells were stained with 0.1% crystal violet (Sigma Aldrich) for 30 minutes followed by extensive washing with water.

For growth curves 7 x 10^3 cells were seeded in duplicates in 24 well plates. Cells from two wells were fixed the day after plating (day 0) and every second day thereafter for a period of 12 days. Fixed cells were stained as described above. Stainings were quantitatively analysed by extracting the crystal violet with 10% acetic acid (Fisher Scientific) and measuring the absorbance of the acetic acid crystal violet solution at 595 nm (A_{595}) using a Bio-Rad 680XR microplate reader. To obtain the growth curves, the absorbance, representing cell density, was plotted against elapsed time.

2.3.2 BrdU (5-Bromo-2'-deoxyuridine) incorporation assay

3.5 x 10^3 Cells were plated in duplicates into 96 well plates and pulsed with 50 µM BrdU (Sigma Aldrich) for 16 hours the following day (Gratzner, 1982). Cells were then washed with PBS and fixed for 10 minutes with 4% (w/v) paraformaldehyde (PFA, Sigma Aldrich) at room temperature.

For immunofluorescence staining the following steps were performed at room temperature. Cells were permeabilised with 0.2% (v/v) Triton X-100 in PBS (Sigma Aldrich) for 10 minutes and incubated in 1 x blocking solution (0.5% (w/v) BSA (Calbiochem), 0.2% (w/v) fish skin gelatin (Sigma Aldrich)) for 30 minutes. Staining with rat anti-BrdU antibody, clone 3H579 (1:100, Santa Cruz) was performed in the presence of DNase I (0.5 U/µl; Sigma Aldrich) and $MgCl_2$ (3 mM, Sigma Aldrich) in 1 x blocking solution for 1 hour. Cells were washed three times with PBS and incubated with the secondary Alexa Fluor 488 goat anti-rat antibody (1:1000 in PBS, Invitrogen) for 30 minutes. Finally, cells were washed with PBS and incubated with DAPI (1.5 µM, Invitrogen (Schnedl et al., 1977)). Plates were read by the IN Cell Analyzer and High Content Analysis was performed to discriminate positive BrdU nuclei and total nuclei (see below for the description of High Content Analysis).

2.3.3 Senescence associated ß-galactosidase (SA-ß-gal) assay

To analyse enhanced SA-ß-gal activity cells were washed once with PBS, fixed with 0.5% (w/v) glutaraldehyde for 10 minutes at room temperature and washed twice with PBS (pH6) supplemented with 1 mM $MgCl_2$ (PBS/$MgCl_2$). Fixed cells were stained overnight at 37°C with an X-gal staining solution containing 1 mg/ml X-Gal (Invitrogen), 5 mM $K_3Fe(CN)_6$ (Sigma Aldrich) and 5 mM $K_4Fe(CN)_6 \cdot 3H2O$ (Sigma Aldrich) in PBS/$MgCl_2$. Staining was stopped by washing the cells with distilled water when sufficient staining was visible in IMR90 H-RASG12V control cells. Pictures were taken on an Olympus IX inverted microscope and the percentage of SA-ß-gal positive cells was scored.

2.4 Immunofluorescence (IF) staining and antibodies

Cells were plated in 96 well plates at a density of 3.5 x 10^3 cells per well. Two days later cells were fixed with PFA and permeabilised as described above. Incubation of primary antibodies was performed in 1 x blocking buffer (see section 2.3.2) at room temperature for 1 hour. After consecutive rinses in PBS, cells were incubated with secondary antibody for 30 minutes. Nuclei were stained as previously described.

The following primary antibodies were used: mouse anti-$p16^{INK4a}$ antibody, clone JC8 (1:500, Santa Cruz); mouse anti-$p21^{Cip1}$, clone P1484 (1:100, Sigma Aldrich) and mouse anti-p53, clone DO-1 (1:100, Santa Cruz). Goat anti-mouse Alexa488-conjugated antibody (1:1000, Invitrogen) was used as secondary antibody.

Images of stained cells were acquired using the IN Cell Analyzer 1000 automated high-throughput microscope (GE Healthcare) with 10x (for BrdU staining) or 20x (for $p16^{INK4a}$, $p21^{Cip1}$ and p53 staining). For each condition images were acquired at an excitation wavelength of 360 nm and emission wavelength of 460 nm (corresponding to DAPI) and at an excitation wavelength of 480 nm and emission wavelength of 535 nm (corresponding to primary antibody/Alexa Fluor® 488 secondary antibody) respectively. For analyses of $p21^{Cip1}$ and p53 levels in CKS positive cell subsets, additional incubation steps with a rabbit anti-CKS1/2 FL79 antibody and a goat anti-rabbit Alexa Fluor® 594-conjugated antibody (1:1000, Invitrogen) were necessary. Alexa Fluor® 594 was detected using an excitation filter of 565 nm and emission filter of 620 nm wavelength.

Images were analysed and quantified via High Content Analysis (HCA) with the help of the IN Cell Investigator (v1.7) software (GE Healthcare). First, DAPI images were used to identify the nuclear area of each cell and thus the cell itself and the number of all cells in one image. A nucleus was defined as region with a defined DAPI intensity and an area of at least 100 μm^2. To identify the single cells, a collar segmentation routine was used with a diameter of 1 µm around the nucleus. Then the average intensity of pixels in the second channel (AlexFluor®488) for the nuclear region was determined. A negative control, usually the empty vector control, and a positive control, which express low levels and high levels of the visualised protein respectively, were first analysed. The nuclear intensity values for the specific protein of all cells were mapped into a histogram. Using the histogram, a threshold filter was set up, which allowed for distinguishing between cells with high levels (positive) or low levels (negative) of the specific protein. After the analysis of all images in one experiment the software generated a mean percentage for both cell populations (positive and negative) in all images acquired per well. This procedure was repeated for the third channel

(AlexFluor®594) where applicable. In the case of double stainings with an antibody against CKS and against $p21^{Cip1}$ or p53, a decision tree was set up to identify the percentage of cells negative for CKS but positive for checkpoint protein as well as double positive and double negative cells.

For the quantification of cells positive for Senescence associated heterochromatic foci (SAHF), 20x images of DAPI stained cells were acquired at two different intensities using two different exposure times. The nucleus was defined in the image with the lower intensity. In the image with the higher intensity SAHF were identified by using the organelle parameter in the IN Cell Investigator Software. SAHF were defined as spots with a high fluorescence intensity and an area of 1 to 3 µm. After comparing empty vector control cells and *RAS* oncogene control cells, a threshold number of SAHF for the cells to be counted as positive for SAHF formation was defined.

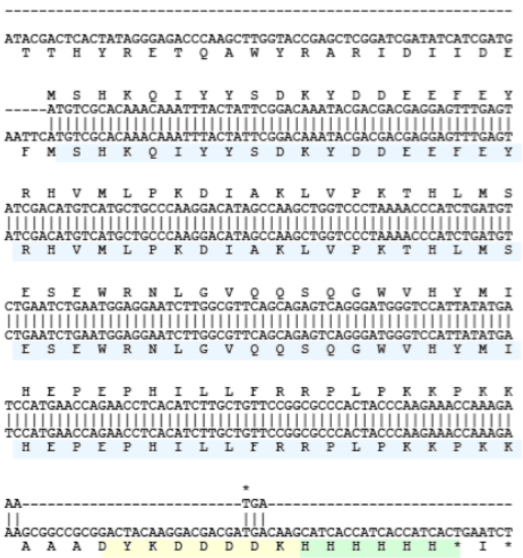
2.5 Generation of viral vectors for the transduction of fibroblasts

2.5.1 Retroviral vectors used for continuous protein expression

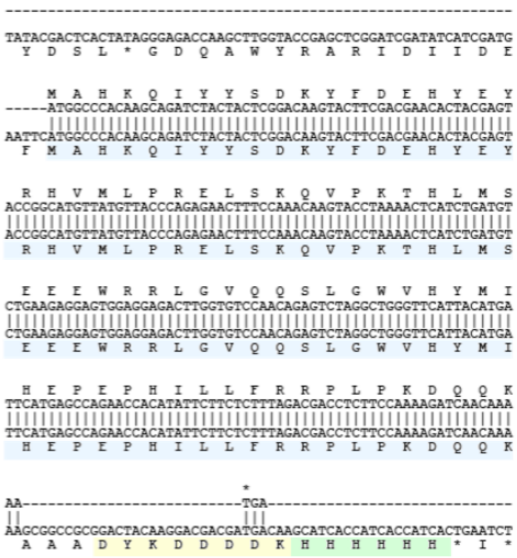
Constructs for the stable expression of human *CKS1wt*, *CKS1E63Q*, *CKS2wt* and *CKS2E63Q* were prepared by Mattia Frontini. Briefly, cDNA was prepared and the sequence coding for *CKS1wt* and *CKS2wt* was PCR-amplified and cloned into pSP72. *CKS1E63Q* and *CKS2E63Q* were obtained by PCR site-directed mutagenesis of the wt counterparts. FLAG and HIS tags were introduced. The sequence encoding for all *CKS* variants coupled to the FLAG and HIS tag were then PCR-amplified from pSP72 plasmids with primers containing BamHI and SalI sites for cloning into the pBABE PURO retroviral vector (Figure 4.1 A).

Using the pBABE CKS1wt and CKS2wt PURO as template, pBABE CKS1 K11E S51E K71A and CKS2 K11E S51E K71A PURO were generated by PCR site directed mutagenesis by Roman Holic. pBABE HRASG12V PURO, pMaRX c-MYC PURO, LXSN E6 and E7 NEO have been available in the lab and were described previously (Banito et al., 2009). pWZL hTERT BLAST was kindly provided by Martin Teichmann (University of Bordeaux, France). Constructs for inducible *CKS* expression are described below.
The accuracy of *CKS* sequences in pBABE as well as the inducible vectors was verified by sequencing (Figure 2.1):

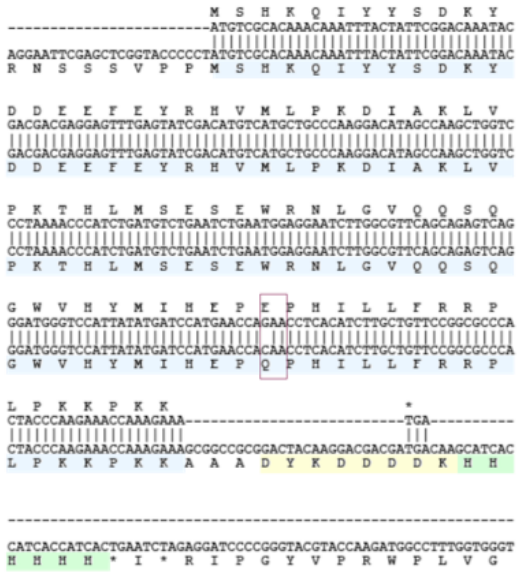
CKS1



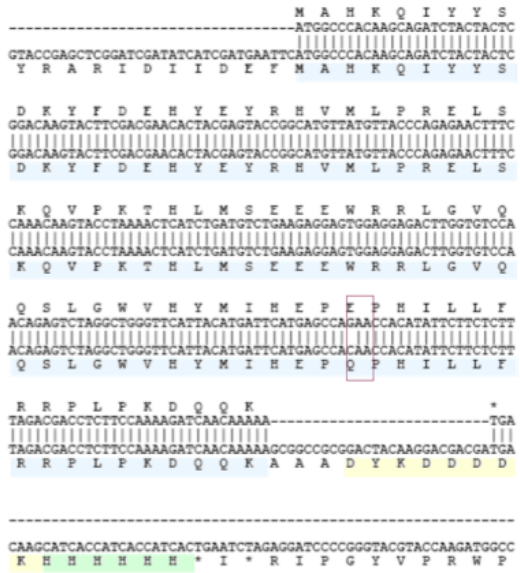
CKS2



CKS1E63Q



CKS2E63Q



CKS sequence

FLAG-Tag

Polyhistidine-Tag

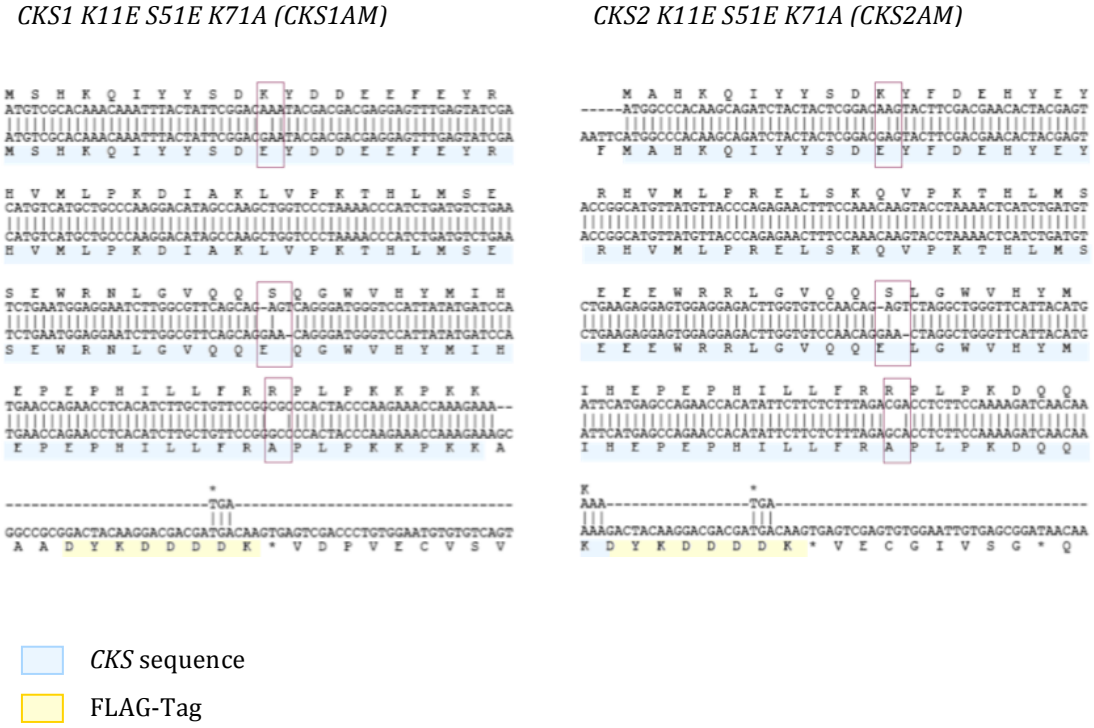


Figure 2.1 **Verification of *CKS* sequences in pBABE PURO vectors.** Sequences obtained from pBABE CKS PURO analyses were aligned with the respective *CKS* c-DNA sequences to verify their accuracy. The alignments show the published cDNA sequences for the CKS variants (upper sequence) aligned with that obtained from the sequencing of the produced pBABE CKS PURO DNA constructs (lower sequence). Red boxes highlight the mutational changes in the sequence leading to the insufficiency of CDK (E63Q) or phospho-protein (AM) binding in the CKS variants.

2.5.2 Generation of viral vectors used for induced protein expression

2.5.2.1 TetO-FUW CKS ZEOCIN constructs

The TetO-FUW ZEOCIN vector was obtained from Addgene (Boston, MA). CKS FLAG Tag constructs were used as templates in a PCR reaction to obtain the *CKS* fragments with EcoRI restriction sites on both ends. Sequences of the primers used were as follow:

FLAG_EcoRI Reverse: TGTAGAATTCTCAGTGATGGTGATGGTGAT
CKS2_EcoRI Forward: AACTGAATTCATGGCCCACAAGCAGAT
CKS1_EcoRI Forward: CGACGAATTCATGTCGCACAAACAAATTTACTAT

PCR conditions used:

Initial denaturing: 95°C 5 min	
Denaturing: 98°C 45 s Annealing: 55°C 45 s Elongation: 72°C 30 s	5 times
Denaturing: 98°C 45 s Annealing: 60°C 45 s Elongation: 72°C 30 s	25 times
Final elongation: 72°C 5 min	

PCR products and TetO-FUW vector were both cut on their *EcoRI* sites (Figure 3.1 B). For ligations the T4 ligase and buffer (NEB) were used according to the recommended conditions.

2.5.2.2 pBABE sin tet CKS PURO constructs

The pBABE sin tet PURO vector was provided by Ana Banito (Cell Proliferation Group, CSC MRC). The CKS FLAG Tag sequences were cut out from the pSP72 CKS FLAG Tag constructs using *EcoRI* and *XhoI* restriction enzymes and cloned into the *EcoRI/XhoI* restriction site of the pBABE sin tet PURO vector (Figure 3.2 B).

2.5.2.3 Additional constructs for induced protein expression

The tetracycline inducible activator was expressed via the MSCV M2rtTA HYGRO, which was provided by Ana Banito (Cell Proliferation Group, CSC MRC). The pLV-tTRKRAB NEO was coding for the tetracycline responsive repressor and was provided by the Didier Trono laboratory (École polytechnique fédérale de Lausanne).

2.5.3 Transformation of *Escherichia Coli*

For cloning and plasmid recovery, chemically competent *Escherichia coli* (*E. coli*) strain DH5α was used. A stock of competent cells was obtained as follow: *E. coli* were picked and a starter culture was grown in 5 ml lysogeny broth (LB) overnight at 37˚C. 1 ml of starter culture was added to 500 ml LB and the culture was grown until an $OD_{595\,nm}$ of 0.375 to 0.5 was reached. Cells were centrifuged at 4˚C for 5 minutes at 4000 g. Resulting pellets were resuspended in ice cold 0.1 M $CaCl_2$ and incubated on ice for 20 minutes. Cells were centrifuged again, resuspended in 10.5 ml ice cold 0.1 M $CaCl_2$, 22% (v/v) glycerol and dispensed in 100 µl aliquots. Aliquots were snap frozen on dry ice and stored at -80˚C.

For transformation, competent cells were thawed on ice and mixed with the ligation product (see below) or 10 ng of plasmid DNA by gently flicking the tube. Cells were then incubated 30 min on ice, heat shocked for 40 seconds in a 42˚C water bath and returned to ice for another 5 minutes. 500 µl of LB medium without antibiotics was added and the transformation mixture was grown for 1 hour at 37˚C. Approximately 100 µl of each transformation was spread on LB agar plates containing the appropriate antibiotic. Plates were incubated overnight at 37˚C and several colonies were picked and grown in 2 ml of LB medium containing antibiotics overnight. Ampicillin was used for all vectors described at a concentration of 100 µg/ml. To verify cloning, plasmid DNA was extracted, digested with appropriate restriction enzymes to check for the correct insert and sequenced. To obtain a sufficient amount of plasmid for retroviral infections of IMR90, 2 ml cultures were added to 250 ml LB broth medium containing the appropriate amount of antibiotics and grown at 37˚C with shaking at 150 rpm overnight.

2.5.4 Plasmid DNA Extraction from *Escherichia coli*

From 2 ml overnight cultures 1 ml was pelleted in 1.5 ml eppendorf tubes and plasmid DNA was extracted using the YORBIO Plasmid DNA Purification Kit. Buffers and Columns provided with the kit were used and DNA was isolated according to manufacturer's instructions, based on a modified alkaline lysis procedure.

250 ml overnight cultures were pelleted by centrifugation at 6000 g for 15 min at 4˚C. Plasmid DNA was then purified using the Qiagen HiSpeed® Plasmid Purification kit. The purification protocol is also based on a modified alkaline lysis procedure. Briefly, cell pellets were resuspended in buffer P1 containing EDTA to protect DNA from degrading enzymes and RNase to remove contaminating RNA. Lysis of the cells was achieved by adding an alkaline lysis buffer P2 containing SDS. Pre-chilled neutralisation

buffer P3 was added to renature plasmid DNA and precipitate genomic DNA and proteins. The soluble fraction containing the plasmid DNA was applied to an anion-exchange resin. Impurities were removed by washing the resin with isopropanol-containing buffer QC. Plasmid DNA was eluted from the column using a high salt buffer QF with a pH of 8.5. The DNA was precipitated from the high salt solution with Isopropanol, collected using the QIAprecipitator and eluted from the precipitator with a Tris-EDTA buffer. DNA concentration was determined by measuring the absorbance at 260 nm (A260) with a NanoDrop® ND-1000 UV-Vis spectrophotometer.

2.5.5 PCR

PCR was performed in 25 µl of 1x Phusion HF buffer using 0.4 units of Phusion polymerase (New England Biolabs), 0.5 µM primers and 200 µM dNTPs (Roche) and 20 ng of the desired plasmid as template DNA. The reaction took place in a DNA Engine Dyad Peltier Thermal Cycler (Bio Rad) using the appropriate program (see above).
For cloning purposes the PCR reactions of 6 reaction tubes were combined and cleaned using the QIAquick PCR Purification Kit (Qiagen). The kit contains silica-gel-membrane columns, which bind DNA in the presence of a high salt binding buffer. First, 5 volumes of the binding buffer PB were added to the combined PCR products. The solution was mixed by inverting the tube and transferred to the silica-gel-membrane column. After spinning the column for 15 seconds at high speed the flow-through was discarded and 700 µl of washing buffer PE was added then centrifuged for 1 minute. The flow through was discarded and the column was centrifuged again for 1 minute. The purified DNA was eluted with 42 µl of buffer EP.

2.5.6 Restriction enzyme digest

PCR products and target vectors were digested with restriction enzymes to receive the desired overhanging basepairs. All enzymes used were obtained from New England Biolabs (NEB) and the appropriate reaction buffer was chosen according to the recommendation provided by NEB. Digestion of 5 µg of vector DNA or PCR products were performed in 50 µl of the appropriate buffer supplemented with 40 units of each enzyme for the recommended time at 37°C.

To avoid self-ligation of the resulting ends of the vector, the phosphate group of the 5' end of the vector was removed by adding 6.5 units of antarctic phosphatase with the corresponding buffer (NEB), incubated for 30 minutes at 37°C.

2.5.7 Agarose gel electrophoresis

To analyse PCR product size and separate the products of a restriction enzyme digest, DNA was subjected to electrophoresis using 1% agarose gels. Electrophoresis was performed in 1xTAE (diluted from 50x stock: 2 M Tris-acetate, 50 mM EDTA, pH8.0) at a constant 100 volts in Mini Sub GT tanks (Biorad). DNA was visualised using the DNA intercalator, ethidium bromide, and a Geldoc transilluminator (Biorad).

2.5.8 DNA recovery following agarose gel electrophoresis

Resolved DNA fragments were visualised using UV light. The desired DNA fragments were excised from the Gel and the DNA extracted from the Gel using the QIAquick Gel Extraction Kit (Qiagen). This involves first dissolving the agarose gel in three volumes of buffer QG at 50˚C for 10 minutes followed by binding of the DNA to a silica-gel-membrane similar to the membranes used in the PCR purification kit. Further clean up of the DNA was performed as described for the purification of PCR products.

2.6 Analysis of gene expression

2.6.1 RNA extraction from IMR90 fibroblasts

RNA extraction was performed using TRIzol reagent (Ambion). The method used is based on the guanidium thiocyanate-phenol-chloroform extraction first described by Piotr Chomczynski (Chomczynski and Sacchi, 1987). TRIzol is an acidic phenol solution containing guanidinium isothiocyanate. Guanidinium isothiocyante denatures proteins including RNases. After adding chloroform to the acidic phenol and a centrifugation step RNA resides in the resulting upper aqueous phase.

For RNA isolation, cells were plated on 6 cm culture dishes and grown until approximately 70% confluent. Cells were homogenised by adding 1 ml of TRIzol and the resulting suspension was transferred to an eppendorf tube. Chloroform was added and the mixture vortexed, incubated for 3 minutes and centrifuged for 15 minutes at 4°C and highest speed. The RNA-containing aqueous phase was transferred into a new tube and precipitated by adding 400 µl of isopropanol and incubating at -20°C overnight. RNA was pelleted by centrifugation for 30 minutes at highest speed and 4°C. The pellet was washed with 75% ethanol and resuspended in RNase free water.

2.6.2 Quantitative RT PCR

Quantitative PCR was carried out using SensiMix One-Step (Quantace) as directed in the accompanying manual. The SensiMix buffer contains the reverse transcriptase for cDNA-synthesis from mRNA, the DNA-polymerase, nucleotides and magnesium. Additionally provided were SYBR Green I solution, which is incorporated during DNA synthesis and a RNase inhibitor. In a 20 µl reaction, 10 ng of RNA was added plus primers at a final concentration of 200nM. The primers used for determination of CKS expression were:

CKS1 Forward: CTAGCAAACCGAGCGATCAT
CKS1 Reverse: TTTAGGGACCAGCTTGGCTA
CKS2 Forward: CCCAAAACTCATCTGATGTCC
CKS2 Reverse: TGGAAGAGGTCGTCTAAAGAGAA

Thermocycling was performed using the BioRad CFX 96 Real Time system C1000 Thermal Cycler and the following conditions:

cDNA synthesis: 50°C 30 min

Initial denaturing: 95°C 15 s
Denaturing: 94°C 15 s
Annealing: 60°C 30 s
Elongation: 72°C 30 s
} 40 times
Plate read
Melting curve

2.6.3 Protein extraction

Cells were washed once with ice cold PBS, scraped from the culture dish by using 1 ml of PBS per 10 cm dish and briefly centrifuged. Lysis of the resulting cell pellet was performed using lysis buffer (50 mM Tris-Cl pH 8.0 (Sigma Aldrich), 0.5% Nonidet P-40 (NP-40, Roche), 0.1% EDTA (Sigma Aldrich), 10% glycerol (BDH) and protease inhibitors (Complete EDTA-free, Roche). Protein concentrations were determined using the RC DC Protein Assay kit (Bio rad), which is based on the Lowry protocol (Lowry et

al., 1951) and measured with the Nanodrop 1000 Spectrophotometer using the Lowry protocol of the ND-1000 V3.3.1 Software.

2.6.4 SDS-PAGE

Electrophoresis was performed using the discontinuous buffer system introduced by Laemmli (Laemmli, 1970). Samples corresponding to 40 µg of proteins were prepared by adding 5x Laemmli buffer (1x Laemmli buffer final concentration: 60 mM Tris-Cl pH 6.8, 2% SDS (Sigma Aldrich), 10% glycerol, 5% 2-Mercaptoethanol (Sigma Aldrich) and 0.01% bromophenol blue (Sigma Aldrich)) and boiling it for 5 minutes. The samples were loaded into the wells of the gel and proteins were separated using 100 volts to allow stacking of proteins and constant 130 volts thereafter until appropriate resolution was achieved. All SDS-PAGE was performed using the Mini-Protean® electrophoresis system from Bio-Rad.

2.6.5 Western blot analysis

The protocol used for transfer and blotting was based on the original technique described by Towbin and Burnette (Burnette, 1981; Towbin et al., 1979). The transfer was performed using the Mini-PROTEAN® transfer system from Bio-Rad with Tris/Glycine transfer buffer (25 mM Tris, 192 mM glycine (Sigma Aldrich), 10% methanol (Fisher Scientific), 0.1% SDS). Proteins were electrophoretically transferred for 1.5 hours to a Hybond-P PVDF membrane (GE Healthcare) using a constant 100 volts. Membranes were blocked for 1 to 2 hours in blocking solution (5% skimmed milk and 0.1% Tween-20 (Sigma Aldrich) in PBS) and probed with primary antibodies (Table 2a) overnight at 4˚C in blocking solution. After 4 washes with PBS-tween (0.1% Tween in PBS) for 5 minutes at room temperature HRP-conjugated secondary antibody (Table 2b) incubation was conducted for 1 hour at room temperature in blocking solution. Following a further 4x5 minute washes in PBS, antibody bound proteins were detected using ECL Plus reagents and exposed to Hyperfilm-ECL from GE Healthcare.

Table 2a **Primary antibodies for Westen blot and immunofluorescence analysis**

Antibody	**Clone**	**Source**	**Company**	**Concentra-tion in WB**
anti-CKS1/2 (FL79)	poly	rabbit	Santa Cruz	1:250
anti-γ-tubulin (T3559)	poly	rabbit	Sigma Aldrich	1:2000
anti-Flag	M2	mouse	Sigma Aldrich	1:500
anti-Telomerase reverse	poly	rabbit	Abcam	1:250
anti-$p21^{Cip1}$	P1484	mouse	Sigma Aldrich	1:500
anti-p53	DO-1	mouse	Santa Cruz	1:250
anti-$p16^{INK4a}$	JC8	mouse	Santa Cruz	-
Anti-BrdU	3H579	rat	Santa Cruz	-
anti-PCNA	PC10	mouse	Cell Signalling	1:1000
anti-cyclin A (H-432)	poly	rabbit	Santa Cruz	1:250
anti-cyclin B1	GNS1	mouse	Santa Cruz	1:250
anti-cyclin D1 (H-295)	poly	rabbit	Santa Cruz	1:250
anti-cyclin E	E-4	mouse	Santa Cruz	1:250
anti-CDK2	poly	rabbit	Santa Cruz	1:500
anti-RPA32/RPA2	9H8	mouse	Abcam	1:500
anti-MCM2 (d7g11)	poly	rabbit	Cell Signalling	1:1000
anti-γH2AX	JBW301	mouse	Milipore	1:250
anti-$p27^{Kip1}$ (C-19)	poly	rabbit	Santa-Cruz	1:500

Table 2b **Secondary antibodies for Westen blot**

Antibody	**Source**	**Company**	**Concentra-tion in WB**
HRP conjugated anti-mouse	goat	Santa Cruz	1:250
HRP conjugated anti-rabbit	goat	Sigma Aldrich	1:2000
HRP conjugated anti-goat	donkey	Sigma Aldrich	1:500

2.7 Analysis of CDK2 activity

2.7.1 Transfection of HEK 293T cells

293T cells were transiently transfected using polyethylenimine (PEI). Here the polycation PEI forms positively charged complexes with the DNA, which should be integrated into the cell (Boussif et al., 1995). These complexes bind to anionic cell surface components and are taken up by the cell via endocytosis. For the transfection confluent 293T cells were split 1:4 in 10 cm culture dishes. The transfection solution was prepared by mixing 20 µg of vector (pBABE CKS1 PURO, pBABE CKS2 PURO or empty pBABE PURO) with 1 ml of serum-free DMEM and 75 µl of PEI and incubating it for 20 minutes. The PEI-DNA solution was added drop wise to the cells. Cells were then kept as usual at 37°C, 5% CO_2 overnight before the medium was changed and cells harvested for protein isolation, immune precipitation and kinase assay the same day.

2.7.2 Transduction of IMR90 cells

IMR90 cells used for kinase assay experiments were virally transduced with pBABE CKS1 PURO, pBABE CKS2 PURO and empty pBABE PURO as described before. The day after infection with the retroviral supernatant, cells were either harvested immediately for further processing or kept in puromycin containing medium for additional seven days.

2.7.3 Immunoprecipitation

CKS1, *CKS2* or empty vector pBABE PURO transduced IMR90 and 293Tcells were cultured in 10 cm dishes and harvested at different time points after transformation. Therefore cells from one plate were washed with 5 ml ice-cold PBS and collected with 1 ml PBS using a cell scraper. Cells were then pelleted by centrifugation at 2000 g for 1 minute. Cells were resuspended in approximately 2 to 5 times the volume of the resulting cell pellet of ice cold RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X 100, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, complete protease inhibitor tablets (Roche, 1 tablet/50 ml)). Cells were lysed in RIPA buffer for approximately 15 minutes on ice and subsequently centrifuged at 4°C at 16 000 g for 10 minutes. The total cell extract, which was present in the supernatant, was collected and an aliquot was frozen down for further analysis by Western blotting (WB). The protein concentration was measured using the RC DC Protein Assay kit (Bio Rad). 1 µg of the desired antibody was added to 1 µg of cell lysate (for cyclin A-CDK2 CoIP: Anti-cyclin A; for cyclin E-CDK2 CoIP: Anti-cyclinE). The mixture was rotated slowly for 2 hours at 4°C.

30 µl of protein G coated magnetic beads (Dynabeads, Invitrogen) were added and the mixture was rotated again for 1h at 4°C to allow the conjugation of the antibodies to the beads. The protein-bound beads were washed three times in RIPA buffer and finally resuspended in 30 µl of 1 x reaction buffer A (provided with the CDK2-cyclin A Kinase Enzyme System; Promega). Kinase assays were performed immediately after immune precipitation and an aliquot was frozen down to determine the amount of CDK2 bound to the cyclins via Western blot.

2.7.4 Kinase assay

Kinase assays were performed using the ADP-Glo™ Kinase Assay Kit (Promega). Here, the kinase activity is measured by the amount of ADP, which is produced during a kinase reaction. Histone H1 is used as a substrate for the immunoprecipitated CDK2 complex. In the assay the kinase converts added ATP into ADP. After the reaction is stopped, remaining ATP is depleted. Then the ADP is converted into ATP, which is measured using a luciferase/luciferin reaction in which ATP is necessary as cofactor.

The assay was performed by following the instructions provided with the kit. Briefly, reactions were set up in a white 96 well plate. In a first step the components for the kinase reaction were added to the plate consisting of the following in a final volume of 25 µl: 10 µl of the immunoprecipitated kinase complex or 10 µl of 1 ng/µl purified active CDK2-cyclin A as positive control (CDK2-cyclin A2 Kinase Enzyme System, Promega), 5 µl of 1 mg/ml Histone H1 (CDK2-cyclin A2 Kinase Enzyme System, Promega), 5 µl of 2xbuffer (CDK2-cyclin A2 Kinase Enzyme System, Promega) and 5 µl of 250 µM ATP in 1xbuffer. The reaction was incubated for 15 minutes at 30°C while shaking. To terminate the reaction and deplete the remaining ATP, 25 µl of ADP-Glo™ Reagent was added and incubated for another 40 minutes at room temperature while shaking. For the luciferase reaction 50 µl of kinase detection reagent was added and the reaction mixture was incubated for 30 minutes at room temperature while shaking. The Luminescence was measured on a GloMax® Microplate Luminometer (Promega) by using the Kinase-Glo™ Luminescence Protocol.

Chapter 3 - Induced expression of CKS proteins in IMR90 fibroblasts using a Tet-On expression system

CKS proteins were to be over-expressed in fibroblasts to study the role of CKS in cancer development and to investigate the molecular processes, which lead to a possible advantage of *CKS* expression in cancer cells. Over-expression was to be achieved by viral transduction using retroviral vectors containing *CKS*.

Vectors generally used for the stable over-expression of proteins carry a selection marker, which confers resistance against a selection agent in a successfully transfected cell. Cells transduced with the desired viral construct need to be selected for this marker. When using regular viral transfection vectors the protein of interest is over-expressed during this selection process. If any cellular toxicity is associated with the over-expression of the target protein this will effectively be a selection disadvantage, thereby significantly complicating the selection of stably over-expressing cells.

An initial test using the retroviral vector pBABE for the stable over-expression of *CKS* resulted in growth arrest of the transduced IMR90 fibroblasts (see Chapter 4). Thus, a stable over-expression of CKS proteins and a possible oncogenic effect on cells caused by CKS impairs the study of the early events upon elevation of CKS protein levels.

An inducible system was introduced to overcome the complications of CKS-related toxicity during selection of transfected cells and to facilitate the study of the early events upon elevation of CKS protein levels. The expression of the target protein was inducible via the addition of doxycycline. This allowed for selection to take place without immediate over-expression of *CKS* and has the advantage that *CKS* is expressed only during the course of the desired experiment.

3.1 TetO FUW ZEOCIN

For the controlled induction of CKS proteins a Tet-On inducible system was generated. *CKS1*, *CKS2*, the non CDK binding mutants *CKS1E63Q* and *CKS2E63Q* as well as the anion binding site mutants *CKS1AM* and *CKS2AM* were cloned into the TetO FUW vector as described in Material and Methods. The TetO FUW vector contains a Tetracycline responsive promoter element (TRE). The *TRE* contains seven direct repeats of the *tetO* operator sequence, upstream from a minimal CMV promoter. This sequence is derived from Tet-operons in bacteria, which confer resistance against antibiotics from the tetracycline family, such as doxycycline. Only in the presence of doxycycline do the

bacteria transcribe the necessary efflux protein responsible for the resistance. This is due to Tet repressors (TetR), which are bound as homodimers to the *tetO* sequence in the absence of doxycycline, blocking the function of the promoter and thus suppressing expression of the efflux protein gene (Hillen and Berens, 1994; Welman et al., 2007). Binding of doxycycline to the TetR homodimer changes its conformation, which leads to a lower affinity of the repressor towards *tetO* and transcription can take place. Another Tet repressor, rTetR, whose binding properties were the reverse of TetR was subsequently identified (Berens and Hillen, 2003). The rTetR would bind to the *tetO* sequence in the presence of doxycyline but not in its absence. And by fusion of a transcription activating domain to the rTetR, a reverse activator (rtTA) was engineered, which would activate gene expression in the presence of doxycycline (Gossen and Bujard, 1992; Gossen et al., 1995).

In the constructed *CKS*-expressing TetO FUW vectors, the cloned CKS constructs are under the control of the CMV promoter, which is in turn controlled by the Tetracycline responsive element (Figure 3.1 A and B).

The Vector bears a zeocin resistance for stable selection in mammalian cells. The reverse transactivator M2rtTA is used to induce *CKS* expression (Figure 3.1 A). In comparison to rtTA, M2rtTA is more sensitive to doxycycline, more stable in eukaryotic cells and shows a reduced basal activity (Urlinger et al., 2000).

Cells were first transduced with a vector containing M2rtTA (MSCV M2rtTA HYGRO), selected for the appropriate period of time with hygromycin and subsequently transduced with the TetO FUW CKS ZEOCIN constructs. Due to the high number of cycles of cell growth and division associated with the two stable selection processes to produce the inducible protein-expressing cell lines, immortalised IMR90 fibroblasts with an introduced telomerase function, IMR90 TERT, were used as the parental cell line. After the transduction of IMR90 TERT M2rtTA cells with the TetO FUW CKS ZEOCIN vector, cells were selected for about three weeks in the presence of zeocin. Zeocin is a member of the bleomycin/phleomycin family of antibiotics. It is thought to intercalate into DNA and cleave it (Oliva-Trastoy et al., 2005). During and after the selection process, cells, which were successfully transduced with TetO FUW CKS ZEOCIN were viable. Nevertheless, zeocin selection led to a remarkable change in the appearance of the IMR90 TERT cells (Figure 3.1 C). Cells became elongated and formed spindle-shaped branches at low levels of zeocin (25 µg/ml). This phenotype became more profound at high levels of zeocin. The toxicity of zeocin despite the used selection resistance gene has been previously described in human cells (Oliva-Trastoy et al., 2005). To test the functionality of the CKS induction via doxycycline, cells, selected in

medium containing low levels of zeocin, were treated for 48 hours with 10 µg/ml of doxycycline (Figure 3.1 D). Without doxycycline the IMR90 TERT M2rtTA TetO FUW CKS1 and CKS2 cells showed already a basal expression of CKS proteins. Upon induction with doxycycline, *CKS1* expression could slightly be increased. Cells transduced with the CKS2 construct showed a higher level of induction. Due to the high sensitivity of the cells to zeocin as well as the observed background expression, the inducible system needed further optimization.

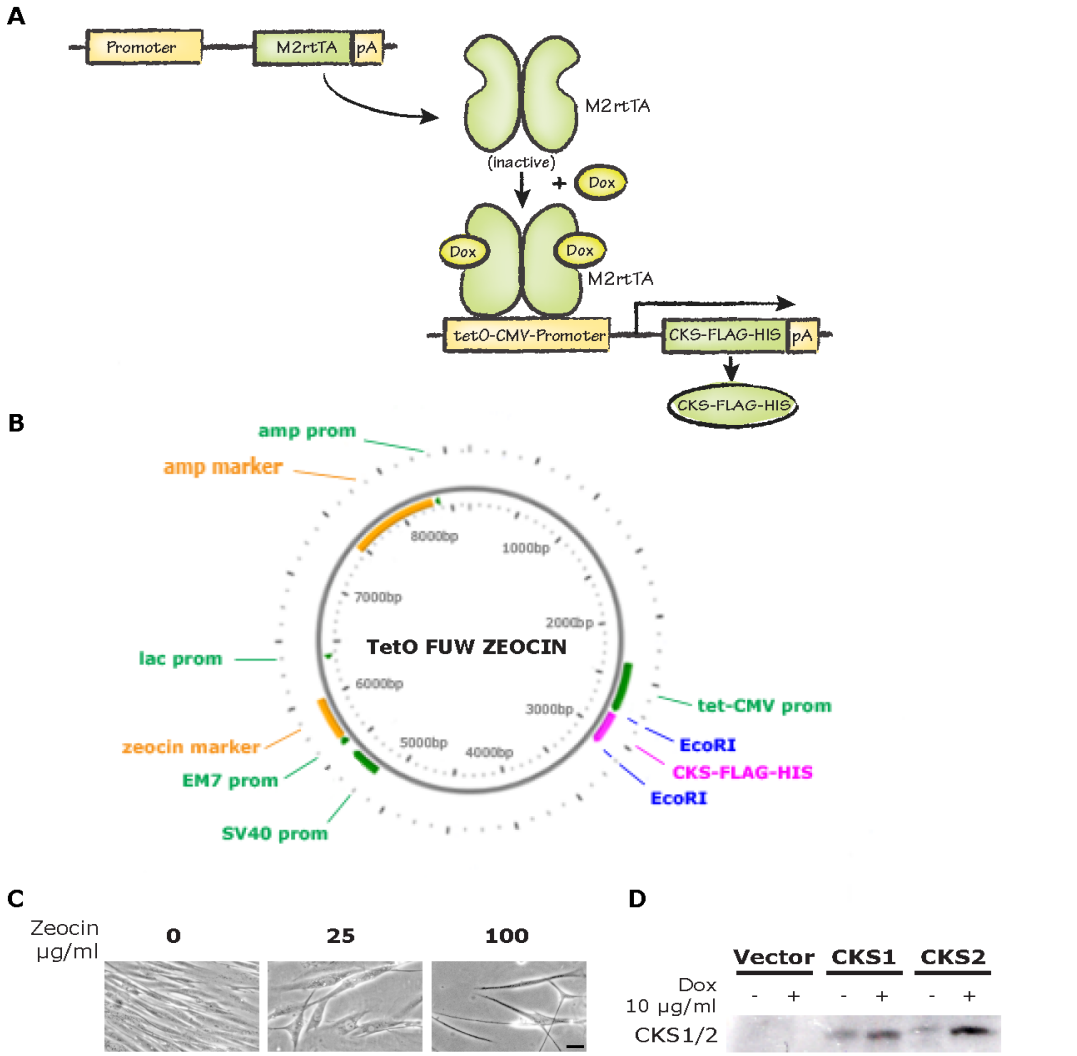


Figure 3.1 **Using the TetO-FUW vector with the zeocin selection marker for the induced expression of CKS proteins results in cell stress for IMR90 TERT cells.** (A) Model displaying the mechanism by which *CKS* is expressed using a Tet-On inducible expression system: the reverse transactivator M2rtTA is transcribed. In the presence of doxycycline, M2rtTA binds as homodimer to the tetO sequence of the TetO FUW vector and activates the transcription of the inserted CKS constructs via the CMV promoter. (B) Map depicting the TetO FUW ZEOCIN vector with inserted CKS constructs (total size: 8655 bp). (C) Brightfield images showing the phenotype of IMR90 TERT cells, which were cultured for 7 days in medium containing different concentrations of Zeocin. (D) Western blot showing the *CKS* expression in IMR90 TERT cells transduced with TetO FUW ZEOCIN CKS, selected and kept in medium containing a low concentration of Zeocin. CKS expression was assessed after adding doxycyline for 48 h.

3.2 pBABE sin tet PURO

In retroviral vectors, the expression cassette is flanked by the virus' long terminal repeats (LTR). In the corresponding virus, these 5' and 3' LTR embed the viral genome. They are necessary for the integration of the dsDNA, which results from the reverse transcription of viral RNA, into the host chromosome. They are also the control centre for viral gene expression and contain, for example, the viral enhancer and promoter sequences (Uren et al., 2005).

In Tet-On inducible retroviral expression vectors, activation of transcription occurs via the reverse activator binding to the tetO operator sequence. In the absence of doxycycline the reverse activator does not bind the operator sequence. Therefore transcription of the target protein is not activated via this promoter. Transcription can still occur to a certain degree via the LTR flanking the cassette. To inhibit this transcription, vectors were developed in which the enhancer and promoter region of the 3'-LTR are deleted. Upon transcription of the viral genome, reverse transcription of the resulting mRNA and integration of the DNA copy into the host cell genome, 5' and 3' LTR do not contain the promoter sequence anymore and the genes of interest have to be expressed from internal promoters. This deletion also minimises the possibility that cellular oncogenes in proximity to the integrated virus DNA could be transactivated. Vectors, in which the expression of genes is inactivated by the deletion of LTR regions are called self inactivating (sin) vectors (Yu et al., 1986).

To decrease the leaky expression of CKS proteins in the absence of doxycycline, pBABE sin tet PURO was chosen as retroviral expression vector. Here, the cloned *CKS* genes are under the control of the CMV promoter, which is in close proximity to the *tetO* operator sequence (Figure 3.2 A and B). A reverse activator activates *CKS* expression in the presence of doxycycline. To further inhibit residual leaky expression of the CKS proteins in the absence of doxycycline, cells were also transduced with a vector coding for a tetracycline controlled transcriptional silencer (tTRKRAB). The silencer is a fusion of a Tet repressor and the potent repressor domain KRAB (Krüppel associated box), derived from the human kidney protein Kid-1 (Freundlieb et al., 1999). In the resulting system, tTRKRAB binds to *tetO* in the absence of doxycycline and represses the activity of the adjacent CMV promoter. Conversely, in the presence of doxycycline the reverse activator M2rtTA engages with the *tetO* sites and the promoter is activated (Figure 3.2 A).

IMR90 TERT cells were first transduced with MSCV Hygro M2RtTA, followed by pLV-tTRKRAB and finally with the pBABE sin tet CKS PURO constructs. Each round of

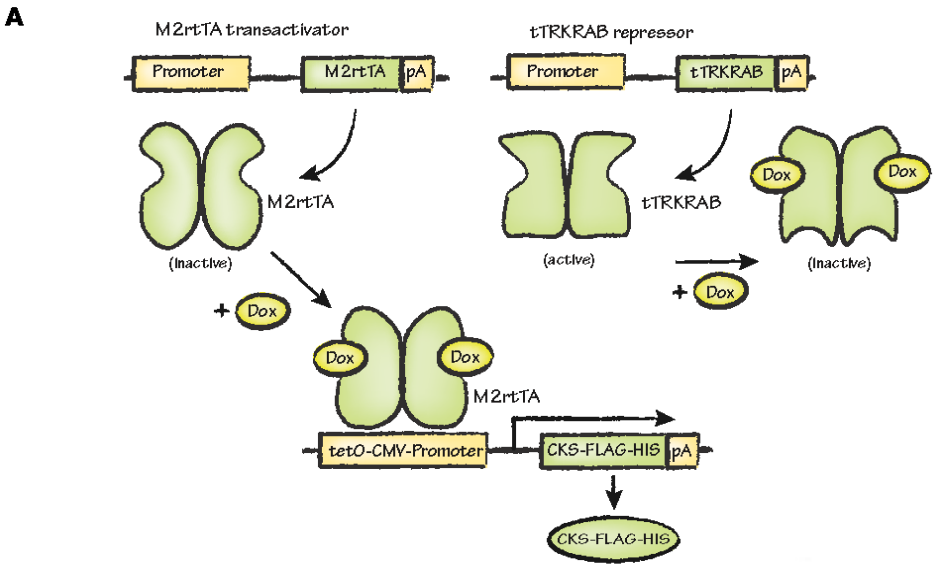
viral transduction was followed by selection of stably transduced cells with the selection agent to the transduction marker from the most recently added vector. This took place over a time period shown to allow for complete selection of stably transduced cells over cells that are not (table 1). The pBABE PURO vector is selected using puromycin, which allows cells to be selected relatively quickly and is in comparison to zeocin, well tolerated by cells bearing the resistance marker.

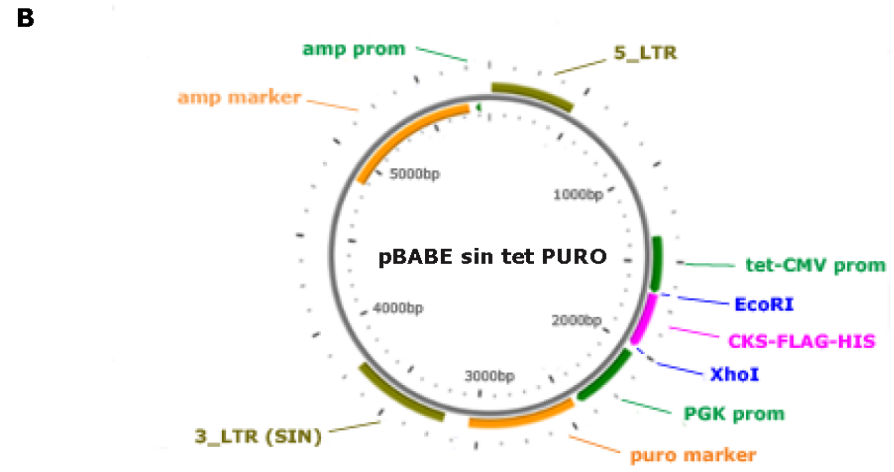
To test the efficiency of this inducible system, IMR90 TERT sin tet CKS cells were treated with increasing concentrations of doxycycline. After 48 hours cells were collected and *CKS* expression was evaluated by Western blot analysis (Figure 3.2 C). Almost no ectopic *CKS* expression was observed in the absence of doxycyline. In CKS1, CKS1E63Q and CKS2 IMR90 TERT cells 1 µg per ml of doxycycline led to a moderate expression of CKS proteins with CKS1 cells showing the most pronounced expression. The expression of protein increases with the doxycycline concentration, effectively peaking at a concentration of 4 µg per ml of doxycycline or higher. Cells transduced with *CKS2E63Q* bearing constructs had little or no expression of the protein even after induction with 8 µg of doxycycline. Thus pBABE sin tet CKS2E63Q PURO was not used for further analysis. As 4 µg of doxycycline induced peak protein expression all subsequent experiments were performed using this concentration for *CKS* induction. Next the ectopic *CKS* expression upon induction was observed over a period of five days. Doxycycline was added at timepoint 0 and cells were collected for protein isolation and analysis at 8 hour timepoints. At 48 and 96 hours the medium was changed and fresh medium with doxycycline added. Moderate *CKS* expression was observed at 16 hours post induction, with the level of *CKS* expression increasing with time until peaking at 48 hours (Figure 3.2 D). At the point that new media with doxycycline is added, there appeared to be a slight decrease in *CKS* expression, but levels were generally stable thereafter.

In IMR90 cells transduced with vectors for stable *CKS* expression, cell growth was impaired to a high degree (see Chapter 4). Next, I tested whether IMR90 TERT cells transduced with the pBABE sin tet CKS showed the same phenotype upon CKS induction as observed in the stably *CKS* expressing IMR90 cells. Therefore the proliferation of untreated IMR90 TERT cells was compared to IMR90 TERT cells, which were treated with doxycycline for the induction of *CKS* expression. Protein expression was induced for 48 hours and cells were plated for the various assays performed to determine proliferative capacity (Figure 3.2 E). A BrdU uptake assay did not show any difference in the amount of cells, which were actively proliferating after the induction of *CKS* expression. In a colony formation assay no difference in the proliferative potential of

CKS induced and non-induced cells could be observed as well. This was confirmed using 40 µg/ml instead of 4 µg/ml of doxycycline to induce *CKS* expression (Supplemental figure 7.1). Thus *CKS* induction in cells transduced with the inducible system did not lead to growth arrest.

Growth arrest is induced by checkpoint proteins upon the activation of oncogenes. To see whether checkpoints were activated in this setting, cells were treated with doxycycline for 5 days, fixed and stained for checkpoint proteins. Here $p21^{Cip1}$ was chosen as representative growth arrest inducing checkpoint protein (Figure 3.2 F). A large up-regulation of $p21^{Cip1}$ was observed in CKS induced cells. 30% of cells over-expressing *CKS2* and twice as many *CKS1* over-expressing cells were $p21^{Cip1}$ positive. In contrast almost no $p21^{Cip1}$ positive non-induced cells were observed. The discrepancy of induced expression of CKS proteins up-regulating $p21^{Cip1}$ checkpoint proteins but not resulting in growth arrest in IMR90 cells might be due to the process in which these cells were generated.

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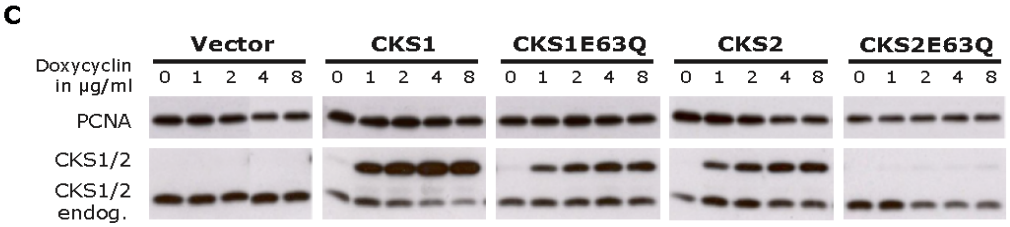
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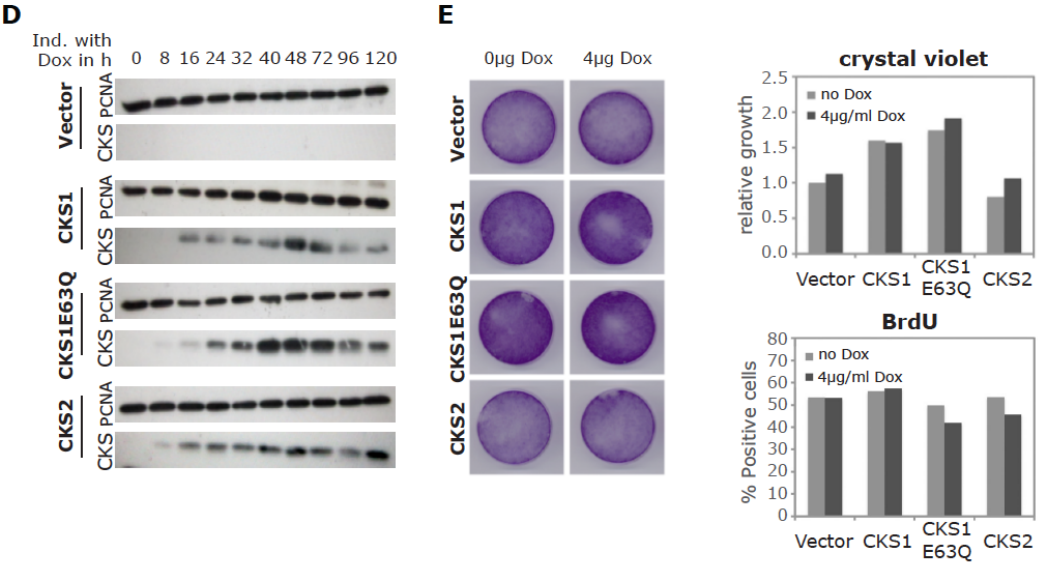
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% Positive cells

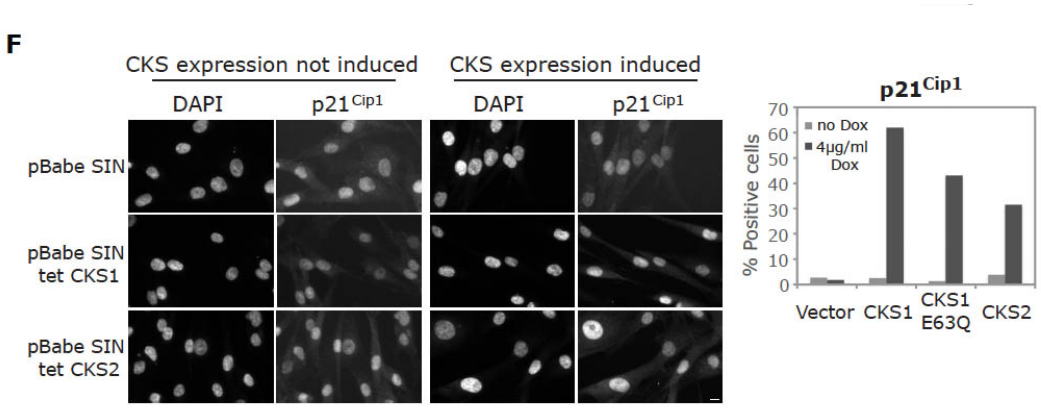

Figure 3.2 **Using pBABE sin tet PURO for the induced expression of CKS proteins in IMR90 TERT cells ensures a good expression of recombinant CKS proteins and results in p21^{Cip1} activation.** (A) Model showing the mechanism by which *CKS* is expressed using a Tet-On inducible expression system including a Tet reverse activator and a Tet repressor: In the absence of doxycycline the Tet repressor tTRKRAB would bind as homodimer to the *tetO* sequence of pBABE sin tet PURO whereas the reverse activator M2rtTA would not be able to bind. Thus the transcription of the tetO-CMV-promoter regulated gene is suppressed. When doxycycline binds to the activator as well as the repressor, both change their conformation. tTRKRAB is no longer able to bind to the tetO-CMV promoter of pBABE sin tet PURO and can not inhibit the transcription of the inserted CKS construct. Additionally binding of M2rtTA to the tet responsive promoter element activates the expression of *CKS*. (B) Map displaying the pBABE sin tet PURO vector with inserted CKS constructs (Total size: 5867 bp). (C, D) Western blot analyses showing the expression of CKS proteins in IMR90 TERT cells transduced with the pBABE sin tet CKS constructs. CKS expression was induced with increasing concentrations of doxycycline for 48 h (C). Additionally the *CKS* expression was monitored over a period of 120 h (D). For this purpose the medium containing 4 µg/ml of doxycycline was changed every 48 h. (E) The proliferative capacity of IMR90 TERT CKS cells was assessed via crystal violet staining and BrdU incorporation assay. pBABE sin tet CKS PURO transduced IMR90 TERT cells, in which *CKS* expression was induced and non-induced control cells were seeded at a low density and stained with crystal violet two weeks later. Images of stained cells (left) were acquired and the staining was quantitatively analysed by dissolving the crystal violet dye with acetic acid and measuring the absorbance as indicated in Material and Methods. The absorbance representing the non induced Empty Vector control was set as one (upper panel). To assess the percentage of actively replicating cells, IMR90 TERT CKS cells were incubated with BrdU overnight and stained with an anti BrdU antibody thereafter (lower panel) Images of BrdU positive cells were acquired using the IN Cell Analyzer 1000. The percentage of positive cells was assessed via high content analysis as described in Material and Methods. (F) To test for checkpoint activation by induced *CKS* expression, IMR90 TERT CKS cells were subjected to immunofluorescence using an antibody recognizing p21^{Cip1}. Left panels show representative images of cells subjected to immunofluorescence 5 days after CKS protein induction using 4 µg/ml of doxycycline or left untreated respectively. Percentage of positive cells was determined by high content analysis as described in the methods.

Primary IMR90 cells were first transduced with pWZL hTERT BLASTICIDIN, selected and expanded. This process was repeated with MSCV M2rtTA HYGRO, pLV tTRKRAB NEO and pBABE sin tet CKS PURO constructs. In each of these steps alterations in the cellular genome can lead to changes in the checkpoint pathways. Abrogation of a fully functional checkpoint pathway has been previously shown for hTERT transduced human fibroblasts. In one example, in VH25 hTERT fibroblasts p53 was shown to be up-regulated during later passages without cell cycle arrest (van Waarde-Verhagen et al., 2006).

To avoid possible alterations in the checkpoint pathways, the long process of consecutive rounds of stable transfection and subsequent selection was shortened to a minimum. To achieve that, primary IMR90 cells were directly cotransduced with the minimal amount of constructs necessary for induced *CKS* expression, namely MSCV M2rtTA HYGRO and pBABE sin tet CKS PURO. Following a selection process of three weeks, the resulting cells were treated in the same way as described before for IMR90 TERT sin tet CKS cells. After the induction with 4 µg/ml of doxycycline for 5 days, cells were subjected to immunofluorescence staining to determine their level of $p21^{Cip1}$ expression. No up-regulation of $p21^{Cip1}$ expression was observed in these fibroblasts following induced expression of *CKS1* or *CKS2*, contrasting with the induced $p21^{Cip1}$ expression shown previously in the IMR90 TERT sin tet CKS cells (Figure 3.3 A). Up-regulation of the checkpoint protein and transcriptional activator of $p21^{Cip1}$, p53 as well as cell cycle arrest could also not be observed (Supplemental figure 7.2). Western blot analysis showed a satisfactory up-regulation of CKS1 proteins after 48 hours of incubation in doxycycline containing medium (Figure 3.3 B). Up-regulation of CKS2 was lower than expected. Immunofluorescence staining with an anti-CKS antibody revealed however that only 45% of *CKS1* and 26% of *CKS2* transduced cells were positive for high CKS levels (Figure 3.3 C). It therefore appears that transducing the IMR90 cells with the various vectors of the inducible system resulted in a heterogeneous cell population, where despite all the transduced cells being resistant to the selection markers not all of the resulting cells could express CKS proteins when treated with doxycycline. This could be due to the silencing of the inserted CKS construct in some of the population. Heterogeneous induction of CKS expression may also have impacted on the cell proliferation studies reported here. It is possible that variable expression of CKS proteins resulted in variable expression of checkpoint proteins, such as $p21^{Cip1}$, amongst the whole cell population. In some cells, $p21^{Cip1}$ may have caused growth arrest, but this could have been masked by cells where CKS proteins were not expressed or expressed in insufficient quantities, allowing them to continue to proliferate.

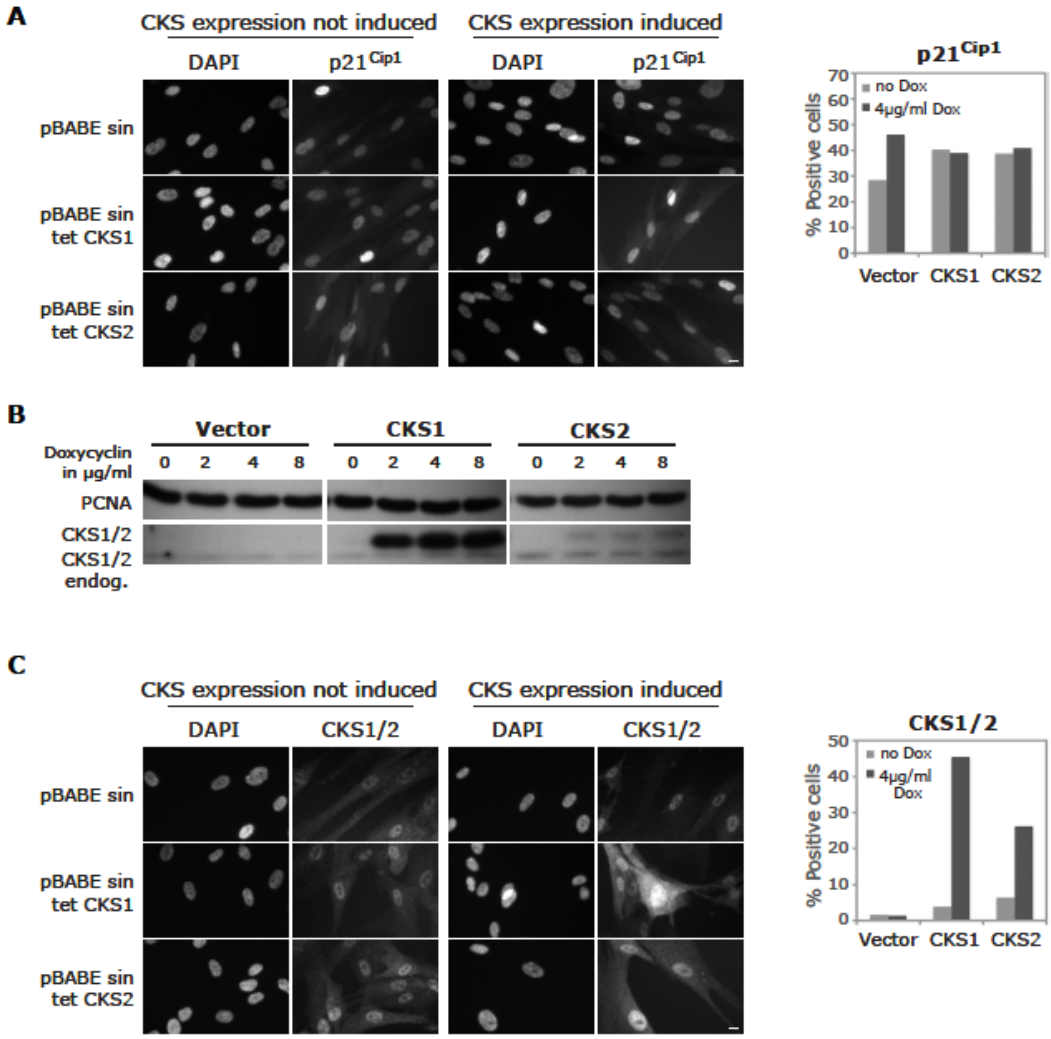

Figure 3.3 **Transduction of primary IMR90 fibroblasts with pBABE sin tet CKS results in a heterogeneous cell population regarding CKS expression.** (A) IMR90 cells transduced with pBABE sin tet CKS PURO were kept in medium either with or without doxycycline, fixed and stained for $p21^{Cip1}$ thereafter. (B) *CKS* over-expression in pBABE sin tet CKS PURO transduced IMR90 was confirmed by Western blot analysis after *CKS* expression was induced with different concentrations of doxycycline for 48 h. (C) The percentage of IMR90 CKS cells expressing a high level of *CKS1* or *CKS2* respectively was assessed by immunofluorescence staining with an antibody against both human paralogs of CKS. (A and C) Representative images of immunofluorescence stainings, which were performed 5 days after induction of the cells with 4 µg/ml of doxycycline, are shown in the left panel. Images were acquired with the IN Cell Analyzer 1000 and quantitatively evaluated using high content analysis as described in Material and Methods. Resulting graphs are shown in the right panel. Scale bar, 10 µm.

To avoid a heterogeneous population of IMR90 cells regarding the ability to express CKS proteins, possible genetic variations of the cells which might have occurred during the infection and selection process should be minimised. To obtain a homologues cell

population IMR90 TERT sin tet CKS1 cells were produced by transfecting IMR90 cells with the appropriate vectors and selecting for successfully transfected cells as described before. After the selection process cells were seeded at a very low density to enable the outgrowth of single clones containing cells with the same genetic background. Clones were picked and cultured for several weeks until cell numbers were sufficient to test for *CKS* induction. Cells were treated with doxycycline for 5 days and *CKS* induction was determined via immunofluorescence staining (Figure 3.4 A). None of the isolated four clones tested showed a homogeneous up-regulation of CKS proteins upon induction. In two clones no up-regulation of CKS proteins could be observed at all, while in the remaining two clones only a quarter or a third of the cells expressed high levels of CKS. No up-regulation of $p21^{Cip1}$ was observed in any of the four clones (Figure 3.4 B).

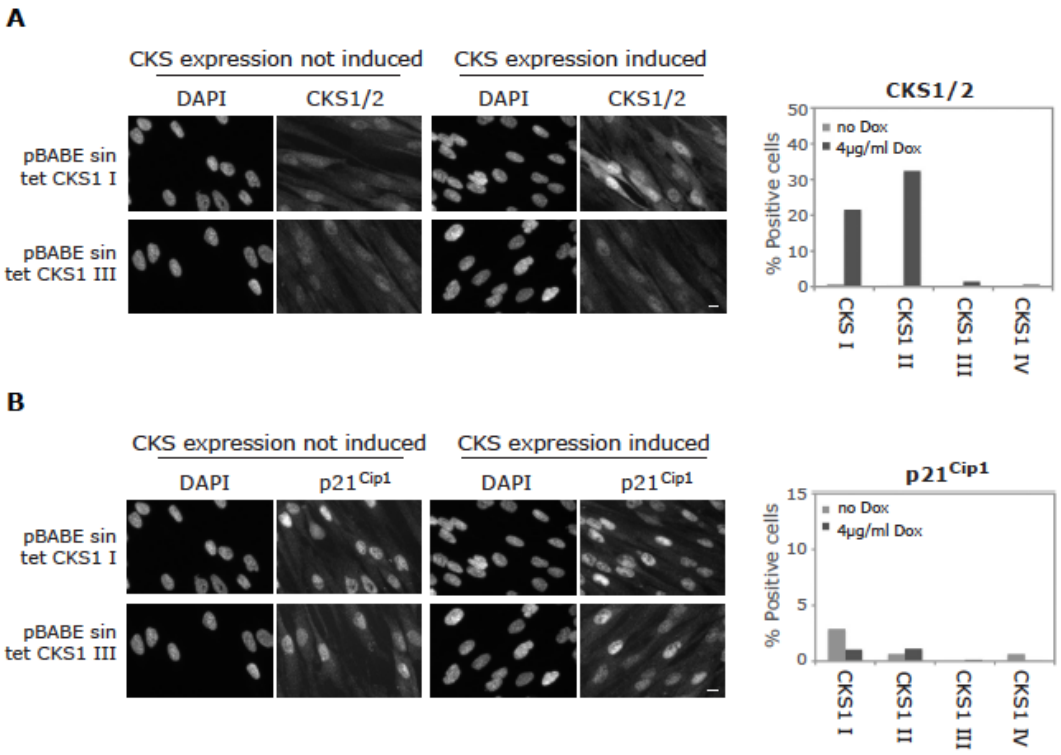

Figure 3.4 **Clones picked from pBABE sin tet CKS PURO transduced IMR90 TERT fibroblasts also give rise to heterogeneous populations.** IMR90 TERT cells were virally transfected with the CKS1 bearing inducible constructs and selected with puromycin. Clones were picked and cultured. The resulting cell lines (CKS I, II, III and IV), were kept in medium containing no or 4 µg/ml of doxycycline for 5 days, fixed and objected to immunofluorescence staining with antibodies recognising CKS (A) and $p21^{Cip1}$ (B). Representative images are shown in the left panel. Graphs showing the percentage of CKS or $p21^{Cip1}$ positive cells respectively are given in the right panel. Intensity thresholds determining positive cells were set and percentage of positive cells determined using the IN Cell Analyzer 1000 software as described in Material and Methods. Scale bar, 10 µm.

Previously, the ability of induced CKS proteins to stimulate $p21^{Cip1}$ checkpoint protein expression was analysed as a percentage of the total population of cells. However, it is possible that the relationship between CKS protein expression and checkpoint protein expression might be stronger in the subset of the heterogeneous cells that strongly express the CKS proteins when induced. To examine this, cells were treated with doxycycline for five days, fixed and double stained for CKS and $p21^{Cip1}$ (Figure 3.5 A). Approximately one third of fibroblasts showed an up-regulation of CKS1. Whereas only 18% of the cells in the total cell population were $p21^{Cip1}$ positive (upper right panel), 32% of the cells with up-regulated CKS1 levels were positive for $p21^{Cip1}$ (lower right panel). In contrast only 11% of cells were $p21^{Cip1}$ positive in the CKS negative subset. 15% of all IMR90 TERT cells transduced with the pBABE sin tet CKS2 vector and treated with doxycycline were positive for $p21^{Cip1}$. In the CKS2 positive subset, 32% of the cells and in the CKS2 negative subset only 10% of the cells showed an up-regulation of $p21^{Cip1}$. Additionally, levels of p53, a checkpoint protein acting upstream of $p21^{Cip1}$, were investigated (Figure 3.5 B). After CKS induction in IMR90 TERT sin tet CKS1 cells, 37% of the cells were p53 positive. The proportion of p53 positive cells was 48% in the CKS1 positive subset and 24% in the CKS1 negative. In IMR90 TERT sin tet CKS2 cells 30% of all cells were p53 positive. Almost half of the CKS2 positive cells were p53 positive whereas in only 14% of the CKS2 negative cells the p53 pathway was activated. This result indicates that the cells, which were transduced with the vectors of the inducible system and in which CKS is strongly expressed, act as anticipated and were more likely to express both $p21^{Cip1}$ and p53 checkpoint proteins. Using a method, which enables the identification of the subset of cells high in CKS, would make it possible to study the effect of high levels of CKS proteins in a heterogeneous population. In conclusion, high levels of CKS expression result in an increase of $p21^{Cip1}$ and p53 expression. The high-content image analysis method that I developed and describe here, allows for the first time to assess the role of CKS1 in tumour progression at a single-cell level. This technique overcomes problems with cell population heterogeneity and addresses these questions in a physiological context, i.e. primary fibroblasts. It remains to be shown how hTERT expression – which was necessary to achieve the described inducible expression – contributes to the observed phenotypes. One possibility is that expression of hTERT overcomes CKS-induced growth arrest.

In the following chapters, a possible oncogenic effect of CKS proteins in IMR90 fibroblasts is studied in more detail. This includes the influence of CKS proteins on growth and cell cycle of the whole cell population. Therefore it was important to

perform growth analyses using a homologous population rather than a subset of a heterogeneous population. Thus the formation of a heterogeneous cell population regarding CKS levels should be avoided. The approach used to prevent this was the use of IMR90 fibroblasts shortly after they were stably transfected with pBABE CKS constructs and express *CKS* in a continuous manner.

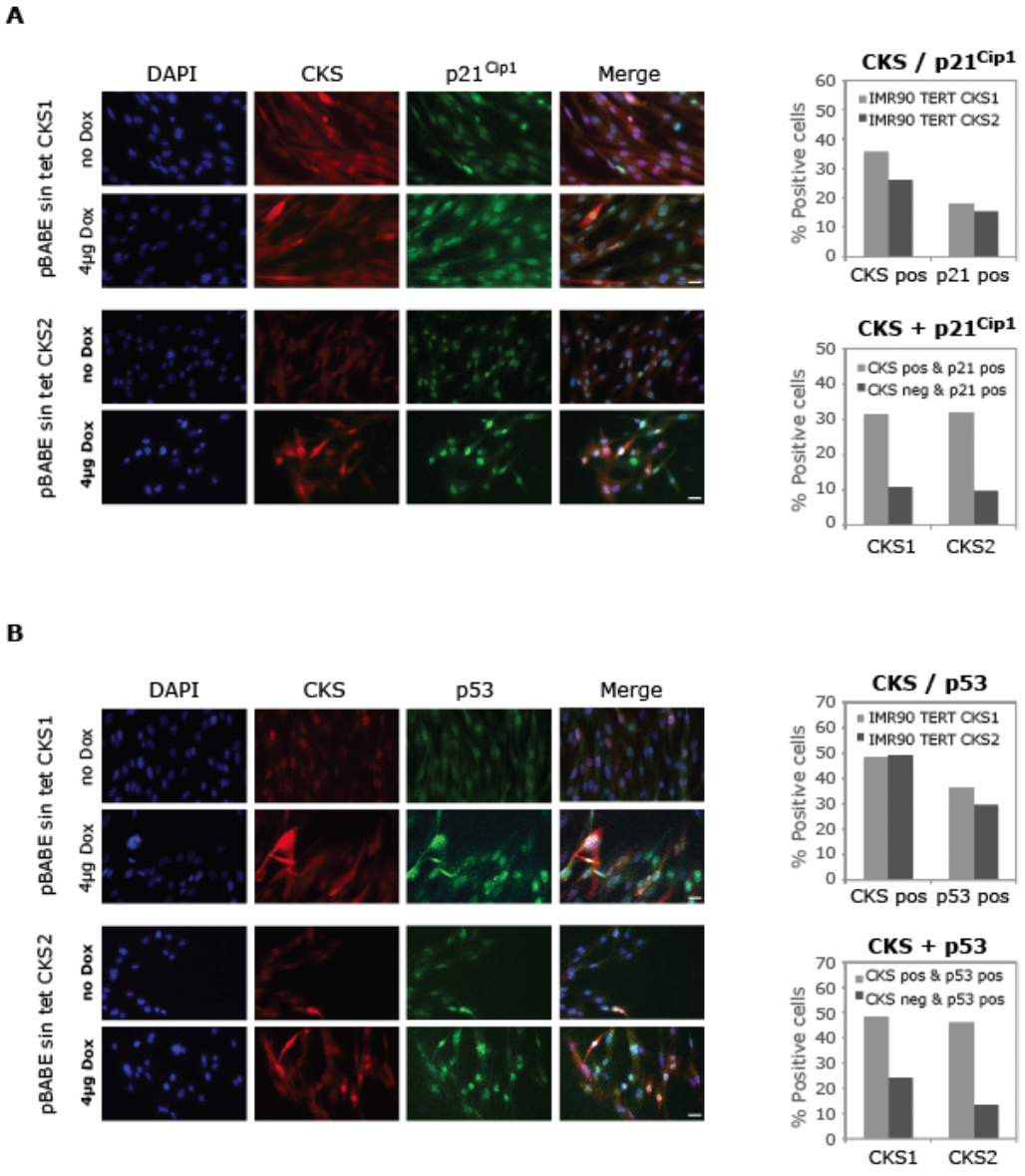

Figure 3.5 **The population of IMR90 TERT cells expressing high levels of *CKS* upon induction with doxycycline displays a higher percentage of cells positive for checkpoint proteins.** After the induction of *CKS* expression in IMR90 TERT CKS cells for 5 days with 4 µg/ml of doxycycline, cells were fixed and stained for CKS (Alexa Fluor 594) and $p21^{Cip1}$ (Alexa Fluor 488) (A) or CKS (Alexa Fluor 594) and p53 (Alexa Fluor 488) respectively (B). The percentage of CKS positive cells as well as $p21^{Cip1}$ or p53 positive cells was identified using high content analysis as described in Material and Methods and presented in the according upper right panel. Additionally, the percentage of cells double positive for CKS and $p21^{Cip1}$ or CKS and p53 as well as of cells negative for CKS but positive for $p21^{Cip1}$ or p53 was determined (lower right panel). Scale bar, 10 µm.

3.3 Discussion

To study the effect of *CKS* over-expression in normal fibroblasts a system for the regulated expression of CKS proteins was to be established. The aim was to create an IMR90 fibroblast cell line in which the expression of CKS proteins could be induced within a short period of time. This would facilitate the analysis of immediate and direct effects of high levels of CKS proteins in primary fibroblasts. Initial analysis performed with fibroblasts constitutively expressing CKS proteins revealed that their expression impairs cell growth. Thus, a constant expression might not be tolerated and, if possible, evaded by the cells (counterselection). This process occurs when the protein of choice is expressed during the process of selection after viral transduction with the vectors coding for the respective protein as well as for the selection marker and could be circumvented when the protein would be not present or inactive during the selection and culturing process. A more practical advantage of an inducible system is that cells can be expanded to the number needed in experiments after introducing the inducible vector and transfection of a huge number of cells with stable expression vectors can be avoided.

Here a system was chosen, in which the treatment of cells with the tetracycline derivate doxycycline induces the expression of CKS proteins. This Tet-On inducible system is based on the activation of transcription. Doxycycline binds to and changes the conformation of the activator M2rtTA, which thereafter is able to bind the tetracycline responsive element within the promoter regulating the expression of the desired gene. Both, M2rtTA activator and the actual construct containing the *CKS* gene under the control of the tetracycline responsive element containing CMV promoter were introduced into IMR90 fibroblasts by transduction. This had the advantage that the delivery efficiency is high, the desired gene is directly integrated into the genome and thus long outgrowth periods of single clones are not necessary. The inducible Tet-On vector (TetO-FUW) used initially in hTERT immortalised IMR90 fibroblasts, carries a zeocin selection marker and cells had to be selected for approximately three weeks. The long selection process is a probable reason for the stressed phenotype of the IMR90 TERT cells. Zeocin binds and cleaves DNA and has been described to be toxic to cells even when they do express the resistance marker Sh ble, which binds to zeocin and prevents it from binding to the DNA (Oliva-Trastoy et al., 2005). The suboptimal selection process might also have favoured the basal expression of ectopic CKS proteins, which was observed in IMR90 TERT TetO FUW CKS ZEOCIN cells before the induction with doxycycline. A rather long period of selection and DNA damage could have led to

the alteration of the regulatory element responsible for transcription of the CKS proteins. In a next step toxicity and leakiness was reduced by replacing the TetO FUW CKS ZEOCIN vector by the pBABE sin tet CKS PURO vector, which contains a puromycin selection marker. Puromycin, which inhibits protein translation, acts quicker on cells that do not bear the selection marker unlike zeocin. Thus long periods of cells in culture, which would allow ageing and alterations of the genome, are avoided. Furthermore cells bearing the resistance gene are viable and retain intact DNA. Also I replaced the vector with one that contains self-inactivating long terminal repeats (LTR). Thus the LTR cannot act as promoter after the integration of the construct into the genome of the transduced cells reducing the leakiness of the system. To further inhibit basal expression a repressor was inserted, which inhibited transcription of the introduced CKS construct in the absence of doxycycline. Using these constructs in IMR90 TERT cells, background expression of ectopic CKS proteins was successfully reduced. After induction with doxycycline *CKS* over-expression was satisfactory in all but the IMR90 TERT cells transfected with pBABE sin tet CKS2E63Q PURO. In comparison to CKS2, CKS2E63Q has a reduced ability to bind to CDK. Sequencing verified the accuracy of the CKS2E63Q construct (Material and Methods). A possible reason for the very low *CKS2E63Q* expression could be a possible toxicity of the protein, which might lead to its down-regulation. Quantitative real time PCR revealed that the variant of *CKS2* was over-expressed but at lower levels than CKS2 in pBABE CKS2 PURO transduced cells (see chapter 5). A lower transcription rate might be due to epigenetic suppression of CKS2E63Q.

Additionally later analysis revealed that treatment of the cells with a proteasome inhibitor led to a moderate up-regulation of CKS2E63Q (see chapter 5). This supports the idea that CKS2E63Q might have a toxic effect on cells, which should be discussed later in this thesis. Further investigations into the suitability of the pBABE sin tet PURO vector were concentrating on the over-expression of the wild-type forms of *CKS1* and *2*. Upon induction of *CKS 1* and *2* expression, $p21^{Cip1}$ was up-regulated in IMR90 TERT cells. Unexpectedly however the proliferation of *CKS* induced IMR90 TERT cells was not impaired as seen in stably *CKS* expressing fibroblasts. Initially the immortalisation of IMR90 cells by the introduction of a telomerase function as well as long periods in culture and selection were assumed to be the underlying reasons. Each transfection step requires a selection process, which implicates the occurrence of stress in the cells and endangers their genomic integrity. This might lead to changes in tumour suppressor genes and insufficient checkpoint activation upon the activation of oncogenic stress. Thus the transfection process was shortened leaving out hTERT and the repressor

tTRKRAB. Introducing this minimal inducible system into primary IMR90 cells however did not even lead to a noticeable up-regulation of $p21^{Cip1}$, ruling out the possibility that solely alterations in checkpoint proteins might have led to the inability of the cells to activate pathways leading to growth arrest. Using immunofluorescence to detect CKS positive cells on a single cell level, a probable reason for the insufficient checkpoint activation was identified. Only a subset of the cells transduced and viable in selection medium, do express CKS proteins. In this subset however $p21^{Cip1}$ expression is indeed elevated. Since it is assumed that CKS proteins act as oncogenes and apparently lead to checkpoint activation in non cancerous fibroblasts it can be suspected that the outgrowth of cells with lower levels of *CKS* is favoured. Additionally, cells expressing higher levels might die. Another possibility might be that CKS proteins are degraded by cellular mechanisms. It is to say that CKS proteins are regulated in a cell cycle dependent fashion. This might lead to low levels of CKS proteins in early G1 cells although *CKS* is under the control of the constitutively active CMV promoter in the inducible constructs. In situ hybridization could show, whether transcription of *CKS* is already impaired in the cells lacking CKS proteins. Furthermore a proteasome inhibitor could be used to see whether CKS proteins are degraded to a high extent.

The generated cell line for the induced expression of CKS proteins will be valuable for further studies on early and late effects of *CKS* over-expression despite its inhomogeneity. By carefully choosing the expression vectors regarding a well tolerated selection marker and a tight expression control mechanism the cell line generated grows normally and appears morphologically consistent with the parental cell in the absence of doxycycline. Additionally the inducible cell line shows good levels of CKS protein expression upon induction with doxycycline. I developed a method, which makes it possible to study the impact of CKS proteins on a single cell level. To analyse the effect, which *CKS* over-expression has on a particular protein of interest, a marker for both, the target proteins and CKS proteins can be used simultaneously. These analyses can be easily performed using imaging techniques. Additionally flow cytometry and cell cycle analyses can be performed in a similar way.

For techniques looking at the behaviour of the overall cell population however, a homogenous cell population would be required. The attempt to obtain such a population by picking single clone colonies of pBABE sin tet CKS1 PURO vector transduced IMR90 TERT fibroblasts was not successful in the first instance. To further optimise the inducible system regarding homogenous levels of CKS protein upon induction of expression, a higher number of clones could be selected and analysed. Other systems of regulated expression could be tried out alternatively. Fusion of *CKS* to

the ligand binding domain of the estrogen receptor (ER) might be one possibility (Littlewood et al., 1995). Here CKS would be kept bound to the chaperone in the cytoplasm until 4-hydroxytamoxifen (4OHT) is added. 4OHT would bind to the ER part of the fusion protein, which leads to a conformational change so that the protein is released. Since the proteins are already expressed at the point of treatment with 4OHT, the up-regulation of available CKS proteins upon induction is assumable quicker than in transcription regulated systems. It still needs to be explored whether the constant expression of CKS proteins does not lead to unwanted available CKS even without 4OHT treatment. Another possibility of induced expression would be the use of the Cre-lox system. Here the *CKS* gene would be under the control of a constitutive promoter. The transcription however is inhibited by the insertion of a "stop" sequence consisting of several stop codons. This sequence is flanked by Lox P sites. After the activation of a Cre recombinase these Lox P sites are recombined, leading to the excision of the stop sequence (Dale and Ow, 1991). Only then *CKS* would be transcribed. The cre-recombinase itself could be controlled by a heat shock factor (HSF) regulated promoter (Pelham, 1982). Here incubation of the cells at a higher temperature would lead to the trimerisation of HSF and its binding to the promoter, which activates transcription. The stop sequence should prevent the expression of CKS in the absence of the Cre recombinase completely. How tightly the induction of the recombinase can be controlled would need to be investigated.

Chapter 4 – High levels of CKS proteins lead to growth arrest, which can be bypassed by the inhibition of checkpoint proteins

4.1 Response to CKS over-expression in primary human fibroblasts

Oncogenes such as *RAS*, are known to induce different checkpoint pathways in the cell, which result in specific cellular phenotypes (Bihani et al., 2004). I analysed the effect of over-expression of the potential oncogene *CKS* in the primary fibroblast cell line IMR90. It was tested whether a phenotype can be observed in the cells, which is similar to the one known after oncogene over-expression.

In order to study the effect of CKS over-expression on cell growth and checkpoint arrest, a retroviral system was applied to introduce stable expression of the *CKS* genes. The pBABE PURO vector, containing the virus LTR as a promoter, was used as retroviral backbone vector. Genes cloned into the vector are thus expressed constitutively. For the selection of infected cells the pBABE vector carries a puromycin resistance gene. Only cells, which integrate the vector DNA into their genome, survive the selection with puromycin. *CKS1*, *CKS2* and their non-CDK binding mutants *CKS1E63Q* and *CKS2E63Q* were cloned into pBABE PURO (Figure 4.1 A). IMR90 cells were infected with the resulting retroviral vectors to over-express the CKS proteins. Cells were selected for one week and plated for analyses thereafter (Figure 4.2 B).

Correct expression of CKS proteins was assayed by Western blot analysis (Figure 4.1 B). *CKS* over-expression was satisfactory for all *CKS* variants except for *CKS2E63Q*.

To ensure that lower protein expression is not due to imperfections in the CKS2E63Q vectors, both, the pBABE PURO as well as the pBABE sin tet CKS2E63Q PURO vector were sequenced (Figure 2.1). Since no irregularities could be detected, the low expression of *CKS2E63Q* is most likely to be explained by a lower stability of the protein, a higher sensitivity of cells in response to the protein or a mechanism of the cells to down-regulate the gene. The lower levels of expression were therefore always considered when results obtained from the *CKS2E63Q* expressing cells were analysed.

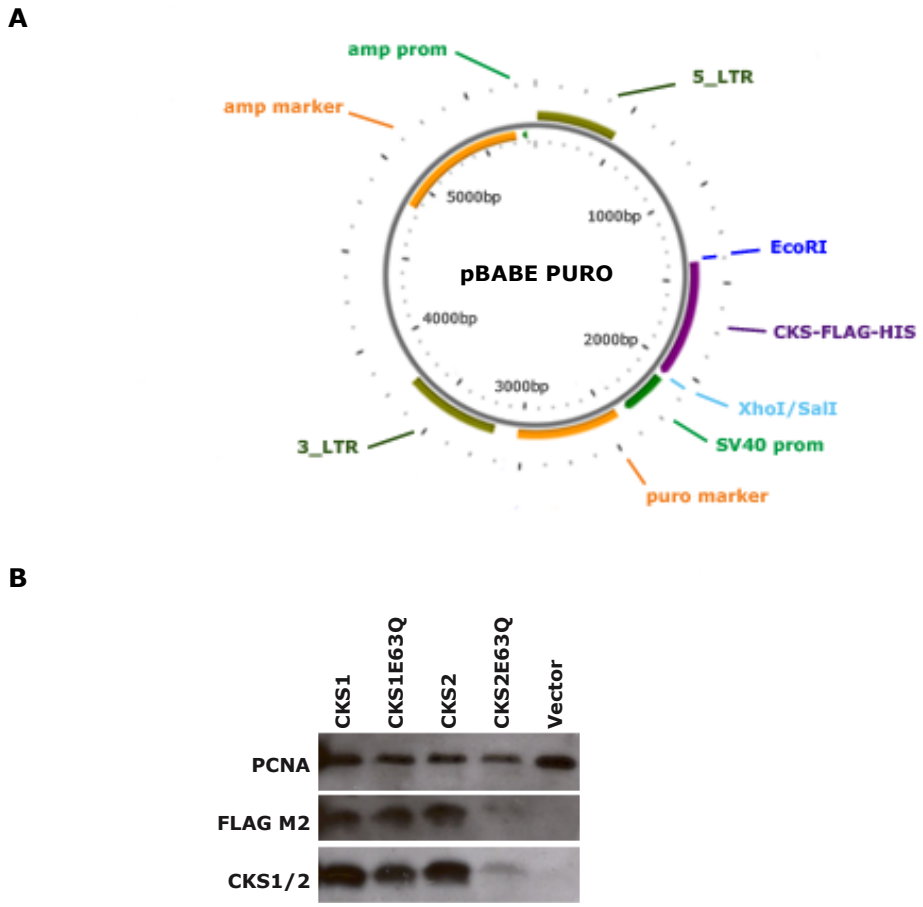

Figure 4.1 **pBABE CKS PURO vector for the expression of the different CKS proteins.** (A) The sequences encoding for each of the *CKS* variants *CKS1*, *CKS1E63Q*, *CKS2* or *CKS2E63Q*, which were FLAG and His tagged, were cloned separately into the retroviral vector pBABE. The vector contains a puromycin resistance (total size of the vector: 5845 bp). (B) The expression of the indicated *CKS* variants in IMR90 was confirmed by Western blot analysis. IMR90 fibroblasts were transduced with the appropriate pBabe CKS PURO vector and selected for puromycin resistance for seven days. Cells transduced with an empty vector were used as control. Cell extracts were made and proteins separated by SDS PAGE. CKS proteins were detected using antibodies either against the FLAG tag or an antibody that recognises both CKS1 and CKS2. Ectopic *CKS* expression was satisfactory in all but the IMR90 CKS2E63Q cells.

4.1.1 *CKS* transduced IMR90 cells show features of cellular senescence

After transduction with the pBABE CKS PURO vectors, possible oncogenic effects of CKS were studied using a simplified cancer development model (Figure 4.2 A). The starting point for this model is unaltered primary IMR90 cells. In the first step *CKS* over-expression was established in these unaltered primary fibroblasts. If CKS proteins had

the expected oncogenic effect on cells, tumour suppressor pathways would be activated in these checkpoint competent cells. Pathway activation would result for example in apoptosis or growth arrest like senescence. Growth arrest that is caused by oncogenes can be bypassed by disrupting the pathway, which counteracts the oncogenic effect (Braig and Schmitt, 2006; Hahn et al., 1999). To see whether this was the case in *CKS* over-expressing IMR90 cells, the two important tumour suppressor genes, *p53* and *RB*, were down-regulated by introducing the human papilloma virus proteins E6 and E7. The effect of *CKS* over-expression on proliferation in these checkpoint-inhibited cells was assessed and the ability of CKS proteins to transform these cells was analysed (Figure 4.2 A).

For the initial analyses in primary cells, IMR90 human fibroblasts were transduced with an empty vector control or with the *CKS* bearing constructs. As a tumour suppressor pathway inducing control, oncogenic *RAS (HRASG12V)* was over-expressed. *HRASG12V* expression is known to result in senescence in human and mouse cells (Serrano et al., 1997). Three days after transduction with CKS and control constructs, cells were selected by adding puromycin for approximately 7 days and subsequently analysed regarding checkpoint activation and proliferative capacity (Figure 4.2 B).

As evaluated by colony formation assay and BrdU incorporation assay, *CKS* expression caused a decrease in the number and growth of IMR90 cells. For the colony formation assay, cells were plated at a low confluence (around 10%) and cultured for approximately 14 days. Cells were fixed and stained with the DNA dye crystal violet (Figure 4.2 C). As expected, in the negative control panel (labelled, Vector), cells proliferated to a high extent. Introduction of the positive control oncoprotein, HRASG12V, inhibited proliferation of these cells almost completely. Wild-type CKS1 similarly inhibited proliferation, as did CKS2 and the E63Q variants. The BrdU incorporation assay confirmed a decrease in proliferation since less *CKS* over-expressing cells were able to take up BrdU, indicating a lower number of actively replicating cells. When compared to the number of BrdU incorporating empty vector cells (set as 1) only a fraction of CKS1 (0.67) and CKS2 (0.57) positive cells was able to replicate and enter S-phase. The effect of both E63Q variants (0.76 for CKS1E63Q and 0.85 for CKS2E63Q) does not seem to be as strong as the one of the wild-type CKS proteins (Figure 4.2 D). As described before oncogenic *RAS* over-expression led to the highest inhibition of replication in IMR90 fibroblasts (0.38).

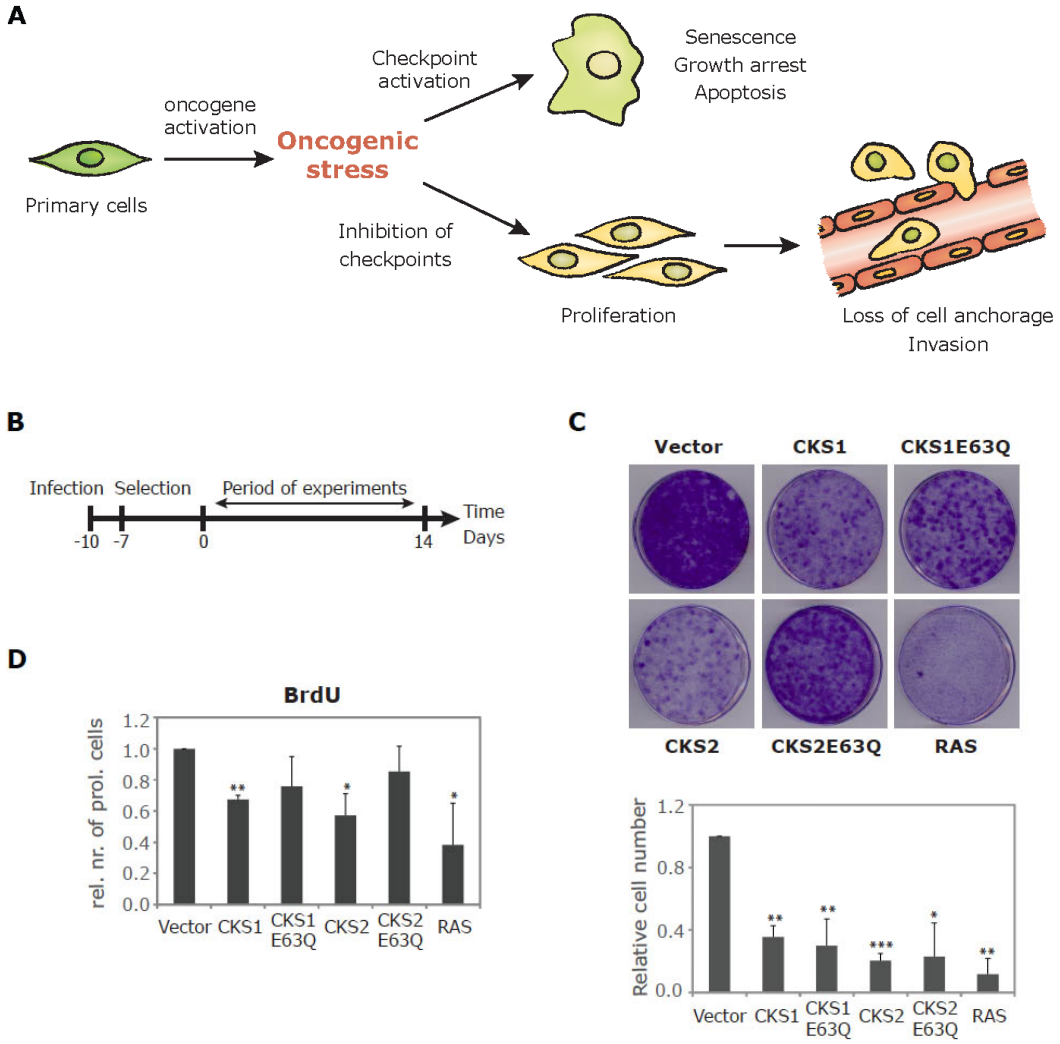

Figure 4.2 **Over-expression of CKS proteins induces growth arrest in IMR90 fibroblasts.** (A) Model displaying the approach used to test the oncogenic effect of CKS proteins: Primary IMR90 fibroblasts are subjected to *CKS* as well as control oncogene over-expression. Oncogene activation causes the activation of checkpoint proteins, which might result in senescence or apoptosis. Inhibition of the responsible checkpoint proteins consequently leads to the alleviation of the suppressive phenotype and favours the transformation of primary cells towards cancer cells. (B) Timeline of the experiments presented in figure 4.2, figure 4.3 and figure 4.4. IMR90 fibroblasts were transduced with the retroviral vectors bearing the sequences coding for CKS1, CKS1E63Q, CKS2, CKS2E63Q or the control oncogene *RAS*. 3 days after transduction, cells were selected for 7 days and subsequently seeded for experiments. (C) Quantification of BrdU incorporation in IMR90 cells transduced with CKS constructs and the RAS oncogene control. Cells were incubated with 50 µM BrdU over night and stained with an anti BrdU antibody. Images were acquired using the IN Cell Analyzer 1000 and the percentage of BrdU positive cells determined using high content analysis as described in Material and Methods. The percentage of BrdU positive empty vector transduced control cells was set as one. (D) Images and quantification of crystal violet staining in the indicated IMR90 CKS cells. Cells were seeded at a low density and stained with crystal violet two weeks later. For quantification the dye was solubilised using 10% acetic acid and the absorbance of the resulting solution measured at 595 nm. The absorbance of the empty vector control was set as one. Images correspond to one

representative experiment (n=3). Error bars in C and D correspond to three independent experiments. An unpaired *t*-test was performed to test significance of the down-regulation of proliferation in CKS or RAS over-expressing IMR90 cells in comparison with control vector transduced cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

The *RAS* oncogene is known to arrest cells and cause a senescent phenotype when over-expressed in primary cells in a process known as oncogene-induced senescence (Serrano et al., 1997). Senescent cells typically have a flat and enlarged appearance. Thus I monitored cell growth and analysed the morphology of *CKS* over-expressing IMR90 fibroblasts regarding these phenotypical characteristics of senescence (Figure 4.3 A). IMR90 cells bearing the control vector were rather small. *CKS* transduced cells displayed the morphology that resembled that of senescent cells. Additionally *HRASG12V* expressing cells often displayed a high number of vacuoles. To further investigate whether the CKS induced growth arrest showed any other characteristics of senescence, cells were stained for senescence-associated β-galactosidase activity (SA-β-Gal), which is a widely used marker for the identification of senescent cells (Dimri et al., 1995). 95% of the IMR90 cells, which were infected with *HRASG12V* displayed SA β-gal activity. *CKS* over-expression still led to an elevated β-galactosidase activity in around three-quarter of the transduced cells.

Additionally the presence of senescence associated heterochromatic foci (SAHF) was determined. SAHF were detectable in more than twice as many *CKS* over-expressing cells (CKS1: 44.9%; CKS1E63Q: 43.4%; CKS2: 41.7%) than in the vector control cells (17.5%). In the less efficiently expressing *CKS2E63Q* cells SAHF formation could be observed in 32.5%. Together these results suggest that *CKS* over-expression induces senescence in human diploid fibroblasts with intact tumour suppressor pathways.

4.1.2 Levels of senescence mediators during CKS induced senescence

To identify the pathways responsible for senescence induced by high levels of CKS proteins, the expression of different senescence effectors was analysed by immunofluorescence staining followed by high content analysis. Two important regulators of senescence, p53 and its downstream effector $p21^{Cip1}$, were up-regulated in *CKS* transduced cells (Figure 4.4 A and B). Whereas only 8% of empty vector control cells were positive for p53, above 60% of cells expressing one of the *CKS* variants showed elevated levels of this crucial initiator of senescence. $p21^{Cip1}$ levels were up-regulated as well but were more variable between experiments.

The other pathway leading to cell cycle arrest and senescence is regulated by $p16^{INK4a}$. $p16^{INK4a}$ however was only up-regulated in the *HRASG12V* oncogene control cells (Figure 4.4 C). Thus, senescence as a result of *CKS* over-expression is mainly triggered by the p53 pathway.

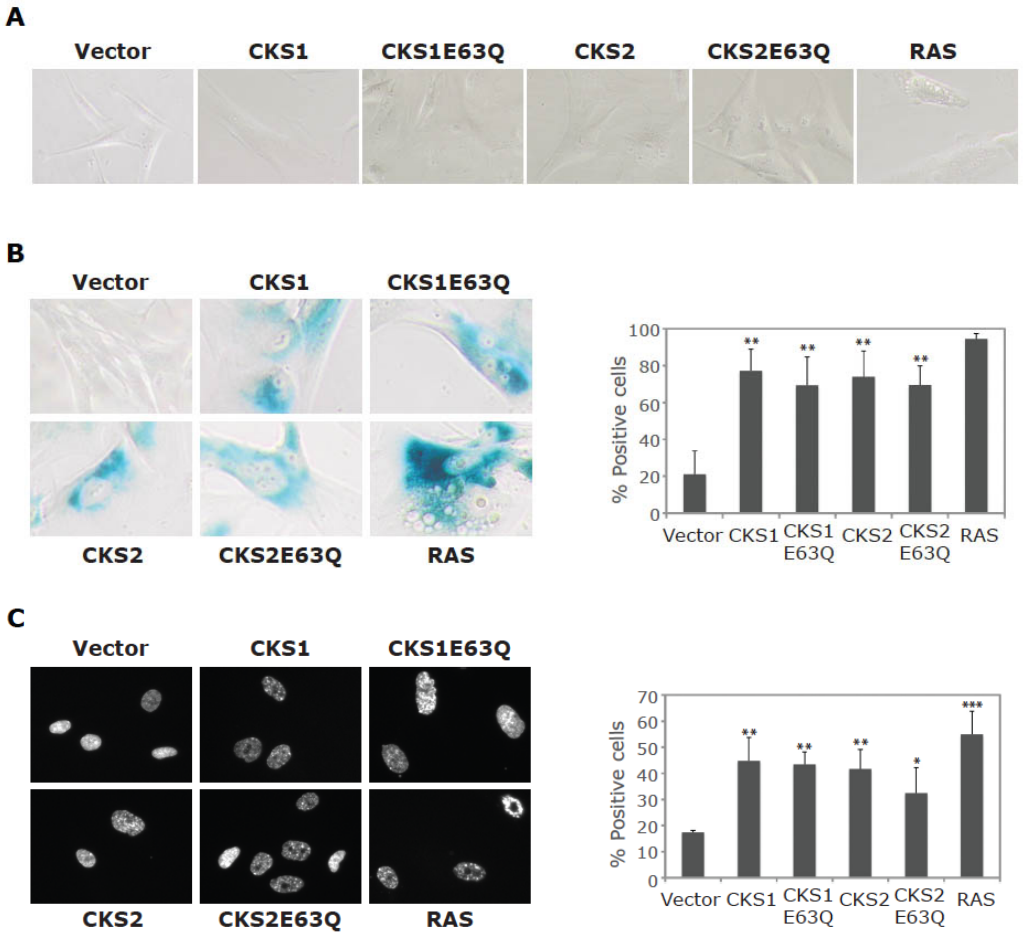

Figure 4.3 **IMR90 cells over-expressing CKS proteins present characteristics of senescence.** (A) Bright field images showing the morphology of primary IMR90 fibroblasts transduced with the empty vector control, CKS constructs and RAS control oncogene after being kept in puromycin containing medium for 10 days. (B) Representative images and quantitative analysis of stainings for elevated senescence-associated β-galactosidase activity (SA β-Gal) in IMR90 cells transduced with the indicated vectors. Transduced cells were selected for seven days, fixed with glutaraldehyde and stained with X-gal solution as described in Material and Methods. For the quantification approximately 200 cells were counted in each of three independent experiments. Scale bar, 30 µm. (C) Images display DAPI stainings of virally transduced IMR90 fibroblasts illustrating the formation of senescence-associated heterochromatic foci (SAHF) in *CKS* and *RAS* over-expressing cells. Quantification of SAHF was performed for three independent experiments using IN Cell Analyser 1000 and the IN Cell Investigator software. Areas of DAPI stained nuclei with high intensity were identified using a minimal area of 0.5 µm. Nuclei containing more than 5 high intensity areas were scored as positive. An unpaired *t*-test was performed to test significance of the up-regulation of senescence markers in *CKS* or *RAS* over-expressing IMR90 cells in comparison with control vector transduced cells (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

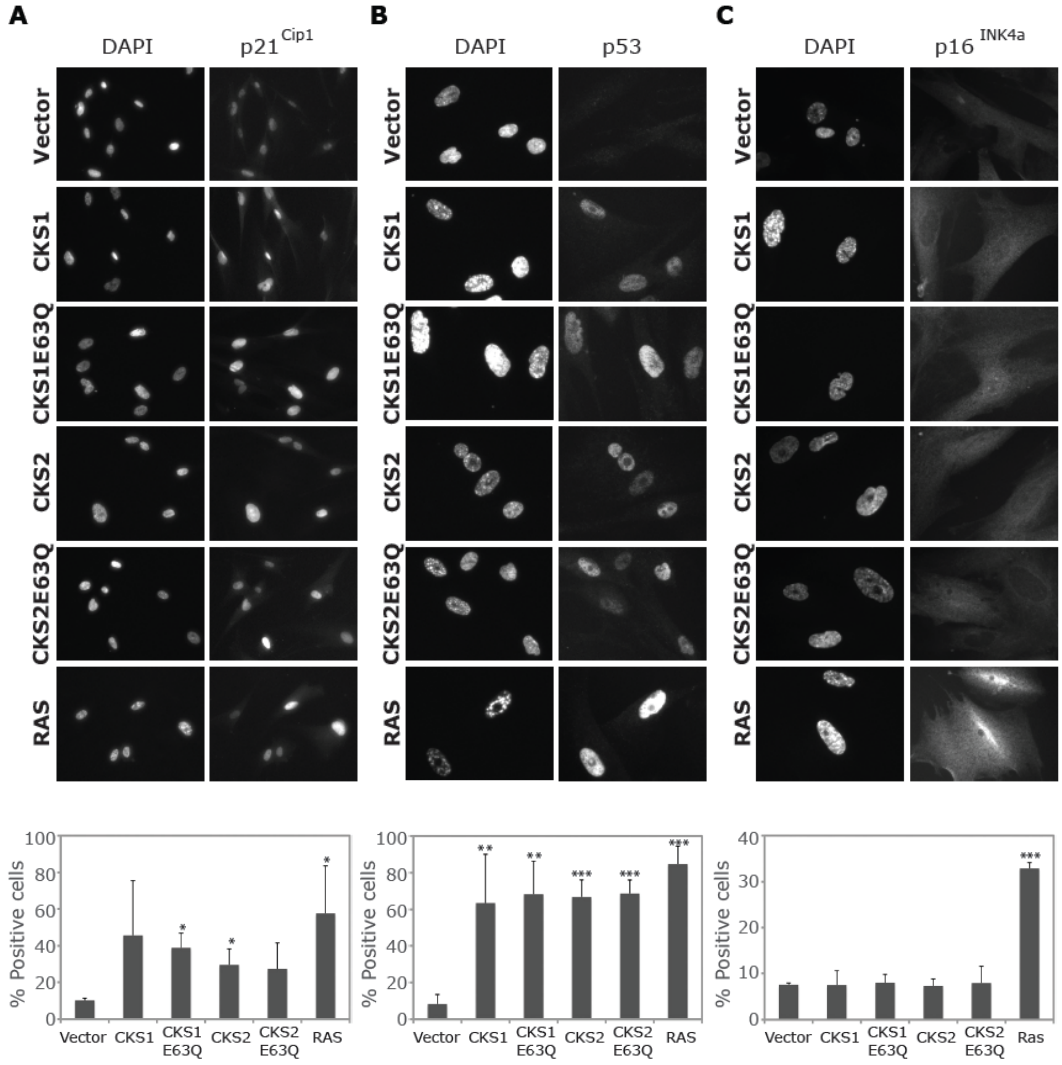

Figure 4.4 **The p53/p21^{Cip1} pathway is activated upon *CKS* over-expression.** (A-C) p21^{Cip1}, p53 and p16^{INK4a} stainings of IMR90 fibroblasts transduced with the empty vector control, CKS constructs and RAS control after seven days of selection. Images were acquired using the IN Cell Analyzer 1000 (upper panels). Percentage of positive cells was determined by high content analysis as described in the methods (lower panels). Error bars correspond to three independent experiments. An unpaired *t*-test was performed to test significance of the up-regulation of p21^{Cip1}, p53 and p16^{INK4a} in *CKS* or *RAS* over-expressing IMR90 cells in comparison with control vector transduced cells (*P < 0.05; **P < 0.01; ***P < 0.001).

4.2 Response to *CKS* over-expression in human fibroblasts with inhibited tumour suppressor proteins

4.2.1 Inhibition of checkpoint proteins alleviates growth arrest in IMR90 cells

Growth arrest, which is caused by oncogene activation, can be bypassed by disrupting the pathway, which counteracts the oncogenic effect (Braig and Schmitt, 2006; Hahn et al., 1999). To identify whether this is the case for the growth arrest observed in *CKS*

over-expressing cells, regulators of senescence typically activated by oncogenes were down-regulated in IMR90 cells.

IMR90 cells enter a state called replicative senescence after about 58 population doublings (Ouellette et al., 1999). This is caused by telomere shortening. To avoid the onset of replicative senescence in an experimental setting, telomerase activity was induced using a viral vector coding for human telomerase reverse transcriptase (hTERT) (Lee et al., 2004). The resulting IMR90 TERT cell line was now immortalised and able to replicate independently of telomere shortening. As the immunofluorescence analysis suggested that p53 is one of the up-regulated tumour suppressors in *CKS* over-expressing IMR90, p53 as well as one of its downstream regulators, RB, were down-regulated in the IMR90 TERT cells. Down-regulation of these tumour suppressors was achieved by the infection of the IMR90 TERT fibroblasts with retroviral vectors coding for E6 and E7 (Figure 4.5 A). E6 and E7 are human papillomavirus oncoproteins, which bind and inactivate p53 and RB respectively. Upon tumour suppressor down-regulation, *CKS* over-expressing cells were able to resume growth (Figure 4.5 B,C). Cells do not show a senescent morphology anymore and show no impairment of growth in the colony formation assay. These results suggest that high levels of CKS lead to a growth arrest induced by the activation of tumour suppressor genes in primary IMR90 cells. Down-regulation of these tumour suppressor genes lifts the growth inhibition.

4.2.2 Transforming ability of CKS

In cancer cells, p53, RB or upstream and downstream regulators of these tumour suppressors are commonly impaired. In this setting oncogenes are able to unfold their whole transformational potential. To see whether CKS proteins are able to transform IMR90 E6 E7 TERT cells into cancer-like cells, I looked for the presence of typical markers of transformation. First, the effect of CKS proteins on proliferation was assessed. I analysed the growth of *CKS* over-expressing primary IMR90 fibroblasts and checkpoint-inhibited IMR90 E6 E7 TERT cells (Figure 4.6 A). Growth curves were obtained by measuring the relative cell number on every second day over the course of twelve days for IMR90 cells and eight days for IMR90 E6 E7 TERT cells. The number of empty vector control cells on the last day of the time course was set as one. In IMR90 cells, proliferation slowed down moderately upon up-regulation of all *CKS* variants. The proliferation rate of the single CKS variant expressing IMR90 fibroblast does not differ significantly from each other. Only cells transduced with pBABE CKS2 PURO showed an even more pronounced growth inhibition than the others. In comparison to the IMR90

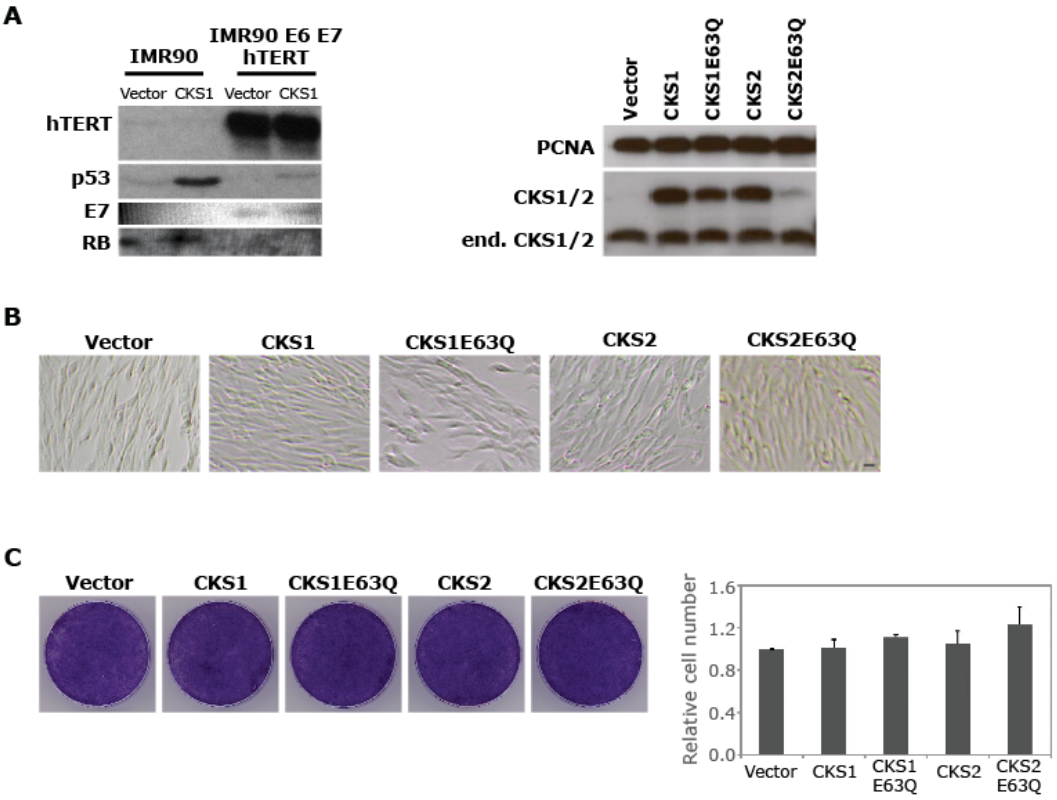

Figure 4.5 **Inhibition of checkpoint proteins alleviates the growth arrest in *CKS* over-expressing IMR90**. Fibroblasts were transduced subsequently with pWZL hTERT BLAST, LXSN E6 E7 NEO and pBABE CKS PURO, selected for the appropriate amount of time and plated for the particular experiments. (A) Western blot analyses showing the expression of hTERT and E7 as well as the down-regulation of p53 and RB in IMR90 E6 E7 hTERT cells transduced with the vector control or pBABE CKS PURO (left panel). Additionally ectopic *CKS* expression was checked in IMR90 E6 E7 hTERT cells after they have been transduced with the appropriate pBABE CKS PURO vector and selected for 7 days (right panel). (B) Brightfield images were taken of IMR90 E6 E7 hTERT CKS cells growing in culture. Scale bar, 100 µm. (C) To assess the proliferative capacity of IMR90 E6 E7 hTERT CKS cells, they were plated at a low density and stained with crystal violet 14 days later. Representative images were taken (n=3) and the staining quantitatively analysed as described in Material and Methods. Error bars correspond to 3 independent experiments.

fibroblasts, *CKS* over-expression in IMR90 E6 E7 TERT cells led to a slightly increased growth compared to the vector control. Again, the CKS variants among one another do not differ significantly.

A more prominent characteristic of cancer cells than an increased proliferative capacity is the ability of cells to exhibit anchorage independent growth (Jove and Hanafusa, 1987). I tested whether CKS proteins are able to induce anchorage independent growth in IMR90 TERT E6 E7 cells using a soft agar assay for colony formation (Figure 4.6 B). In this assay, the *CKS* over-expressing cells were seeded in a viscous agar and cultured for 3 weeks. Colony formation was observed for IMR90 TERT

E6 E7 cells which expressed the oncogenes *small T* (Yu et al., 2001), *HRASG12V* and *c-MYC*. Cells over-expressing the *CKS* variants were not able to form colonies. Therefore *CKS* expression in this background leads to a proliferative advantage but is not sufficient to induce anchorage independent growth.

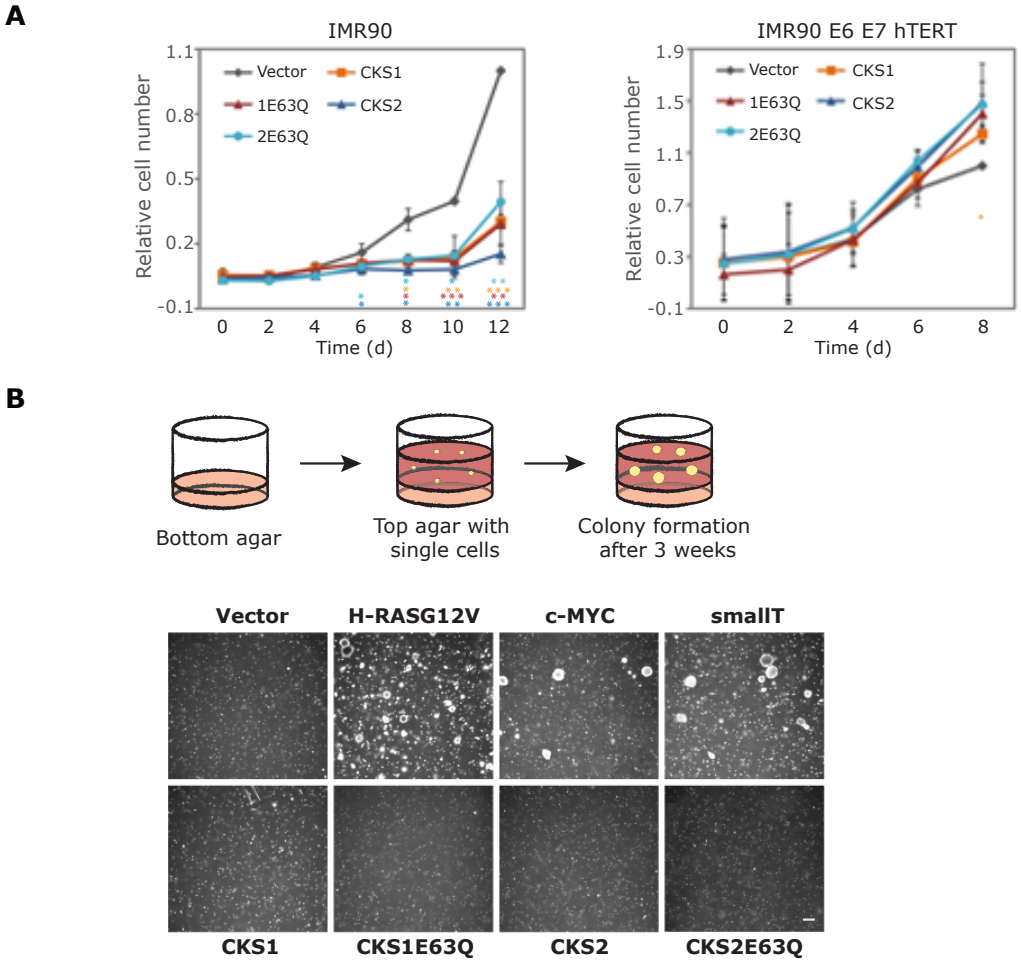

Figure 4.6 **Transforming ability of CKS**. (A) The proliferative capacity of *CKS* over-expressing IMR90 (left panel) and IMR90 E6 E7 hTERT fibroblasts (right panel) was compared after both cell lines were transduced with the indicated CKS constructs and selected for seven days. Growth curves were obtained by seeding the transduced cells in duplicates in 24 well plates. One plate was fixed every two days and stained with crystal violet at the end of the time course. The staining was quantified as described in Material and Methods. The relative cell number corresponds to the absorbance at each time point relative to the absorbance of the empty vector control at the end of the time course. Error bars correspond to two independent experiments. Levels of significance (unpaired *t*-test) for the altered proliferation of CKS over-expressing cells in relation to the vector control transduced cells are illustrated by * for $P < 0.05$, ** for $P < 0.01$ and *** for $P < 0.001$. (B) A colony formation assay was performed with *CKS* and control oncogene over-expressing IMR90 E6 E7 hTERT cells. A bottom agar was poured into six well plates and a top agar was added containing the transfected IMR90 E6 E7 hTERT cells. Cells were incubated for three weeks before assessing colony formation (schematic diagram is shown in upper panel). Colonies were only observed in the fibroblasts over-expressing the control oncogenes H-RAS, c-MYC and small T. Brightfield images shown are representative of two independent experiments. Scale bar, 1 mm.

4.3 Discussion

Previously an oncogenic effect of CKS proteins was predicted since they were reported to be over-expressed in a variety of cancers. So far, studies were mostly restricted to gene and protein analysis of tissue samples from cancer patients or *in vitro* and *in vivo* assays with cancer cells showing a lower proliferation rate when inhibiting *CKS* expression. In a recent study, *Cks1* was over-expressed in primary B cells derived from a bone marrow transplant in mice. Here *Cks* over-expressing B cells did not lead to any increase in proliferation or showed any other kind of transformational change (Kratzat et al., 2012). Therefore the first objective of this study was to investigate whether it was only the lack of the cell cycle protein CKS, which gave cancerous cells from previous studies a proliferative disadvantage or whether CKS itself could contribute to the transformation of primary cells and act as an oncogene. For this purpose cancer development was mimicked commencing with primary IMR90 fibroblasts and finishing with immortalised p53 and RB inhibited cells. In this background *CKS* over-expression was imposed on the cells and the ability of CKS to transform the cells was assessed. Previously the effect of the *RAS* oncogene was studied in this kind of setting. Whereas RAS leads to oncogene induced senescence in primary cells, it leads to transformation in immortalised p53 and RB inhibited cells (Hahn et al., 1999; Hahn and Weinberg, 2002). Nevertheless attention needs to be paid, since different cells and different oncogenes behave in different ways. Using young IMR90 fibroblasts, a well-established cell line in the field of oncogene studies, facilitated the interpretation of results. In these cells *CKS* over-expression led to reduced growth as well as characteristics of oncogene induced senescence like senescence-associated β-galactosidase and senescence-associated heterochromatic foci formation. Thus *CKS* seems to act like a typical oncogene by inducing oncogenic stress and senescence in cells with intact checkpoints.

The control oncogene *HRASG12V* showed the most pronounced features of senescence in all sets of experiments performed. HRASG12V is a mutated form of the HRAS protein, often found in skin cancer. Its mutation leads to a low GTPase activity and a strong ability to bind GTP (Pylayeva-Gupta et al., 2011). These changes lead to a constitutively active HRAS. It acts as an oncogene in several ways. It promotes proliferation by acting on a variety of pathways leading to the activation of growth factor signalling cascades, up-regulation of growth factors themselves, inhibition of TGFβ and cyclin dependent kinase inhibitors and the stimulation of cyclin D1 expression (Pylayeva-Gupta et al., 2011). These proliferative signals alone result in a high level of replicative stress and thus a strong activation of checkpoint proteins. CKS

however might only influence proliferation by direct interaction with cell cycle proteins and their regulators and therefor induces checkpoint pathways to a moderate but less pronounced degree than active RAS does.

In contrast to RAS, CKS proteins did not activate the $p16^{INK4a}$ pathway. As part of the RAS-MAP kinase pathway, RAS leads to the up-regulation of a variety of transcription factors including for example the Ets family of transcription factors. Ets1 and Ets2 in turn increase transcription of $p16^{INK4a}$ (Rayess et al., 2012). It was also shown that oncogenic RAS leads to an up-regulation of the JMJD3 demethylase, which in turn contributes to the epigenetic control of the *INK4a/ARF* locus (Barradas et al., 2009). p53 again is mainly activated by replicative stress and DNA damage, which activates the ATM/ATR pathways (Mallette and Ferbeyre, 2007). This would support the idea that CKS proteins activate oncogene induced senescence by their direct influence on the cell cycle rather than having an impact on growth factor signalling pathways which lead to the up-regulation of transcription factors.

In the assays performed to show lower proliferation and onset of senescence in *CKS* over-expressing cells, the *E63Q* variants of *CKS1* and *2* showed a slightly but not significantly weaker reaction. CKS binding to CDK2 was shown to be reduced by approximately 80% in the E63Q variants compared to the wild-type proteins (Watson et al., 1996). A slightly less pronounced ability to induce oncogene induced senescence in *CKSE63Q* over-expressing IMR90 fibroblasts might implicate a role of CKS CDK interaction in an oncogenic function of CKS. That the difference between mutant and wild-type proteins is not very distinctive might be due to the amount of mutant CKS still interacting with CDK. On the other hand it might be possible that CDK dependent and independent functions lead to the induction of senescence.

As seen before in the attempt to establish an inducible system for *CKS* expression in IMR90 fibroblasts, the expression of *CKS2E63Q* was to a high degree less pronounced when compared to all other *CKS* variants. Apart from the induction of senescence associated heterochromatic foci however the *CKS2E63Q* expressing cells hardly differed from *CKS1E63Q* expressing cells. Thus the lower expression is sufficient to induce the senescent phenotype in the primary IMR90 cells. CKS2E63Q might even have a stronger impact on the cells than the CKS2 wild-type protein has, leading to its rapid degradation. This could be the case when a CDK independent function of CKS has a strong effect on the cells. Not being able to bind CDK, more CKS2 would be available to conduct this task. This might be a function similar to the one of CKS1 in the SCF^{Skp2} ubiquitin ligase, which is involved in the degradation of the CDK2 inhibitor $p27^{Kip1}$ (Spruck et al., 2001). Loss of $p27^{Kip1}$ then leads to an up-regulation of CDK2 activity and

consequently replicative stress. Another possibility would be that CKS2E63Q has a dominant negative effect on a function of CDK2. For example cyclin A is ubiquitinated by the APC/C with the help of CKS. Here cyclin A binds to CDK2 which is in a complex with CKS and CKS in turn binds via its anion binding site to the phosphorylated APC complex and thus brings cyclin A into close proximity to the ubiquitin ligase APC/C (Di Fiore and Pines, 2010). When CKS is not able to bind CDK but APC this CKS variant might block the access to the APC for functional CKS-CDK-cyclin A complexes. This would lead to excessive cyclin A, replicative stress and checkpoint activation, which the cells might want to evade by degrading this CKS variant. In this particular case a CKS variant with CDK and anion binding site mutation would give the answer to whether a function in which CKS acts as adaptor protein between CDK complexes and phosphoproteins is disrupted by the E63Q mutation.

Following the observation that *CKS* over-expression leads to a senescent phenotype, which is accompanied by high p53 levels, the p53 pathway should be switched off, to see whether growth arrest can be circumvented. p53 itself and RB, the negative transcriptional regulator downstream of p53 and $p16^{INK4a}$, have been down-regulated in my setting. When both main checkpoint proteins involved in the onset of senescence were not functional, *CKS* over-expression did not lead to a growth arrest in the fibroblasts. Therefore it can be assumed that the up-regulation of p53 was at least one of the main reasons for arresting IMR90 cells with high CKS levels. Checkpoint inhibited IMR90 TERT CKS cells showed a slightly increased growth rate in comparison to the control cells. In a transformation assay, which examined the ability of the cells to grow anchorage independent, *CKS* over-expression did not lead to the acquisition of this feature. Whether the slight growth advantage is the only reason leading to p53 activation would need to be investigated. A migration assay for example could reveal whether CKS might allow the p53 and RB negative cells to migrate.

That CKS proteins were not able to transform the cells completely is not surprising since the development of cancer is a multistep process and was simplified in the model used. An oncogene like *RAS*, which acts on multiple pathways, is able to transform cells completely in this setting. Other oncogenes, like possibly *CKS*, have more restricted actions, like cell cycle progression or the ability to invade other tissues. Here a variety of other changes have to take place for a cell to become cancerous.

Chapter 5 – Molecular analyses of the mechanism underlying checkpoint activation in *CKS* over-expressing IMR90 fibroblasts

5.1 CDK2 activity in *CKS* over-expressing senescent cells

Analysis of *CKS* over-expression in primary IMR90 cells showed that high CKS levels cause senescence and growth arrest. p53 up-regulation was proposed to be the underlying reason. This was confirmed when *CKS* over-expressing cells resumed growth upon down-regulation of p53 and its downstream effector RB. Cells, in which inhibition of the p53 pathway was introduced, actually had a slightly higher proliferative capacity after *CKS* over-expression. As part of the G1/S-phase checkpoint, p53 is often activated by replicative stress which causes stalled or collapsed replication forks. Replicative stress might be due to cell cycle deregulation and incorrect regulation of replication (Halazonetis et al., 2008). In my next set of experiments I wanted to investigate, whether this is the reason for checkpoint activation and p53 up-regulation in primary IMR90 cells. Progression through the cell cycle and activation of specific checkpoints were examined by determining protein levels of cell cycle regulators in *CKS* over-expressing fibroblasts. Additionally, the activity of CDK2 was analysed. CDK2 is the cyclin dependent kinase initiating and regulating replication (Halazonetis et al., 2008). CDK2 was also reported to interact with and be kept active by CKS proteins (Bourne et al., 1996; Liberal et al., 2011).

Regulation of replication was investigated in *CKS* over-expressing IMR90 cells as follows. IMR90 fibroblasts were transduced with retroviral vectors bearing CKS or empty vector controls as described in Material and Methods. After seven days of selection, proteins were isolated for Western blot analysis. Protein lysates at this time point were also used for the isolation of the cyclin A/CDK2/CKS complex by immunoprecipitation (IP) using cyclin A antibodies. The success of the IP was verified by checking the presence of CDK2 in the immunoprecipitated complex (Figure 5.1 A). For this purpose the complexes immunoprecipitated from empty vector control cells and *CKS1* and *CKS2* over-expressing cells were separated by SDS page and analysed by Western blot analysis using a CDK2 antibody. These complexes were also used in kinase activity assays with Histone 1 (H1) as a substrate. These kinase assays should provide information about what effect *CKS* over-expression has on CDK2 activity. *CKS1* and *CKS2* over-expressing cells showed a reduced kinase activity in comparison to empty vector control cells (Figure 5.1 B). Ectopic *CKS2* expression led to a slight decrease in CDK2

activity (P = 0.28, t-test). The CDK2 activity in *CKS1* over-expressing fibroblasts was even more affected with a significant reduction of activity of 40% (P = 0.04, t-test). Western blot analyses revealed that p53 and $p21^{Cip1}$ were up-regulated in these IMR90 cells upon *CKS* over-expression for one week (Figure 5.1 C). This confirms the data obtained by immunofluorescence staining (Figure 4.4). Therefore, a lower CDK2 activity could be due to CDK2 inhibition by the checkpoint proteins p53 and $p21^{Cip1}$ and a general G1 arrest. To verify this, the kinase assay was repeated with CDK2 complexes immunoprecipitated using an anti-cyclin E antibody. Cyclin E activates CDK2 during G1/S transition (Woo and Poon, 2003) and an observed inhibition of the kinase complex in cells with high CKS levels could indicate a general problem in the progression from G1 to S-phase.

Results obtained with cyclin E-CDK2 complexes were variable (Figure 5.1 B). CKS over-expression led to a non-significant decrease in CDK2 activity (P_{CKS1} = 0.19; P_{CKS2} = 0.8, t-test) The kinase isolated from *CKS1* over-expressing cells was moderately less active than the one from control cells. *CKS2* over-expressing cells showed a 5% reduction in CDK2-cyclin E activity. The lower activity of CDK2 in complex with cyclin A and the slightly less pronounced cyclin E associated CDK2 activity support the idea that *CKS* over-expression in primary fibroblasts leads to checkpoint activation and consequently G1 arrest in primary fibroblasts.

5.2 A short period of *CKS* over-expression leads to the activation of the G1/S-phase checkpoint in primary fibroblasts

In a next step it was investigated whether checkpoint activation and CDK2 inhibition is set early after initiation of *CKS* over-expression or only after extended periods of over-expression. For these studies analysis was performed the day after transduction of the IMR90 cells with the CKS1 and 2 constructs. The analysis of various cell cycle regulators pointed out that one day after expression of ectopic CKS1 and CKS2, p53 was already considerably elevated (Figure 5.2 A). $p21^{Cip1}$ also showed a slight up-regulation. These results indicate that expression of CKS proteins leads to an acute checkpoint activation in primary IMR90 cells. p53 and $p21^{Cip1}$ are effector proteins of the G1/S-phase as well as intra S-phase checkpoints, which impair the progression from early stages of the cell cycle through S- and towards G2- and M-phase (Bartek and Lukas, 2001; Gartel et al., 1996). This is in accordance with a slight elevation of cyclin D1, a G1 phase cyclin, that I have observed in these studies in *CKS1* and *2* over-expressing IMR90 cells (Figure 5.2 A). Cyclin B1 in contrast, an important cyclin during late S and G2 phase, was slightly

decreased. No change could be observed in the overall amount of the S-phase cyclins E and A as well as MCM2, which is part of the pre-replication complex.

A slightly lower activity of CDK2-cyclin A complexes also indicated the activation of the G1/S phase checkpoint upon one day of *CKS* over-expression in IMR90 cells (Figure 5.2 B and C). However CDK2 activity in *CKS1* over-expressing cells was not yet significantly lower than in control cells (Figure 5.2 C).

Together with the elevation of G1 cyclins as well as p53 and $p21^{Cip1}$ up-regulation after one day of *CKS* over-expression the data points out that one cell cycle is already enough to activate the G1/S checkpoint in primary fibroblasts.

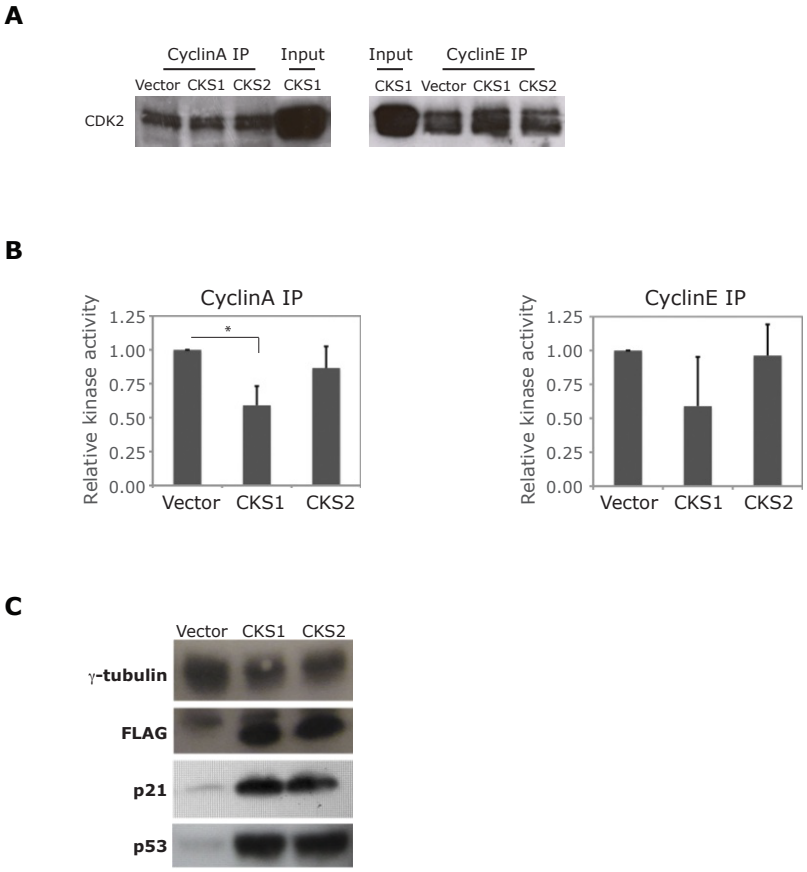

Figure 5.1 ***CKS* over-expression leads to a lower CDK2 activity with the onset of senescence.** (A-C) IMR90 cells were transduced with a retroviral CKS1, CKS2 or control vector and selected in puromycin containing medium for seven days. Proteins were extracted and cyclin A-CDK2 or cyclin E-CDK2 containing complexes immunoprecipitated using an anti-cyclin A or anti-cyclin E antibody as described in Material and Methods. (A) The presence of CDK2 in the immunoprecipitated complexes was verified via Western blot analysis. The upper band shows CDK2, the lower band the binding of the anti-CDK2 antibody to G protein used for the IP. (B) Immunoprecipitated cyclin A-CDK2 and cyclin E-CDK2 complexes of protein extracts derived from IMR90 fibroblasts over-expressing *CKS1* or *CKS2* were used in kinase activity assays. Histone H1 served as target protein and the assays were performed using the Kinase-Glo protocol (promega). Error bars correspond to three independent experiments. Significance testing was performed using a paired *t*-test ($P < 0.05$*)(C) The expression of checkpoint proteins as determined by Western blot performed with whole cell lysates from the cells used for IP.

It was shown in previous publications that the G1/S-phase checkpoint can be activated by stress occurring early during replication or DNA damage, accompanied by high levels of RPA and γH2AX (Boye et al., 2009; Liu et al., 2012). The RPA protein binds to single stranded DNA, which is present in higher ratios at stalled forks during replicative stress (Oakley and Patrick, 2010). Stalled forks also lead to the creation of DNA double strand breaks and phosphorylation of histone H2AX (γ-H2AX) (Fillingham et al., 2006).

To determine if the G1/S phase checkpoint was activated in *CKS* over-expressing cells by replicative stress, RPA and γ-H2AX levels were analysed. Levels of RPA and γ-H2AX in *CKS* over-expressing cells however were not altered (Figure 5.2 A). Thus, the reason for p53 and $p21^{Cip1}$ up-regulation needs to be further investigated.

To study whether *CKS* over-expression affects cell cycle regulation and CDK2 activity via mechanisms independent to the down-regulation of CDK activity by G1/S checkpoint activation, I used HEK 293T cells as this cell line is deficient in the G1/S checkpoint (Ahuja et al., 2005; DuBridge et al., 1987). HEK 293T cells are derived from human embryonic kidney cultures and contain chromosomal abnormalities as well as gene expression for the SV40 Large T-antigen (SV40 TAg). SV40 TAg inhibits the activity of p53 and RB (An et al., 2012). For the inhibition of p53 SV40 TAg binds to p53 and inhibits it transcriptional activity. Transcriptional targets like $p21^{Cip1}$ are therefor not transcribed. Via the binding to p53 TAg stabilises p53. SV40-transformed cells thus harbour generally high p53 levels. Inhibition of RB leads to the general expression of E2F transcription factor regulated genes (An et al., 2012). As expected, no p53 or $p21^{Cip1}$ up-regulation could be observed when *CKS* was over-expressed in these cells (Figure 5.2 A). Kinase assays were performed with complexes isolated via cyclin A IPs in the same way as described above, with Histone H1 as the CDK2 substrate. The ability of CDK2 to phosphorylate Histone H1 was neither significantly up- or down-regulated upon *CKS* over-expression. As observed before in IMR90 fibroblasts, RPA and γ-H2AX levels were not increased (Figure 5.2 A).

The level of the CDK2 inhibitor $p27^{Kip1}$ was also determined in 293T cells. It is known that $p27^{Kip1}$ is marked for degradation by ubiquitination via the SCF^{Skp2} ubiquitin ligase. During this process CKS1 acts as a linker protein between the ubiquitin ligase and $p27^{Kip1}$ and therefore facilitates its degradation (Spruck et al., 2001). Previous studies in cancer cells show that high CKS1 levels are often correlated with low $p27^{Kip1}$ levels. Thus $p27^{Kip1}$ down-regulation was suggested to be one mechanism by which CKS1 promotes proliferation in cancer cells (Kitajima et al., 2004; Lee et al., 2011; Wang

et al., 2009). In my setting $p27^{Kip1}$ levels were unchanged after one day of CKS up-regulation in 293T cells.

In experiments on G1/S checkpoint deficient 293T cells, *CKS* over-expression for one day had no direct influence on the CDK2 activity. Likewise an indirect effect on CDK2 activity due to $p27^{Kip1}$ down-regulation can be excluded.

Overall, cell cycle protein analyses performed confirmed the activation of the G1/S checkpoint in IMR90 fibroblasts. Checkpoint activation supports the idea of CKS exhibiting oncogenic activity. p53 activation is in agreement with the observed reduction in CDK2 activity in primary cells. Studies in checkpoint deficient 293T cells did not show a significant effect of CKS on CDK2 activity. At a time point of one day after *CKS* over-expression the effect this over-expression has on cells seems to be $p27^{Kip1}$ independent.

5.3 Phospho-protein binding by CKS proteins

Earlier in the project, the up-regulation of senescence markers upon *CKS* over-expression was presented. In these experiments the CKSE63Q mutants, which have a reduced ability to bind to CDK1 and 2, showed a slightly less pronounced phenotype for the appearance of SAHF, inhibition of proliferation and β-galactosidase activity compared to wt CKS proteins. The lower levels of senescence markers in *CKSE63Q*-expressing cells, might suggest that a CDK-dependent oncogenic effect of CKS proteins cannot be excluded.

To determine whether CKS might support CDK activity by functioning as an adaptor protein between CDK and its phosphorylated targets, mutated forms of *CKS1* and *2* were constructed by site directed mutagenesis. These mutants are not able to bind to phospho-proteins via their anion-binding site and are thus called CKS anion mutant (CKS1AM and CKS2AM). As in the CKS wt constructs, *CKS1AM* and *CKS2AM* have been FLAG-tagged. To test for the efficient expression of these constructs in IMR90 cells, quantitative real time PCR as well as Western blot analyses were performed. Although transcription levels of *CKS1AM* and *CKS2AM* were higher than that observed for their wild-type and E63Q forms (Figure 5.3 A), the FLAG-tagged CKSAM proteins could not be detected by Western blot.

DNA sequences of the vectors were obtained to ensure accuracy of the constructs (Figure 2.1, Material and Methods). As transcription levels of *CKS1AM* and *CKS2AM* were satisfactory a higher degradation rate was assumed as a reason for the lack of detectable CKSAM proteins. To test whether these *CKS* mutants were targeted to a

higher degree for proteasomal degradation, cells were incubated with the proteasome inhibitor MG132. Upon treatment for 5 hours, both CKS1AM and CKS2AM were clearly detectable (Figure 5.3 B). It is worth mentioning, that levels of the CKS2E63Q protein, which showed only low expression levels before, could also be increased by proteasome inhibition. I therefore conclude that the inability of CKS2 to bind CDK and the lack of the phosphoprotein binding site in both CKS homologues results in an enhanced proteasomal degradation of the proteins.

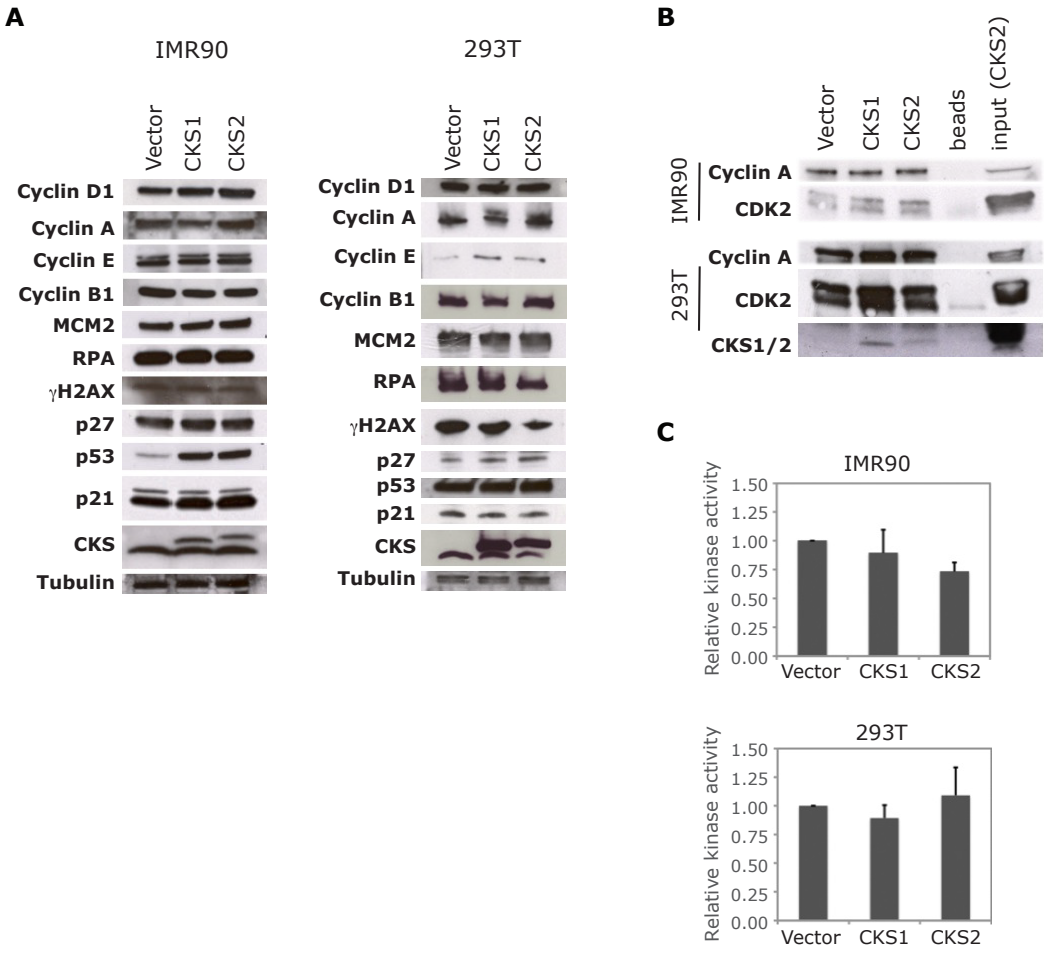

Figure 5.2 ***CKS*** **over-expression leads to checkpoint activation and reduced kinase activity within one day in primary IMR90 fibroblasts but not in transformed HEK 293T cells.** IMR90 fibroblasts as well as 293T cells were transduced with pBABE CKS1 or CKS2 PURO. The following day proteins were isolated as described in Material and Methods and subjected to Western blot analysis and immunoprecipitation for subsequent kinase activity analysis. (A) The level of various checkpoint and cell cycle proteins was analysed via Western blot analysis in the initial protein extracts of IMR90 as well as 293T cells. (B) Cyclin A antibodies and protein G coated magnetic beads were used to immunoprecipitate the cyclin A-CDK2-CKS complex. The presence of the components of this complex was verified by Western blot analysis. The lower band in the CDK2 blot shows unspecific binding of the CDK2 antibody to protein G. (C) Kinase glo activity assays were performed with the immunoprecipitated cyclin A-CDK-CKS complex as described in Material and Methods. Error bars correspond to three independent experiments.

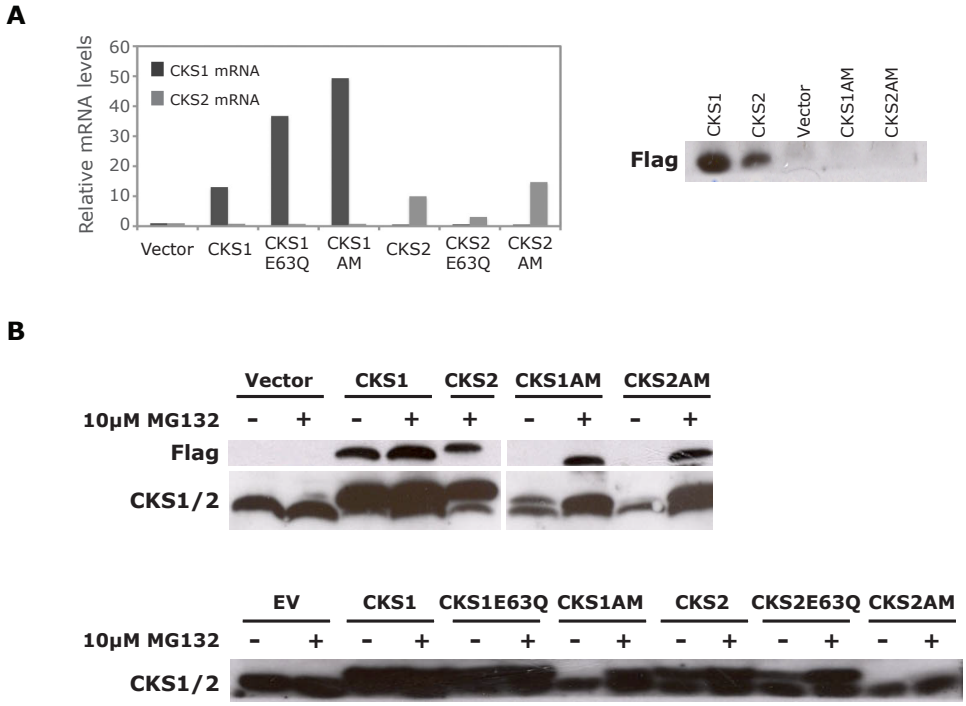

Figure 5.3 **Mutations in the anion-binding site of CKS1 and 2 lead to degradation of CKS proteins via the proteasome.** IMR90 fibroblasts were transduced with retroviral constructs bearing the sequences for *wt CKS1* and *2*, *CKS1E63Q* and *CKS2E63Q* as well as *CKS1AM* and *2AM* which code for CKS variants unable to bind phospho-proteins. Cells were selected for seven days in medium containing puromycin. (A) Quantitative RT-PCR showing mRNA of ectopic CKS1 and 2 in IMR90 fibroblasts over-expressing the indicated CKS variants (left panel). Western blot analysis detecting the FLAG-tagged CKS proteins in protein samples derived from the indicated cell lines after seven days in selection. (B) Before isolation of proteins for Western blot analysis cells were treated for 5 hours with 10 μM MG132. IMR90 cells transduced with the empty vector control, CKS1 and 2 vectors as well as CKS1AM and CKS2AM vectors were examined regarding the expression of FLAG- tagged protein and overall CKS1 and 2 (upper panel). The experiment was repeated for all CKS variants using an antibody detecting CKS1 and 2 (lower panel).

5.4 Discussion

Initial analyses have been conducted to elucidate how high levels of CKS proteins might cause G1/S checkpoint activation. In previous studies G1/S checkpoint activation was a result of deregulated initiation of replication via an altered CDK2 activity (Branzei and Foiani, 2009; Kilbey et al., 1999; Neganova et al., 2011; Zhao et al., 2012). Therefore CDK2 kinase activity assays were performed. In the first instance the suspected down-regulation of the activity of the cyclin A-CDK2-CKS complex upon a long period of *CKS* over-expression in primary fibroblasts was confirmed. The lower CDK2 activity was a

result of the onset of senescence in these cells. Earlier in this study I showed that primary IMR90 fibroblasts have entered a senescent state already one week after transduction with the CKS constructs. This state is accompanied by the expression of CDK2 inhibitors like $p21^{Cip1}$, which was confirmed here by Western blot analysis.

To study the effect of *CKS* over-expression on CDK2 activity upon a short period of time, an earlier time point for the kinase activity assays was chosen. After only one day of ectopic *CKS* expression, IMR90 cells showed a reduced CDK2 activity. This is an interesting observation as previous studies suggested that CKS has a positive influence on CDK2 activity possibly by keeping CDK2 in an active form (Liberal et al., 2011) or down-regulating one of its inhibitors (Spruck et al., 2001). However those studies were not conducted in primary human cell cultures. In the primary IMR90 cells used in my study, an up-regulation of p53 and $p21^{Cip1}$ could be observed already the day after transfecting the cells with CKS constructs. High p53 and $p21^{Cip1}$ levels therefore contributed to the low kinase activity.

Checkpoint activation suggests preceding DNA damage or other events caused by deregulated replication like stalled and collapsed forks. H2AX, a marker for DNA damage, becomes phosphorylated by kinases such as ATM at the appearance of a DNA break. ATM binds to the DNA upon double strand breaks and is then able to phosphorylate the histone variant, which is then designated γ-H2AX. Whereas this has been well documented for double strand break formation upon radiation, the involvement of γ-H2AX in other replication stress responses has not been proven (de Feraudy et al., 2010; Revet et al., 2011). Therefore, the lack of an increase in γ-H2AX after initial *CKS* expression might not indicate a lack of impaired replication in *CKS* over-expressing cells. Thus another marker for the detection of replicative stress, RPA, was tested. During unperturbed replication RPA binds to single stranded DNA and keeps it from winding back (Wold and Kelly, 1988). During replicative stress RPA binds to the accumulating long stretches of single stranded DNA and is believed to take part in replication stress responses like fork stabilization, fork restart and finally checkpoint activation (Fanning et al., 2006; Zou et al., 2006). In the IMR90 cells used here, *CKS* over-expression did not lead to an overall accumulation of RPA. During replicative stress response, RPA is hyperphosphorylated on specific residues by members of the phosphatidylinositol 3-kinase-like kinases like ATR (Block et al., 2004). In further experiments, antibodies specific for these phosphorylation sites could be used to detect potential replicative stress induction caused by *CKS* over-expression in IMR90 cells. Furthermore, IMR90 cells expressing different oncogenes, which result in replicative stress should be used as controls to facilitate the analyses. Additionally the presence of

other markers for replication stress like other phosphorylated downstream effectors of ATR could be analysed. Cell cycle studies via flow cytometry and protein expression analyses in the single cell cycle phases would help to detect exactly when in the cell cycle *CKS* over-expressing cells arrest.

In transformed HEK 293T cells, in which the TAg inhibits G1/S phase checkpoint activation as described above, p53 and $p21^{Cip1}$ up-regulation was not observed. Kinase activity in *CKS* over-expressing 293T cells was not down-regulated indicating once more that G1/S checkpoint activation led to CDK2 inhibition in fibroblasts with intact checkpoints.

CDK2 activity in *CKS* over-expressing 293T cells did also not significantly exceed that measured for empty vector control cells. This might suggest an effect of CKS, which is independent of direct interaction with CDK2. It needs to be mentioned however, that the used kinase activity assay was mainly designed to detect CDK activity per se. The assay might not be suitable to detect specific varieties of enhanced CDK activity. In my studies the kinase complex was isolated via immunoprecipitation from *CKS* over-expressing cell lysates and measured by its ability to phosphorylate histone H1. The assay can therefore detect altered CDK2 activity when it is due to conformational changes of CDK2 as it was proposed earlier (Liberal et al., 2011), regulation of inhibitory or activating phosphorylation of CDK2 or direct inhibition by CDK inhibitory proteins. One possibility is if CKS enhances CDK2 activity by acting as an adaptor protein between CDK2 and certain phospho-proteins, this would not be recognised by the assay, unless the substrate for which the phosphorylation by CDK2 is facilitated, happens to be histone H1. To test whether CKS facilitates the phosphorylation of certain substrates by acting as an adaptor protein, different CDK2 target proteins could be used in a kinase assay screen. Alternatively the phosphorylation status of these target proteins could be analysed via Western blot analysis.

Investigations into the importance of phospho-protein binding by the CKS proteins should have been performed in the present study as well. Therefore CKS variants (CKS1AM and CKS2AM) were produced, which are not able to bind phospho-proteins due to mutations in their anion-binding site. This would presumably prevent CKS from functioning as an adaptor protein between enzymes like CDK2 or the SCF^{Skp2} ubiquitin ligase and their phosphorylated target proteins. The aim was to investigate a possible role for phospho-protein binding in the induction of senescence in primary cells and enhanced proliferation in transformed, checkpoint-inhibited cells. Protein analysis revealed however, that neither the CKS1AM nor CKS2AM transduced cells were able to express the protein. The anion binding site mutant was quickly degraded by the

proteasome as observed before for the CKS2E63Q variant. Only the addition of the proteasome inhibitor MG132 prevented the degradation of CKS2E63Q as well as the CKSAM mutants. The cells do not seem to tolerate the expression of the proteins and promote their fast degradation. This may be a result of the mutant proteins disrupting essential pathways in the cells by preventing binding of not previously described phospho-proteins. Another cell damaging effect might be caused by excessive amounts of CKS proteins, which are available for anion binding site independent functions of CKS. Boosting these anion binding site independent functions might not be tolerable for the cells. Further experiments would be necessary to reveal the importance of phospho-protein binding in the oncogenic function of CKS. The use of the proteasome inhibitors in cell cycle analyses however would impair the analysis. Additionally, experiments running for longer periods of times, like the ones necessary to investigate the onset of senescence, are not applicable. Instead, experiments in which the anion binding site mutant is expressed for a short time in the presence of the proteasome inhibitor might reveal further functions involving the ability of CKS proteins to bind to phospho-proteins. These experiments would involve the analyses of cell cycle protein levels as well as their phosphorylation and ubiquitination status.

Chapter 6 – Final discussion

6.1 *CKS* as oncogenes

CKS1 and *CKS2* over-expression has been described in a variety of cancers (Calvisi et al., 2009; Chang et al., 2010; Chen et al., 2011; de Wit et al., 2005; Fonseca et al., 2006; Inui et al., 2003; Lan et al., 2008; Lee et al., 2011; Li et al., 2004; Liu et al., 2008; Lyng et al., 2006; Masuda et al., 2003; Shapira et al., 2005; Slotky et al., 2005; van 't Veer et al., 2002; Wang et al., 2009; Westbrook et al., 2009; Zhan et al., 2007). In addition to expression analysis in tumour samples, studies in which a potential oncogenic role for CKS proteins was investigated were performed *in vitro*. The *in vitro* systems used however, were almost exclusively based on existing cancer cell lines expressing high levels of CKS proteins. In these cell lines, the role of CKS proteins as oncogenes has been mainly studied by down-regulating *CKS1* and *CKS2*. In cell cultures derived from oral squamous cell carcinomas it was shown for example, that a down-regulation of *CKS1* leads to a decrease in proliferation *in vitro*. A lower proliferation was also observed in tumours derived from mice, which have been injected with these cells (Kitajima 2004).

Similarly, Lan and colleagues showed that down-regulation of *CKS1* in otherwise CKS1-high AT3 prostate cancer cells led to a decrease in cell growth. They also described an inhibition of anchorage-independent growth due to the loss of CKS1. In the same study down-regulation of *CKS2* in prostate tumour cells derived from transgenic adenocarcinomas of the mouse prostate (TRAMP), led to an enhanced apoptotic phenotype of the cells. CKS2 inhibition in TRAMP cells, which were transplanted into mice, resulted in a decreased tumorigenicity in comparison to *CKS2* expressing TRAMP transplants (Lan 2008).

Wang et al. also showed a reduced growth and invasion ability of MDA-MB-231 breast cancer cells after *CKS1* knockdown by RNA inference (Wang 2009).

In a later study *CKS1* knock-down in hepatocellular carcinoma cells was shown to result in a lower proliferation rate as well as decreased tumorigenicity, which was associated with down-regulation of IL8 (Lee 2011). These studies all indicate that higher levels of CKS proteins confer proliferative advantage for many cancer cells. As it was shown that CKS proteins have various functions within the cell cycle, this was to be expected. The observed reduction in the ability of invasion and anchorage independent growth when CKS is decreased could also be a result of a slower proliferation.

Therefore it is not understood whether *CKS* acts as a real oncogene, contributing to the transformation process of primary cells to cancer cells.

During my studies I approached this question by over-expressing CKS proteins in primary IMR90 fibroblasts, which were undergoing a transformation process from primary fibroblasts to tumour suppressor-inhibited cells. I have shown that high levels of CKS proteins lead to an impact on G1/S phase transition and the activation of tumour suppressor pathways in primary IMR90 fibroblasts resulting in a senescent state. Induction of senescence in otherwise unperturbed cells also occurs upon the activation of a range of oncogenes such as *RAS*, *RAF*, *AKT* and *cyclin E* (Bartkova et al., 2006; Michaloglou et al., 2005; Minamino et al., 2004; Serrano et al., 1997). CKS proteins thus have the same impact as oncogenes on cells with normal tumour suppressor gene function. In the next step I was able to show that inhibition of tumour suppressor pathways in *CKS* over-expressing cells alleviates growth arrest and leads to a modest proliferative advantage in *CKS* over-expressing checkpoint inhibited cells. Complete transformation of the cells, leading to the ability to grow in an anchorage independent way, however could not be achieved by *CKS* over-expression alone. These results suggest that CKS proteins act as oncogenes by their positive influence on proliferation. CKS therefore may act early in the oncogenic pathway during multistage tumorigenic transformation (Figure 6.1). Together with events like the activation of other growth sustaining signalling pathways as well as the activation of TERT and inhibition of growth inhibiting tumour suppressor genes, the up-regulation of CKS proteins contributes to the high and indefinite proliferative capacity of neoplastic cells. For the further establishment of the growing tumour additional steps are necessary (Hanahan and Weinberg, 2011). To allow sufficient supply of nutrients and oxygen, growing tumours undergo an angiogenic switch induced by the activation of VEGF (Carmeliet, 2005; Hanahan and Weinberg, 2011). In the next step of multistage tumorigenesis, neoplastic cells acquire the ability to invade their surrounding tissues and form metastases in distant organs. This program, which is referred to as epithelial-mesenchymal transition (EMT), is characterised by the expression of matrix degrading enzymes like Matrix metalloproteinases and loss of E-cadherin, which leads to loss of adherent junctions (Compagni and Christofori, 2000; Hanahan and Weinberg, 2011). Whereas oncogenic *RAS* has been reported to be involved in the regulation of EMT (Janda et al., 2002a) and is thus able to transform neoplastic cells completely, the function of CKS in cancer development seems to be restricted to its contribution to enhance proliferation. As a high proliferative rate is one of the main characteristics of aggressive cancers, CKS proteins may provide a therapeutic target in cancer treatment.

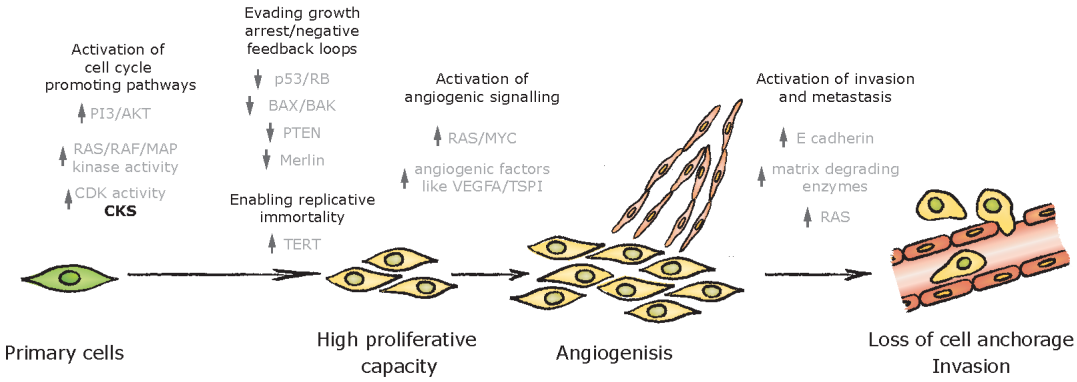

Figure 6.1 **Simplified model indicating the role of CKS proteins during multi stage carcinogenesis.** The process of cell transformation is a multi-step process and involves several alterations within the signalling networks of a cell. During early events cells gain the ability of unrestricted growth. By mutations in cell cycle activating signalling pathways they become independent of growth signals. Often proliferation is accelerated. One component contributing to this accelerated growth of these neoplastic cells are high levels of CKS. At the same time mechanisms evolve in the cell to evade growth suppression and negative feedbackloops to sustain high proliferation (Hanahan and Weinberg, 2011). An activated telomerase ensures the preservation of telomers and prevents replicative senescence (Belgiovine et al., 2008). The growing tumour tissue ensures its supply with nutrients and oxygen by the activation of angiogenic signalling which results in the formation of tumour associated neovasculature (Hanahan and Weinberg, 2011). To be able to invade other tissues and to grow anchorage independent, the tumour cell finally needs to undergo the process of epithelial-mesenchymal transition. Here adherens junctions are lost and the cell receives the ability to migrate and to grow anchorage independent (Hanahan and Weinberg, 2011). Whereas the well studied oncoprotein RAS is involved in multiple steps in this model of multistage carcinogenesis (Hanahan and Weinberg, 2011; Janda et al., 2002a; Janda et al., 2002b), the effect of high CKS levels emerges early during tumorigenesis.

6.2 The effect of *CKS* over-expression is dependent on cellular context

The observations of my study that high levels of CKS proteins confer a proliferative advantage in checkpoint inhibited but not in primary fibroblasts go along with studies conducted by members of the Keller laboratory (Keller et al., 2007; Kratzat et al., 2012). They showed that *CKS1* over-expression alone does not necessarily lead to higher proliferation of B cells transplanted into wild-type mice and is unable to induce lymphoma development. However, knocking out *CKS1* in precancerous B cells as well as lymphoma cells derived from mice, did lead to a decrease in proliferation (Keller et al., 2007; Kratzat et al., 2012). Thus the studies of the Keller laboratory as well as my study indicate that the effect of high levels of CKS is dependent on the cellular context. My

model showed that in primary cells with intact checkpoints, *CKS* over-expression leads to cell cycle deregulation and cell cycle arrest and CKS therefore cannot execute its cell cycle promoting activity. Upon checkpoint inhibition however, high levels of CKS lead to a proliferative advantage for the cells. This is in accordance with the discussed work in cancer cell lines with high CKS levels, in which *CKS* down-regulation leads to the decline of their proliferative capacity (Kitajima et al., 2004; Lan et al., 2008; Lee et al., 2011; Wang et al., 2012).

A study presented by the Reed laboratory (Liberal et al., 2011) showed that *CKS* over-expression in transformed HEK293A cells and immortalised human mammary epithelial cells did not have an impact on the cell cycle. They observed however that in comparison to non *CKS* over-expressing HEK293A cells, those cells which did over-express *CKS* did not properly arrest in G1 after treatment with the ribonucleotide reductase inhibitors thymidine or hydroxyurea (HU). This study therefore suggests, that not only the tumour suppressor gene background of a cell, but also the conditions the cell is under are important to the effect high levels of CKS have on the cell.

The Reed laboratory showed, that, although checkpoint proteins are activated in the *CKS* over-expressing HEK293A cells upon treatment with HU, origin firing was greatly elevated when compared to HU-treated control cells. Thus, *CKS* over-expressing HEK293A cells showed a proliferative advantage by overriding the G1/S checkpoint upon replicative stress caused by ribonucleotide reductase inhibitors. They suggested a model in which CKS1 and CKS2 help to override this checkpoint by keeping CDK2 in an active form. To show that CDK2 from *CKS* over-expressing cells possess a high activity despite checkpoint activation by dNTP pool deprivation, they performed kinase activity assays with CDK2 isolated from *CKS2* over-expressing or control cells upon thymidine block. In their assay, the ability of CDK2 to phosphorylate RB was higher in the presence of the CKS proteins. As the observed phenotype was shown in *CKS2* over-expressing cells and CKS2 is not part of the SCF^{Skp2} ubiquitin ligase, they excluded an effect, which is due to lower $p27^{Kip1}$ levels. They did not observe any effects on the cell cycle in *CKS* over-expressing cells without the addition of thymidine. In my study, *CKS* over-expression alone led to checkpoint activation in primary IMR90 fibroblasts without thymidine treatment. This checkpoint activation led to CDK inhibition and cell cycle arrest. If the model suggested by the Reed laboratory holds true, it only applies to the context of a ribonucleotide reductase inhibitor related S-phase checkpoint in the context of a transformed cell line like HEK293A or possibly cancer cells.

6.3 Possible domains involved in CKS related transformation

I confirmed the suspected oncogenic effect of CKS proteins, which results in replicative stress and checkpoint activation. I also showed that high expression is of advantage for cells lacking certain tumour suppressor pathways. In the next step I set out to identify the underlying pathway involving CKS. The domains of CKS proteins important in causing G1/S deregulation should be revealed and possible substrates important in CKS related transformation identified.

Previous studies often suggested the involvement of CKS1 in the down-regulation of the CDK2 inhibitor $p27^{Kip1}$ as the main reason for the proliferative advantage of CKS1-high cancer cells (Kitajima et al., 2004; Lee et al., 2011; Masuda et al., 2003; Wang et al., 2009). Initially $p27^{Kip1}$ is phosphorylated by CDK2 on Threonine 187. As part of the SCF^{Skp2} ubiquitin ligase, CKS1 but not CKS2 recognises phosphorylated $p27^{Kip1}$ and binds to it, together with the F box protein Skp2. This is followed by ubiquitination and degradation of $p27^{Kip1}$ (Spruck et al., 2001). Studies in cancer cell lines often show an accumulation of $p27^{Kip1}$ upon *CKS1* knockdown, which is accompanied by a decreased proliferative capacity (Kitajima et al., 2004; Lee et al., 2011; Wang et al., 2009). Results obtained during my work indicate that $p27^{Kip1}$ down-regulation is not the sole reason for faster growth in cancer cells with high CKS levels. In my studies *CKS1* and *CKS2* show similar effects upon over-expression in primary IMR90 cells. Additionally, checkpoint activation in these IMR90 cells was already observed one day after transfection with the CKSwt constructs, a time point at which $p27^{Kip1}$ down-regulation could not yet be detected. Furthermore, CKS2, which is not involved in $p27^{Kip1}$ degradation, leads to a slightly more increased proliferation in checkpoint inhibited IMR90 E6 E7 TERT cells than CKS1 does. Thus, an oncogenic function exists, which leads to an elevation of proliferation in transformed cells and is likely to be independent of the degradation of $p27^{Kip1}$. This would be in good agreement with observations made by Höllein et al. Höllein saw that MEFs lacking CKS1 showed a lower proliferative capacity characterised by less actively replicating cells in S-phase and cells accumulating in G1 and G2. Loss of $p27^{Kip1}$ could not reverse this S-phase defect but led to a reduction of the cells, which accumulated in G2 (Hoellein et al., 2012). His results would suggest that degradation of $p27^{Kip1}$ with the help of CKS1 is more important for G2/M transition and that the effect of CKS1 on G1/S transition is mediated by other mechanisms.

Another mechanism by which CKS proteins could lead to a deregulated G1/S transition might be by direct interaction with CDK. This interaction is well known and might change CDK activity possibly by supporting binding to specific substrates (Bourne et al., 1996). To address the question of whether CKS proteins do affect CDK activity by directly interacting with the kinase in my settings, CKSE63Q mutants were used. The binding capacity of CKSE63Q mutants towards CDK was previously shown to be decreased to 20% of the wild-type protein (Bourne et al., 1996). In the experiments performed during my study cells, over-expressing the *E63Q* or *wt* variants were compared with empty vector control cells.

Over-expression of *CKSwt* as well as the *CKSE63Q* mutants led to checkpoint activation and a pronounced growth arrest in IMR90 fibroblasts. This suggests that a function of CKS proteins, which is independent of direct CDK binding, contributes to the development of deregulated cell cycle progression during early steps of tumorigenesis. A function of CKS, which is independent of direct CDK binding, is the discussed role of CKS as adaptor protein between the SCF^{Skp2} ubiquitin ligase and its substrate $p27^{Kip1}$. Moreover the ubiquitination of cyclin A by the APC/C ubiquitin ligase is facilitated via CKS proteins (Di Fiore and Pines, 2010). In both cases the anion-binding site of CKS is required. My studies to whether the anion-binding site is of importance in the induction of deregulated replication by CKS remained inconclusive. Assuming that the anion-binding site is essential and endogenous CKS acts as an adaptor protein, excessive CKS protein levels might counteract the actual function of CKS as adaptor. Whereas a subset of CKS proteins binds solely to the target protein another subset binds to the ubiquitin ligase consequently hindering enzyme and target interaction (Figure 6.2). Thus high levels of CKS proteins may exert a dominant negative mechanism leading to a G1 cell cycle arrest for which the mechanism is not known yet. The competition for substrates by excessive CKS proteins might not only effect the well studied functions of CKS within protein degradation or regulation of CDK activity but also metabolic pathways (Radulovic et al., 2010) or other yet unknown CKS involving pathways.

A possible dominant negative effect of CKS proteins is independent of a direct interaction of CKS proteins with CDK and might explain the growth arrest, which was observed in *wt CKS* as well as *CKSE63Q* mutant over-expressing cells. When the effect of wt CKS proteins and CKSE63Q mutant proteins on primary IMR90 fibroblasts were compared however, little differences in the resulting phenotype of IMR90 fibroblasts were noticed. A slightly less pronounced phenotype for growth inhibition as well as for other senescent markers like SA-β-Gal activity was observed for cells expressing the

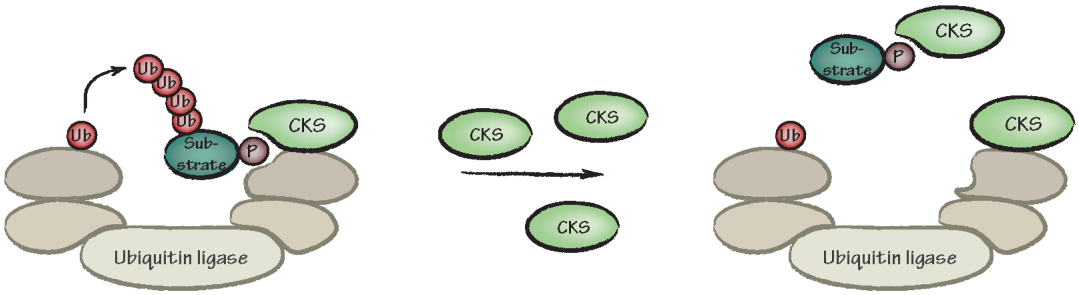

Figure 6.2 **Model illustrating a possible dominant negative effect of excessive CKS using the example of CKS dependent substrate ubiquitination.** When CKS protein levels are tightly regulated, CKS function as adaptor proteins to promote the binding of specific substrates to their ubiquitin ligases. Upon an increase of CKS availability in the cell, the access of the specific substrate to the ubiquitin ligase is blocked as excess of CKS proteins causes the substrates being sequestered by CKS that are not able to interact with the ligase due to the fact that their docking site is taken by other CKS (right).

E63Q variant. Thus checkpoint activation upon *CKS* over-expression in IMR90 fibroblasts could be partly due to the ability of CKS to directly bind CDK. This would go along with studies conducted in yeast showing the importance of CKS for the activity of CDK-G1 cyclin complexes (Reynard et al., 2000). It would also be in agreement with the study discussed earlier in which *CKS* but not *CKSE63Q* over-expression leads to higher CDK2 activity (Liberal et al., 2011).

A CDK2-dependent function of CKS proteins was also shown by Di Fiore and Pines, who proposed a model for the ubiquitination of cyclin A. In their model cyclin A, which is in complex with CDK/CKS, binds the APC/C cofactor CDC20 and is directed to the APC/C via the interaction of CKS with the phosphorylated APC/C (Di Fiore and Pines, 2010). According to this, CKS proteins act as adaptor proteins between CDK complexes and the phosphorylated ubiquitin ligase APC/C promoting the function of APC/C. A similar model would be conceivable for a CDK promoting function of CKS. Here CKS could interact with phosphorylated target proteins of CDK thus bringing CDK into close proximity to their target proteins. In my model I could not detect a significantly elevated CDK2 activity after over-expression of CKS even when using transformed 293T cells. As mentioned before, this could be due to the design of the kinase assay in which histone H1 was used as target protein. When CKS proteins might increase CDK activity by acting as an adaptor between the CDK complex and its substrate, CKS would show a higher affinity towards specific targets. Thus histone H1 might not be a specific target for the

potential adaptor protein CKS within the CDK2-cyclin A complex. In the Reed laboratory, where *in vitro* assays showed that CDK2 was kept active by addition of CKS proteins, RB was used as target instead. Thus, an adaptor function within the CDK2-cyclin A complex might lead to the phosphorylation of proteins like RB and therefore would promote S-phase. My studies of whether phospho-protein binding is involved in a cancer promoting function of CKS failed however, due to the fast degradation of the phospho-protein binding mutant CKS AM by the proteasome.

6.4 Future work

Further studies would need to be made to investigate the involvement of the phospho-protein binding site of CKS in CKS related transformation.

Preferably these studies should be conducted using an inducible system for *CKS* expression. In this way, effects of *CKS* over-expression could be examined within hours upon the induction of the protein and furthermore degradation of the mutant forms might be avoidable. Initially cell cycle studies, including flow cytometry and Western blot analyses of cell cycle proteins, could be conducted to see whether wt *CKS* and anion binding site mutant expression in primary IMR90 fibroblasts had an effect on progression into and through S-phase. An initial experiment was performed using the IMR90 TERT cells, transduced with tetracycline inducible CKS1 vectors resulting in a heterogeneous cell population for *CKS1* expression. Upon synchronisation by contact inhibition and induction of CKS expression for two days, these cells showed a slightly delayed entry into S-phase upon re-plating at low density (Supplemental figure 7.3). This effect is possibly due to the described checkpoint activation and might be avoidable when early time points are chosen.

If over-expression of the anion binding site mutant would not lead to checkpoint activation a possible dominant negative effect of CKS proteins involving the blockage of enzyme binding to their phosphorylated substrates could further be investigated.

Also a possible function of CKS as adaptor protein for CDK2 and their target proteins could be further analysed in a kinase assay screen with different CDK2 target proteins as substrates.

6.5 Concluding remark

Since their discovery, CKS proteins have been studied extensively. They exhibit an important function in the degradation of cyclin A via the APC/C and the degradation of $p27^{Kip1}$ via the SCF^{Skp2} ubiquitin ligases. Despite these well-established roles, CKS

proteins are involved in a variety of cell cycle processes for which the underlying molecular mechanism still needs to be revealed.

In previous studies inhibition of CKS protein expression in cancer cells has been shown to impair cell proliferation and tumorigenicity. In case of CKS1 the positive effect on proliferation in cancer cells was assumed to be dependent on the role of CKS in $p27^{Kip1}$ down-regulation by the SCF^{Skp2} ubiquitin ligase.

The presented study supports a role of *CKS* as oncogene. This role seems to be solely due to the effect of CKS on the cell cycle. The oncogenic effect does not seem to involve cyclin A or $p27^{Kip1}$ degradation. High levels of CKS might either enforce the function of CKS as adaptor protein or lead to a dominant negative effect and blockage of CKS functions, which remain to be identified.

It has to be noted that although *CKS1* and *CKS2* over-expression in primary fibroblasts resulted in checkpoint activation and senescence it is possible that they act on different pathways within the cell to cause cell cycle deregulation. CKS1 and 2 proteins might regulate the function of several enzymes in different ways and the converged action of all, results in the observed phenotype of cell cycle arrest in primary cells. Further investigations will provide further understanding into which CKS dependent enzymes and substrates are involved during this early step of the oncogenic pathway when *CKS* is over-expressed.

Supplemental Data

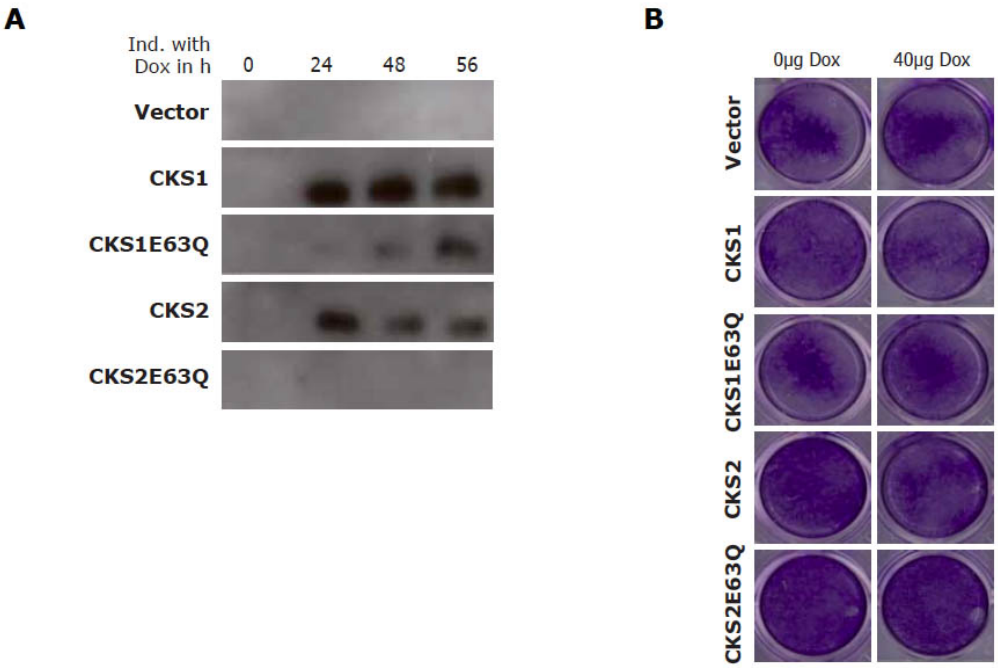

Figure 7.1 **Induction of CKS protein expression in IMR90 TERT cells using the pBABE sin tet PURO system does not lead to cell cycle arrest.** (A) Western blot analysis displaying ectopic CKS expression upon viral transduction of the IMR90 TERT cells with the pBABE sin tet CKS construct. Expression was induced with 40 µg/ml of doxycycline and cells were harvested for Western blot analysis at the indicated time points. Over-expressed CKS proteins were detected using an anti-Flag antibody. (B) IMR90 TERT cells with induced CKS expression were compared with non-induced cells regarding their proliferative capacity. Cells were seeded at a low density and fixed with glutaraldehyde two weeks later for crystal violet staining.

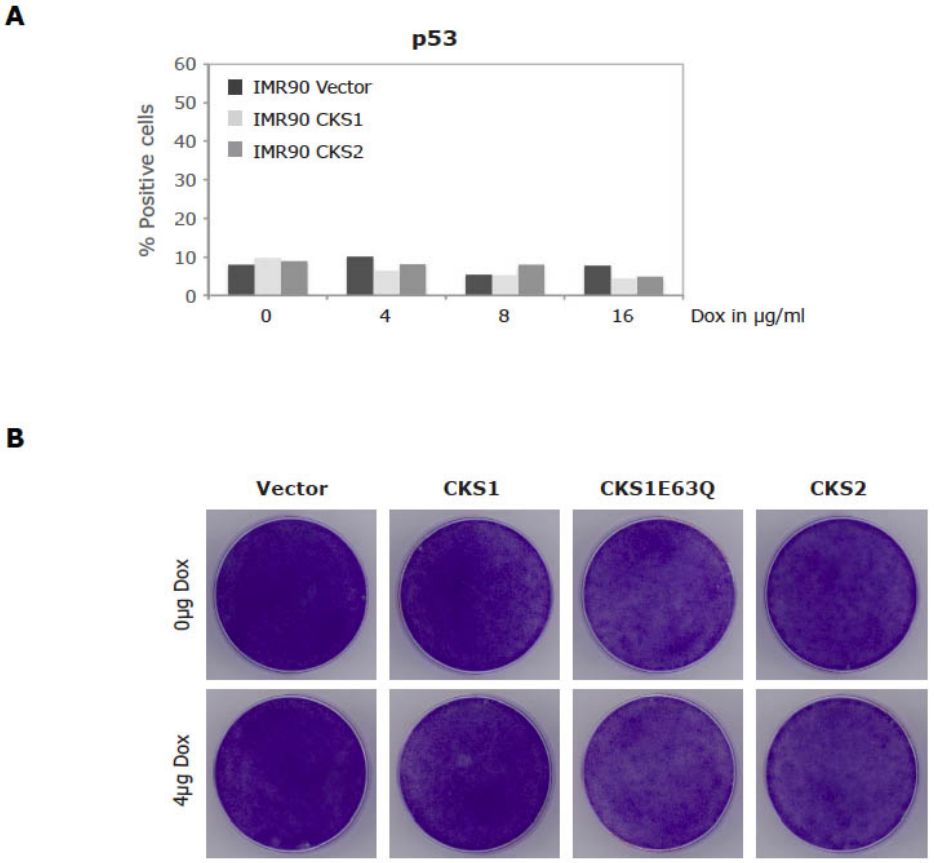

Figure 7.2 **Over-expression of CKS proteins in primary IMR90 cells via the pBABE sin tet CKS PURO system does not lead to checkpoint activation and cell cycle arrest.** (A) Primary IMR90 cells were transduced with the pBABE sin tet CKS PURO constructs, selected and CKS expression was induced using the indicated concentrations of doxycycline. Cells were plated the following week and fixed the day after. Immunofluorescence staining was performed with an antibody against p53. Images were acquired with the IN Cell Analyzer 1000 and quantitatively evaluated using high content analysis as described in Material and Methods. (B) The proliferative capacity of pBABE sin tet CKS PURO transduced IMR90 cells was assessed using a crystal violet assay. Therefor CKS expression was induced using 4 µg/ml of doxycycline. Cells were plated at a low density the following week and fixed for crystal violet staining two weeks later.

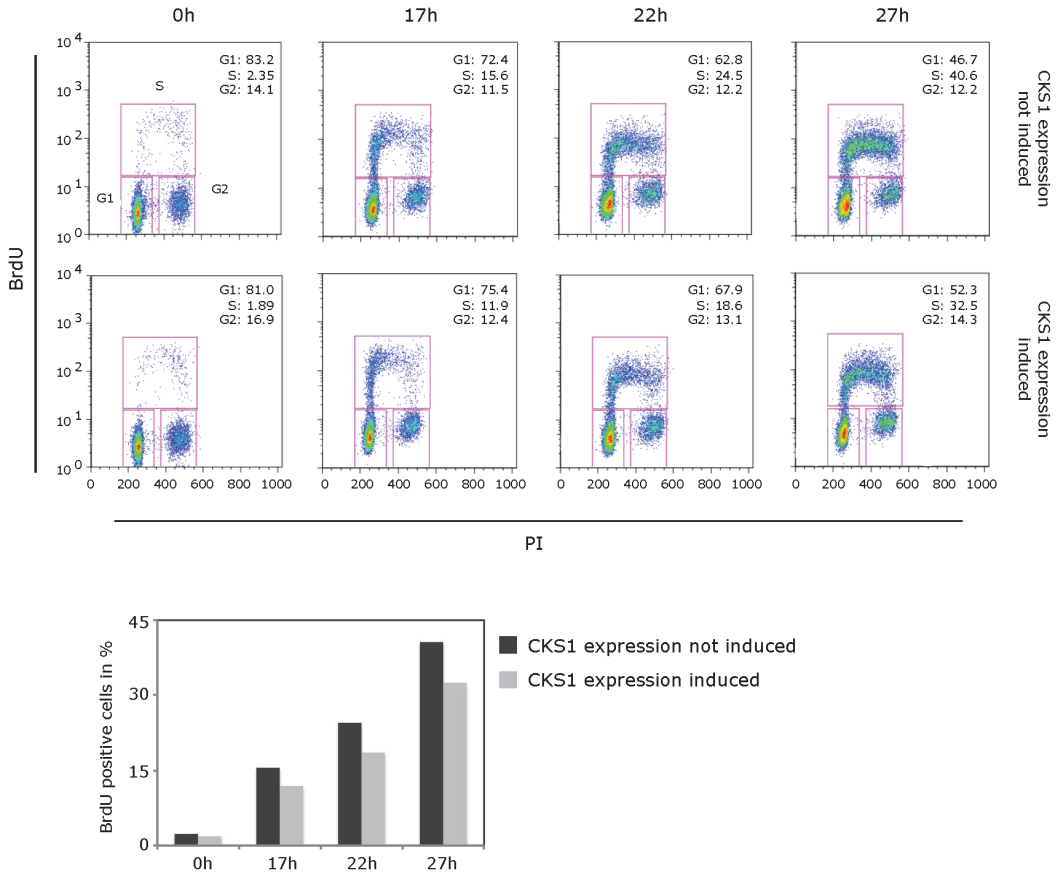

Figure 7.3 **Cell cycle analysis upon induction of *CKS* expression.** IMR90 TERT cells transduced with pBABE-sin-tet-Puro-CKS were kept at 100% confluence for 5 days to allow cells to arrest in G1. Cells were kept in medium either with or without doxycycline from day 4 onwards. To allow progression from G1 through the cell cycle, cells were seeded at a lower density. Cells were pulsed with BrdU for one hour at the given time points, fixed and stained with propidium iodid and an anti-BrdU antibody thereafter. Flow cytometry was performed (upper panel) and cell populations were gated into G1, S and G2-phase. The diagram presented (lower panel) shows the proportion of CKS1 induced and non induced IMR90 TERT cells in S-phase at the given timepoints.

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