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Single cell analysis of checkpoints in G₁

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Science is facts; just as houses are made of stones, so is science made of facts; but a pile of stones is not a house and a collection of facts is not necessarily science.

Henri Poincaré (1854-1912)
French mathematician and physicist

Abstract

It is becoming evident that defective cell cycle regulation is an important property of the cancer cell. The restriction point (R), a G₁ checkpoint after which cell cycle progression becomes independent of extracellular growth factors, is often aberrant in cancer cells. The molecular mechanism governing R is unknown, but hyperphosphorylation and inactivation of the tumour suppressor retinoblastoma (pRb) has been suggested as a potential candidate. We examined the temporal relationship between R and pRb phosphorylation using methods for detailed single cell analysis. We found that that phosphorylation of pRb occurs after passage through R and therefore is not the mechanism regulating passage through R. Instead, phosphorylation of pRb seems to constitute a separate checkpoint in late G₁ which possibly regulates S phase entry.

Cyclin D-cyclin dependent kinase (cdk) 4/6 phosphorylates pRb in response to growth factor stimulation and we studied the timing of cyclin D and pRb colocalisation in relation to the R point and pRb phosphorylation. We found that cyclin D and pRb only colocalise in late G₁, after passage through R, with kinetics similar to pRb phosphorylation. This strongly suggests that intra-nuclear translocation of cyclin D to pRb is involved in regulation of pRb phosphorylation and not in passage through R. This further supports our finding that pRb phosphorylation is not the mechanism behind the restriction point.

R occurs at a set time after mitosis, implying that the R point mechanism might be related to events that occur during mitosis. In support of this notion, time-lapse video-microscopy analysis revealed that early post mitotic cells, prior to passage through R, undergo a transient change in cell shape associated with cell cycle exit when subjected to serum starvation. However, addition of growth factors was shown to counteract both the change in cell shape and cell cycle exit induced by serum withdrawal. In conclusion, our data suggest that reorganisation of the cytoskeleton and/or cell adhesion to ECM after exit from mitosis is involved in R point passage.

To study cell growth in relation to pRb phosphorylation we inhibited rRNA synthesis through treatment with actinomycin D. This induces cell cycle arrest in G₁ cells, but not in S phase cells. This G₁ arrest was shown to be associated with inhibited pRb phosphorylation. We also observed that protein synthesis was not inhibited in actinomycin D treated, G₁ arrested, cells. This indicates that ribosome biogenesis is involved in the regulation of a late G₁ checkpoint that is separate from the R point and involves pRb phosphorylation, but not mass accumulation.

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List of publications

The thesis is based on the following articles and manuscripts, which will be referred to by their roman numerals:

- I. **Martinsson H-S***, Starborg M*, Erlandsson F and Zetterberg A
Single cell analysis of G1 check points - The relationship between the restriction point and phosphorylation of pRb
Exp Cell Res, 2005;305(2):383-391
- II. **Martinsson H-S**, Zickert P, Starborg M, Larsson O and Zetterberg A
Changes in cell shape and anchorage in relation to the restriction point
J Cell Physiol, 2005;203(1):27-34
- III. **Martinsson H-S**, Erlandsson F, Zetterberg A and Starborg M
Changes in intra-nuclear translocation of cyclin D in relation to the restriction point and pRb phosphorylation
Manuscript
- IV. **Martinsson H-S**, Zetterberg ACF, Lundin P and Zetterberg A
Cell cycle block in late G₁ – Effect of Actinomycin D on pRb phosphorylation and cell growth
Manuscript

*authors contributed equally

List of abbreviations

| | |
|--------------------|---|
| 3D | three-dimensional |
| Akt | gene product of the human homolog of murine thymoma viral oncogene, also know as protein kinase B (PKB) |
| AMD | Actinomycin D |
| AO | Acridine Orange |
| ATM | the ataxia telangiectasia mutated gene product |
| ATP | adenosine triphosphate |
| ATR | the ataxia telangiectasia and Rad3 related gene product |
| ARF | alternative reading frame |
| BrdU | bromodeoxyuridine |
| BRG-1 | brm/SWI2 related gene product 1 |
| brm | brahma gene product |
| CAK | cdk activating kinase |
| CCD | charge-coupled device |
| cdc | cell division cycle |
| cdk | cyclin dependent kinase |
| Chk | checkpoint kinase |
| CIP | cdk interacting protein |
| CKI | cyclin dependent kinase inhibitor |
| DAPI | 4',6'-diamidino-2-phenylidole |
| DMEM | Dulbecco's modified Eagle's Medium |
| DNA | deoxyribonucleic acid |
| DP | E2F dimerisation partner |
| <i>E1A</i> | early region 1A of adenovirus |
| E2F | E2 promoter binding factor |
| ECM | extracellular matrix |
| EGF | epidermal growth factor |
| ERK | extracellular signal-regulated kinase |
| FAK | focal adhesion kinase |
| FBS | foetal bovine serum |
| FITC | fluorescein isothiocyanate |
| G ₀ | quiescent state after exit from the cell division cycle |
| G ₁ | the first gap phase of the cell division cycle |
| G ₁ -pm | the post-mitotic part of G ₁ |
| G ₁ -ps | the pre-DNA synthetic part of G ₁ |
| G ₂ | the second gap phase of the cell division cycle |
| GDP | guanosine diphosphate |
| GF | growth factor |
| GSK-3 β | glycogen synthase kinase-3 β |

| | |
|--------------|--|
| GTP | guanosine triphosphate |
| Grb2 | growth factor receptor-bound protein 2 |
| HAT | histone acetyltransferase |
| HDAC | histone deacetylase complex |
| HDF | human diploid fibroblasts |
| IGF-1 | insulin like growth factor 1 |
| INK4 | inhibitor of cdk4 |
| KIP | cdk inhibitory protein |
| M | the mitotic phase of the cell cycle |
| MAPK | mitogen-activated protein kinase |
| MEK | MAPK/ERK kinase |
| MEM | minimum essential medium |
| MDM2 | mouse double minute 2 |
| Myt-1 | membrane associated tyrosine- and threonine-specific cdc2-inhibitory kinase |
| NY | Naphthol Yellow |
| PBS | phosphate buffered saline |
| PDGF | platelet derived growth factor |
| PI3K | phosphatidylinositol-3-OH kinase |
| pRb | the retinoblastoma gene product |
| R | the restriction point |
| Raf | gene product of the human homolog of the transforming gene of murine sarcoma virus 3611 |
| Ras | retrovirus associated sequence gene product |
| <i>RB</i> | the retinoblastoma susceptibility gene |
| RNA | ribonucleic acid |
| rRNA | ribosomal RNA |
| RTK | receptor tyrosine kinase |
| S | the DNA synthetic phase of the cell division cycle |
| Ser | serine |
| SH2 | src homology 2 domain |
| Shc | SH2 containing-protein |
| Sos | son of sevenless |
| <i>src</i> | human homolog of Rous sarcoma virus oncogene |
| SWI/SNF | switching/sucrose non-fermentation gene product |
| TGF- β | transforming growth factor β |
| Thr | threonine |
| Tyr | tyrosine |
| TLV | time-lapse video-microscopy |
| Wee-1 | gene product of the human homolog of the yeast gene Wee-1 which causes a small (i.e. "wee") phenotype if mutated |

Introduction

Cancer

Cells in a multicellular organism are under strict regulation of proliferation, differentiation and apoptosis in a way that is advantageous for the organism, but not necessarily for the single cell. Most cells in the human body are terminally differentiated and have lost the ability to proliferate, except for a few cells that retain this ability. They can divide if necessary, for example in the case of wound healing. In response to stress, for example DNA damage or oncogenic activation, cells have the possibility to undergo programmed cell death, apoptosis. They commit suicide in order to save the organism. Loss of regulation of the processes that control proliferation and apoptosis might ultimately result in cancer and death of the organism.

It is believed that cancer arises from a single somatic cell that through mutation has gained a proliferative advantage over the neighbouring cells. This cell will divide more often than surrounding cells and eventually produce a homogenous clone. Expansion of the clone is followed by genetic and/or epigenetic changes that result in altered physiological properties than those of normal, unchanged cells. The transformed cells are under constant selection pressure, which forces the population of altered cells to evolve in a way that allows cells to disregard the normal proliferative and apoptotic control mechanisms (Ponder 2001). The tumour development process follows the rules of classical Darwinian evolution: the genesis of a cell with properties advantageous for survival and reproduction results in clonal expansion and ultimately a tumour. The tumour cells may eventually kill its host by invasion, destabilisation and erosion of normal tissue (Evan and Vousden 2001). The standard mutation rate is not high enough to account for the large number of mutations observed in cancer cells. One theory is that events during tumourigenesis increase the genetic instability of the transformed cells, thereby increasing the risk for subsequent genetic changes. Consequently tumours tend to become more and more aggressive as they develop (Evan and Vousden 2001; Hanahan and Weinberg 2000). An alternative idea is that genetic instability is a very early event in tumourigenesis, and that it provides the cell with the opportunity to change genetically and obtain all

the necessary characteristics of a cancer cell (Cahill *et al.* 1999). A common trait of all cancer cells is that they differ from normal cells in aspects concerning regulation of cell proliferation and cell death (Evan and Vousden 2001; Green and Evan 2002). Tumour cells become self-sufficient in growth signalling, insensitive to anti-growth signals, acquire the ability to avoid apoptosis and gain potential for limitless replication, as well as possibility for angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg 2000).

The cell division cycle

The order and timing of cell cycle events are critical for accurate propagation of chromosomes to daughter cells and deregulation of these processes is an important step in tumourigenesis. Therefore, increased knowledge about regulation of the cell cycle is advantageous in the battle against cancer.

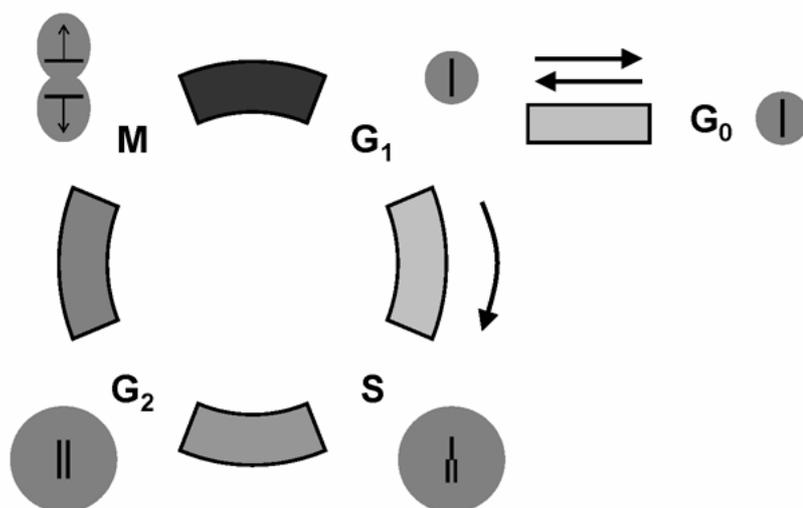


Figure 1. The cell cycle consists of four phases G₁, S, G₂ and M. In G₁, the cell grows in size and prepares for DNA synthesis. During this phase, the cell interprets extracellular and intracellular signals and decides whether or not to continue the cell cycle. If the conditions are unfavourable for proliferation the cell can choose to exit the cell cycle and enter a quiescent state termed G₀. In S phase the genetic material is duplicated. G₂ is committed to checking the DNA integrity. During M, the

The eukaryotic cell division cycle of somatic cells is divided into four phases (Figure 1). The cell cycle starts with the first gap phase (G₁), during which the cell grows in size and prepares for DNA replication. G₁ is followed by the DNA synthesis (S) phase, which results in a duplication of the chromosomes. Thereafter the second

gap phase (G₂) follows, and during G₂ the cell checks the integrity of the DNA and prepares for cell division. Together, the G₁, S and G₂ phases are referred to as interphase. The last phase in the cell division cycle is mitosis (M), during which cytokinesis and chromosome segregation occur, resulting in the generation of two daughter cells that enter G₁ phase. This completes one round of the cell division cycle is completed. If the surrounding environment is not favourable for proliferation, early G₁ cells exit the cell cycle and enter a quiescent state, denoted G₀ (Pardee 1974; Temin 1971; Zetterberg and Larsson 1985). If the surrounding conditions once again become advantageous for proliferation cells will re-enter the cell division cycle, but only after several hours in the presence of a proliferation promoting environment (Zetterberg and Larsson 1985). Cell cycle arrest can also occur at certain checkpoints in G₁ and G₂, where the cell verifies for example its DNA integrity (Andreassen *et al.* 2003; Kastan and Bartek 2004).

Cyclins and cyclin dependent kinases drive cell cycle progression

Central processes of the cell division cycle are regulated by protein complexes comprised of a regulatory unit, cyclin, and a catalytic unit, cyclin dependent kinase (cdk). The cyclins were initially discovered in *Arbacia punctulata* (sea urchin) embryos as proteins that accumulated in interphase and were destroyed during mitosis (Evans *et al.* 1983). The catalytic cdk:s were originally discovered as cell cycle regulators in the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Hartwell *et al.* 1974; Nurse 1975). Cdk:s are serine/threonine protein kinases that phosphorylate target proteins essential for cell cycle progression (Pavletich 1999). They are expressed throughout the cell cycle and their activity is tightly regulated at no less than four levels: (i) Synthesis and degradation of the regulatory cyclins (Pavletich 1999). As reflected in their name the majority of the cyclins are accumulated and degraded in a cyclic fashion. They bind and activate cdk:s as well as influence cdk substrate specificity. (ii) Phosphorylation of a conserved cdk threonine residue of the cyclin-cdk complex by cdk activating kinase (CAK) further increases the kinase activity of the complex 100-fold (Pavletich 1999). (iii) Inactivating phosphorylation by the kinases Myt-1 and Wee-1 takes place at Thr¹⁴ and Tyr¹⁵ of the cdk respectively, and reduces the kinase activity of the complex (Lee and Yang 2001). To oppose the action of Myt-1 and Wee-1, dephosphorylation of these sites by a phosphatase called cell division cycle protein 25 (cdc25) leads to reactivation of the

cyclin-cdk complex (Lee and Yang 2001). (iv) Binding of cdk inhibitors (CKI:s) also impairs kinase activity (Pavletich 1999). There are two families of CKI:s, the INK4 (inhibitor of cdk4) and the CIP/KIP (cdk interacting protein/cdk inhibitory protein) inhibitors. The INK4 inhibitors include p16^{INK4a}, p15^{INK4b}, p18^{INK4c} and p19^{INK4d} and only restrain activity of cdk4 and cdk6. The CIP/KIP inhibitors include p21^{Cip1} and p27^{Kip1} and interact with a broader range of cdk:s.

G₁ phase

In G₁ cells decide whether to divide or to exit the cell cycle. The decision is made during the first phase of the cell division cycle since it would be disadvantageous and potentially dangerous to spend large quantities of energy on DNA duplication, ribosome biogenesis and protein synthesis unless the cell will complete the cell division cycle correctly and divide into two daughter cells, each with an intact genome. The decision is based on availability of nutrients and growth factors in the external environment surrounding the cell during early G₁. Thereafter the cell traverses a G₁ checkpoint called the restriction point (R) and becomes independent of extracellular growth factors for cell cycle progression (Pardee 1974; 1989; Temin 1971). Progression through G₁ is controlled by cyclin D-cdk4/6 and cyclin E-cdk2 activity (Weinberg 1995). Phosphorylation of their main substrate, the retinoblastoma tumour suppressor (pRb), is essential for G₁ progression. The molecular background for the R point is not known, but phosphorylation of pRb by cyclin D-cdk4/6 has been suggested as a possible mechanism (Bartek *et al.* 1996; Lukas *et al.* 1996; Weinberg 1995). The work presented in this thesis was performed to increase the understanding of the control mechanism involved in G₁ progression.

The retinoblastoma pathway

The retinoblastoma (RB) pathway is a major regulatory pathway of the G₁ phase (Figure 2). Retinoblastoma is a rare childhood cancer of the eye and in the eighties a common lesion of retinoblastomas, located on chromosome 13, was discovered. The corresponding gene, *the retinoblastoma susceptibility gene (RB)*, was the first tumour suppressor gene to be characterised (Friend *et al.* 1986; Fung *et al.* 1987; Lee *et al.* 1987). Since then it has been discovered that the *RB* gene is involved in a pathway that largely regulates progression through G₁ phase (Bartek *et al.* 1997) and

consequently is referred to as the RB pathway. Mutations of the tumour suppressors and proto-oncogenes involved in the RB pathway occur in more than 80% of human cancers, and mutations or deletions leading to constitutive activity of the RB pathway has been suggested as a requirement for tumour formation (Hahn and Weinberg 2002; Ortega *et al.* 2002; Sherr and McCormick 2002). The retinoblastoma gene product (pRb) prevents premature cell cycle progression by repressing the transcription of genes necessary for progression through G₁ and for S phase entry (Bartek *et al.* 1997).

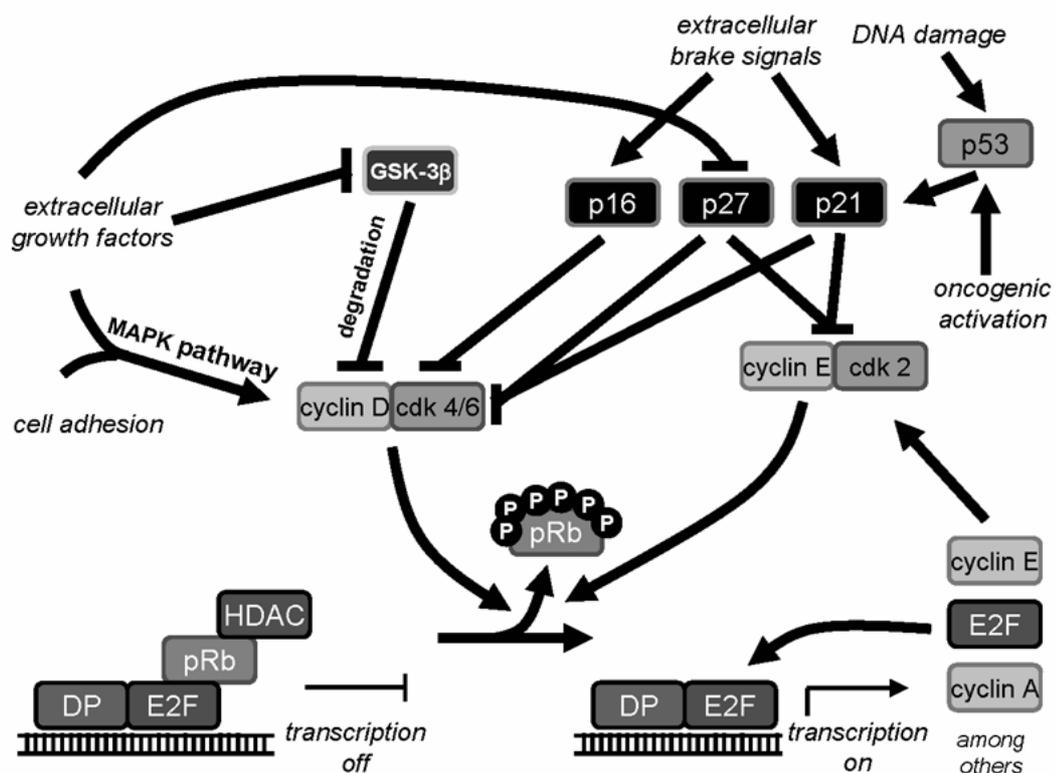


Figure 2. The RB pathway regulates progression through the G₁ phase. Progression through G₁ is controlled by phosphorylation of pRb by cyclin D-cdk4/6 and cyclin E-cdk2. In early G₁ pRb is hypophosphorylated and active as a transcriptional repressor of E2F regulated genes, through interaction with E2F and chromatin remodelling complexes (here represented by HDAC). In late G₁ pRb becomes hyperphosphorylated and S phase entry is triggered. Cell adhesion and extracellular growth factors cause accumulation of cyclin D-cdk4/6 complexes through the MAPK pathway, promoting cell cycle progression. Absence of growth factors causes degradation of cyclin D and cell cycle arrest. Progression through the cell cycle can also be halted through the action of CKI:s, induced by extracellular brake signals or DNA damage.

The retinoblastoma tumour suppressor protein

Transcriptional repression by pRb is to a large extent achieved through physical interaction of pRb with the E2 promoter binding factor (E2F) family of transcription factors (Bartek *et al.* 1997; Flemington *et al.* 1993; Harbour and Dean 2000)

(Figure 2). E2F was originally discovered as a cellular factor required for the early region 1A (*E1A*) transforming protein of adenovirus to activate transcription of the *E2* promoter (Kovesdi *et al.* 1986; 1987; Loeken and Brady 1989). It was later discovered that E2F, together with its heterodimeric partner DP (E2F dimerisation partner), controls expression of various cellular proteins, including proteins involved in (i) cell cycle regulation such as cyclin E (DeGregori *et al.* 1995; Geng *et al.* 1996; Ohtani *et al.* 1995), cyclin A (DeGregori *et al.* 1995; Schulze *et al.* 1995), cdk1 (also known as cell division cycle protein 2 [*cdc2*]) (DeGregori *et al.* 1995; Furukawa *et al.* 1994), *cdc25A* (Chen and Prywes 1999) and E2F itself (Neuman *et al.* 1994; Sears *et al.* 1997) (Figure 2), (ii) nucleotide biosynthesis (DeGregori *et al.* 1995; Hiebert *et al.* 1991; Karlseder *et al.* 1996; Ogris *et al.* 1993; Wade *et al.* 1995) and (iii) the DNA replication machinery (DeGregori *et al.* 1995; Duronio *et al.* 1998; Ohtani *et al.* 1996; Ohtani *et al.* 1999; Pearson *et al.* 1991; Yan *et al.* 1998).

pRb can directly block the ability of E2F to activate transcription by binding the transactivating domain of E2F (Bartek *et al.* 1997; Harbour and Dean 2000; Helin *et al.* 1993). Interaction between pRb and E2F does not inhibit the binding of E2F to DNA and therefore, pRb-E2F complexes form at promoters where pRb can actively repress transcription by blocking activity of surrounding enhancers on the promoter (Adnane *et al.* 1995; Bremner *et al.* 1995; Harbour and Dean 2000; Neuman *et al.* 1994; Sellers *et al.* 1995; Weintraub *et al.* 1995; Weintraub *et al.* 1992). The active transcriptional repression is achieved through two different mechanisms. The first mechanism is repression of transcription through inhibiting activity of adjacent transcription factors (Luo *et al.* 1998; Ross *et al.* 1999). The second mechanism is through recruitment of chromatin remodelling proteins (Figure 2). In this case E2F only works as a tether attaching pRb to the promoter (Bremner *et al.* 1995; Sellers *et al.* 1995; Weintraub *et al.* 1995). You could say that pRb, in this case, works as a molecular equivalent of double-sided adhesive tape, which binds E2F and chromatin remodelling proteins simultaneously. Chromatin remodelling is an important mechanism in activation and inactivation of gene transcription (Kingston and Narlikar 1999) that can be achieved in several different ways.

One mechanism of chromatin remodelling in which pRb is involved is deacetylation of histones (Kingston and Narlikar 1999). Acetylation of histones, performed by histone acetyltransferases (HATs), is most commonly associated with transcriptional activation, since it allows transcription factors to access the promoter

(Harbour and Dean 2000; Kingston and Narlikar 1999). Histone deacetylase complexes (HDACs), on the other hand, remove acetyl groups from histones leading to repression of gene expression (Grunstein 1997; Hassig and Schreiber 1997; Kingston and Narlikar 1999). pRb has been associated with HDAC activity *in vivo* and binds three of the known HDACs (HDAC1-3) *in vitro* (Brehm *et al.* 1998; Harbour and Dean 2000; Lai *et al.* 1999; Luo *et al.* 1998) indicating a role for HDACs in pRb associated transcriptional repression.

In addition, pRb is also involved in chromatin remodelling by ATP dependent nucleosome remodelling complexes. These complexes appear to influence the accessibility of chromatin to transcription factors through altering the structure and position of nucleosomes (Eberharter and Becker 2004; Muchardt and Yaniv 1999; Schnitzler *et al.* 1998; Tyler and Kadonaga 1999). The SWI/SNF (switching/sucrose non-fermentation gene product) complex is an ATP dependent nucleosome remodelling complex (Muchardt and Yaniv 1999). The helicase-like subunits brm (brahma gene product) and BRG-1 (brm/SWI2 related gene product 1) of the human SWI/SNF complex interact with pRb, and have been implicated in the transcriptional repression induced by pRb (Dunaief *et al.* 1994; Muchardt and Yaniv 1999; Singh *et al.* 1995; Strober *et al.* 1996; Trouche *et al.* 1997).

The two modes of active transcriptional repression described above do not exclude one another. On the contrary, there seems to be a role for both HDAC and SWI/SNF in pRb function, and it has been reported that pRb can interact with HDAC and SWI/SNF simultaneously (Zhang *et al.* 2000).

Mutations of *RB* is a prerequisite for the childhood cancer retinoblastoma and are also observed in a multitude of adult tumours (Sherr 1996; Sherr and McCormick 2002). *RB* mutations are especially common in small cell lung cancer, but are also frequently present in non-small cell lung cancer and sarcomas, as well as in bladder and breast carcinomas (Nevins 2001; Sherr 1996; Sherr and McCormick 2002; Weinberg 1995). Members of the E2F family of transcription factors are, in contrast to pRb, not commonly altered in human malignancies (Nevins 2001).

Cell cycle dependent regulation of pRb function

The activity of pRb as a regulator of G₁ progression is controlled by a series of serine/threonine phosphorylations, performed by the cyclin D-cdk4/6 and cyclin E/cdk2 complexes (Bartek *et al.* 1997) (Figure 2). Hypophosphorylated pRb

functions as a transcriptional repressor, and inactivation of pRb by hyperphosphorylation is required for cell cycle progression. There are 16 cyclin-cdk phosphorylation sites on pRb (Adams 2001; Harbour and Dean 2000) and complete inactivation of pRb requires both cyclin D-cdk4/6 and cyclin E-cdk2 activity (Adams 2001; Lundberg and Weinberg 1998). Cyclin D-cdk4/6 complexes phosphorylate pRb prior to cyclin E-cdk2 complexes, and cyclin D related pRb phosphorylation is moreover required for de-repression of cyclin E transcription and accumulation of cyclin E (Geng *et al.* 1996; Le Cam *et al.* 1999; Ohtani *et al.* 1995; Zhang *et al.* 2000). Once pRb is in its hyperphosphorylated, inactive state, it remains inactivated through the rest of the cell division cycle, presumably through kinase activity of cyclin A-cdk2 and cyclin B-cdk1, until cyclin-cdk complexes are inactivated in mitosis (Adams 2001; Harbour and Dean 2000).

Additionally, alterations of the proteins involved in control of pRb function also leads to deregulation of G₁ progression, and although these kinds of changes are not as common as inactivating mutations of pRb itself, they are worth mentioning. Cyclin D1 is commonly overexpressed in many malignancies, including breast carcinomas, B cell lymphoma and squamous cell carcinomas (Sherr 1996; Sherr and McCormick 2002; Weinberg 1995). Also amplification of cdk4 has been reported for some types of cancers, as well as mutations of cdk4 preventing its interaction with the INK4 CKI p16 (Sherr 1996; Weinberg 1995). Cyclin E overexpression occurs in carcinomas, lymphomas, leukaemia and sarcomas and is generally associated with an aggressive phenotype and poor clinical outcome (Hwang and Clurman 2005; Lindahl *et al.* 2004; Nielsen *et al.* 1999).

Cyclin D functions as a sensor of extracellular growth factors

The G₁ cyclins were initially discovered as regulators of G₁ progression in *Saccharomyces cerevisiae* (Cross 1988; Hadwiger *et al.* 1989; Richardson *et al.* 1989) and human cyclin D was identified through its ability to complement defective G₁ cyclins in *S. cerevisiae* (Lew *et al.* 1991; Xiong *et al.* 1991). In mammalian cells, three closely related D-type cyclins, designated D1, D2 and D3, have been identified (Sherr 1993). Cyclin D3 is expressed in most cell types, whereas cyclin D1 and D3 are somewhat lineage specific (Inaba *et al.* 1992; Matsushime *et al.* 1991).

Cyclin D represents the link between growth factors and cell cycle progression during G₁. When cells enter the cell cycle from quiescence, cyclin D is rapidly

accumulated and remains expressed as long as mitogen activation prevails (Sherr 1995). Not only the expression of cyclin D is dependent on growth factor signalling, but so is also assembly of the active cyclin D-cdk4 complex (Sherr 1995; Sherr and Roberts 2004).

Many extracellular growth factors are detected by membrane bound receptor tyrosine kinases (RTKs), which are comprised of two extracellular α -subunits and two transmembrane β -subunits. Upon ligand binding, the β -subunits become autophosphorylated, which activates receptor tyrosine kinase function (Lopez-Illasaca 1998). The active receptors phosphorylate a range of cytoplasmic target molecules, including signalling molecules that are part of complex signalling cascades or signalling networks. One important network involved in RTK signalling, resulting in cyclin D expression, is the mitogen-activated protein kinase (MAPK) pathway (Kolch *et al.* 2002; Pouyssegur *et al.* 2002).

The MAPK signalling pathway (Figure 3) starts with recruitment of the cytosolic guanine nucleotide exchange protein Sos (son of sevenless) to the activated membrane bound receptor through the docking protein Grb2 (growth factor receptor-bound protein 2). Grb2 binds phosphorylated tyrosine residues of the activated receptor via its src homology 2 domain (SH2), while it also binds Sos. Interaction of Sos with the G protein Ras (retrovirus associated sequence gene product) leads to activation of Ras through exchange of GDP to GTP (Kolch *et al.* 2002; Lopez-Illasaca 1998). Upon activation, Ras interacts with downstream effector molecules. One of the important effector molecules in the pathway leading to expression of cyclin D is the cytoplasmic serine/threonine kinase Raf, which is recruited to the cell membrane and activated by GTP-bound Ras. Active Raf subsequently phosphorylates MAPK/ERK kinase (MEK), which in turn phosphorylates and activates extracellular signal-regulated kinase (ERK), which is also referred to as p42/p44 MAPK (Lopez-Illasaca 1998; Pouyssegur *et al.* 2002). Activated ERK, which is a prerequisite for proliferation of fibroblasts (Pages *et al.* 1993), is thereafter transported to the nucleus where it exerts its effects primarily by phosphorylating a variety of transcription factors (Yang *et al.* 2003). Accumulation of active cyclin D-cdk4/6 complexes is one important downstream effect of the Ras/Raf pathway (Albanese *et al.* 1995; Lavoie *et al.* 1996).

Due to their short half lives, D-type cyclins require continuous growth factor stimulation for accumulation, and they are rapidly down-regulated upon serum withdrawal (Hunter and Pines 1994). Degradation of cyclin D occurs through ubiquitin-dependent proteolysis, which depends on glycogen synthase kinase-3 β (GSK-3 β) phosphorylation of cyclin D1 on Thr-286 (Diehl *et al.* 1998; Diehl *et al.* 1997). The activity of GSK-3 β is also regulated through the Ras signalling pathway (Diehl *et al.* 1998). In this signalling cascade, activated Ras cooperates with the phosphatidylinositol-3-OH kinase (PI3K)-Akt pathway, which down-regulates GSK-3 β activity and causes stabilisation of cyclin D (Diehl *et al.* 1998).

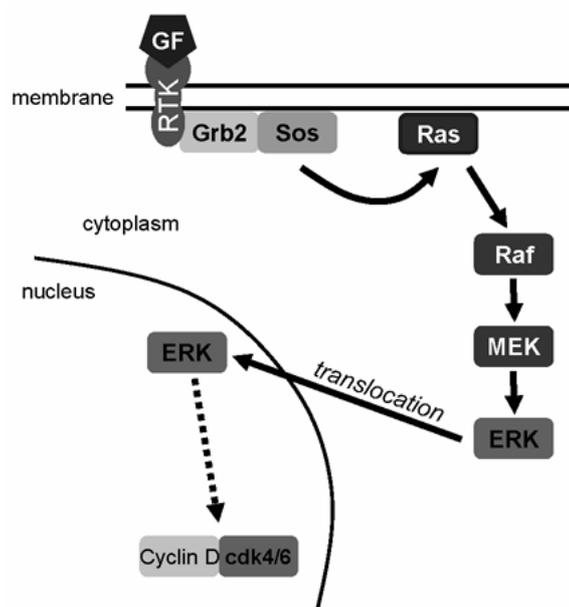


Figure 3. The MAPK pathway regulates accumulation of cyclin D-cdk4/6 complexes. Growth factors (GF) activate receptor tyrosine kinases (RTK). Grb2 binds the phosphotyrosines of RTKs and recruits Sos to the plasma membrane. Sos activates Ras, which starts signalling through Raf, MEK and ERK. Upon activation ERK gets translocated to the nucleus, where it phosphorylates a variety of transcription factors. Activated ERK eventually leads to accumulation of active cyclin D-cdk4/6 complexes.

Overexpression of cyclin D, which is mentioned above, is far from the only alteration of the growth factor signalling pathways involved in tumour formation. In addition, about 25% of human cancers have a Ras-mutation that makes mitogenic Ras signalling constitutively active, independent of the presence of growth factors. Autocrine stimulation by growth factor synthesis or overexpression of RTK:s to increase growth factor sensitivity is also common (Hanahan and Weinberg 2000).

Phosphorylation and inactivation of pRb by cyclin E

The human homolog of cyclin E was discovered in experiments similar to the ones performed when cyclin D was discovered (Koff *et al.* 1991; Lew *et al.* 1991). Accumulation of cyclin E, which is essential for S phase entry (Ohtsubo *et al.* 1995) commences after phosphorylation of pRb by cyclin D-cdk4/6 (Geng *et al.* 1996; Le Cam *et al.* 1999; Ohtani *et al.* 1995; Zhang *et al.* 2000). Cyclin E accumulates during late G₁ phase and complexes with cdk2 (Dulic *et al.* 1992; Koff *et al.* 1991). Cyclin E peaks briefly at the G₁/S transition and is degraded by ubiquitin-mediated proteolysis in early S phase (Dulic *et al.* 1992; Ekholm *et al.* 2001; Strohmaier *et al.* 2001; Won and Reed 1996).

CKI titration of cyclin-cdk complexes during G₁

In addition to phosphorylation of pRb, cyclin D-cdk4/6 complexes also promote G₁ progression through a different mechanism. By sequestering Cip/Kip type CKI:s from cyclin E-cdk2 complexes, accumulating cyclin D-cdk4/6 complexes help to activate cyclin E-cdk2 kinase activity (Sherr and Roberts 1999). A noteworthy feature is that cyclin D-cdk4/6 complexes retain their kinase activity even when associated with Cip/Kip inhibitors (Blain *et al.* 1997; Cheng *et al.* 1999). Accordingly, redistribution of Cip/Kip in this manner facilitates cell cycle progression through activation of cyclin E-cdk2 complexes and by allowing cyclin D-cdk4/6 complexes to remain active even when bound to the inhibitor. However, cyclin D-cdk4/6 activity is not entirely unaffected by Cip/Kip, but the regulation seems to be stoichiometric. High levels of Cip/Kip inhibitors causes each cyclin D-cdk4/6 complex to associate with more Cip/Kip molecules leading to inhibition of the cdk kinase activity (Blain *et al.* 1997; Kato *et al.* 1994). Conversely, if cdk4/6 specific CKI:s of the INK4 type accumulate in the cell, they interact with cdk 4/6 and disrupts the cyclin D-cdk4/6 complexes, leading to inactive cdk4/6 and liberation of Cip/Kip. The released inhibitors are then allowed to associate and inhibit cyclin E-cdk2 complexes leading to cell cycle arrest (Reynisdottir *et al.* 1995).

The restriction point

The fact that mammalian cells are dependent upon external mitogens for cell cycle progression during G₁ has been known for a long time. This requirement is only present until a certain point in G₁ (Temin 1971), denoted the restriction point (R)

(Pardee 1974; 1989), which occurs 3 to 4 hrs after exit from mitosis (Zetterberg and Larsson 1985; 1991). The R point divides G_1 into two functionally distinct sub-phases: (i) The part of G_1 that lasts from M until R is referred to as the post mitotic part of G_1 (G_1 -pm) and has a constant length of 3 to 4 hrs (Figure 4). During this period the cell assesses the amount of extracellular growth factors present in the surroundings. (ii) The part of G_1 that lasts from R until S phase is called the pre-DNA-synthetic part of G_1 (G_1 -ps). The G_1 -ps interval is presumably used for preparation for the upcoming S-phase and once the cells have passed R and entered G_1 -ps they have become committed to initiate DNA replication. This interval varies greatly in length, ranging from less than 1 hr to more than 10 hrs and accounts for most of the variability of cell cycle duration (Zetterberg and Larsson 1985; 1991) (Figure 4). The variations in G_1 -ps length is a considerable obstacle in cell cycle research since cells that are experimentally synchronized by for example mitotic shake-off or serum starvation, will only progress through G_1 -pm in a synchronized fashion and will subsequently lose synchrony after passage through R point. If the levels of extracellular growth factors are insufficient during G_1 -pm cells will not pass the R point. Instead they will exit the cell cycle and enter the quiescent state G_0 . Cells in G_0 have ceased progression through the cell cycle and only re-enters G_1 if the levels of extracellular growth factors in the surroundings increase, and then only after a considerable time period (Zetterberg and Larsson 1985).

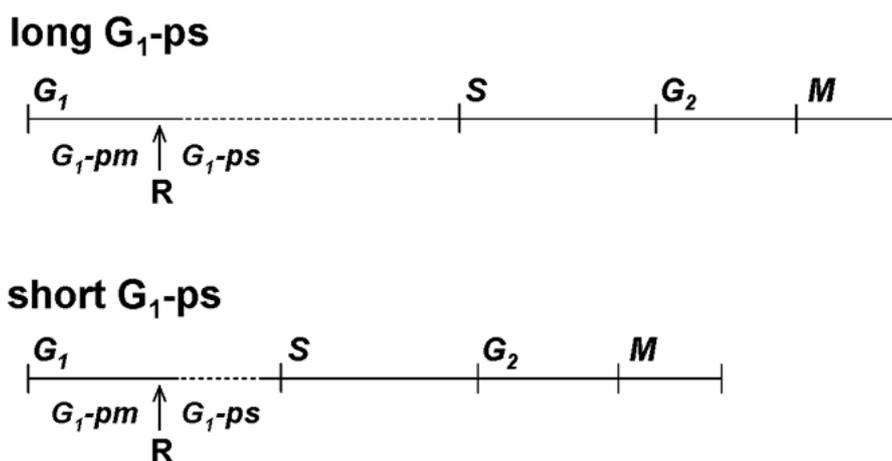


Figure 4. The R point divides the G_1 phase in two sub-phases: G_1 -pm and G_1 -ps. Most of the variability of the cell cycle time occurs during G_1 -ps, which can vary greatly in time.

The molecular mechanism behind the restriction point is unclear. It has been proposed that accumulation of a labile restriction point protein is the molecular basis for passing this checkpoint (Pardee 1974; Rossow *et al.* 1979). Both cyclin D (Bartek

et al. 1996; Lukas *et al.* 1996; Weinberg 1995) and cyclin E (Dou *et al.* 1993; Zetterberg *et al.* 1995) have been suggested as the R point protein since they are involved in the phosphorylation of pRb. However, it has been shown that cyclin E is not likely to be involved (Ekholm *et al.* 2001). Cyclin D remains a candidate regulator of the R point since it functions as a connection between the extracellular environment and cell cycle progression. The R point is often lost or aberrant in cancer cells and therefore it is of importance to elucidate its regulation on the molecular level.

Cell adhesion

Apart from soluble extracellular growth signals most normal cells also require attachment to the extracellular matrix (ECM) for cell cycle progression. Attachment is mainly mediated through a family of transmembrane receptors called integrins, which are composed of α and β chains that heterodimerise in distinct combinations that confer ligand specificity (Kumar 1998; Ruoslahti 1991). They bind ECM components, but can also bind soluble ligands or adjacent cells. Integrins link molecules of the ECM to the cytoskeleton and are involved in regulating cell shape, spreading and survival (Kumar 1998). Anchorage to ECM is involved in control of progression through G1 and entry into S through accumulation of cyclin D, ultimately leading to phosphorylation of pRb (Assoian and Zhu 1997; Bohmer *et al.* 1996).

The signalling pathways of integrins and RTK:s are intertwined and form complex networks (Schwartz and Assoian 2001). The integrin $\alpha\beta3$ is one example. It binds a number of matrix proteins associated with cell proliferation (Schwartz and Assoian 2001). The extracellular domain of $\beta3$ integrin interacts with the platelet derived growth factor (PDGF) receptor, which is a RTK, and enhances its signalling (Schneller *et al.* 1997; Schwartz and Assoian 2001). The αv subunit of the $\alpha\beta3$ integrin interacts with Shc (SH2 containing protein) which recruits Grb2 and Sos to the plasma membrane and leads to signalling via the Ras-Raf-MEK-ERK pathway (Pawson and Scott 1997; Schwartz and Assoian 2001). In addition to specific interactions of integrin with proteins involved in signalling, adhesion to ECM also causes more general effects. Integrins aggregate into organelles called focal adhesion complexes (Boudreau and Jones 1999; Kumar 1998), which leads to integrin dependent autophosphorylation of a non-receptor tyrosine-kinase called focal adhesion kinase (FAK) (Kumar 1998). Phosphorylation of FAK enables it to interact

with a number of adaptor proteins, including Grb2/Sos, thereby linking integrins to activation of the Ras-Raf pathway (Boudreau and Jones 1999; Kumar 1998). Moreover, it has been shown that a number of RTK:s, including receptors for PDGF and epidermal growth factor (EGF), are recruited to focal adhesion complexes, resulting in increased sensitivity of the cell to extracellular growth factors (Boudreau and Jones 1999).

If normal cells loose attachment, integrin signalling is deactivated, which leads to decreased signalling through the Ras-Raf-pathway, and eventually a G₁ arrest is induced. As described, signalling through both RTK:s and integrins induce the Ras-Raf-pathway, and the sustained activation necessary for cyclin D expression requires joint signalling of both integrins and RTK:s (Schwartz and Assoian 2001).

As mentioned previously, Ras is often mutated in cancer leading to permanent activation of the Ras/Raf pathway. In addition, cancer cells also change the types of integrins they express to those that favour cell cycle progression (Hanahan and Weinberg 2000).

Stopping G₁ progression

Cells have to be able to stop cell cycle progression in response to certain intrinsic or extracellular signals, and this is generally achieved by accumulation of CKI:s. However, the exact molecular response varies with the different brake signals. Absence of extracellular growth factors leads to ubiquitin-dependent proteolysis of cyclin D (Diehl *et al.* 1998; Diehl *et al.* 1997; Hunter and Pines 1994), which holds back cell cycle progression through G₁. Lack of growth factors also leads to accumulation of p27^{Kip1} by inhibiting its degradation (Hengst and Reed 1996; Pagano *et al.* 1995), which leads to inhibition of cdk activity and cell cycle arrest (Figure 2).

The cell also responds to extracellular growth inhibitory signals, e.g. interferon- α and transforming growth factor β (TGF- β), which both induce both the Cip/Kip and INK4 families of CKI:s (Figure 2). Interferon- α induces G₁ arrest by upregulation of p21^{Cip1}, p15^{INK4b} and p16^{INK4a} (Sangfelt *et al.* 1997) while TGF- β causes activation of p21^{Cip1} and p15^{INK4B} and (Kang *et al.* 2003; Seoane *et al.* 2001) (Figure 2).

G₁ progression can also be halted in response to DNA damage. DNA damage is detected by ATM (ataxia telangiectasia, mutated gene product) and ATR (ataxia

telangiectasia and Rad3 related gene product), which phosphorylates a variety of substrates, including p53 and Chk2 (Barzilai and Yamamoto 2004; Kastan and Lim 2000) (Figure 2). The activated Chk2 further phosphorylates p53. Phosphorylation of transcription factor p53 inhibits its interaction with MDM2 (mouse double minute 2), which normally targets p53 for degradation. Phosphorylation also makes p53 transcriptionally active, which leads to accumulation of p21^{Cip1} and G₁ arrest (Figure 2).

Oncogenic activation leads to induction of two genes at the INK4a locus: p16^{INK4a} and p14ARF (Serrano *et al.* 1997). p16^{INK4a} inhibits cdk activity and p14ARF inhibits binding to p53, which causes general inhibition of cdk kinase activity by induction of p21^{Cip1} (Figure 2).

Cell growth and cell proliferation

Cell growth is a process different from cell proliferation and it occurs by accumulation of mass through biosynthesis of macromolecules. The two separate processes of cell growth and cell proliferation are generally well coordinated during normal division of a somatic cell in order to preserve cell size (Killander and Zetterberg 1965). Deviation from this coordination leads to abnormal cell size, with cells that are either larger or smaller than normal, eventually resulting in cell death (Neufeld and Edgar 1998). In order to produce enough protein to maintain cell size proliferating cells spend as much as 80% of their energy on ribosome biogenesis (Schmidt 1999). In addition, control of rRNA synthesis, which is the rate limiting step in biosynthesis of ribosomes, seems to be linked to control of cell proliferation (Grummt 2003). The rate of rRNA synthesis occurs at about double the rate at the end of the cell cycle as compared to the beginning (Zetterberg and Killander 1965a), which is explained by the fact that the cell duplicates its DNA during S phase and thereby also doubles the number of templates available for rRNA synthesis (Neufeld and Edgar 1998). Since the rate of protein synthesis and accumulation is limited by the existing number of ribosomes (Zetterberg and Killander 1965b) the rate of protein synthesis closely follows the rate of ribosome synthesis and also becomes close to doubled during the course of the cell cycle (Zetterberg and Killander 1965a).

The link responsible for coordination of the processes of cell growth and cell proliferation is still not fully elucidated, but some connections have been made. The

G₁ cyclins D and E have been shown to regulate transcription of rRNA (Voit and Grummt 2001; Voit *et al.* 1999) and the mitotic cyclin B is important for repression of rRNA synthesis during M phase (Heix *et al.* 1998; Kuhn *et al.* 1998; Sirri *et al.* 2000). The important G₁ regulator pRb has also been ascribed a role in regulating rRNA biosynthesis through obstructing assembly of the transcription initiation complex (Ciarmatori *et al.* 2001; White 2005; Voit *et al.* 1997). Moreover, a number of cell cycle regulatory proteins, among them cyclin D and cdk4, have complicated 5' mRNA leader sequences and are translationally controlled in such a way that they are only efficiently expressed in conditions that favour cell growth (Neufeld and Edgar 1998).

Aim of the study

The purpose of this study was to perform a biological and molecular characterisation of G₁ checkpoints.

The specific aims were:

1. To examine if major phosphorylation of pRb is the molecular mechanism behind the R point.
2. To investigate if the R point mechanism is related to events that occur during mitosis.
3. To study physical interactions between cyclin D and pRb in relation to the R point and to phosphorylation of pRb.
4. To investigate the connection between rRNA synthesis, cell growth and cell cycle control.

Material and methods

A detailed description of all materials and methods used in this thesis are presented in Paper I-IV. Therefore, the following chapter only lists some methods together with brief comments.

Cell culture

Early passage human diploid fibroblasts (HDF) of skin origin were used in Paper I-IV. Mouse Swiss 3T3 fibroblasts were used in Papers II and III. Cell lines were maintained in a humidified atmosphere containing 7.5% CO₂. HDF cells were cultured in a 1:1 mixture of Minimum Essential Medium (MEM) and Ham's F-12 supplied with 10% foetal bovine serum (FBS) and antibiotics. Swiss 3T3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplied with 10% FBS and antibiotics.

Reagents

The thymidine analogue bromodeoxyuridine (BrdU) was incubated with cells at a concentration of 20 μM to label newly synthesized DNA. Incorporated BrdU was thereafter detected using immunofluorescence.

To inhibit biosynthesis of ribosomes we used Actinomycin D (AMD), which is a cytostatic drug that specifically blocks synthesis of ribosomal RNA (rRNA) when administered at low doses.

Immunofluorescence staining

Immunofluorescence labelling was used for determination of levels of protein, protein phosphorylation and BrdU incorporation in cells. Staining was performed using standard protocols. The major methodological drawback of this method is that it is only semi-quantitative. There is only a rough correlation between fluorescence staining intensity and antigen content since epitope availability is affected by variations in fixation and sample preparation. Fixation and permeabilisation are

critical steps and were optimised for detection of each antigen. All immunofluorescently labelled cells were mounted in medium containing 4',6'-diamidino-2-phenylidole (DAPI), which produces a blue fluorescence when bound to DNA.

Fixation and permeabilisation for immunocytochemistry

The following fixations were used:

Methanol fixation: fixation in methanol for 60 min at room temperature.

4% formalin fixation: fixation in 4% formalin for 10 to 60 min at room temperature followed by permeabilisation in 0.2% Triton X-100 in phosphate buffered saline (PBS) for 2 min.

1% formalin fixation: fixation in a solution containing 1.0% formalin, 100 mM phosphate buffer (pH 7.5) and 1.0 mM MgCl₂ for 10 min, followed by 70% ethanol over night at 4°C. Permeabilisation was thereafter performed using 0.2% Triton X-100 in PBS for 2 min.

Methanol acetone fixation: fixation in methanol for 5 minutes followed by acetone for 2 min, both at -20°C.

Table 1. Summary of fixations used.

| <i>antigen</i> | <i>fixation</i> | <i>Paper</i> |
|---------------------------------|---------------------------|--------------|
| pRb | methanol fixation | I |
| | 4% formalin fixation | I and IV |
| | 1% formalin fixation | III |
| phospho-Ser ⁷⁹⁵ -pRb | 4% formalin fixation | I and IV |
| | methanol acetone fixation | I |
| phospho-Ser ⁷⁸⁰ -pRb | 4% formalin fixation | I |
| cyclin E | methanol fixation | IV |
| | 4% formalin fixation | I |
| cyclin A | methanol fixation | I and IV |
| BrdU | methanol fixation | I |
| cyclin D1 | 1% formalin fixation | III |
| | methanol acetone fixation | III and IV |
| cyclin D3 | 1% formalin fixation | III |
| | methanol acetone fixation | III and IV |

Organic solvents such as alcohols or acetone dehydrate cells, precipitate proteins and remove lipids, whereas formalin fixation forms intermolecular crosslinks through free aminogroups, which results in a network of linked antigens. Crosslinking preserves the structural integrity better than organic solvents, but may reduce antigenicity of proteins and require a permeabilisation step to allow access of antibody to the antigen. For an overview of which fixation method was used for each of the specific antigens refer to Table 1.

For detection of incorporated BrdU a denaturing treatment of the fixed cells was required for the antibody to access the antigen. The cells were therefore incubated with a denaturing solution containing 70 mM NaOH in 15% acetone-15% methanol-water mixture for 45s at 4°C and thereafter washed briefly with a neutralising solution containing 70 mM HCl.

Analysis of immunofluorescently labelled cells

Fluorescence intensities of the labelled cells in Paper I, III and IV were analysed in one of two ways. The first and most straightforward method of analysis was visual inspection in a fluorescence microscope. The second, more objective, method of analysis involved digital image analysis, where the relative levels of protein or protein phosphorylation were calculated from images of the stained cells. This procedure is further described below. Protein localisation was determined in Paper III using three-dimensional (3D) deconvolution microscopy (described below).

Time-lapse video-microscopy

We used time-lapse video-microscopy (TLV) to investigate cell cycle behaviour of single cells. This technique was also used to study cell shape and for immunofluorescence intensity measurements to determine protein levels or protein phosphorylation status, in relation to the cell division cycle. TLV enables analysis of single cells in an unperturbed, asynchronously growing cell population. The advantage of single cell analysis over synchronisation methods, where population averages are measured and valuable information about cell variability is lost, is that single cell analysis allows for detection of intercellular variability in cell cycle progression.

To facilitate TLV analysis, cells were seeded at a relatively low density to keep the cells well separated. Cells were grown on coverslips with an engraved grid to allow identification of single cells (Figure 5). Cells were TLV recorded using an inverted microscope equipped with a CCD camera and a scanning table. The equipment was placed in an incubator. Humidity was omitted to protect the equipment and the petri-dish containing the coverslips with the cells was wrapped with parafilm to avoid evaporation. Thereafter 9 (3×3) or 25 (5×5) images were recorded every 4 or 6 min for the duration of the experiment. This allowed the analysis of 200 to 500 individual cells from every film.

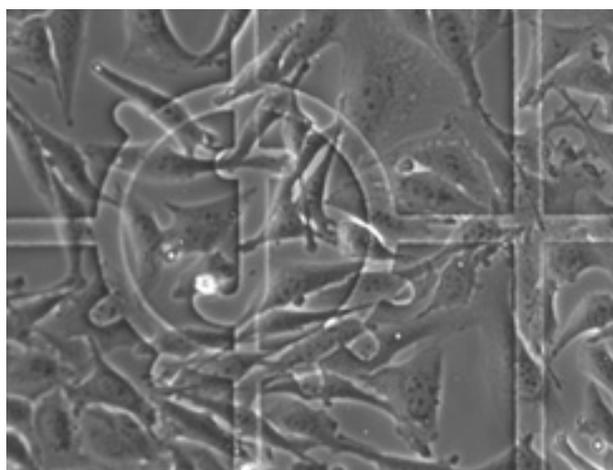


Figure 5. Image from a TLV film. Cells were seeded at low density onto gridded coverslips and images are acquired every 4 or 6 min.

Cell cycle analysis

To analyse changes in cell cycle behaviour of single cells (Papers I, III and IV), cells cultured in normal medium were filmed for 24 hrs and thereafter subjected to a change of medium. The medium was changed to either normal medium, to medium with low/no FBS and/or to medium containing a drug. Subsequently the cells were filmed for an additional 48 hr period. From the acquired film the cell age post mitosis at the change of medium and the time lapsed to the next mitosis (generation time) was analysed (Figure 6).

A similar TLV analysis was also performed in Paper II, but in this case, cells were filmed while subjected to treatment with medium containing low FBS or medium with low FBS supplemented with PDGF for a limited time (4 hrs) and thereafter returned to normal cell medium.

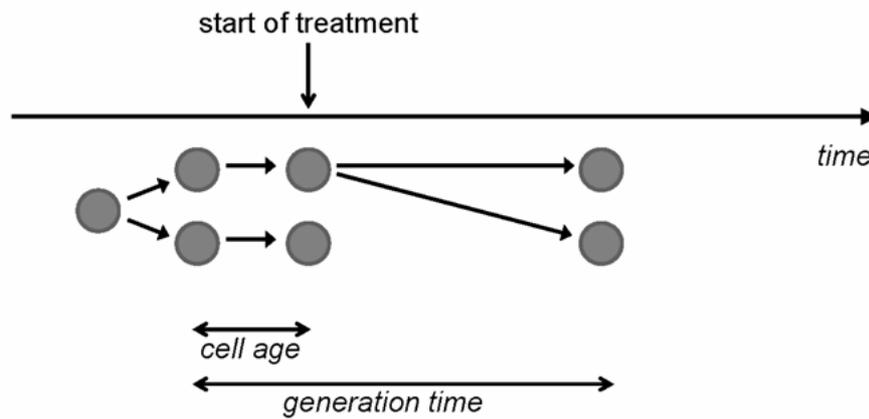


Figure 6. Cell cycle analysis. Cell age (post mitosis) at start of treatment and time to next mitosis (generation time) was calculated from the TLV film.

Analysis of cell shape

Analysis of cell shape in Paper II was performed on cells that were filmed for various times. The cells were subjected to normal cell medium, medium with low or no FBS or medium with low FBS supplemented with either (i) PDGF at 50 ng/mL, (ii) insulin like growth factor 1 (IGF-1) at 10 ng/mL or (iii) epidermal growth factor (EGF) at 25 ng/mL during the time of the TLV experiment. Cell shape and projected cell area in relation to cell age post mitosis in response to the different treatments was investigated.

Age determination before analysis of fluorescence intensity

For semi-quantitative analysis of protein levels (Paper I, III and IV) or protein phosphorylation (Paper I and III) in relation to the cell age, cells were filmed for 17 hrs and subjected to fixation in accordance with the analysed antigen (see Table 1). The cell age of each single cell at the time of fixation was calculated from the TLV film (Figure 7). Immunofluorescence staining was performed to assess levels of proteins or protein phosphorylation in relation to cell age post mitosis. Digital images of the stained and age-determined cells were acquired for image analysis as described below.

In situ extraction of hyperphosphorylated pRb

The hypophosphorylated form of pRb, which is active as a transcriptional repressor, is tightly associated with the nuclear structure and is not easily extracted. As pRb

becomes phosphorylated its association with the nuclear structure weakens and hyperphosphorylated pRb is easily eluted from cells using a hypotonic buffer. This property of pRb was used to determine phosphorylation status of pRb in Papers I and IV. Cells were washed in PBS at 37°C, rinsed briefly in distilled water at room temperature and then incubated on ice together with cold (4°C) hypotonic buffer containing 10 mM HEPES-KOH (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol and 0.1% Triton X-100 for 10 min. After the extraction procedure, the cells were fixed and subjected to immunofluorescence staining with an anti-pRb antibody, which in this case detected hypophosphorylated pRb that remained tightly bound to the nuclear structure after the *in situ* extraction.

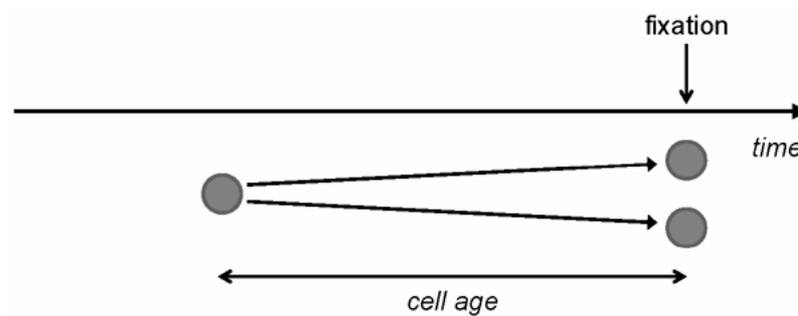


Figure 7. Age determination of fixed cells. Cell age post mitosis at the time of fixation was calculated from the TLV film.

Determination of cytoplasmic RNA levels

Acridine Orange (AO) is a dye used for staining intracellular nucleic acids. It binds both RNA and DNA, but due to differences in binding, fluorescence of the dye occurs at different wavelengths depending on which nucleic acid it is bound to. In Paper IV we used AO to determine the amount of RNA that was present in the cytoplasm after treatment with AMD, as compared to untreated cells. Cells were either fixed in 4% formalin prior to the staining, or fixed as the final step in the staining procedure by adding formalin to the staining solution to a final concentration of 1.5%. Results from both fixation methods were in good agreement. The first step in the staining procedure was permeabilisation for 45 s using a solution containing 0.08 M HCl, 0.15 M NaCl and 0.1% Triton X-100. The second step was incubation for 20 min with the staining buffer, which contained 0.1 M/0.2 M citrate-phosphate buffer, 2.0 mM EDTA and AO at a concentration of 8 µg/mL. If fixation was performed as part of the staining procedure, incubation for 10 min was performed together with the

fixative. After mounting, images of the stained cells were obtained and analysed digitally as described below.

Image acquisition and digital image analysis

Images of cells stained by immunofluorescence or stained with AO were acquired by using a CCD-camera coupled to a fluorescence microscope. Images of labelled cells in asynchronously growing populations (Paper III and IV) were acquired from randomly chosen areas. For TLV filmed and labelled cells, images were obtained of cells that had been age determined using the TLV data (Paper I, III and IV).

Intensity measurements

For intensity measurements of immunofluorescently stained cells in Paper I, III and IV, two or three wavelength images (DAPI-Cy3, DAPI-FITC or DAPI-Cy3-FITC) were acquired for one focal plane. Specialised computer software was used to assess the relative protein levels from the fluorescence intensities. The software segmented the nuclei from the DAPI image and thereafter, both the Cy3 and FITC fluorescence intensity over each single nucleus was calculated. Since all proteins analysed were nuclear, measurement of the intensity in the nucleus was sufficient to determine protein levels.

In order to measure RNA levels using AO staining (Paper IV), two wavelength images (DNA and RNA) were acquired from one focus plane. The images were analysed using software specially designed for the task. The user defined cells where the cytoplasms did not overlap and the software segmented the nucleus from the DNA image and the whole cell (nucleus plus cytoplasm) from the RNA image. The segmented area representing the nucleus was thereafter subtracted from the segmented area representing the whole cell, rendering a segment representing the cytoplasm. The segmented area representing the cytoplasm was thereafter used to calculate the cytoplasmic RNA specific fluorescence from the original image, giving one RNA fluorescence value for each single cell.

Deconvolution microscopy

Deconvolution microscopy is a 3D microscopy technique that uses a computational method to reduce out-of-focus fluorescence. This technique was employed for the

localisation studies described in Paper III. The advantage of deconvolution microscopy over other 3D microscopy techniques, like for example confocal microscopy, is that image data can be collected at very low light intensity levels, allowing image acquisition at multiple focal-planes over long periods of time.

Images of a number of focal planes at 0.2 μm distance from each other were captured using a Delta Vision system, creating what is called a z-stack. The z-stack was thereafter processed via a constrained iterative deconvolution algorithm using the Delta Vision Softworks package.

Determination of cell ploidy and total protein content

Measurement of cell ploidy (DNA content) and total protein content of single cells in Paper IV was carried out by Feulgen and Naphthol Yellow (NY) staining respectively. Feulgen staining was performed either alone or before NY staining, while NY staining always was performed in sequence with Feulgen staining. Both Feulgen and NY stained cells were analysed using image absorption cytometry of single cells. Cells were visualised on a computer screen using a prototype high resolution instrument with a CCD camera coupled to a microscope. An image analysis program was used to segment single cells defined by the user and to measure the absorbance of the cells.

Results and discussion

Paper I

The restriction point, which is the G₁ serum checkpoint, is often lost or defective in cancer cells. The mechanism that controls the R point at the molecular levels is not known. However, it has been suggested that the molecular mechanism behind R point passage is hyperphosphorylation of pRb (Bartek *et al.* 1996; Lukas *et al.* 1996; Weinberg 1995). This suggestion is based on studies of cell populations, which only allows comparison of the population averages. However, information about single cells and cellular variations, that can be useful when studying kinetics in relation to the cell cycle, is lost in the averaging process. Single cell analysis, on the other hand, gives high resolution when investigating temporal relationships of G₁ events, and also provides information about cellular variation. Therefore we decided to perform a single cell study investigating the temporal relationship between passage through the restriction point and hyperphosphorylation of pRb. This was done using a combination of TLV and semi-quantitative immunocytochemistry. Four different criteria for pRb phosphorylation were analysed in age-determined cells: (i) Phosphorylation status of the cyclin D-cdk4/6 dependent phosphorylation sites Ser⁷⁹⁵ and Ser⁷⁸⁰ were investigated using two sets of phosphospecific antibodies directed towards these sites. We discovered that cyclin D associated phosphorylation of pRb on Ser⁷⁹⁵ and Ser⁷⁸⁰ occurred in cells that had passed the R point and the kinetic observed for the two phosphorylation events were similar (Figure 8A). (ii) Since interaction of pRb with the E2F family of transcription factors is abrogated upon phosphorylation of pRb we employed an E2F-1 antibody that detects the pRb binding domain of E2F-1. This antibody only recognises E2F-1 that is not bound to pRb, and works as a more functional assay of pRb phosphorylation status. Free E2F-1 was detected in an increasing fraction of G₁-ps cells (i.e. cells after R) (Figure 8B) (iii) The degree of association of pRb to the nuclear structure, which is a property of pRb that is closely related to phosphorylation status, was used to study the phosphorylation status of pRb through *in situ* extraction of hyperphosphorylated pRb. We observed that the presence of hypophosphorylated, active pRb was detected in a decreasing number of cells that had passed R (Figure 8C) (iv) As a final measure of

pRb phosphorylation we examined accumulation of cyclin E, which is a process that is dependent on pRb phosphorylation by cyclin D-cdk4/6. As observed previously (Ekholm *et al.* 2001) cyclin E accumulated in cells that had already passed R (Figure 8D). In summary, all four different criteria show that passage through the R point and phosphorylation of pRb are temporally separated events in G₁. We found that R point passage precedes pRb phosphorylation and that a gradually increasing number of G₁-ps cells display phosphorylated pRb as the cells progress through the G₁ phase.

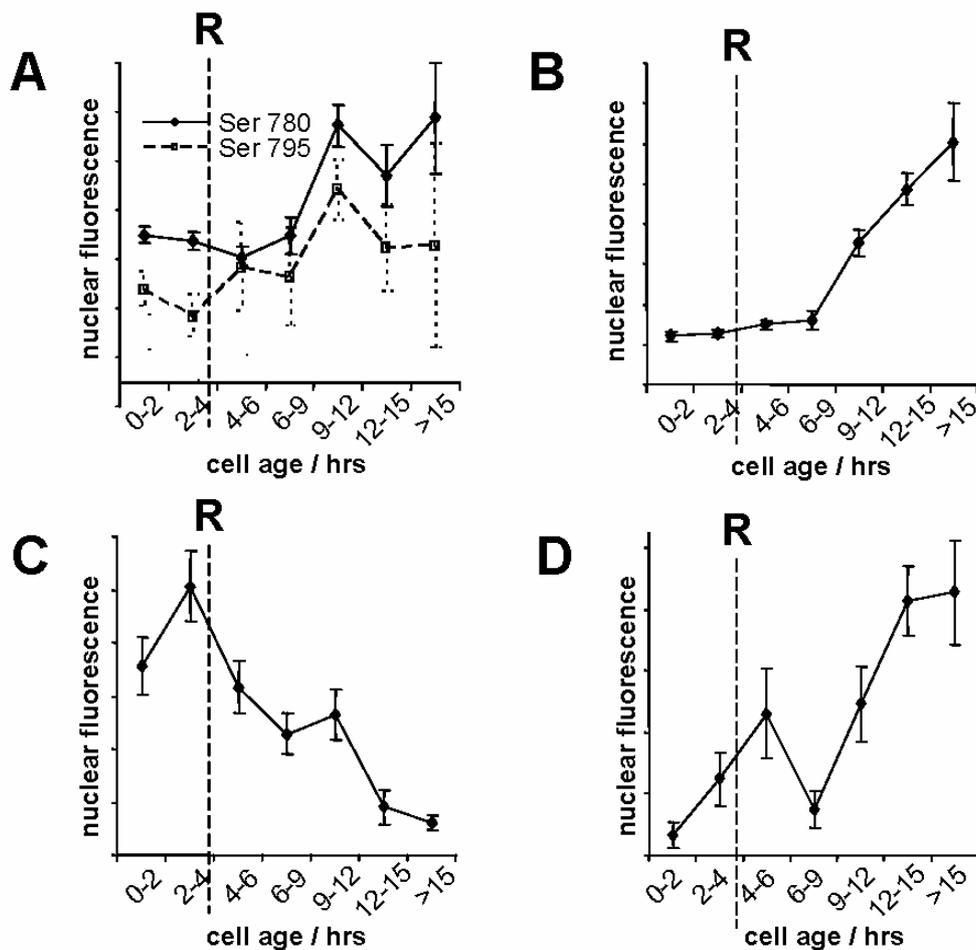


Figure 8. Phosphorylation of pRb occurs after passage through the restriction point. Phosphorylation on residues Ser⁷⁹⁵ and Ser⁷⁸⁰ is displayed in (A), detection of the pRb binding domain of E2F-1 is shown in (B), the amounts of tightly bound, hypophosphorylated pRb are illustrated in (C) and the accumulation of cyclin E is presented in (D). The x axes show the cell age after mitosis and the y axes show the average nuclear fluorescence of all measured cells within an age interval. R together with a broken line illustrates passage through the R point.

The main conclusion from this study is that pRb phosphorylation is an event downstream of R point passage, and that pRb phosphorylation therefore is not

involved in the molecular mechanism governing the G₁ serum checkpoint. Instead, analysis of pRb phosphorylation together with cyclin A as a marker of S phase entry (Erlandsson *et al.* 2000), indicates that pRb phosphorylation may be related to S phase entry (as illustrated in Figure 9). In fact, the R point and pRb phosphorylation seems to constitute two separate checkpoints in G₁, and consequently the mechanism behind the restriction point remains to be elucidated.

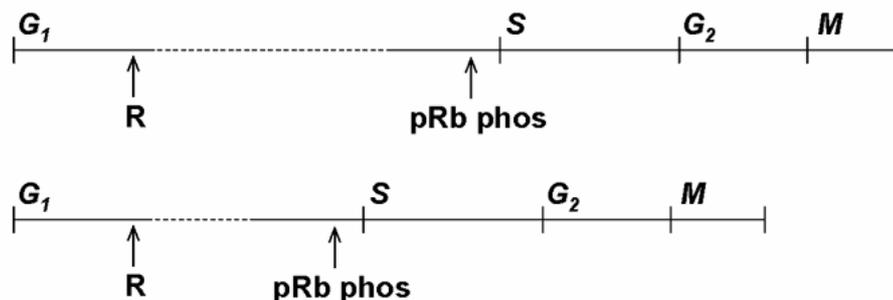


Figure 9. Phosphorylation of pRb seems to be closely connected to S phase entry. Here the timing of passage through the restriction point and phosphorylation of pRb are illustrated for a long and a short cell division cycle. Most of the cell cycle variability occurs in G₁ as illustrated by the broken line. Passage through the restriction point occurs at a fixed time after mitosis while phosphorylation of pRb is variable in relation to exit from mitosis, but fixed in relation to S phase entry.

Paper II

The fact that cells pass the R point at a set time (3 to 4 hrs) after mitosis (Larsson *et al.* 1989; Zetterberg and Larsson 1985; 1991; Zetterberg *et al.* 1995) implies that passage through R might be related to metabolic and/or structural changes that occur during the previous mitosis. Therefore we decided to look for a possible mechanism for R that was related to mitosis. In a study of single cells by TLV we discovered that serum starvation of cells caused a transient change of shape in a subpopulation of cells. These cells changed shape from flat to spherical in the absence of serum and partially lost contact with the ECM (compare Figure 10A and B). To further examine this phenomenon we used the projected cell area as a measure of cell shape based on the fact that a flat cell will display a larger projected cell area than a spherical cell. We found that in a population of cells growing in medium supplied with serum, cells have small projected areas only during and around mitosis (Figure 10C). In contrast, in a cell population subjected to serum starvation for 60 min, cells that have not passed the R point display small projected cell areas, whereas cells after the R point

have projected areas equivalent to cells supplied with serum (Figure 10C and D). In conclusion this morphological property only occurs for G₁ cells that have not passed the R point (i.e. G₁-pm cells) and these cells exit the cell cycle upon serum withdrawal. The shape of cells that have progressed past R (i.e. cells in G₁-ps, S and G₁) is unaffected, and these cells also continue progression through the rest of the cell division cycle.

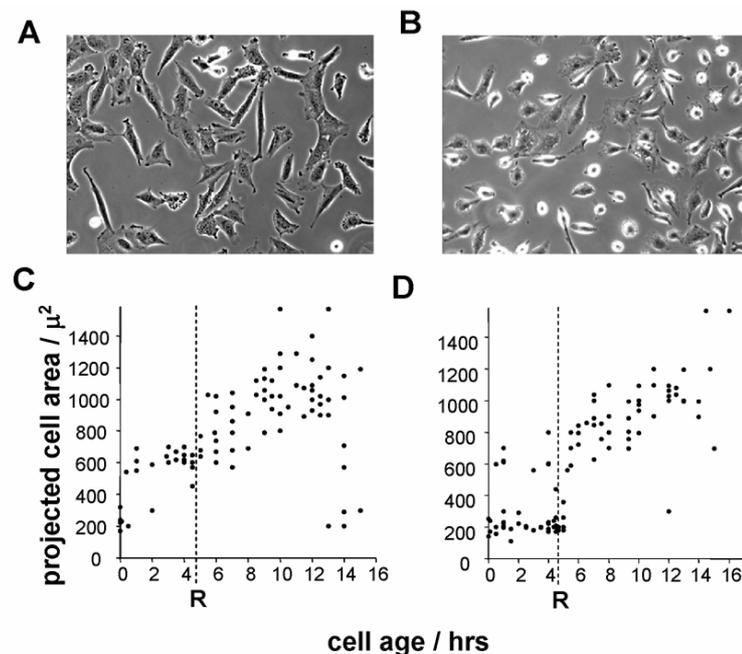


Figure 10. A change in cell shape of G₁-pm cells is associated with serum starvation.

Most cells growing in medium supplied with serum are flat (A) whereas a substantial subpopulation of serum deprived cells transiently change shape and become more or less spherical (B). Cells grown in the presence of serum display projected cell areas that increase with increasing age after mitosis, and only mitotic cells (or cells close to entry to or exit from mitosis) display the small projected cell areas of spherical cells (C). Cells that are deprived of serum display for 60 min small projected cell areas for cells that have not passed the R point, whereas cells that have passed R have projected cell areas corresponding to cells of the same age that are supplied with serum (D). In (C) and (D) the x axis represents the cell age and the y axis represents the projected cell area.

Since addition of PDGF during serum starvation counteracted cell cycle exit, we wanted to examine its effect on the up-rounding of G₁-pm cells after serum withdrawal. Interestingly, we observed that addition of PDGF counteracted the change in cell shape of G₁-pm cells induced by serum withdrawal. EGF and IGF-1 partially counteracts cell cycle exit in the absence of serum, and we found that addition of either of these growth factors to the starvation medium only partially counteracted the change of cell shape of G₁-pm cells. Together these data show that

there is a direct link between changes in cell shape, passage through the R point and cell cycle exit upon serum starvation. A possible explanation for this link could be that the growth-promoting anchorage required for cell cycle progression is established during the G₁-pm window (i.e. from the time the cell exits mitosis until it passes the R point) and that this establishment of sufficient contact with the ECM is mediated or controlled by growth factors. This explanation is in agreement with the reduced requirement for adhesion observed for tumour cells that lack a restriction point and therefore have the ability to proliferate in low or no serum conditions (Pardee 1974; 1989; Zetterberg and Larsson 1985; 1991). Finally, we propose that reorganisation of the cytoskeleton and/or the degree of cell adhesion to the ECM after exit from mitosis are involved in regulating passage through the restriction point.

Paper III

D-type cyclins are an important link between the extracellular environment and cell cycle progression (Albanese *et al.* 1995; Hunter and Pines 1994; Lavoie *et al.* 1996; Sherr 1995; Sherr and Roberts 2004). They are only expressed in the presence of growth factors (Hunter and Pines 1994; Sherr 1995) and regulate cell cycle progression through G₁ by phosphorylating and inactivating pRb (Bartek *et al.* 1997). In the absence of growth factor signalling cyclin D is rapidly downregulated. These observations lead us to investigate two different aspects of cyclin D in relation to two G₁ checkpoints: (i) the R point and (ii) pRb phosphorylation.

The fact that pRb phosphorylation seems to constitute a second G₁ check point, which is separate from the R point does not exclude that cyclin D is involved in the mechanism governing the R point passage. For this reason we decided to study changes in colocalisation of cyclin D and pRb within the nucleus in relation to the R point and pRb phosphorylation. Our study revealed that although both pRb and cyclin D were present in cell nuclei throughout G₁, they were colocalised (within the nucleus) in an age dependent manner. In early post mitotic G₁ cells (i.e. G₁-pm cells) the two proteins were in the nucleus, but remained separated (Figure 11A). This situation was changed in cells that had passed the restriction point, and the two nuclear proteins became colocalised (Figure 11B) with kinetics similar to cyclin D-dependent phosphorylation of pRb. This indicates that translocation of cyclin D

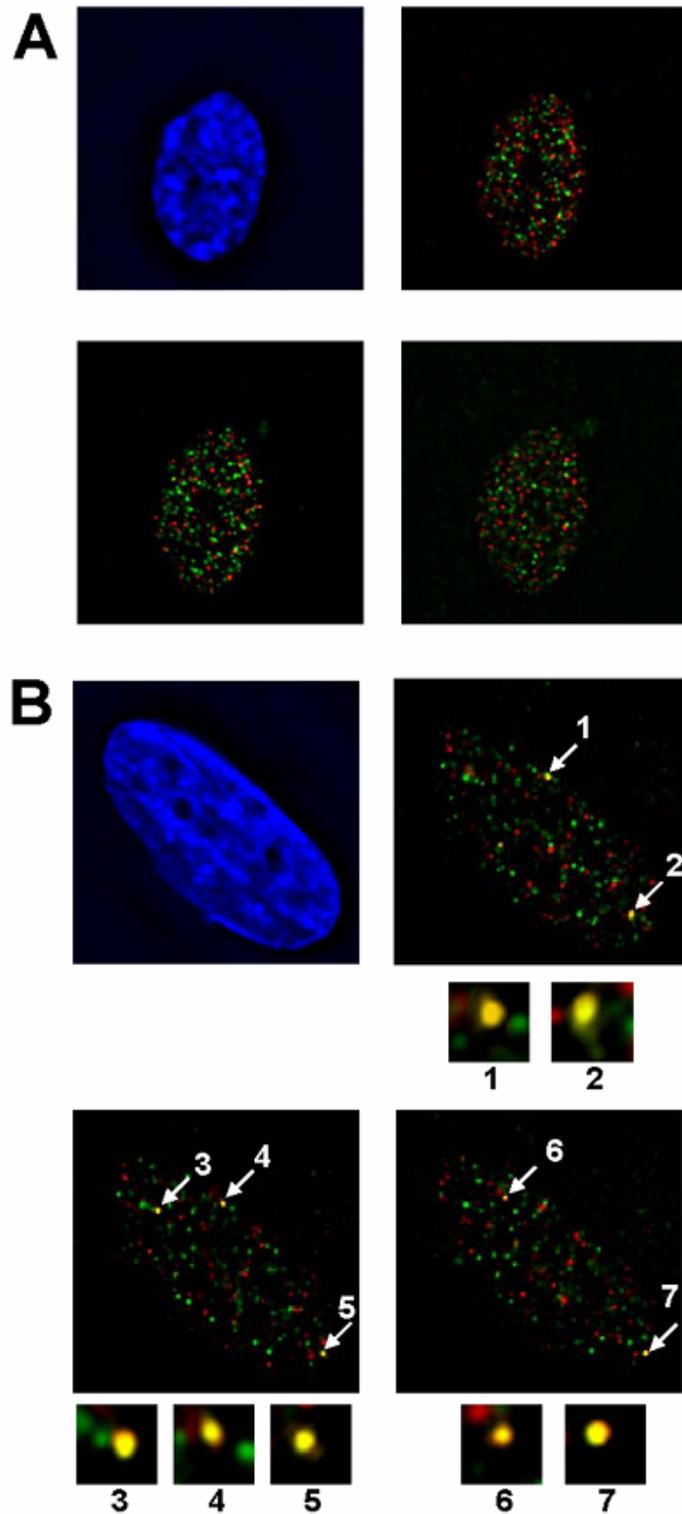


Figure 11. Colocalisation of cyclin D and pRb only occur in a subset of cells. In young post mitotic G₁ cells before R no colocalisation between the two proteins occur (A), whereas cells that have passed the R point display colocalisation (B). In (B) the colocalisation spots are numbered and an enlargement of each spot is displayed under the corresponding image. The DAPI image is displayed in blue, the pRb is displayed in red and cyclin D1 is displayed in green.

within the nucleus may be involved in regulating phosphorylation of pRb, but not in the regulation of R point passage.

We furthermore investigated downregulation of cyclin D upon serum starvation from a cell cycle perspective. This was done in order to search for an answer to whether the rapid cell cycle exit of G₁-pm cells in response to serum starvation (Zetterberg and Larsson 1991) was caused by preferential or more rapid downregulation of cyclin D in these cells as compared to cells that has progressed past R in the cell cycle. We compared levels of cyclin D1 and D3 in untreated cells and cells that had been serum starved for 4 hrs and found that in the serum starved cells levels of cyclin D were reduced as compared to untreated cells. We also observed that the reduction occurred in all phases of the cell division cycle. Consequently the downregulation of cyclin D does not occur preferentially or more rapidly in cells that have not passed R than in cells after R.

In conclusion this study shows that the rapid cell cycle exit of G₁ cells before R is not caused by preferential downregulation of cyclin D in these cells. Nevertheless, cyclin D downregulation may exert its effect in G₁-pm cells, but not in cells in other parts of the cell cycle. More importantly we also present evidence suggesting that translocation of cyclin D to pRb in a cell cycle dependent manner may be involved in pRb phosphorylation, but not in the mechanism governing R point passage. This finding gives further strength to our previous study (Paper I), in which we show that passage through the R point and phosphorylation of pRb seems to constitute two separate G₁ checkpoints.

Paper IV

A low dose of the cytostatic drug actinomycin D (AMD) blocks biosynthesis of rRNA (Perry 1962) and since rRNA synthesis is a rate limiting step in ribosome biogenesis (Grummt 2003) a low dose of AMD accordingly blocks ribosome synthesis. We used this property of AMD to examine the relationship between ribosome biosynthesis, cell cycle control and cell growth.

Treatment with AMD has been reported to induce a cell cycle block (Caspersson *et al.* 1965) and we investigated the temporal location of the induced block. This was done using two techniques: we used (*i*) TLV to determine the timing of cell cycle arrest induced by AMD in relation to mitosis, the R point and S phase

entry and (ii) Feulgen staining to analyse the ploidy of the cells which gives information about cell cycle phase. We observed that a low dose of AMD blocked cell cycle progression of virtually all G₁ cells, including cells that had passed the R point (Figure 12A). Cells that had entered S phase, on the other hand, were not arrested, and completed DNA replication in the presence of AMD (Figure 12B). We also report that the cell cycle arrest was associated with a block in pRb phosphorylation (Figure 12C). The cell cycle regulating function of pRb is closely related to phosphorylation status (Adams 2001; Harbour and Dean 2000; Harbour *et al.* 1999) and the observed block of pRb phosphorylation is most likely involved in the molecular mechanism behind the late G₁ cell cycle arrest induced by AMD. This is in agreement with a previous study that reports that impaired ribosome biogenesis activates p53 which in turn leads to upregulation of p21^{Kip1} and p27^{Cip1} which results in a block of pR phosphorylation and cell cycle arrest (Pestov *et al.* 2001).

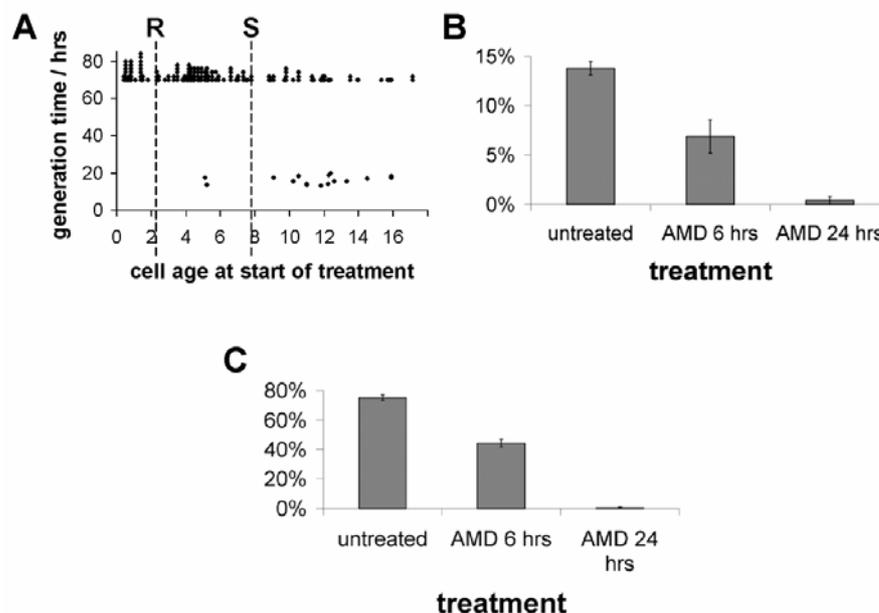


Figure 12. A low dose of AMD affects cell cycle progression and pRb phosphorylation status. TLV analysis of cells treated with AMD showed that cell cycle progression was blocked in virtually all G₁ cells (A). However, cells percentage of cells in S phase decreased after AMD treatment, showing that AMD did not affect DNA synthesis (B). Furthermore, cyclin D-cdk4/6 preferred phosphorylation was blocked in the presence of AMD (C). In (A) the x-axis represents cell age after mitosis and the y axis represents the generation time. In (B) and (C) the x-axis gives information about the treatment of the cells and the y-axis gives the percentage of positive cells (as judged by eye).

A notable observation was that even though the impaired rRNA synthesis causes a cell cycle block, cells continue to synthesise protein and accumulate mass in

the presence of AMD. This finding is in good agreement with a report showing that abrogated ribosome biogenesis *in vivo* hinders cell cycle progression but allows accumulation of mass (Volarevic *et al.* 2000). In summary our results show that inhibiting ribosome biogenesis causes a cell cycle arrest in late G₁ after the restriction point. This G₁ checkpoint is likely to involve pRb phosphorylation status and affects cell proliferation but not cell growth.

Conclusions

The four studies included in this thesis were performed to increase the understanding of G_1 progression. Many of the molecules involved in regulation of G_1 progression are mutated or have altered expression patterns in tumour cells as compared to normal somatic cell. Cell cycle progression during G_1 is dependent on growth factors until 3 to 4 hours after mitosis when the cell passes a G_1 checkpoint called the restriction point (R), thereafter the cell can progress through the rest of cell division cycle in the absence of extracellular growth factors. The retinoblastoma tumour suppressor protein (pRb) regulates progression through G_1 by repressing transcription of genes involved in S phase entry. Inactivating phosphorylation of pRb by cyclin D-cdk4/6 has been found to occur at roughly the same time as the cell passes the R point, and therefore phosphorylation of pRb has been suggested to constitute the molecular mechanism behind R point passage.

In Paper I we investigated the temporal relationship between passage through the restriction point and phosphorylation of pRb. We show that passage through the R point occurs before phosphorylation of pRb takes place and that pRb phosphorylation takes place at variable times in late G_1 (after passage through R), close to S phase entry. These results strongly suggests that phosphorylation of pRb does not constitute passage through R, but instead represents a separate G_1 checkpoint that occurs later in G_1 than the R point.

Paper II was devoted to the search for an alternative mechanism for R point passage based on the observation that passage through the R point takes place at a fixed time after exit from mitosis. We show that upon exit from the cell cycle to a quiescent state due to serum deprivation, G_1 -pm cells (i.e. cells before R) undergo a transient change in cell shape which reduces their attachment to the ECR. This morphological phenomenon is only observed for G_1 cells that have not passed the R point, and is counteracted by addition of growth factors to the same extent as they prevent cell cycle exit. Altogether, these data imply an alternative mechanism for R point passage involving reorganisation and/or degree of cell adhesion to ECM after exit from mitosis.

In Paper III we studied different aspects regarding cyclin D during the G_1 phase. We demonstrate that the rapid exit from the cell cycle of cells that have not

passed R is not due to preferential or more rapid degradation of cyclin D in early post mitotic G₁ (G₁-pm) cells, but that down-regulation of cyclin D occurs to the same extent in all cells, independent of cell cycle position. More importantly we studied the colocalisation of cyclin D and pRb, and report that intra-nuclear translocation of cyclin D seems to be involved in the regulation of pRb phosphorylation but not in passage through the restriction point. Our data from this study further strengthens our previous observation that passage through R and phosphorylation of pRb constitutes two temporally, and possibly functionally distinct checkpoints in G₁.

In Paper IV we investigated the relationship between ribosome biogenesis, cell cycle progression and cell growth. We discovered that blocking of rRNA synthesis causes cell cycle arrest of virtually all G₁ cells, but that cells in S phase continue their cell cycle progression. The induced G₁ cell cycle block is associated with impaired pRb phosphorylation. We also show that abrogation of rRNA synthesis does not abolish protein synthesis. In conclusion our results indicate that impaired ribosome synthesis blocks cell cycle progression through a checkpoint in late G₁, separate from the R point, which occurs in early to mid G₁. The mechanism controlling this late G₁ checkpoint involves phosphorylation status of pRb. Moreover, the late G₁ checkpoint induced by AMD only seems to affect cell proliferation but not cell growth. The data from this study gives strength to our finding in Paper I that the R point and pRb phosphorylation are two different G₁ control points.

Altogether we want to emphasise that passage through the R point and phosphorylation of pRb are two temporally separated events that occur during the G₁ phase. They both seem to constitute G₁ checkpoints, but with different functions. R point passage seems to be related to cellular changes that take place during mitosis and relies on extracellular growth factors. pRb phosphorylation on the other hand appears to be related to S phase entry and is dependent on intracellular events like ribosome biogenesis.

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