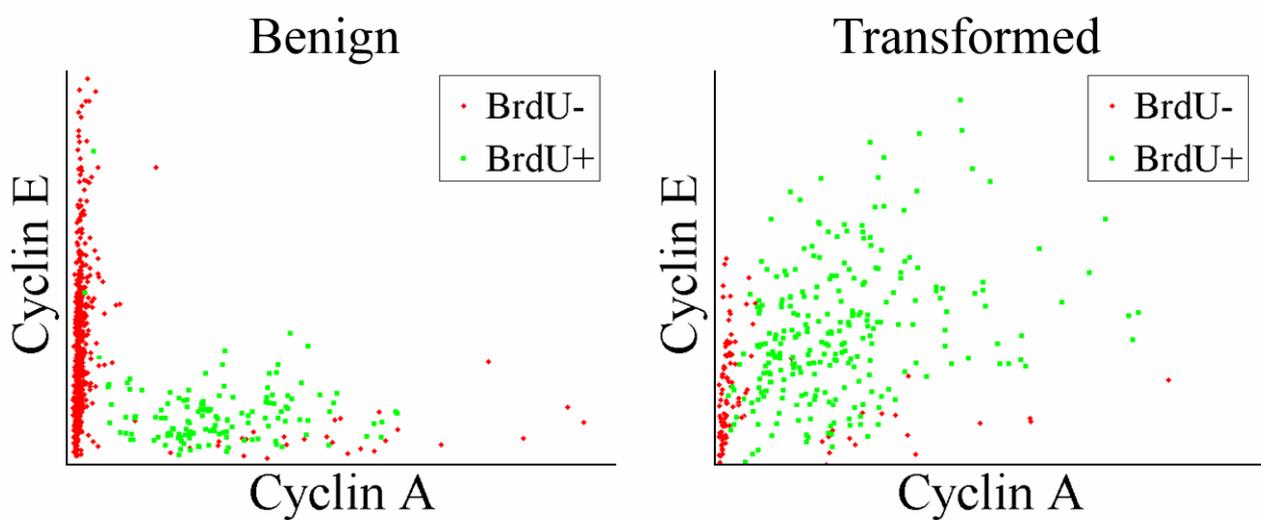


Immunofluorescence studies on the cell cycle expression of cyclin A and cyclin E



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Stockholm 2004

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on the cell cycle expression of
cyclin A and cyclin E**

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To my beloved family

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Abstract

Cell cycle progression is tightly regulated in normal eucaryotic cells. Cancer is caused by the transformation of cells, and is always associated with defects in the cell cycle regulatory machinery. An important result of a defective cell cycle regulation is genetic instability, as the cell with a deficient cell cycle control fails to correctly duplicate and split its genome. Genetic instability leads to the acquisition of new characteristics, and is thought to cause tumour progression. Cyclin Dependent Kinases (CDKs), and their regulatory subunits, the cyclins, are central in cell cycle regulation. Cell cycle progression is accomplished by the successive activation and deactivation of the various cyclin-CDK complexes. Cyclin-CDK complexes act by phosphorylating a range of target proteins appropriate for the cell cycle phase in which the cyclin-CDK complex is active. Cyclin E-CDK2 is active in late G₁ and phosphorylates proteins central to the progression through late G₁ and entry into S-phase. Cyclin A-CDK2 is active in S and G₂, and cyclin A-CDK2 activity is essential from progression through S-phase, and entry into mitosis.

The study of the cell cycle machinery puts high demand on the methodological approach, as cell cycle progression often is altered, or even abrogated, by the treatment of the cells required by many of the traditional biochemical methods. In order to study the intricate cell cycle regulatory mechanisms a novel method based on immunofluorescence staining was developed which allowed the semi-quantitative measurement of the levels of a large number of proteins in individual cells in culture or tissue samples.

Cyclin A and cyclin E are both central in the regulation of DNA replication. Hence a deregulated expression of either cyclin could potentially cause genetic instability. In order to study the expression patterns of cyclin A and cyclin E over the cell cycle in normal and transformed cells a triple immunofluorescence staining protocol was utilised. The position in the cell cycle of each individual cell could then be established, and correlated with the immunofluorescence staining intensity for cyclin A or cyclin E.

Nuclear cyclin A accumulation was shown to begin virtually exactly as the cells entered S-phase, i.e. very close to the G₁/S-transition. The cyclin A accumulation began at the same point in the cell cycle and progressed with similar kinetics in both normal and transformed cells. The data suggests that deregulation of cyclin A expression is not commonly occurring in transformed cells, possibly because it is deleterious.

Cyclin E accumulation, on the other hand, was shown to be highly different in normal and transformed cells. In normal cells cyclin E levels were found to rise after progression through the R-point, peak in late G₁-phase, and then decrease as the cells entered S-phase. In transformed cells cyclin E accumulation commonly continued throughout S-phase, and cyclin E was often not completely degraded until in mitosis. Therefore the clinical implications of a deregulated cyclin E expression pattern in cervical carcinoma lesions was investigated. It was found that a highly deranged cyclin E expression over the cell cycle was associated with poor survival. The data are well in line with the results presented by others, which have shown that a deregulated cyclin E expression can cause genetic instability.

List of publications

This thesis includes the following papers:

- I. Wählby C¹, **Erlandsson F**¹, Bengtsson E, and Zetterberg A. Sequential immunofluorescence staining and image analysis for detection of large numbers of antigens in individual cell nuclei. *Cytometry* 2002;47(1):32-41.
¹ The two first authors contributed equally.
- II. **Erlandsson F**, Linnman C, Ekholm S, Bengtsson E, and Zetterberg A. *A detailed analysis of cyclin A accumulation at the G₁/S border in normal and transformed cells.* *Exp Cell Res* 2000;259(1):86-95.
- III. **Erlandsson F**, Wählby C, Ekholm-Reed S, Hellström A-C, Bengtsson E, and Zetterberg A. *Abnormal expression pattern of cyclin E in tumour cells.* *Int J Cancer* 2003;104(3):369-375.
- IV. **Erlandsson F**, Martinsson H-S, Wallin K-L, Andersson S, Hellström A-C, and Zetterberg A. *Cell cycle expression of cyclin E in neoplastic lesions of the uterine cervix.* Manuscript 2004.

Related work

In addition to the papers included in this thesis the author has also contributed to the following publications:

Wählby C, **Erlandsson F**, Nyberg K, Lindblad J, Zetterberg A, and Bengtsson E. *Multiple tissue antigen analysis by sequential immunofluorescence staining and multi-dimensional image analysis.* In I Austvoll, editor. *Proceedings of the 12th Scandinavian Conference on Image Analysis (SCIA)*, pages 25-32, Bergen, Norway, June 2001.

Wählby C, Sintorn I-M, **Erlandsson F**, Borgefors G, Bengtsson E. *Combining intensity, edge, and shape information for 2D and 3D segmentation of cell nuclei in tissue sections.* Under publication in *Journal of Microscopy*.

Zhang A, Zheng C, Hou M, Lindvall C, Li KJ, **Erlandsson F**, Björkholm M, Gruber A, Blennow E, Xu D. *Deletion of the telomerase reverse transcriptase gene and haploinsufficiency of telomere maintenance in Cri du chat syndrome.* *Am J Hum Genet* 2003;72(4):940-948.

Zhang A, Wang J, Zheng B, Fang X, Ångström T, Liu C, Li X, **Erlandsson F**, Björkholm M, Nordenskjöld M, Gruber A, Wallin K-L, Xu D. *Telomere attrition predominantly occurs in precursor lesions during in vivo carcinogenic process of the uterine cervix.* Under publication in *Oncogene*.

Abbreviations

APC/C	Anaphase Promoting Complex/Cyclosome
ARF	The Alternate Reading Frame gene located at the INK4a locus
ATM	The Ataxia Telangiectasia Mutated gene product
ATR	The ATM Related gene product
BrdU	Bromodioxuridine
CAK	Cyclin Activating Complex
CCD	Charge-Coupled Device
CDC	Cell Division Cycle
CDK	Cyclin Dependent Kinase
CKI	CDK Inhibitor
DABCO	1,4-Diazabicyclo(2,2,2)octane, C ₆ H ₁₂ N ₂
DAPI	4',6-Diamidino-2-Phenylindole, a stain for double stranded DNA
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine Tetraacetic Acid, C ₁₀ H ₁₆ N ₂ O ₈
EGF	Epidermal Growth Factor
ERK	Extracellularly Regulated Kinase
FAK	Focal Adhesion Kinase
FITC	Fluorescein Isothiocyanate
G ₁ -pm	G ₁ -post mitosis (the part of G ₁ occurring before R)
G ₁ -ps	G ₁ -pre S-phase (the part of G ₁ occurring after R)
HDAC	Histone Deacetylase
IC	Initiation Complex
IGF	Insulin-like Growth Factor
NGF	Nerve Growth Factor
MAPK	Mitogen Activated Protein Kinase
MEK	MAPK or ERK Kinase
ORC	Origin of Replication Complex
PBS	Phosphate Buffered Saline
PDGF	Platelet-Derived Growth Factor
Plk	Polo Like Kinase
Post-RC	Post-Replication Complex
pRb	The Retinoblastoma gene product protein
Pre-RC	Pre-Replication Complex
RC	Replication Complex
SIFS	Sequential Immunofluorescence Staining
TRIS	Tris(hydroxymethyl)aminomethane, NH ₂ C(CH ₂ OH) ₃

Introduction

Background

Already in 1858 did the German pathologist Rudolf Virchow formulate the cell doctrine: "Where a cell arises, there must be a previous cell, just as animals can only arise from animals and plants from plants." The life cycle of the living cell is therefore a necessary requirement for the perpetuation of life, and it has continued in an unbroken chain from the appearance of life and the first cell some 3 billions of years ago until today. Thus all living things on Earth, plants and animals alike, are related.

A well regulated cell cycle is an absolute requirement in order for a cell to ensure the survival of its progeny. The genetic material present in each cell at the beginning of the cell cycle, which contains the full blueprint for the whole organism, must be copied exactly once, and then correctly divided (segregated) in two identical copies. The cell must double its size, and its content of organelles, during the course of the cell cycle. Furthermore, the molecular machinery regulating cell cycle progression must be able to respond to adverse intracellular events such as DNA damage by halting cell cycle progression while the damage is repaired. Finally the cell must be able to react to environmental signals either by halting or reassuming cell cycle progression. The complex machinery regulating the cell cycle progression manages all of these tasks with an astonishing high degree of accuracy and redundancy.

In single cell organisms a seriously disrupted cell cycle regulation leads to the death of the organism. In multicellular organisms, on the other hand, does failure by the regulatory machinery generally only lead to the death of the damaged cell by programmed cell death (apoptosis) in order to save the organism. In large organisms with a long life span do however individual cells occasionally escape the control mechanisms and cause disease, most notably cancer. The main hallmarks of the cancer cell are caused by a disrupted cell cycle regulation. Genetic instability is the result when the cell on the road to transformation fails to correctly duplicate and segregate its genetic material. Passage through the cellular damage checkpoints in the face of DNA damage is ensured by mutations of essential components of the cell cycle machinery. Hyper-proliferation ensues as the cancer cell becomes independent of extra-cellular signals for growth. Cell sizes and shapes vary considerably in the expanding clone, giving rise to the morphology of malignant tissues. A single cell with a disrupted cell cycle regulation can occasionally kill the whole host organism through clonal expansion if left unchecked. The severity of the deficiency in the cell cycle machinery will generally dictate the course and progression (the malignancy) of the disease. The



Figure 1: Rudolf Virchow (1821-1902) formulated the cell doctrine in 1858 by stating that "Where a cell arises, there must be a previous cell, just as animals can only arise from animals, and plants from plants.

potential to improve the treatment and detection of human tumours have more than anything else provided a strong incentive for cell cycle research.

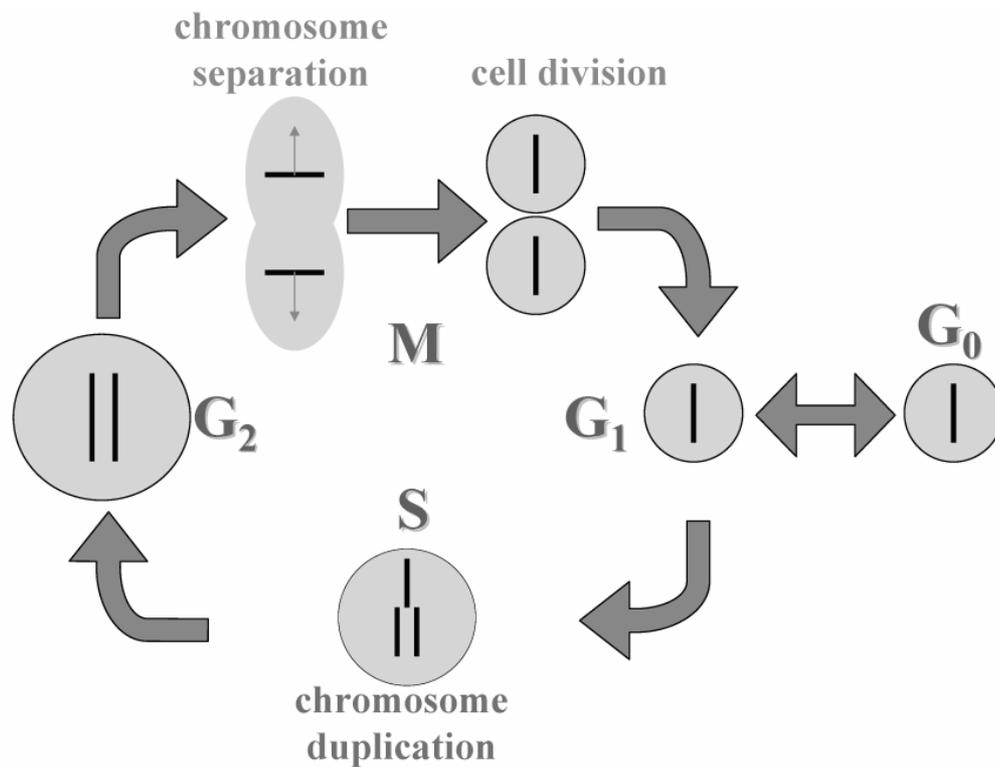


Figure 2: The cell cycle consists of the phases G₁, S, G₂ and M (mitosis). During G₁ the cell decides whether or not to continue through the cell cycle. In S-phase the cell duplicates its DNA. In G₂ the cell checks that the DNA synthesis has been completed successfully. During M-phase (mitosis) the cell divides the genome, and then splits into two daughter cells.

The phases of the cell cycle

The cell cycle has traditionally been divided in four distinct phases (fig 2). The duplication of the genome by DNA synthesis takes place during S-phase (S for synthesis). The physical division of the cell in two daughter cells takes place during the M-phase (M for mitosis). The two gaps between each M- and S-phase have been designated G₁ and G₂ (G for gap). Thus a newborn cell first enters G₁. During G₁ the cell evaluates its size and the extra-cellular signals it is exposed to. When certain conditions are met the cell traverses the second part of G₁, and begins synthesising DNA. After S-phase the cell enters G₂, during which it checks that the DNA is ready to be divided, before finally splitting in two during M-phase. If the conditions for continuous growth are not met in G₁ the cell instead enters the quiescent state G₀.

Molecular mechanisms behind the regulation of cell cycle progression

The main molecular components of the cell cycle machinery are the cyclin dependent kinases (CDKs) and the cyclins. The CDKs were originally discovered in two highly evolutionary divergent species of yeast. Leland Hartwell used *Saccharomyces cerevisiae* (baker's yeast) to discover CDC28 (Hartwell, 1974),

and Paul Nurse independently discovered *cdc2* (Nurse et al., 1976) in *Schizosaccharomyces pombe* (budding yeast). Both genes are necessary for cell progression through the cell cycle in their respective organisms. The genes were found to encode very similar proteins, and antibodies against their gene products were found to cross react with a 34kDa human protein eventually named CDK1 (Draetta et al., 1984). The first cyclin was discovered by Tim Hunt in fertilised sea urchin eggs as a protein that was degraded at each cell division (Evans et al., 1983).

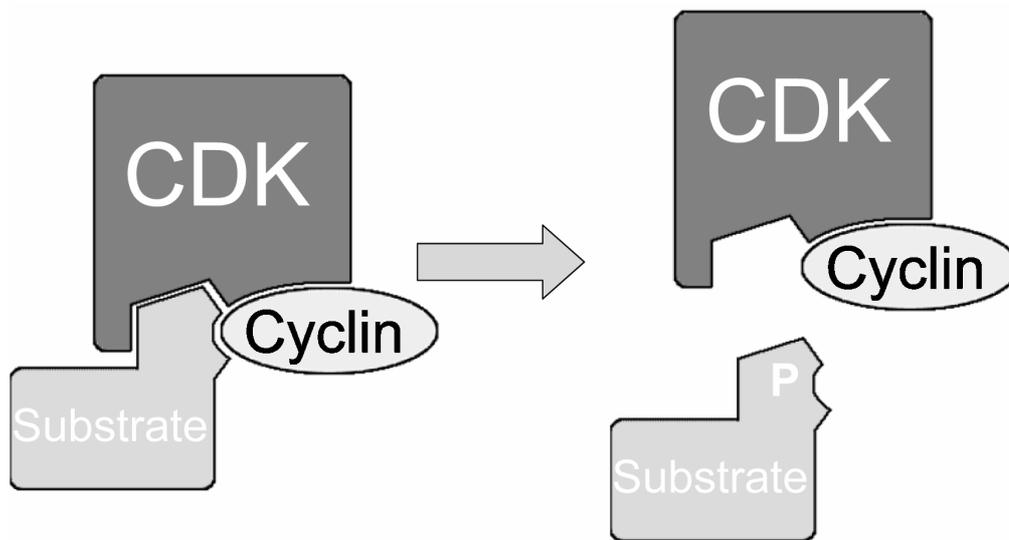


Figure 3: Cyclin and the Cyclin Dependent Kinase (CDK) forms a complex with kinase activity. Substrate specificity is incurred both by the cyclin and the CDK.

Cyclin Dependent Kinases, the cell cycle engine

The group of serine/threonine protein kinases named cyclin dependent kinases (the CDKs) are one of the two main components of the cell cycle machinery. When activated they phosphorylate target proteins essential for the progression of the cell cycle. The activity of the CDKs is tightly regulated in at least four ways (fig 4).

The main mode of regulation of the CDKs is the binding to a cyclin. Different cyclins are present in the nucleus during different parts of the cell cycle (fig 5). Apart from activating the CDKs the cyclins also confer substrate specificity to the cyclin-CDK complex by affecting the substrate binding site.

The activity of the cyclin-CDK complex is also regulated by reversible phosphorylation. In order to be activated the CDKs need to be phosphorylated by the CDK activating complex (CAK), which consists of cyclin H, CDK7 and Mat1 (Fisher and Morgan, 1994; Yee et al, 1995). The regulatory functions of CAK, if there are any, are unclear at best (Taylor and Stark, 2001). It seems likely that CAK is always active in all cells, except maybe in cells with high levels of p53 (Schneider et al., 1998). There are also a few phosphorylation sites in the substrate binding site, which when phosphorylated inhibits the activity of the cyclin-CDK complex. The sites are phosphorylated by a group of kinases that include the Wee1 kinase, but the phosphate groups can also be removed by the Cdc25 group of phosphatases. The Wee1 and Cdc25 activity varies greatly in response to intracellular events such as DNA damage.

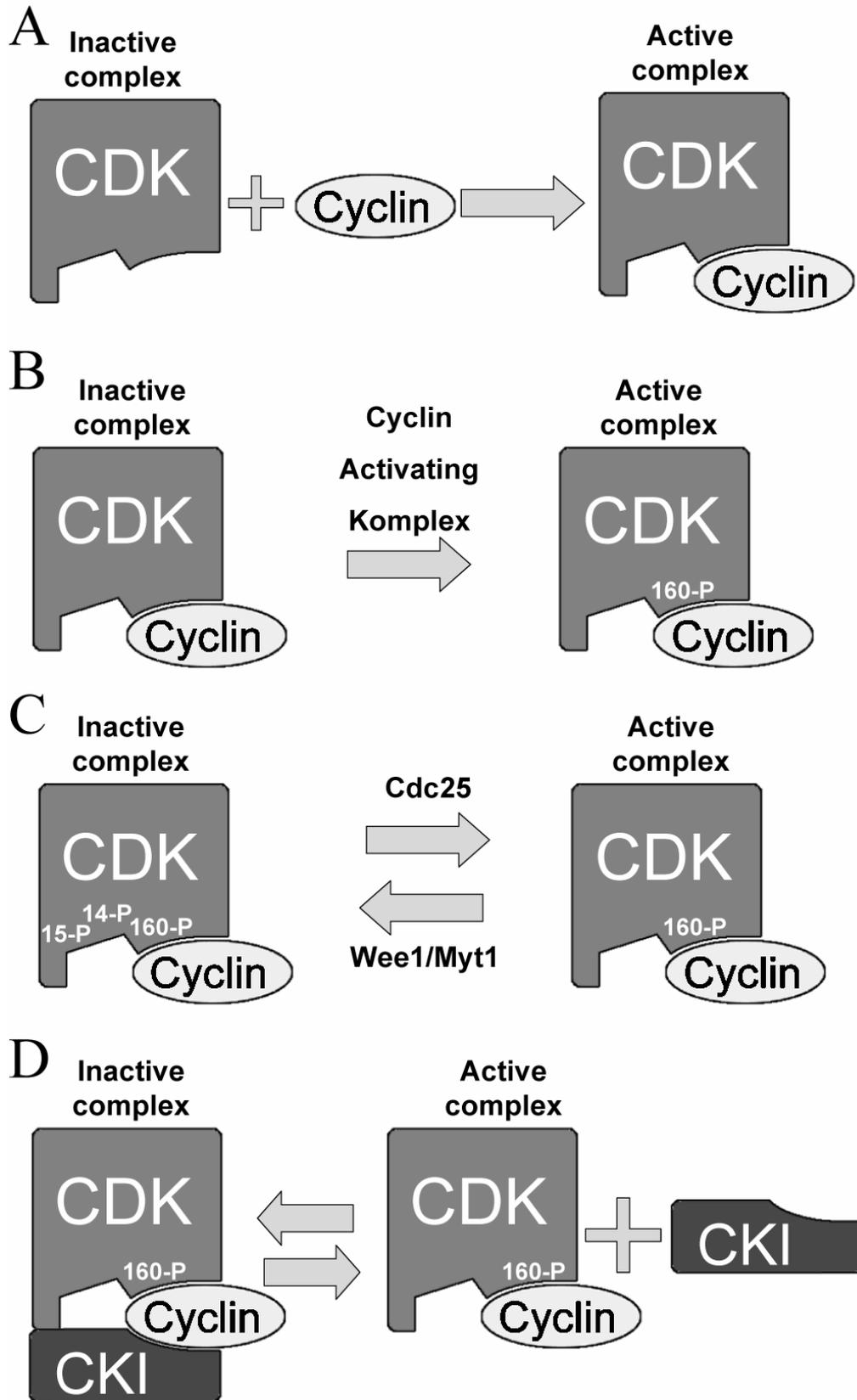


Figure 4: The four main modes of CDK regulation. A: Without being bound to a cyclin the CDK complex is void of kinase activity. B: After cyclin binding the complex can be phosphorylated by the CAK, and rendered fully active. C: Further phosphorylation at inhibitory sites renders the complex inactive. D: CDK inhibitors (CKIs) can inhibit the complex by blocking the active site. The phosphorylation sites refers to the CDK2 phosphorylation sites.

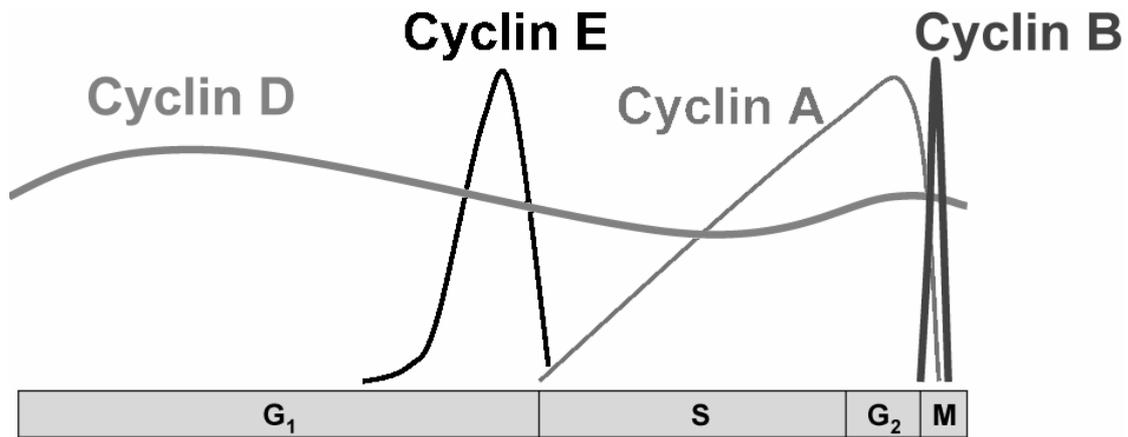


Figure 5: The different cyclins are expressed during different phases of the cell cycle. Cyclin D associates with CDK4/6 and is expressed throughout the cell cycle, but the levels are dependent of the mitogenic signalling the cell receives. Cyclin E is peaks in late G₁, and associates with CDK2. Cyclin A is accumulated during S, G₂, and M, and initially associates mainly with CDK2, but later with CDK1. Cyclin B appears in the cell nucleus around entry into M, associates with CDK1, and is degraded in mid-M.

Finally some cyclin-CDK complexes can be inhibited by binding to a CDK inhibitor (CKI). The CKIs consist of two groups of proteins, the INK4-group and the CIP/KIP-group of inhibitors. The INK4 inhibitors are p15INK4b, p16INK4a, p18INK4c and p19INK4d, and they exert their effect by associating with CDK4 and CDK6, thereby rendering them inactive by preventing their association with cyclin D (Carnero and Hannon, 1998). The CIP/KIP group consists of p21CIP1/WAF1, p27KIP1 and p57KIP2, and can bind to cyclin-CDK complexes containing CDK1, CDK2, CDK4, and CDK6 (Morgan, 1995). Surprisingly the CIP/KIP CKIs under some circumstances increase the kinase activity of the cyclin D-CDK4/6 (Sherr and Roberts, 1999).

The G₁-phase

In G₁ the cell has to make the decision whether or not it should continue dividing, as it would be suboptimal to continue into S-phase and duplicate the DNA unless the cell intends to complete the cell cycle and divide. The cell therefore makes the decision whether to divide or not already in G₁, at the restriction point.

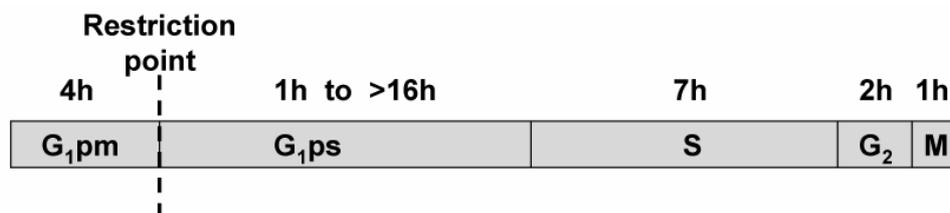


Figure 6: The restriction point occurs approximately 3.5 to 4h into G₁. After passage through the restriction point the cell progresses through the full cell cycle independently of the level of growth factors at the cell membrane. The restriction point splits G₁ in G₁pm (post mitosis) and G₁ps (pre S).

The restriction point

It has long been known that mammalian cells commit to DNA-duplication, and thereby cell division, already a few hours into G₁ (Temin, 1971). The concept of the restriction point (R-point) was first introduced by Art Pardee (Pardee, 1974; Pardee, 1989). The restriction point is the point in the cell cycle after which the cell is independent of extra-cellular signals for the completion of its cell cycle (fig

6). The restriction point has been shown to be passed about 3.5 to 4h after cell birth (Zetterberg and Larsson, 1985). It divides the G_1 -phase in two functionally very distinct parts, G_1 -pm (post-mitosis) and G_1 -ps (pre-DNA synthesis). During G_1 -pm the cell evaluates the growth factors present in its surroundings. If the right growth factors are present in sufficient amounts the cell proceeds into G_1 -ps. Cells in G_1 -ps will continue all the way through the cell cycle to the next G_1 -pm, even if all growth factors in the environment are depleted, as passage through R has rendered the cell independent of extra-cellular signals.

If insufficient amounts of growth factors are provided to the cell during G_1 -pm the cell will not pass the restriction point. It will instead enter a quiescent state called G_0 . In G_0 the cell has stopped all progression through the cell cycle, and it will only return to G_1 when the levels of growth factors rises, and then only after a considerable lag phase of several hours (Zetterberg and Larsson, 1985). The vast majority of cells in the body are terminally differentiated, and are thus in a more or less permanent G_0 state.

G_1 -pm is about 3.5 to 4 hours long in all normal mammalian cells studied, but there is considerable variability in the length of G_1 -ps between cells (Zetterberg and Larsson, 1985). Presumably G_1 -ps is used for preparation for the upcoming S-phase, such as accumulation of the necessary building blocks and enzymes. These preparations take different lengths of time in different cells, probably depending on how fast the previous cell cycle was completed, and by how much of a stock each of the newborn cells received from its ancestor. The length of G_1 -ps has been shown to be closely related with cell size (Killander and Zetterberg, 1965). The large variations in G_1 -ps length is a considerable obstacle in cell cycle research as cells synchronised in M- or G_1 -phase do not enter S-phase simultaneously.

Detection of extra-cellular growth signals

Cells of multicellular organisms are highly sensitive to their environment, since the cells of the organism must coordinate their life and deaths for survival. Growth signal independence is one of the most important features of the transformed cell, hence virtually all components of the molecular pathways detecting extra-cellular growth factors are potential proto-oncogenes. There are numerous different growth factors with distinct signalling cascades such as insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and nerve growth factor (NGF).

Many of the growth factors are detected by tyrosine kinase receptors located at the cell membrane. The receptors consist of two extracellular α -subunits which binds the growth factor, and two transmembrane β -subunits. The β -subunits autophosphorylate when a growth factor is bound by the receptor. Autophosphorylation activates the tyrosine kinase activity of the β -subunits.

The activated receptors subsequently phosphorylate tyrosine residues on a range of target proteins, including signalling molecules at the top of intricate signalling cascades. The multi-step signalling process ensures the amplification of the detected signal. One of the best studied signalling cascades consists of the p42/p44 MAPK (mitogen activated protein kinase) module (reviewed in depth in Pouyssegur et al., 2002). Via the G-protein Ras the serine/threonine kinase Raf is

recruited to the cell membrane and activated. Raf then phosphorylates and thereby activates MEK (MAPK or ERK kinase), which phosphorylates and activates ERK (extracellularly regulated kinase). The activation of 5% of the cell content of Ras is sufficient to activate the entire cellular pool of ERK (Hallberg et al, 1994), which shows the efficiency of the signalling cascade. Activated ERK is essential for cell proliferation in fibroblasts (Pages et al, 1993). ERK exerts its effects mainly by phosphorylating a range of transcription factors (reviewed in Yang et al., 2003). A major downstream event from the Ras to ERK signalling cascade is the formation of active cyclin D-CDK4/6 complexes (Lavoie et al., 1996).

Other similar signalling cascades exist, such as the p38 MAPK module, the BMK1/ERK5 module, and the JNK module. However, p42/p44 MAPK and BMK1/ERK5 are the modules affected by extracellular growth factors, the p38 MAPK and the JNK modules are mainly involved in stress response signalling (Yang et al., 2003).

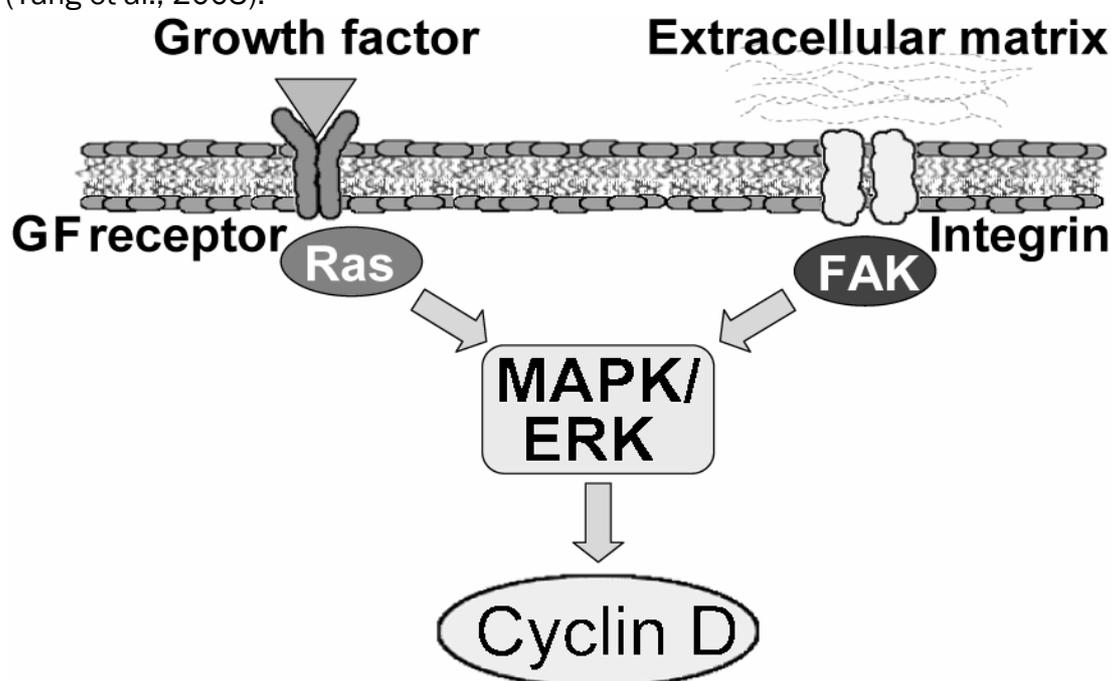


Figure 7: The detection of cell adhesion and extracellular growth signals occurs through growth factor receptors and integrins at the cell membrane. The signals are amplified through the MAPK/ERK signalling cascades, and converge to increase the activity of the cyclin D-CDK4/6 complex.

Detection of cell adhesion

Apart from surveying the surroundings for growth signals in the form of growth signals the cell also ensures that it is securely attached before progressing through G₁. In analogy with growth factor dependence tumour cells commonly lose their dependence on cell attachment during transformation.

Integrins connect the extra-cellular matrix with the intracellular cytoskeleton. The integrins are transmembrane heterodimers with a lower ligand affinity but higher concentration than other cell membrane receptors, such as growth factor receptors. The extracellular domain binds to various extracellular matrix proteins, while the intracellular domain binds to components of both the actin and the intermediate filaments. Integrins also activate intracellular receptors when bound to their ligands, most notably through activation of the focal adhesion kinase

(FAK). FAK activation leads to cell cycle progression by decreasing the levels of CDK inhibitor p21 (Zhao et al, 1998) and by activation of ERK, leading to accumulation of cyclin D (Zhao et al, 2001).

Cyclin D integrates the extracellular signals

Hence extracellular signals such as growth factors and cell adhesion converge on the formation of active cyclin D-CDK4/6 complexes, as shown in fig 7. The importance of cyclin D expression levels has been shown in overexpression experiments. Cyclin D1 overexpression in vitro renders cells anchorage independent (Resnitzky, 1997), and speeds up the return to the cell cycle from quiescence in cells exposed to serum starvation (Resnitzky et al., 1994).

Molecular mechanisms of G₁ progression

Early G₁ (G₁-pm) is the only phase of the cell cycle when there is virtually no CDK activity present. However, if the cell is securely attached to the extracellular matrix, and if there are sufficient amounts of growth factors in its surroundings cyclin D associated CDK4/6 activity rapidly increase in early G₁.

When activated the cyclin D-CDK4/6 complexes phosphorylate targets necessary for progression into S-phase. The main substrate is assumed to be the retinoblastoma gene product pRb, as shown in fig 8. Unphosphorylated pRb binds and thereby inactivates the E2F group of transcription factors (Flemington et al., 1993; Helin et al., 1993). Phosphorylation by cyclin D-CDK4/6 and cyclin E-CDK2 renders pRb unable to sequester E2F, and the released E2F can enter the cell nucleus and affect transcription of proteins necessary for DNA replication to be initiated, among other cyclin E and cyclin A (Geng et al., 1996; Ohtani et al., 1995; Botz et al., 1996; DeGregori et al., 1995; reviewed in Dyson et al., 1998; Harbour et al., 2000).

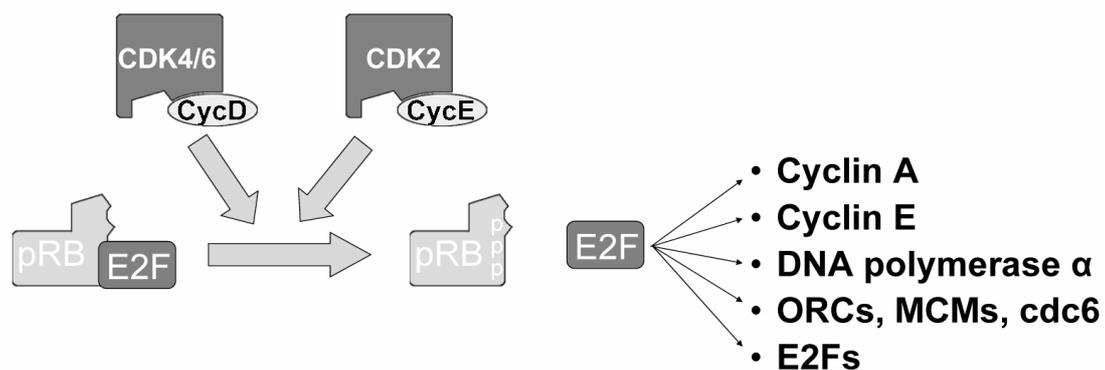


Figure 8: Phosphorylation of pRb by the cyclin D-CDK4/6 and cyclin E-CDK2 complexes releases E2F. E2F is a family of transcription factors that increase the expression of genes encoding proteins necessary for S-phase entry and progression. Since E2F increases cyclin E expression it has been proposed that cyclin E-CDK2 - pRb - E2F - cyclin E forms a positive feedback loop, making the progression into S-phase irreversible.

The regulatory loop triggered by cyclin D-CDK4/6 activity and perpetuated by phosphorylated pRb increasing cyclin E levels, which in its turn increases pRb phosphorylation, has been widely assumed to constitute the molecular basis for the restriction point. As soon as the extracellular conditions were sufficient the phosphorylation of pRb would reach a threshold level after which cyclin E would

assume responsibility for pRb phosphorylation, and thereby rendering the cell serum and attachment independent. More recent data have proven that this popular model is not true, as both cyclin E accumulation and pRb phosphorylation have been shown to occur after passage through the restriction point (Ekholm et al., 2001; Hanna-Stina Martinsson, data submitted for publication).

In late G₁ cyclin E peaks briefly, heralding the accumulation of cyclin A, and entry into S-phase. The sequential expression of cyclin E and cyclin A is tightly regulated. Apart from being transactivated by members of the E2F family the synthesis of cyclin E is regulated by the pRb-hSWI/SNF-HDAC complex, which represses cyclin E transcription (Zhang et al., 2000). Cyclin D-CDK4/6 phosphorylation of pRb displaces HDAC from the complex, thereby allowing cyclin E to be synthesised. The remaining pRb-hSWI/SNF complex has been proposed to repress cyclin A activation until pRb is further phosphorylated by cyclin E-CDK2 (Zhang et al., 2000). When cyclin A levels increase the cell enters S-phase.

Thus the normal cell progresses through G₁ by first accumulating cyclin D in response to extracellular signals, then accumulating cyclin E until the levels are high enough to allow cyclin A synthesis, which finally leads to S-phase entry.

Cyclin E prepares the cell for S-phase

Cyclin E associates with CDK2 (Koff et al., 1991; Dulic et al., 1992). The cyclin E-CDK2 kinase activity in late G₁ has been proposed to be involved in regulating several essential cellular functions, among other the transformation of the origin of replication complexes (ORCs) into pre-replication complexes (pre-RCs), which prepares the cell for DNA-replication, and the induction of centrosome duplication (Matsumoto et al., 1999), which is necessary for successful completion of the cell cycle.

Braking G₁ progression

G₁ arrest is mediated by an increase in the level of either of the members of the two CDK inhibitor (CKI) families. At least four different cues can cause G₁ arrest, growth factor deprivation, DNA damage, extracellular signals, and oncogenic activation.

Growth factor deprivation

Growth factor deprivation leads to the accumulation of CDK inhibitor p27 through the inhibition of p27 degradation (Pagano et al., 1995; Hengst et al., 1996). The CIP/KIP family member p27 can block both CDK4/6 and CDK2 activity. Increased levels of p27 are needed for efficient blocking of G₁ progression in G₀ cells (Rivard et al., 1996; Coats et al., 1996). It is likely that the two main factors keeping G₀ cells in G₀ are low levels of cyclin D, and high levels of p27, which renders both CDK4/6 and CDK2 inactive (Agami and Bernards, 2002).

Extracellular brake signals

Apart from extracellular growth signals there are also extracellular growth inhibitory signals. One example is interferon-alpha, which causes G₁ arrest by upregulating CDK inhibitors p21, p15, and p16 (Sangfelt et al., 1997).

DNA damage

The mechanisms behind DNA damage detection are not completely known. However, double strand breaks induced by ionising radiation are detected by ATM, the gene product of the ataxia telangiectasia gene. Activated ATM phosphorylates Chk1, Chk2 and p53, among others (Kastan et al., 1992; review in Kastan et al., 2000). The phosphorylation of Chk2, also known as Cds1, leads to its activation and further p53 phosphorylation and activation. The transcription factor p53 is normally kept in check by MDM2, which binds to p53 and thereby renders it both inactive and available for degradation. Phosphorylation of p53 leads to release from MDM2 control and makes p53 transcriptionally active. One of the results of p53 activation is p21 synthesis and accumulation. The CDK inhibitor p21 inhibits CDK2 activity, and thereby causes G₁ arrest.

DNA damage may also by a p53 dependent pathway result in inhibition of CAK activity, which is necessary in order to activate all CDKs, but the data are contradictory (discussed in Taylor and Stark, 2001).

If the DNA repair enzymes succeeds in repairing the double strand brakes the ATM activation of p53 ceases, and p21 is degraded. The cell is then free to proceed through the cell cycle. If the DNA repair fails activated p53 can drive the cell to programmed cell death (apoptosis).

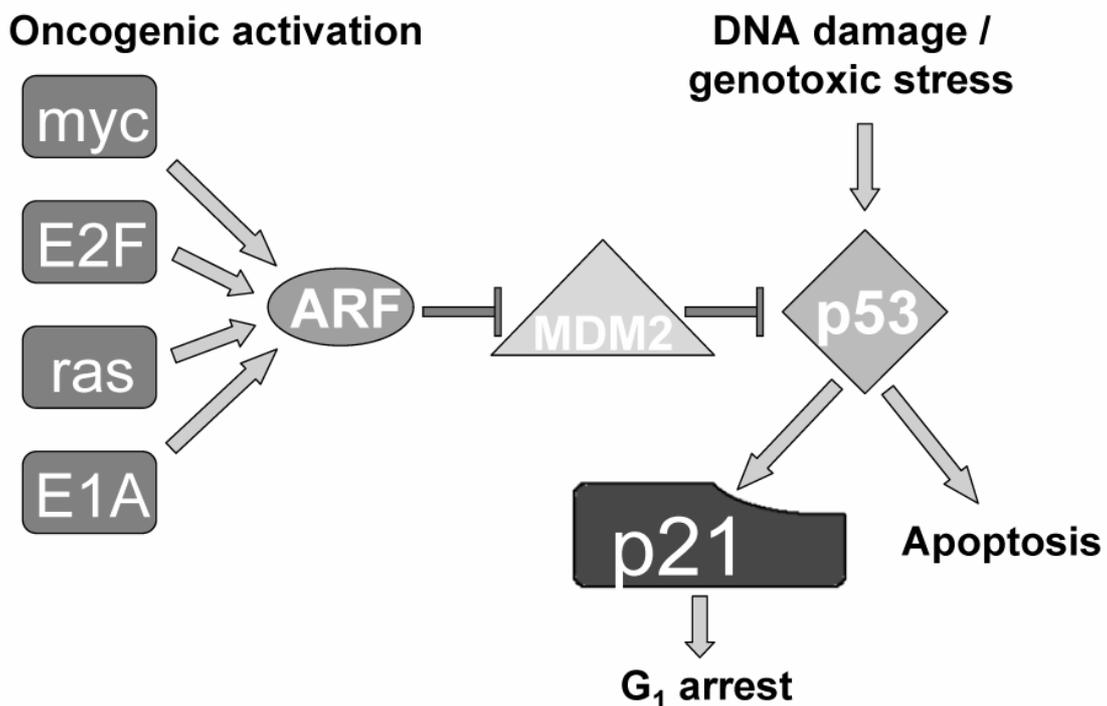


Figure 9: Oncogenic activation, genotoxic stress, or DNA damage can cause G₁ arrest by p53 dependent pathways. If the levels of any a wide range of proteins promoting G₁ progression increases above normal levels ARF is induced and inhibits MDM2, which releases p53 activity. DNA damage or genotoxic stress also increases p53 levels and activity. By mechanisms largely unknown p53 induces either apoptosis or arrests cell cycle progression by increasing the levels of CDK inhibitor p21.

Oncogenic activation

G₁ progression can be braked by oncogenic activation as a fail-safe against transformation, see fig 9. When one of the components of the extracellular

signalling cascades, such as Ras, has been rendered constitutively active the cell cycle machinery receives sustained hyperproliferative signals. These signals leads to the induction of the two genes at the INK4a-ARF locus (Serrano et al., 1997). The INK4a gene encodes p16, a CDK inhibitor which can arrest cells in G₁ by inhibiting cyclin D-CDK4/6 activity. The ARF gene product p19ARF activates p53 by inhibiting MDM2, and thereby causes apoptosis and/or G₁ arrest.

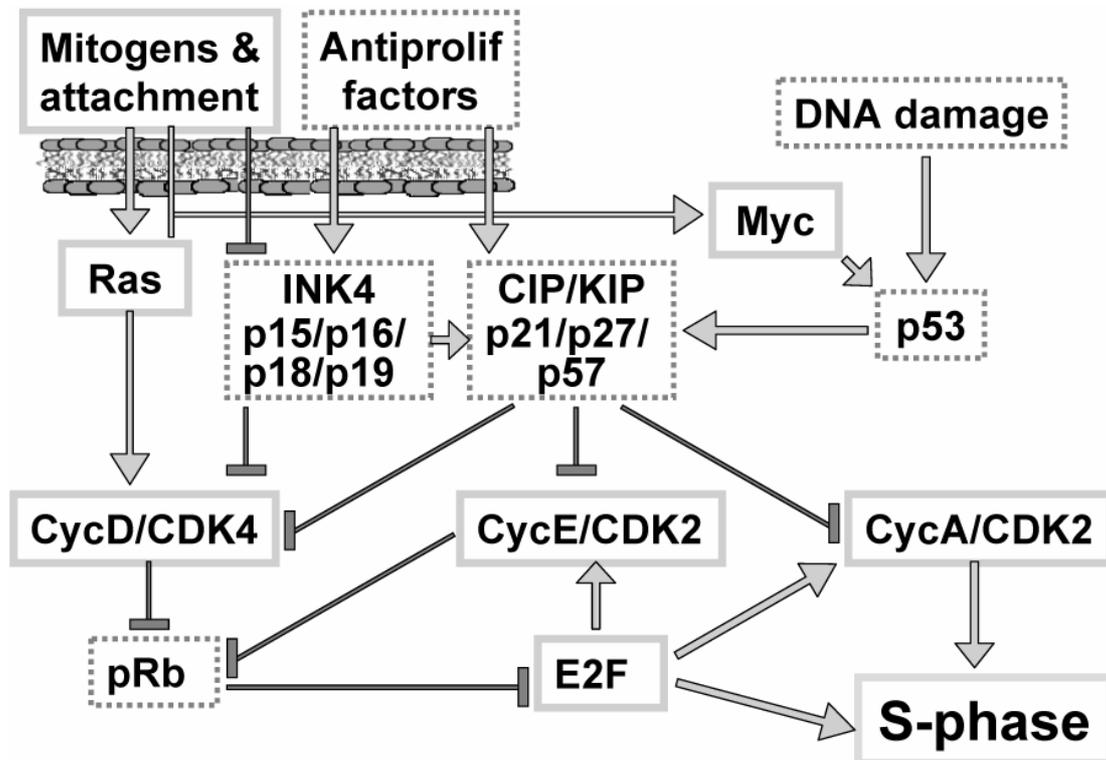


Figure 10: A simplified schematic of the regulation of G₁ progression. See the text for details.

The two regulatory axes of G₁

A schematic overview of the mechanisms regulating G₁ progression is shown in figure 10. It has been proposed that there are two G₁ signalling axes which both need to be altered in order for a cell to be transformed (among others Sherr, 2000). The first axis consists of the extracellular growth factor signalling cascade – cyclin D-CDK4/6 – pRb – E2F – cyclin E-CDK2 – cyclin A-CDK2. An aberration in this axis, or in the CDK inhibitors affecting it, can result in continuous proliferation and independence of cell attachment. The other axis consists of ATM/ATR/CHK2/p19ARF – MDM2 – p53 – p21. When this axis is silenced the cell can no longer detect the oncogenic activation created by an aberration in the first axis and react accordingly by entering apoptosis. The cell also becomes unable to detect DNA damage, and may progress through the cell cycle also in the face of grave genetic aberrations.

The S-phase

S-phase is defined as the part of the cell cycle during which the cell synthesises DNA. With few exceptions each of the two daughter cells should contain an exact copy of the DNA contained in the mother cell. Each of the 3.2×10^9 bases of the 46 chromosomes in the human cell must be copied once, and only once, during the cell cycle. Hence DNA synthesis is closely regulated.

DNA licensing

Cell fusion experiments have shown that a G₁ cell fused with a cell in S-phase would initiate DNA replication prematurely, while a G₂ cell fused with a cell in S-phase would be arrested in G₂ until the S-phase nucleus finished synthesising DNA (Rao and Johnson, 1970). The experiments indicated that only the DNA contained in cells in G₁ or S-phase was able to be replicated. This led to the conclusion that DNA had to be “licensed” to be replicated, and that once a piece of DNA was duplicated it lost its license.

DNA replication starts at the binding sites of proteins called ORCs (Origin of Replication Complex). ORCs are bound to DNA through the cell cycle. Several of the components necessary for DNA synthesis bind to the ORC in late M or early G₁, forming a pre-replication complex (pre-RC), and thereby rendering the DNA licensed for replication. At the G₁/S border the last factors necessary to initiate DNA replication are added, transforming the pre-RC into an initiation complex (IC). When the IC fires and the replication forks move away, many of the components of the IC are removed from the DNA, and only a post-replication complex (post-RC) remains at the site of initiation. The post-RC can not fire another round of DNA synthesis until it is again converted into a pre-RC in the next G₁.

The ORC proteins form complexes at the sites of DNA initiation

In *S cerevisiae* the initiation sites for DNA replication have been extensively studied. Six proteins designated Orc1 to Orc6 form complexes at specific sites defined by the DNA sequence. The protein complex remains bound to the DNA throughout the cell cycle (Bell and Stillman, 1992) and defines the location of the initiation sites for DNA replication. Human homologues of each of the 6 yeast Orc proteins have been found, but not all of the human homologues (hsOrcs) are associated to DNA throughout the cell cycle (Landenberger et al., 2002; Abdurashidova et al., 2003). However, a core structure is formed by hsOrc2, hsOrc3, and hsOrc4 in HeLa cells (Vashee et al., 2001). The importance of the detachment and reattachment of some of the hsOrcs during the cell cycle remains to be elucidated. It is also unclear how the human ORC binding sites are defined, as the human DNA replication initiation sites seem to lack similarities in DNA sequence (Vashee et al., 2003). It therefore has been proposed that epigenetic factors may decide where ORC is bound in human cells.

Events resetting the post-RC into a pre-RC after the conclusion of M

The ORC functions as a docking station for Cdt1, Cdc6, and Noc3p, which are loaded onto the ORC as the CDK activity drops when cyclin B is degraded in late mitosis (Cocker et al., 1996; Nishitani et al., 2000; Zhang et al., 2002). Throughout S and G₂ Cdt1 is unable to bind to ORC as it is tightly associated to geminin (Wohlschlegel et al., 2000). After Cdt1, Cdc6, and Noc3p are attached to ORC the Mcm2-7 proteins are able to attach to the complex. After the Mcm2-7 proteins have been loaded onto the ORC the DNA is licensed for another round of replication, at least in *Xenopus* (Kubota et al., 1997). Mcm10 is also attached to the complex by interacting with Mcm7 in G₁, but it is unclear whether this event occurs before or after the complex becomes licensed in human cells (Gerbi et al., 2002).

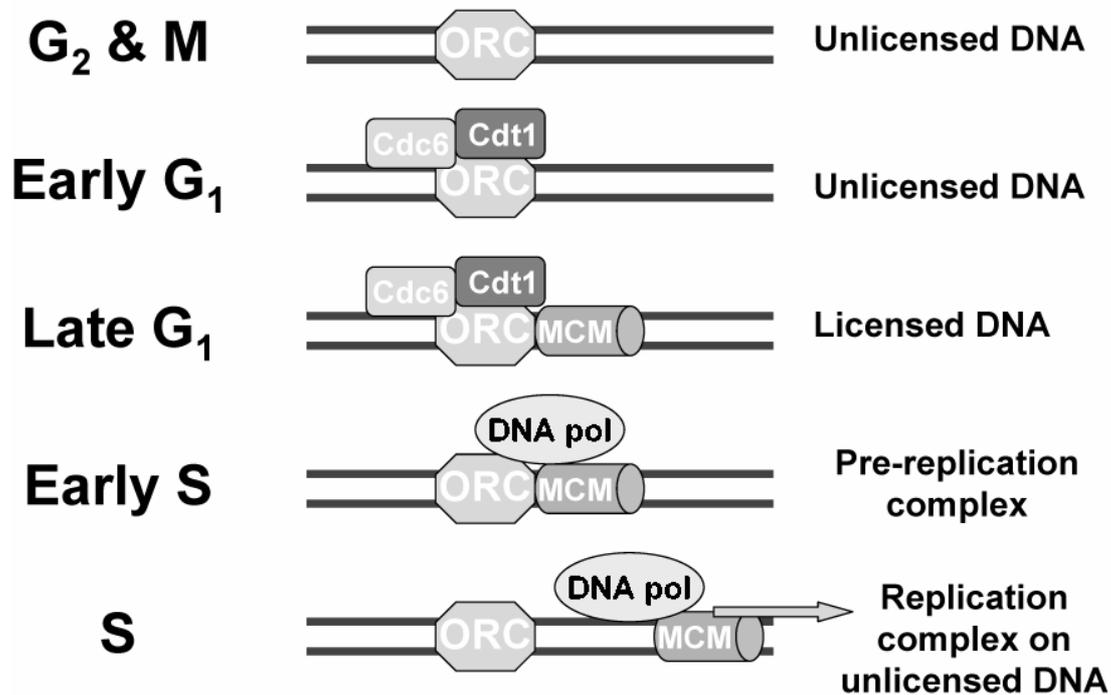


Figure 11: DNA licensing is organised around the ORC complexes and ensures that DNA is replicated only once per cell cycle. Unlicensed DNA can not load the proteins necessary to construct a pre-replication complex. Only where pre-replication complexes are localised the DNA replication can be started. The stepwise preparation of DNA for DNA replication is regulated by the activity of several cyclin-CDK complexes, see the text for details.

Turning the pre-RC into an IC and firing off DNA replication

Around the G₁/S border additional molecular events are needed in order to turn the pre-RC into an initiation complex. As cyclin A begins to accumulate the cyclin A-CDK2 dependent phosphorylation displaces Cdc6 from the nucleus, thereby effectively preventing relicensing of fired ICs (Petersen et al., 1999). Cdt1 is also displaced from the pre-RC around the G₁/S border (Maiorano et al., 2000). Instead Cdc45 is attached to the pre-RC (Zou and Stillman, 1998). Cdc45 binding is dependent on the kinase activity of the Dbf4p-Cdc7p complex (Zou and Stillman, 2000). The activation of the Dbf4p-Cdc7p complex is dependent on the presence of an active S-phase cyclin-CDK complex, at least in *S cerevisiae* (Nougarede et al., 2000). Replication protein A (RP-A) associates with the pre-RC simultaneously as Cdc45 (Zou and Stillman, 2000). Then the pre-RC finally has been turned into an IC ready to fire, as Cdc45 and RP-A binding leads to the unwinding of the DNA (Walter and Newport, 2000). Cdc45 also interacts with DNA polymerase (Kukimoto 1999; Mimura, 1998), which can be loaded onto the DNA with the help of Dpb11 (Masumoto et al., 2000). When the DNA polymerases have been loaded the initiation complex “fires” and DNA replication proceeds bidirectionally from site of initiation. DNA synthesis occurs continuously on the leading strand, and by the formation of Okazaki fragments on the lagging strand. After firing only the ORC remains at the site of initiation, whereby re-replication prior to M-phase completion is rendered impossible.

Organisation of DNA replication

Given the average distance between the ICs and the speed of the replication fork the human cell theoretically should be able to complete S-phase in about one hour. However, in real life S-phase lasts about six to eight hours in all mammalian cells. The delay occurs since not all ICs fire simultaneously. Instead DNA replication is organised in replication units consisting of about 60-80 closely located ICs which fire simultaneously. The replication unit firing order is similar between successive cell divisions. Replication units located at the sites of transcriptionally active genes have been shown to fire in early S-phase, while replication units consisting of the more condensed chromatin containing transcriptionally silent genes fire late in S-phase. This organisation of DNA replication gives rise to the BrdU staining patterns used to identify cells in early, mid, and late S-phase in paper II.

Sustaining DNA synthesis

Data from experiments utilising anti-cyclin A antibody injection at different points in the cell cycle indicated that cyclin A-CDK2 activity is necessary in order to sustain DNA synthesis (Pagano et al., 1992). The results could easily be explained by a need for the ICs to be exposed to cyclin A-CDK2 activity in order to fire, but the exact mechanism is not yet known. The dependence on cyclin A-CDK2 activity for the successful completion of S-phase is the basis for a regulatory system ensuring that DNA synthesis is halted in the face of DNA damage. Double stranded DNA breaks induced by ionising radiation are detected by ATM in S-phase, as well as in G_1 (see G_1 DNA damage section above). ATM-dependent phosphorylation of Chk1 and Chk2 activates their kinase activity, and leads to the subsequent phosphorylation of residues on the Cdc25A protein, thereby priming Cdc25A for degradation (Sorensen et al., 2003; Falck et al., 2001). Cdc25A is a phosphatase necessary for the cyclin A-CDK2 complex to remain active. Hence double stranded DNA breaks can stop progression through S-phase by an ATM, Chk1 and Chk2 dependent mechanism commonly referred to as the “S-phase checkpoint” (Falck et al., 2001; Kastan and Lim, 2000; Sorensen et al., 2003).

The G_2 -phase

G_2 is defined as the part of the cell cycle which occurs after DNA synthesis is completed, but before DNA condensation begins, which marks the entry into M-phase. G_2 is quickly traversed, in general only about one to two hours are spent in G_2 . The G_2 checkpoint ensures that all DNA is replicated and intact prior to M-phase entry.

During late G_1 , S, and G_2 cell cycle progression is driven by CDK2-dependent phosphorylation. In late G_2 the complex containing CDK1, also known as CDC2, and cyclin B are activated and imported to the nucleus. Cyclin B-CDK1 activity then triggers entry into mitosis (see below). Hence the activation of G_2 arrest is achieved when the cyclin B-CDK2 activity is abolished. The mechanisms detecting DNA damage in G_2 are similar as in G_1 and S, except that the target is inhibition of cyclin B-CDK1 instead of cyclin A/E-CDK2. This can be achieved by both a transcription dependent, and a transcription independent pathway. The transcription dependent pathway is essential for sustaining a G_2 -block, but takes several hours to come to full effect. The transcription independent pathway can

respond much faster to DNA damage, but is not sufficient for a sustained G₂ block.

The transcription dependent pathway

Also in G₂ do ATM and ATR detect DNA damage and activate p53. The transcription factor p53 then represses the genes encoding cyclin B and CDK1. In addition p53 activates genes encoding proteins that inhibit the activity of the cyclin B-CDK1 complex. Such proteins are the CDK inhibitor p21, which can render cyclin B-CDK1 complexes inactive by binding to them (Li et al., 1994), 14-3-3 σ , which can anchor CDK1 in the cytoplasm (Hermeking et al., 1997), and Gadd45, which can dissociate cyclin B from CDK1 by binding to it (Kastan et al., 1992; Zhan et al., 1999).

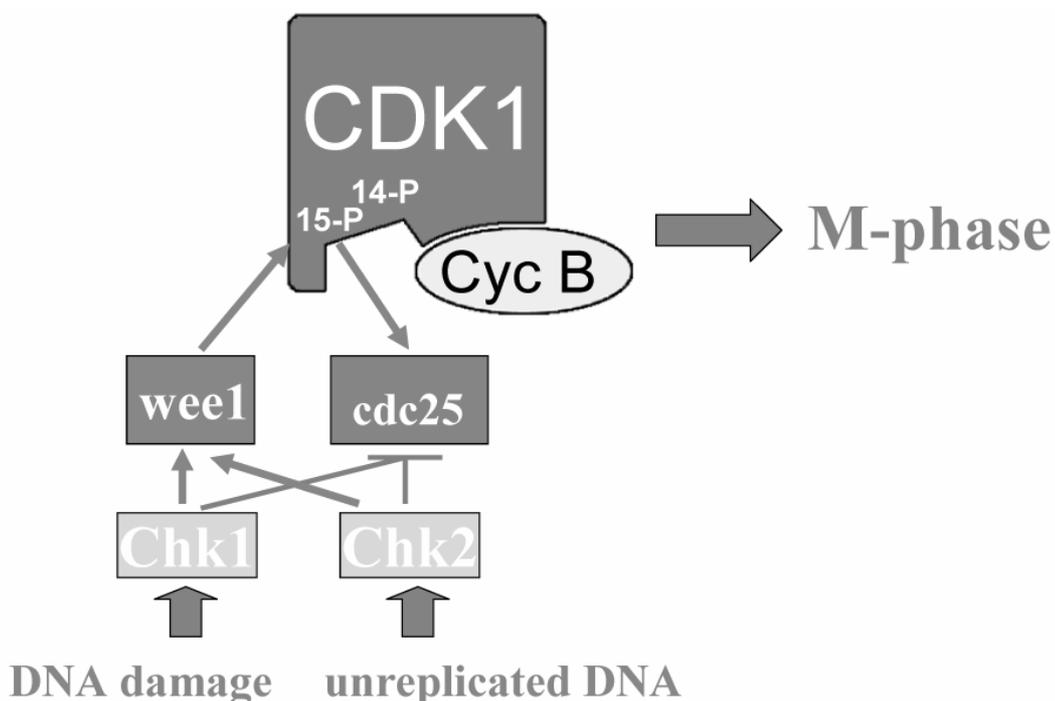


Figure 12: Phosphorylation and dephosphorylation of cyclin B-CDK1 regulates entry into M-phase. DNA damage and the presence of unreplicated DNA can halt progression through G₂ and into M-phase.

The transcription independent pathway

Cyclin B-CDK1 is kept inactive during G₂ by inactivating phosphorylation of CDK1 by the Myt1 and Wee1 kinases (Liu et al., 1997; Booher et al., 1997). The cyclin B-CDK1 complex can however be de-phosphorylated by the Cdc25 family of phosphatases. Apart from activating p53, ATM/ATR activation also leads to the phosphorylation and activation of Chk1 and Chk2 (Chaturvedi et al., 1999; Furnari et al., 1997). Chk1/2 subsequently phosphorylates members of the Cdc25 family (Sanchez et al., 1997). In human cells there are three members of the family, Cdc25A, which probably has its main functions during G₁ and S-phase, and Cdc25B and Cdc25C, which fill their main function as regulators of entry into M-phase. Both Cdc25B and Cdc25C can promote mitosis, but are likely to have slightly different functions (Karlsson et al., 1999). When Cdc25C is phosphorylated it can be sequestered by members of the 14-3-3 protein family (Peng et al., 1997). Hence ATM/ATR activation leads to CHK1/2 activation

resulting in Cdc25 inactivation and subsequent inactivating hyper-phosphorylation of CDK1 by Wee1/Myt1. Cyclin B-CDK1 becomes inactivated and remains in the cytosol, and the cell arrests in G₂.

The detection of unreplicated DNA

In both *S cerevisiae* and *S pombe* the Chk2 homologues are responsible for arresting cell cycle progression at the G₂/M checkpoint if there is unreplicated DNA present in the cell (Rhind and Russel, 1998). However, in mammalian cells both Chk1 and Chk2 are able to respond to the presence of unreplicated DNA (Zheng et al., 1998). Once Chk1 or Chk2 are activated, the G₂ cell is arrested by the same mechanisms as when exposed to DNA damage (see *The transcription independent pathway* above).

The M-phase

M-phase, or mitosis, is the most dramatic part of the cell cycle. During M-phase the DNA, which has been copied during S-phase, must be correctly divided. Each half of the DNA is then drawn to each of the two poles of the cell, before the cell is divided.

Mitosis consists of several steps. During prophase the centrosomes begin to form the mitotic spindle consisting of microtubuli, and the DNA condenses. Prometaphase begins as the nuclear envelope breaks down. The chromosomes can then attach to the microtubuli of the mitotic spindle via their kinetochores and begin to move. When all the chromosomes have lined up halfway between the centrosomes the metaphase ensues. In anaphase the sister chromatids of each chromosome breaks apart and begin to move toward separate poles. The chromatids arrive at the poles in telophase, and two new nuclear membranes are formed around each of the two now completely separated sets of chromatids. Finally the cytosol of the cell is divided in two during cytokinesis by the pinching action of a contractile ring of actin and myosin filaments.

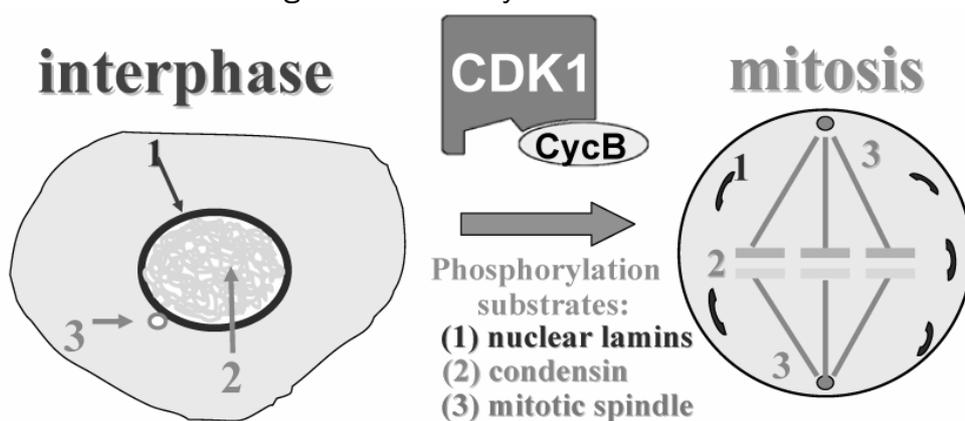


Figure 13: Progression through the early phases of G₂ is dependent upon the kinase activity of the cyclin B-CDK1 complex. Phosphorylation of lamins causes nuclear envelope breakdown. Other targets for cyclin B-CDK1 phosphorylation cause chromosome condensation and formation of the mitotic spindle.

Cyclin A is needed for progression through early M-phase (Furano et al., 1999). The main M-phase cyclin is however cyclin B, which associates with CDK1, also known as cdc2. The activity of the cyclin B-CDK1 complex leads to the degradation of the nuclear envelope, the condensation of the chromosomes, and

their subsequent alignment to form a metaphase plate. Before the cell can progress from metaphase to anaphase cyclin B has to be degraded. The loss of cyclin B-CDK1 activity leads to the migration of the chromosomes towards the poles, and the division of the cell in two.

Preparing for mitosis by keeping chromatids together

In order to split the DNA into two identical halves the cell must keep the sister chromatids together during interphase. In G₁ a protein complex called cohesin is attached to the DNA strands (Michaelis et al., 1997; Schmiesing et al., 1998; Hakimi et al., 2002). The main components of the cohesin complex are Scc1, Scc3, Smc1, Smc3 (Freeman et al., 1999). Cohesin is thought to form a ring around the strand and as it is replicated in S-phase both copies of the strand are kept within the ring, hence sister chromatids are attached to each other throughout G₂ (Haering et al., 2002; Haering and Nasmyth, 2003).

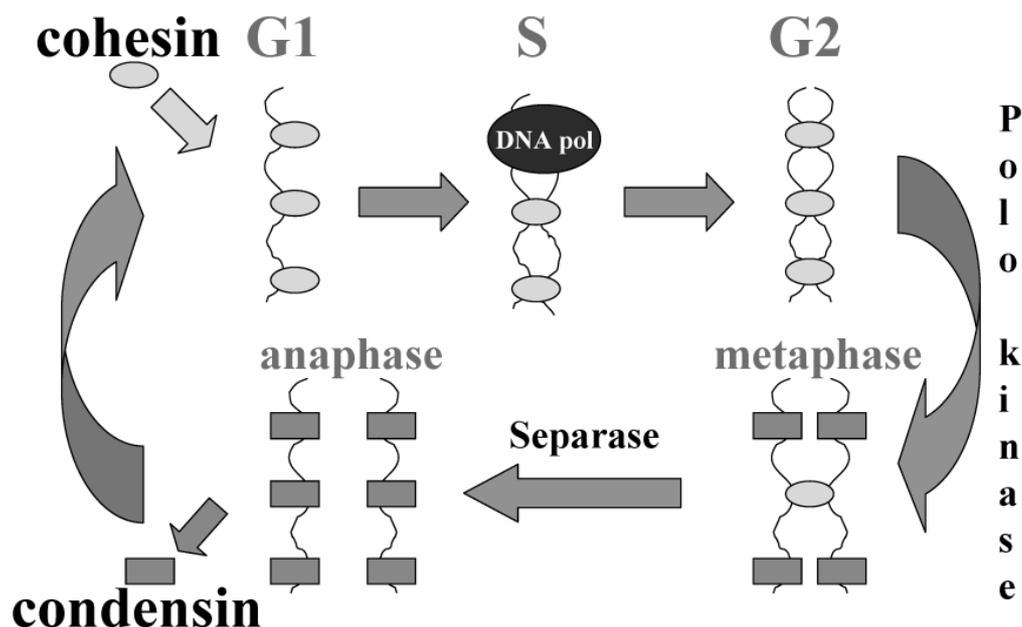


Figure 14: Sister chromatids are kept together from S-phase until anaphase. Cohesin is loaded onto the G₁ chromosomes and keeps the sister DNA strands resulting from DNA replication together. As the cell enters mitosis all cohesin not located at the kinetochores is replaced with condensin, which enhances chromosome condensation. The remaining cohesin is cut by separase at the metaphase/anaphase transition. Finally the condensin is removed in late M-phase, and the DNA is decondensed.

Molecular regulation of prophase events

In prophase the newly activated cyclin B-CDK1 complex ensures that the transition from interphase to mitosis is irreversible by phosphorylating cdc25 family members. The inhibitory effects of cdc25B and cdc25C on the cyclin B-CDK1 complex are thereby diminished, causing a positive feedback loop. Hence the activation of cyclin B-CDK1 irrevocably causes interphase exit.

Cyclin B-CDK1 complexes also phosphorylate, and thereby activate, the polo like kinase (Plk). Activated Plk also contribute to the inactivation of cdc25, thereby reinforcing the positive feedback loop. Furthermore, Plk recruits γ -tubulin and activates Asp. Asp is located at the centrosomes and help focus the minus ends

of the microtubuli. Thus active Plk is crucial in the formation of the bipolar spindle in the early phases of mitosis. Activated Plk also removes the cohesin molecules keeping sister chromatids together (Sumara et al., 2002). However, at the kinetochores the cohesin remains and thereby ensures that the sister chromatids are kept together until the end of metaphase.

As cohesin is removed the protein complex condensin takes its place. Condensin is a protein complex highly similar to cohesin. However, instead of trapping two strands from different sister chromatids it attaches to the same chromatid in two places, thereby forcing it to form a loop (Haering and Nasmyth, 2003). Condensin attachment to DNA is dependent on the kinase activity of cyclin B-CDK1, and leads to the condensation of the chromosomes occurring in prophase (Kimura et al., 1998).

The nuclear envelope consists of a lipid bi-layer supported by the nuclear lamina, a mesh of proteins. The nuclear lamina contains members of the lamins protein family, which can be phosphorylated by cyclin B-CDK1. Following phosphorylation of the lamins the nuclear lamina is disrupted, and the nuclear envelope breaks down (Peter et al., 1990; Dessev et al., 1991). As nuclear envelope breakdown is achieved the cell enters prometaphase.

Molecular regulation of prometaphase events

In prometaphase the microtubuli of the mitotic spindle gain access to and connect with the kinetochores of the chromosomes, as the nuclear envelope is removed. HsEg5 is phosphorylated and thereby activated by cyclin B-CDK1. HsEg5 is a motor protein associated with the microtubuli and is involved in creating the pulling force exerted upon the kinetochores of the chromosomes by the microtubuli in prometaphase, metaphase and anaphase (Blangy et al., 1995).

During prometaphase only the cohesin at the kinetochores keep the sister chromatids together as they are being pulled apart by the microtubuli of the spindle and while they are aligned at the equator of the spindle. Separase is a protein which can cleave the Scc1 component of cohesin, but it is kept inactive by phosphorylation by the cyclin B-CDK1 complex (Zou et al., 1999; Stemmann et al., 2001). The activity of separase is during prometaphase further inhibited by binding to securin, which covers its active site (Yanagida, 2000). However, as soon as separase is released the sister chromatids can be separated (Hauf et al., 2001).

Detection of chromosome alignment in metaphase

In metaphase the chromosomes are aligned at the equator of the mitotic spindle. The sister chromatids remain attached to each other, but are being pulled apart by the microtubuli. Before the remaining cohesin can be cut the cell must ensure that both sister chromatids of all the chromosomes are attached, and that they are attached to microtubuli pulling them towards different poles (amphitelic attachment). Otherwise separase activation would cause non-disjunction as both sister chromatids of one or more chromosomes would be pulled to the same pole.

Successful amphitelic attachment leads to a rise in tension between the kinetochores of sister chromatids. Lack of tension between the chromatids of as little as one chromosome results in arrest in metaphase (Rieder et al., 1995). The

exact molecular mechanism detecting tension is a matter of debate (Chan and Yen, 2003), but it has been shown in lower order species that the genes Mad1-3, Bub1, Bub3, and BudR1 are necessary for metaphase arrest. The gene products localise to unattached kinetochores. It has been proposed that Bub1 or BubR1 may in the absence of tension prime Mad2 to sequester cdc20 (Shannon et al., 2002). When Mad2 is bound to cdc20 the activity of the Anaphase Promoting Complex/Cyclosome (APC/C) is inhibited (Li et al., 1997; Fang et al. 1998).

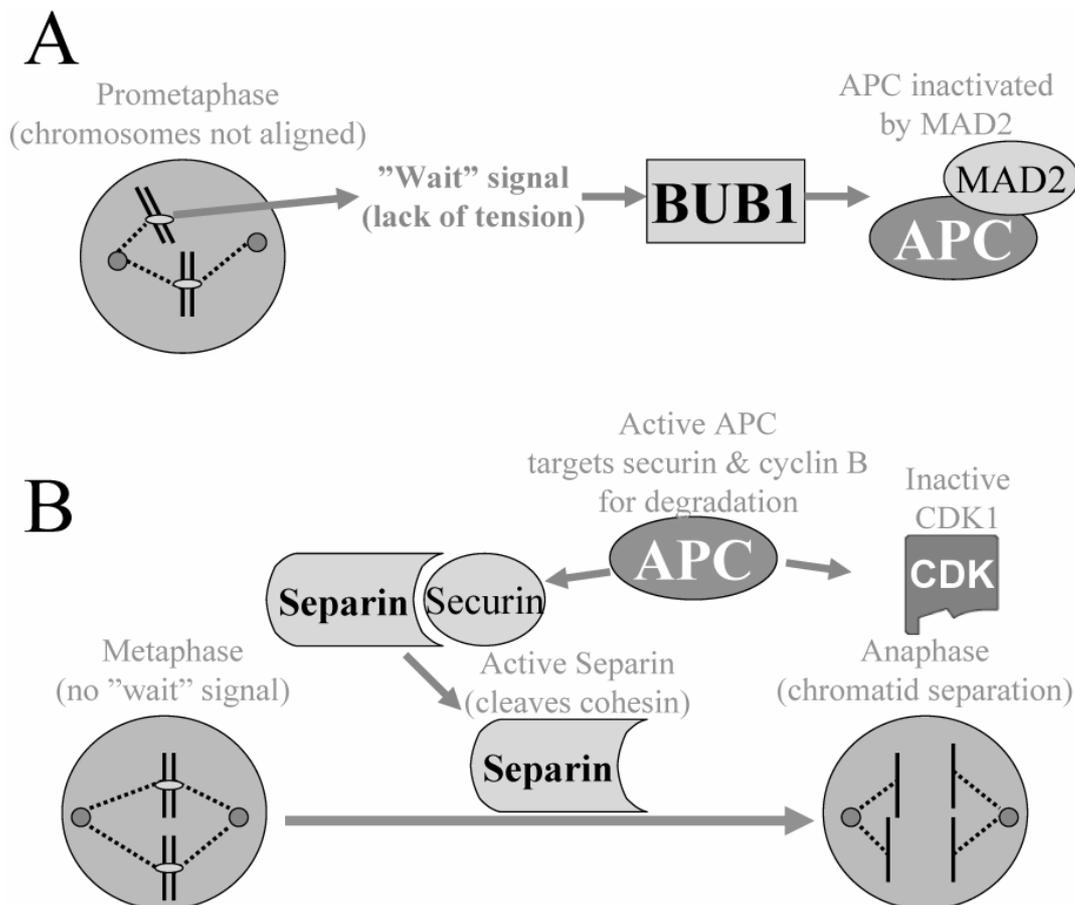


Figure 15: Unless all chromosomes are correctly attached to the mitotic spindle progression from metaphase into anaphase is halted. A: As long as a single chromosome lacks tension between the sister chromatids APC is inhibited by a Bub1-Mad2-dependent pathway. B: Once the chromosomes are correctly attached between the poles of the mitotic spindle the APC inhibition is removed and cyclin B and securin can be degraded. Securin degradation leads to the release of separin, which cuts the remaining cohesin, thereby releasing the sister chromatids, which then proceeds to migrate towards different poles.

Anaphase initiation through APC/C activation

Soon after all chromosomes have achieved amphitelic attachment the Bub-Mad mediated inhibition of APC/C is lifted. The APC/C is a ubiquitin ligase that targets both cyclin B and securin for destruction once it is activated by binding to cdc20 (Irniger et al., 1995; Zachariae and Nasmyth, 1996; Tugendreich et al., 1995; King et al., 1995).

Ubiquitination of a protein is a multistep process and leads to the addition of a chain of ubiquitin to a protein, which subsequently can be detected and degraded by the 26S proteasome (Finley et al., 1984; Chiechanover et al., 1984). Ubiquitin

dependent degradation is central to the regulation of cell cycle progression as many of the cyclins, their inhibitors, as well as their activators, are degraded by the ubiquitin pathway in a highly regulated manner (reviewed in Reed, 2003).

When cyclin B and securin has been degraded separase becomes active. It cuts the remaining cohesin, and the anaphase is initiated as the chromatids begin to migrate towards opposite poles of the mitotic spindle.

Telophase and cytokinesis are caused by lack of cyclin B

In telophase the lack of cyclin B-CDK1 activity causes the lamins phosphorylated during prophase to return to their original state, and thereby the nuclear lamina can reform, resulting in the formation of two nuclei (Wheatley et al., 1997). Furthermore is the decondensation of the DNA during telophase dependent on the degradation of cyclin B in late metaphase/early anaphase (Wheatley et al., 1997).

Similarly the actions by the structural proteins causing cytokinesis are inhibited by cyclin B-CDK1 activity. As it is diminished cytokinesis ensues, resulting in the division of the cytosol, and the formation of the two daughter cells (Echard and O’Farrell, 2003). As soon as the daughter cells have been completely divided mitosis is finished and the cells enter G₁.

Please refer to the section *The G₁-phase* above for an overview of the regulation of the cell cycle progression of each of the two daughter cells.

Life cycle of the centrosome

The centrosome is the principal microtubuli organising center in all animal cells. Similar to DNA the centrosome must be duplicated once, but no more than once, each cell cycle. Hence also the life cycle of the centrosome is tightly regulated. In early G₁ only one centrosome exists in the cell, but the centrosome contains a pair of centrioles. In late G₁ the centrioles separate under the influence of cyclin E-CDK2 activity (Hinchcliffe et al., 1999; Lacey et al., 1999; Matsumoto et al.,

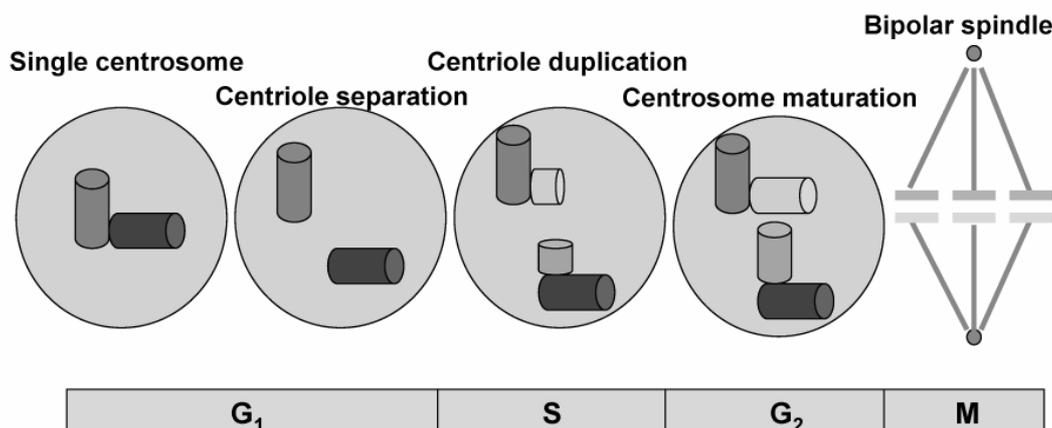


Figure 16: The life cycle of the centrosome. In late G₁ the centrioles of the centrosome separates. During S-phase the centrioles are duplicated, and during G₂ the centrosome matures as the elongation of the new centrioles is completed. During mitosis the centrosome splits to form two asters, each of which forms a pole of the bipolar mitotic spindle.

1999). The duplication of the centrioles is however also dependent on the presence of cyclin A-CDK2 activity (Lacey et al., 1999), which appears in S-phase. In G_2 each of the pairs of centrioles mature, but still remain within the same centrosome. As the cell enters mitosis the centrosome splits, and each of the pairs of centrioles form an aster. The asters migrate to opposite sides of the cell and form the two poles of the mitotic spindle. As the cell divides each daughter cell receives one aster containing two centrioles that form a centrosome.

Cells containing an aberrant number of centrosomes are likely to fail at separating the sister chromatids properly in mitosis, resulting in unequal separation of the genome. Endoreplication of DNA (two rounds of DNA replication without an intervening M-phase) followed by an aberrant mitosis due to a deranged number of poles has been proposed to be the mechanism behind the grave chromosomal aberrations seen in many aneuploid tumours. The regulation of the life cycle of the centrosome is therefore important for the maintenance of genetic stability.

The cell cycle and cancer

The first discovered oncogenes were genes coding for proteins involved in signal transduction, such as Ras, Myc, and Raf. This led to the popular misconception that cancer cells misinterpreted extracellular cues, but had a normal cell cycle progression. Today we know that a hallmark of cancer is genetic instability, which arises only when the regulation of the cell cycle is deranged, and not through solely constitutive activation of signalling cascades.

Genetic instability and cancer

A major characteristic of the malignant tumour cell is its ability to rapidly acquire new characteristics. Such characteristics essential to the tumour cell are the ability to penetrate basal lamina and invade surrounding tissues, the ability to enter and exit the bloodstream, the ability to grow elsewhere in the body than in the tissue of origin, the ability to stimulate endothelial cells to form new blood vessels to support the increasing metabolic requirements of a tumour, and the ability to become resistant to treatment. The only way for the transformed cell population to quickly acquire all those abilities is through genetic instability.

The importance of genetic instability for the progression of cancer is emphasised by the fact that highly aneuploid tumours, that is tumours containing cells with highly aberrant amounts of genetic material, progress faster and more often kill their hosts than diploid tumours. Hence one of the holy grails of cell cycle research is to establish the molecular basis for genetic instability, as it would open the field for the development of novel treatment strategies and better prognostic tools.

Several mechanisms for the rise of genetic instability have been proposed. Cells defective in their repair systems fail to correctly repair DNA damage and correct replication errors, and therefore develop numerous point mutations. Cells failing to maintain the integrity or number of their chromosomes, for instance by failing to correctly duplicate their centrosomes, by prematurely losing sister chromatid cohesion, or by undergoing endoreplication, commonly exhibit gross abnormalities in their karyotype. Finally are cells with a slowly progressing replication fork more prone to acquire gene amplification or deletion. Different tumours commonly

exhibit different types of genetic damage, suggesting that even tumours arising from the same type of tissue do not share the same cell cycle or DNA repair defects.

Detection of genetic damage

Although the mechanisms behind genetic instability varies considerably between tumours all tumours cells must fail to detect and/or correctly react to the genetic damage incurred by genetic instability. As discussed above (see *The two regulatory axes of G₁*) the ATM/ATR/CHK2 – MDM2 – p53 – p21 pathway is very often, maybe always, muted in transformed cells. Consequently the p53 gene is the most commonly altered gene in human solid tumours, occurring in at least 50% of the tumours.

Cyclin A and cyclin E in cancer

Both cyclin A and cyclin E are intricately involved in the regulation of the DNA synthesis. Both proteins have also been implicated in the regulation of the life cycle of the centrosome. Finally can successive rounds of cyclin A and cyclin E accumulation cause endoreplication in megakaryoblasts. Hence there is a definitive potential for both cyclin A and cyclin E to cause genetic instability by several independent mechanisms simply by being expressed during the wrong phases of the cell cycle.

Aims of the study

The general aim of this thesis was to investigate the expression over the cell cycle of selected cell cycle regulatory proteins, in the hope of making new discoveries, preferably with clinical implications, regarding the aberrations in the cell cycle regulation seen in tumour cells. Aberrations in the cell cycle machinery may cause genetic instability, which generally leads to rapid tumour progression and potential fatal outcome for the patient.

The first goal of the study was to develop a method by which the location in the cell cycle of individual cells could be ascertained, as well as the cell content of a investigated protein. Since methods traditionally used to investigate cell cycle dependent protein expression, such as Western blots, require cell synchronisation they are less reliable. Cell synchronisation can potentially cause cell cycle aberrations, and therefore we instead choose to focus on methods in which individual cells could be studied. Furthermore are tumour cell populations highly heterogeneous, which further emphasises the importance of single cell studies.

Second we set out to investigate the expression pattern of cyclin A in normal and transformed cells. Cyclin A is involved in driving DNA synthesis, as well as the regulation of the duplication of centrioles, and hence could a deregulated expression of cyclin A potentially cause genetic instability.

Third we choose to investigate the expression of cyclin E over the cell cycle, as cyclin E is also involved in regulating DNA synthesis, especially in the licensing of DNA. Cyclin E had also been previously implicated in driving endoreplication in megakaryoblasts and had been shown to be overexpressed in tumours. Finally cyclin E-CDK2 activity is crucial for centriole separation. A heterochronic expression of cyclin E (the expression of cyclin E during the wrong phases of the cell cycle) thus could potentially lead to genetic instability.

Finally a case-control retrospective clinical study was performed with the intent to investigate the implications a heterochronic cyclin E expression have for the clinical outcome for patients with neoplastic disease.

Materials and methods

A range of different methods were employed during the experimental work underlying this thesis. Many experimental approaches were tried but failed to generate reproducible and original data in sufficient amounts for publication. Instead of describing all methods that were tried irrespective of outcome this thesis focuses on immunofluorescence staining coupled with image cytometry techniques based on digital image analysis, as that was found to be the most successful and original approach.

The choice of methods used in this thesis

The time spent in G_1 varies considerably between normal cells, even when they are exposed to identical conditions, and in tumour cell populations the intercellular differences in cell progression are even greater. Hence it is advantageous to study individual cells when investigating the cell cycle machinery. An added advantage gained by investigating individual cells is that also the degree of intercellular heterogeneity can be studied. Unfortunately few methods lend themselves to the single cell approach. Different blotting techniques generally require more material than can be collected from a single cell or cell nucleus. Instead methods by which the cells are stained, and the amount of staining evaluated, can be used. Examples of such methods are immunohistochemical or immunofluorescence staining methods, and *in situ* hybridisation protocols. Evaluation can be done by visual evaluation, or by automated methods. The protocols relying on fluorescent fluorophores for detection, i.e. immunofluorescence staining and fluorescence *in situ* hybridization (FISH), are better suited for automatic evaluation. They are also considered to be more quantitative than histochemical detection methods relying on enzymatic reactions.

Measurement of the fluorescence emitted by individual cells can be done either by flow or image cytometry. Flow cytometry has the advantage of producing large amounts of data with less effort, while image cytometry is considerably more tedious with the equipment available today. However, flow cytometry can only be applied to cells in suspension. When cells are extracted from a tissue sample and suspended in buffer the spatial relationship between the studied cells is lost. It is also impossible to trace the origin of individual measurements when using flow cytometry. Image cytometry have the benefits that it can be applied to tissue sections, and thus a small clone of cells in near proximity with an aberrant behaviour can be detected and further investigated, as the area of the tissue section containing the cells can be localised.

The methods mainly utilised in this thesis in order to study the expression of cell cycle regulatory proteins over the cell cycle were immunofluorescence staining, digital image acquisition and image analysis. A novel method called sequential immunofluorescence staining, which makes it possible to semi-quantitatively measure the levels of a large number of different antigens in individual cell nuclei, was also developed.

Preparation of cell material

In order to acquire normal and tumour cells to be studied cell cultures were used. Both primary cell cultures consisting of normal cells and cell lines consisting of tumour derived transformed cells were utilised.

All cell cultures were maintained in an incubator maintaining a 5% CO₂/95% air mix and 100% humidity at 37 °C. Cells were transferred to new bottles before reaching confluence. When transferring cells a solution based on Puck's modified saline A and containing 0.5mM EDTA and 0.25% (w/v) trypsin was used. Prior to staining the cells were seeded on cover slips in petri dishes at a density of approximately 3000 cells/cm². The cells were allowed to attach to the cover slips and then cultured for approximately 24h prior to fixation.

In some experiments incorporation of bromodeoxyuridine (BrdU) was used in order to mark newly synthesised DNA. BrdU is incorporated in DNA during DNA synthesis in S-phase cells. The longer the time between the addition of DNA to the cell medium, the more BrdU is incorporated. If BrdU incorporation time is kept short, and the cells are fixed right after incorporation, then the BrdU staining pattern shows the location of active replicons. During the experiments performed in the work described in this thesis BrdU was usually added to a concentration of 20µM for 5-10 minutes.

Many different methods of fixation were used, and even more tried, during the experimental work behind this thesis. Different types of fixation can potentially lead to the exposure of different antigenic sites on the studied proteins, hence the fixation protocol is of paramount importance to the outcome of the subsequent staining. Fixation in formalin is the standard method for fixating tissue samples in pathology laboratories. Formalin fixation does however result in higher background autofluorescence, therefore 4% freshly prepared paraformaldehyde was mostly used instead. An alternative fixation agent commonly used for cultured cells was methanol. After fixation, generally for about an hour, the cells were stored in 70% ethanol at 4 °C. Storage times were kept as short as possible.

Preparation of tissue samples

Paraffin embedded tissue samples were acquired from the pathology archives at Karolinska University Hospital in Stockholm, Umeå University Hospital in Umeå, Akademiska Hospital in Uppsala, and the archives of the Stockholms läns landsting. Tissue slices usually 4µm thick were cut and melted onto glass slides by incubation overnight at approximately 47 °C. After storage at -20 °C the slides were stepwise rehydrated in graded alcohols. The slides with the tissue sections were then rinsed in water or washing buffer (0.3mM NaCl and 0.02% Tween 20 in a buffer consisting of 0.05mM TRIS-HCl at pH 7.6).

Material fixed in formalin (both cultured cells and tissue samples) was usually exposed to antigenic recovery. Antigenic recovery removes some of the bonds introduced by formalin fixation, thereby uncovering antigenic sites for the primary antibodies. The main method of antigenic recovery was by boiling for 2-3x15 minutes in 0.1M citrate buffer at pH 6.0, although overnight treatment at 70 °C in

an EDTA solution was more successful for some tissue types such as breast tumour samples. After antigenic recovery the cells were again washed in washing buffer.

Immunofluorescence staining

All incubations below were performed in a covered moisture chamber at room temperature unless stated otherwise. In order to block unspecific protein binding of IgG molecules prior to the exposure to the primary antibodies the tissue sections were preincubated with blocking buffer (1% bovine serum albumin and 0.5% Tween 20 in PBS) for 15-30 minutes. Next the slides were incubated with the primary antibodies diluted between 1:10 and 1:1000 (depending on the antibody used) in blocking buffer for 8 to 48 hours. The slides were then washed immersed in washing buffer on a shaker for 3x10-45 minutes. Longer washing times result in less background staining, but excessive washing can also lower staining intensity, as seen in paper I.

Primary antibody detection was performed by preincubating the slides with 4% donkey serum diluted in blocking buffer for 15-60 minutes. Fluorophore or biotin conjugated secondary antibodies diluted between 1:100 and 1:1000 in blocking buffer with 4% donkey serum were then added for 0.5-1 hour, followed by another round of washing for 3x10-45 minutes in washing buffer. If biotin conjugated secondary antibodies were used the slides were incubated in fluorophore conjugated streptavidin for 30-60 minutes, and then washed for 3x15 minutes in PBS.

Generally cell nuclei were visualised by DNA staining using 4',6-diamidino-2-phenylindole (DAPI). DAPI was either added to the washing solution during the second last round of washing, or included in the mounting medium. Using mounting mediums containing DAPI was convenient, but resulted in slightly more background staining and staining intensity was harder to control.

Mounting, used microscope, and image acquisition

The slides with tissue sections or cover slips with cultured cells were finally mounted for microscopy. Either the Vectashield mounting medium H-1200 from Vector Laboratories or the DABCO mounting medium (25mg/ml Kodak DABCO and 0.1xPBS in spectroglycerol, pH set to 8.6 using HCl) was used for mounting. The mounting mediums were designed to ensure photo bleaching was minimised, and to optimise the optical conditions for microscopy.

The mainly used image acquisition system consisted of a DeltaVision fluorescence 3D microscope system with either a Zeiss Plan-Apochromat 63x/NA1.4 lens or a Zeiss Plan-Neofluar 40x/NA1.3 lens. A mercury lamp was used as light source, and image acquisition was achieved using a water-cooled monochrome CCD camera from Roper. Image resolution was 0.2µm along the X and Y axes and 0.4 along the Z axis using the x63 lens, and 0.5µm along the X and Y axes and 1.0 along the Z-axis using the x40 lens. Selective excitation and emission filters designed for each of the used fluorophores were used. Hence a picture over an area stained using the fluorophores DAPI, FITC and Cy3 actually consisted of three greyscale pictures, each acquired using different sets of excitation and emission filters.

Digital image analysis

Digital image analysis generally consists of the steps pre-processing, registration, segmentation, feature extraction, and data analysis.

Pre-processing

Pre-processing may be needed to reduce effects on the image caused by imperfections in the image acquisition system. The pre-processing most frequently utilised in this thesis is the DeltaVision calibration system. The DeltaVision calibration system is based on knowledge about the set up of the microscopy acquired through the use of images acquired under very specific circumstances. Images taken with the excitation shutter closed allows the system to calculate the dark current of the CCD. Images of a microscope slide containing nothing but a homogeneous fluorophore solution is used to compensate for imperfections in the light distribution. Finally are images acquired with a range of different exposure times used to measure the response of each pixel unit in the CCD. The information regarding dark current, light distribution, and linear response are then integrated and applied to subsequent image acquisitions in order to compensate for the imperfections in the system set up. However, the calibration has to be performed for each of the sets of excitation and emission filters (i.e. for each fluorophore to be used), and it must be repeated each time the optical conditions in the microscope are changed, such as after a dramatic shift in the ambient air temperature, or after a light bulb replacement.

Many algorithms and theories have been developed to allow pre-processing without the type of prior knowledge that the DeltaVision system requires (comparison in Lindblad and Bengtsson, 2001). The advantage with such methods is that they can compensate for intensity nonuniformities also when the nonuniformities are caused by the investigated tissue specimen, such as when there are variations in the thickness of the tissue section or in the autofluorescence caused by extracellular matrix variations.

If a series of images of the same object are acquired and the relationship between the camera and the object changes between the image acquisitions image registration is required. Image registration is also called spatial matching, and is the process of calculating the correct relationship between subsequently acquired images or volumes. The intent commonly is to make it possible to use the same segmentation mask for each of the images. An example of image registration is described in paper I, in which the same area of a tumour tissue section is photographed several times after different treatments. The subsequently acquired volumes were matched with regard to x, y and z translation, as well as z rotation to the first acquired image. The DAPI stain was used for the registration, as the other channels were stained differently between subsequent volumes. After image registration the images were transformed using trilinear interpolation according to the result of the spatial matching. Hence the differences between the volumes resulting after different treatments could be measured reliably, as the same segmentation mask could be used for all volumes. The exact registration algorithm used in paper I has been described elsewhere (Wählby et al., 2001).

Segmentation

Image segmentation is the division of an image into studied objects and background. Segmentation is commonly the most demanding part of the image analysis process. However, the better the quality of the image data, the easier the segmentation step becomes. In papers II and III most image segmentation was achieved by plain greyscale thresholding. The nuclei of cultured cells are very easily distinguished from the background when stained for DNA, as they only rarely overlap, and since the background staining intensity is very low due to the lack of an extracellular matrix. Furthermore could the fixation step be optimised to yield the lowest possible background.

Greyscale thresholding is one of the simplest segmentation techniques available. A threshold staining intensity value is defined that separates pixels belonging to the background and to objects (i.e. cell nuclei). Each contiguous area of the image consisting of pixels with higher intensity than the threshold can then be considered to be one object. Objects containing less or more than a set number of pixels can be assumed to consist of either artefacts, incomplete objects or several overlapping objects, and may be excluded after careful consideration.

Images from tissue sections were segmented in paper I, III, and IV. Such images are considerably more demanding to segment and require more efficient algorithms such as the watershed algorithm (Vincent and Soille, 1992). In the simplest case used in this thesis the watershed algorithm used two sliding thresholds (described in Wählby et al., 2001, and used in paper I). In brief, the upper threshold was initially set to the highest intensity in the image. Each found pixel in the image with an intensity higher than the upper threshold was considered part of a new object. The object was assumed to consist of all 8-connected pixels with staining intensities above the lower threshold. The difference between the upper and lower threshold therefore defined the highest acceptable difference between the highest pixel intensity in an object and the lowest pixel intensity in an object. The thresholds were then lowered by one and the process repeated, until a pre-set background intensity level was reached. Hence the version of the watershed algorithm developed and implemented by C Wählby for paper I differs from the original watershed algorithm by incorporating a simple merging step to reduce oversegmentation (the segmentation of one object into two or more objects). Several alternative versions of the algorithm were tried during the work underlying this thesis, and the most refined has been described elsewhere (Wählby et al., 2004).

Unfortunately were even the most advanced automated segmentation algorithms unable to result in completely reliable segmentation masks due to limitations in image quality and the diversity in tumour cell morphology. Therefore the segmentation masks suggested by the algorithms commonly had to be visually inspected and manually corrected. Much of the image segmentation described in this thesis should therefore be considered as semiautomatic.

Feature extraction

After pre-processing, registration and segmentation, the feature extraction is comparatively easy. It involves calculating the needed features of the objects defined by the segmentation mask. The features mainly used in this thesis were

object size (i.e. cell nucleus size) and average pixel intensity in each channel (i.e. the average nuclear staining intensity for each of the fluorophores). Occasionally the P2A value of the objects (a measurement of how round the nuclei were) or the distribution of intensity values within each object were used in order to identify artefacts that had managed to slip by the inspection and correction stage of the semiautomatic segmentation.

Data analysis

The data resulting from the feature extraction was displayed in graphs or further analysed using a widely spread spreadsheet software or a more flexible but not quite as widely spread language of technical computing.

A commonly occurring problem was to define which cell nuclei that stained for the investigated antigens, and which did not. A threshold value was used to separate the cell population into positive or negative with respect to each stain. A novel algorithm developed by Joakim Lindblad was used to calculate thresholds (Lindholm et al., 2000). In brief terms the algorithm created a function which approximated the distribution of the measurements as plotted in a histogram. Maxima in the second derivative of the function are then calculated and proposed as thresholds. The algorithm generally performed well in producing threshold with a few exceptions. For instance did it not manage to reliably separate cells staining negative from positive when applied to cell populations where the vast majority of cells stained positive. A potential further development would be to include a texture feature in the threshold setting algorithm, as the staining texture is very important to the human observer trying to separate negative cells from positive.

Sequential immunofluorescence staining

As part of this thesis a novel method was introduced based on a combination of immunofluorescence staining and image analysis (see paper I). By repeatedly staining, photographing the specimen, and then removing the stain, it is possible to evaluate the distribution of many more antigens in the same tissue section than was possible ever before. The method was named sequential immunofluorescence staining (SIFS), and is further described in the results section below, as the developed protocol must be considered a result in itself.

Study design

Paper IV, and to some extent paper III, describe small clinical studies aimed at evaluating the clinical implications of a defined aberration in the cell cycle dependent regulation of the nuclear levels of cyclin E. The paramount factors in the choice of study design was study efficiency, and the use of tissue material from patients with a well known outcome. Study efficiency was very important as the evaluation of the tissue samples from a single patient was tedious and time consuming. Routinely collected archive material had to be used in order to be able to relate the results to clinical outcome with an acceptable follow up time.

In order to optimise efficiency the case-control study model with highly matched pairs was utilised. Each case, a patient which died within a few years after the date of diagnosis, was matched with a control, a patient with at least 5 years of disease free follow up after treatment. The factors matched were age, stage, grade, and lymphnode status, as these are previously known important factors for

patient outcome. A traditional cohort or case-control study would certainly delivered considerably more data, but would have required the evaluation of many more tissue samples with respect to cyclin E expression pattern in order to achieve sufficient statistical power.

Results and discussion

Paper I

The intricate signalling networks and complicated mechanisms underlying the cell cycle regulation makes it attractive to be able to study the levels of a large number of different proteins in individual cells. Staining the investigated cell populations for two or three proteins using immunofluorescence techniques is fairly straightforward, but to achieve four or more different stains have rarely, if ever, been done. The maximum number of stains that can be achieved is in practice limited by the number of different fluorophores and corresponding excitation and emission filter sets available, as well as the availability of primary antibodies from different species.

In order to overcome the traditional hurdles of multiple immunofluorescence staining an attempt was made to develop a method to remove previously applied fluorophores as well as primary and secondary antibodies, without destroying the antigenicity of the studied tissue sample. The investigated tissue could then be stained several times, and images acquired from different rounds of staining compiled to give a complete picture of the distribution of all the investigated antigens in the tissue sample.

A thorough review of the in many cases several decades old literature gave a few tips. Paper I describes the evaluation of elution at pH 2.0 of the antibodies and fluorophores, as well as denaturation of the antibodies and fluorophores by microwave cooking.

Elution can remove the fluorophores, but not all primary antibodies

Elution using a glycine buffer at pH 2.0 was tried as it had been previously successfully used to remove primary antibodies in a immunostaining protocol (Nakane, 1968). When utilised in immunostaining the enzymatic end product (i.e. the stain) remained, but when applied to immunofluorescence staining the fluorophores were successfully removed after 1 to 2 hours of elution. However, a small amount of the primary antibodies remained, even after 3 hours of elution (see figure 1 of paper I). Hence elution alone was enough to remove the fluorophores, but not the firmly attached primary antibodies.

Elution does not destroy the antigenicity of the sample

Surprisingly the second round of staining even generated stronger staining than the first round following elution. Particularly short elution times generated strong stains in the second round of staining (see figure 1, paper I). The strong staining in the second round even when no elution had been performed at all (0 elution time in figure 1, paper I) is most likely caused by the additional signal amplification achieved by primary antibodies of the second staining round binding to the remaining free arms of the previously bound secondary antibodies of the first staining round. The increased staining intensity is actually quite close to the theoretically predicted doubling of the signal amplification. The seen effect is a confirmation of the usefulness of the common practise to simply try staining the sample again if the first round of staining failed or was too weak.

However, also after two and three hours of elution the second round of staining is considerably stronger than the first, even though the amount of remaining secondary antibodies must be very low, as only barely detectable levels of primary antibodies remain, and as no remaining fluorophores could be detected. An alternative and intriguing explanation to the increased second round of staining is that the elution treatment improved the antigenic recovery.

Elution followed by denaturation can remove or destroy all primary antibodies

Another technique to allow sequential immunoenzymatic staining rounds is the denaturation of the primary antibodies applied in the first round of staining (Len et al., 1995). As only low levels of primary antibodies remained after elution the denaturation of the remaining antibodies through microwave cooking was attempted. The results are shown in figure 2 in paper I. After only 2x5 minutes of microwave cooking the remaining primary antibodies were removed or lost their antigenicity.

Elution and denaturation does not destroy sample antigenicity

Also when elution was followed by denaturation the second round of staining was considerably better than the first. The antigenic recovery performed prior to the first round of staining consisted of 2x5 minutes of microwave cooking, but obviously the staining was considerably stronger after another 2x5 minutes of microwave cooking and 2 hours of elution, probably due to improved antigenic recovery.

The most probable reason why the fluorophores and primary antibodies can be completely removed or destroyed by elution and denaturation but the antigenicity remains, is probably the stabilisation of the molecules of the sample provided by the fixation.

Denaturation alone or followed by elution could not remove the antibodies and fluorophores applied in the first round of staining. A possible explanation is that the denaturation made the secondary antibodies more resistant to elution due to conformational changes. Similarly could not the biotin-streptavidin amplification be used, as the resulting complexes were resistant to both elution and denaturation.

More than two rounds of staining can be performed

The crossover experiment shown in figure 3 of paper I proves that at least three rounds of staining can be performed. As much as four rounds have been successfully performed, allowing the straightforward staining of as much as eight antigens (data not shown).

As part of the work underlying paper I the removal and restaining using a large number of antigens was attempted. The majority of antibodies tested behaved similarly, i.e. they were successfully removed or destroyed by elution and denaturation, and the antigenicity of their antigens persisted. However, a small fraction of the primary antibodies were more resistant to elution and denaturation, and another small fraction of antibodies could not detect their antigens after elution and denaturation. Hence any attempt to perform a

sequential staining experiment should be carefully prepared, and the order in which the different antibodies are to be used carefully considered.

Today there are several alternative methods to staining a large number of antigens. Alternatives to the traditional fluorophores with considerably more narrow excitation and emission spectrums are coming into use, and the excitation and emission filters are continually being improved. However, the new technology is still quite expensive, and has to my knowledge still not achieved the simultaneous staining of 8 different antigens. The second problem of multistaining, the availability of primary antibodies from different species can also be at least partially circumvented by blocking techniques, or by producing novel primary antibodies from a long range of different species. This is however also quite resource demanding. The SIFS protocol described in paper I does on the other hand only require a glycine buffer, a microwave oven, and a camera equipped fluorescence microscope, apart from a couple of days preparative work.

The fairly large amounts of data that can be collected using the described sequential staining protocol must be carefully weighted against the considerable preparation needed for a successful staining sequence. Occasionally the SIFS protocol was used during the work underlying papers III and IV, for instance in an evaluation of whether a subpopulation of the cells with a G₁ content of DNA but with a very high content of cyclin E were senescent or cycling, but unfortunately the results were never interesting enough to warrant publication. Hence the first published actual application of the SIFS protocol still remains to be seen.

The main conclusion of paper I is that sequential immunofluorescence staining, SIFS, is a feasible method that can be reliably utilised with some preparation, and doesn't require much resources.

Paper II

As described in the introduction and in the aims sections above cyclin A levels begin to rise in late G₁ or early S-phase. Cyclin A associates with CDK2, and cyclin A-CDK2 kinase activity is probably crucial to S-phase initiation and progression. Prior to the publication of paper II there was some controversy regarding if cyclin A accumulation began before or after entry into S-phase, as conflicting results had been published. Furthermore was it not entirely clear whether the increased fraction of cells staining for cyclin A in transformed cell populations compared to normal cell populations was caused by a change in the expression of cyclin A over the cell cycle, or if it was simply due to increased proliferation.

Cyclin A accumulation begins precisely at the G₁/S transition

By utilising a triple immunofluorescence staining protocol the location in the cell cycle of each individual cell could be pinpointed. Cells at the G₁/S border could then be studied and the statistical analysis lead to the conclusion that detectable nuclear levels of cyclin A appears just as the cells enter S-phase, as shown in figure 3 and 4 in paper II.

Also in transformed cells did the nuclear accumulation of cyclin A begin precisely when the cells entered S-phase, but the interpretation of this fact is not uncomplicated. The obvious interpretation is that the timely expression of cyclin A

is central for cell survival, and that tumour cells therefore must retain a correctly regulated cyclin A expression. An alternative, but equally interesting, interpretation is that nuclear cyclin A accumulation is the trigger event that fires off the pre-replication complexes both in normal and transformed cells.

Cyclin A accumulates throughout S-phase

In order to study the accumulation of cyclin A during S-phase the nuclear levels of cyclin A was compared with the levels of newly synthesised DNA. The cyclin A nuclear levels were found to be linearly related to the level of newly synthesised DNA, i.e. the cyclin A accumulation and DNA synthesis progressed with similar kinetics. There are many potential implications of this result, but they remain highly speculative without further data.

Tumour cells exhibit large intercellular variations in BrdU staining intensity

When the BrdU staining intensity was plotted against DNA content, as in figure 7 in paper II, it was obvious that the transformed cells exhibited much more variations in the BrdU staining intensity. The obvious interpretation is that the rate of DNA synthesis varies considerably in tumour cell populations. However, that interpretation is based on the assumption that all cells in the population have the same intercellular nucleotide content, as the rate of BrdU incorporation is directly related not only to the rate of DNA synthesis, but also to the relationship between the BrdU concentration and the size of the endogenous nucleotide pool. The data is however a clear indication that either the rate of DNA synthesis or the size of the nucleotide pool varies considerably within tumour cell populations, which either way may have an effect on the genetic stability of the population.

The first main conclusion of paper II is that the nuclear cyclin A level is intimately linked to the DNA synthesis, well in line with other studies that have established a link between cyclin A-CDK2 kinase activity and the regulation of DNA synthesis. The second main conclusion is that the relationship between cyclin A and DNA synthesis is the same in tumour and normal cell populations.

Paper III

Cyclin E accumulation begins in late G₁, after passage through the restriction point. When the cyclin E levels remain high throughout the cell cycle chromosomal aberrations are induced (Spruck et al., 1999). In addition the level of cyclin E expression in breast cancer tumours is correlated with survival (Porter et al., 1997). Therefore we set out to study the expression of cyclin E over the cell cycle in normal and transformed cell populations.

Normal cells commonly degrade their cyclin E in early S-phase

The cover picture shows the cyclin E staining intensity plotted against cyclin A staining intensity in a normal (benign) cell population. Green dots represent cells in S-phase, and red dots to the right (i.e. cells not staining for BrdU and with a high cyclin A content) are cells in G₂. The figure indicates that the majority of the normal cells degrade cyclin E in late G₁ or early S, and that the cyclin E levels are low in S and G₂.

Cyclin E levels remain high throughout S-phase in many tumour cells

The cover picture also shows a cell population consisting of transformed cells. The cyclin E expression pattern of the transformed cells is strikingly different. Cyclin E levels remain high throughout S-phase, but also in the transformed cells is the cyclin E degraded prior to G₂ entry, indicating that the cyclin E degradation machinery remained intact. Other cell lines than Hs578T, which is displayed on the cover, had high cyclin E levels also in G₂ cells. Unfortunately the cyclin E levels in M-phase cells could not easily be investigated in the cultured cells, as the rounded up M-phase cells commonly are lost during the handling necessary for fixation and immunofluorescence staining.

As cyclin E is implicated in the regulation of many functions necessary for genomic stability, such as the licensing of DNA, and the duplication of centrosomes, the heterochronic expression (the expression during the wrong parts of the cell cycle) of cyclin E may be a common step in the development of the genetic instability of cancer cells. Furthermore do the results support the data by others (among others Porter et al., 1997) that there is commonly more cyclin E in tumour tissue than in normal tissue, as paper III shows that cyclin E levels remain high throughout a larger part of the cell cycle in transformed cells. The increased levels of cyclin E in tumours seen by others is not merely the result of an increased proliferation.

The expression pattern of cyclin E in cervical carcinoma tumours varied

In order to investigate whether the heterochronic expression pattern of cyclin E existed also in tumour cells *in vivo* we investigated tissue sections from a few cervical carcinomas. The cyclin E expression over the cell cycle in the studied tumours varied considerably. In some tumours the cyclin E expression pattern was very similar to the expression pattern of benign cells in culture, while in other tumours the expression pattern was highly abnormal, similar to the transformed cell lines, as shown in figure 4 of paper III. When the survival of patients with tumours exhibiting either pattern was compared we found that a heterochronic cyclin E expression pattern may be coupled with poor prognosis, as is shown in figure 5 of paper III.

The data presented in paper III is well in line with the notion that ubiquitous cyclin E expression can cause chromosomal aberrations (Spruck et al., 1999). The genetic instability in tumours that have a heterochronic cyclin E expression may lead to a more rapid progress of the cancer disease with earlier metastasis and death. Unfortunately the number of tumours available to us was small, hence a larger study was necessary to firmly establish the link between a heterochronic cyclin E expression pattern and poor outcome.

The main conclusions of paper III were that the expression pattern of cyclin E over the cell cycle is commonly abnormal in transformed cell lines, and that the same expression pattern occurs *in vivo* in human tumours. The data also indicated that a heterochronic cyclin E expression pattern could be associated with a poor prognosis.

Paper IV

Since the pilot study on the impact on outcome of heterochronic cyclin E expression in cervical carcinoma lesions described in paper III yielded promising

results a larger study was initiated. The study was designed as a case-control study in which each case was a patient that died within a few years after being diagnosed with a cervical carcinoma stage Ib or IIa. Each case was matched with a control patient of similar age that had a similar tumour with respect to stage and grade, but which had experienced more than five years of disease free follow up.

Heterochronic cyclin E expression is associated with poor prognosis

Also in the larger study a highly variable cyclin E expression pattern was seen in different cervical carcinoma tumours. Figure 2 of paper IV exhibits three different cyclin E patterns seen in the tumours. Furthermore, the expression patterns were found to be related to patient survival. In 15 out of the 18 patient pairs (a total of 36 patients were included) the deceased patient had considerably higher nuclear cyclin E expression levels in S- or G₂-cells, indicating that a heterochronic expression of cyclin E is associated with poor prognosis.

Other papers have been published regarding the relationship between the total cyclin E level in the tumours, or the fraction of cells containing cyclin E (see paper III and IV for references), but the data presented in paper IV are the first to show a link between a heterochronic cyclin E expression pattern and poor outcome. However, the causal relationship between a heterochronic cyclin E expression and poor outcome remains to be established.

Cyclin E expression in adenocarcinomas and precancerous lesions

In order to further study the frequency of heterochronic cyclin E expression patterns a line of tissue samples from cervical precancerous lesions, as well as from cervical adenocarcinomas were also studied. The data from the precancerous lesions was hard to interpret, as only some of the lesions exhibited slightly but not significantly elevated S-phase cyclin E levels. The most probable interpretation is that cyclin E deregulation commonly occurs late in the transformation process, but more data would be needed to warrant that conclusion. The adenocarcinomas did on the other hand exhibit some highly deranged cyclin E expression patterns, although there were also some tumours with normal cyclin E expression patterns, as seen in the squamous cervical carcinomas.

Cyclin E levels are not elevated in M-phase cells in cervical carcinoma

To investigate whether or not cyclin E was degraded prior to M-phase entry the studied tumour samples were searched for cells in mitosis staining for cyclin E. Not a single mitosis cell with detectable cyclin E levels could be found in the more than 50 investigated tumours and lesions. Hence it seems likely that even the tumour cells with a heterochronic cyclin E expression pattern retains the ability to degrade cyclin E prior to or around M-phase entry. Interestingly a very recent paper found increased cyclin E levels in M-phase and a subsequent defect in the transmission of chromosomes in colorectal tumours (Rajagopalan et al., 2004). It remains to be seen whether other tumours developed without the influence of HPV displays a similar phenotype.

The main conclusions of paper IV is that a heterochronic cyclin E expression pattern is commonly occurring both in squamous carcinomas and in

adenocarcinomas of the uterine cervix, and that the presence of such an expression pattern is linked with poor outcome at least in squamous cell carcinomas. Finally paper IV shows that the tumour cells retain their capability to degrade cyclin E, as it is missing in M-phase cells.

Conclusions

Cancer have been viewed as caused by the overexpression or hyperactivation of oncogene gene products, and by the deletion or silencing of tumour suppressor genes, resulting in a lack of functional suppressor gene products. However, models based on this simplified view have yet to manage to explain the genetic instability seen in cancer cells.

Through many recent studies, as well as the papers included in this thesis, a more refined view of transformation is emerging. Defects in cell cycle regulation can cause genetic instability and transformation. In order to achieve a deficient cell cycle regulation it is sufficient to express normal levels of a normal protein during the wrong phases of the cell cycle (heterochronic expression). Traditional methods used in cancer research are not able to detect heterochronic expression of a protein. This thesis shows that heterochronic expression of cell cycle regulators can be investigated using immunofluorescence staining techniques, if coupled with digital image acquisition and image analysis, and providing carefully selected antigens are used.

The usefulness of the approach used in this thesis is confirmed by the results in paper IV, as well as recent papers by others (Rajagopalan et al., 2004). The expression pattern of cyclin E over the cell cycle is related to both the genetic instability of the tumour cells, as well as patient outcome. Therefore future studies regarding the impact heterochronic expression of cyclin E and other proteins have on cell transformation and tumour progression are highly desirable.

Future prospects

A natural extension of this thesis would be to perform larger cohort studies, and maybe even prospective studies, on the cyclin E expression pattern in various types of human cancers. Much remains to be learned regarding the development and progression of the cancer disease in humans, and it is not entirely impossible that the evaluation of the cyclin E expression pattern could reach clinical practice, as the cyclin A staining fraction has.

Larger clinical studies would require much more automated evaluation methods. It would be an exhilarating challenge to develop the antibodies, staining protocols, instruments, and software needed for high throughput screening for heterochronic cyclin E expression.

Another exciting potential future project would be to investigate the mechanisms behind the connection between the cyclin E expression pattern and genetic instability, as well as the mechanisms behind the connection between the cyclin A levels and DNA synthesis.

It would also be nice to optimise a SIFS staining protocol including antibodies against a range of proteins regulating progression through G_1 . G_1 is to me the most complex and intriguing part of the cell cycle, and new knowledge about the regulation of G_1 progression in normal and transformed cells is likely to be useful in clinical practice in a not too distant future.

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