Regulation of Cell Cycle Progression and Cellular Survival in Primary Rat Hepatocytes

by

Lene Wierød

UNIVERSITY OF OSLO

FACULTY OF MEDICINE

Laboratory for Toxicopathology

Institute of Pathology

Rikshospitalet Radiumhospitalet Medical Centre

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Oslo, January 2007

Lene Wierød

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ABBREVIATIONS

CAK	Cdk-activating kinase
CDK	Cyclin-dependent kinase
CKI	Cyclin-dependent kinase inhibitors
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ERK	Extracellular signal regulated protein kinase
GDP	Guanosine diphosphate
GEF	Guanine-nucleotide exchange factor
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
MAPK	Mitogen-activated protein kinase
MEK	Mitogen/ERK kinase
NES	Nuclear export sequence
NLS	Nuclear localization sequence
PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosphatidylinositol (3,4)-diphosphate
PIP_3	Phosphatidylinositol (3,4,5)-triphosphate
PKB	Protein kinase B
Rb	Retinoblastoma protein
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
RSK	Ribosomal S6 kinase
TGF-β	Transforming growth factor- β

PUBLICATIONS INCLUDED

I Wierød L, Rosseland CM, Lindeman B, Oksvold MP, Grøsvik H, Skarpen E, and Huitfeldt HS. Activation of the p53-p21^{Cip1} Pathway is Required for CDK2 Activation and Proliferation in Primary Rat Hepatocytes. Submitted November 2006.

II Wierød L, Rosseland CM, Lindeman B, Oksvold MP, Grøsvik H, Skarpen E, and Huitfeldt HS. CDK2 Regulation through PI3K and CDK4 is Necessary for Cell Cycle Progression of Primary Rat Hepatocytes. Submitted November 2006.

III Rosseland CM, Wierød L, Oksvold MP, Werner H, Østvold AC, Thoresen GH, Paulsen RE, Huitfeldt HS, and Skarpen E (2005). Cytoplasmic Retention of Peroxide-Activated ERK Provides Survival in Primary Cultures of Rat Hepatocytes. Hepatology, Vol. 42, No 1, 200-207.

IV Rosseland CM, Wierød L, Flinder LI, Oksvold MP, Skarpen E, Huitfeldt HS. Distinct Functions of H-Ras and K-Ras in Proliferation and Survival of Primary Hepatocytes Due to Selective Avtivation of ERK and PI3K. Submitted December 2006.

INTRODUCTION

Background

The liver is an excellent tissue for the study of growth regulation because of its ability to regenerate by a process of compensatory growth following surgical resection or toxic injury. Much of the investigation on the mechanisms of hepatic growth has been done in partially hepatectomized animals *in vivo* or in primary cultures of isolated hepatocytes. Furthermore, primary hepatocytes can be maintained in a serum-free medium with defined growth factors such as epidermal growth factor (EGF) [1, 2] and can respond to growth factor stimulation by expressing many of the same cell cycle regulatory proteins that are expressed in the liver and that drive liver regeneration *in vivo*.

Liver models are also well suited for studies of the early phases of carcinogenesis, because the growth of preneoplastic, cancer-prone lesions can be synchronously induced and are easily identifiable. Typically, carcinogen exposure results in p53 induction and growth inhibition. Initiated, preneoplastic cells are resistant to the cytotoxic effects of carcinogen exposure, compared to normal cells, and therefore escape this growth inhibition. Thereby, the cells may develop further towards neoplasia. Our laboratory has previously shown changes in the growth factor regulated phase of the cell cycle in carcinogen-exposed liver. In cultured hepatocytes, growth arrest was accompanied by altered signalling through the EGF receptor and a subsequent reduced nuclear accumulation of activated ERK [3]. Furthermore, carcinogen exposure led to delayed induction of cyclin D1 and abridged nuclear accumulation of cyclin dependent kinase 2 (CDK2) and CDK4 [4]. During these studies we decided to further explore the physiological mechanisms that regulate CDK2 and CDK4 nuclear trafficking in normal cells. The work

presented also describes growth factor- and stress activated ERK and its role in intracellular signalling.

Intracellular signalling

Hepatocytes from adult animals are constitutively resting cells, and growth factor stimulation is necessary to advance their cell cycle recruition and progression, until they become growth factor independent just prior to DNA synthesis. EGF, TGFα and HGF are all potent liver mitogens [5]. The former two bind to and activate the plasma membrane receptor tyrosine kinase EGF receptor (EGFR). The best characterized intracellular mitogenic signal thus initiated is the Ras-Raf-MEK-MAPK pathway [6, 7] (figure 1). The interaction between ligand activated EGFR and Shc/Grb2, either through Grb2 binding alone or as a preformed Shc/Grb2 complex, results in the recruitment of Sos to the plasma membrane [8-10]. Sos operates as a guanosine triphosphate (GTP) exchange factor converting an inactive guanosine diphosphate (GDP)-bound Ras to an active GTP-bound Ras [11, 12]. Ras is a membrane-localized G-protein, functioning as molecular switches in a variety of signalling pathways, including MAPK. There are three major isoforms of classical Ras; H-Ras, K-Ras and N-Ras, sharing a high degree of sequence homology [13].

Activated Ras mediates activation of the serine/threonine kinase Raf [14, 15]. The activation of Raf is complex and the process is not yet fully understood. Raf resides in the cytosol in an inactive state in complex with heat shock proteins and 14-3-3ζ [16]. A binding site for Raf is created on the GTP-bound Ras upon ligand activation of EGFR. When bound to the Ras effector loop at the membrane, Raf then interacts through its zink finger domain with a second epitope on Ras, which must be prenylated [16, 17]. Raf is phosphorylated on serine and tyrosine

residues, and it is suggested that this stabilizes an open, active Raf configuration, enabling its release from Ras-GTP into the cytosol [17, 18]. Raf then phosphorylates and activates the threonine/tyrosine kinases MAPK/ERK kinase (MEK) 1 and 2 by serine phosphorylation. MEK then activates the serine/threonine kinases MAPK ERK1 and ERK2 through phosphorylation on threonine and tyrosine residues [19].

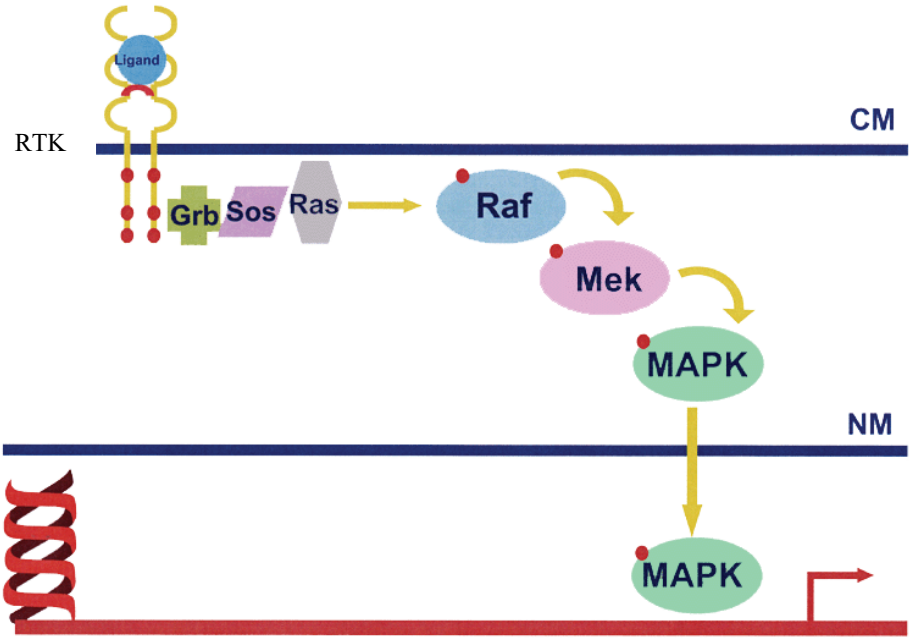

Figure 1: A schematic illustration of the MAPK pathway. Ligand-binding of Receptor Tyrosine Kinase (RTK) leads to autophosphorylation of the receptor and assembly of a signalling complex including Grb2, Sos and Ras. Activated Ras recruits Raf to the membrane. Raf then starts a cascade of phosphorylation through MEK and MAPK. Activated MAPK translocates to the nucleus where it phosphorylates a number of substrates.
CM=cellular membrane, NM=nuclear membrane.
Figure from http://jorde-lab.genetics.utah.edu/people/reha/Reha.html

Upon activation, ERK can translocate to the nucleus and phosphorylate substrates important for growth progression [20]. The induction of cyclin D1 mRNA has most frequently been attributed to the

activation of ERK [21]. In most cases, activation of Ras-Raf-MEK-ERK cascade induces cyclin D1 gene expression. Several studies have shown that the induction of cyclin D1 requires only moderate ERK activity, but the activity must be sustained for several hours [22]. Sustained ERK signalling in resonse to integrin and RTK synergism [21] may therefore explain the joint growth factor plus ECM requirement for cyclin D1 expression. However, it appears that MAPK pathway is not the only signalling pathway required for accumulation of cyclin D1 in G1-phase.

Phosphatidylinositol-3 kinase (PI3K) is an another important modulator of cell survival, mitogenesis, cytoskeletal remodelling, metabolic control and vesicular trafficking in various cell systems [23]. PI3Ks are grouped into three classes (I-III) according to their substrate preference and sequence homology. Different classes of PI3K have distinct roles in cellular signal transduction, as do the different isoforms that can exist within each class. Class I PI3Ks are divided into two subfamilies, depending on the receptors to which they couple. Class 1A PI3Ks (PI3Kα, β and δ) are activated by growth factor receptor-tyrosine kinases (RTKs), whereas class 1B PI3K (PI3Kγ) is activated by G protein-coupled receptors. Both type 1A and type 1B PI3Ks are also activated by Ras [24]. Class 1A PI3Ks are heterodimers composed of a p110 catalytic subunit and a p85 regulatory subunit. PI3K is activated by direct interaction between the p110 subunit and GTP-bound Ras [25], or by association of the p85 subunit to activated growth factor receptors [26, 27]. In rat hepatocytes, Gab2 has been identified as a key molecule responsible for EGF-induced PI3 kinase activation [28]. Following, all class 1 PI3Ks phosphorylate phosphatidylinositol (3,4)-diphosphate (PIP_2), a constitutive membrane component, to create the lipid second messenger phosphatidylinositol (3,4,5)-triphosphate (PIP_3) (figure 2).

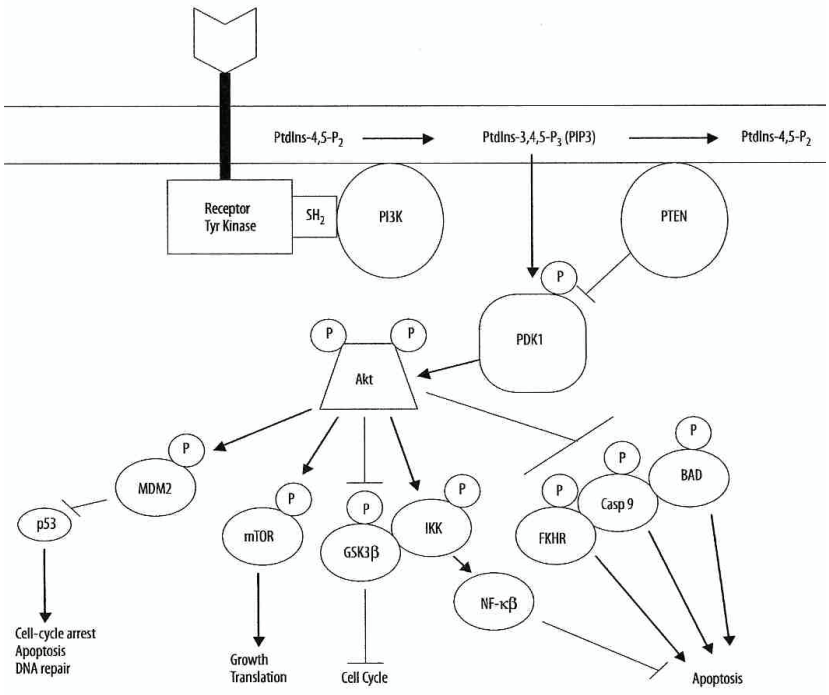

Figure 2: A schematic illustration of the PI3K signalling pathway. Aktivation of Receptor Tyrosine Kinase (RTK) leads to autophosphorylation on tyrosine residues and transphosphorylation of adaptor proteins with SH_2-domain. PI3K binds directly to phosphotyrosine residues on growth factor receptors or to adaptor proteins. Following activation, PI3K phosphorylates PtdIns-4,5-P_2 to create the lipid second messanger PtdIns-3,4,5-P_3 (PIP_3). PIP_3 phoshorylates PDK1. Activated PDK1 phosphorylates Akt, whereas Akt next phosphorylates a number of effector proteins, leading to complex events like cell cycle progression, survival or apoptosis. PTEN dephosphorylates PIP_3 and therefore terminates PI3K signalling.
Figure from http://www.benbest.com/health/cancer.html

The PIP_3 signal is mediated by its binding to the plextrin homology (PH) domain of target proteins, which induces their membrane translocation and/or conformational changes. Known PIP_3 targets are numerous and from a variety of enzyme classes, e.g. protein kinases, phospholipases and guanine-nucleotide exchange factors (GEFs). These PIP_3 targets together regulate a huge list of essential cellular functions including transcription, translation, protein synthesis,

cell survival, cell cycle entry and the structure of the actin cytoskeleton [29]. PTEN (phosphatase and tensin homologue) dephosphorylates PIP_3 and therefore terminates PI3K signalling [29].

The best known downstream target of PI3K is Akt, which transmit survival signals from growth factors [30-32]. Akt inhibits GSK3β and FoxO transcription factors, which in turn control cell cycle regulators like cyclin D1 and $p27^{Kip1}$. [33-37]. Inhibition of PI3K activity blocks both cyclin D1 mRNA and protein expression [38]. Requirement for PI3K for mammalian cell cycle progression was first recognized by studies adopting platelet-derived growth factor (PDGF) receptor mutants that lack phosphoacceptor sites required for binding of PI3K p85 [39, 40], as well as "add-back" mutants with selective restoration of these sites [41]. Inhibitors of PI3K, LY294002 and wortmannin, have also been shown to inhibit S-phase entry in a variety of cell types [42-45].

Cell-cycle progression and regulation

Cell cycle progression is an exactingly regulated process where the fundamental task is to ensure that DNA is faithfully replicated and that sister chromatides are correctly segregated during mitosis. To ensure this, the cell has several cell cycle checkpoints. When the cells contain DNA damages that have to be repaired, cells activate DNA damage checkpoint that arrests the cell cycle.

The periods of the cell cycle associated with DNA replication (S-phase) and mitosis (M phase) are separated by gaps of varying lengths called G1 and G2 (figure 3).

Progression of cells through the cell cycle is facilitated through the sequential activation of CDK-complexes [46, 47]. These complexes consist of a regulatory subunit (cyclin) and a catalytic subunit (CDK),

which are assembled and activated at specific points of the cell cycle. In many regards, the control of cell cycle progression by cyclins and CDKs is remarkably well conserved throughout eukaryotic species [48].

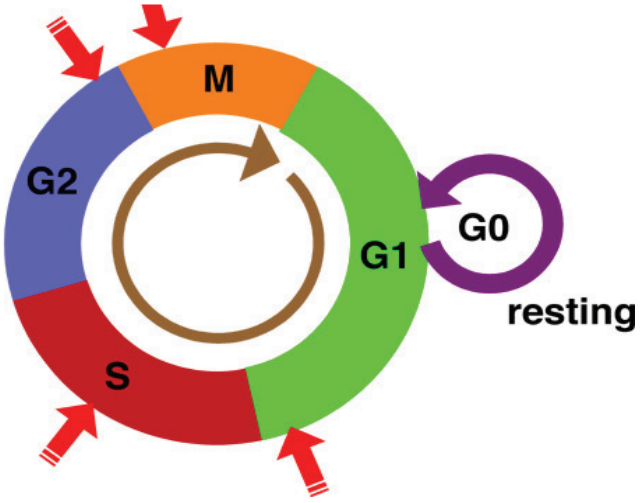

Figure 3: Regulation of the mammalian cell cycle. The periods of the cell cycle associated with DNA replication (S-phase) and mitosis (M-phase) are separated by gaps of various lengths called G1- and G2-phase. The G0 phase is a period in the cell cycle where cells exist in a quiescent state. To maintain genomic integrity, the cells are equipped with several cell cycle checkpoints (red arrows).
Figure from homepage.mac.com/enognog/checkpoint.htm.

Cyclin-dependent kinases and cyclins

Cyclin dependent kinases are the catalytic subunits of a large family of heterodimeric serine/threonine kinases whose best-characterized members are involved in controlling progression through the cell cycle (figure 4). According to the latest versions of the human and mouse genomes, there are 11 genes encoding CDKs and 9 other genes encoding CDK-like proteins [49].

Studies using sea urchin eggs identified a series of proteins that were synthesized and destroyed at each cleavage division. On the basis of this feature, these proteins were designed cyclins [50]. Analysis of the human genome has identified at least 29 genes encoding related proteins that share a conserved stretch of 150 amino acid residues termed cyclin-box. This domain is formed by five helical regions and is responsible for binding to CDKs [49]. Transcriptional control [51] and ubiquitin-mediated degradation [52] ensure the proper and irreversible timing of cyclin expression. Cyclin binding activates CDKs by inducing conformational changes in their structure [53, 54]. Cyclins have also been suggested to determine the substrate specificity of CDKs [55-58].

The G1 and S phases

Progression through G1 is regulated by a complex mechanism that can involve at least three CDKs – CDK4, CDK6 and CDK2 – and their regulators [59, 60]. Mitogenic signalling induces synthesis of the D-type cyclins and possibly the proper folding and transport of CDK4 and /or CDK6 to the nucleus. Both the PI3 kinase – and MAPK pathways are required for hepatocyte cyclin D1 induction in response to mitogens [61].

In studies of mammalian cells, a clear theme has emerged concerning the role of the D-type cyclins during G1 phase. They seem to act as intracellular "sensors" of extracellular stimuli that promote proliferation [60, 62]. However, the role of the D-type cyclins appears to vary significantly between cell types. For example, whereas cyclin D1 is a pivotal mediator of G1 progression in many cell types including hepatocytes, it does not appear to mediate proliferation in lymphocytes, in which cyclin D2 appears to perform a similar function [63, 64].
The role of cyclin D3 is highly dependent upon the cellular context. It appears to promote cell cycle progression in some cell types and growth

arrest or differentiation in others. In hepatocytes, cyclin D3 is expressed in quiescent cells and is only modestly responsive to EGF, insulin or PI3 kinase signalling, suggesting that it does not play a critical role in regulating progression through G1 phase in response to these signals [61].

Positive regulation of CDK activity occur in two steps: phosphorylation on a central threonine residue (Thr160 and Thr172 for CDK2 and CDK4, respectively) by CDK activating kinase (CAK) and dephosphorylation of the threonine (Thr14) and tyrosine (Tyr15) residues by the Cdc25 phosphatase family [65, 66].

Active complexes of CDK4 or CDK6 and D-type cyclins phosphorylate members of the retinoblastoma (Rb) protein family, which includes pRb, p107 and p130. These proteins contain multiple sites for phosphorylation by CDKs, only some of which can be recognized by the CDK4-cyclin D and/or CDK6-cylin D complexes. The Rb family members have been the only demonstrated substrates for CDK4 for many years. However, recently it has been demonstrated that Smad3, a key mediator for TGF-β antiproliferative responses, is a target for inhibitory phosphorylation by both CDK4 and CDK2 [67, 68].

Although the initial phosphorylation of Rb proteins is mediated by the CDK4–cyclin D and/or CDK6–cyclin D kinases, the irreversible inactivation of these proteins is presumed to be carried out by CDK2–cyclin E. This process is believed to render cells independent of mitogenic signals and corresponds to the restriction point [59]. The restriction point has been defined as the stage during G1 in which cells no longer require mitogenic stimuli to undergo cell division.

Progressive phosphorylation of Rb by CDK4/6-cyclin D and CDK2-cyclin E releases E2F and promotes S-phase entry [69, 70]. E2F is a transcription factor that regulates the expression of genes involved in S phase (reviewed in ref. [71]). Cyclin E itself is an E2F target gene and the cyclin E promoter contains defined E2F binding sites [72-74]. This

describes a growth factor dependent cyclin E induction in late G1 phase. Thus, as CDK2-cyclin E phosphorylates and inactivates Rb, cyclin E may reinforce its own expression through a positive feedback loop [75]. In addition to Rb, other proteins that regulate cell division are also CDK2-cyclin E substrates. NPAT/p220 is a transcription factor that controls cell cycle-dependent histone gene transcription, and phosphorylation of NPAT by CDK2-cyclin E stimulates histone mRNA synthesis [76, 77]. CDK2-cyclin E also phosphorylates $p27^{Kip1}$, CBP/p300 and centrosomal proteins like nucleophosmin and CP110 [78-81]. Cyclin E cooperates with CDC6 as well, and this allow prereplication complexes to form during the G1-S transition [82]. Inactivation of pRb also participates in promoting the transcription of genes that are necessary for subsequent phases of the cell cycle, including those encoding the A-type and B-type cyclins. Although A-type cyclins accumulate during S phase, synthesis of B-type cyclins is not evident until the G2–M transition. Indeed, current models propose that once CDK2 is no longer associated with cyclin E, it interacts with the newly synthesized A-type cyclins, A1 and A2. While cyclin A1 is limited to male germ cells, cyclin A2 is widely expressed [83, 84]. In cultured cells, cyclin A2 functions during both G1-S and G2-M phases [85-87]. Cyclin A2 can bind and activate CDK1 and CDK2 [88-90]. CDK2–cyclin A complexes have been reported to phosphorylate numerous proteins that are thought to be required for proper completion and exit from S phase. These proteins include upstream regulators of Cyclin A (pRb), transcription factors (E2F1, B-Myb), proteins involved in DNA replication, DNA repair, histone modification, ubiquitine-mediated proteolysis and cell cycle checkpoints (p53, $p21^{Cip1}$, MDM2) [60].

The G2 and M phases

At the end of S phase, A-type cyclins associate with CDK1. CDK2-cyclin A and CDK1-cyclin A complexes share several substrates and whether these complexes have differential roles during the S to G2 phase remains obscure. During G2, A-type cyclins are degraded, whereas the B-type cyclins are actively synthesized. As a consequence, CDK1 binds to B-type cyclins – an association believed to be essential for triggering mitosis [91].

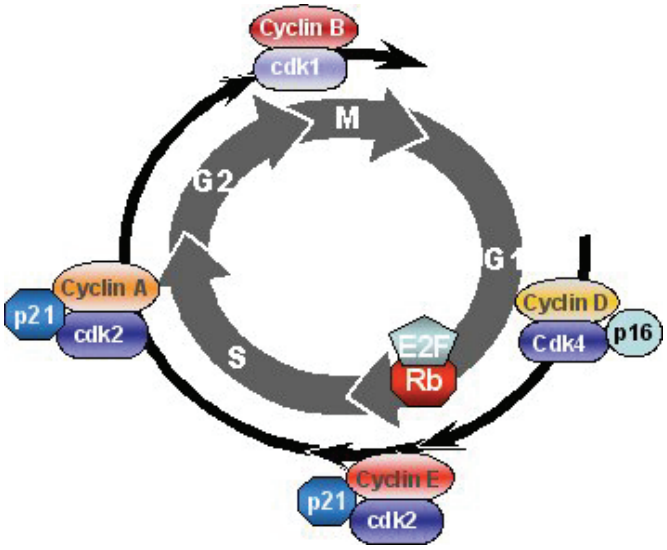

Figure 4: Regulation of the mammalian cell cycle. CDK4-Cyclin D complexes, together with CDK2-cylin E complexes, regulate G1 progression. In S-phase, CDK2-cyclin A complexes ensure transition to the G2 and M-phase, whereas CDK1-cyclin B complexes, in M-phase, make the cycle complete. p16 and p21 are CDK inhibitors, which also regulate cell cycle progression.
Figure adapted from www.cicr.uq.edu.au/cancerBiology/cellCycle.html

CDK activating kinase

CDK7 together with cyclin H and the assembly factor MAT1 forms the CAK complex, responsible for the activating phosphorylation of CDK1, CDK2, CDK4 and CDK6 [92-98]. CAK is located in the nucleus, mainly in coiled bodies [99, 100]. CDK7 activity is invariant during cell cycle, but levels and activity of CDK7 are both reduced in quiescent G0 cells [100, 101].

CDK inhibitors

It is generally agreed that CDK inhibitors play a critical role in regulating cell cycle progression and exit during morphogenesis and differentiation and/or in maintaining cell quiescence [60, 102, 103]. Cyclin dependent kinase inhibitors (CKIs) have been assigned to one of two families based on their structures and CDK targets. The first class includes the INK4 proteins (inhibitors of CDK4), so named for their ability to specifically inhibit the catalytic subunits of CDK4 and CDK6. Four such proteins, $p16^{INK4a}$ [104], $p15^{INK4b}$ [105], $p18^{INK4c}$ [106, 107] and $p19^{INK4d}$ [108] are composed of multiple ankyrin repeats and bind only to CDK4 and CDK6, and not to other CDKs or to D-type cyclins. The second class of inhibitors includes $p21^{Cip1}$ [109-114], $p27^{Kip1}$ [115-117] and $p57^{Kip2}$ [118, 119] all of which contain characteristic motifs within their amino-terminal moieties that enable them to bind to cyclin and CDK subunits [120-125]. In hepatocytes, $p21^{Cip1}$ is induced by growth factors during G1 phase, whereas p27 expression do not vary substantially during the cell cycle and is not dependent upon growth factors [126]. Based largely on *in vitro* experiments and *in vivo* overexpression studies, CKIs of the Cip/Kip family were initially thought to interfere with the activities of cyclin D-, E- and A-dependent kinases. More recent work has altered this view and revealed that

although the Cip/Kip proteins are potent inhibitors of cyclin E and A-dependent CDK2, they also act as positive regulators of cyclin D-dependent kinases [60, 127, 128].

Nuclear translocation

Little is known about the exact mechanisms that control nuclear import and export of cyclin-CDK complexes. CDK4-Cyclin D1 is imported into the nucleus in G1 phase, possibly by piggy-backing on the $p21^{Cip1}$/$p27^{Kip1}$ CDK-inhibitors [128, 129], and is then exported in S phase concomitant with phosphorylation by GSK3 and subsequent proteolysis [130].

The CDK2 sequence does not contain a classical nuclear localization signal (NLS), but CDK2 is a small protein and may thus enter the nucleus by diffusion. However, efficient nuclear translocation of CDK2 is likely to depend on association with a carrier protein. Its regulating partner, Cyclin E, contains NLS, thus cyclin E – CDK2 complexes may translocate to the nucleus independently of $p21^{Cip1}$ piggy-backing. Moreover, it has recently been suggested that the NLS of cyclin E is not functional [55, 131]. In addition, our studies suggest that nuclear translocation of G1 CDKs rely on $p21^{Cip1}$ expression (submitted data).

The mechanisms responsible for promoting nuclear translocation of ERK and controlling is durability are largely unknown. In quiescent cells, ERK is located in the cytoplasm, in complex with its activator MEK [132]. Because of its nuclear export sequence, MEK localizes in the cytoplasm irrespectively of its activation state [133]. ERK itself does not contain a nuclear export sequence or a nuclear import sequence [134]. In spite of this, ERK accumulates in the nucleus following activation by a potent mitogen [20]. Nuclear export of dephosphorylated

ERK is also mediated by MEK, which shuttles in and out of the nucleus as a result of its nuclear export sequence [135].

Cellular growth arrest

Growth arrest is a reversible halt of the cell cycle that occurs at either the G1/S or G2/M barrier. Growth arrest in G1 is a conserved response to a wide spectrum of cellular stresses like oxidative damage, radiation, heat stress, DNA damage due to carcinogens etc. Deregulation of the G1 checkpoint in cancer is rather a rule than an exception [47].

p53

The p53 gene, first described in 1979, was originally believed to be an oncogene, but genetic and functional data obtained ten years after its discovery showed it to be a tumor suppressor. Just as a car's brakes regulate its speed, properly functioning tumor-suppressor genes act as brakes to the cycle of cell growth, DNA replication and division into two new cells [136].

The p53 protein is known to act biochemically as a transcription factor and biologically as a tumor suppressor. The biological functions of p53 are mainly mediated through transcriptional regulation of its downstream target genes. For example, p53-induced growth arrest is achieved primarily by transactivation of $p21^{Cip1}$ for G1 arrest (figure 5) and of 14-3-3s for G2 arrest [137].

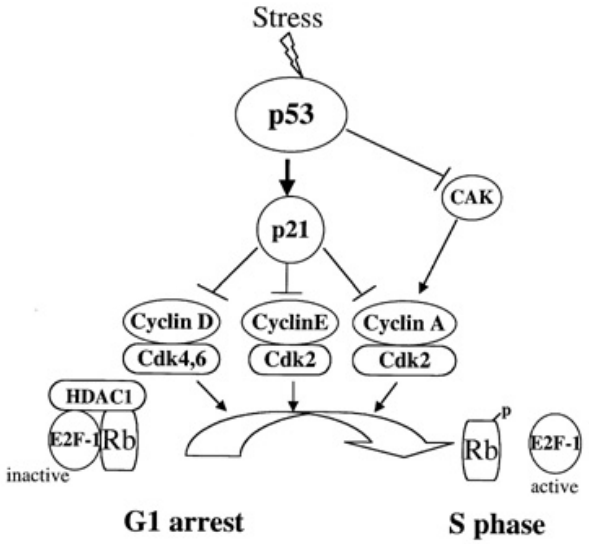

Figure 5: p53-mediated growth arrest in G1. Stress-activated p53 induces $p21^{Cip1}$. $p21^{Cip1}$ then bind to CDK-cyclin complexes and inhibits CDK-activity, a process that leads to G1-arrest. p53 is also able to inhibit CAK directly to inhibit cell cycle progression.
Figure modified from www.eurekah.com/dbimages/blagosklonny_6_2.jpg

Among the signals that activate the p53 protein, damage to the integrity of the DNA template is the most studied. Gamma or UV irradiation, alkylation of bases, depurination of DNA or reaction with oxidative free radicals all alter the DNA in different ways, and for each damaging agent, a different detection and repair mechanism is employed by the cell [139-141]. The activation of the p53 protein in response to stresses is mediated and regulated by protein kinases, histone acetyl-transferases, methylases, ubiquitin and sumo ligases. In addition, the activated p53 protein appears to interact with a number of proteins that are important for its transcriptional activity such as promyelocytic leukaemia (PML) bodies and the Werner helicase [142-144].

CAK also has a role in response to DNA damage. Two different studies showed that p53 is a substrate of CDK7 *in vitro* [145, 146]. Phosphorylation of p53 enhanced the ability of p53 to bind to the

sequence-specific p53-responsive DNA element. In addition, a direct interaction between p53 and cyclin H has been observed both *in vitro* and *in vivo*, and binding of p53 to CAK reduces CAK activity [138].

MDM2 prevents p53-dependent gene expression through diverse mechanisms. It inhibits transactivation by binding and occluding the p53 N-terminal transactivating domain, preventing the interaction of p53 with the basal transcription machinery [147-149]. Various stresses result in the acetylation of p53 by the histone acetyl transferases PCAF and p300/CBP; however, this too can be blocked by the assosoiation of p53 with MDM2 [150-154]. In addition to these direct mechanisms of transcriptional inhibition, MDM2 can indirectly inhibit p53-dependent gene expression by ubiquitinating and degrading p53 [155, 156]. MDM2 consists of several potential PI3 kinase binding sites of which Ser17 and Ser395 are phosphorylated *in vitro* and *in vivo*, respectively [157, 158]. Akt has been suggested to regulate the intracellular localization of MDM2 by phosphorylation on serines 166 and 186 [159].

Oxidative stress

Oxidative stress may be defined as an imbalance between cellular production of reactive oxygen species (ROS) and antioxidant defence mechanisms [160]. ROS are defined as partially reduced metabolites of molecular oxygen and include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$). They are highly reactive, and may cause oxidative damage to cellular macromolecules [161]. H_2O_2 has a relatively long half-life and is soluble in both lipid and aqueous media, thus it easily diffuses to its cellular targets [162].

Growth factors and cytokines are capable of generating ROS that participate in cell signalling [161, 163, 164]. In agreement with this, Carmona-Cuenca et al showed that both EGF and TGF-β increased ROS

production [165]. It is well established that membrane-localized NADPH oxidase is responsible for generation of ROS triggered by receptor binding of numerous peptide growth factors such as PDGP, HGF, insulin, VEGF and TNFα triggering cell proliferation, differentiation and apoptosis [166-168]. Rac-1 GTPase is the upstream signal enzyme for NADPH oxidase dependent ROS generation [169].

In normal hepatocytes, the major source of ROS production is toxic byproducts of oxidative phosphorylation in mitochondria. In addition, detoxification of hepatotoxins in the endoplasmatic reticulum or peroxisome compartments induces an endogenous production of ROS. A number of defence systems have evolved to combat the accumulation ionof ROS. These include various non-enzymatic molecules (e.g. glutathion, vitamins A, C and E and flavenoids) as well as enzymatic scavengers of ROS (e.g. superoxide dismutases (SOD), catalase and glutathione peroxide). When the cellular production of ROS exceeds the antioxidant capacity of the cell, oxidative damage of lipids, proteins or DNA will occur, resulting in oxidative stress, hepatic injury and possibly cell death [170].

It has been shown that oxidative stress leads to substantial activation of ERK and that growth factor receptors play an important role in mediating this effect. Expression of inactive mutant forms of various growth factor receptors reduces activation of ERK by oxidative stress [171], while overexpression of certain normal growth factor receptors results in enhanced activation of ERK by hydrogen peroxide [172].

Cell survival or apoptosis

The balance between positive and negative signals determines the decision between life and death. An imbalance can result in diseases

linked to increased apoptosis or decreased cell survival. The extent of DNA damage and the level of different proteins contribute to the life or death decision-making process.

In hepatocytes, growth and survival signals are initiated by EGF binding and mediated through the EGF receptor/Ras/Raf/MEK/ERK signalling pathway. In addition, PI3K provides a universal survival signal largely due to its ability to activate Akt, but PI3K is also able to block ROS production from the NAPDH oxidase activation, thereby promoting survival [165].

Some proteins in the cell cycle and apoptotic pathways, have dual roles following stimulation. Both p53, Ras, Akt, pRb, CDKs, cyclins and CKIs may induce cell proliferation, cell cycle arrest or cell death; the different outcomes depends on diverse variables [173].

The p53 protein is widely recognized as a “guardian of the genome” protein functioning during the cell cycle and apoptosis [174, 175]. The factors and mechanisms underlying the decision to undergo growth arrest or apoptosis in response to p53 are not fully understood. The decision is likely complex and governed by multiple factors that depend on both extrinsic and intrinsic factors that are additionally cell type-specific. p53 induced apoptosis is mediated by activation of genes involved in two major apoptotic pathways: (1) the mitochondrial pathway, with genes such as BAX, NOXA, PUMA, p53AIP1, PIGs, and APAF-1; and (2) the death receptor pathway, with genes such as KILLER/DR5, FAS and PIDD. p53 regulates these processes by transactivating genes involved in different cellular functions, but p53 also activates transcription-independent mechanisms of apoptosis [136, 174, 176, 177]. It has also been demonstrated that MAPK signalling pathways, active in transformed cells, can differentially regulate the cellular response to p53 activation and suggest that the Ras/MEK pathway may be a specific determinant of the decision to undergo p53-dependent apoptosis or G1 arrest [137].

The anti-apoptotic effects of Ras can be mediated by up-regulation of Bcl-2 and other anti-apoptotic members of the Bcl-2 family [178]. Downstream of Ras, activated Raf, MEK and ERK contribute to the inhibition of apoptosis by phosphorylating and inactivating Bad. Activation of PI3K by Ras also inhibits apoptosis by activating Akt, which also results in phosphorylation and inactivation of Bad [179]. Akt also phosphorylates and inactivates caspase-9 [180]. However, Akt can also affect cell survival by exerting its effect on the transcription factor NF-κB via phosphorylation of the NF-κB regulator IκB [181].

pRb inhibits cell cycle progression by interacting with transcription factors such as E2F. In addition pRb has been shown to suppress apoptosis. For instance, pRb-deficient embryos show defects in fetal liver haematopoiesis, neurogenesis and lens development, and extensive apoptosis was observed in these tissues [182, 183]. The mechanisms by which pRb/E2F influences apoptosis remain unknown, but E2F has been shown to induce the expression of the pro-apoptotic factor Apaf-1 and evidence suggests a role for E2F in apoptosis following DNA damage [184, 185]. E2F cannot induce apoptosis when pRb is coexpressed and pRb possibly has an anti-apoptotic effect through the inhibition of E2F [186, 187].

The role of CDKs and cyclins in cell proliferation is widely known, but contradictory results exist about the role of CDKs in apoptosis. Some studies report a pro-apoptotic activity for CDKs; they show requirement of activated CDKs during apoptosis of thymocytes [188, 189]. In addition, CDKs seem to be required for neuronal cell death [190]. Some apoptosis-inducing agents can cause induction of CDK1 and CDK2 activity prior to cell death [191]. Dominant negative mutants of CDK1, CDK2 and CDK3 suppress apoptosis, induced by staurosporine and TNF-α [192]. Contrary, inhibition of CDK2 has been shown to protect thymocytes from apoptosis, mitochondrial changes and caspase activation [189].

CDK inhibitors have been suggested to be indirectly involved in apoptosis through regulation of CDK. Improper regulation of CDK can send conflicting signals for cell division and cell cycle arrest. $p21^{Cip1}$ can have anti or pro-apoptotic properties, for example. Overexpression of $p21^{Cip1}$ inhibits radiation-induced apoptosis in human colorectal carcinoma cells, while an inducible expression of $p21^{Cip1}$ sensitizes EJ tumor cells to mitomycin C-induced apoptosis [193, 194]. $p27^{Kip1}$ may also have both pro- and anti-apoptotic effects [195-197].

TGF-β is an important growth inhibitor and inducer of apoptosis in different cell types [198]. Apoptosis induced by TGF-β has been linked to an oxidative stress process and production of ROS [199-201]. However, ROS may also induce adaptive responses through activation of proteins that mediate the survival of cells. ERK is an essential mediator of survival signals through phosphorylation of ribosomal S6 kinase (RSK), which positively influence the transcription of pro-survival genes [202]. A role for ERK in survival after ROS exposure has been shown in both cell lines and in hepatocytes [203-207]. In line with this, we found that ROS mediated adaptive responses to environmental stress through activation of ERK, providing increased cell survival [208].

AIMS OF THE STUDY

The background for the present work was studies in our laboratory showing changes in the growth factor regulated phase of the cell cycle in carcinogen-exposed liver. In cultured hepatocytes, growth arrest was accompanied by altered signalling through the EGF receptor and a subsequent reduced nuclear accumulation of ERK. Furthermore, carcinogen exposure led to reduced induction of cyclin D1 and abridged nuclear accumulation of CDK2 and CDK4. To clarify those mechanisms, it became apparent that we needed a better characterization of growth factor regulation of the G1 phase of the cell cycle in normal hepatocytes. Thus, we defined the following aims for this project:

1. Define CDK2-cyclin E regulation of growth induction in normal cells.

2. Define p53 functions in normal cell proliferation.

3. Characterize the regulation of ERK nuclear trafficking after growth factor- or stress activation.

METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of the methods are given in the respective articles (appendices I-IV). The following methodological considerations will discuss the choice of model system and methods, some of their advantages and limitations, and describe some problems encountered during the course of the experimental work performed in this thesis.

Cell cultures

Cell cycle progression of normal hepatocytes was analysed by using a well-characterized *in vitro* model of primary rat hepatocytes stimulated to proliferate by addition of EGF [209, 210]. Primary hepatocytes isolated from adult liver are recruited from a resting G0 state into G1 phase of the cell cycle during cell isolation and plating [211]. Thus, these cells are effectively synchronized before receiving a further proliferative stimulus. Hepatocyte mitogens like EGF, TGFα and HGF effectively induce DNA synthesis in this model. Greater stimulation of DNA synthesis can be achieved in cultured hepatocytes from young, rather than old rats, apparently reflecting differences in signal transduction down-stream from growth factor receptors and/or in expression of genes regulating the cell cycle [212, 213]. Although mouse hepatocytes have the capacity to proliferate in the absence of growth factors in medium, proliferation activity of rat hepatocytes is tightly dependent on stimulation with mitogens. Thus, rat hepatocytes in culture represent a more appropriate model to study mitogen-dependent entry and progression along the cell cycle. However, under these conditions, the cells have limited lifespan *in vitro* (5-7 days) and usually die after one round of the cell cycle. The quality of the final suspension was checked by trypan blue exclusion as an indication of viability, and cell preparations with viability of 60-100 % were used.

Transfection

Primary hepatocytes are not easy to transfect. Thus, we tried several methods and transfection reagents to find an efficient and reproducible DNA-mediated gene transfer that did not interfere with the biological processes of interest. Viral transfection, calcium-phosphate and Lipofectamin, Transmessenger and DOTAP were evaluated considering transfection efficiency, toxicity and influence on proliferation. DOTAP, which is a special reagent for primary cells, showed highest transfection efficiency (30-60 %) and had little side effects and was therefore used in articles I, II and IV.

To quantify transfection efficiency, a PEYFP-Mem Vector, which is a fusion protein consisting of the N-terminal 20 amino acids of neuromodulin and a yellow-green fluorescent variant of the enhanced green fluorescent protein (EGFP), was detected by fluorescence microscopy in viable cells. In some experiments it seemed like the majority of the cells were transfected. This might indicate that many cells express low levels of transfected genes. However, transfection efficiencies might be underestimated because of the high autofluorescence of hepatocytes.

Immunological methods

The high specificity of immunological methods makes them useful tools in cell biology. These methods are based on recognition between antigen epitope and antibody and were used to study relative protein levels, protein phosphorylation and cellular protein localization. The specificity of the antibodies used is very important for the reliability of the results.

Western blotting was used to study protein levels and phosphorylation status. Protein dephosphorylation can happen fast. To inhibit dephosphorylation, lysis buffers contained inhibitors of

dephosphorylation in addition to inhibitors of protein degradation. When phosphorylation was examined, the samples were not stored, but immediately subjected to SDS-PAGE.

We made use of nuclear fractionation and immunofluorescence microscopy studies to characterize the cellular localization of the various proteins. One of the major concerns with immunofluorescence microscopy is antibody cross-reactions. Immunological multi-staining requires crossover controls to ensure absence of immunological cross-reactions. Nonspecific binding of antibodies cannot be totally eliminated, but it can be limited by preincubation of cells in blocking solution, antibody preabsorbtion and sufficient dilution of the antibody. The inherent possibilities of antibody cross-reactions, which in many cases are difficult to control for, underscores the need to supplement immunofluorescence microscopy with other techniques. We have therefore applied several different immunological methods to support our microscopy results. All antibodies used in immunofluorescence microscopy were tested by Western immunoblotting.

Co-localization of proteins detected by confocal microscopy is only indicative of distribution to identical subcellular compartment and possible direct interaction. To avoid bleed-through from the two fluorochromes used in dual staining, the images were taken sequentially. By merging the red fluorochrome image with the green fluorochrome image, possible colocalization of the proteins are visualized as yellow signals.

Measurement of proliferation

Primary hepatocytes can be maintained in a serum-free medium with defined growth factors, such as EGF, and respond to stimulation by expressing many of the same cell cycle regulatory proteins that are expressed in the liver and drives liver regeneration *in vivo*. To determine

the EGF dependent proliferation, we measured the incorporation of ^{3}H-thymidine into DNA by a liquid scintillation counter. ^{3}H-thymidine incorporated gives an accumulated measurement of the amount of cells in S-phase. To discriminate whether reduced thymidine incorporation was due to reduced proliferation or cell death, we used CPM per milligram protein as a measurement of proliferation.

^{3}H-thymidine incorporation is frequently used to measure hepatocyte proliferation, since the cells have a short lifetime in culture and are not viable throughout the mitosis. An alternative method might be flow cytometri, but this is difficult due to the high autofluorescence of hepatocytes.

Cell death assay

Survival mechanisms in hepatocytes exposed to H_2O_2 (article III) and TGF-β (article IV) was determined by trypan blue exclusion assay in the first article and caspase assay in the latter. In necrosis, bleb rupture and breakdown of the plasma membrane permeability barrier leads to uptake of dyes like trypan blue that enters and label nuclei of nonviable cells. One of the major concerns with this method is that dead cells loose their adhesion capacity and might be washed away before counting. This can lead to a false high viability.

In the caspase assay, cleavage of the fluorescent caspase-3 substrate Ac-DEVD-amc was measured. Caspase-3 mediates apoptotic cell-death, thus this method might also give a false high viability since it does not detect caspase-independent cell death.

SUMMARY OF ARTICLES

Article I

Activation of the p53-p21^{Cip1} Pathway is Required for CDK2 Activation and Proliferation in Primary Rat Hepatocytes.

In this study we found that p53 and p21^{Cip1} were sequentially induced by EGF in primary rat hepatocytes, and that both proteins were regulated through PI3 kinase. p53 inactivation, by transfection with a dominant negative mutant resulted in inhibited CDK2- and CDK4 nuclear accumulation, CDK activation and cell proliferation. These negative effects were neutralized by ectopic expression of p21^{Cip1}. Thus, we demonstrated that EGF-induced p53-p21^{Cip1} was important for activation of both CDK2 and CDK4 and for proliferation in primary hepatocytes.

Article II

CDK2 Regulation through PI3K and CDK4 is Necessary for Cell Cycle Progression of Primary Rat Hepatocytes.

The aim of this work was to clarify the molecular mechanisms involved in mitogen regulation of CDK2 in primary rat hepatocytes. We used two methods to knock out the CDK4 kinase activity in our cells. Indirectly, by inhibiting the PI3 kinase activity, and thus cyclin D1 induction, and directly by overexpressing a kinase-defective mutant of CDK4. We found that cyclin E was induced in hepatocyte cultures independently of growth factor stimulation and PI3K signalling, but this was not sufficient for CDK2 nuclear translocation and pRb-phosphorylation. We also demonstrated that CDK4 activity was essential

for CDK2 activation and for hepatocyte proliferation. In addition, our results suggested that PI3 kinase was involved in regulating CDK4 activity, independently of cyclin D1 induction. These results are consistent with the hypothesis that contribution of CDK2-cyclin E complexes to pRb phosphorylation requires prior action by CDK4-cyclin D1 complexes.

Article III

Cytoplasmic Retention of Peroxide-Activated ERK Provides Survival in Primary Cultures of Rat Hepatocytes.

In this study we describe a role for cytoplasmically retained, redox-activated ERK in survival during oxidative stress in primary hepatocytes. We showed that H_2O_2 induced a dose-dependent activation of ERK that was transient but equally intense as phosphorylation induced by the growth factor EGF, both being MEK-dependent. However, whereas EGF-induced phosphorylated ERK accumulated in the nucleus and induced proliferation, H_2O_2-activated ERK was arrested in the cytoplasm and had no impact on cell proliferation. Instead, inhibition of H_2O_2-induced ERK activation with the MEK inhibitor U0126 reduced cell survival during ROS exposure significantly. Also, the cytoplasmic ERK substrate RSK, a known pro-survival protein, was activated and translocated to nucleus in H_2O_2-exposed hepatocytes.

Article IV

Distinct Functions of H-Ras and K-Ras in Proliferation and Survival of Primary Hepatocytes Due to Selective Avtivation of ERK and PI3K

In this study we showed that the isoforms H-Ras and K-Ras differentially activated the downstream effectors ERK and PI3K, thereby having distinct implications on proliferation and survival of primary hepatocytes. We observed that transient transfection of constitutive active H-Ras as well as constitutive active K-Ras, significantly reduced TGF-β1-mediated apoptosis in primary hepatocytes. However, whereas H-Ras mediated survival through activation of ERK and PI3K, K-Ras induced survival relied on PI3K only. Also, H-Ras but not K-Ras mediated activation of the survival protein RSK, which is a known substrate of ERK. Further, transient transfection of constitutive active H-Ras, but not K-Ras, potentiated EGF- induced proliferation and expression of dominant negative H-Ras, but not K-Ras reduced ERK phosphorylation and excluded ERK from the nucleus following EGF.

DISCUSSION OF RESULTS

Our group has previously studied growth inhibition during early carcinogenesis, and our main focus has been to determine how this contributes to cancer development. In hepatocytes, DNA-damaging carcinogen exposure led to an extensive p53 induction and growth inhibition. However, $p21^{Cip1}$ induction, which could have explained the growth inhibition, was not observed in cell cultures, and $p21^{Cip1}$ was not found in complex with CDK2 in an *in vivo*, carcinogen-inhibited liver regeneration model [4, 214]. Instead, cytoplasmic retention of CDK2 and CDK4 appeared to be a central mechanism related to growth inhibition. Furthermore, previous studies indicated that EGFR-ERK1/2 signalling was altered in carcinogen-exposed hepatocytes [3], which possibly could explain the alterations in CDK2 and CDK4 regulation. Since our findings could not easily be explained by current concepts of hepatocyte growth regulations, we decided to further characterize growth factor regulation of CDK2, CDK4 and p53 in normal, proliferating hepatocytes.

p53 dependent proliferation

Generally, the major role of p53 is to induce cell cycle arrest, DNA reparation or apoptosis in response to cellular stress, particularly DNA damage [139]. However, new evidences support the concept that p53 might play key roles in extracellular matrix homeostasis and inflammation [215]. In addition, Inoue and coworkers have suggested that p53 is HGF regulated and indispensable for induction of hepatocyte proliferation [216].

Our studies of growth factor-induced cell cycle progression demonstrated an EGF-dependent induction of p53 and $p21^{Cip1}$ in

hepatocytes. In line with previous data in other cell types [217], p53-$p21^{Cip1}$ induction was a sequential event (article I).

Previous studies have shown that PI3K regulates p53-Mdm2 pathway on multiple levels under stress, induced by UV-radiation and proteasome dysfunction [218]. Recently, Bar and coworkers demonstrated that induction of p53 by Cisplatin in cultured cells required the presence of serum factors. In addition, the induction of p53 was blocked by the PI3K inhibitor LY294002, implicating the PI3K pathway as a critical mediator of the positive role of serum factors in p53 activation [219]. Also in cultured hepatocytes, we found growth factor induction of p53 to be regulated through PI3K.

When p53 was inactivated, by transfection with a dominant negative mutant or by the p53 inhibitor pifithrin-α, hepatocyte proliferation was significantly reduced. This was in accordance with studies from Inoue et al. and implicated that growth factor induced p53 was critical for cell cycle progression in hepatocytes [216].

Our observation that CDK2- and CDK4 nuclear accumulation and kinase activation was abolished when p53 was inhibited may explain why these cells did not proliferate. It is generally accepted that these two CDKs have to be transported to the nucleus to be activated and to inactivate pRb for further cell cycle progression [46, 220]. The mechanism behind nuclear transport of G1 CDK-cyclin complexes is not well understood, but piggy-backing on $p21^{Cip1}$ has been suggested [128, 129, 221]. Since hepatocytes with an inactive p53 did not express $p21^{Cip1}$, wild type $p21^{Cip1}$ was ectopically expressed in these cells. This coexpression almost completely abolished the negative effects of p53 inactivation. Thus, our results demonstrated that p53 was involved in regulating CDK2- and CDK4 activity through $p21^{Cip1}$ expression, initiated by an EGF induced PI3 kinase activity. Furthermore, the results showed that $p21^{Cip1}$ was necessary for nuclear translocation of CDK2 and CDK4, confirming its role as a piggy-backing nuclear transporter.

CDK2 regulation in G1 phase

In carcinogen exposed hepatocytes, reduced induction of cyclin D1 and abridged nuclear accumulation of CDK2 and CDK4 was observed [4]. Based on these findings and our observations in article I, we decided to further explore the physiological mechanisms that regulate CDK2 and CDK4 nuclear trafficking in normal cells.

During G1 phase in most experimental systems, the D- and E-type cyclins are induced and bind to their respective CDK partners to form active pRb kinases [222, 223]. In many cell types, cyclin D1 is the first cyclin to be up-regulated by growth factors during G1 phase. In hepatocytes, it has been reported that cyclin D1 induction corresponded in time with passage through the restriction point [126, 224, 225]. Previous studies have suggested that cyclin D1 plays an important role in hepatocyte proliferation [1, 38, 126, 225-228]. In accordance with Rickheim and coworkers we found that PI3K activation was required for hepatocyte cyclin D1 expression in response to mitogens [61]. On the other hand, cyclin E expression was detectable in the absence of growth factor, and its induction was not dependent upon an active PI3K (article II). In line with previous studies, we observed three cyclin E isoforms, and the two lower forms were up-regulated after EGF-stimulation [126].

Since PI3K inhibition abolished cyclin D1 induction, we expected CDK4 to be retained in the cytoplasm, even after EGF-stimulation. Our observations that both CDK4 and CDK2 was retained in the cytoplasm, implicated that mitogen-induced CDK2 nuclear translocation was dependent on an active PI3K. If this effect was mediated through CDK4 activity or not was explored by overexpression of a dominant negative mutant of the protein. Mutant $CDK4^{N158}$ contains an Asp-to-Asn mutation at amino acid residue 158, a position conserved in all cyclin dependent kinases. This residue is involved in the binding of Mg^{2+}-ATP, a necessary step for enzymatic activity of these kinases. It has been demonstrated in both insect cells and $Rb^{-/-}$ 3T3-cells that CDK4-

inactivation lead to delayed cell cycle entry after serum starvation [229, 230]. One way to verify that CDK4 activity is inhibited might be Western blotting with a Thr172-phospho-specific antibody. Such an antibody has been used in a recent study [231], but this was just a test preparation which is currently not commercial. Thus, inhibition of CDK4 activity was confirmed by showing reduced pRb phosphorylation at a CDK4-specific site (Thr826). In hepatocytes with an inactive CDK4 kinase, we also demonstrated reduced Thr160-phosphorylation of CDK2 and reduced pRb phosphorylation at a CDK2-specific site (Ser807/811). This was confirmed by data which showed that after CDK4 inactivation, the CDK2-cyclin E complex was retained in the cytoplasm. Correspondingly, we also found proliferation of these cells to be significantly reduced. It is generally accepted that both CAK and pRb are located in the nucleus, thus if CDK2 is retained in the cytoplasm, it can neither be activated by CAK nor function as a pRb kinase. From our observations in cells with an inactive CDK4, we then demonstrated that CDK2 nuclear accumulation and CDK2 kinase activity are dependent on a prior CDK4 activity.

Lately, it has been an ongoing discussion whether mammalian cells need both CDK2 and CDK4 kinase activity for induction of S-phase [232-238]. Other CDKs may compensate for the ablation of a particular cyclin-CDK complex in the context of knock-out mice. Whether this can occur if CDKs are knocked out after cellular differentiation remains unexplored. In cultured cells, it has been suggested that phosphorylation of pRb by CDK4-cyclin D1 initiates a subsequent round of phosphorylation of the tumor suppressor which is completed by CDK2-cyclin E in late G1 phase [239, 240]. This is in accordance with our data and suggests that CDK4 and CDK2 do not have overlapping functions in differentiated hepatocytes.

Because cyclin D1 is up-regulated in many human malignancies, the response to its overexpression has been studied extensively in cell

culture systems. In general, cyclin D1 alone does not promote cell cycle progression in the absence of mitogen, but it shortens the duration of G1 phase in response to growth factors [60]. However, Nelson and coworkers have demonstrated that transient expression of cyclin D1 was sufficient to promote hepatocyte replication and liver growth [126, 227]. Conversely, we found that ectopic cyclin D1 expression did not induce CDK4 nuclear translocation, CDK2 activation or proliferation in the absence of growth factor. EGF-stimulation through PI3K was required even in this situation. Whether these differences reflects variations between our cell culture model and liver regeneration in an intact organ, or can be attributed to the adenoviral transfection method applied in the latter, remains unresolved. Our observations indicated that in addition to cyclin D1 induction, PI3K activity was necessary for translocation of CDK4- cyclin D1 complexes to the nucleus, and that this process was a prerequisite for nuclear translocation and functional activation of CDK2.

In our p53-studies (article I), we found PI3K-dependent $p21^{Cip1}$ expression important for CDK4 and CDK2 nuclear translocation. Therefore, we explored if overexpression of both $p21^{Cip1}$ and cyclin D1 was sufficient for CDK nuclear translocation or to initiate proliferation in our system. Preliminary data did not show proliferation in cells cotransfected with wild type $p21^{Cip1}$ and wild type cyclin D1 in the absence of EGF, nor could CDK4 or CDK2 nuclear accumulation be observed (unpublished results). Thus, growth factor receptor signalling seemed to have additional effects critical in hepatocyte proliferation than cyclin D1 and $p21^{Cip1}$ induction.

Also, in the light of article I, we have cotransfected hepatocytes with wild type $p21^{Cip1}$ and dominant negative CDK4. Preliminary data from these studies showed CDK2 nuclear accumulation and increased EGF-induced proliferation, compared to $CDK4^{N158}$ transfected cells. Furthermore, we have lately observed reduced p53 induction in CDK4

inactivated hepatocytes. This might indicate that the effect of CDK4 kinase activity on CDK2 is to induce $p21^{Cip1}$.

Considering the conclusions in article I and II and the preliminary results described above, we suggest that PI3K-dependent CDK4 activity is necessary for p53- $p21^{Cip1}$ induction, and that $p21^{Cip1}$ is essential for the subsequent CDK2 nuclear translocation and activation.

ERK in survival and proliferation

Our group has earlier found that ERK do not translocate to the nucleus in carcinogen-exposed hepatocytes. Thus, the mechanisms regulating ERK nuclear trafficking in normal hepatocyte proliferation and during hepatocyte stress appeared to be relevant to early carcinogenesis.

Oxidative stress by ROS may cause severe damage and cell death. However, ROS may also induce adaptive responses through activation of proteins that mediate survival of cells. Particularly, a role for ERK in survival after ROS exposure has been shown both in cell lines and in primary hepatocytes [203-207]. Whether ERK is activated in a highly regulated manner or through interference with growth regulatory pathways is still unknown. We therefore studied the potential role of ERK during adaption of hepatocytes to oxidative stress in a hydrogen peroxide environment. H_2O_2 has been suggested to act as a paracrine mediator of apoptosis and has widely been used as a model of exogenous oxidative stress. In accordance with earlier findings [203, 204], we found a strong activation of ERK during H_2O_2 exposure. We also showed that this ERK-activation was independent of an active EGFR kinase activity. Thus, H_2O_2 can activate ERK directly or via upstream activators.

MEK is the upstream activator of ERK in the signalling pathway from EGF receptor. In quiescent cells, ERK is located in the cytoplasm

where MEKs can serve as cytoplasmic anchors for ERK through direct binding interaction. Upon mitogen stimulation, the MEK-ERK association is disrupted and a proportion of ERK translocates to the nucleus [132]. Several studies show activation of MEK after ROS exposure. Also in hepatocytes, we found MEK to be the activator of ERK. However, ERK did not accumulate in the nucleus after H_2O_2 exposure, as seen after EGF stimulation. To exclude the possibility of a rapid export of the MEK/ERK complex, H_2O_2 exposed cells were pretreated with leptomycin B, a specific inhibitor of CRM1/exportin. Also in this case, ERK was exclusively located in the cytoplasm. Thus ERK activated by H_2O_2 did not enter the nucleus, but was arrested in the cytosol.

Specific intracellular localization of ERK has been shown to determine cell faith. Generally, ERK trapped in the cytoplasm blocks DNA synthesis and cell proliferation, whereas forcing an active ERK into the nucleus promotes proliferation and oncogenic transformation [241-243]. Activated ERK can provide survival through several downstream mediators, including RSK [202], which neutralizes the pro-apoptotic protein Bad [244]. We found a strong reduction in the survival of hepatocytes when activation of ERK was inhibited. Second, we found RSK to be activated, via ERK, by both oxidative stress and growth factor stimulation. Activated RSK was located in the nucleus. Thus, to conclude this article we hypothesized that survival in hepatocytes was mediated by redox-activated ERK from the cytoplasmic compartment through activation and nuclear transport of RSK.

Considering the results in article III, we next explored one of the upstream activators of ERK, Ras. Ras has been suggested as an upstream H_2O_2 acceptor [206]. By activating both the MAPK signalling cascade and the PI3K pathway, Ras has a role in cell proliferation as well as in inhibition and promotion of apoptosis [245].

TGF-β1 plays an important role in mediating hepatocyte death *in vivo*, thereby controlling liver size [246]. TGF-β1 does not activate ERK or PI3K [247], therefore we used TGF-β1-exposure as a model to induce cell death. The apoptotic process induced by TGF-β1 is mediated by ROS. Moreover, it includes the activation of caspases 2, 3, 7 and 8 [248]. Several growth factors, including EGF, are able to oppose the apoptotic process. In agreement with previous studies, we found that both ERK- and PI3K activation suppressed TGF-β1–induced apoptosis [247, 249]. However, PI3K had a more potent anti-apoptotic effect on TGF-β1-exposed primary hepatocytes.

The C-terminal portion of Ras proteins, encompassing the last 25 amino acids, is unique for each isoform and has therefore been termed the hypervariable region (HVR). The function of HVR is to mediate the association of Ras proteins with cellular membranes. This function is accomplished through post-translational modifications of HVR. Whereas H-Ras undergoes both farnesylation and palmitoylation, K-Ras undergoes only a single lipid modification – farnesylation [250]. Several studies have reported that H-Ras is retained in the endosomes to a much higher extent than K-Ras, and that the latter recycles faster to the plasma membrane due to ineffective sorting into the early endocytic compartment [251, 252]. We found endogenously expressed H-Ras and K-Ras internalized and co-localized with EGF receptor in endosome-like compartments after stimulation with EGF, suggesting an equal endosomal sorting of the two isoforms.

Recently, it has been suggested that the two homologues H-Ras and K-Ras may have different biological effect. When overexpressed in COS-cells, K-Ras has been reported to be a more potent activator of the ERK pathway, whereas H-Ras was a more potent activator of PI3K [253]. However, contradictory results have been reported, showing that oncogenic K-Ras expressed *in vivo* did not activate the Raf/ERK pathway in unstimulated cells [254]. In accordance with the latter, we

found that expression of constitutive active K-Ras (K-Ras V12) did not activate ERK in unstimulated cells. Conversely, in the absence of EGF, expression of constitutive active H-Ras led to phosphorylation, but not nuclear translocation of ERK. Still, H-Ras is critical for ERK phosphorylation and nuclear transport, since the dominant negative mutant (H-Ras N17) abolished EGF induced ERK nuclear accumulation.

To promote cell cycle progression, ERK has to be activated and translocated to the nuclear compartment [241]. However, Ras-independent pathways also need to be initiated before cells respond mitogenically to EGF [255]. This is in accordance with our findings that constitutive active H-Ras, or the combination of constitutive active H-Ras and K-Ras, were not enough to mimic growth factor stimulation and induce cell cycle progression.

Toulany and his group have shown that K-Ras mediate radio-resistance in carcinoma cell lines by activating PI3K [256]. In line with this, we found that K-Ras mediated an anti-apoptotic activity through PI3K, but not through ERK, whereas H-Ras promoted hepatocyte survival through both ERK and PI3K. Thus, according to the results in article III and IV, we suggested that redox-activated ERK promoted survival through H-Ras in primary rat hepatocytes.

CONCLUDING REMARKS

We have demonstrated that in primary rat hepatocytes, CDK4 activity was necessary for CDK2 nuclear translocation and proliferation, and that growth factor activation of p53-$p21^{Cip1}$ was necessary for CDK4 and CDK2 trafficking. Our results were in accordance with the notion that in cells without DNA damage, p53-$p21^{Cip1}$ activation is a positive regulator of physiological cellular proliferation. In addition to regulation of cyclin D1, PI3K controlled proliferation through p53 induction. It also appeared that an additional growth factor signalling pathway was necessary, since ectopic expression of cyclin D1 and $p21^{Cip1}$ was not sufficient for growth induction.

Cytoplasmic retention of activated ERK remains a putative mechanism of carcinogen-induced growth inhibition and thus cell cycle progression. Although this was observed following both H_2O_2 and carcinogen (2-acetylaminofluorene) exposure, the former appeared to affect ERK differently, since it phosphorylated ERK by itself. From the cytoplasmic compartment, redox-activated ERK was able to mediate survival. Such signalling may possibly contribute to the carcinogenic development. Our results demonstrated that hepatocyte proliferation was H-Ras dependent, since K-Ras did not activate ERK. In addition, H-Ras promoted survival through both ERK and PI3K, whereas K-Ras mediated an anti-apoptotic activity through PI3K.

For further experiments, this thesis pinpoints the following problems to be addressed:

- Characterize the unknown signals, which are critical for hepatocyte cell cycle progression, in addition to cyclin D1 and $p21^{Cip1}$ induction.

- Characterize if ectopically expressed $p21^{Cip1}$ can recover the growth arrest in carcinogen-exposed hepatocytes.
- Characterize the upstream activator of MEK following H_2O_2-exposure.

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