Cellular signaling in human cholesteatoma

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Chapter 2

2

Molecular pathways in human cholesteatoma

A comprehensive review of signaling pathways investigated in this thesis.

Cellular signaling by cytokines, growth factors and peptide hormones is generally mediated through membrane-bound receptors, whereas smaller and more lipophilic signaling molecules such as steroid hormones and some vitamins often signal through nuclear receptors. General features of cytokines and growth factors are pleiotropism and redundancy. Pleiotropism refers to the ability to act on different cell types, which reflects that different cell types may have the same type of receptors on their membrane surface. Redundancy refers to the property of multiple cytokines and growth factors having the same functional effect, which can be explained by the fact that the same receptor or receptor type can bind different cytokines or growth factors[1].

This overview will concentrate on signal transduction pathways and proteins that regulate the balance between keratinocyte cell proliferation, survival, apoptosis and differentiation, with a particular emphasis on the role of the mitogen-activated protein kinase-(MAPK), PI3Kinase/ Akt-, TGFß-signaling and the p53 and $p21^{cip1/waf1}$ protein.

MAPK signaling

The classic MAPkinase cascade consists of three sequental intracellular activation steps and is initiated upon ligand binding with the appropriate receptor. The three MAPK routes are initiated by different stimuli through different receptors but the architecture of the signaling cascades is in general similar. After ligand binding usually a set of adaptors such as sonic hedgehog (Shc) and growth factor receptor-bound protein 2 (GRB2) bind and recruit the guanine nucleotide exchange factor (GEF) SOS to the plasma membrane in proximity to Ras to exchange the GDP for GTP on Ras proteins (Ras, Rac, Rho), which in turn activate the first member of the cascade: a MAPKK kinase (MAPKKK or MEKK) (Fig.1)[2]. A MAPKKK is a serine/ threonine kinase that phosphorylates and activates MAPK kinases (MAPKK or MEK).

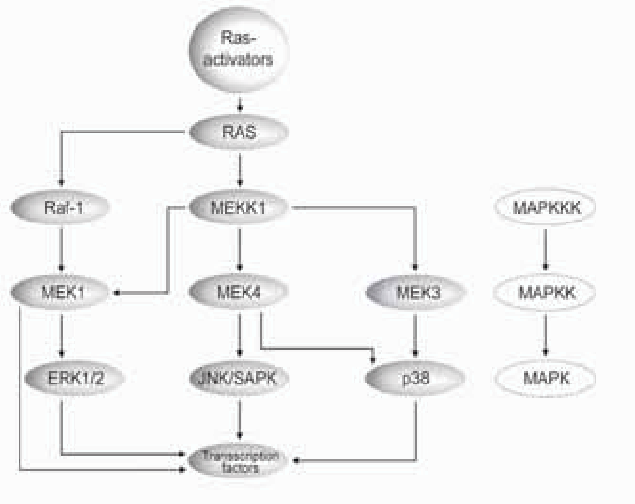

Figure 1.
The classic MAPKinase cascade consists of three intracellular activation steps and is initiated when the first member, MAPKKK, is activated. MAPKKK activates MAPKK, subsequently, MAPKK activates a MAPK. There are three MAPK pathways downstream including Ras/ Raf/ MEK1/ ERK, Ras/ MEKK/ JNK/ SAPK and the Ras/ MEKK1/ p38 MAPKinases. Crosstalk between different MAPK pathways may occur.

Subsequently, MAPKK activates a MAPK by dual phosphorylation on adjacent threonine and tyrosine residues. All three MAPK families are activated by dual phosphorylation on both adjacent threonine and tyrosine residues separated by a single amino acid, forming a tripeptide sequence. The second amino acid for MAPKs for extracellular regulated kinase (ERK) is glutamate (Thr- Glu- Tyr), for the p38 family Glycine (Thr- Gly- Tyr) and for the c-Jun N terminal-kinase (JNK) family Proline (Thr- Pro- Tyr)[3].

Several studies describe extensive crosstalk between these cascades in which a particular kinase in one pathway affects a kinase activity in another pathway. This illustrates that MAPKs are a highly interdependent regulatory network in which the cellular outcome is likely to be dependent on the balance between regulatory inputs. Full specificity is ensured through docking interactions by kinases that recognize a distinct site on their substrates. E.g., the JNK docking region of c-Jun recognizes JNK and determines thereby its specific phosporylation at ser63 and ser73[4]. Scaffold proteins can provide an assembly site for such specialized protein interactions. These scaffold proteins usually do not contain any intrinsic enzymatic activity but possess a structure that enables them to recruit different factors of a specific pathway simultaneously[5]. But, although scaffold proteins increase specifity of individual signaling cascades, they act at the expense of signal amplification. Spatial localization of signaling molecules is another device to augment specificity in signal tranduction. E.g., MEKK1 colocalizes with elements of the cytoskeleton and cytoskeletal rearrangements may stimulate MEKK1 activity[4]. Finally, the duration of the signal can strongly influence the direction of the various pathways[6]. However, once activated, the MAPK cascade enables the cell to respond to environmental changes in a prompt and ordered fashion[3].

The Ras/ Raf/ MEK1/ ERK1/2-pathway.

The classic ERK1/2 cascade may serve as a prototype of the other MAPK cascades. It is discussed extensively here to improve the understanding of the basic principle of MAPK signal transduction pathways. The Ras/ Raf/ MEK1/ ERK1/2 cascade is activated in response to many mitogenic stimuli, such as EGF, PDGF, thromboxane A2, angiotensin II, TGF, insulin, LPS, osmotic stress and adherence of monocytes and endothelial cells.

Ras is a small guanosine tri phosphatase- (GTP-ase) that is activated through its interaction with the Grb2- Sos (son of sevenless) complex, where Sos catalyzes the dislocation of GDP with the subsequent formation of Ras- guanosine tri phosphate (GTP) complex. Different Ras isoforms and mutations have been observed, which possess varying abilities to activate downstream signaling pathways. In its GTP-bound state, Ras recruites Raf to the membrane. The known members of the mammalian Raf gene family are Raf-1, A-Raf and B-Raf. Raf is one of the MAPKKK kinases and subsequently activates its downstream MAPKK, MEK1. Raf, however, is not the only inducer of MEK1 and ERK1/2 activation. All MAPKKK family members, with the exception of MEKK4, have the potential to activate MEK1. By contrast, Raf is unable to activate other MAPKKs[5]. The ERKs are characterized in two isoforms, ERK1 and ERK2 (ERK1/2), which are sometimes referred to as p44/42 MAP kinases.

Because ERKs, like other MAPKs, are activated by phosphorylation, protein phosphatases dephosphorylating MAPKs are key elements in controlling ERK1/2 activity[1]. The duration as well as the magnitude of the ERK1/2 signal is a critical factor in determining the response of a certain type of cell to changes of the extracellular environment, i.e. a transient ERK1/2 signal activates other transcription factors than a sustained ERK1/2 activity[7]. In keratinocytes, a mitogenic stimulus such as provided by growth factors results in a relatively strong, transient activation of Raf/ ERK1/2. Then, after minutes, the signaling is downregulated. Such a transient signaling may dictate modulation in the level of $P21^{cip1/waf1}$ expression, resulting in cell cycle progression[7]. It has been reported that a sustained ERK1/2 signaling may occur independently of Ras, through a newly discovered GTP-ase, Rap1. Convergence towards ERK1/2 activation occurs on the level of Raf. Sustained activation may also occur through the cooperative activation of different receptors e.g., the EGF- and the integrin receptor. It has been demonstrated in keratinocytes that such a sustained Raf/ ERK1/2 activation, induces a persistently high level of $p21^{cip1/waf1}$ and a subsequent G1 arrest[7]. In the nucleus, ERKs can phosphorylate and activate a number of transcription factors such as c- fos, Elk-1, NFκB and Jun. Fos and Jun family members homo- or heterodimerize to an AP1 transcription promotor complex. Dependent on the composition of the complex, differential gene transcription and protein production occurs.
Once ERK1/2 have been activated they can also target cytoplasmic - or cytoskeletal proteins. Cross talking between other MAPKs such as p38, but also with other signaling pathways like the TGFβ/ SMAD and the PI3K/ Akt have been reported[8-10]. By its broad spectrum activation program, the Ras/ c-Raf1/ MEK1/ ERK1/2/ MAPK signal transduction pathway is involved in most cellular processes like proliferation, cell cycle arrest and apoptosis.

The Ras/ MEKK1/ MEK4/ JNK/ SAPK pathway
The c-Jun N-terminal kinase (JNK) was originally identified as the UV-induced factor responsible for phosphorylation and thus activation of the transcription factor c-Jun[11]. JNKs are also characterized as stress-activated protein kinases (SAPK) on the basis of their activation in response to inhibition of protein synthesis[12]. Three genes JNK1, JNK2 and JNK3 with 12 possible isoforms derived from alternative splicing products have been described[13]. Environmental stress, radiation, growth factors and endotoxins induce activation of JNK/SAPKs. Regulation of the JNK/ SAPK pathway is extremely complex and is influenced by many MAPKs. There are e.g., 13 MAPKKs that can regulate the JNK/SAPKs. This diversity allows a wide range of stimuli to activate this MAPK pathway[14]. JNK/SAPKs are considered to be important in controling programmed cell death or apoptosis.

The Ras/ MEKK1/ MEKK6/ p38 pathway
P38 is recognized as the MAPK that is activated in response to physiologic stress, osmotic stress, LPS and UV exposure[3]. The p38 MAPK family has been shown to consist of four different isoforms, p38α, -β, -γ and –δ[15]. Different activation and substrate specifity of each p38 isoform results in their different physiological

functions. It has been shown that p38α and p38β are ubiquitously expressed, while p38γ and p38δ are expressed in a more tissue specific manner. p38γ expression, e.g., has not been detected in the epidermis, while p38δ plays a key role in epidermal differentiation[8].
Besides activation via the MAPK signaling cascade, the p38α, but also the JNK kinase have been shown to be activated by the protein complex TAK1/2/ TAB1, which is part of the interleukin-1 cytokine signaling pathway[6,16]. Because TAB1 has no known catalytic activity, it appears to be an adaptor or scaffolding protein[14]. This is an important observation, which indicates that inflammatory cytokines, or other adaptor proteins, may be involved in the activation of different MAPK pathways through one signaling module. Complex formation e.g., between two MAPKs, p38 and ERK, has also been reported, underlining the complexity of the system[8]. A number of downstream targets of p38 have been demonstrated. Cytoplasmic substrates of p38 include different protein kinases which act on the translational as well as the transcriptional level. In the nucleus, p38 regulates the activity of a number of transcripion factors such as ELK1, p53, NFκB and AP-1[5,8,17]. However, p38 is generally considered to be the MAPKinase, which is dominant in the regulation of keratinocyte terminal differentiation via the AP-1 human involucrin promoter[8,18].

pAkt signaling

Recent studies have revealed a burgeoning list of Akt/ PKB substrates implicated in oncogenesis, nutrient metabolism, transcriptional regulation and cell survival[19]. Among its pleiotrophic effects, activated Akt/ PKB is a well established survival factor and in this chapter we will discuss the molecular mechanism of its function in regulating cell survival particularly. In mammals, three Akt/ PKB genes have been identified, termed Akt1/ PKBα, Akt2/ PKBβ and Akt3/ PKBγ. Akt/ PKB are the downstream effector kinases of phosphoinositide 3 kinase (PI3K), which is activated by growth factors via a tyrosine kinase receptor and via the G-protein-coupled receptors (Fig.2). Following ligand binding, PI3K is recruited to the cell membrane and activated. Then PI3K interacts with and phosphorylates phosphatidylinositoldiphosphate (PIP2), which results in generation of phosphatidylinositoltriphosphate (PIP3). PIP3 does not activate Akt/ PKB directly but instead appears to recruit Akt/PKB to the plasma membrane and to alter its conformation. This allows subsequent phosphorylation by phosphoinositide-dependent kinase-1 (PDK1). The Akt/ PKB protein has two phosphorylation sites, Thr308/309 and Ser473/474, of which Thr308/309 phosphorylation is necessary for Akt/ PKB activation while Ser473/474 is required for maximal activity[20]. Activated Akt/ PKB is then released from the membrane and translocates to both the cytosol and the nucleus[21].
Different reports have suggested that pAkt/ PKB can be activated in a PI3K-independent way. It has been shown that cyclic adenosin mono phosphate (cAMP) inducing agents such as prostaglandin-E1 were able to activate Akt/ PKB through Protein Kinase A (PKA). Although the mechanism is not quite clear, it appears that dual phosphorylation of Akt/ PKB is not required for PKA mediated pAkt/ PKB activation. It has also been shown in vitro that Akt/ PKB can be activated by Ca2+/

calmodulin-dependent kinases[19]. Moreover, it has been indicated that pAkt/ PKB can be activated by cellular stress and heat shock proteins[19]. Once activated, the control of cellular survival of Akt/ PKB occurs via direct and indirect effects on the apoptotic pathway. These effects are: post transcriptional regulation, activation of transcription factors and metabolic interaction.

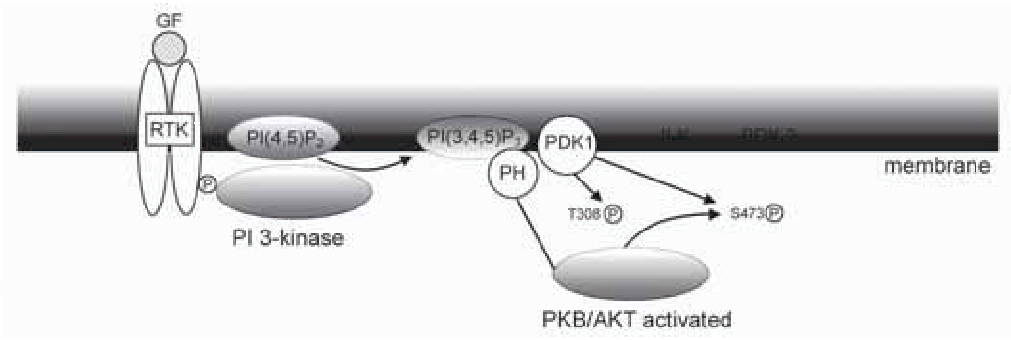

Figure 2.
Akt signaling pathway, activation of different cell-surface receptors, such as tyrosin kinase receptors, induce production of second messengers like PIP3, phosphatidylinositol 3,4,5-trisphosphate, that convey signals to the cytoplasm from the cell surface. PIP3 signals activates the kinase PDK1, 3-phosphoinositide-dependent protein kinase-1, which in turn activates the kinase Akt, also known as protein kinase B. The Akt/ PKB protein has two phosphorylation sites, Thr308/309 and Ser473/474.

There are numerous post-transcriptional Akt/ PKB-mediated cellular survival mechanisms of which the most important are discussed below. One important mechanism includes phosphorylation and de-activation of different pro-apoptotic proteins such as the BAD protein[22], caspase-9[23], and apoptosis- mediating MAPKinases. Akt/ PKB also initiates indirect de-activation of pro-apoptotic proteins such as apoptosis signal-regulating kinase (ASK1)[24]. Akt/ PKB-induced phosphorylation may also comprise activation of anti-apoptotic proteins of which murine double minute 2 (MDM2) is the most essential one[25]. Other mechanisms include activation of anti-apoptotic proteins like NFκB, by binding to and phosphorylation of their inhibitors.
BAD is a pro-apoptotic member of the BCl-2 family, phosphorylation of BAD may occur at two critical sites, Ser112 and Ser136. Phosphorylation at each site is sufficient for association of BAD to 14-3-3. However, full inactivation of the protein is only induced when both serines are phosphorylated[22]. This synergistic phosphorylation is driven by the MAPK signaling pathway at Ser112- and by Akt/ PKB-mediated phosphorylation on the Ser136-site[22].
Caspase-9 is called a death protease and acts as one of the direct effectors of apoptosis. Akt/ PKB-induced phosphorylation of caspase-9 has been shown to diminish the activation of its target execution caspases[23].
ASK1 is one of the MAPKKKs that interacts with and is phosphorylated by Akt/ PKB on Ser83. This results in a decreased ASK1 mediated signaling to JNK/ SAPK and a suppressed susceptibility to apoptosis[24].

Indirect inactivation of ASK may also prevent apoptosis. The cell growth inhibitory activity of $p21^{Cip1/WAF1}$ is strongly correlated with its nuclear localization. However, Zhou et al. have shown that Akt/PKB phosphorylation of Thr145 in $p21^{Cip1/WAF1}$ triggers its cytoplasmic localization[25]. Cytoplasmic $p21^{Cip1/WAF1}$ then forms a complex with ASK1 which, indirectly, results in resistance to apoptosis[26].
MDM2 is an oncoprotein localized in the cytoplasm in a complex with Akt/ PKB. After growth factor stimulation, Akt/ PKB phosphorylates MDM2 on two residues Ser166 and Ser 186. Then MDM2 dissociates from the complex and enters the nucleus where it binds to p53. The MDM2-p53 complex subsequently shuttles to the cytoplasm where p53 is targeted for ubiquitine proteasome-mediated degradation[27]. Under certain circumstances like cellular stress or UV-radiation, p53 has been reported to mediate cell death. Therefore, Akt/ PKB could support cellular survival by promoting degradation of p53.
It has been shown that Akt can activate the transcription factor NF-κB and that this blocks apoptosis induced by certain stimuli. The mechanism whereby Akt activates NF-κB has been controversial, with evidence supporting induction of nuclear translocation of NF-κB via activation of IκB kinase activity and/or the stimulation of the transcription function of NF-κB[28-31]. It has also been demonstrated that Akt targets the transactivation function of NF-κB in a manner that is dependent on IκB kinase activity and on the MAPK p38[32]. These disparate observations point to deficiencies in the understanding of the Akt/ PKB-mediated NF-κB activation. However, it is generally accepted that Akt/ PKB is involved in NF-κB transcription of pro-survival proteins, such as Bcl-xL and caspase inhibitors[19].
Recent studies have shown that Akt/ PKB can regulate cellular survival through transcriptional factors that are responsible for pro- as well as anti-apoptotic genes. The most known are Forkhead (FH). Akt/ PKB can directly phosphorylate all four isoforms of FH. The phosphorylated FH proteins can promote cell survival by inhibiting the activity of a number of FH target genes. These target genes are usually extracellular ligands and important in promoting apoptosis. The most common are the FAS-ligand, TNF-related apoptosis-inducing ligand (TRAIL) and TNF receptor type 1 associated death domain (TRADD)[33].
A major physiologic function of Akt/ PKB is the regulation of cell metabolism. When high levels of insulin are present, Akt/ PKB phosphorylates glycogen synthase 3 (GSK-3), which inhibits its function. This promotes the storage and utilization of glucose. It has been hypothesized that the inhibition of GSK3 is protective against growth factor-deprived apoptosis[21].

TGFβ signaling

Transforming growth factor β (TGFβ) is the prototype of a large family of cytokines that regulate a wide variety of cellular processes including cell proliferation, cell differentiation, cell motility and extracellular matrix production. The TGFβ family includes a large number of related proteins including bone morphogenetic proteins (BMP). The effects of TGFβ on cell growth control are complex and can vary dramatically depending on the target cell type and the presence of other growth factors[34]. TGFβ-related factors signal through ligand binding to the type II TGFβ

receptor and, after forming a heterodimer with the type I TGFβ receptor, signal propagation occurs by phosphorylation of the receptor-specific (R) Smads (Fig.3).

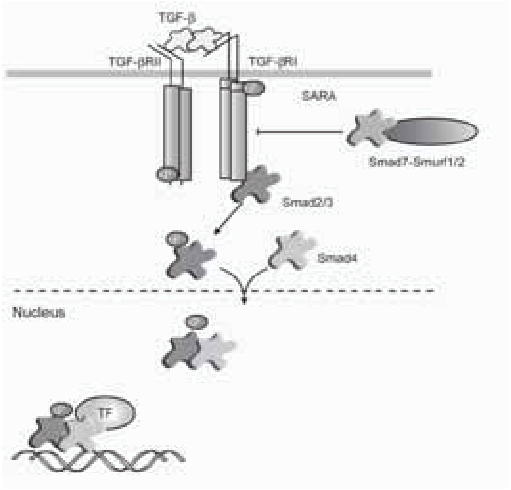

Figure 3.
TGFβ-related factors signal through ligand binding to the type II TGFβ receptor and, after forming a heterodimer with the type I TGFβ receptor, signal propagation occurs by phosphorylation of the receptor-specific (R) Smads. The TGFβ part of the family mediates signaling by activation of the R-Smads, Smad2 and Smad3. The phosphorylated R-Smads then oligomerise Smad4 and translocate to the nucleus, where they regulate the transcription of target genes. The inhibitor Smads (I-Smads), Smad6 and Smad7, function as a broad spectrum intracellular antagonists of TGFβ family signaling. I-Smads bind to the activated receptor complex, in competition with the R-Smads. In addition, Smad7 mediates docking of the ubiquitine ligases Smurf1 and Smurf2 to the TGFβ receptor. This causes ubiquitination and proteasomal degradation of the receptor.

The TGFβ part of the family mediates signaling by activation of the R-Smads, Smad2 and Smad3. BMP signals through activation of the other R-Smads: Smad1, Smad5 and Smad8[35]. The phosphorylated R-Smads then oligomerise with the common-mediator Smad (co-Smad, Smad4), translocate to the nucleus and regulate the transcription of target genes[36]. The inhibitor Smads (I-Smads), Smad6 and Smad7 function as broad spectrum intracellular antagonists of TGFβ family signaling[37]. Expression of inhibitory Smads is induced by multiple stimuli, including EGF and various members of the TGFβ family. I-Smads bind to the activated receptor complex, in competition with the R-Smads. In addition, Smad7 mediates docking of the ubiquitine ligases Smurf1 and Smurf2 to the TGFβ and BMP receptors. This causes ubiquitination and proteasomal degradation of the receptors. Smad7 counters most of the TGFβ-regulated processes in the cell like growth inhibition via $p21^{Cip1/Waf1}$ and production of extracellular matrix proteins. Contradictory, Smad7 expression is necessary for TGFβ-induced apoptosis[38]. The association of the Smad complexes with transcription factors and transcriptional co-activators/co-repressors in the nucleus regulates transcriptional control by TGFβ. TGFβ modulates several other signaling pathways such as the JNK MAPK, which can either be activated or inhibited by TGFβ[39]. Differential activation of ERK1/2 and p38 by TGFβ has also been reported [39,40]. Moreover, in keratinocytes, epidermal mesenchymal transition (EMT) is induced by TGFβ 1 through the activation of both ERK1/2 and p38 MAPKs[41]. Interestingly, co-treatment of cells with EGF enhanced the activation of these MAPKs[41]. In this report, phosphorylation of JNK could not be detected. However, specific inhibitors of MEK1, p38 and JNK all blocked EMT, indicating that activation of all three pathways

was required for TGFβ 1-induced EMT[41]. Crosstalking to the PI3K-Akt pathway has also been reported. It has been demonstrated that TGFß phosphorylates Akt in a PI3K-dependent manner, leading to TGFβ-mediated EMT and cell motility[42].

p53 in repair

Activation of p53

The p53 tumour suppressor protein plays a pivotal role in essential cellular processes like apoptosis, cell cycle control, senescence, differentiation and neoplastic transformation. The function of p53 involves mainly the prevention of the accumulation of genetic alterations by initiating signaling pathways to either DNA repair by growth arrest/ senescence or by eliminating cells by apoptosis. The p53 gene can be activated in many stress responses like DNA damage, illegitimate activation of oncogenes, hypoxia and inflammatory cytokines triggering[43]. In the absence of cellular stress p53 expression is maintained at a low level. At present, multiple lines of evidence indicate that one of the key mechanisms by which p53 functions is regulated through control of the MDM2 protein. MDM2 has been shown to inhibit p53 activity in at least two distinct molecular mechanisms. This may occur by binding to the N-terminal transactivation domain of p53, which blocks interactions with other proteins necessary for p53-dependent regulation of gene expression[43]. Another mechanism, essential under non-stress conditions, is the targeting of p53 for ubiquitination which leads to proteasomal degradation[45,46]. Since p53 can also associate to p53 binding sites within the MDM2 promoter, it can trigger MDM2 expression. This is an important negative autoregulatory feedback mechanism in p53-MDM2 interaction[43,44]. In some cancer cells, mutant p53 does not induce MDM2 gene expression. In that situation, mutant p53 is not degraded and its half-life in cells is prolonged[47]. The regulation of p53 stability is a complex process that is dependent of many different forms of stress. Many pathways can be used to allow stabilization of p53, such as phosphorylation, inhibition of MDM2 synthesis or cytoplasmic sequestration of p53[45,48]. Another important regulatory mechanism of p53 stabilization via MDM2 inhibition is the binding of the alternative reading frame (ARF). This binding inhibits the p53- targeting for ubiquitination[49-51]. The consequence of ARF expression is the efficient stabilization and activation of p53. ARF plays an important role in the induction of p53 in response to oncogenic activation, eliminating cells with proliferative abnormalities[43]. In addition to the inhibition of MDM2 by ARF, it has recently been established that the AKT/PKB kinase can also be engaged in MDM2 inhibition (see Akt section). There are also MDM2-independent mechanisms for p53 degradation, of which JNK, by targeting p53 for ubiquitination, appears to be the most important[52] .

Activation by p53

The molecular basis for the differential activation of particular sets of target genes by p53 is not fully understood. Multiple molecular mechanisms most certainly contribute to p53 target gene selectivity. Studied intensively is the covalent modification of the p53 protein by phosphorylation. The most important kinases

involved in p53 phosphorylation include casein kinase, CHK1 and 2, DNA-dependent protein kinase (DNA-PK) and JNK[53-55]. These kinases also phosphorylate MDM2 in vitro, suggesting a regulatory role for these modifications[56-58]. It has been suggested that the phosphorylated p53 protein undergoes some conformational changes, which alters its DNA-binding specifity. In line with this, it has been demonstrated that phosphorylation on specific residues of p53 alters its DNA binding preference in vitro[43]. But, the in vivo relevance of this finding has been questioned[43]. Besides modifications of the p53 protein, the transcription of particular target genes appears to be determined by interaction of p53 with other proteins. These proteins may be transcriptional coactivators like p300, CBP, PCAF and E2F1 which have been shown to enhance p53-mediated transcription[59]. The need for additional p53 partners may be of particular importance for genes with a low-affinity p53 binding site (p53BS). It is of interest that many, if not all, pro-apoptotic p53 target genes harbour p53BS of rather low binding affinity. Thus, this subclass of genes may rely more heavily on cooperation of p53 and its co-activators, whereas cell cycle inhibitory genes may be turned on by p53 as a default option[43]. The question how p53 chooses between induction of apoptosis versus induction of a viable growth arrest has received great attention. As it appears now, p53 is not the only determinant that influences this choice. The phenotype of the cell, the extra cellular stimuli and the type of stress and its intensity are of great importance for the direction of p53 transcriptional activities[60]. With respect to the phenotype, different cell types may respond to the same apoptotic stimulus with either apoptosis, or cell cycle arrest[61]. This might be due to their differential ability to induce pro-apoptotic proteins of the Bcl-2 family, like Bax, Noxa and Puma[43]. It might also be possible that the difference in apoptotic threshold is a reflection of the biology of the cells involved. Cells with a high turnover, like T cells, must respond quickly to death stimuli in order to limit the immunological response[60]. In general, activation of p53 in normal cells results in cell- cycle arrest or senescence, whereas in malignantly transformed cells p53 usually promotes apoptosis[61]. Moreover, extracellular stimuli, such as cytokines and growth factors can protect cells from apoptotic response to cell death stimuli or DNA damage by p53-promoted growth arrest[62].

The ability of p53 to promote apoptosis has been studied extensively and multiple pathways have been identified. However, to what extent each pathway contributes to the apoptotic activity of p53 remains a controversial matter. One of the most prominent pathways is the mitochondrial pathway which is involved in the transcription of the pro-apoptotic proteins Bax, Noxa and Puma and their transport to the mitochondria[43,63]. This action promotes loss of the mitochondrial membrane potential and cytochrome c release, which results in the formation of the apoptosome, a holoenzyme consisting of cytochrome c, APAF1 and pro-caspase 9[63]. Pro-caspase 9 is one of the members of a family of programmed cell death executioner cysteine proteases, which are called caspases[63]. The apoptosome promotes cleavage of pro-caspase 3 to its active form, activated caspase 3. Activated caspase 3 is a so-called effector caspase, which cleaves the inactive part from

caspase-activated DNAse (CAD). This is the final step in DNA degradation[63]. Among the caspases, activated caspase 3 is considered to be an important marker of ongoing apoptosis[64]. Another important pro-apoptotic activity of p53 implies the membrane death receptor induced pathway of apoptosis. Expression of at least two of the death receptors FAS/APO1 and DR5/KILLER and one of the death receptor ligands FASL, have been observed to be upregulated by p53[65,66]. Activation of death receptors by their ligands (FAS by FASL and TRIAL by DR5) results in trimerization and recruitment of intracellular adaptor molecules which initiate the caspase cleavage cascade and apoptosis. Moreover, p53 can also promote apoptosis by the negative regulation of the integrin-associated survival signaling[67,68]. A differential role in p53-mediated apoptosis is played by the transcription factor NF-κB. Its positive effects have been demonstrated[69], but it is also known as a mediator for expressing survival genes[70].
The function of p53 in the control of cell cycle arrest appears to be primarily mediated by genes, dominated by $p21^{waf1/cip1}$, and will be discussed below. p53 can also trigger growth arrest in a p21-independent way, by the preventing the activation of the cyclin-dependent kinase (CDK)2/cyclin A kinase, which is required for G1/S transition[71]. Another p21-independent, p53 mediated growth arrest is the induction of 14-3-3s, and to some extent that of the GADD45 gene[43,72], which both lead to a G2 arrest[43].

$p21^{cip1/waf1}$ in cell cycle control

The $p21^{cip1/waf1}$ protein has been shown to play an essential function in mediating G1 arrest in response to DNA damage as well as in blocking the re-entry of G2 cells into S phase. $p21^{cip1/waf1}$ transcription is usually p53-dependent, but p53-independent upregulation of $p21^{cip1/waf1}$ has also been reported[73]. $p21^{cip1/waf1}$ levels are also regulated post-transcriptionally. $p21^{cip1/waf1}$ is subject to proteosome-dependent degradation. This degradation can be prevented by interactions with proteins, that can bind to the c-terminal domain of $p21^{cip1/waf1}$, i.e., the binding site of the proteasome[74]. There are different proteins with this binding capability such as PCNA, cyclins or MAPKs[73,74]. Increased $p21^{cip1/waf1}$ expression is not necessarily linked to growth arrest, as it also occurs when cells are growth-stimulated, e.g., after growth factor exposure[75]. These apparently conflicting findings can be reconciled by the fact that $p21^{cip1/waf1}$ plays a dual function as both an inhibitor of cyclin/ CDK activity, and a positive modulator of cyclin/ CDK complex formation and nuclear localization. During physiological mitogenic stimulation, expression of D-type cyclins is induced and gives rise to the formation of cyclin D/ CDK4 and cyclin D/ CDK6 complexes (Fig. 4). These complexes are phosphorylated by cyclin activating kinase (CAK) and become components from a tetrameric complex which consists of the cyclin D, CDK4/6, $p21^{cip1/waf1}$ and PCNA. The active cyclin D complex phosphorylates the retinoblastoma protein (Rb). Rb is subsequently double phosphorylated by the cyclin E/ CDK2/ $p21^{cip1/waf1}$ / PCNA complex, which on its turn has been phosphorylated by CAK. Once Rb has become hyperphosphorylated, the transcription factor E2F1 is released from inhibition by Rb and the expression of genes required for the S phase is induced[73].

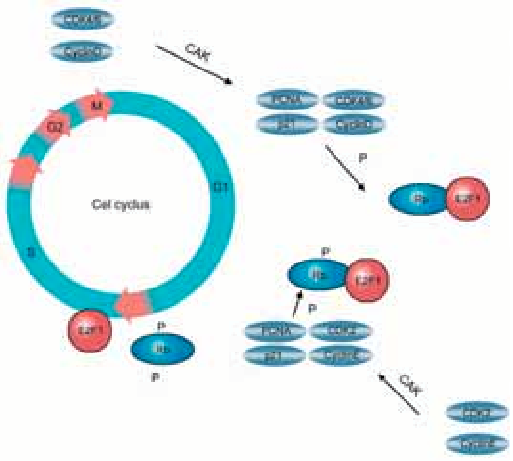

Figure 4.
The cell cycle and the involvement of different protein complexes in its progression. When expression of D-type cyclins is induced, the formation of cyclin D/ CDK4 and cyclin D/ CDK6 complexes is initiated. After phosphorylation of these complexes by cyclin activating kinase (CAK) they form a tetrameric complex with $p21^{cip1/waf1}$ and PCNA. This complex phosphorylates the retinoblastoma protein (Rb). Rb is subsequently double phosphorylated by the cyclin E/ CDK2/ $p21^{cip1/waf1}$/ PCNA complex. This complex has also been formed after phosphorylation by CAK. Once Rb has become hyperphosphorylated, the transcription factor E2F1 is released from inhibition by Rb and the expression of genes required for the S phase is induced.

Thus, at low levels, $p21^{cip1/waf1}$ functions as an assembly factor for cyclin D/ CDK4/ 6/ PCNA complexes.
The induction of D-type cyclins in early G1 recruits $p21^{cip1/waf1}$ into these active complexes. By insertion into this complex, free $p21^{cip1/waf1}$ is prevented from its inhibitory effects on cyclin E/ CDK2/ PCNA, which promotes progression through G1[73]. Consistent with its requirement for progression through G1, $p21^{cip1/waf1}$ expression level is very low in quiescent cells. High and sustained levels of $p21^{cip1/waf1}$, however, inhibit the activity of all CDKs, especially the cyclin E/ CDK2 complexes. In contrast to cyclin E/ CDK2, $p21^{cip1/waf1}$ is unable to inhibit cyclin E/ CDK4 at equimolar amounts and may require higher stoichiometric amounts to achieve inhibition[73]. The growth arresting activity of $p21^{cip1/waf1}$ is consistent with its induction during cell differentiation, in growth arrest by TGFβ and in senescent cells.
The amino-terminal domain of $p21^{cip1/waf1}$ is both necessary and sufficient to inhibit cyclin/ CDK activity in vitro and in vivo. The unique carboxy-terminal domain of $p21^{Cip1/waf1}$ associates with the proliferating nuclear antigen (PCNA), a subunit of δ DNA polymerase. Besides involvement in the quaternary complex of cyclin/ CDK/ $p21^{cip1/waf1}$ and PCNA, $p21^{cip1/waf1}$ has also been shown to form a binary complex with PCNA. This binary $p21^{cip1/waf1}$-PCNA complex is capable to regulate DNA replication directly, without affecting DNA repair. The association of the carboxy-terminal domain of $p21^{cip1/waf1}$ with the proliferating cell nuclear antigen (PCNA) competes with the interaction of the PCNA-δ DNA polymerase complex with DNA- metabolizing enzymes, like (DNA cytosine-5) methyltransferase (MCMT) and FEN1(Flap endonuclease 1)[74]. $p21^{cip1/waf1}$ is involved in different anti-apoptotic processes. Inhibition by $p21^{cip1/waf1}$

of CDKs have been reported to be a necessary step to prevent apoptosis, either upstream or downstream of caspase activation[74] The possibility that the anti-apoptotic effects of $p21^{cip1/waf1}$ are due to cyclin/ CDK inhibition is supported by the fact that similar effects can be observed with expression of CDK dominant negative mutants. However, a second mechanism by which $p21^{cip1/waf1}$ could protect cells from apoptosis is through its effects on molecules more specifically involved in the apoptotic process, such as caspases 8 and -10, caspase 3, SAPKs or MEKKs (ASK1)[74]. Thus, the induction of $p21^{cip1/waf1}$ in response to apoptotic stimuli arrests cell cycle progression via the inhibition of CDKs, but may also help to prevent apoptosis.
The $p21^{cip1/waf1}$ protein has also been shown to have a role in irreversible cell cycle arrest: senescence, which may result from critically shortened telomeres[76]. Some of these functions are at least partially exerted through activation of the p53 transcription factor. However, senescence and p21 transcriptional induction also occur in p53-defective HaCaT (immortalized keratinocyte) cells[77].
Besides the functions of $p21^{cip1/waf1}$ in cell cycle control, apoptosis and senescence, this molecule plays an unexpectedly complex role in differentiation. Different studies in which cellular differentiation has been induced e.g., by increasing cellular calcium levels, or by interruption of cell-extracellular matrix contacts or by 12-o-tetradecanoylophorbol-13-acetate (TPA application) have shown a concomitant increase in $p21^{cip1/waf1}$ expression[78,79]. However, $p21^{cip1/waf1}$ null mice develop normally[80], which may indicate that other proteins such as p27, may complement $p21^{cip1/waf1}$ function.
Recently, it has been reported that $p21^{cip1/waf1}$ plays a key role in restricting the number of keratinocyte stem cell populations, as well as further downstream, determining the irreversibility of stem cell differentiation[81]. The interconnection of $p21^{cip1/waf1}$ and the Notch family of cell surface receptors, inserts the $p21^{cip1/waf1}$ protein in the stem cell/ transit amplifying cell regulatory mechanism[82].
Additionally, endogenous Notch1 activity is required for induction of $p21^{cip1/waf1}$ expression in differentiation. In fact, in mouse keratinocytes, the p21 gene is a direct transcriptional target of Notch1 activation.
Altogether, $p21^{cip1/waf1}$ can considered to be a mediator in the transition between growth and growth arrest. This may imply that $p21^{cip1/waf1}$ is likely to play an essential role in restricting the number of keratinocytes with high growth potential.

A central question of differential signaling in a given system, is whether multiple substrates can be activated simultaneously. Thus, several downstream signaling pathways may function in parallel, perhaps acting as a multipathway signaling unit. The proteins and protein signaling pathways described in this thesis may also be subjected to such complex interactions.

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