CHAPTER 1

General Introduction

CONTENTS

Signal transduction

In a multicellular organism a proper balance needs to be established between proliferation, differentiation and cell death in order to maintain tissue homeostasis. To achieve this, a signaling network has evolved by which cells can communicate. To allow communication between cells, in addition to cell-cell contact, mechanisms have evolved that include secretion of growth factors and cytokines which can bind to their cognate receptor on the outside of target cells. Ligand-receptor interaction triggers a cascade of intracellular events, which can ultimately lead to a plethora of responses, including survival or apoptosis (programmed cell death), proliferation, differentiation and modulation of activities of differentiated cells. This cascade of events, which is triggered upon ligand-receptor interaction, is termed signal transduction.

Signal transduction is characterized by the coordinate action of kinases and phosphatases, as well as the release of second messengers. This can result in posttranslational modification, as well as relocalization of proteins, thus altering their activity. The importance for regulation of proliferation, differentiation and apoptosis is illustrated in that dysregulation of this balance is associated with cancer, chronic inflammatory diseases and degenerative diseases as a result of inappropriate cell death. To understand the mechanisms underlying these diseases, ultimately allowing clinical interference, it is important to study signal transduction and how its dysregulation is associated with disease. In this thesis, research investigating mechanisms underlying proliferation and survival by cytokines in inflammatory cells is described. We focused on cytokines of the IL-3, IL-5 and GM-CSF family, which play a crucial role in proliferation, survival and differentiation of cells of the myeloid lineage[1].

The IL-3, IL-5 and GM-CSF family: structure and function

Cytokines of the interleukin (IL)-3, IL-5 and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) are secreted amongst others by activated T cells and mast cells and promote survival, proliferation and differentiation of cells of the hematopoietic lineages. The receptors for IL-3 and GM-CSF are expressed on a broad range of hematopoietic cells, whereas the expression of the IL-5 receptor is more restricted, being present on eosinophils and basophils in man, as well as on B cells in mice[2,3].

Receptors for IL-3, IL-5 and GM-CSF consist of a cytokine-specific alpha chain, IL-3α, IL-5 α and GM-CSFα, glycoproteins of 60-80 kD, and a common beta chain (βc), a 120 kD glycoprotein, which is shared by these receptors. The βc does not bind cytokine itself, but forms a high-affinity receptor with the alpha subunit upon ligand binding[4]. Receptors for IL-3, IL-5 and GM-CSF belong to the cytokine receptor superfamily, which consists of a variety of receptors that are structurally and functionally related[2]. The extracellular domain of the α chains and βc contains structural motifs that are conserved throughout this superfamily. These motifs consist of 4 cysteines and a WSXWS repeat, both of which are involved in ligand binding (Fig. 1). The intracellular domain of α subunits is short (~50 amino acids) and contains a proline-rich motif. In contrast, the intra-

cellular domain of βc is relatively large (436 aa in man and 432 aa in mouse), containing structurally conserved membrane-proximal regions, known as "box 1" and "box 2", and six tyrosine residues in the membrane-distal region. Whereas in man all α subunits bind to βc, in mice an additional β subunit is present, βIL-3, that binds exclusively to IL-3Rα [5]. βIL-3 is highly homologous to βc and has probably arisen by gene duplication[6]. Analysis in knockout mice, however, has revealed no additional function of βIL-3 compared to βc[7]. Six tyrosine residues in βc are conserved from mouse to man, although the murine βc has an additional tyrosine, of which the relevance for signal transduction remains to be established. Ligand binding results in receptor dimerization, triggering subsequent signaling events, transduced through the βc. Although some signal transducing activity has been attributed to the alpha subunit, in particular the proline-rich region, there are conflicting data as to whether this contributes to cell proliferation or survival[8-12]. Given their cytokine-specificity, the alpha subunits might be involved in mediating differentiation of target cells, although this is still largely unconfirmed.

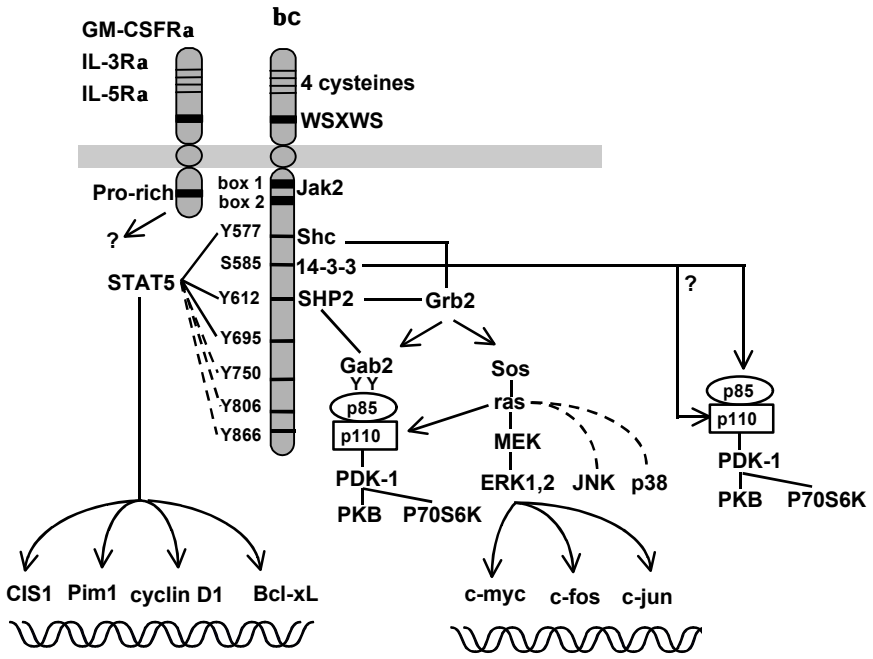

Figure 1. Activation of signaling pathways by the IL-3, IL-5 and GM-CSF receptor family. Binding of IL-3, IL-5 and GM-CSF to their cytokine-specific alpha chain triggers heterodimerization with the βc, resulting in activation of JAK2. JAK2 then phosphorylates tyrosine residues in the βc, which recruits binding of cytoplasmic signaling proteins. These proteins either directly promote transcription (STAT5) or in turn recruit other signaling proteins to the βc, resulting in the activation of multiple signaling pathways.

Intracellular signals elicited by IL-3, IL-5 and GM-CSF

Initial studies on signal transduction of IL-3, IL-5 and GM-CSF revealed that they elicit similar patterns of tyrosine phosphorylation of cytosolic proteins[13,14]. As is true for many cytokine receptors, neither the α chains nor βc possess intrinsic tyrosine kinase activity, but instead a tyrosine kinase, JAK2, is associated with the box 1 region of βc[15,16]. Receptor-ligand-induced dimerization of βc subunits promotes cross-phosphorylation and activation of JAK2, which then phosphorylates tyrosine residues in the cytoplasmic tail of βc (see Fig. 1). Activity of JAK2 is both necessary and sufficient for the initiation of signaling, since mice deficient in JAK2 fail to respond to either IL-3 or GM-CSF[17]. Moreover, constitutive activation of JAK2, by constructing a chimeric receptor (βc /JAK2), consisting of βc extracellular and transmembrane regions fused with JAK2, abrogates the need for cytokines for proliferation or survival[18]. Deletion analysis of βc revealed that signaling through the membrane proximal region induces a transient proliferative response, potentially mediated by induction of the serine threonine kinase pim1 and transcription factor c-myc[19], whereas the membrane distal region is required for cell viability[20,21].

The membrane distal region of βc also contains tyrosine residues that are phosphorylated upon the action of IL-3, IL-5 and GM-CSF[14]. These phospho-tyrosines are capable of recruiting cytoplasmic signaling molecules through their Src-homology 2 (SH2) domain. Subsequently, relocalization of these signaling molecules promotes their activation, triggering subsequent signaling events. Furthermore, the distal region of βc is also important for activation of kinases of the Src family, such as Lyn, Hck and Fyn, which may occur independently of βc tyrosine phosphorylation. Interestingly, this family of kinases has also been proposed to contribute to tyrosine phosphorylation of βc[22-24].

Activation of STAT5 through bc.

One signaling protein that can bind directly to phospho-tyrosines on the βc is the transcription factor STAT5 (Signal Transducer and Activator of Transcription). Binding of STAT5 to the βc results in its subsequent phosphorylation and dimerization that then translocate to the nucleus and promote transcription of target genes which include pim1, CIS1, Bcl-xL and cyclin D1 (reviewed in [25]; Fig. 1). There is some redundancy in βc-mediated activation of STAT5, since STAT5 can bind to multiple phosphorylated tyrosine residues, including Tyr577, Tyr612, Tyr695, and Tyr750 on the human βc[26]. Moreover, Tyr806 and Tyr866 have also been described to promote its activation, but direct binding of STAT5 to these residues has not been demonstrated[27].

Activation of the small GTPase p21ras and its intracellular targets.

Other signaling routes initiated through the distal region of the tyrosine-phosphorylated βc involve the activation of the small GTPase p21ras[28]. Downstream effectors of p21ras activation include the dual specificity kinase MEK and its target ERK (MAPK), resulting in induction of the transcription factors c-fos and c-jun[28,29]. Besides activating MEK-ERK, p21ras can also induce PI3K activity by direct binding to its p110 catalytic subunit[30]

(see below). Finally, signaling through βc can also activate two other members of the MAPK family, p38 and JNK, although their role in transducing a proliferative response and the precise mechanism of their activation still remains to be resolved[31-33].

The activation of p21ras through the βc is complex and requires intermediate proteins. Two proteins that function as adaptor proteins and can bind directly to phosphorylated tyrosine residues on βc are Shc and the protein tyrosine phosphatase SHP2 (also known as PTP-1D, PTP-1C, SH-PTP2 or Syp). Shc binds to phosphorylated Tyr577 of the βc, whereas SHP2 binds Tyr612. Both proteins become tyrosine-phosphorylated upon binding to βc[34-36]. Subsequently, the phosphorylated tyrosine residues on Shc and SHP2 serve as docking sites for the SH2 domain of another adaptor protein, Grb2[37]. In addition to its SH2 domain Grb2 possesses two SH3 domains that bind to the proline-rich region of the GTP-exchange factor Sos. Recruitment of Sos to the plasma membrane subsequently activates p21ras (Fig. 1).

Regulation of phosphatidylinositol 3-kinase activation

Another mechanism by which signals from the distal part of the βc may promote cell survival is through activation of the lipid kinase phosphatidyl inositol 3-kinase (PI3K)[21,38]. The PI3K family consists of several isoforms (reviewed in [39]), of which the classical form consists of two subunits, a p85 regulatory subunit, containing two SH2 domains, and a catalytic subunit, p110. Recruitment of p85 to the βc results in membrane targeting, allowing phosphorylation of the phosphatidylinositol (PI) lipids, phosphatidylinositol-4-phosphate (PI-4P) and phosphatidylinositol-4,5-bisphosphate PI4,5-P_2 on the D3 position (reviewed in [40]). Following this phosphorylation, activation of PI(3,4,5)-trisphosphate dependent kinase (PDK-1) is promoted upon binding to PI(3,4,5)P_3[41]. Downstream targets of PDK-1 include the serine-threonine kinase Protein Kinase B (PKB, also known as Akt)[42], and p70S6K[42-44] (see Fig. 1).

In addition to direct activation by p21ras[30], there are several alternative mechanisms by which PI3K can be activated through βc, which again involve intermediate adaptor proteins. The mechanism of cytokine-mediated PI3K activation has been elusive until the recent discovery of involvement of the adaptor protein p97/ Gab2[45]. Gab2 can be recruited to the βc through association with either tyrosine-phosphorylated SHP2 or Grb2. Following recruitment to βc Gab2 is itself tyrosine-phosphorylated and these tyrosines serve as docking sites for the p85 subunit of PI3K[46] (Fig 1). Interestingly, serine phosphorylation of the βc has also been reported to be critical for PI3K activation[47]. Recently, IL-3 stimulation was demonstrated to result in the phosphorylation of Ser585 in the human βc, and site-directed mutagenesis of this residue specifically impaired the PI3K signaling with a concomitant reduction in cell survival[48]. Ser585 is conserved from mouse to man, and is located in a consensus phosphorylation site for PKA, serving as a docking site for 14-3-3 proteins. 14-3-3 proteins are regulatory molecules that can bind various signaling molecules and might thus serve as adaptor proteins (reviewed in [49]). Binding of 14-3-3 to this site has been suggested to recruit the p85 subunit of PI3K,

either directly or via an intermediate, promoting PI3K activation[47]. This suggests that there are multiple signaling pathways that could contribute to the proliferative and anti-apoptotic signals through βc.

Turning off the signal

Phosphatases

Following IL-3, IL-5 or GM-CSF binding, a transient increase in tyrosine phosphorylation of βc and intracellular proteins is observed. Addition of phosphatase inhibitors both elevates and extends the duration of this tyrosine phosphorylation, resulting in enhanced proliferation and survival[13,14]. This suggests that the action of phosphatases is necessary to regulate a transient signal, and that dysregulation of these phosphatases might be linked to aberrant proliferation or survival. Several phosphatases have been implicated in IL-3, IL-5 or GM-CSF-induced signaling. These include SHP1 (also referred to as PTP-1C, HCP or SH-PTP1), SHP2, SHIP (SH2-containing inositol 5-phosphatase)[36,50,51] and PTEN (Phosphatase and Tensin homolog). SHP1 binds to βc, presumably to Tyr-612, to which it might compete for binding with SHP2[36]. Overexpression of SHP1 decreased IL-3-induced proliferation and was effective in dephosphorylating the βc in vitro[50], suggesting that SHP1 can directly abrogate recruitment of signaling molecules to βc. Little is known regarding the functional significance of SHP2 phosphatase activity in βc-mediated signaling, although it has been proposed to dephosphorylate Gab2[45] and demonstrated to dephosphorylate STAT5 *in vitro*[52]. Another phosphatase involved in inhibiting βc-generated signals is SHIP, an inositol 5' phosphatase that hydrolyzes the PI3K product $PI(3,4,5)P_3$ to $PI(3,4)P_2$[53,54], making it a direct modulator of downstream signaling of PI3K. Indeed, SHIP is important for inhibiting activation of PKB, since SHIP*(-/-)* mice exhibited a prolonged PI3K-dependent $PI(3,4,5)P_3$ accumulation and PKB activation in response to IL-3 and GM-CSF[55]. Moreover, as SHIP binds to tyrosine-phosphorylated SHP2[56] and Shc[57,58], SHIP might interfere with the propagation of βc signals by competing with Grb2 and Gab2 for binding. SHIP-deficient mice exhibit a chronic hyperplasia of myeloid cells which are more resistant to apoptosis[55], suggesting a role for this phosphatase in oncogenesis. The lipid phosphatase PTEN can directly counteract PI3K action by dephosphorylating 3-phosphorylated phosphoinositides on the D3 position[59]. Besides the recent observation that phosphorylation of PTEN at the C-terminus increases its stability to proteasome-mediated degradation[60,61], little is known regarding the regulation of its phosphatase activity. The importance of PTEN in downregulating signals is illustrated in *PTEN (+/-)* mice. These mice were investigated instead of *PTEN (-/-)* mice deletion of both *PTEN* alleles caused early embryonic lethality. *PTEN (+/-)* mice developed a lethal autoimmune disorder accompanied by decreased apoptosis and increased proliferation upon activation in T lymphocytes from these mice[62]. Furthermore, loss of PTEN activity resulting in an elevation of PKB activity has been observed in tumor cells. Reintroduction of PTEN in these cells reduced PKB activity[63] and abrogated their tumorigenicity[64,65].

SOCS proteins

Another mechanism through which cytokine-induced signaling can be abrogated is through downregulating its own signal. The signals that are generated upon cytokine binding to its receptor can promote transcriptional upregulation of proteins that inhibit the further transduction of signals, thus providing a cytokine signaling-induced feedback loop. One family of proteins involved in "turning off" cytokine-induced signals is the SOCS/CIS/SSI (Suppressor Of Cytokine Signaling/ Cytokine Induced SH2 containing protein/ STAT-induced STAT Inhibitor) family (reviewed in [66]). All members of the SOCS/CIS/SSI family contain a conserved domain at their C-terminus, called the SOCS-box, which interacts with components of the ubiquitin system. Thus, SOCS proteins may act as adaptor molecules that target activated cell signaling proteins to the protein degradation pathway[67]. STAT5 transcriptionally upregulates CIS1, which can bind to the tyrosine-phosphorylated βc and thereby reduce the IL-3-induced growth rate upon overexpression[68]. Higher levels of CIS1 inhibit STAT5 activity possibly through "shielding" the βc tyrosines or alternatively, ubiquitin-mediated degradation of the βc by the proteasome[69]. This mechanism of CIS1-induced abrogation of cytokine-induced signaling has been suggested for the erythropoietin receptor[70]. With respect to this it is interesting that treatment of cells with proteasome inhibitors prolonged IL-3-induced activation of JAK/STAT pathway, as well as ERK phosphorylation[71]. Two other SOCS-family members, JAB (Jak Binding protein, also known as SOCS-1 or SSI-1) and SOCS-3 (or Ssi-3, CIS3 OR JAB2) directly bind to JAK2 tyrosine kinase domain, and inhibit its kinase activity[72,73].

A further cytokine-induced inhibitor of proliferation is p56(dok-2), which inhibits phosporylation of Shc through an as yet unidentified mechanism[74]. Altogether, cytokine receptors possess a variety of means to "turn off" cytokine-induced signaling, illustrated in Fig. 2. As discussed above, deletion or mutation of some of these proteins, has recently been demonstrated to play a role in oncogenesis.

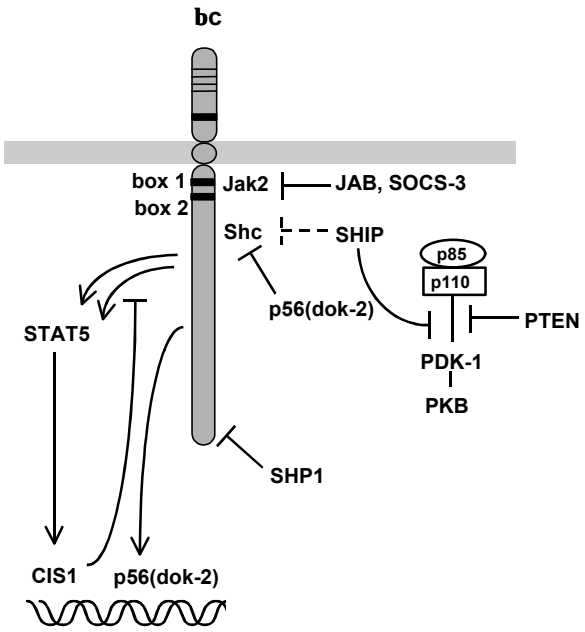

Figure 2. Turning off IL-3, IL-5 and GM-CSF-induced signals. Signals elicited through the βc can be abrogated through the action of phosphatases, SHP1, SHP2, SHIP and PTEN. Alternatively, signal transduction through the βc also promotes transcription of inhibitors, such as CIS or p56(dok-2) that interfere with βc-generated signals.

Mechanisms of growth factor and cytokine-induced proliferation

To get a complete picture as to how cytokines and the signaling pathways that they activate regulate proliferation, one needs to understand the complex process of cell division. Growth factors and cytokines regulate cell proliferation through a network of signaling cascades, regulating assembly and activation of components of the cell cycle machinery, which are described below.

Progression through the cell cycle

The events occurring in the eukaryotic cell cycle include duplication and condensation of the chromosomes, their migration into a spindle network and segregation into two daughter cells (reviewed in [75,76]). With respect to these events, the cell cycle can be divided into four phases: an S (synthesis) phase, in which the chromosomes are duplicated, and M phase (mitosis) separated by two intervals, G_1 (gap 1) the interval between M and S and

G_2, the interval between S and M (Fig. 3). In addition to these phases there is also G_0, representing the quiescent state of cells that are not cycling, which include terminally differentiated cells. Before initiating the cell cycle, controlling systems ensure that the cell only divides when it has attained the proper size and only when it receives positive signals from its environment. In addition, there are regulatory mechanisms to ensure correct chromosome replication and proper alignment before undergoing mitosis. These controlling systems include the so-called "checkpoints", present in G_1 (R, for Restriction point), or G_2 and M (also called spindle formation checkpoint). In this section, focus is on R as passage through this checkpoint is an important means by which growth factors and cytokines are able to override the intracellular controls that block cell cycle progression.

The sequential events of G_1-S-G_2-M are coordinated by so-called "cyclins" and their associated catalytic subunit cyclin-dependent kinases (CDKs). The regulated expression of cyclins and their association with distinct CDKs varies through each phase of the cell cycle (Fig. 3), resulting in an altered CDK substrate specificity[77]. The distinct substrates that are phosphorylated during the different phases of the cell cycle promote the specific events associated with each phase. The cyclin/CDK complexes that are present in G_1 and promote the progression through G_1 and the G_1-S transition are the D-type cyclins, associated with CDK4 and CDK6, and the E-type cyclins, associating with CDK2. The induction of S-phase by cyclin D(1,2,3)/CDK(4,6) and cyclin E/CDK2 is largely dependent on their ability to phosphorylate members of the Rb (retinoblastoma) family, consisting of Rb, p107 and p130[78] (reviewed in [79]). Rb proteins associate with E2F transcription factors and hereby inhibit their activity. Phosphorylation of Rb proteins by G_1 cyclin/CDK complexes results in the release of E2F, allowing transcription of its targets, including cyclin E and cyclin A[80,81], the predominant cyclin in S phase. This allows further progression through G_1 and initiation of S phase (see Fig. 3).

The cyclin-dependent Kinase Inhibitors (CKIs)

In addition to the Rb family, there is another class of proteins that negatively regulates G_1 progression, the Cyclin-dependent Kinase Inhibitors (CKIs). These associate to, and thus inhibit, the activity of cyclin/CDK complexes. CKIs can be divided into two classes, one being the p16/INK4 family, inhibitors of D-type cyclin/CDK4,6, and members of the $p21^{CIP1}$, $p21^{KIP1}$, $p57^{KIP2}$ family (for review, see [82]). The p16/INK4 family will not be discussed further here. The $p21^{CIP1}$, $p27^{KIP1}$, $p57^{KIP2}$ family can inhibit cyclin D, as well as cyclin E- associated kinase activities. When present at lower concentrations however, $p21^{CIP}$ and $p21^{KIP1}$ are also involved in the assembly of CDK/ cyclin complexes, without actually inhibiting their activity[83]. $p21^{CIP1}$ promotes DNA-damage induced cell cycle arrest, when its levels are transcriptionally elevated in a p53-dependent manner (reviewed in [84]). In response to growth factors, levels of $p27^{KIP1}$ dramatically decrease[85-87], which appears to be a critical mechanism by which growth factors are capable of inducing cell cycle progression. Moreover, antisense inhibition of $p27^{KIP1}$ expression pre-

vents cell cycle arrest in response to growth factor depletion[85]. This suggests that upregulation of $p27^{KIP1}$ might be a key mechanism by which cell cycle arrest is induced in response to growth factor deprivation. Interestingly, decreased levels of $p27^{KIP1}$ have also been described to be associated with a poor prognosis in cancer[88,89]. An important means by which expression of $p27^{KIP1}$ is regulated is via posttranslational mechanisms; phosphorylation on Thr-187 results in subsequent targeting for degradation by the ubiquitin system[85,90,91]. This phosphorylation is mediated by cyclin E/CDK2[91,92], providing a negative feedback control through cell cycle progression (Fig. 3).

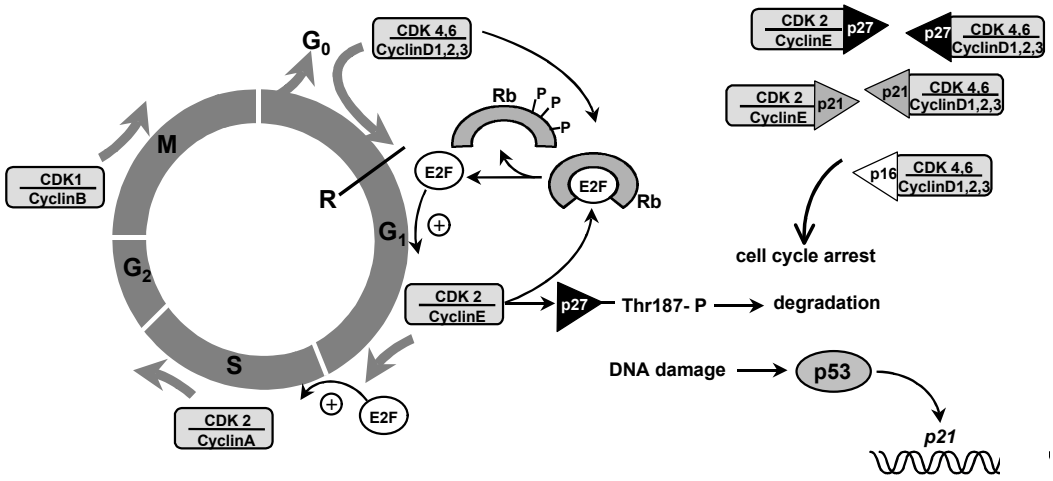

Figure 3. Regulation of the cell cycle. CDK/cyclin complexes promote progression through the different phases of the cell cycle, G_1-S-G_2-M. The initial onset of the cell cycle requires phosphorylation of Rb, thus allowing transcription factor E2F to promote transcription of components of the cell cycle machinery. Activity CDK/cyclin complexes can be inhibited by CKIs. The regulation of their expression includes transcriptional upregulation (p21) or posttranslational modification (p27). Adapted from [1].

Molecular mechanisms regulating the onset of proliferation

Given that cytokines and growth factors can promote proliferation, considerable effort has been invested in elucidating how and which signal transduction pathways are involved in this process. Signaling pathways promoting proliferation generally act in concert and one should keep in mind that there is considerable crosstalk between those pathways. However, it is possible to determine what the relative contribution of each pathway is to inducing proliferation. For this purpose, either pharmacological inhibitors or overexpression of active or dominant-negative forms of components of a signaling pathway are utilized.

Growth factor and cytokine-induced signaling rapidly induces transcription of so-called immediate-early genes, such as c-fos, c-jun and c-myc, all of which have been associated with the induction of a proliferative response[29,93]. One of the earliest events in growth factor-induced proliferation is the synthesis of cyclin D. Higher levels of cyclin D are able to override the inhibitory effects of CKIs, resulting in the activation of associated CDK4,6, cyclin E/CDK2, and the onset of proliferation (reviewed in [79]). Upregulation of cyclin D is cannot be attributed to one specific signaling pathway, since elevation of cyclin D1 has been associated with STAT5[94], p21ras[95], MEK [95,96], as well as PI3K activity[97]. PI3K-promoted entry into S-phase may be mediated through its downstream target p70S6K, which has been demonstrated to be essential for G_1 progression and elevation of cyclin D1[98,99]. The importance for PI3K in proliferation is demonstrated by the observation that proliferation is impaired in B cells that were derived from mice deficient for the p85 subunit of PI3K[100]. PKB has also been implicated in promoting cyclin D expression by enhancing its translation[101], possibly through promoting the phosphorylation and subsequent inhibition of a repressor of mRNA translation, 4E-BP1[102].

Elevation of cyclin D levels might also be linked to the transcription factor c-myc, as growth factor stimulation increases levels of c-myc through transcriptional and posttranscriptional mechanisms involving p21ras[103,104]. Moreover, c-myc has been demonstrated to rapidly induce cyclin E/ CDK2 kinase activity through promoting sequestration of $p27^{KIP1}$ via elevation of cyclin D levels[105,106].

Mechanisms of growth factor and cytokine-induced rescue from apoptosis

Cellular proliferation induced by growth factors or cytokines can only occur in the presence of distinct survival signals. Cells receiving proliferative signals, in the absence of survival signals do not proliferate, but rather die, by a process termed apoptosis. One example illustrating this is that ectopic expression of c-myc alone, which can act as an inducer of proliferation, drives cells into apoptosis in the absence of p21ras and PI3K activity[107-109]. In this way apoptosis may be an important mechanism for the elimination of cells harboring mutations in cell cycle regulatory proteins, which may otherwise create an imbalance in normal cell proliferation. Thus, oncogenesis is promoted by aberrant proliferative signals together with deregulated apoptosis, allowing uncontrolled cell division (reviewed in [110,111]). Programmed cell death is also required during the normal development of a multicellular organism, in the turnover and renewal of cells in the adult body and in the elimination of cells that do not or inappropriately respond to signals from their intracellular environment.

Characteristics of apoptotic cell death

Apoptosis is a process characterized by morphologically distinct features including blebbing and shrinking of the nucleus and the cytoplasm, loss of mitochondrial transmembrane potential, cleavage of intracellular proteins and degradation of chromosomal DNA. DNA is cleaved into fragments of distinct size, called "DNA laddering", a hallmark often used to characterize apoptotic cells[112]. Furthermore, the lipid content on the outside of apoptotic cells changes, resulting in an increase in phosphatidylserine, which can be recognized by macrophages, which phagocytose dying cells. These events are distinct from necrosis, cell death caused by injury, resulting in swelling and bursting of the cell and random degradation of chromosomal DNA.

The specific cleavage of proteins and induction of DNA laddering in cells undergoing apoptosis is carried out by cysteine proteases or "caspases" that cleave specific sites, carboxy-terminal to an aspartate residue (reviewed in [113]. Caspase activity also results in the activation of CAD (caspase-activated deoxyribonuclease), which is responsible for chromosomal DNA degradation during apoptosis[114,115]. Caspases exist as inactive pro-enzymes in the cell and possess an N-terminal prodomain, containing protein-interaction motifs, together with large and small subunits. The prodomain generally targets the caspase for activation, which occurs through proteolytic cleavage, resulting in the release of the prodomain and rearrangement of the large and small subunits to form an active caspase. Initial caspase activity in apoptosis is induced through the recruitment of caspase pro-enzymes to activator molecules, promoting pro-caspase oligomerization and auto-activation. These activated "initiator caspases" then cleave and activate downstream caspases, resulting in cleavage of apoptotic substrates (reviewed in [116,117]).

Molecular events regulating apoptosis or cell survival

An important family of proteins involved in the control of apoptosis is the Bcl-2 family (reviewed in [118]). The first member, Bcl-2, was identified due to its deregulation in B cell lymphomas bearing the t(14;18) translocation, resulting in constitutive Bcl-2 expression[119,120]. Many more Bcl-2 members have been identified and characterized since, based on homology in conserved regions, called Bcl-2 homology (BH 1-4) domains. Bcl-2 proteins are conserved though evolution, and in the nematode worm *Caenorhabditis elegans*, Bcl-2 homolog CED-9 is able to suppress apoptosis by preventing activation of the caspase, CED-3 (reviewed in [121]). Analogous CED-9 members in mammals include Bcl-2, Bcl-xL, A1 and Mcl-1[122]. Further dissection of the apoptotic pathway in *C. elegans* led to the identification of a CED-9 antagonist, EGL-1[123]. EGL-1 binds to and inactivates CED-9, and activates CED-3 through a mechanism involving CED-4, the homolog of mammalian Apaf-1, apoptosis activating factor-1 (Fig. 4).

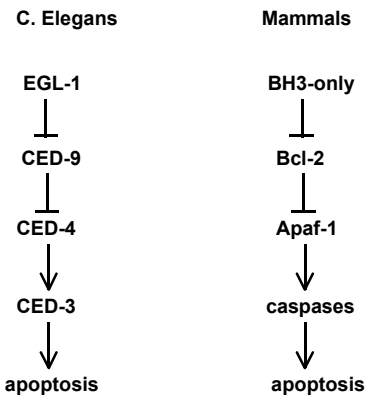

Figure 4. Evolutionary conservation of the apoptotic program. In C. elegans, as well as in mammals, induction of apoptosis requires the activation of caspases. One mechanism of activating caspases can be mediated by Apaf-1/ CED-4 and can be inhibited by the action of Bcl-2/ CED-9. Activity of BH3-only proteins/ EGL-1 can result in induction of apoptosis through the inhibition of Bcl-2/ CED-9.

Interestingly, EGL-1 also belongs to the Bcl-2 family, but of the conserved BH domains in the Bcl-2 family, it only contains the BH3 domain. EGL-1 homologs in mammals have also been identified, including Bad, Bid, Bik, Bim and Hrk (harakiri), which have been termed BH3-only proteins. In addition, mammals also contain other pro-apoptotic Bcl-2 proteins that can induce apoptosis independent of binding to Bcl-2, such as Bax[122]. Interestingly, antiapoptotic Bcl-2 family members were found to inhibit apoptosis by preventing the release from cytochrome c from mitochondria[124]. The mechanism by which cytochrome c could promote activation of caspases has been a mystery until the identification of a cofactor, Apaf-1, which can form a complex with caspase-9 in the presence cytochrome c and dATP, resulting in its activation[125,126] (Fig. 5).

Homology to bacterial toxins with pore-forming activities and experiments in vitro suggested that Bax, but also anti-apoptotic Bcl-2 members form pores in the outer membranes of mitochondria, with Bax promoting release of cytochrome c[127]. Conversely, anti-apoptotic Bcl-2 family proteins prevent mitochondrial changes associated with cytochrome c release[124,128], also through inhibition of Bax channel-forming activity[129].

Maintaining an appropriate balance between pro- and anti-apoptotic Bcl-2 family members is a crucial mechanism by which cellular homeostasis is achieved. Growth factors and cytokines have been demonstrated to be able to promote survival through transcriptional upregulation of anti-apoptotic Bcl-2 family members or inactivation of pro-apoptotic Bcl-2 proteins[130-132]. Interestingly, caspases have also been shown to cleave

Bcl-2, thus inhibiting its anti-apoptotic activity and this way provide a positive feedback loop in executing the apoptotic program[133,134]. With respect to Bcl-2 regulation in lymphocytes an important observation was made in *Bim (-/-)* knockout mice. Lymphocytes derived from these mice failed to undergo apoptosis in response to cytokine withdrawal[135]. This suggests that Bim is indispensable for hematopoietic homeostasis and that there is a unique role for Bim in promoting cytokine withdrawal-induced apoptosis. With respect to this, it is interesting that Bcl-2 deficient mice develop lymphopenia, providing genetic evidence of the importance of maintaining the balance between pro- and anti-apoptotic Bcl-2 family members[136].

Other evolutionarily conserved players in mediating survival are IAPs (Inhibitors of Apoptosis Proteins), that suppress apoptosis by directly binding to and inhibiting the activity of caspases[137], (reviewed in [138]). Although their regulation by growth factors has been suggested in endothelial cells[139], regulation in lymphocytes remains to be demonstrated.

In addition to growth factor deprivation-induced activity of pro-apoptotic members of the Bcl-2 family, there is another mechanism of initiating apoptosis, which occurs through the activation of so-called "death receptors", such as Fas (also known as CD95 or Apo-1) and results in a rapid induction of the apoptotic program. Fas-induced apoptosis has been described as an important mechanism of eradicating activated T cells (reviewed in [140,141]). Ligation of Fas with Fas ligand, FasL, recruits caspase-8 (also known as FLICE) into a multimeric complex via adaptor protein FADD, which triggers its activation (reviewed in [140]; see Fig. 5). Caspase-8 promotes induction of apoptosis though direct cleavage of apoptotic substrates, but can also activate other caspases by cleaving BH3-only protein Bid. Cleaved Bid is then recruited to the mitochondria and inhibits anti-apoptotic Bcl-2 family members, resulting in the activation of further caspases (reviewed in [140,141], also Fig. 4). Cytokines have been reported to interfere with death receptor-induced apoptosis via induction of c-FLIP (FLICE-inhibitory protein), which competes with caspase-8 in binding to FADD[142].

Finally, a third mechanism by which apoptosis can be initiated is through DNA damage. DNA damage results in activation of transcription factor p53, which can trigger apoptosis through transcriptional upregulation of Bax[143], as well as BH3-only protein Noxa[144] (see Fig. 5).

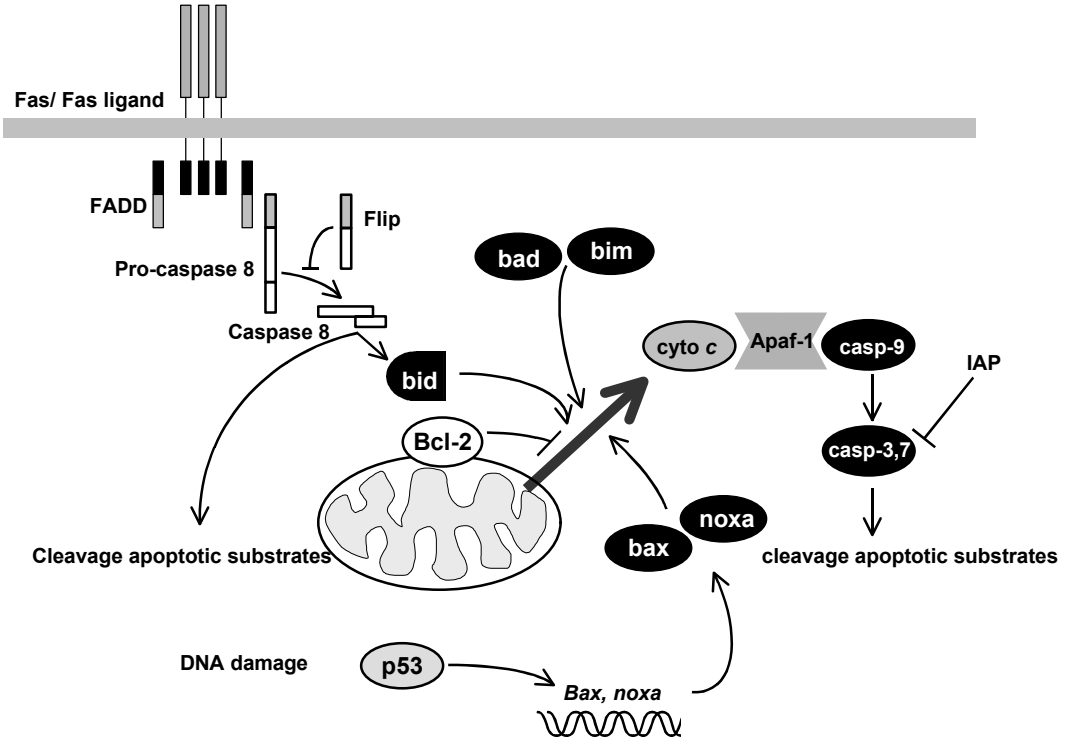

Figure 5. Regulation of apoptosis in higher eukaryotes. Induction of the apoptotic program can be induced through regulation of members of the Bcl-2 family, consisting of anti-apoptotic members, such as Bcl-2 (white) or pro-apoptotic members (black). Enhancement of the activity of the pro-apoptotic Bcl-2 family members results in the release of cytochrome c, which, together with Apaf-1, promotes caspase activation. An alternative way of activating caspases is triggered through Fas/ Fas ligand signaling, resulting in the direct activation of caspase-8. The action of caspase-8 also involves activation of other caspases via cleavage of Bid.

Signal transduction pathways regulating cell survival

As discussed, signaling through growth factors and cytokines can trigger proliferation, which requires simultaneous regulation of components of the cell cycle machinery, as well as inhibiting apoptosis. A predominant mechanism of promoting survival is through enhancing the anti-apoptotic and inhibiting the pro-apoptotic activity of the Bcl-2 family members.

A direct requirement for STAT5-induced Bcl-xL in anti-apoptotic signaling[145] through the EpoR was confirmed in STAT5 deficient mice which had low numbers of erythroid progenitors, showing higher levels of apoptosis[146]. Another anti-apoptotic tar-

get of STAT5 is the serine-threonine kinase pim1[147], which may regulate the expression or activity of pro- and anti-apoptotic members of the bcl-2 family, although this still remains to be demonstrated[148].

Utilizing ectopic expression of an oncogenic p21ras mutant, it was demonstrated that p21ras could also contribute to rescue from apoptosis through upregulation of Bcl-2 and Bcl-xL[149]. This might be mediated by its effector ERK, which can phosphorylate Bcl-2 and increase its anti-apoptotic potential[150,151], possibly by making it more resistant for degradation[152]. Furthermore, p21ras activity has also been linked to the transcription factor Aiolos, described to induce Bcl-2 in T cells[153], and NFIL3 (E4BP4), which can rescue cells from apoptosis[154], although its transcriptional targets remain to be identified.

One protein that has recently received considerable attention as a suppressor of apoptosis is the PI3K effector PKB. Thus far, several mechanisms have been demonstrated by which this serine-threonine kinase mediates cell survival. First, PKB activity can affect the balance of the Bcl-2 family by transcriptionally upregulating anti-apoptotic Bcl-2 family member Mcl-1[155], which is an essential component of the survival response conferred by GM-CSF[156]. Furthermore, phosphorylation of BH3-only protein Bad by PKB inhibits its activity[42,157], however, whether this is a critical target for PKB-mediated rescue from apoptosis is still debatable[158]. Inhibitory phosphorylation of caspase-9 by PKB might also be a way of inhibiting apoptosis in some but not all systems[159,160]. Recently, PKB activity has also been linked to inhibition of death receptor-induced apoptosis through the increase of c-FLIP expression[161]. The importance of PKB in attenuating death receptor-induced apoptosis is supported by the observation that in *PTEN (+/-)* mice Fas-induced apoptosis was impaired[62].

Given that STAT5, p21ras and also PI3K regulate transcription of genes involved in proliferation, as well as rescue from apoptosis, it is no surprise that these signaling molecules or their downstream targets may mediate cellular transformation. Although there is little evidence for a direct oncogenic potential of STAT5, it has been described to contribute to oncogenesis[162,163]. p21ras was initially identified as a molecule that was mutated in certain tumors, including hematological abnormalities, resulting in its constitutive activation, and is one of the most common contributors to induction of malignancies (reviewed in [164]). Increased PI3K activity has also been proposed in lymphoproliferative disorders[165], and inactivation of its inhibitory phosphatase PTEN has been extensively reported in cancer[166,167].

Scope of this thesis

Signal transduction in response to growth factors or cytokines enables cells to appropriately respond to their environment and thus establish a balance between cell proliferation, differentiation and apoptosis in order to maintain homeostasis. Dysregulation of this balance is often correlated with changes in activity of components of the signaling work, which can result in malignancies or inflammation. This study focuses on the mechanisms by which IL-3, IL-5 and GM-CSF mediate proliferation and survival of hematopoietic cells. Utilizing pharmacological inhibitors of signal transduction pathways, we analyzed which signaling pathways are indispensable for cytokine-mediated proliferation and survival. We identified PI3K as the crucial target of IL-3, IL-5 and GM-CSF in mediating proliferation and survival and PKB as the PI3K target involved in mediating the PI3K-induced survival signal. Therefore, we analyzed activation of the PI3K-PKB pathway in response to IL-3, IL-5 and GM-CSF and how this pathway can promote survival and proliferation.

In chapter 2, we analyzed which tyrosine residues of the βc are important for the activation of PKB. Next, we set out to identify novel targets by which PI3K might promote proliferation and survival. This has resulted in the identification of Forkhead transcription factor FKHR-L1, a novel inhibitory target of PI3K-PKB signaling through the βc, whose activity promotes apoptosis, as well as cell cycle arrest (Chapter 3). Upon analyzing how transcriptional activity of FKHR-L1 could result in these events, two FKHR-L1 targets were found (Chapter 3 and 4). Next, we analyzed by which intracellular mechanisms FKHR-L1 could promote apoptosis (Chapter 5). In addition, we provide evidence that cytokine withdrawal-mediated induction of apoptosis is mediated by FKHR-L1. Finally, to identify additional FKHR-L1 targets, we compared gene array filters derived from cells in which FKHR-L1 activity was present or absent (Chapter 6).

Altogether, our findings provide a novel mechanism by which PI3K-PKB can induce rescue from apoptosis, as well as promote induction of proliferation.

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