
**cAMP Signalling in Modification of DNA Damage
Responses in Lymphoid Cells – Implications for
Development and Treatment of BCP-ALL**

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2 LIST OF PAPERS INCLUDED

- I. **Naderi EH, Findley HW, Ruud E, Blomhoff HK, Naderi S.** Activation of cAMP signaling inhibits DNA damage-induced apoptosis in BCP-ALL cells through abrogation of p53 accumulation. *Blood*. 2009 Jul 16;114(3):608-18.
- II. **Naderi EH, Naderi S, Jochemsen AG, Blomhoff HK.** Activation of cAMP signalling interferes with stress-induced p53 accumulation in ALL-derived cells by promoting the interaction between p53 and HDM2. Submitted for publication.
- III. **Kloster MM, Hafte TT, Moltzau LR, Naderi EH, Dahle MK, Skalhegg BS, Gaudernack G, Levy FO, Naderi S, Blomhoff HK.** EBV infection renders B cells resistant to growth inhibition via adenylyl cyclase. *Cell Signal*. 2008 Jun;20(6):1169-78.
- IV. **Naderi EH, Ugland HK, Diep PP, Josefsen D, Sandnes D, Myklebost O, Ruud E, Naderi S, Blomhoff HK.** Selective inhibition of cell death in malignant versus normal B cell precursors: Implications for cAMP in development and treatment of BCP-ALL. Manuscript.

3 ABBREVIATIONS

4-OOH-CP	4-hydroperoxycyclophosphamide
9-1-1 complex	Rad9-Rad1-Hus1 complex
AC	adenylyl cyclase
AKAP	A-kinase anchoring protein
ALL	acute lymphoblastic lymphoma
ALT	alternative lengthening of telomeres
AML	acute myeloid leukaemia
APAF-1	apoptotic protease activating factor-1
ARF-BP1/Mule	ARF-binding protein 1/MCL-1 ubiquitin ligase E3
ASPP	ankyrin-repeat-, SH3-domain- and proline-rich-region-containing proteins
A-T	ataxia-telangiectasia
ATF-1	activating transcription factor-1
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia and Rad3 related
ATRIP	ATR interacting protein
β -AR	β -adrenergic receptor
BCL-2	B cell leukaemia/lymphoma 2
B-CLL	B cell chronic lymphocytic leukaemia
BCP	B cell precursor
BCR	B cell receptor
BH	BCL-2 homology
BL	Burkitt lymphoma
BLM1	Bloom syndrome protein 1
BM	bone marrow
BMME	bone marrow microenvironment
BRCA1	breast cancer associated gene 1
bZIP	basic region leucine zipper
c-ABL	cellular Abelson murine leukemia
<i>c-myc</i>	cellular myelocytomatosis oncogene
C subunit	catalytic subunit
CAK	CDK-activating kinase
cAMP	3'-5'-cyclic adenosine monophosphate
CARD	caspase recruitment domain
Caspase	cysteine-aspartic protease
CBD	cAMP binding domain
CBP	CREB binding protein
CCS	clear cell sarcoma
CDC25	cell division cycle 25
CDC6	cell division cycle 6
CDK	cyclin-dependent kinase
CDKN1A	cyclin-dependent kinase inhibitor 1A
CDKN2A	cyclin-dependent kinase inhibitor 2A
<i>ced</i>	cell death abnormal
CHK1	checkpoint kinase 1
CHK2	checkpoint kinase 2
Cip/Kip	CDK-interacting protein/Kinase inhibitory protein

CK1	casein kinase 1
CKI	cyclin-dependent kinase inhibitor
CLP	common lymphoid progenitor
CNG ion channel	cyclic nucleotide-gated ion channel
CNS	central nervous system
COP1	constitutively photomorphogenic 1
COX2	cyclooxygenase 2
CREB	cAMP-response element binding protein
CREM	cAMP response element modulator
CSR	class switch recombination
Cx	connexion
$\Delta\psi_m$	mitochondrial membrane potential
DBD	DNA-binding domain
DC	dendritic cell
DED	death effector domain
DLBCL	diffuse large B cell lymphoma
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DSB	double strand break
E6AP	E6-associated protein
EBI2	Epstein-Barr virus-induced receptor 2
EBV	Epstein Barr virus
EMT	epithelial-mesenchymal transition
EP receptors	PGE ₂ receptors
EPAC	Rap1-guanine nucleotide exchange factor protein directly activated by cAMP
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
FL	follicular lymphoma
G1 phase	gap 1 phase
G2 phase	gap 2 phase
G _i	inhibitory G protein
G _s	stimulatory G protein
<i>GADD45</i>	growth arrest and DNA damage 45
GC	germinal centre
GEF	guanine nucleotide exchange factor
GF	growth factor
GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone releasing hormone
GPCR	G protein-coupled receptor
GSK-3 β	glycogen synthase kinase-3 β
<i>gsp</i>	G _s protein oncogene
HAUSP	herpesvirus associated ubiquitin specific protease
HBV	hepatitis B virus
HCL	hairy cell leukaemia
HCN channel	hyperpolarization-activated cyclic nucleotide-gated channel
HDM2	human homologue of Mdm2
HL	Hodgkin's lymphoma
HLA	human leucocyte antigen
HPV	human papilloma virus

HSC	haematopoietic stem cell
HTLV-1	human T cell lymphotropic virus type 1
ICE	interleukin-1 β -converting enzyme
Ig	immunoglobulin
INK4	inhibitor of CDK4
IR	ionizing radiation
IRES	internal ribosome entry site
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
KO	knock out
LCL	lymphoblastoid cell line
M	mitosis
MALT	mucosa-associated lymphoid tissue
MCL	mantle cell lymphoma
MCM complex	mini chromosome maintenance complex
Mdm2	mouse double minute 2
MEF	mouse embryonal fibroblast
MIC-1	macrophage inhibitory cytokine-1
MM	multiple myeloma
MNC	mononuclear cell
MRD	minimal residual disease
MRN complex	Mre11/Rad50/Nbs1 complex
MYT1	myelin transcription factor 1
MZL	marginal zone lymphoma
NES	nuclear export signal
NF- κ B	nuclear factor kappa B
NHEJ	non-homologous end joining
NK	natural killer cell
NLS	nuclear localization signal
NOPHO	Nordic Society of Paediatric Haematology and Oncology
OMM	outer mitochondrial membrane
p38MAPK/MK2	mitogen-activated protein kinase/ mitogen-activated protein kinase-activated protein kinase 2
PARP	poly(ADP-ribose) polymerase
PAI-1	plasminogen activator inhibitor-1
PDE	phosphodiesterase
PGE ₂	prostaglandin E ₂
PI	propidium iodide
PI3K	phosphoinositide 3-kinase
<i>PIG3</i>	p53-inducible gene 3
PJS	Peutz-Jegher's syndrome
PKA	protein kinase A
PL	plasmablastic lymphoma
pRB	retinoblastoma protein
PS	phosphatidylserine
PTB	polypyrimidine tract-binding protein
PTEN	phosphatase and tensin homologue
PTLD	post-transplant lymphoproliferative disease
PTM	post-translational modification
PUMA	p53 up-regulated modulator of apoptosis

R point	restriction point
R subunit	regulatory subunit
RAG	recombination activating gene
RE	response element
RGS	regulator of G-protein signalling
RhoA	Ras homolog gene family member A
RPA	replication protein A
RS cell	Reed-Sternberg cell
S phase	synthesis phase
sAC	soluble adenylyl cyclase
SH3 domain	Src homology domain
SHM	somatic hypermutation
shRNA	short hairpin RNA
siRNA	small interfering RNA
SLL	small lymphocytic lymphoma
SSB	single strand break
ssDNA	single stranded DNA
SV40	Simian virus 40
T reg	T regulatory lymphocyte
TAD	transactivation domain
TERT	telomerase reverse transcriptase
TdT	terminal deoxynucleotidyl transferase
TGF- β	transforming growth factor β
TIP60	Tat interacting protein 60
TM	transmembrane
tmAC	transmembrane adenylyl cyclase
TNF	tumour necrosis factor
TopBP1	topoisomerase 2-binding protein 1
<i>TP53</i>	tumour protein p53 (human p53 gene)
<i>Trp53</i>	transformation related protein 53 (mouse p53 gene)
TSH	thyroid stimulating hormone
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling
UTR	untranslated region
VEGF	vascular endothelial growth factor
WM	Waldenström's macroglobulinaemia
wt	wild type
XIAP	X-linked inhibitor of apoptosis protein
YY1	Yin Yang 1

4 INTRODUCTION

4.1 Cancer

Multicellular organisms are composed of individual cells that are all derived from the fertilized egg, but which have differentiated according to a strict developmental programme, giving rise to a variety of specialized cell types. Most of these carry a complete organismic genome encoding far more information than any one of these cells will ever require. This endows the cells with versatility and the vital possibility to adapt to the ever changing needs of the organism as a whole, for instance by retaining the ability to proliferate and participate in tissue morphogenesis in response to injury. However, it also poses a danger to the organism, as the individual cells may erroneously gain access to information that should normally be denied to them. Specifically, the genomic sequences are vulnerable to somatic mutations and epigenetic changes which in some cases can change the behaviour of the cell.

When such genomic alterations occur that result in a changed pattern of growth, favouring cell division and disfavoured cell death, a neoplasm can arise. Neoplasia literally means ‘new growth’ and can be used interchangeably with tumour, a Latin term meaning ‘swelling’. As long as a tumour respects the normal boundaries of the tissue of origin, it is said to be benign. However, upon the acquisition of additional genomic changes, a tumour can progress to invade and destroy neighbouring tissue structures and spread to distant sites where it can establish new tumours termed metastases. These are the common characteristics of malignant tumours, collectively referred to as cancers.

4.1.1 The hallmarks of cancer

It has long been suggested that tumour development proceeds via a process adhering to the principles of Darwinian selection, in which a succession of genetic changes conferring growth advantages are selected for, resulting in the progressive development of malignant traits [1]. In 2000 D. Hanahan and R. A. Weinberg published a seminal paper titled “The Hallmarks of Cancer”, suggesting a collection of six such traits or capabilities that are shared by most and perhaps all types of human cancers, and which should thus be considered the cornerstones of selective pressures acting upon a developing tumour [2]. These capabilities are self-

sufficiency in growth signals, insensitivity to anti-growth signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Figure 1). Later, the evasion of immune surveillance has been suggested as an additional hallmark [3].

Self-sufficiency in growth signals

Normal cells usually exist in a quiescent state requiring mitogenic growth signals from their surroundings to reenter the cell cycle. These signals can be provided as diffusible growth factors (GFs), by extracellular matrix components, or by neighbouring cells, and are generally transmitted into the cell by transmembrane receptors, often belonging to the tyrosine kinase receptor family. Self-sufficiency in these signals can be achieved through autocrine supply of GFs, overexpression of receptors, or alterations in components of downstream cytoplasmic signalling [2].

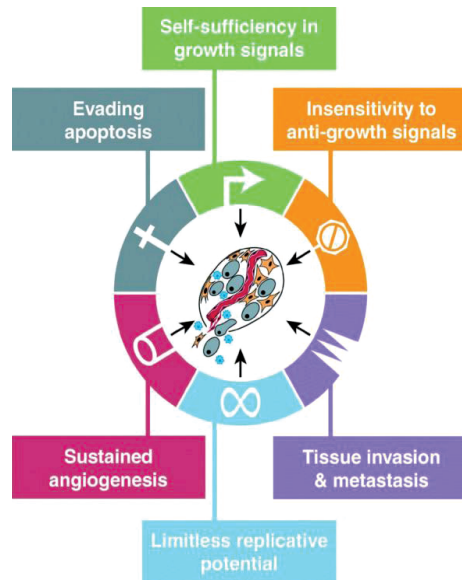


Figure 1. The hallmarks of cancer as suggested by Hanahan and Weinberg. Adapted from [2].

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Insensitivity to anti-growth signals

The quiescent state of resting cells is not only maintained by the absence of mitogenic signals, but also by the presence of signals actively repressing progression through the cell cycle. Such signals are transmitted to the cell through transmembrane receptors paralleling the situation for growth signals, and the cellular response is often funnelled through effects of inhibitors of the cell cycle machinery such as the retinoblastoma protein (pRB). Insensitivity to these signals can be achieved through downregulation of receptors, direct inactivating mutations of *RB*, or mutations of components of the cell cycle machinery [2].

Evasion of apoptosis

Cell proliferation is balanced by cell death in normal homeostatic tissues. As a safeguard against untimely proliferation induced by the activation of oncogenes, the cell's innate response is the activation of apoptosis. This commonly occurs through p53-dependent

activation of the so-called intrinsic apoptotic pathway (see section 4.3.2), and inactivation of this pathway through direct p53 mutation or deregulation of upstream or downstream p53 signalling is thought to be an almost universal occurrence in tumour cells [4].

Limitless replicative potential

The number of replicative cycles a cell can encounter is normally limited by the erosion of telomeres associated with every replication cycle, resulting in the inability to protect the ends of chromosomal DNA after 60-70 doublings. The maintenance of telomeres in tumour cells can be achieved by the expression of the telomerase reverse transcriptase (TERT), whose activity is normally limited to a very select subset of somatic cells such as stem cells [5]. In addition, about 10% of tumours can engage an alternative strategy to maintain telomeres, termed 'alternative lengthening of telomeres' (ALT) [6;7].

Sustained angiogenesis

The diffusion capacity of nutrients, metabolites and gases limits the distance from a vascular capillary at which a cell can survive to approximately 100 μm . Thus, an expanding tissue mass must be able to support the ingrowth of new vessels if it is to exceed a certain size. The activation of this so-called angiogenic switch can be accomplished by increased expression and secretion of soluble factors which can bind to receptors on endothelial cells stimulating the sprouting of novel vessels. Examples include vascular endothelial growth factor (VEGF) and fibroblast growth factors (FGFs). Additionally, the secretion of inhibitory factors can be downregulated [2].

Tissue invasion and metastasis

The ability of cancer cells to grow not only in an expansive manner, but to expand growth beyond its natural boundaries, thereby infiltrating neighbouring structures, is a defining characteristic of a malignant tumour. This allows cancer cells to enter into the blood stream (intravasate), be transported to distant tissues where it can lodge in microvessels, and in some cases extravasate and successfully form new colonies of cancer cells. These distant metastases are responsible for the vast majority of cancer-related deaths [8]. However, this hallmark stands out from the other five in that it is not intuitively apparent why such a capability should be selected for. Recent evidence supports the notion that metastatic dissemination indeed does not depend on the acquisition of additional genetic alterations, but rather is an inadvertent side-effect of primary tumour formation. In particular for carcinomas, *i.e.* malignant tumours

arising from epithelial tissues, a process termed the ‘epithelial-mesenchymal transition’ (EMT) is suggested to be crucial. EMT is a developmentally programmed process, involving the activation of a specific set of transcription factors, enabling epithelial cells to transiently enter a mesenchymal state allowing it to cross the basal lamina and invade the extracellular matrix of the underlying stroma. It is envisioned that tumour-associated stroma can supply tumour cells with heterotypic signals activating EMT, thus enabling invasion, intravasation and consequent dissemination of cancer cells. The successful survival and proliferation of these cells at new locations resulting in the formation of macroscopic metastases, is thought to be a very rare event when compared to the number of tumour cells that originally disseminate, and the mechanisms governing such colonization are currently unresolved (reviewed in [9]).

Additional hallmark: Evasion of immune surveillance

In addition to the six hallmarks of cancer originally suggested by Hanahan and Weinberg, a seventh hallmark, evasion of immune surveillance, has later been suggested [3]. It is clear that our immune system has evolved to employ numerous strategies to suppress cancer development, and consequently cancer cells will have to select for properties that allow them to evade this immune surveillance if the cancer is to succeed. The interplay between tumour cells and the cells of the innate and adaptive immune system is highly complex. However, three main strategies have been suggested to be employed by cancer cells to reduce the pressure imposed by the immune system. First, cancer cells can ‘hide’ from the T lymphocyte-mediated response by downregulating their expression of human leucocyte antigen (HLA) type I molecules. Secondly, cancer cells can actively repress the induction of the extrinsic apoptotic pathway involved in some forms of T lymphocyte-mediated cancer cell killing. Thirdly, cancer cells can induce an immune subversion, *i.e.* the active suppression of the immune response (reviewed in [3]).

4.1.2 The nature of genetic perturbations in cancer cells

Through comprehensive investigation into signalling pathways involved in the development of the hallmarks outlined above, numerous oncogenes and tumour suppressor genes have been identified and characterized. Some of these are found mutated frequently, examples of which include *PI3K* (phosphoinositide 3-kinase), *RAS*, *TP53* (tumour protein p53), *PTEN* (phosphatase and tensin homologue), *RB*, and *CDKN2A* (cyclin-dependent kinase inhibitor 2A, encoding p16^{INK4a} and p14^{ARF}) [10]. When large-scale sequencing of multiple cancer

genomes became available, there was considerable expectation that novel high-frequency mutations would be discovered, providing insight into new pathways important for tumour development. However, such studies have failed to identify previously unrecognized commonly mutated genes. Rather they conclude that every tumour harbours a complex combination of low-frequency mutations that drive the tumour phenotype [11-16]. In other words, even though the genetic changes driving the tumour phenotype might result in a relatively limited number of common functional hallmarks, the genetic changes themselves display tremendous complexity and heterogeneity. This has led to the definition of cancer as “a complex collection of distinct genetic diseases united by common hallmarks” [10].

4.1.3 The tumour microenvironment

The genetic and phenotypic changes described above, all refer to the primary tumour cells *per se*. It has, however, become increasingly acknowledged that the tumour stroma plays a key role in supporting tumour growth. This stroma consists of extracellular matrix as well as various non-transformed cells such as fibroblasts, myofibroblasts, leukocytes, myoepithelial and endothelial cells [17].

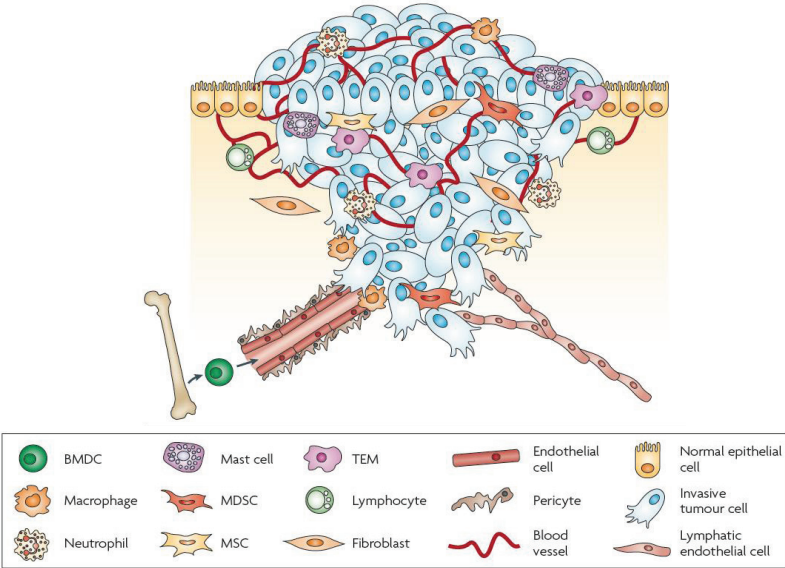


Figure 2. The tumour microenvironment. The cancer cells are surrounded by a microenvironment comprised of endothelial cells from blood and lymphatic vessels, stromal fibroblasts, and several bone marrow-derived cells (BMDCs) including macrophages, myeloid-derived suppressor cells (MDSCs), TIE2-expressing monocytes (TEMs) and mesenchymal stem cells (MSCs). Adapted from [18].

A normal stromal environment has been shown to exert an inhibitory role on tumour growth, but evidence suggests that heterotypic interactions between the transformed cells and the surrounding stroma induce substantial gene expression and epigenetic changes in the latter part, leading to phenotypic changes better suited to support the tumour [10;19]. In addition, there is evidence that tumour cells can recruit bone marrow (BM)-derived mesenchymal cells and haematopoietic cells to the tumour site, which subsequently establish as an integral part of the tumour stroma [20;21]. This novel microenvironmental niche created by the tumour cells not only serves as a more permissive neighbourhood, but is thought to play an active role throughout cancer initiation, progression and metastasis through the action of secreted factors and cell-cell interactions.

4.2 Cell proliferation

Most cells in a multicellular organism at a given time exist in a quiescent state, requiring specific stimulation of growth signals to enter a proliferative state. These signals act on components of the cell cycle, which describe the distinct stages a cell passes through to prepare for and execute cell division. Originally, cell division was divided into two stages: mitosis (M) and interphase. Later the interphase stage of cell division was divided into three distinct phases: the gap 1 (G1) phase in which the cell prepares for DNA synthesis, the synthesis (S) phase in which DNA is replicated, and the gap 2 (G2) phase in which the cell prepares for mitosis [22]. The progression through the phases of the cell cycle can mainly be attributed to the action of two classes of proteins, the cyclins and the cyclin-dependent kinases (CDKs), whereas negative regulation is provided by various cyclin-dependent kinase inhibitors (CKIs).

4.2.1 Cell cycle progression

CDKs are serine/threonine protein kinases, expressed at relatively stable levels throughout the cell cycle. Their activation requires the binding of cyclins that confer both substrate specificity and regulation of the CDK enzymatic activity [23]. The cyclin levels rise and fall in a coordinated manner throughout the cell cycle (Figure 3). This leads to periodical activation of the CDKs, laying the grounds for the ordered induction of downstream events associated with progression through the different cell cycle phases. The human genome contains 13 loci encoding CDKs and 25 loci encoding cyclins [24]. However, only subsets of

these are directly involved in driving the cell cycle, including the interphase CDK2, CDK4 and CDK6; the mitotic CDK1; and ten cyclins belonging to four different classes termed A-, B-, D- and E-type cyclins.

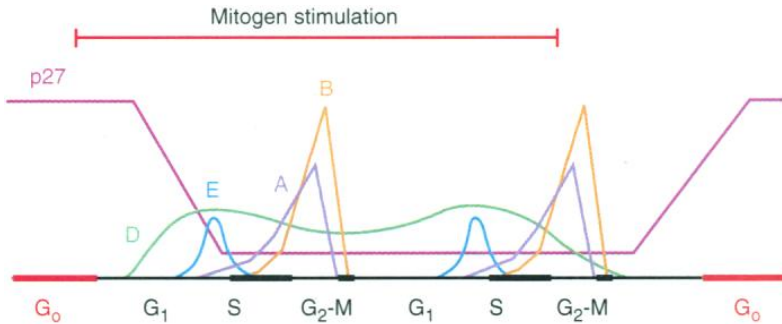


Figure 3. The fluctuation of cyclins during the cell cycle. The figure depicts how D-type cyclins are expressed in a mitogen-dependent manner. This initiates signalling resulting in the sequential and transient expression of E-, A- and B-type cyclins in restricted phases of the cell cycle. Adapted from [25].

The entrance of a cell into the G1 phase of the cell cycle is marked by the induction of D-type cyclins. Transcriptional induction of these cyclins occurs in response to several mitogenic stimuli [26-32]. Anti-proliferative stimuli on the other hand, can repress their expression [33-37]. In addition, levels of D-type cyclins are under post-transcriptional control, with regulation of their degradation through the ubiquitin/proteasome pathway partly depending on phosphorylation events imposed by glycogen synthase kinase-3 β (GSK-3 β) [38-42]. Once induced, D-type cyclins bind to and activate CDK4 and CDK6. One of the targets of CDK4/6-cyclin D is pRB, which upon partial phosphorylation releases members of the transcription factor family E2F, leading to transcription of E2F-responsive genes required for further propagation through the cell cycle [43;44]. Amongst the earliest E2F-induced genes are E-type cyclins [45]. This enables activation of CDK2 by cyclin E in late G1 phase, resulting in further pRB phosphorylation [45;46]. The resulting hyperphosphorylation of pRB defines the so-called restriction point (R) at which the cell does not need further stimulation by mitogenic signals to complete its cell cycle. During late G1/early S phase, E2F activity induces transcription of A-type cyclins, enabling the formation of CDK2-cyclin A complexes which play an active role in DNA synthesis through phosphorylation of proteins involved in DNA replication such as cell division cycle 6 (CDC6) [47;48]. Towards the end of interphase,

cyclin A levels increase further, allowing its association with CDK1, and this complex is thought to be essential for the initiation of prophase, marking the G₂/M phase transition [49]. Following the nuclear envelope breakdown, cyclin A is degraded, and this facilitates the formation of CDK1-cyclin B complexes which actively participate in and complete mitosis [24] (Figure 4).

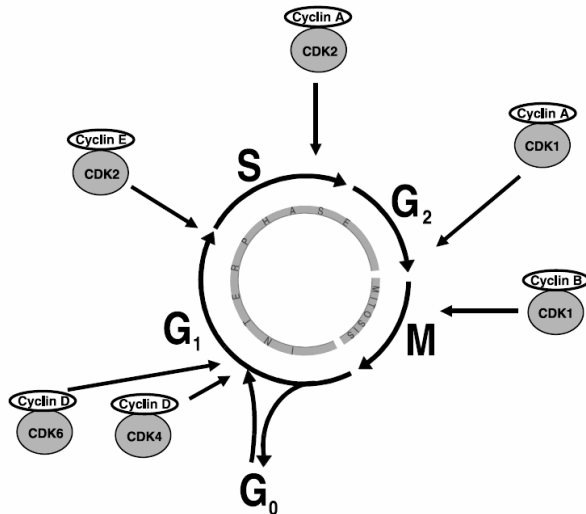


Figure 4. The cell cycle stages. The various stages of interphase and mitosis are shown with the site of activity of regulatory CDK-cyclin complexes indicated. G₀ phase represents a resting/quiescent phase entered by the cell upon lack of mitogenic signals or the presence of anti-mitogenic signals. Cells in G₀ account for the major part of the non-growing, non-proliferating cells in mammals. Adapted from [50].

The cell cycle model outlined above represents the so-called ‘classical model’, in which the basic concept is that each phase is driven by specific CDK-cyclin complexes. This model has recently been challenged by numerous systematic knock out (KO) studies in mice, demonstrating that CDK2, CDK4 and CDK6 are redundant for cell cycle progression in most cell types, as their function can generally be replaced by CDK1 [51]. CDK1 on the other hand, is absolutely required to drive the embryonic cell division, as its elimination prevents embryos from developing beyond the two cell stage [51]. A similar situation has been demonstrated for cyclins, with cyclin A2 and B1 emerging as the most non-redundant family members (reviewed in [23;52]). While these studies have revealed previously unrecognized redundancy in cell cycle regulation, the general principle of the classical model is still thought to be valid in most cell types in the presence of the full complement of CDK and cyclin family members. However, as deletion models have demonstrated, different CDKs and cyclins can have tissue- or cell-specific functions in adult tissues [23].

4.2.2 Cell cycle checkpoints

As already illustrated by figure 4, the cell cycle is organized much like a timer that triggers the events of the cell cycle in a set sequence. The CDK-cyclin complexes can be viewed as biochemical switches in this timer, ensuring the timely activation of events. Upon this basic programme, a number of checkpoints are imposed that can turn the switches on or off according to the state of the cell.

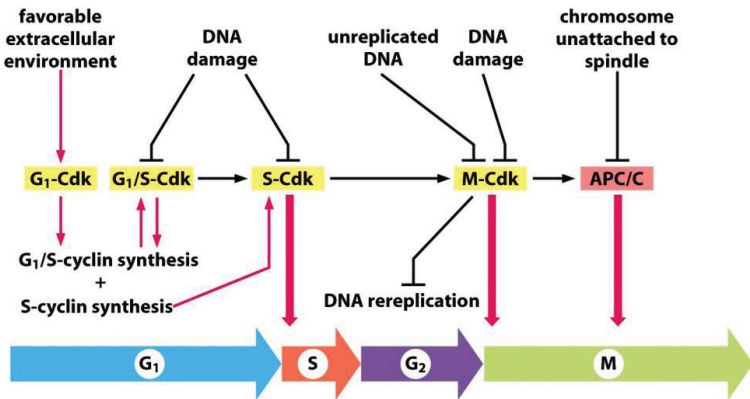


Figure 5. The cell cycle control system. The figure illustrates some of the crucial steps in the progression through and regulation of the cell cycle. Major check points are indicated with thick arrows. (APC/C: anaphase-promoting complex/cyclosome). Adapted from [53].

Examples of biochemical events providing such modulation of CDK-cyclin activity are mentioned in section 4.2.3. There are many ways of defining cell cycle checkpoints; however, in its simplest sense it can be divided into three major regulatory transitions. The first is the already mentioned restriction point at which mitotic and anti-proliferative signals are integrated to decide whether the cell shall indeed start replicating its DNA. The second is the G₂/M checkpoint in which the cell ensures that DNA replication is complete before committing to mitosis. The third is the metaphase-to-anaphase transition in which the cell ensures that all chromosomes are correctly aligned and attached to the spindle before the cell proceeds to cytokinesis [53]. In addition to the sensing of events intrinsic to the progression of the cell cycle, there are multiple points at which the cell cycle can arrest in response to DNA damage, ensuring time for the damage to be repaired before further propagation through the cycle. DNA damage halts the cell cycle primarily during G₁ phase, at the G₁/S transition, or at the G₂/M transition (Figure 5) and will be described in more detail in sections 4.4.3.2 and 4.5.3.2.

4.2.3 Regulation of CDK activity

As has already been mentioned, CDK activation requires the availability and binding of a proper cyclin. In addition, CDK activity is regulated by post-translational modifications (PTMs) and CKIs.

Post-translational modifications

Full activation of a CDK-cyclin complex requires phosphorylation on specific threonine residues: T161 in CDK1, T160 in CDK2 and T172 in CDK4 [54;55]. Such activating phosphorylation is ensured by the enzyme CDK-activating kinase (CAK) [56]. Other kinases, such as WEE1 and myelin transcription factor 1 (MYT1) catalyze phosphorylation of threonine and tyrosine residues resulting in inhibition of CDK-cyclin complexes even in the presence of activating phosphorylation [57-60]. Inhibitory phosphorylation of CDKs is assumed to be constitutive, suggesting that the regulation lies in the activity of the CDC25 family of phosphatases, which can specifically remove these phosphate groups [61-63].

Cyclin-dependent kinase inhibitors

There are two main families of cell cycle inhibitors: the INK4 (inhibitor of CDK4) family (p15^{INK4b}, p16^{INK4a}, p18^{INK4c}, and p19^{INK4d}), and the Cip/Kip (CDK-interacting protein/Kinase inhibitory protein) family (p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}) [64]. Members of the INK4 family specifically inactivate the G1-phase CDKs by complexing with CDK4 and CDK6 prior to their association with D-like cyclins, thereby preventing CDK-cyclin complex formation [65]. This blocks pRB phosphorylation and E2F release, resulting in G1 arrest. Members of the Cip/Kip family can inhibit the already formed CDK-cyclin complex. They generally display high affinity for G1- and S-phase complexes, but can additionally inactivate the M-phase complex CDK1-cyclin B [66-69].

The expression of CKIs varies according to extrinsic and intrinsic cell signals. For instance, the *CDKN1A* (cyclin-dependent kinase inhibitor 1A) locus encoding p21^{Cip1}, is under the transcriptional control of p53 [70]. p53 is positioned as an integrator of several stress sensing signalling pathways in the cell, being activated in response to DNA damage (see section 4.4.3.1). The resulting transcription of p21^{Cip1} arrests the cell in G1, allowing time for DNA repair [71]. In addition, p21^{Cip1} is able to induce G2- and S-phase arrest [72-74], and has been implicated in terminal differentiation [75] and senescence [76]. An example of cell cycle

inhibition resulting from extrinsic anti-proliferative signalling is the expression and activation of p15^{INK4b} and p27^{Kip1} in response to binding of transforming growth factor β (TGF- β) to its receptor [77;78].

There exists an intimate interplay between CDK-cyclin complexes and CKIs. One example is provided by CDK4/6-cyclin D complexes which, in addition to facilitating E2F-mediated cyclin E transcription, also ensures titration of p21^{Cip1} and p27^{Kip1} from the CDK2-cyclin E complexes, thereby relieving their inhibition (reviewed in [79;80]). In response, active CDK2-cyclin E phosphorylates p27^{Kip1}, priming it for degradation and thus further amplifying the CDK2 activity necessary for the G1/S transition [81].

4.3 Apoptosis

In 1842 Carl Vogt published his work on the developmental history of the common midwife toad (“Untersuchungen über die Entwicklungsgeschichte der Geburtshelferkröte [Alytes obstetricians]”). This probably constitutes the first mention of naturally occurring cell death. During the next 120 years, cell death other than damage-induced necrosis received little attention. The study of the phenomenon then started to gain some pace, with the realisation that cell death can be a programmed, normal morphogenic event in the development of multicellular organisms [82;83]. Further morphological characterization by light microscopy led to the distinction between coagulative necrosis and so-called shrinkage necrosis in 1965, both phenomena being observed in parallel in the ischemic liver [84].

In the following years, the distinction between these two forms of ‘necrosis’ was described in further detail using electron microscopy, preparing the grounds for a paper by J. F. Kerr, A. H. Wyllie and A. R. Currie published in British Journal of Cancer in 1972 [85] in which the term apoptosis was coined. Here, recent advances by their and other research groups describing a new mode of cellular death with ultrastructural features consistent with an active and controlled phenomenon is described, culminating in the important conclusion that it represents a “basic biological phenomenon with wide-ranging implications in tissue kinetics”.

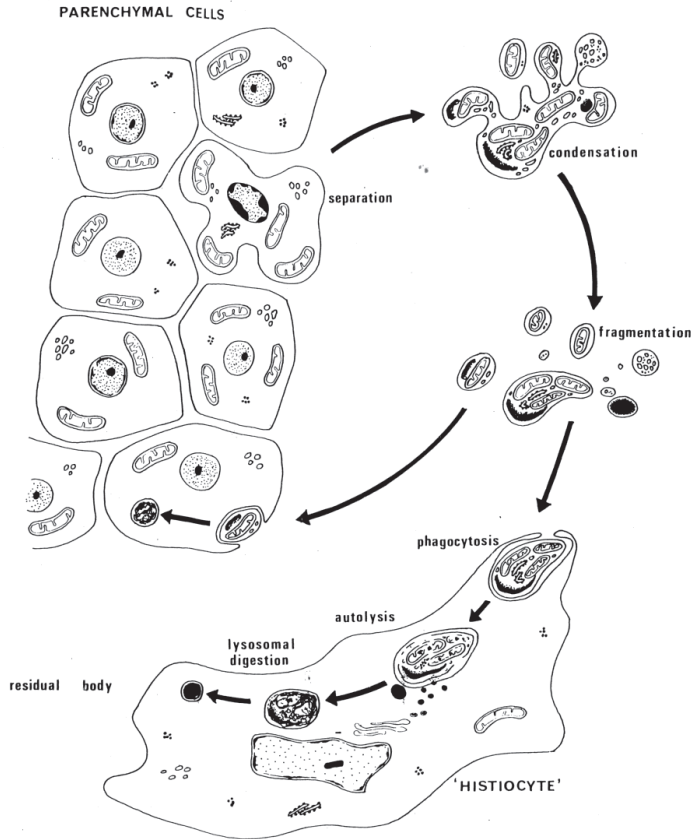


Figure 6. Illustration of the apoptotic process *in vivo*. The figure illustrates the separation of the apoptotic cell from its surrounding cells followed by condensation, fragmentation and subsequent engulfment by neighbouring cells. Adapted from [85].

Due to the associations of the term ‘necrosis’ with cell death imposed by noxious agents, the authors did not favour the continued use of ‘shrinkage necrosis’ to describe a process which was conceived as essential in the normal development and physiology of multicellular organisms. The word ‘apoptosis’ was therefore proposed. The word is taken from Greek, originally describing the ‘dropping off’ or ‘falling off’ of petals from flowers, or leaves from trees. In the context of a multicellular organism, this parallels the separation of the dying cell from the surrounding cells, which is followed by its condensation, fragmentation, and phagocytic engulfment by neighbouring cells (see illustration from the original publication, figure 6). The authors conclude that apoptosis is “well suited to a role in tissue homeostasis” and refer to experiments demonstrating the occurrence of significant levels of apoptosis in healthy tissues, during embryogenesis, teratogenesis, atrophy/involution, and in growing

malignant neoplasms. Until this point the deregulation of mitosis had received much attention when seeking to explain the mechanistic basis for tumour development, but importantly the authors now postulated that deregulation of apoptosis might be equally important.

4.3.1 The signalling pathways involved in apoptosis

Several years passed between this important definition of apoptosis on the basis of electronmicroscopic features and the unravelling of its molecular basis. In 1980 A. H. Wyllie provided the first report of the association between the observed chromatin condensation and activation of an endogenous endonuclease, resulting in internucleosomal double-strand cleavage of DNA, producing the characteristic laddering of DNA upon agarose electrophoresis [86].

Four years later, the B cell leukaemia/lymphoma 2 (*BCL-2*) gene was identified based on its position at the break point of the t(14;18) translocation commonly found in follicular lymphoma and occasionally in acute lymphoblastic leukaemia (ALL) [87;88]. Because this translocation placed *BCL-2* in close association with the enhancer region of the human μ chain gene, it was immediately hypothesized that this would lead to an increase in *BCL-2* protein expression that might be directly involved in B cell oncogenesis. In 1988 the first report that gave experimental support to this hypothesis was published, showing that *BCL-2* could cooperate with cellular myelocytomatosis oncogene (*c-myc*) to promote proliferation of B cell precursors, some of which became tumourigenic [89]. Importantly, this paper also showed that if indeed *BCL-2* was to qualify as a proto-oncogene, it would stand out from other proto-oncogenes known until then, as it seemed to provide a survival advantage rather than a drive for proliferation. This was soon confirmed by several papers from S. J. Korsmeyer's group, reporting survival advantages in the absence of growth advantage in cells overexpressing *BCL-2* [90-92]. The same group could also show that *BCL-2* localizes to the inner mitochondrial membrane [93]. Eventually, *BCL-2* turned out to be the first identified member of a large family of proteins characterized by the presence of *BCL-2* homology (BH) domains. This group consists of both pro- and anti-apoptotic members, and plays a pivotal role in the regulation and execution of the intrinsic apoptotic pathway.

In parallel with the unravelling of the function of *BCL-2* emanating from work on human and murine systems, studies of the genetic control of apoptosis in the nematode *Caenorhabditis*

elegans (*C. elegans*) provided important clues to other crucial components of mammalian apoptosis. In 1992, a *C. elegans* homologue to BCL-2 was identified with the discovery of the gene *ced-9* (cell death abnormal) which acted to inhibit apoptosis [94]. However, already in 1986, two other genes; *ced-3* and *ced-4* were shown to be crucial for developmental apoptosis in *C. elegans* [95]. Seven years later, *ced-3* was cloned, and the predicted Ced-3 protein was found to show similarity to the already known mammalian interleukin-1 β (IL-1 β) -converting enzyme (ICE) and Nedd-2 [96]. ICE had an already identified function as a cysteine protease, responsible for cleaving and activating IL-1 β . Consequently it was proposed that Ced-3 may act as a cysteine protease in the initiation of programmed cell death in *C. elegans*, and that cysteine proteases also function in programmed cell death in mammals. Within three years, a total of ten human homologues had been identified, and subsequently termed cysteine-aspartic proteases (caspases) [97]. It was soon confirmed that many of the caspases indeed played a central role in apoptosis in humans.

As opposed to the case for Ced-3, no human homologue for the putative Ced-4 protein was known at the time of the cloning of *ced-4* in 1992 [98]. In 1997, the human homologue, termed apoptotic protease activating factor-1 (*APAF-1*) was cloned, and it was concurrently demonstrated that APAF-1 binds cytochrome c [99]. Shortly before this discovery, the release of cytochrome c from the mitochondrial intermembrane space to the cytosol had been identified as a crucial event in the induction of the apoptotic programme [100]. In the subsequent year, it was found that cytochrome c release was linked to caspase-3 activation [101], and that cytochrome c release could be inhibited by BCL-2 [102;103]. This contributed to a more coherent outline of the mammalian apoptotic pathway as we know it today. Finally, in 1998 the gene product of *egl-1*, a gene identified in egg-laying abnormal *C. elegans* 15 years earlier [104], was cloned. The resulting protein, Egl-1 was shown to act in a proapoptotic manner, interacting physically with Ced-9, and to contain a region similar to the BH3-domain found in known pro-apoptotic members of the BCL-2 family in humans [105]. Consequently, the following sequence of events was proposed for the apoptotic process in *C. elegans*: Egl-1 can activate apoptosis by binding to and directly inhibiting the activity of Ced-9, thus releasing the cell death activator Ced-4 from an inhibitory Ced-9/Ced-4-containing complex. This model has since been acknowledged as the general outline of *C. elegans* apoptosis, and the homologies of this highly conserved process in the nematode and mammals, can be represented in a simplified manner as demonstrated in figure 7.

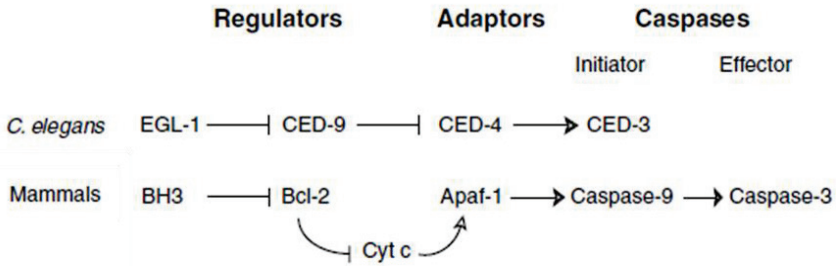


Figure 7. The homologies of the highly conserved process of apoptosis. The figure shows the parallel function of the homologous proteins in the apoptotic process in *C. elegans* and the intrinsic apoptotic pathway in mammals. Modified from [106].

The mammalian pathway outlined above, corresponds to the intrinsic/mitochondrial apoptotic pathway. In addition, there exists an extrinsic apoptotic pathway elicited by the binding of ligands to a subset of receptors belonging to the tumour necrosis factor (TNF) superfamily. The activation of these so-called death receptors initiate a cascade of events either culminating directly in caspase-3 activation, or resulting more indirectly in caspase activation through effects on the mitochondrial apoptotic machinery (Figure 8) (reviewed in [107]).

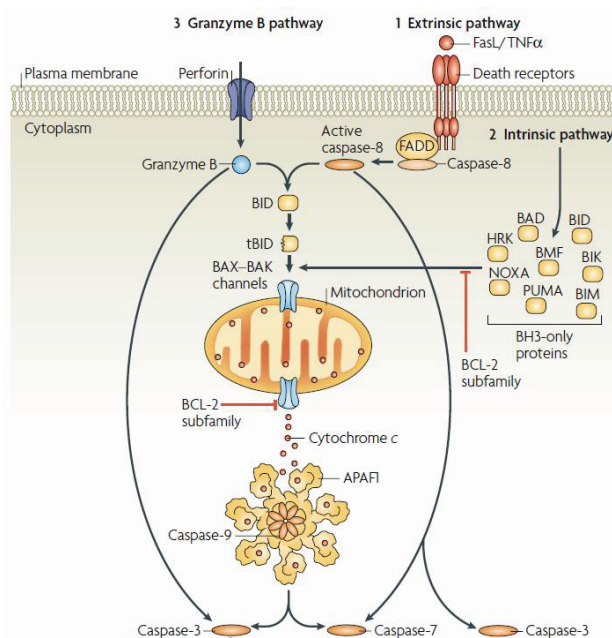


Figure 8. Overview of mammalian apoptotic pathways. 1) The extrinsic pathway. Binding of ligands to death receptors leads via adaptor proteins to the activation of caspase-8, which can either activate effector caspases directly or activate the intrinsic pathway through cleavage of the BCL-2 protein BID. 2) The intrinsic pathway. Cellular stress leads to increased activity of the pro-apoptotic members of the BCL-2 family (BH3-only proteins), and this facilitates permeabilization of the outer mitochondrial membrane by BAX and BAK. Released cytochrome c joins APAF-1 and procaspase-9 to form the apoptosome, resulting in activated caspase-9 which can further activate effector caspases. 3) The granzyme B pathway. Cytotoxic T lymphocytes and natural killer cells can induce apoptosis in target cells by releasing the pore forming protein perforin and granzyme B. Upon entry into the cell, granzyme B can either activate caspase-3 directly or activate the intrinsic apoptotic pathway through BID cleavage. Modified from [108].

4.3.2 The intrinsic apoptotic pathway

The intrinsic apoptotic pathway is initiated by cellular stressors such as increased intracellular reactive oxygen species, DNA damage, unfolded proteins, and deprivation of growth factors [109]. In this pathway members of the BCL-2 family are crucial regulators of the mitochondrial membrane integrity. Upon permeabilization, cytochrome c is released into the cytosol, permitting activation of the initiator caspase-9 on the scaffold protein APAF-1. Caspase-9 then further cleaves and activates the effector caspases, caspase-3 and caspase-7, which directly mediate the development of an apoptotic phenotype resulting in the demolition of the cell (Figure 8).

4.3.2.1 The BCL-2 family

The BCL-2 family is characterized by the presence of BH domains and comprises three functionally distinct subfamilies (Figure 9). The anti-apoptotic subfamily is made up of the originally described BCL-2 protein, in addition to BCL-X_L, BCL-W, MCL-1, BCL-2A1 (A1), and BCL-B. They contain four BH domains, and most of them also a transmembrane (TM) domain. The pro-apoptotic members are divided into two subfamilies. The first consists of BAX, BAK, and the little-studied BOK. These proteins contain three BH domains and share significant homology with members of the anti-apoptotic subfamily. The second pro-apoptotic subfamily is termed BH3-only proteins and comprises BIK, HRK, BIM, BAD, BID, PUMA, NOXA, and BMF. As the name implies, these proteins only contain one BH domain, the BH3. Apart from this domain, they share little homology with members of the other subfamilies or members within their own subfamily (reviewed in [108]).

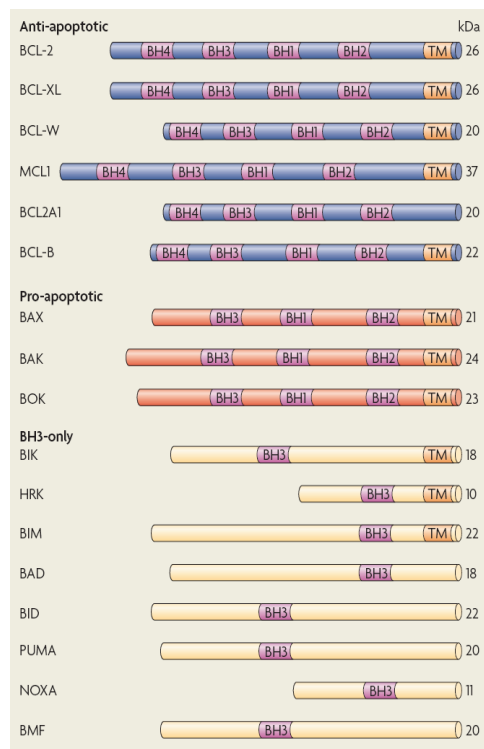


Figure 9. The BCL-2 protein family. A schematic overview of the members of the three subfamilies of BCL-2 proteins. (BH: BCL-2 homology domains, TM: transmembrane domain). Adapted from [108].

The simplified version of how these three subfamilies exert their control over the intrinsic apoptotic pathway is that upon activation by a stress signal, the BH3-only proteins insert their BH3 domain, an amphipathic α -helix, into a hydrophobic groove on the anti-apoptotic members. This releases pro-apoptotic members from inhibition, subsequently resulting in the insertion of BAX and/or BAK oligomers in the outer mitochondrial membrane (OMM) with consequent permeabilization. This general order of events with BH3-only proteins acting upstream of BAX and BAK is firmly established, as BH3-only proteins cannot induce apoptosis in cells lacking both BAX and BAK [110;111]. It is also clear that BH3-only proteins can bind and inhibit members of the anti-apoptotic subfamily, and that there exists differential affinity between members of these subfamilies. BIM, PUMA, and tBID can bind to all anti-apoptotic BCL-2 proteins, whereas NOXA binds only MCL-1 and A1, and BAD binds only BCL-2, BCL-X_L, and BCL-W (Figure 10) [112].

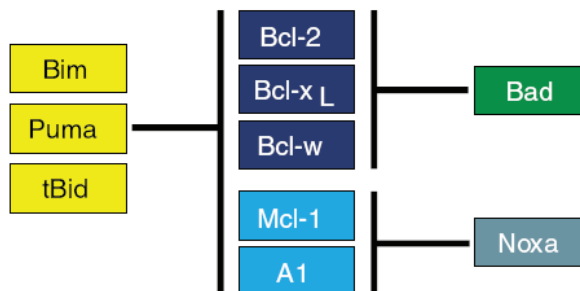


Figure 10. Selective inhibitory capacity of BH3-only proteins. The promiscuous inhibitors BIM, PUMA and tBID can inhibit all anti-apoptotic BCL-2 family members, whereas BAD and NOXA exhibit selective inhibition. Adapted from [113].

4.3.2.1.1 Activation of BAX and BAK

The exact mechanism by which BH3-only proteins activate BAX and BAK is still a subject of debate. In healthy cells, BAX exists predominantly as a cytosolic monomer [114] with its BH3 domain buried, a conformation that precludes its association with other BCL-2 class members [115]. Upon a death stimulus, BAX then translocates to intracellular membranes, including OMM [114;116], where it assembles into high molecular weight oligomers [117]. In contrast to BAX, BAK, with its BH3 domain buried [118], is constitutively localized at OMM [119]. Two competing models termed the ‘direct activation model’ and the ‘indirect

activation model' have been proposed to explain how the anti-apoptotic BH3-only members of the BCL-2 family regulate the activation and polymerization of BAX and BAK.

The direct activation model

In this model, the BH3-only members are further subdivided into 'activators' and 'sensitizers'. The 'activators' include BIM, tBID, and possibly PUMA and are proposed to bind directly to BAX and BAK to induce a conformational change, thereby unveiling their BH3 domain and promoting their activation. The role of the 'sensitizers' is to bind anti-apoptotic members, thereby displacing and releasing any bound 'activators'. This model thus implies that certain BH3-only members can bind directly to BAX and BAK, as have been demonstrated in some experimental settings [120-124]. The reason why BIM, tBID, and PUMA are the most potent apoptosis-inducers in the BH3-only group can in this model be explained by the fact that they hold the role as 'activators' (Figure 11). Although not a BCL-2 family protein, cytoplasmic p53 has also been given a possible role in this model, with a dual function both as an 'activator' and as a 'sensitizer' [125].

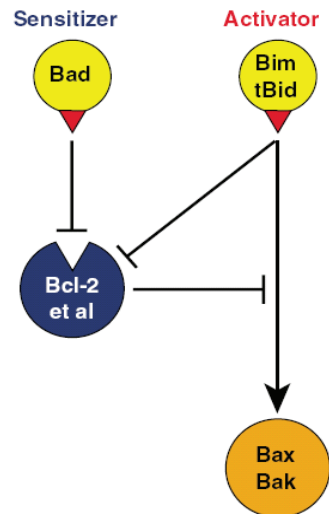


Figure 11. The direct activation model. A schematic outline of the direct activation model in which BH3-only proteins are divided into sensitizers and activators. Adapted from [126].

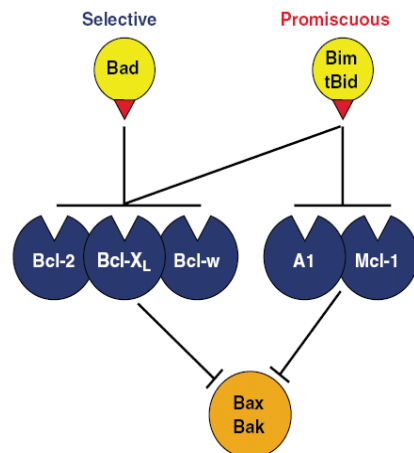


Figure 12. The indirect activation model. A schematic outline of the indirect activation model in which BH3-only proteins are divided into selective and promiscuous inhibitors of the anti-apoptotic BCL-2 subfamily members. Adapted from [126].

unknown signal supplied by an apoptotic stimulus, their conformation change into a ‘primed’ state exposing their BH3-domains. This enables the oligomerization of BAX and BAK; however, such oligomerization will initially be inhibited through BH3-domain-mediated interaction with anti-apoptotic members of the BCL-2 family [127-130]. BH3-only members induced by the apoptotic stimulus then subsequently displace members of the anti-apoptotic subfamily, thereby releasing BAX and BAK from their inhibition. The reason why BIM, tBID, and PUMA are the most potent apoptosis-inducers in the BH3-only group, can in this model be explained by their promiscuous nature, *i.e.* they can engage and inhibit all members of the anti-apoptotic subfamily (Figure 12).

The permeabilization of the outer mitochondrial membrane

Exactly how the conformationally changed BAX and BAK proceed to oligomerize is not certain. It has been demonstrated that exposure of the BH3-domain in BAX can lead to homodimerization [131], and models proposed both by advocates of the ‘direct’ and the ‘indirect’ model, seem to envisage a higher order assembly of such dimers through an as yet unidentified interaction surface which can lead to the formation of large oligomer structures (Figures 13 and 14).

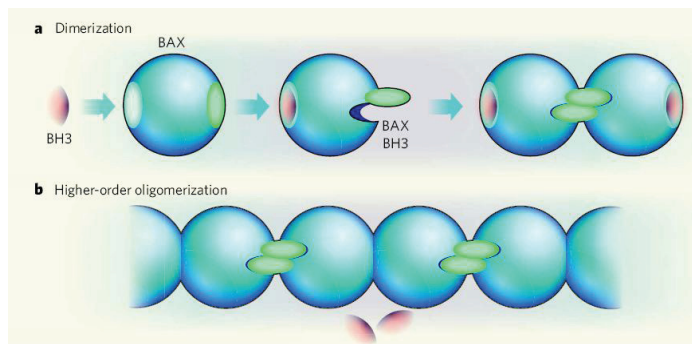


Figure 13. Activation of BAX oligomerization according to the ‘direct activation model’. The figure shows how a BH3-only activator protein is thought to induce conformational change in BAX through a ‘hit-and-run’ mechanism, enabling homodimerization of BAX. This in turn is thought to facilitate higher order oligomerization of BAX dimers. Adapted from [132].

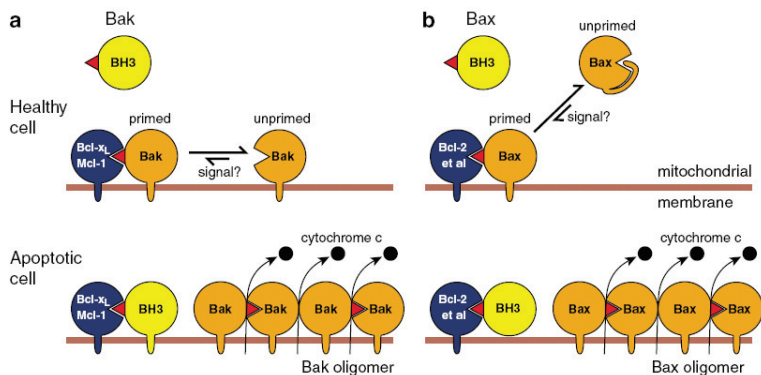


Figure 14. Activation of BAX and BAK oligomerization according to the 'indirect activation model'. The figure shows how BAX and BAK are thought to change from an unprimed to a primed configuration upon an apoptotic stimulus. The primed forms are kept in check by binding to anti-apoptotic members of the BCL-2 family until displaced by BH3-only proteins. This enables homodimerization of BAK and BAX, facilitating higher order oligomerization of dimers. Adapted from [113].

4.3.2.2 The formation of the apoptosome

APAF-1 is a multidomain protein with three functional regions: an N-terminal caspase recruitment domain (CARD), a nucleotide-binding and oligomerization domain (NOD/NB-ARC), and a C-terminal string of WD40 repeats. The function of CARD is to bind procaspase-9 by homotypic interaction, and the WD40 repeats bind cytochrome c [133]. In the absence of cytochrome c, APAF-1 exists in a monomeric form. However, upon binding of

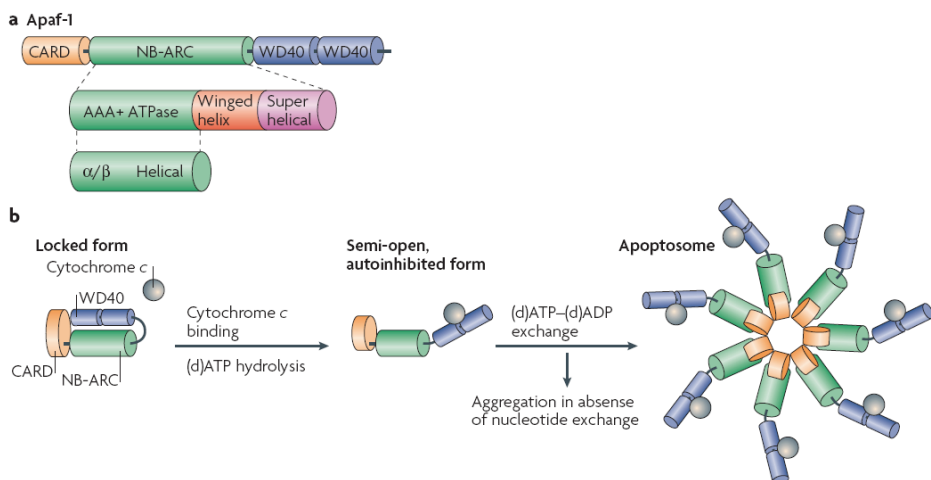


Figure 15. The structure of APAF-1 and the formation of the apoptosome. (a) The structure of APAF-1 with CARD, NB-ARC (also called NOD) and WD40 repeats. (b) The stepwise formation of the apoptosome upon cytochrome c release. Adapted from [133].

cytochrome c, an intrinsic ATPase in the NOD hydrolyses bound ATP or dATP (hereafter commonly referred to as (d)ATP) to (d)ADP, resulting in a conformational change of APAF-1. It is thought that (d)ADP – (d)ATP exchange is then necessary to enable the monomers to polymerize into an heptameric wheel-like structure which constitutes the functional apoptosome [133]. In this structure, the seven CARDS constitute the centre of the wheel, located in close proximity to each other (Figure 15). This proximity is thought to be crucial for activation of procaspase-9 which now has access to the CARDS of the apoptosome.

4.3.2.3 The caspase family

As previously mentioned, the term caspase is derived from cystein asparsate specific protease. It refers to a conserved cystein side chain of the enzyme which is critical for catalysis, and a stringent specificity for cleaving protein substrates containing aspartate, a rare specificity among proteases. All caspases have an almost identical primary specificity pocket which is perfectly shaped to accommodate an aspartate side chain [134]. The caspases identified in humans are schematically depicted in figure 16. Common to all caspases is that they are produced as inactive precursors termed zymogens. These display very low catalytic activity, which is kept in check by regulatory molecules. The N-terminal prodomain varies in length and motif. The long prodomains consist of protein-protein interaction domains termed CARD or death-effector domain (DED) which mediate recruitment of these zymogens to specific signalling complexes displaying similar interaction domains. The zymogens are further made up of a large and a small subunit, and the cleavage between these subunits typically

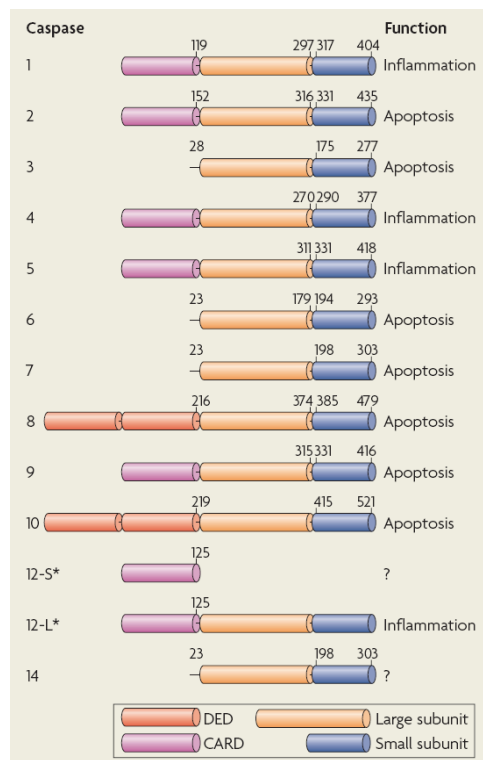


Figure 16. The caspase family. The figure gives a schematic overview of the structure and function of known human caspases (12-S* is the common truncated catalytically inactive form of caspase-12, 12-L* is the full length caspase-12 expressed in a subset of humans of African descent). Adapted from [108].

generates the active caspase which exists as a heterotetramer composed of two heterodimers derived from two zymogens. As will be discussed below, the proteolytic processing is not obligatory for activation of all caspases. The mammalian caspases are primarily involved in apoptosis or inflammation. However, many of the caspases with clearly defined roles in apoptosis or inflammation may have additional roles in other cellular processes (reviewed in [135]). The apoptotic caspases can be subdivided into initiator and effector caspases.

The initiator caspases

The initiator caspases (caspase-2, -8, -9, and -10) are characterized by long N-terminal prodomains that recruit them to signalling complexes, thus linking upstream signalling to downstream caspase-mediated effects through cleavage and activation of effector caspases. Caspase-8 is an essential initiator caspase of the extrinsic apoptotic pathway [136], whereas the possible physiological roles of caspases-2 and -10 are still a matter of debate [108].

Caspase-9 is a key component of the intrinsic apoptotic pathway. Via its N-terminal CARD, procaspase-9 binds to APAF-1-CARD in the centre of the septameric wheel structure of the apoptosome. Procaspase-9 does not need proteolytic cleavage to activate its catalytic site. As opposed to most other procaspases which exist as inactive homodimers before cleavage, procaspase-9 exists as an inactive monomer and its proteolytic site is activated by dimerization [137]. The function of the apoptosome is therefore to create a platform on which the CARD of individual APAF-1 proteins are brought in close proximity and can recruit several procaspase-9 molecules. There is evidence to suggest that this facilitation of local concentration is sufficient to promote dimerization, an event termed 'induced-proximity activation' of procaspase-9 (reviewed in [138]). However, it has also been argued that an additional interface between the apoptosome and procaspase-9 is involved in inducing a conformational change of procaspase-9 which facilitates its dimerization. This is termed the 'induced conformation model' (reviewed in [138]). Irrespective of how dimerization is facilitated, it leads to internal proteolysis between the large and small subunit as a secondary event, resulting in partial stabilization of the activated dimers [139]. *cas9* KO mice display embryonic or perinatal lethality depending on the strain of mice [140;141]. The most prominent phenotypic feature is hyperplasia of the brain which is shown to result from decreased apoptosis, and mouse embryonal fibroblasts (MEFs) from *cas9*-null mice show resistance to apoptosis induced by irradiation as well as cytotoxic drugs. However, haematopoietic cells display a more varied sensitivity to apoptotic stimuli, suggesting that the

requirement for caspase-9 in the intrinsic apoptotic pathway might vary according to cell type and insult [140;141].

The effector caspases

Caspase-3 is regarded as the main effector caspase responsible for the cleaving the majority of cellular substrates associated with apoptosis. It is activated by proteolytic cleavage by caspase-9, -8, or -10, but is not a substrate for caspase-2 [142]. *casp3* KO mice have a very similar phenotype as *casp9* KO mice in some genetic backgrounds [143;144], but show normal development in others [145], suggesting it might be redundant for most developmental cell death. Cells derived from *casp3*-null animals are defect in DNA fragmentation and chromatin condensation when exposed to cytotoxic agents, but most cells eventually die exhibiting the other typical morphological hallmarks of apoptosis [144].

Caspase-7 is structurally similar to caspase-3, is activated by the same initiator caspases, and has similar substrate specificity [106]. However, *casp7* KO mice develop normally, and cells derived from these animals retain sensitivity to apoptosis [145]. *casp7/casp3* double KO mice, on the other hand, die perinatally, suggesting that caspase-3 can compensate for caspase-7 [145].

Although structurally similar to caspase-7 and -3, caspase-6 has different substrate specificity [146]. *casp6* KO mice develop normally [147], and it is not established that caspase-6 plays a role in apoptosis, although it is often found classified as an apoptotic effector caspase.

4.3.2.4 Caspase substrates

The consensus substrate specificity sequence of caspases is found in a wide array of proteins, and many of these can be shown to be cleaved by caspases *in vitro*. However, only a few proteins have been shown to be caspase substrates *in vivo* with established relevance to the apoptotic process. It is therefore thought that many of the assumed substrates are either innocent bystanders or are not actually cleaved at all *in vivo* [134]. Some of the established substrates of initiator caspases have already been mentioned, such as caspase-3 and -7, and BID. In addition, pRB is cleaved in its C-terminal region in a conserved caspase cleavage site during apoptosis, and this cleavage is necessary for TNFR1-induced but not DNA damage-induced apoptosis to proceed [148]. Caspase-3 cleaves ICAD, an inhibitor/chaperone of CAD,

the nuclease responsible for double-strand cleavages during apoptosis. This allows CAD to dimerize, forming the catalytically competent form [149-151]. Poly(ADP-ribose) polymerase (PARP) is a substrate of caspase-3 and -7. PARP normally catalyses poly(ADP-ribose) ligation to acceptor proteins in response to DNA damage, and its cleavage prevents this action [152]. Finally, caspase-9 is itself a substrate for caspase cleavage at two distinct sites in the interchain linker region. The first cut is catalyzed by itself, creating a novel N-terminal epitope which is essential for the binding of caspase-9 to its inhibitor X-linked inhibitor of apoptosis protein (XIAP) [153]. This is assumed to function as a temporary brake on the apoptotic process. The second cut is mediated by the effector caspases and creates yet another N-terminal epitope which renders caspase-9 resistant to XIAP inhibition. By releasing caspase-9 from inhibition, this cleavage event creates a positive feedback loop whereby effector caspases increase the proteolytic potential of caspase-9 [134].

4.3.3 Defining apoptosis

When cell death through apoptosis was defined morphologically in 1972, it was contrasted to the only defined mode of cell death at the time, namely necrosis. Necrosis was viewed as an accidental death in a physiological sense, induced by overwhelming noxious stimuli that resulted in a more or less uncontrolled destruction of the cell. Apoptosis, on the other hand, could either be part of a normal physiological process or a result of noxious stimuli. However, the resulting process in the cell was conceived as orderly, controlled and purposeful, clearing the dying cell without trace by preparing it for phagocytosis by neighbouring cells. More recently, accumulating evidence has suggested the existence of a more complex picture of cell death. Consequently, a Nomenclature Committee on Cell Death has been established with the aim of critically evaluating names describing distinct modalities of cell death, and formulating recommendations on their definition and use. In their updated recommendations from 2009 [154], they suggest strict definitions of when a cell can be considered 'dead'. In the absence of a specific biochemical event shown to define a 'point-of-no-return' in the cell death process, the following morphological definitions are still recommended: 1) the cell has lost the integrity of its plasma membrane, as defined by the incorporation of vital dyes *in vitro*; 2) the cell, including its nucleus, has undergone complete fragmentation into discrete bodies; and/or 3) its corpse (or its fragments) has been engulfed by an adjacent cell *in vivo*.

The committee defines four distinct modalities of cell death (Table 1). In addition, they describe tentative definitions of ‘atypical cell death modalities’ including mitotic catastrophe, anoikis, excitotoxicity, Wallerian degeneration, paraptosis, pyroptosis, pyronecrosis, and entosis. What characterizes these atypical modalities is that they display morphological or biochemical features of one or more of the established modes of cell death. Consequently, it is not currently clear whether they can be said to represent truly novel and distinct modes of cell death.

Cell death mode	Morphological features	Notes
Apoptosis	Rounding-up of the cell Retraction of pseudopodes Reduction of cellular and nuclear volume (pyknosis) Nuclear fragmentation (karyorrhexis) Minor modification of cytoplasmic organelles Plasma membrane blebbing Engulfment by resident phagocytes, <i>in vivo</i>	‘Apoptosis’ is the original term introduced by Kerr <i>et al.</i> ¹⁴ to define a type of cell death with specific morphological features. Apoptosis is NOT a synonym of programmed cell death or caspase activation.
Autophagy	Lack of chromatin condensation Massive vacuolization of the cytoplasm Accumulation of (double-membraned) autophagic vacuoles Little or no uptake by phagocytic cells, <i>in vivo</i>	‘Autophagic cell death’ defines cell death occurring with autophagy, though it may misleadingly suggest a form of death occurring by autophagy as this process often promotes cell survival. ^{15,16}
Cornification	Elimination of cytosolic organelles Modifications of plasma membrane Accumulation of lipids in F and L granules Extrusion of lipids in the extracellular space Desquamation (loss of corneocytes) by protease activation	‘Cornified envelope’ formation or ‘keratinization’ is specific of the skin to create a barrier function. Although apoptosis can be induced by injury in the basal epidermal layer (e.g., UV irradiation), cornification is exclusive of the upper layers (granular layer and stratum corneum). ^{17,18}
Necrosis	Cytoplasmic swelling (oncosis) Rupture of plasma membrane Swelling of cytoplasmic organelles Moderate chromatin condensation	‘Necrosis’ identifies, in a negative fashion, cell death lacking the features of apoptosis or autophagy. ⁴ Note that necrosis can occur in a regulated fashion, involving a precise sequence of signals.

Table 1. The four defined modes of cell death. The Nomenclature Committee on Cell Death’s definition of apoptosis, autophagy, cornification, and necrosis based on accepted morphological features. Adapted from [154].

With respect to apoptosis, the committee stresses that morphological criteria still apply as the only defining features, and that ‘apoptosis’ hides a major degree of biochemical and functional heterogeneity. Although numerous studies have demonstrated that apoptosis is associated with several biochemical criteria, including DNA fragmentation and caspase activation, a number of reports indicate that these changes are not always required for the execution of the cell death programme after typical apoptotic stimuli. For instance, inhibition of caspase activation has been shown to be insufficient to prevent cell death (reviewed in [155]). As already mentioned in section 4.3.2.3, caspase-3 activation may be necessary for the development of some of the typical morphological and biochemical signs of apoptosis such as chromatin condensation and DNA fragmentation. Therefore, inhibition of caspase activity can prevent the appearance of some of the apoptotic features, and delay but often not inhibit cell death (reviewed in [156]). In conclusion, the absence of caspase activity and DNA fragmentation cannot exclude ongoing apoptosis-like cell death. However, due to the

morphological definition of apoptosis, and the role played by effector caspases in the formation of some of the morphological traits, it may be argued that in the strictest sense classical apoptosis is caspase-dependent. G. Kroemer and S. J. Martin have suggested dividing the relationship between caspase activation and cell death as illustrated in figure 17 [155].

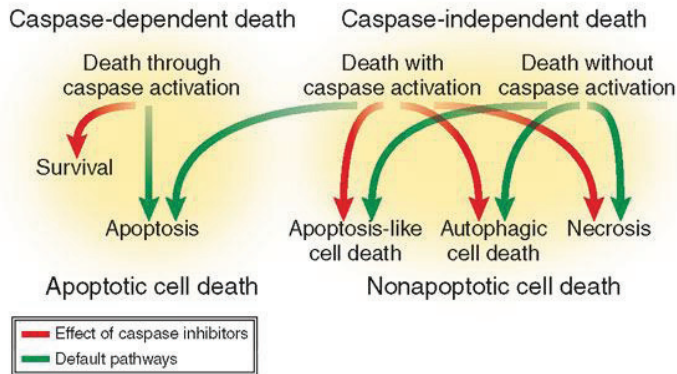


Figure 17. The relationship between caspase dependence and effect of caspase inhibitor on cell death. The figure illustrates how dependence on caspase activation can differ between cell types and between death stimuli. Effects of caspase inhibition will consequently have different effects on whether cell death can still occur, and on the morphological features of the resulting cell death. Adapted from [155].

Having discussed all the possible pitfalls of attempts to correlate measurements of biochemical markers to the occurrence of apoptosis, the Nomenclature Committee on Cell Death, ends their most recent report by discussing whether the present morphology-based strict definition of different forms of cell death is indeed expedient. Firstly, they argue that much of the work performed to delineate the biochemical pathways involved in cell death has been performed *in vitro* on immortalized cell lines. In intact tissues, however, dying cells are usually engulfed well before signs of advanced apoptosis become detectable. It may therefore still be acceptable to consider caspase activation and/or DNA fragmentation sufficient to diagnose apoptotic cell death *in vivo*. Secondly, as there are numerous examples of cell death displaying mixed features of apoptosis and necrosis or autophagy, it may not be meaningful to operate with clear-cut and absolute distinctions based on historical morphological criteria. Thirdly, they argue that it would be preferable to replace the morphological aspects with biochemical/functional criteria. As there does not seem to exist perfect correlations between morphology and biochemistry in this field, the committee anticipates that the morphological terms will eventually be displaced by biochemical definitions, probably resulting in a more differentiated classification of cell death. However,

they argue that such classification will only become meaningful once it can have a predictive impact, that is to predict possibilities to pharmacologically/genetically modulate cell death and/or predict consequences of cell death *in vivo* [154].

4.4 The DNA damage response

Maintenance of genomic integrity is a fundamental feature of life. As a result, several distinct surveillance mechanisms, collectively termed the DNA damage response (DDR) machinery, have evolved to detect and initiate appropriate responses to diverse genotoxic insults. These insults are in part imposed from the environment, examples of which include ultraviolet (UV) and ionizing radiation (IR) as well as various chemicals. In addition, reactive oxygen species (ROS) are constantly produced inside the cell as a result of normal metabolic processes. The DDR machinery components are hierarchically organized into sensors, transducers and effectors. Upon recognition of a lesion, these pathways will initiate DNA repair processes (reviewed in [157-159]). If a lesion is complex, the DDR machinery also ensures delay in cell cycle progression through activation of cell cycle checkpoints to allow time for repair (reviewed in [160;161]). In the case of extensive lesions or failure to repair, the DDR machinery can activate pathways that will eliminate the cell from the proliferative pool by inducing senescence or cell death (reviewed in [162-164]), thereby avoiding the fixation of potentially deleterious mutations.

4.4.1 DDR sensors

Different types of DNA damage attract different sets of damage-sensing proteins. With respect to DNA double strand breaks (DSBs) it is assumed that the earliest events probably involve alterations in chromatin structure [165-167]. Through mechanisms that are still poorly understood, this recruits a mediator complex consisting of Mre11, Rad50, and Nbs1 (MRN) and also results in the phosphorylation of variant H2A histones (H2AX) [168] in the vicinity of the DSB. These two events act as a signal amplifier that recruits additional signalling molecules to the lesion, amongst them the crucial upstream transducer kinase ataxia telangiectasia mutated (ATM) [169]. In addition to the MRN complex, a heterodimeric complex consisting of Ku70 and Ku86 subunits binds to the free DSB ends [170;171].

An example of another type of sensor complex is provided by the exposure of stretches of single stranded DNA (ssDNA). This can result from replication fork stalling during S phase due to the continued unwinding of the DNA template by helicases, but is also thought to be a secondary event in DSBs associated with the endonuclease and/or exonuclease activity of Mre11/CtIP complexes [172;173]. Stretches of ssDNA are rapidly coated by replication protein A (RPA) [174], an event that attracts the upstream transducer kinase ATR (ATM- and Rad3-related) through binding of its regulatory subunit ATR-interacting protein (ATRIP) [175].

4.4.2 Activation of kinase transducers

ATM-CHK2

ATM is a PI3K-related kinase that normally exists as an inactive multimer, being activated upon dissociation into monomers [176]. It is known that interaction with the MRN complex plays an important role in its efficient and rapid activation, but the molecular details of this process are not fully understood. Activation of intermolecular autophosphorylation on S1981 is thought to be an essential event [176], and in addition autophosphorylation on S367 and S1893 might be of importance [177]. More recently, it has been reported that acetylation of K3016 by the acetyl transferase Tat-interacting protein 60 (TIP60) might be crucial to ATM activation [178;179]. Both phosphorylation and acetylation events of ATM occur within minutes of the induction of DNA DSBs [176;178;179], allowing for swift kinase activity towards an array of ATM substrates.

A central ATM target is the checkpoint kinase 2 (CHK2) which is phosphorylated in an ATM-dependent manner on T68 [180-182]. This event is probably not sufficient for full CHK2 activation; however, it is thought to trigger additional autophosphorylation events on T383 and T387, ultimately resulting in CHK2 activation [180;183] (Figure 18). Additionally, ATM can activate the stress response signalling complex p38MAPK/MK2

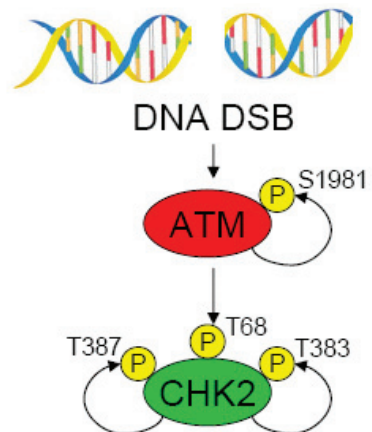


Figure 18. Activation of kinase transducers involved in DNA double-strand break signalling.

(mitogen-activated protein kinase/mitogen-activated protein kinase-activated protein kinase 2), which has been demonstrated to play a role in DNA damage-induced cell cycle arrest [184].

ATR-CHK1

ATR, like ATM, is a PI3K-related kinase, but the events leading up to its activation are less well characterized than for ATM. They are thought to involve ATR-ATRIP interaction with another DNA-associated complex composed of Rad9-Rad1-Hus1 (9-1-1) and DNA topoisomerase 2-binding protein 1 (TopBP1) [185;186]. Activated ATR recruits a mediator protein called claspin to the site of damage, which enables the phosphorylation on S317 and S345 of the main ATR downstream kinase checkpoint kinase 1 (CHK1), resulting in its activation [187] (Figure 19). Similar to ATM, ATR can also activate p38MAPK/MK2.

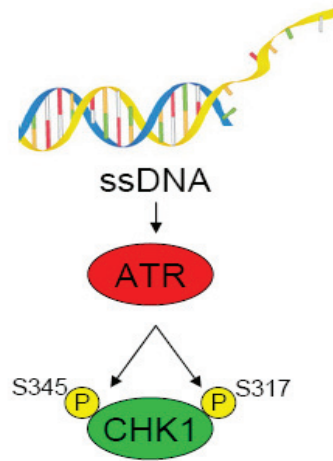


Figure 19. Activation of kinase transducers involved in single-stranded DNA signalling.

DNA-PKsc

A third PI3K-related kinase recruited to sites of DNA damage is DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PKcs is recruited to the site of damage through binding to the Ku heterodimer, which also activates the kinase activity of DNA-PKcs (reviewed in [188]).

4.4.3 DDR effector proteins and outcomes

4.4.3.1 p53 activation

DDR activation leads to accumulation and activation of the tumour suppressor p53, a protein that plays an important role in several of the DNA damage-induced effector pathways, including DNA repair, cell cycle arrest, senescence and apoptosis. The p53 signalling involved in the execution of these pathways will be explained in more detail in the section on

p53 (see section 4.5.3), whereas this paragraph mainly will focus on the mechanistic background for p53 activation upon DDR signalling.

p53 is a direct substrate for ATM, with ATM activation resulting in rapid phosphorylation of p53 on S15 [189;190]. ATR is thought to maintain S15 phosphorylation at later stages during the DDR [191]. S15 phosphorylation has been proposed to be a prerequisite for subsequent T18 phosphorylation by casein kinase 1 (CK1) [192] which has been demonstrated to negatively affect p53 binding to its negative regulator HDM2 (see section 4.5.2.1.1) [193]. CHK1 and CHK2 can further phosphorylate p53 on S20 [194;195]. Whether phosphorylation of S15 and S20 contributes to p53 accumulation through effects on HDM2 binding is currently debated. Alternatively, they have been suggested to contribute to increased p53 transcriptional activity [189;196-198]. ATM has also been indirectly implicated in phosphorylation of p53 on S46 [199;200], and in its dephosphorylation at S376 [201].

In addition to effects on the p53 phosphorylation status, the DDR machinery contributes to DNA damage-induced p53 accumulation by acting on two of the crucial negative p53 regulators: HDM2 and HDMX (see sections 4.5.2.1.1 and 4.5.2.2.1). ATM directly phosphorylates HDM2 on S395, an event that impairs nuclear export and degradation of p53 [202;203]. ATM also indirectly affects HDM2 through activation of the tyrosine kinase c-ABL (Abelson murine leukemia) which binds to and phosphorylates HDM2 on Y394, impairing the inhibition of p53 [204-206]. Finally, ATR can phosphorylate HDM2 on S407, resulting in reduced HDM2-dependent export of p53 to the cytoplasm [207]. With regards to HDMX, ATM directly phosphorylates it on S403, and this contributes to its DNA damage-induced ubiquitination and degradation [208].

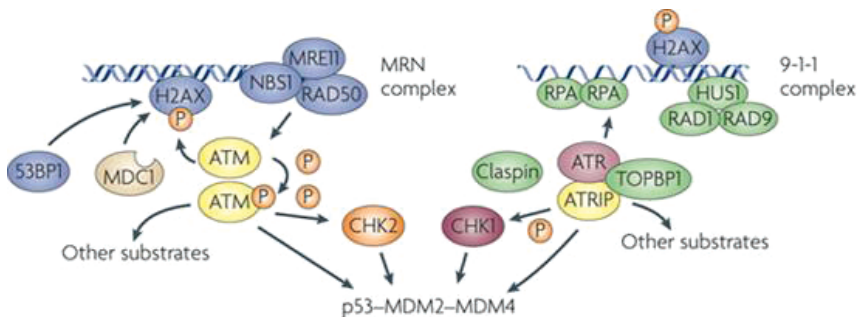


Figure 20. Activation of p53 by DDR. The figure illustrates some of the key events involved in DNA damage-induced activation of the ATM-CHK2 and ATR-CHK1 signalling pathways, and how they converge to activate p53 through direct and indirect effects. Adapted from [209].

4.4.3.2 p53-independent DDR outcomes

DNA repair

ATM and ATR have been shown to directly affect the activity of several players in the DNA repair machinery through their kinase activity. Examples include the DNA helicase Bloom syndrome protein 1 (BLM1), breast cancer associated gene 1 (BRCA1), histone H2AX, the replication fork associated mini chromosome maintenance (MCM) complex, as well as the MRN complex itself [210]. In addition, DNA-PKcs plays a direct role in non-homologous end joining (NHEJ) in response to DSBs (reviewed in [211]).

Cell cycle arrest

With respect to p53-independent DDR-induced cell cycle arrest, both the ATM-CHK2 and ATR-CHK1 pathways converge to inactivate CDC25 family members (Figure 21).

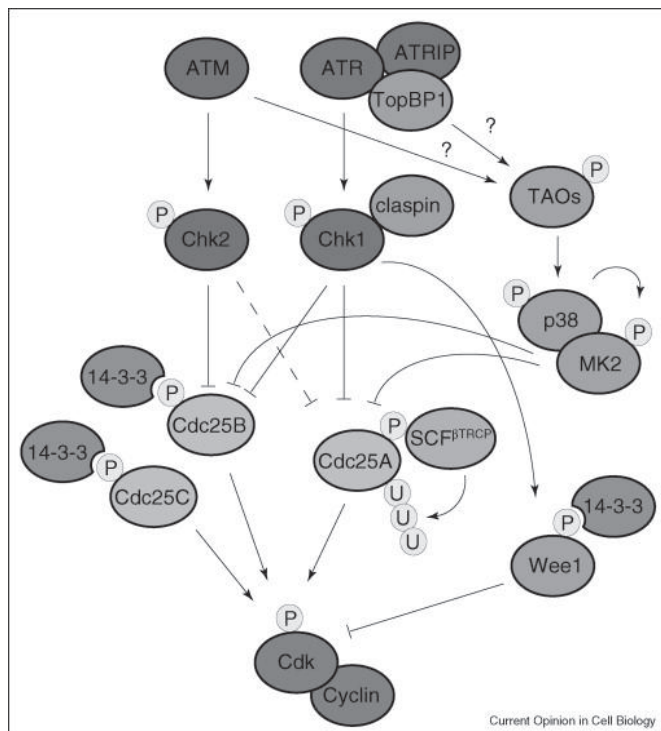


Figure 21. Pathways involved in p53-independent DNA damage-induced cell cycle checkpoints. The figure summarizes the various pathways involved in DDR kinase-induced inhibition of CDK-cyclin complexes. Adapted from [184].

As mentioned in section 4.2.3, CDC25 is a family of CDK-directed phosphatases, contributing to CDK activation through removal of inhibitory phosphate groups. CDC25A is an important substrate for CHK1 and possibly CHK2. Phosphorylation on S76 primes it for ubiquitination and subsequent proteasomal degradation [212], which can contribute to both a p53-independent G1 arrest [213], S-phase delay [214], and G2 arrest [215]. At the G2/M boundary CDC25B plays an important role in the activation of CDK1 which upon binding to cyclin B can further activate CDC25C, creating a positive feedback loop [216]. CDC25C can be phosphorylated by both CHK1 and CHK2 on S216, creating a binding site for 14-3-3 proteins [217-219], probably resulting in functional inactivation of the phosphatase by cytoplasmic sequestration [220;221]. Similarly, CDC25B is functionally inactivated by phosphorylation on S309 and S323 mediated by CHK1, CHK2 or p38MAPK/MK2 [184;222]. Additionally, CHK1 can phosphorylate WEE1 on S624 and thereby activate its function as an inhibitory CDK kinase [223;224].

4.4.4 Consequences of deregulated DDR

There are several diseases that result from germline mutations of components of the various DDR pathways. Depending on the exact role of the defect component, these diseases display phenotypes typically involving developmental defects, severe immunodeficiency, neurodegeneration, premature ageing, or cancer predisposition, and thus illustrate the importance of high fidelity genome maintenance for normal development and health [161]. One striking example is provided by mutations of the *ATM* gene, resulting in the autosomal recessive disorder ataxia-telangiectasia (A-T). Obvious clinical signs of the disease are cerebellar ataxia and oculocutaneous telangiectasias, which provided the disease with its name long before the mutated gene was recognized and termed accordingly. The ataxia is caused by progressive neurodegeneration principally affecting the Purkinje cells of the cerebellar cortex, which is probably caused by defective responses to DNA DSBs. In addition, the disease is characterized by immunodeficiency caused by the lack of resolution of DNA DSBs generated during V(D)J recombination, a process vital for generating the versatile repertoire of T cell receptors and immunoglobulin chains (see section 4.6.1). Both T and B lymphocyte precursors in these patients have a high frequency of abnormal hybrid joins which has occurred during inversion rearrangements, and this also predisposes them to develop lymphoid neoplasias. As expected, they also exhibit hypersensitivity to ionizing radiation (for a comprehensive review on A-T and ATM function, see [225]).

Although it is becoming increasingly clear that germline mutations and genetic polymorphisms play an important role in cancer predisposition, most of the accumulated changes driving the malignant phenotype in a fully developed cancer are the result of somatic mutations. Cancer cells generally display increased mutational frequencies and chromosomal aberrations indicating severe attenuation of the processes normally involved in ensuring the fidelity of the genome. Indeed, aberrations of the ATM-CHK2-p53 cascade and other DDR components are almost universal features of cancer cells. The selective forces driving such a DDR-defective phenotype are not yet completely delineated. However, emerging candidates are two anti-cancer barriers involving the DDR machinery: oncogene-induced DNA replication stress and telomere shortening. Both of these processes converge on DNA damage signalling and are proposed to be of major importance for the selection of mutations in the DDR pathway during carcinogenesis (reviewed in [161]).

4.5 p53

The discovery of p53 is attributed to two publications from 1979. First, D. P. Lane and L. V. Crawford reported a 53 kDa host protein which co-precipitated with the Simian virus 40 (SV40) large T antigen [226]. Soon after, D. I. Linzer and A. J. Levine reproduced this finding, and could also report that this protein could be found in uninfected carcinoma cells, implicating it as a tumour antigen independent of SV40-infection [227]. However, almost simultaneously the group of L. J. Old reported that antisera raised against a chemically induced mouse sarcoma recognized a 53 kDa protein in transformed, but not untransformed cells. In this report the name 'p53' appeared for the first time [228].

For several years after its discovery, p53 was regarded as a probable oncoprotein, as it was found overexpressed in many virally transformed cells and cell lines derived from human tumours [229;230]. This notion was strengthened when the mouse p53 cDNA was cloned in 1984 [231], as cells co-transfected with plasmids harbouring this p53 cDNA and the activated *ras* oncogene underwent transformation [232;233]. However, it later became clear that these initial p53 cDNAs contained mutations, and that wild type (wt) p53 was not able to cooperate with an activated *ras* gene to transform cells. Rather, wt p53 was found to suppress *ras* mediated transformation [234;235]. Analysis of tumour materials from cancers of various sites, showed frequent occurrence of mutations in the *TP53* gene, and in accordance with

Knudson's two-hit hypothesis for tumour suppressor gene inactivation, it was found that in many cases the non-mutated allele was lost [236]. In 1990, inherited *TP53* mutations were found to cause the familial Li-Fraumeni syndrome, characterized by a diversity of childhood and adult tumours [237;238]. In 1992 *TP53* KO mice were bred, and found to be viable and developmentally normal, but susceptible to spontaneous tumours [239]. Collectively, these findings firmly placed *TP53* not as a proto-oncogene, but as a tumour suppressor gene.

Simultaneously, functional studies shed light on how p53 could exert its function as a tumour suppressor, with the findings that wt p53 was induced upon DNA-damaging treatment and mediated subsequent cell cycle arrest and apoptosis [240-243]. Based on these findings, it was nicked-named 'The guardian of the genome' [244], a term which can still be regarded to grasp the essence of its function. Mechanistic studies into how p53 could exert its effects revealed that p53 was a sequence-specific transcription factor [245-249], and in 1994 the crystal structure of the p53 core domain interacting with DNA was solved [250].

4.5.1 An overview of p53 structure and function

p53 structure

The p53 protein consists of 393 amino acids, and is commonly divided into three functional domains: the N-terminal domain, the central core and the C-terminal domain (Figure 22).

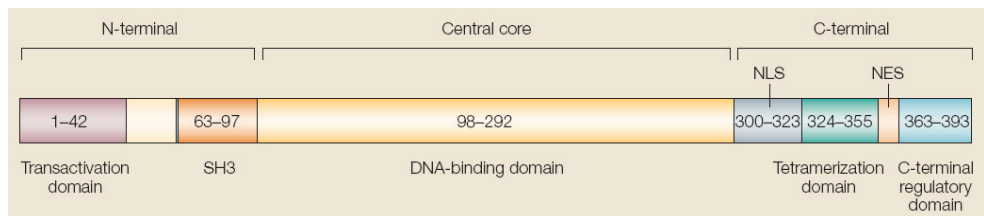


Figure 22. Outline of the p53 protein with its functional domains. The N-terminal part consists of a transactivation domain (TAD) and a Src homology 3-like (SH3) domain. The central core contains the DNA-binding domain (DBD), whereas the C-terminal end contains nuclear localization signals (NLS), a tetramerization domain, nuclear export signals (NES), and a regulatory domain. Adapted from [251].

The N-terminal domain contains the transactivation domain (TAD) which is required for transcriptional activation. The TAD also contains the interaction site with p53's negative regulator HDM2 (see section 4.5.2.1.1). The Src homology (SH3) domain is a proline-rich domain required for the interaction with SIN3, protecting p53 from degradation. The central core contains the sequence-specific DNA binding domain (DBD), and the interaction sites for

most of the p53 interacting proteins. The C-terminal domain contains nuclear localization signals (NLS) and nuclear export signals (NES) in addition to a tetramerization domain required for the oligomerization of p53 monomers. The end of the C-terminal domain (C-terminal regulatory domain) has a high content of basic amino acids thought to play a role in regulating the core DBD [252].

p53 transcriptional activity

p53 is a sequence-specific transcription factor with a target response element (RE) sequence composed of two 10-base decamers and a spacer as shown in figure 23. p53 binds this sequence as a dimer of dimers, in which each p53 subunit contacts three nucleotides of the RRRCW or WGYYY pentamer. This results in conformational changes both of p53 and of the target DNA. Additionally, it has become clear that p53 can also bind to and activate transcription of genes with non-canonical REs, such as half sites, three-quarter sites, and tetrameric REs. Approximately 150 p53 REs have so far been validated [253]. However, genome-wide approaches suggest this could be a gross underestimate of the real number of genes that might be regulated by p53 [254]. In addition, recent evidence indicates that p53 also controls the transcription of several miRNAs such as the miR-34 family (reviewed in [255;256]) and miR-192, -194 and -215 [257-259].

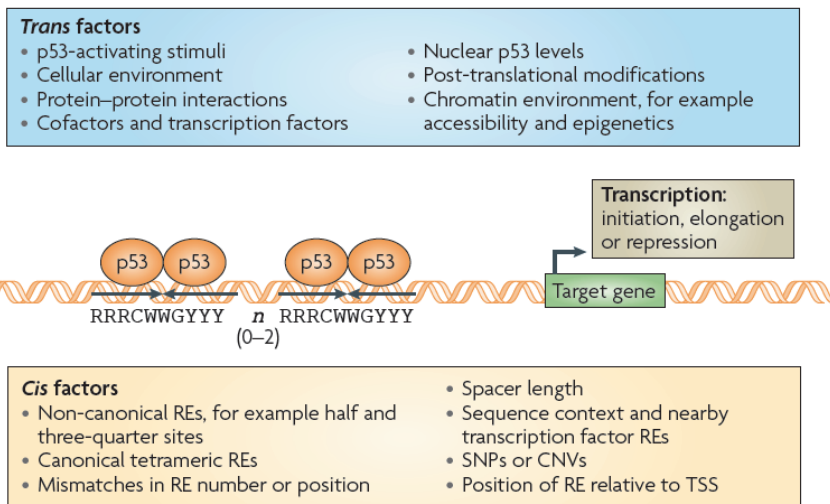


Figure 23. The accepted consensus sequence for p53 binding. The figure illustrates the accepted double decamer consensus sequence for p53 binding (R=purine, Y=pyrimidine, W=A or T, spacer < 3), with listed *trans* and *cis* acting factors that have been shown to affect binding and initiation of transcription. Adapted from [253].

p53 has also been shown to act as a transcriptional repressor. Less is known about the *cis* element requirements in these cases, and many downregulated genes have proposed target sequences that differ from the p53 binding consensus [260;261]. However, recent data indicate that a specific dinucleotide core combination within the CWWG consensus motif could be a key factor in determining whether p53 transcriptionally activates or represses a target gene, thereby identifying approximately 25% of the validated p53 REs to be repressive [262].

p53 non-transcriptional effects

The physiological role of p53 as a transcription factor is well established. However, as early as in the mid 1990's, reports indicated that non-transcriptional effects of p53 might also be of importance. Key findings were that trans-activation deficient mutants of p53 were able to induce apoptosis [263], and that p53-dependent apoptosis could proceed in the absence of RNA and protein synthesis [264]. These findings have later been confirmed and elaborated, and further indications of the importance of transcription independent effects of p53 on apoptosis have accumulated (reviewed in [265;266]). In addition p53 has been shown to have transcription-independent effects on autophagy and DNA repair [267;268].

4.5.2 The regulation of p53

The cell can regulate p53 function at several levels. Two well-established levels of regulation are the control of p53 expression and p53 transcriptional activity. In addition, it has recently become clear that p53 can be expressed as several different isoforms, adding further complexity to the regulation of its function. The latter topic will not be discussed in further detail here (for a recent comprehensive review, see [269]).

4.5.2.1 Regulation of p53 expression

To explain how p53 accumulates after genotoxic stress, the main focus has been on post-translational mechanisms, which certainly plays an essential role through stabilization of the otherwise labile p53 protein. However, there are also studies indicating a contribution of regulation on a transcriptional and post-transcriptional level.

Transcriptional regulation

In 1995, a study concluded that genotoxic insult imposed by different agents lead to an increase in *p53* mRNA levels caused by increased activity at a novel promoter element, termed the *p53* core promoter [270]. Although a κ B motif partly overlapped with this element, nuclear factor kappa B (NF- κ B) was not found to play a role in this process. This was later challenged by another report, stating that NF- κ B could indeed contribute to the regulation of *p53* gene expression after exposure to daunorubicin [271]. More recently, it was reported that *p53* itself is involved in a positive feedback loop whereby DNA damage-induced *p53* or *p73* can bind to a *p53* RE half-site, located at -121 to -112 (relative to the first transcription initiation site), to augment *p53* transcription. By silencing *p53* and *p73*, a significant suppression of *TP53* transcription was observed both under normal and stressed conditions [272].

Post-transcriptional regulation

There have been several reports on regulation of *p53* mRNA translation through different mechanisms. In 1995, it was reported that *p53* itself could bind the 5' untranslated region (UTR) of *p53* mRNA and inhibit its translation, forming a negative feedback loop [273]. In more recent years, it has been demonstrated that HDM2 can bind *p53* mRNA through its RING domain, promoting its translation. Moreover, cancer-derived single silent point mutations could weaken this interaction, providing a means of these mutations to suppress *p53* levels [274]. Another report implicated HDM2 in a more indirect manner by showing that the ribosomal protein L26 can bind *p53* mRNA and augment its translation. HDM2 was shown to bind L26 and prime it for degradation through ubiquitination in addition to attenuating its affinity to *p53* mRNA. This was shown to contribute to the low *p53* levels in unstressed cells. In response to genotoxic stress, the inhibitory effect of HDM2 on L26 was attenuated, enabling rapid increase in *p53* synthesis [275]. Yet another report focused on two internal ribosome entry sites (IRESs) in *p53* mRNA which can direct the translation of *p53* and its N-truncated isoform Δ N-*p53*. A polypyrimidine tract-binding protein (PTB) was shown to bind to both IRESs but with differential affinity augmenting translation of the respective isoforms. Upon doxorubicin treatment, PTB translocated to the cytoplasm facilitating *p53* mRNA translation and possibly contributing to the coordinated expression of the two isoforms [276].

MicroRNAs might also play a role in the post-transcriptional regulation of p53 as miR-125b can bind to a microRNA RE in the 3'UTR of *p53* mRNA, leading to repression of p53 protein levels [277]. Finally, *p53* mRNA stability has been shown to be regulated by a natural antisense transcript of p53 termed Wrap53 (WD40-encoding RNA antisense to p53). This transcript targets the 5' UTR of *p53* mRNA leading to its stabilization, and was found to both maintain basal *p53* mRNA levels and to play a role in stabilizing *p53* mRNA in response to DNA damage [278].

Post-translational regulation

Notwithstanding the existence of mechanisms involved in modulation of *TP53* transcription as well as *p53* mRNA stability and translation, regulation of p53 protein stability appears to constitute the major mechanism dictating p53 protein levels. Under unstressed conditions, p53 is a labile protein with a half-life of less than 30 minutes. However, upon the receipt of stress signals, the half-life of p53 is dramatically increased, allowing for accumulation of the protein. Key to regulation of p53 protein stability is the rate of proteasomal degradation controlled by p53 ubiquitination levels [279;280], an event that is under the control of E3 ubiquitin ligases.

4.5.2.1.1 HDM2

A central component in the p53 regulatory circuit is the HDM2 protein. The murine double minute 2 (*mdm2*) gene was identified in 1987 as one of three genes located on extrachromosomal amplifications in the spontaneously transformed murine cell line 3T3-DM [281]. The Mdm2 protein, termed HDM2 in humans, was later shown to bind to and inhibit p53 [282;283], and was soon recognized as a major negative regulator of p53. Indeed, *mdm2*^{-/-} mice display early embryonic lethality which can be rescued upon concomitant *Trp53* (transformation related protein 53) deletion [284;285], underscoring the importance of Mdm2-mediated regulation of p53, at least during embryogenesis. The negative regulatory effect of HDM2 on p53 has been ascribed partly to its ability to act as an E3 ubiquitin ligase for p53 [286], thus promoting its degradation [287;288], and partly to its function as an inhibitor of p53 transcriptional activity [289;290]. *In vivo* models, however, challenge the importance of HDM2 as a p53 transcriptional inhibitor, and favour the role as a p53 E3 ubiquitin ligase as its major physiological asset [291-293].

It has long been assumed that the accumulation of p53 after genotoxic stress is caused at least in part by the dissociation of the p53-HDM2 complex, leading to reduction in p53 ubiquitination and proteasomal degradation. Whether this is a universal mechanism of DNA damage-induced p53 stabilization, or whether additional factors are involved, is still a matter of debate [294]. Changes in HDM2-dependent ubiquitination of p53 can, in theory, be caused by modulation of its interaction with p53, its enzymatic activity, or its protein levels. There is evidence that regulation occurs at all these three levels.

Modulation of HDM2 – p53 interaction

Due to the proximity between residues 18-26 of p53 known to be essential for HDM2 binding, and several N-terminal serines and threonines shown to be phosphorylated upon DNA damage, it was hypothesized that N-terminal p53 phosphorylation might play a role in p53 activation by disturbing the p53-HDM2 interaction. In particular the ATM-target site S15 and the CHK2-target site S20 of p53 have been in focus, and early reports indicated that phosphorylation of these sites might indeed reduce the affinity of p53 for HDM2 [295-297]. However, these results were soon disputed, with a series of *in vitro* studies showing little or no effect of phosphorylation of p53 on S15 and S20, but instead pointing to T18 phosphorylation as a new candidate for disruption of the p53-HDM2 complex [192;193;298;299]. Phosphorylation of S15 might still be of significance as one study has indicated it as a prerequisite for T18 phosphorylation to take place [192]. The importance of these PTMs has further been investigated in mutant mice that express p53 with phosphorylation-deficient serine to alanine substitutions at S18 and S23 (equivalent to human S15 and S20, respectively). The phenotypes of mice with S18A, or S23A, or the double mutation S18,23A are generally mild, although some apoptotic defects have been observed [300-304]. Importantly, however, p53 protein stability after DNA damage is either not, or only marginally, affected by these mutations, supporting the above-mentioned *in vitro* studies that question the role of S15 and S20 phosphorylation in modulation of p53-HDM2 interaction. Mouse models investigating the effects of the human T18 equivalent are yet to be reported. Based on these and other studies, it has been suggested that p53 N-terminal phosphorylation might play a role under certain conditions and/or in certain tissues, but that p53 stabilization *in vivo* requires a far more sophisticated regulatory network than can be provided by p53 phosphorylation alone [305].

In addition to phosphorylation, acetylation of p53 has recently received attention as a mode of post-translational modification that affects p53-HDM2 interaction. p53 acetylation levels are markedly enhanced upon cellular stress. CREB binding protein (CBP)/p300 can acetylate the six C-terminal lysines of p53 [306]. In addition, two acetylation sites located in the DBD have been described, and it was demonstrated that acetylation of these eight different residues prevents the interaction of p53 with HDM2 [307]. Furthermore, as lysine acetylation and ubiquitination are mutually exclusive, acetylation of p53 on its C-terminal lysines has the additional effect of blocking some of the major p53 ubiquitination sites targeted by HDM2 [308-310].

The interaction between HDM2 and p53 can also be modulated by HDM2-interacting proteins. The *CDKN2A* locus gives rise to two unrelated proteins through the use of alternative reading frames: p16^{INK4a} and p14^{ARF}. p14^{ARF} is a predominantly nucleolar protein which is induced upon activation of oncogenes and serves as an important negative regulator of HDM2. This effect has partly been attributed to the ability of p14^{ARF} to interfere with p53-HDM2 interaction by binding to and sequestering HDM2 into the nucleoli [311], and partly to its ability to directly inhibit the E3 ubiquitin ligase activity of HDM2 [312;313]. In addition, p14^{ARF} can modulate the activity of other p53 ubiquitin ligases, such as ARF-BP1 [314]. Although it is not entirely clear which of these mechanisms of action of p14^{ARF} that plays the most important role in a given cellular context, p14^{ARF} has been convincingly shown to play an important role in p53 regulation [305]. As opposed to p14^{ARF}, the transcription factor Yin Yang 1 (YY1) can enhance the interaction between HDM2 and p53 [315].

Modulation of HDM2 E3 ubiquitin ligase activity

Similar to p53, HDM2 is the target of PTMs. HDM2 harbours several phosphorylation and acetylation sites, and modification of these sites appears to mainly affect its enzymatic activity as an E3 ubiquitin ligase. Phosphorylation of HDM2 on S166 and S186 residues promotes its activity, whereas phosphorylation on S395 and Y394 as well as acetylation events mediated by CBP/p300 inhibit its activity [316-318]. The enzymatic activity of HDM2 can also be modulated by interacting proteins like p14^{ARF}, as well as several ribosomal proteins such as L5, L11 and L23 [319-322].

Modulation of HDM2 levels

HDM2 is a relatively labile protein under unstressed conditions [323]. It is targeted for proteasomal degradation through ubiquitination, and has been suggested to be subject to autoubiquitination. However, mutations abolishing HDM2 E3 ligase activity do not affect HDM2 stability, indicating the need for alternative E3 ligases [293]. HDM2 ubiquitination is counteracted by the deubiquitinating enzyme HAUSP (herpesvirus associated ubiquitin specific protease) [324;325]. DNA damage induces phosphorylation of HDM2, resulting in HAUSP dissociation, accelerating HDM2 proteasomal degradation [208;323]. The consequent HDM2 destabilization has been demonstrated to be necessary for p53 activation [326]. This DNA damage-induced reduction in HDM2 levels is transient, as the increased p53 activity that follows leads to increased transcription of the *HDM2* gene [327]. Thus, in relation to the DNA-damage response, HDM2 levels are tightly regulated by multiple factors both at a transcriptional and post-translational level. The HDM2-related protein HDMX has also been suggested to affect HDM2 stability, but this role of HDMX remains controversial [328].

4.5.2.1.2 Other p53 E3 ubiquitin ligases

In addition to HDM2, other E3 ubiquitin ligases capable of targeting p53 have been identified. These include PIRH2, COP1 (constitutively photomorphogenic 1), and ARF-BP1/Mule (ARF-binding protein 1/MCL-1 ubiquitin ligase E3) [314;329-331]. Like HDM2, PIRH2 and COP1 are both p53 target genes and can therefore be part of a negative feedback loop involving p53. It is presently unclear exactly what role E3 ligases other than HDM2 play in regulating p53 levels in different cellular contexts. As many of the recently discovered p53 isoforms lack the binding site for HDM2, it has been suggested that these ligases might play an important role in the differential regulation of p53 isoform levels [279].

4.5.2.2 Regulation of p53 transcriptional activity

Early on in the studies of p53 transcriptional activity, it was envisioned that for p53 to bind to promoter regions in DNA in a sequence-specific manner, a stress signal resulting in a multitude of post-translational modifications was required. This view has later been challenged by observations that a significant portion of p53 is bound to DNA in unstressed cells [332;333], and that p53 is present at the promoters of its transcriptional targets during

normal homeostasis [334;335]. Recently, a model has been proposed in which p53 activation can be divided into three steps [305] (Figure 24).

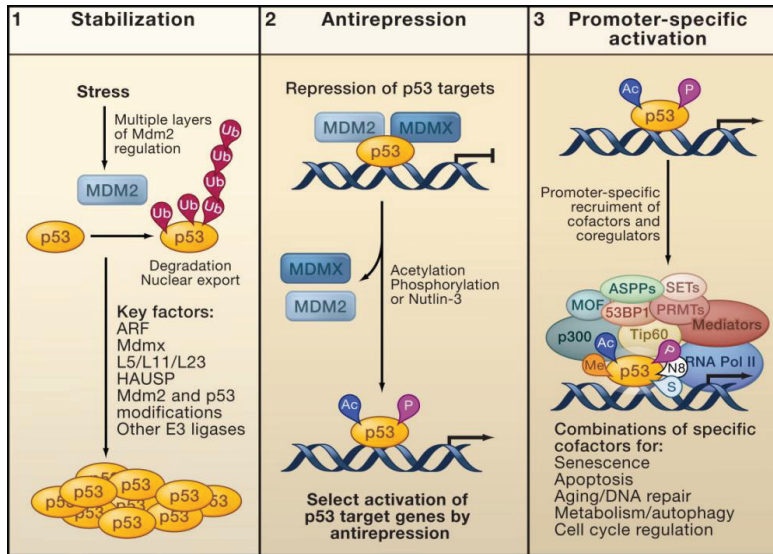


Figure 24. Three-step model for p53 activation in response to stress. (Ac=acetylation; P=phosphorylation; Me=methylation; N8=neddylation; S=sumoylation). Adapted from [305].

The first step involves stabilization of the otherwise labile p53 protein with subsequent accumulation through processes discussed above. This allows for increased amounts of p53 to bind DNA, but its transcriptional activity is actively repressed by HDMX (see below) and possibly HDM2. The second step involves anti-repression through removal of these repressors due to post-translational modifications of p53 and its repressors, enabling select activation of p53 target genes. The third step involves further post-translational modifications of p53 and recruitment of co-factors which direct the final cellular outcome through promoter-specific activation.

4.5.2.2.1 HDMX

The HDMX protein is structurally related to HDM2, with a strictly conserved p53 binding domain [336]. It also contains a conserved RING finger domain, but is not able to act as an E3 ubiquitin ligase towards p53. Rather, this domain is thought to be important for the interaction with HDM2 [337;338]. Transgenic mice models have shown that MdmX (the mouse equivalent of HDMX) acts *in vivo* as an essential, non-redundant, negative regulator of p53

during embryonic development [339-341], and similarly HDMX is assumed to be a crucial regulator of p53 in a variety of cellular settings. The mechanistic details underlying this regulatory effect still remain to be fully elucidated. HDMX has been implicated as a possible regulator of p53 protein stability, but recent evidence has cast doubts on such a role. Currently, it is thought that HDMX does not participate in the regulation of p53 stability independently of HDM2. Whether it does so in an HDM2-dependent manner remains unclear (reviewed in [328]). While the importance of HDMX as a regulator of p53 stability appears to be losing ground, there is accumulating evidence that HDMX is a crucial negative regulator of p53 transcriptional activity [294;342;343].

Transcriptional repression by HDMX and HDM2

HDMX has been demonstrated to interact with p53 at the promoters of specific p53 target genes [307], resulting in inhibition of their transcriptional activation. However, the mechanistic details of how the repression is performed are unresolved. HDMX has been shown to decrease p300-mediated acetylation of p53 [344;345], and this might contribute to its repressive effect. Like HDMX, HDM2 can form a protein complex with p53 at the promoters of its target genes [307;346;347]. The possible role of HDM2 as a transcriptional repressor of p53 is, however, still controversial [291-293].

Anti-repression

The release of p53 from repression by HDMX and HDM2 may occur by two different mechanisms. One involves the stress-induced phosphorylation and acetylation of p53 that alleviate its interaction with HDMX and HDM2. The other is brought about by post-translational modifications of HDMX and HDM2. Phosphorylation of HDMX inhibits its association with HAUSP, resulting in its accelerated turnover [208;323;348]. Similarly, post-translational modifications of HDM2 have been shown to result in increased HDM2 degradation [305]. It should be noted that although anti-repression of p53 might be sufficient to activate p53-dependent transcription at a select set of highly responsive targets, it is thought to be insufficient to induce many of the p53 targets involved in apoptosis [305].

Promoter-specific activation

To activate gene expression patterns involved in particular p53-driven cellular outcomes, it is envisioned that particular combinations of post-translational modifications involving acetylation, methylation, sumoylation, and neddylation, as well as recruitment of specific co-

factors might act as a barcode guiding the select activation of target genes [305]. For instance, acetylation of p53 is not required for transcription of *HDM2* or *PIRH2* involved in feedback and promotion of cell survival. Partial acetylation is sufficient for transcription of *CDKN1A* and *GADD45* (growth arrest and DNA damage 45) involved in transient growth arrest and DNA repair, whereas specific acetylation is required for transcription of pro-apoptotic genes [305]. As an example of the latter, acetylation at K120 occurs after DNA damage or activation of p14^{AFR}, and is essential for transcription of *PUMA* and *BAX* [349;350].

The ASPP (ankyrin-repeat-, SH3-domain- and proline-rich-region-containing proteins) family represents an example of co-factors that influence the promoter specificity of p53. This family consists of two pro-apoptotic members ASPP1 and ASPP2, and one anti-apoptotic member iASPP (reviewed in [351]). ASPP1 and ASPP2 promote binding of p53 to pro-apoptotic target genes such as *BAX*, *PUMA* and *PIG3* (p53-inducible gene 3) [352;353], whereas iASPP prevents the transcriptional activity of p53 bound to pro-apoptotic promoters [354].

4.5.3 Biological functions of p53

The p53 network is normally 'off'. It is activated only when cells are stressed or damaged [355]. Genes shown to be regulated by p53 can be grouped into various biological activities such as apoptosis, cell cycle regulation, senescence, energy metabolism, DNA metabolism, angiogenesis, immune response, cell differentiation, motility and migration, cell-cell communication, and most recently stem cell control (reviewed in [253;356;357]). Among these, only apoptosis and senescence have been directly implicated in mediating the effect of p53 as a tumour suppressor, and these will be discussed in more detail below.

4.5.3.1 Apoptosis

p53 can act at multiple levels of both the intrinsic and extrinsic apoptotic pathways through induction of pro-apoptotic and suppression of anti-apoptotic target genes. In addition it can contribute to the execution of apoptosis through non-transcriptional mechanisms.

Transcription-dependent induction of apoptosis

p53 induces the transcription of a vast number of genes involved in the positive regulation and execution of apoptosis. These include members of the BCL-2 family (BAX, BID, PUMA, and

NOXA), components of the core apoptotic machinery (APAF-1, caspase-8, and caspase-6), components of the extrinsic apoptotic pathway (DR5, FAS, TNFSF10, TNFSF6), and other less-defined factors (AEN, p53AIP, PERP, and PIG3). In addition, p53 can contribute to the transrepression of anti-apoptotic factors such as BCL-2, survivin, ARC, and gelactin-3 [358]. The relative relevance of these and other factors to the apoptotic response unleashed by p53 activation in a given cellular setting is not determined and probably varies according to cell type and cellular context. Even though p53 activation clearly can contribute to the execution of apoptosis through the extrinsic pathway, it is generally assumed that cells that have engaged p53-dependent apoptosis typically follow the intrinsic apoptotic pathway [359].

Of the above mentioned p53 target genes, *PUMA* (p53 up-regulated modulator of apoptosis) has recently received much attention as an important mediator of p53-induced apoptosis, as it in many instances seems to be able to account for virtually all the pro-apoptotic activity of p53. *PUMA* is highly conserved between human and mouse [360], and is also conserved and serves similar functions in more distant vertebrates such as zebrafish [361]. No *PUMA* homologue has been identified in lower eukaryotes, but the BH3-only protein Egl-1 might be its functional counterpart in *C. elegans* [362]. *PUMA* is normally expressed at very low levels [360], but is rapidly induced upon stress. This induction seems to depend solely upon transcriptional activation of *PUMA*. Although the *PUMA* gene promoter has binding sites for multiple transcription factors, studies have indicated that p53 and the p53 binding sites in the *PUMA* promoter are indispensable for its induction by DNA damage [360;363]. *PUMA* has been shown to be essential for p53-dependent apoptosis in a number of cell culture systems [363-367]. In mice, *Puma* is induced in a p53-dependent fashion in tissues such as neurons, thymocytes, haematopoietic cells, spleen and intestinal crypts, and *Puma* KO mice are highly protected from ionizing radiation (IR)-induced apoptosis in all of these tissues [368-371]. These apoptotic defects resemble those observed in *Trp53* KO mice [243;372]. Furthermore, the p53-dependent *PUMA* induction has been demonstrated to be the major mechanism resulting in IR-induced apoptosis in hematopoietic and intestinal stem cells and progenitor cells, contributing significantly to the resulting tissue injury [371;373]. Collectively, these findings place *PUMA* as an essential mediator of p53-dependent apoptosis.

Transcription-independent induction of apoptosis

A fraction of stress-stabilized p53 rapidly translocates to the outer mitochondrial membrane [374;375] where it has been found to interact with several members of the BCL-2 family. By

interacting with and neutralizing BCL-X_L and BCL-2, it can act analogously to a sensitizer BH3-only protein [376;377]. Further it can act analogously to an activator BH3-only protein by binding to BAK and thereby relieving it from MCL-1 inhibition [376;378], and by activating BAX through transient interaction [125]. Even though this mitochondrial action of p53 can proceed independently of its nuclear action, there are also suggestions that the two pathways might cooperate, with p53-induced PUMA being able to release cytoplasmic p53 that is sequestered by BCL-X_L, thereby allowing it to migrate to the mitochondrion [365]. Indications of the *in vivo* relevance of these findings come from whole body IR of mice. In radiosensitive organs, there is a rapid translocation of p53 to the mitochondria that triggers an early wave of apoptosis which is subsequently followed by PUMA induction and a second wave of apoptosis. Lack of p53 accumulation at the mitochondria after IR correlates with cell cycle arrest and the complete absence of apoptosis [379].

4.5.3.2 Cell cycle arrest and senescence

Whereas cell cycle arrest is a reversible process, senescence is by definition a cellular state of irreversible growth arrest. Senescence was originally described in relation to the observed limited proliferation potential of normal cells experiencing accumulating telomere erosion with increasing cell divisions. Such erosion will beyond a critical limit trigger a DNA damage response with subsequent activation of p53, resulting in growth arrest [380;381]. p53-induced senescence can also be initiated by non-telomeric signals such as oncogene activation [382] and DNA damage caused by other events than telomere shortening [383;384].

Central p53 target genes implicated in cell cycle arrest are *CDKN1A* (encoding p21^{Cip1}), *GADD45* and *14-3-3* [385]. p21^{Cip1} is an inhibitor of CDK2-cyclin E activity, whereas *GADD45* and *14-3-3* inhibit CDK1 activity. In addition, p53 can repress the expression of cell cycle progression genes such as *CDK4* and *CCNE2* (encoding cyclin E2), as well as cell cycle phosphatases such as *CDC25A* and *CDC25C* [386-388]. The induction of p21^{Cip1} is also important in p53-induced senescence, but the factors determining the cell's decision between transient and permanent cell cycle arrest remain elusive. One possible mechanism is that the resolution of DNA damage by successful repair will inhibit p53 – p21^{Cip1} signalling, whereas irreparable/sustained DNA lesions or other stimuli leading to sustained p53 and p21^{Cip1} activation will maintain a senescent phenotype [162]. Compared to the relatively well-defined sequence of events involved in p53-mediated apoptosis and cell cycle arrest, the details in

p53-mediated senescence are only partially understood. Beyond the need for p21^{Cip1} induction, *MIC-1* (macrophage inhibitory cytokine-1) has been identified as a p53-responsive gene involved in senescence. MIC-1 is a cytokine of the TGF- β family which might propagate a senescence programme by autocrine or paracrine induction [389]. Also, p53 can stabilize *PAI-1* (plasminogen activator inhibitor-1) mRNA through direct binding [390], thereby facilitating increased translation of PAI-1, an established marker of senescent cells [391;392].

4.5.4 p53 and cancer

Even though p53 has formally been recognized as a tumour suppressor for many years, several aspects regarding how this task is being performed are still unresolved. This is perhaps not surprising taking into account all the different stimuli that can elicit a p53 response, the vast number of genes that can be regulated by such activation, and the many biological endpoints that can ensue (Figure 25). What is known is that there is strong selection against the p53 pathway in almost all cancers, irrespective of cell type or the underlying oncogenic mechanism [4]. Examples of questions that are yet unanswered are: how p53 discriminates between normal and tumour cells; what signals drive selection for loss of p53 function in cancers; when during tumourigenesis p53 is crucially triggered; and which of the many biological functions of p53 are required to forestall tumourigenesis [4].

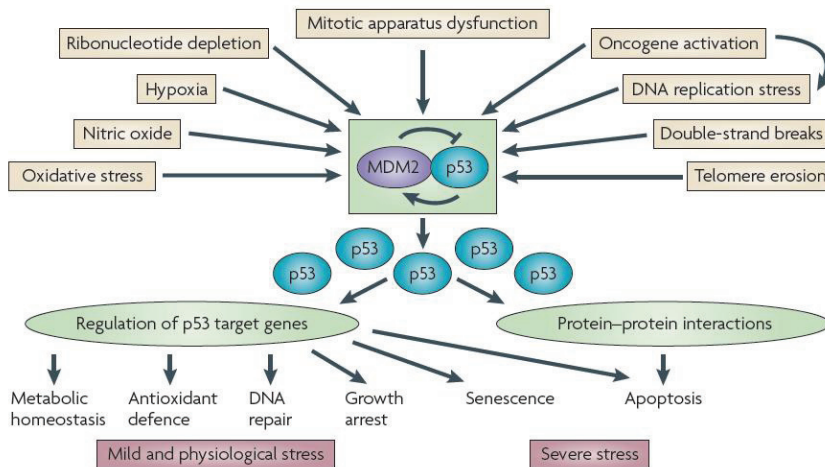


Figure 25. A simplified view of p53 signalling. The figure provides an overview of the various stress factors that affect the p53-HDM2 feedback loop leading to accumulation of p53, with consequent transcriptional and non-transcriptional downstream events leading to different outcomes for the cell depending on the severity of stress. Adapted from [393].

4.5.4.1 Transformation-related activation of p53

With regards to the signalling pathways involved in activating p53 in response to malignant transformation, there have been two leading and contesting theories. The first is the activation of p14^{ARF} in response to activated oncogenes, the second is activation of the DDR pathways.

p14^{ARF}

p14^{ARF} is activated specifically in response to oncogenic signalling [394]. This is a persistent and obligate feature of tumour cells and could therefore be regarded a tumour-specific p53-activating pathway. Several mouse models have recently provided support for an essential role for p14^{ARF} (p19^{ARF} in mice) in p53-mediated tumour suppression, seemingly at the expense of a possible role of the DNA damage pathway. G. I. Evan's group developed mice in which the endogenous *Trp53* was substituted with an inducible p53 (p53ER^{TAM} induced by the administration of 4-OHT) [395]. Mice were divided into two groups, having their p53 activated or not during whole body IR. The p53 status during irradiation did not affect the occurrence of lymphoma in these mice. Activating p53 a while after exposure to IR, on the other hand, did confer protection, but this was dependent on the presence of p19^{ARF}. An inverse approach was employed by L.A. Donehower's group, using mice in which a timed excision of the *Trp53* gene could be performed, also demonstrating that the p53 status at the time of IR had no impact on tumourigenesis [396]. Yet a different approach utilized by J. Serrano's group yielded a similar conclusion. They used 'super-p53' mice harbouring an extra transgenic copy of *Trp53*. These mice showed superior tumour suppression compared to their diploid counterparts, but this protection was lost in an *Arf*-deficient background [397]. Even though these studies provide compelling evidence for the *in vivo* role of ARF and seemingly cast strong doubts on the possible role of DNA damage signalling, two matters of concern have been raised as to their generalizability and interpretation. First, there is the matter of difference between mice and men. Mice lacking p19^{ARF} are highly tumour prone [398], and spontaneous inactivation of *Cdkn2a*^{ARF} is common during tumour progression [399-401]. In humans, tumour-related mutations at the *CDKN2A* locus target mainly p16^{INK4A} and not p14^{ARF} [402;403], raising the possibility that p14^{ARF} might play a more central tumour-suppressive role in mice than in humans. Second, it can be argued that the effect of short-lived, intense DNA damage induced by IR cannot necessarily be compared to the more prolonged and sustained activation of DNA-damage pathways that have been observed in different stages of tumourigenesis.

DDR pathways

Many studies support the notion that DNA damage checkpoints respond to oncogene activation. Analyses of early-stage human tumours show that cells in the earliest precursor lesions show constitutive activation of DNA damage signalling pathways in the absence of signs of chromosomal instability or *TP53* mutation [404-408]. This is in contrast to highly proliferative normal tissues, suggesting that it is a tumour-specific phenomenon. Experimental models have also supported these findings, demonstrating that checkpoint proteins are activated upon controlled increase of oncogene expression in cultured cells [406], and during induction of hyperplasia in human skin xenografts [408]. Signs of DNA damage signalling are also found in more developed and malignant tumours, but they are often lost in advanced tumours, suggesting perhaps a selective pressure to eliminate components of this pathway. Mechanistically, it is proposed that oncogenes, leading to untimely activation of CDKs, might lead to impaired or inappropriately activated origins of replication resulting in DNA replication stress. In support of the notion that oncogene-induced DNA damage is replication-associated, studies have demonstrated that if replication is enforced through the expression of cyclin E or H-Ras, the markers of DNA damage do not appear if the cells are blocked from entering S phase [404;405].

4.5.4.2 Outcomes in p53-mediated tumour suppression

As previously mentioned, the long list of biological outcomes affected by p53 signalling contains several candidates that might be implicated in p53-mediated tumour suppression. However, thus far apoptosis and senescence are the only outcomes that have directly been shown to contribute to this function of p53, and the relative importance of the two probably partly depends on the cell type giving rise to a given tumour. For instance, lymphocytes are known to be very prone to undergo p53-dependent apoptosis, and upon reactivation of p53 in mice with *E μ -myc*-driven lymphomas, rapid and extensive tumour regression due to apoptosis has been described [409-411]. In models of murine sarcomas and hepatocellular carcinomas, on the other hand, p53 restoration induced senescence with consequent tumour regression due to immune clearance [411;412].

4.5.4.3 Modes of p53 inactivation in cancer

p53 mutations

Somatic *TP53* mutations are common in most human cancers, ranging from 5 to 80% depending on type, stage, and aetiology of tumours [413]. Figure 26 shows the prevalence of *TP53* mutations according to tumour site. As mentioned previously, the erroneous use of cDNA from mutant p53 for *in vitro* transfection studies in the 1980's led to the misleading initial conclusion that *TP53* was a proto-oncogene. On the positive side, however, it provided an early clue to the potential of mutant p53 to promote cancer.

TP53 stands out from most other classical tumour suppressor genes with respect to its spectrum of mutations in cancer material. Whereas the classical finding is biallelic inactivation by deletions or truncating mutations, *TP53* most frequently (74%) presents with single monoallelic missense mutations resulting in the formation of a stable full-length protein, often found massively accumulated in cancer cells. The majority of these missense mutations are mapped to the DBD, usually resulting in the abrogation of sequence-specific DNA binding activity [414] (Figure 27).

This has led to speculations that rather than resulting in mere 'loss-of-function', *TP53* mutations might contribute to cancer development through 'gain-of-function' or through dominant-negative effects on the remaining wt p53. Intense studies on the effects of *TP53* mutations both *in vitro*, *in vivo* in mouse models, and from clinical data has not yet clearly concluded how *TP53* mutations exert their tumour-promoting effect (reviewed in [414]). This likely reflects a very complex picture in which 'loss-of-function', 'gain-of-function', and dominant-negative effects might all play a role, perhaps to varying extents depending on the type of *TP53* mutation and the general genetic make-up of the specific tumour in question. In addition to questions regarding the functional role of *TP53* mutations in tumour development, there has been considerable interest in possible effect of *TP53* mutations on cancer prognosis and prediction of treatment response. Early studies using immune histochemistry, in which high levels of p53 in a tumour was interpreted as a sign of the presence of mutated *TP53*, yielded conflicting results. However, more recent studies based on gene sequencing, seem to suggest a general trend in which *TP53* mutations are associated with poor overall and disease-free survival, as well as with poor drug response [415].

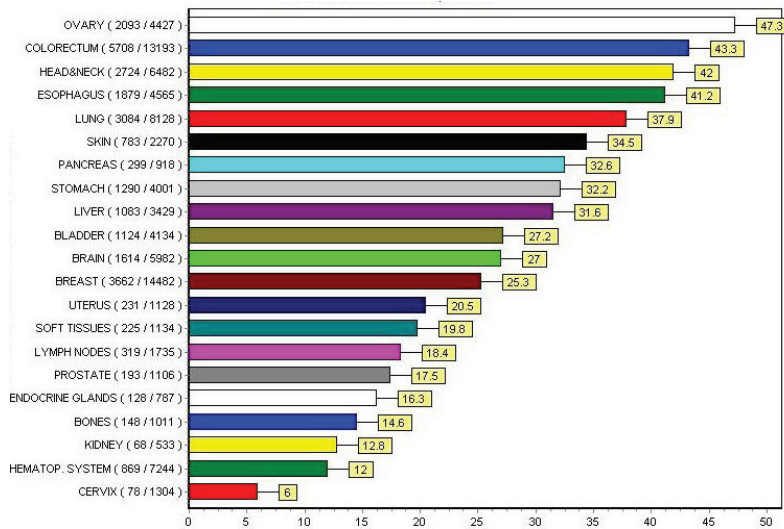


Figure 26. The prevalence of *TP53* mutations by tumour site. The figure shows how the prevalence of *TP53* mutations differ greatly between cancers with the highest prevalence found in ovarian cancer and the lowest prevalence found in cervical cancer. Adapted from [416].

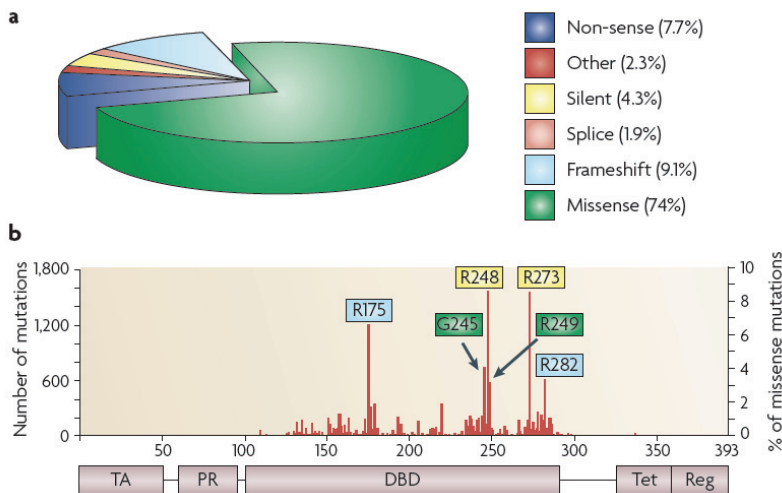


Figure 27. The type and location of cancer-related *TP53* mutations. (a) The pie diagram shows the distribution of cancer related *TP53* mutations according to the type of mutation, with missense mutations being most frequent. (b) The figure illustrates the location of cancer-related *TP53* mutations with the vast majority occurring in the DNA binding domain (DBD), many of which localize to one of six mutational hot spots. Adapted from [414].

Deregulation of wt p53

In spite of the common occurrence of p53 mutations in human tumours, a high proportion still retains wt p53, and it is generally assumed that these tumours must employ strategies to keep their p53 activity in check. Examples of such strategies include HDM2 amplification and overexpression, suppression of p53 by viral products, and loss of p14^{ARF}.

HDM2 is found amplified in 10% of all cancers with the highest incidence of 67% in Hodgkin's lymphoma. In addition, it is found overexpressed in the absence of amplification in many tumours (reviewed in [417]). Specifically, in paediatric ALL, the majority of which retain wt p53, overexpression of HDM2 is a common event [418].

One example of virus-induced deregulation of p53 is found in cervical cancer which is almost always associated with infection with one of several high-risk human papilloma viruses (HPV). High-risk HPVs encode a viral protein, E6 that recruits the cellular ubiquitin ligase E6-associated protein (E6AP), facilitating p53 polyubiquitination and proteasomal degradation [419-423]. Due to the strong association between development of cervical cancer and HPV infection, it is not surprising that cervical cancers have the lowest frequency of p53 mutations when tumours are classified according to site (Figure 26). Other DNA viruses have also been shown to express proteins that can interfere with p53. Examples include SV40 [424], adenovirus [425], and hepatitis B virus (HBV) [426-428]. HBV infection is a well-established risk factor for hepatocellular carcinoma. SV40 infection can cause an array of rodent tumours, and traces of SV40 infection can be found in many human tumours. Whether there is a causal link is uncertain, but findings seem to implicate SV40 at least in the development of mesothelioma [429]. The retrovirus human T cell lymphotropic virus type 1 (HTLV-1), has also been shown to interfere with p53 signalling [430;431], and this virus is a causative factor in the development of adult T cell leukaemia.

Deletion of *CDKN2A* leads to the loss of p14^{ARF}. This unleashes HDM2 activity, resulting in untimely p53 degradation. Such deletions are observed in cancers of various locations such as breast, brain and lung, and are especially frequent in the absence of *TP53* mutation [355].

Polymorphisms in the p53 pathway

Inherited variations in genes of the p53 pathway can theoretically affect p53 levels or activity in response to transformation to influence cancer susceptibility, and later in response to DNA-damaging treatment regimens, to affect therapy response and disease outcome.

With respect to the *TP53* locus itself, numerous single nucleotide polymorphisms (SNPs) and other sequence variations have been identified (reviewed in [432]). The best described is a non-synonymous SNP in the *TP53* coding region at codon 72 located in the SH3 domain of p53, encoding either proline (p53-P72) or arginine (p53-R72). The current view is that p53-R72 is more efficient than p53-P72 at inducing apoptosis and protecting stressed cells from neoplastic development. This has led to the hypothesis that individuals carrying the p53-R72 allele might be protected against cancer relative to individuals carrying the p53-P72 allele. Several studies evaluating the effect of the codon 72 polymorphism on cancer susceptibility have indeed reported statistically significant differences. However, the size and design of these studies have been criticized, suggesting that much larger studies than those undertaken will have to be performed to ascertain a polymorphism-dependent cancer risk difference [432].

In *HDM2*, a T to G SNP at nucleotide 309 in the first intron has been the most intensively characterized [252]. Within this intron lies an estrogen receptor-sensitive promoter/enhancer region for *HDM2*, and the G allele has been shown to increase the affinity to the transcription factor specificity protein 1 (SP1), resulting in increased transcription and expression of *HDM2* [252]. Some clinical studies have demonstrated that the presence of the G allele is associated with lack of response to therapy, poorer survival and outcome in various cancers (reviewed in [433]). Regarding cancer susceptibility, several studies have demonstrated an earlier age of onset or an increased susceptibility to breast cancer in premenopausal women carrying the G allele [252;434;435], whereas other studies show no association [432]. Also, in patients with Li-Fraumeni syndrome, the G allele has been associated with accelerated tumour formation [252;436-438].

4.5.4.4 Prospects for p53 based therapy

The principle of so-called ‘targeted cancer therapy’ has gained substantial interest with the expanding universe of known aberrations in cancer on the molecular level. The most

successful attempts thus far have been directed towards cell surface molecules which can be targeted by antibodies, or enzymes that can be targeted by small molecular enzyme inhibitors. A variety of such compounds are already in common clinical use. As p53 is neither a surface molecule nor an enzyme, the design of drugs aiming to target it has proven more difficult. Two main approaches have been attempted: the use of p53 gene therapy and the use of small molecular compounds with the ability to restore p53 activity.

p53 gene therapy

Regarding gene therapy, there have been two main approaches. The first is delivery of *TP53* via an adenoviral vector. One compound is already in clinical use against head and neck cancer in China [439], whereas another is in phase III clinical trials [440]. An alternative approach is the use of an E1B-deficient lytic virus which can only replicate in p53-deficient cells, thus providing selective cytolysis to p53-deficient tumour cells [441]. This compound has yielded promising results in clinical trials [393].

Small molecular modulators of p53

Regarding small molecular compounds aiming to restore p53 activity, several different strategies have been employed to identify promising candidates. The mechanism of action of these compounds can be divided into three main categories: 1) reactivation of mutant p53, 2) activation of wt p53, and 3) cyclotherapy (for a recent comprehensive review, see [442]). One example of a compound capable of reactivating mutant p53 is PRIMA, which has been shown to interact with mutated p53, causing a change in conformation which can restore wt function [443]. Examples of compounds capable of activating wt p53 are nutlins, MI-219, RITA and tenovins. Nutlins and MI-219 are designed to bind to the p53-interacting surface on HDM2, thereby inhibiting p53 degradation [444;445]. RITA is a compound which has also been found to inhibit p53-HDM2 interaction; however, it appears to do so by binding p53 rather than HDM2 [446]. Tenovins are inhibitors of SIRT1 and SIRT2 deacetylases, preventing p53 deacetylation and thereby promoting p53 stabilisation and transcriptional activity [447]. Whereas activation of p53 is potentially cytotoxic to tumour cells retaining wt p53, it can be used in a protective manner during treatment of tumours harbouring mutated p53. This is the principle behind the concept of cyclotherapy, in which normal cells are placed in a temporary cell cycle arrest by inducing p53 using non-genotoxic drugs such as nutlins. As the cancer cells harbouring mutated p53 would not be affected by this treatment, they continue cycling, and are rendered sensitive to treatment with mitotic inhibitors as opposed to the arrested

normal tissue [442]. In this way, the therapeutic window of the mitotic inhibitor is increased. The clinical potential for the use of small molecular modulators of p53 activity is yet to be determined, as the above-mentioned compounds are all still in the preclinical stages of investigation or in early clinical trials [442].

4.6 B lymphocytes

B (bursal or bone marrow-derived) lymphocytes can be defined as “a population of cells that express clonally diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes” [448]. Along with their close relatives, the T (thymus-derived) lymphocytes, they constitute the major players in our adaptive immune system, and their origin can be traced back to the evolution of adaptive immunity in jawed vertebrates more than 500 million years ago [449]. As implied by its name, the initial development of the B lymphocyte takes place in the BM, which is hence termed a primary lymphoid organ. The immature B lymphocyte is then released into the blood stream, and can upon encounter with an antigen undergo functional maturation in a secondary lymphoid organ such as a lymph node, the spleen or mucosa-associated lymphoid tissues (MALT). The antibody-secreting plasma cell represents the terminally differentiated B lymphocyte, and is the functional endpoint of B lymphocyte development.

4.6.1 B lymphocyte development in the bone marrow

The process of haematopoiesis starts with the haematopoietic stem cell (HSC) which is present within a population of BM cells expressing CD34 [450]. HSC gives rise to a lymphoid-restricted cell termed the common lymphoid progenitor (CLP). CLP is assumed to be CD7⁺CD10⁺, but the phenotype of this cell is not firmly established [451]. In addition to B and T lymphocytes, the CLP can give rise to natural killer cells (NK) and dendritic cells (DC) (Figure 28).

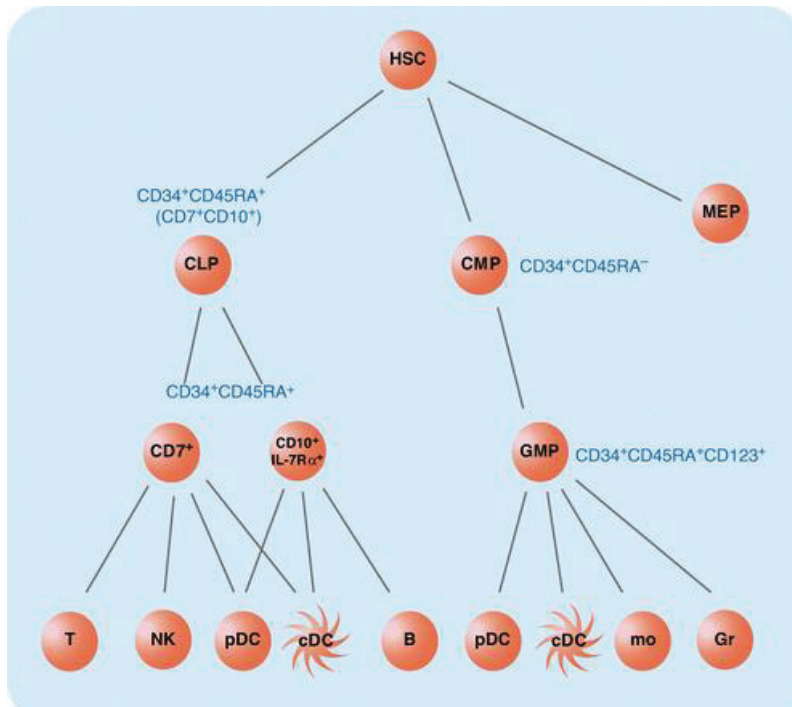


Figure 28. Model for haematopoietic development from the haematopoietic stem cell. HSC: haematopoietic stem cell; CLP: common lymphoid progenitor; T: T lymphocyte; NK: natural killer cell; p/cDC: plasmacytoid/conventional dendritic cell; B: B lymphocyte; CMP: common myeloid progenitor; GMP: granulocyte monocyte progenitor; mo: monocyte; Gr: granulocyte; MEP: megakaryocyte erythrocyte progenitor. Adapted from [451].

The current consensus for the BM development of B lymphocytes is that the B lineage-restricted cell pass through the following stages: early B, pro-B, large pre-BI, large pre-BII, small pre-BII, immature B, and mature B [451] (Figure 29). All B lineage-committed cells prior to immature B lymphocytes are commonly referred to as B cell precursors (BCPs) [452].

An important event during early B lymphocyte development is the formation of the B cell receptor (BCR). Crucial events are recombinations at the variable regions of the heavy (V_H) and light (V_L) chain Ig genes, which lay the basis for generation of primary antibody diversity. Each V_L region is composed of many different long variable gene segments (V) and short joining gene segments (J). The V_H region is similarly constructed, but with an additional diversity gene segment (D). By random recombination events termed V(D)J recombination, one of the many V and J gene segments of the light chain regions, and one V, D and J gene segment in the heavy chain region are combined. This results in a combinatorial

diversification which greatly contributes to antibody diversity. The mechanistic background for V(D)J recombination lies in an enzymatic complex termed RAG which is transcriptionally switched on in two waves corresponding to V_H and V_L rearrangement during B lymphocyte development (Figure 29). RAG functions as an endonuclease, inserting double strand breaks at specific recombination signal sequences between the gene segments. It then initiates rejoining by recruiting enzymes involved in NHEJ. As NHEJ is an error prone process in which a variable number of nucleotides might be lost or inserted, this further contributes to the process of diversification of the V_H and V_L gene regions.

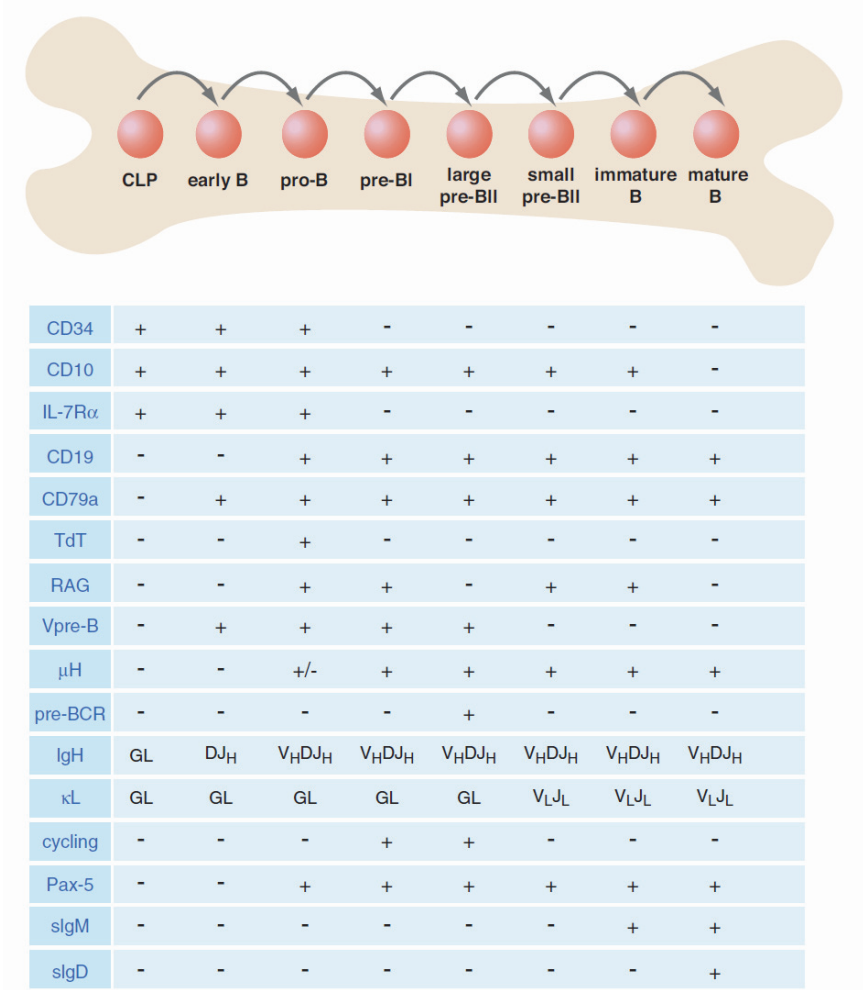


Figure 29. Bone marrow stages of B lymphocyte development. Overview of the different stages of early B lymphocyte development with expression of cell surface markers, enzymes and the state of V(D)J rearrangement. Adapted from [451].

As rearrangement of the V_H locus completes in pro-B lymphocytes, the cells start producing their μ heavy chain, but it is not expressed on the cell surface until the large pre-BII stage. As the recombination at the V_L locus is not completed in these cells, μ associates with a surrogate light chain, forming the pre-BCR. Pre-BCR probably signals through ligand-independent oligomerization [453], and the expression of pre-BCR with subsequent signalling events is a prerequisite for further B lymphocyte development [454]. At the small pre-BII stage, V_L recombination completes, making possible the expression of the true BCR at the immature B stage. Initially, only surface IgM (sIgM) is expressed, but by alternative splicing events of the constant region of the heavy chain (C_H), sIgD is later coexpressed, marking the development into a mature B lymphocyte.

4.6.2 Functional maturation of B lymphocytes

Immature B lymphocytes can respond to certain types of antigens such as lipopolysaccharides in a T lymphocyte-independent manner. This results in differentiation into an IgM-secreting plasma cell, eliciting a rapid but relatively low affinity antibody response [455]. Most mature B lymphocytes reside within secondary lymphoid organs where they encounter and respond to antigens presented by DCs in a T lymphocyte-dependent manner. Such antigenic stimulation results in proliferation and differentiation into IgM-secreting plasma cells. In addition, some of the stimulated cells will enter the germinal centre (GC) where they differentiate into a rapidly proliferating state termed centroblasts. In the GC, the processes of affinity maturation and class switching takes place, greatly increasing the potency of the antibody response towards the antigen in question.

Affinity maturation is the result of an increased mutation rate occurring in the V region coding sequence, termed somatic hypermutation (SHM). Centroblasts undergo repeated cycles of SHM, and only those cells that subsequently express BCRs with increasingly better affinity for the presented antigen will receive survival stimuli, the remaining cells being removed by apoptosis. Thus a clonal selection of the B lymphocytes developing the most efficient antibody takes place. Class switching is a process in which the original IgM and IgD expression is replaced by IgA, IgE or IgG expression. As opposed to the change between IgM and IgD which is a result of alternative splicing of transcripts from unaltered germline DNA, class switching involves genomic recombination, and is thus referred to as class switch

recombination (CSR). During CSR, specific switch sequences flanking the various C regions ensure the deletion of intervening DNA sequences. This results in the joining of the VDJ region to a new C region, encoding the α , ϵ or γ heavy chain instead of the μ or δ heavy chain. Due to functional differences between the different Ig heavy chains, class switching result in a more potent antibody response compared to what is initially achieved through IgM and IgD production.

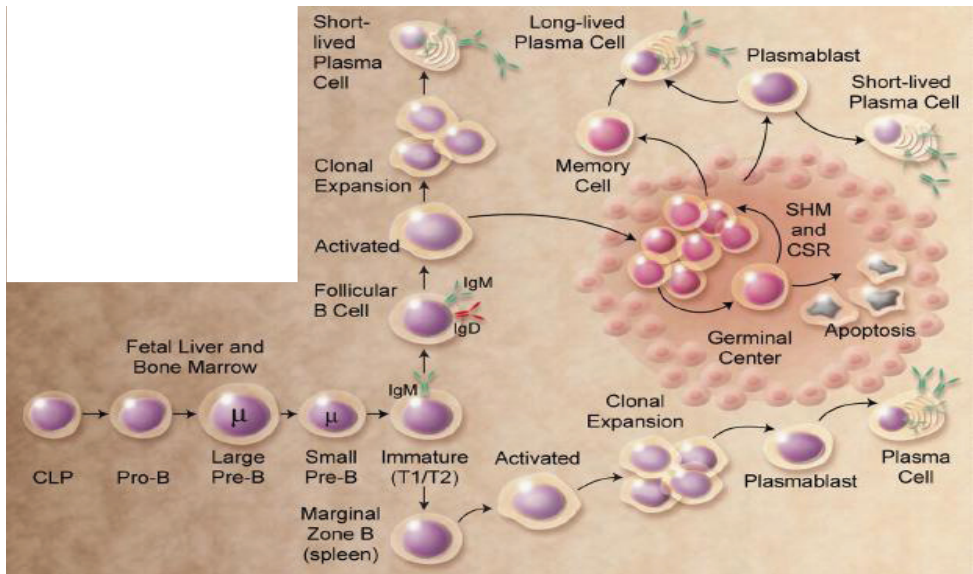


Figure 30. B lymphocyte development, activation and maturation. The figure summarizes the development of B lymphocytes in the bone marrow from the CLP to the immature B lymphocyte which is released into the circulation. There, it can be activated directly in response to certain antigens, or develop into a mature B lymphocyte residing in secondary lymphoid tissues. The mature B lymphocytes will upon encounter with an antigen partly give rise to plasma cells that produce the initial IgM response, and partly move to the germinal centre where they undergo affinity maturation by SHM and CSR, resulting in the delayed IgG/IgE/IgA response and the generation of memory cells providing long-term protection. Modified from [448].

Some of the B lymphocytes successfully surviving the process of clonal selection during a GC response will develop directly into effector cells in the form of Ig-secreting plasma cells. These plasma cells will secrete antibodies with increased affinity and altered biological effect towards the encountered antigen, and thus help booster the response towards the pathogen. Others will develop into memory cells expressing the refined antibody on their surface. These cells can survive in the body for prolonged periods of time, ensuring a rapid and efficient response to the same pathogen upon repeated exposure. Figure 30 summarizes some of the central events in B lymphocyte development, activation and maturation.

4.6.3 B lymphocyte malignancies

The various stages of B lymphocyte development outlined in figure 30 have malignant counterparts reflecting the expansion of a dominant subclone. This results in the development of various types of leukaemias and lymphomas (Figure 31).

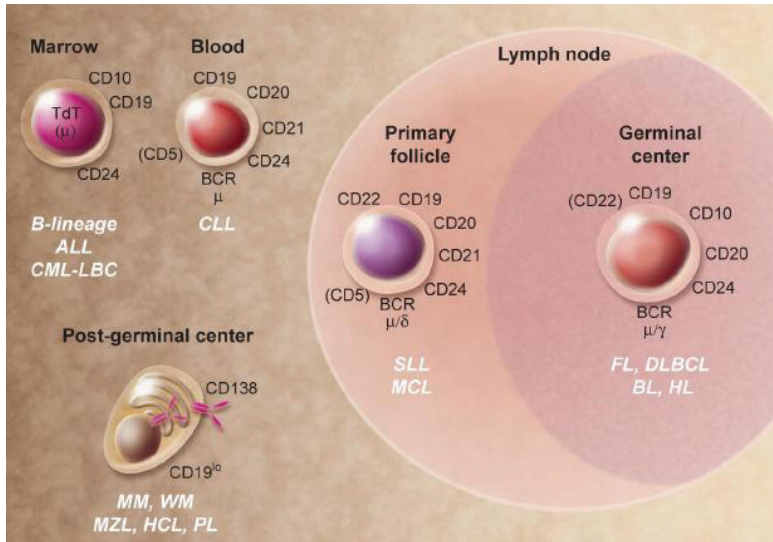


Figure 31. Human B-lymphoid malignancies. The figure provides an overview of the different diseases with corresponding developmental stages of the cell of origin. CML-LBC: chronic myelocytic leukaemia-lymphoid blast crisis; other abbreviations are explained within the text. Adapted from [448].

The term leukaemia literally means ‘white blood’, and strictly speaking refers to a situation in which there is a highly increased amount of malignant white blood cells in the circulation in combination with suppressed erythropoiesis resulting in anaemia. The primary site of affection in many leukaemias such as ALL and chronic lymphocytic leukaemia (CLL) is the BM, with the expansion of transformed clones that are arrested at different stages of differentiation. In many cases of ALL, the spillover of leukaemic blasts into the peripheral circulation is quite low, and leukocytosis is therefore not an obligate finding in ALL. CLL cells on the other hand, phenotypically resemble later stages of B lymphocyte development and typically result in marked leukocytosis. The term lymphoma describes a solid lymphoid tumour mass. Lymphomas typically result from malignancies arising from developmental stages corresponding to B lymphocytes that reside in the secondary lymphoid tissues. The B lineage-derived lymphomas include small lymphocytic lymphoma (SLL), mantle cell lymphoma (MCL), follicular lymphoma (FL), diffuse large B cell lymphoma (DLBCL),

Burkitt's lymphoma (BL), and Hodgkin's lymphoma (HL). Malignancies with phenotypes indicating a post-germinal centre origin, can present both as leukaemias as is the case for hairy cell leukaemia (HCL), and as solid tumours as is the case for marginal zone lymphoma (MZL), plasmablastic lymphoma (PL), multiple myeloma (MM), and Waldenström's macroglobulinaemia (WM). Although applicable to most cases of lymphoid malignancies, the original strict distinction between leukaemias and lymphomas does not always reflect distinct stages of origin, but can in some cases merely reflect different stages of essentially the same disease. This is the case for leukaemia/lymphoma pairs such as CLL and SLL, ALL and lymphoblastic lymphoma, as well as Burkitt's lymphoma/leukaemia. Figure 31 summarizes the different B-lymphoid malignancies according to their developmental stage.

4.6.3.1 B cell precursor acute lymphoblastic leukaemia (BCP-ALL)

ALL is the most common childhood cancer with approximately 200 new cases in the age group under 18 years being diagnosed in the Nordic countries every year. It has a peak incidence at 2 to 5 years of age, but also occurs, albeit with low incidence, in adults and the elderly. Both B and T lymphocyte precursors can give rise to ALL, but BCP-ALL is by far the most predominant subtype in all age groups.

Biology

Historically, ALL has been diagnosed and subgrouped according to the morphological appearance of the blasts in BM smears and biopsies. Typically, ALL blasts are small to intermediary in size with scanty cytoplasm, condensed nuclear chromatin and indistinct or absent nucleoli (Figure 32). However, less commonly blasts may be larger with finely dispersed chromatin and prominent nucleoli [456]. Reflecting the developmental stage of the cell of origin, BCP-ALL cells often express a variety of B lymphocyte-specific surface and cytoplasmic antigens such as PAX-5, CD19, CD22, CD24, and CD79a. A large proportion also expresses CD10, CD34 and terminal deoxynucleotidyl transferase (TdT). Depending on their immunophenotype, BCP-ALLs can be classified into B-I (pro-B), B-II (common B), B-III (pre-B), or B-III (pre-B/B) (Table 2). In addition to the immunophenotypic examination, cytogenetic characterization is used to subgroup BCP-ALLs, yielding further information about biological and pharmacological features that are important in risk stratification of the patients. The two most common genetic abnormalities in paediatric patients are hyperdiploidy and the t(12;21) translocation resulting in a *TEL/AML1* fusion gene, both of

which are favourable prognostic markers. Unfavourable cytogenetic features include t(9;22)(q34;q11) (Philadelphia chromosome) resulting in the *BCR/ABL* fusion gene, t(4;11)(q21;q23) or t(19;11)(p13;q23) resulting in *AF4/MLL* or *ENL/MML* fusion genes respectively, and a hypodiploid karyotype [456].

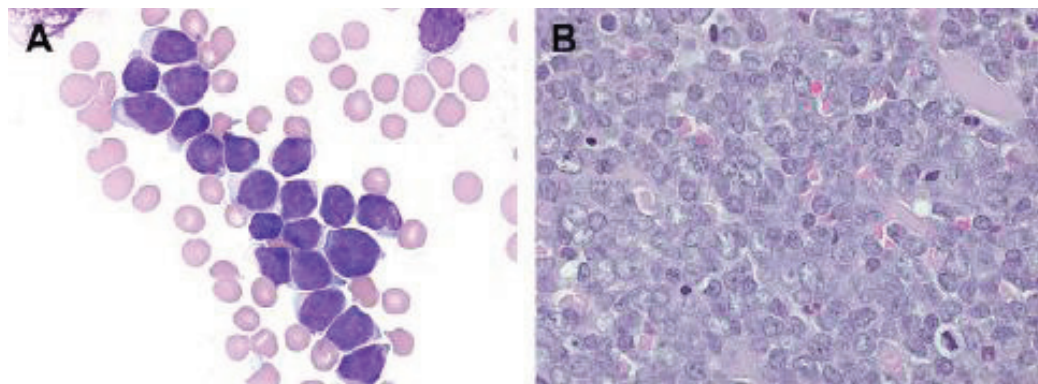


Figure 32. Common morphology of BCP-ALL. (A) Bone marrow smear showing small blasts with a high nuclear to cytoplasmic ratio, condensed nuclear chromatin and indistinct nucleoli. (B) Bone marrow biopsy showing the intense infiltration of the marrow cavity with ALL blasts. Adapted from [456].

	cyCD79	CD19	HLA-DR	CD10	CD20	TdT	cyIgM	sIg
B-I pro-B	+	+	+	-	-	+	-	-
B-II common-B	+	+	+	+	+/-	+	-	-
B-III pre-B	+	+	+	+	+/-	+	+	-
B-III pre-B/B	+	+	+	+	+/-	+	+	+ (κ/λ -)

Table 2. Immunophenotypic classification of BCP-ALL. Based on criteria defined by the European Group for the Immunological Characterization of Leukemias (EGIL) [457;458].

Clinical presentation

ALL often has an acute onset, although some patients present with a more insidious development of symptoms. Symptoms and signs reflect the blast burden and degree of BM replacement. Fever is common, and can either be a direct consequence of the leukaemia or of infections caused by neutropenia. Fatigue and lethargy result from anaemia, and bleeding diathesis from thrombocytopenia. Bone and joint pain are also very common and are a consequence of the expansion of the BM cavity. Frequently involved extramedullary sites of blast infiltration include lymph nodes, liver, spleen, and meninges. In addition, infiltration of

the orbital tissues, testes, tonsils and adenoids can be seen, all yielding symptoms and signs specific for the location of the leukaemic infiltrate. A peripheral blood count commonly reveals anaemia, thrombocytopenia and neutropenia. The total white blood cell count can be suppressed, normal, or elevated depending on the amount of blasts escaping to the peripheral circulation. Only about 15% of patients present with hyperleucocytosis, defined as more than 100×10^9 cells/L [456].

Treatment and outcome

In general, treatment of BCP-ALL involves short-term intensive multimodal chemotherapy with high-dose methotrexate, cytarabine, cyclophosphamide, prednisolone or dexamethasone, vincristine, L-asparaginase, and/or an anthracyclin. This is followed by consolidation therapy with lower intensity regimens over several years. Radiation therapy can be used for localized treatment of central nervous system (CNS) or testicular involvement, or towards other localized infiltrates responding poorly to chemotherapy. Allogenic BM transplantation or high-dose chemotherapy with autologous stem cell support is used for certain patients.

In the Nordic countries, paediatric BCP-ALL is treated according to a protocol developed by the Nordic Society of Paediatric Haematology and Oncology (NOPHO). As children are especially vulnerable to the long-term effects of chemotherapy and irradiation, and as the prognosis in terms of long-term survival is very good for a significant group of the patients, it is vital to achieve good risk stratification. This allows the standard risk group, encompassing more than 50% of all patients, to be treated according to relatively low-intensity protocols. This is expected to yield few and limited long-term side effects, whilst still keeping a cure rate of 90-95%. About 15% of the patients fall into a high-risk group needing treatment with a very intensive protocol to reduce their rate of treatment failure. The remaining intermediate-risk patients are offered intensive therapy balanced against a need for acceptable short- and long-term side effects. CNS irradiation causes serious side effects in children both with regards to endocrine [459] and neurocognitive [460;461] function, as well as predisposing to secondary brain tumours [462]. The NOPHO ALL protocol from 2008 has therefore excluded this treatment modality altogether and replaced it with CNS-penetrating chemotherapy and intensified intrathecal therapy. Risk stratification of patients is initially performed at diagnosis based on white blood cell count and CNS involvement, and is later revised based on karyotype and treatment response, the latter reflected by BM minimal residual disease (MRD) and degree of clearance of CNS involvement [463]. Due to adaptation of therapy according to

risk stratification, improvements in supportive care, and optimization of existing chemotherapeutic drugs, survival rates after treatment for paediatric ALL has improved from less than 10% in the 1960s to 75-80% at present [456]. Figure 33 shows the development in event-free survival in different treatment eras in the Nordic countries.

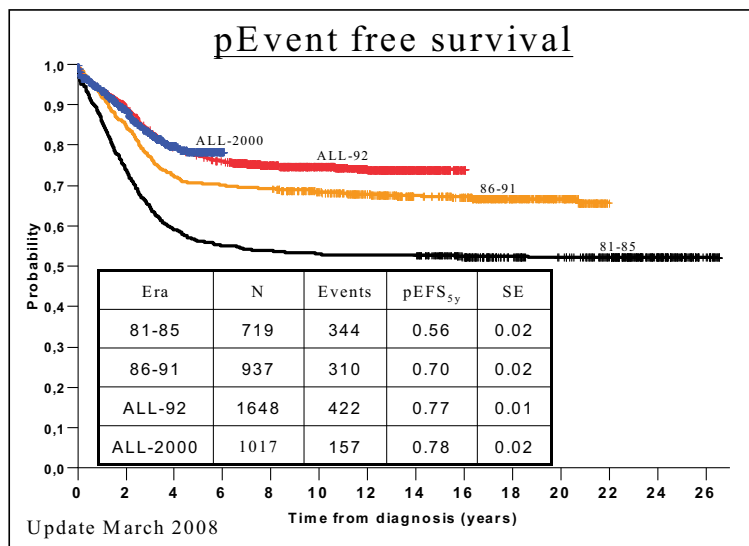


Figure 33. Event-free survival of paediatric ALL in the Nordic countries. Adapted from [463].

Adult ALL patients generally have a poorer prognosis, with overall survival rates of 40-50% [464]. In Norway, these patients are treated with combinatorial chemotherapy regimens according to a protocol termed ‘Hammersmith-82’. Irradiation treatment is still employed against localized infiltrates and recurrence with CNS involvement, and BM transplantation is used more frequently than in paediatric patients [465].

4.6.4 Epstein-Barr virus infection

The Epstein-Barr virus (EBV) is a member of the herpesvirus family of DNA viruses. Herpesviruses are ubiquitous in nature, but humans serve as the only natural host for EBV. Its discovery was facilitated by D. Burkitt, who in 1958 described a common cancer primarily affecting children in specific regions of Africa, which he postulated might be caused by a virus [466]. The disease was termed Burkitt’s lymphoma (BL), and in 1964 the virus was visualized by electron microscopy of a cell line established from BL, thus becoming the first virus clearly implicated in the development of a human tumour [467].

More than 90% of the human population are carriers of EBV. The virus is transmitted from host to host via saliva, and primary infection is thought to begin at the oropharyngeal epithelium with secondary infection of B lymphocytes trafficking in close proximity to these cells [468]. Most individuals are infected during the first decade of life, normally undergoing an asymptomatic infection. However, primary infection during adolescence or adulthood is often accompanied by a self-limiting lymphoproliferative disease known as infectious mononucleosis [469]. After the primary infection, EBV persists in a subset of circulating resting memory B lymphocytes with the viral genome existing in an episomal form at low copy numbers in the cell's nucleus [468].

4.6.4.1 *In vitro* EBV transformation of B lymphocytes

Even though EBV may infect epithelial cells, T lymphocytes, macrophages, granulocytes, and NK cells [468], its preferential target is B lymphocytes. Attachment to the host cell is achieved through the binding of the viral envelope glycoproteins gp350 to CD21 [470] and gp42 to HLA class II molecules [471]. *In vitro*, EBV has the unique ability to transform resting B lymphocytes into permanent, latently infected lymphoblastoid cell lines (LCLs). EBV-transformed LCLs carry multiple copies of the viral episome, and constitutively express a limited set of viral gene products termed latent proteins [472]. The infection also changes the expression pattern of host genes yielding high levels of B lymphocyte activation markers and cell adhesion molecules that are usually absent or expressed at low levels in resting B lymphocytes. This indicates that EBV-induced immortalization can be elicited through constitutive activation of the cellular pathways involved in driving physiological B lymphocyte proliferation [472].

4.6.4.2 EBV-associated malignancies

EBV has been associated with several types of B lymphoid lymphomas in addition to various carcinomas (reviewed in [468]). As opposed to what has been demonstrated for several other DNA viruses with oncogenic potential (see section 4.5.4.3), there is little evidence that EBV encodes viral proteins which inactivate tumour suppressors such as p53 or pRB. Indeed p53 expression and function appear normal in LCLs. Rather, the evidence indicates that EBV subverts downstream parts of normal cell signalling pathways involved in cell growth [473].

The EBV-associated lymphomas can be broadly divided into three groups: lymphomas in immunosuppressed patients, BL and HL. These are described briefly below.

The T lymphocyte-directed immunosuppressive treatment given to patients after transplantations is associated with a greatly increased risk of lymphomas, also termed post-transplant lymphoproliferative disease (PTLD). These are nearly always of B lymphocyte origin and EBV-positive [474]. Studies of V gene mutations and SHM indicate that the PTLD cells have a close relationship to centroblasts [475-477]. They express the full set of latent EBV genes seen in LCL, indicating that EBV plays an important role in driving the proliferation of the infected B lymphocytes in PTLD. Some lymphomas seen in highly immunocompromised AIDS patients show essentially the same phenotype [472].

BL can be divided into two groups, the high-incidence endemic form that affects children in areas of holoendemic malaria, and the low-incidence sporadic form seen in children in the developed world. In the former, EBV is present in all cases, whereas in the latter, EBV is present in about 15% of cases [472]. In the EBV-positive tumours, expression of EBV-encoded genes is much more restricted than is the case for PTLD, so exactly how EBV contributes to the pathogenesis in BL is not clear. However, the phenotype of the transformed cells again points to centroblasts as being the cells of origin. Indeed, translocations of *MYC* into one of the immunoglobulin loci, resulting in constitutive activity, is a hallmark of BL, and the structure of chromosomal breakpoints strongly indicates that the translocations occur as an erroneous result of either CSR or SHM [478;479].

HL is characterized by atypical, large tumour cells known as Reed-Sternberg (RS) cells which typically constitute less than 1% of the tumour mass. EBV is detected in approximately 40% of cases of classical HL in the developed world, but nearly all cases in children from Latin America [480]. As for BL, only a restricted number of EBV-derived genes are expressed, and the role of EBV in the pathogenesis is not established. However, as for the other EBV-associated lymphomas, genetic analysis of RS cells point to GC B lymphocytes as the cells of origin [469].

4.7 cAMP

The cyclic ribonucleotide 3'-5'-cyclic adenosine monophosphate (cAMP), was originally identified in 1957 as a heat-stable factor produced in the presence of epinephrine and glucagon by cell-free liver homogenates, leading in turn to the formation of liver phosphorylase [481]. Soon after, this factor was shown to be identical to an adenine ribonucleotide, produced by degradation of ATP, initially thought to be a cyclic dianhydrodiadenylic acid [482], later corrected to be a monomeric cyclic phosphate [483].

With time, cAMP has become recognized as a prototypical second messenger, being utilized as a signalling molecule in all domains and kingdoms of life [484]. In vertebrates, cAMP signalling is usually initiated through hormone or transmitter binding to G protein-coupled receptors (GPCRs). This leads to activation of the heterotrimeric G protein complex, in which the G_{α} subunit dissociates from the $G_{\beta\gamma}$ subunits. The stimulatory form of G_{α} ($G_{\alpha s}$) then activates an adenylyl cyclase (AC) which catalyzes the conversion of ATP to cAMP. The levels of cAMP are controlled by its rate of synthesis by AC and its rate of degradation by phosphodiesterases (PDEs). For a long time, the only known target of cAMP was protein kinase A (PKA). However, it has now become clear that cAMP can also stimulate the activity of Rap1-guanine nucleotide exchange factor proteins directly activated by cAMP (EPACs), and a subgroup of cyclic nucleotide-gated (CNG) ion channels. Another important realization has been that cAMP does not act by random diffusion from its site of synthesis to its effectors, but that cAMP signalling is organized into signalling units or signalosomes. In these signalosomes the GPCR, AC, PDE, PKA, PKA targets, and other factors are brought in close proximity, providing crucial spatiotemporal control of the many cAMP pathways.

4.7.1 Regulation of cAMP levels

4.7.1.1 G protein-coupled receptors

GPCRs encompass the largest family of membrane proteins and mediate most cellular responses to hormones and neurotransmitters. In addition they play central roles in the transmission of sensory signals involved in vision, olfaction and taste [485]. Structurally, GPCRs are characterized by the presence of seven membrane-spanning α -helices separated by alternating intracellular and extracellular loop regions. These regions provide specificity to the GPCR function by linking the binding of given ligands to effects mediated by given G

protein subtypes as well as G protein-independent signalling. The heterotrimeric G proteins act as switches that turn on or off downstream signalling cascades in response to GPCR activation (Figure 34). They are composed of three subunits: α , β and γ , and are inactive in their heterotrimeric conformation in which G_α binds GDP and the constitutive $G_{\beta\gamma}$ dimer. Activation of GPCRs induces a conformational change which permits G protein binding and catalyzes GDP release from G_α . This results in the formation of a stable, high affinity complex between the receptor and the G protein. Binding of GTP to G_α destabilizes the complex, leading to the release of $G_\alpha(\text{GTP})$ and $G_{\beta\gamma}$, which can both bind to effectors in the cell, contributing to initiation of signalling cascades. The response is then terminated by the hydrolysis by G_α of GTP to GDP and subsequent reassociation of $G_\alpha(\text{GDP})$ with $G_{\beta\gamma}$. This hydrolysis can be accelerated by regulators of G protein signalling (RGSs), which are multi-functional, GTPase-accelerating proteins [486].

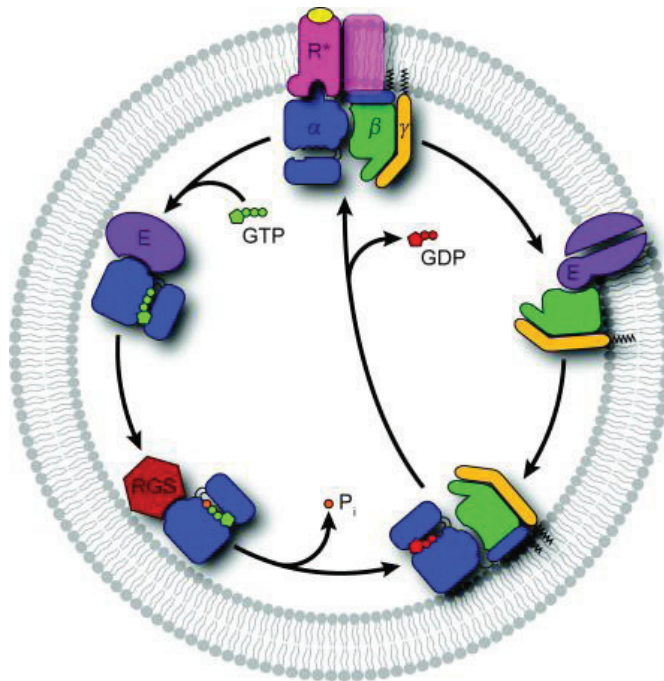


Figure 34. The G protein cycle. Heterotrimers of $\alpha(\text{GDP})$, β and γ subunits are recruited to the activated GPCR (R^*) upon binding of its ligand, resulting in GDP release. Subsequent binding of GTP to G_α causes dissociation of R^* , $G_\alpha(\text{GTP})$ and $G_{\beta\gamma}$. The G protein subunits can then activate various effectors (E) before the signal is terminated by the hydrolysis of GTP to GDP by G_α which may be catalyzed by RGSs. Adapted from [486].

With regards to cAMP generation, two subgroups of G_α family members termed G_s (stimulatory G protein) and G_i (inhibitory G protein) are responsible for the regulation of AC activity upon GPCR activation (Table 3).

G protein family	Members	Canonical effector molecules
G_s	$G\alpha_s, G\alpha_{olf}$	Adenylate cyclase (+)
G_i	$G\alpha_o, G\alpha_{il-3'}, G\alpha_{tl-2'}, G\alpha_{gr}, G\alpha_z$	Adenylate cyclase (-), cGMP phosphodiesterase (+), several $G\beta\gamma$ effectors (e.g., ion channels)
G_q	$G\alpha_q, G\alpha_{11'}, G\alpha_{14'}, G\alpha_{15/16}$	Phospholipase C- β (+)
G_{12}	$G\alpha_{12'}, G\alpha_{13}$	p115RhoGEF

Table 3. G_α protein classification. The table shows the classification of different members of the G_α protein family with corresponding effector molecules. Adapted from [487].

4.7.1.2 Adenylyl cyclases

The generation of cAMP is universally catalyzed by members of the AC family. The catalytic mechanism of ACs is depicted in figure 35.

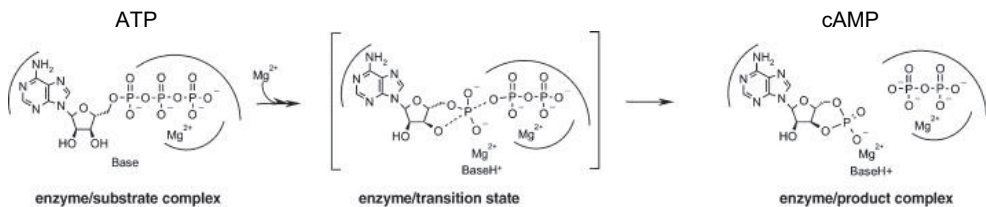


Figure 35. The catalytic mechanism of cAMP generation by ACs. The substrate ATP is converted to a magnesium stabilized penta-covalent transition state in which the incoming and leaving groups are aligned, before completion of the reaction yielding cAMP and pyrophosphate. Modified from [484].

In mammals, nine transmembrane ACs (tmACs) and one soluble AC (sAC) have been identified. tmACs are directly regulated by GPCRs and heterotrimeric G proteins as described above (reviewed in [488]). In addition certain other proteins such as calmodulin, A-kinase anchoring proteins (AKAPs), Ric8a and Snapin can affect the activity of various tmAC isoforms through direct interaction (reviewed in [489]), and they are also subject to regulation

by PTMs [490-492]. sAC on the other hand is regulated by bicarbonate [493] and calcium levels [494;495], and is insensitive to regulation by G proteins [496].

4.7.1.3 Direct cAMP transfer through gap junctions

In addition to the endogenous production of cAMP induced by activation of AC, cAMP can also be provided through transfer from neighbouring cells. As early as in the 1970s it was demonstrated that cAMP could move through junctional channels [497;498]. cAMP has subsequently been shown to be able to pass through gap junctions composed of different classes of connexins (Cx), but whether the permeability of these junctions allows for delivery of sufficient amounts of cAMP to be physiologically relevant has been questioned. Recently, Cx43 was shown to yield particularly favourable permeability to cAMP, resulting in rapid delivery in sufficient quantity to trigger relevant intracellular responses [499]. Regarding the role of such cell-to-cell delivery of cAMP, it has also recently been demonstrated that naturally occurring T regulatory lymphocytes (T regs) harbour high levels of cAMP that can be delivered to responder T lymphocytes via gap junctions, and that this delivery is crucial to T reg-mediated T lymphocyte suppression [500].

4.7.1.4 Phosphodiesterases

cAMP is a relatively stable molecule, and the only means of inactivating the cAMP signal within the cell is to actively degrade it [501]. The only known degradation route for cAMP is its conversion to 5'AMP by PDEs [502]. Mammalian PDEs comprise the products of 21 genes which are categorized into 11 different families based on structural similarity. Members of the PDE5 and PDE6 families are cGMP-specific, whereas the remaining families are comprised of members that are either cAMP-specific or display dual specificity. Additional complexity arises from the use of alternative promoters and alternative splicing resulting in various transcripts from given subfamily genes [503]. All PDEs have a conserved C-terminal catalytic domain with 35 to 50% sequence homology, whereas the unique characteristics of each individual PDE variant are defined by protein domains located in the N-terminal part. The combination of unique tissue-specific expression patterns, gene regulation, enzymatic regulation by phosphorylation and interacting proteins, subcellular localization, and interaction with associated proteins, allows for complex and tight regulation of PDE activity [503]. In haematopoietic cells, PDE4 is the predominant PDE family. It is made out of four genes; *4A*, *4B*, *4C*, and *4D* which encode about 20 distinct isoform members [504]. PDE4A

expression is fundamentally ubiquitous [505;506], whereas PDE4B and PDE4D are the predominant isoforms in cells of the immune system [507;508].

4.7.2 cAMP effectors

4.7.2.1 PKA

PKA was discovered in 1968 as one of the first protein kinases [509]. The holoenzyme is a heterotetramer composed of two catalytic (C) subunits bound non-covalently to a regulatory (R) subunit dimer [510;511]. The enzymatic activity of the C subunit is kept in check by its sequestration by the R subunits, and its release is brought about by a conformational change in the R subunit precipitated by the binding of cAMP (reviewed in [511]). cAMP binds in a cooperative manner to two sites on each R subunit, and the binding of a total of four cAMP molecules results in the dissociation of the R dimer and the two C monomers [512]. The C monomers then act as serine/threonine kinases capable of phosphorylating a wide array of targets within the cell.

There are four genes encoding different R subunits: RI α , RI β , RII α , and RII β , and three genes encoding different C subunits: C α , C β , and C γ (reviewed in [513]). Variants of PKA are divided into two main classes of isozymes: PKAI and PKAII, based solely on their content of RI or RII subunits, respectively. R isoforms are differentially expressed in tissues [514-516], and their subcellular distribution is also distinct [517-520]. PKAII is typically found in a particulate pattern in subcellular structures and compartments caused by its interaction with AKAPs. RI has low affinity for most AKAPs, and PKAI has largely been considered soluble and cytoplasmic; however, some RI-specific and dual-specific AKAPs capable of anchoring PKAI have been identified (reviewed in [521]).

4.7.2.2 cAMP effectors other than PKA

In addition to PKA, two other families of proteins contain a cAMP binding domain (CBD) allowing their activity to be regulated by cAMP: EPAC and CNG ion channels. EPACs were identified in 1998 [522;523], and shown to activate the Ras superfamily small GTPases RAP1 and RAP2 in a cAMP-dependent manner. There are two EPAC isoforms, EPAC1 and EPAC2, of which EPAC1 is ubiquitously expressed whereas EPAC2 is more limited in its

tissue distribution [522;523]. They have later been shown to be involved in a wide variety of cAMP-regulated cellular functions such as cell adhesion, cell-cell junction, exocytosis/secretion, cell differentiation and proliferation, gene expression, apoptosis, cardiac hypertrophy and phagocytosis (reviewed in [524]). CNG ion channels are strictly ligand-gated channels whose opening requires the binding of cAMP or cGMP. They regulate the flux of Na^+ , K^+ and Ca^{2+} , and play an important role in the signal transduction pathways of vision and olfaction. In addition, cAMP and cGMP can facilitate opening of a second group of ion channels termed hyperpolarization-activated cyclic nucleotide-gated (HCN) channels by shifting their voltage dependence (reviewed in [525;526]).

4.7.2.3 Transcriptional regulation by cAMP

Some of the cAMP-dependent cellular effects are mediated through changes in gene transcription, many of which are a result of PKA-mediated phosphorylation and subsequent activation of a subgroup of transcription factors that contain basic region leucine zippers (bZIPs). PKA-activated bZIP-containing factors include cAMP response element binding protein (CREB), cAMP response element modulator (CREM), and activating transcription factor-1 (ATF-1) (reviewed in [527]). In addition, there is evidence that several genes can be regulated by cAMP in a PKA-independent manner, but it is presently not clear which transcription factors are involved in such PKA-independent, cAMP-responsive gene expression. There is some evidence of transcription factor activation downstream of EPAC activation. In addition, other guanine nucleotide exchange factors (GEFs) such as the PDZ-domain containing GEF1 and 2 (PDZ-GEF1/2) are putative cAMP sensors that might be involved in PKA-independent transcriptional regulation (reviewed in [527]).

4.7.3 cAMP signalosomes

With the vast number of GPCRs capable of eliciting cAMP generation by ACs, and with the equally vast number of substrates of EPACs and PKAs, cAMP signalling needs to be carefully compartmentalized in order to obtain the necessary signal specificity. For PKA-mediated effects, the existence of such cAMP signalosomes is firmly established, in which a given ligand binds to its GPCR which signals through associated G proteins to a neighbouring AC resulting in cAMP production. The synthesized cAMP will be restricted to a localized pool due to the existence of anchored PDEs in the vicinity of the AC, and this allows spatial

and temporal control of the cAMP signal. The signal is further delivered to a nearby PKA which is similarly anchored to subcellular structures by the means of AKAPs, thus eliciting the distinct biological outcome intended for the ligand in question (Figure 36).

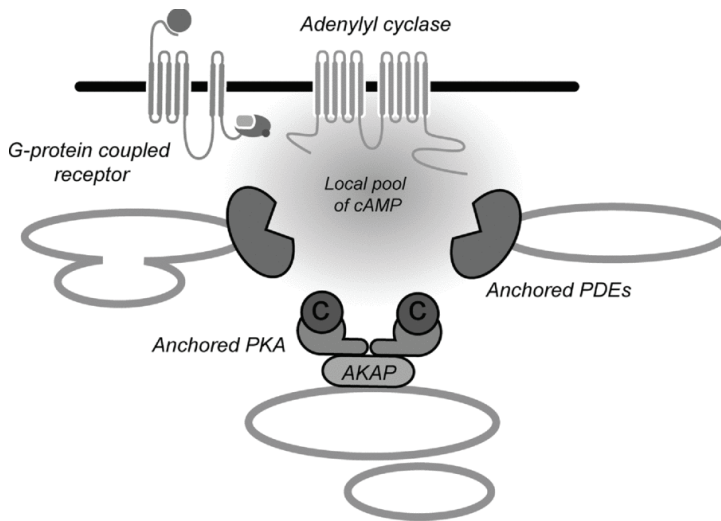


Figure 36. The cAMP signalosome. The figure illustrates the spatiotemporal control of cAMP signalling achieved by the close proximity of the signal input (GPCR), generator (AC), effector (PKA anchored by AKAP) and terminator (PDE). Adapted from [528].

The AKAPs constitute a family of structurally diverse proteins defined by their ability to bind the R subunit of PKA. In addition to their PKA-binding domain, all AKAPs contain a unique targeting domain directing the PKA/AKAP complex to defined subcellular structures, membranes or organelles. Many AKAPs also possess further domains enabling interaction with several other proteins involved in signal transduction, thus enabling them to form multivalent signal transduction complexes (reviewed in [521]).

4.7.4 Biological effects of cAMP

cAMP signalling contributes to the regulation of a wide array of biologically distinct cellular processes, examples of which include cardiovascular function, steroid biosynthesis, reproductive function, metabolism, exocytosis, and immune function (reviewed in [521]). In cells of the immune system, cAMP is well established as an important physiological signal transducer. One example is provided by the stimulation of the T cell receptor/CD3 complex resulting in transient formation of cAMP, which limits further activation of the cells [529] (for

a comprehensive recent review, see [530]). Lymphocytes possess GPCRs for catecholamines and prostaglandin E₂ (PGE₂), and engagement of these receptors by their respective ligands, has been shown to exert a growth-inhibitory effect mediated by the elevation of cAMP levels [531-534]. In addition to its many roles in normal cell physiology, distorted cAMP signalling has been suggested to be exploited by cancerous cells.

4.7.4.1 cAMP signalling in cancer

With the range and complexity of cAMP signalling in normal physiological processes outlined above, it comes as no surprise that the possible role of cAMP signalling in transformed cells has proven to be a complex issue. Indications of involvement of the different levels in the cAMP signalling cascade will be briefly discussed below.

GPCRs

Two well-described classes of GPCRs that can elicit a cAMP response are PGE₂ receptors (EP receptors) and β -adrenergic receptors (β -ARs). Overexpression of cyclooxygenase 2 (COX2), leading to augmented PGE₂ production and EP signalling, has been implicated in the tumourigenesis of a wide variety of cancers (reviewed in [535]). However, EP signalling is complex, and increased cAMP levels have not been directly implicated in the tumour-promoting properties of PGE₂. With regards to β -AR signalling, murine models of ovarian cancer have demonstrated that exposure to chronic stress can promote tumourigenesis, and this effect has been directly associated with increased tissue catecholamines yielding increased β_2 -AR stimulation with subsequent activation of the cAMP-PKA pathway. Human studies have shown that increased β_2 -AR activity in breast cancer patients is associated with accelerated tumour growth. Conversely, males on β -blocker therapy have in some studies been shown to have a reduced risk of prostate cancer (reviewed in [536]).

G proteins

Activating mutations of G_{as} resulting in constitutive AC activation is implicated in the pathogenesis of several endocrine tumours. The reason why cells of a subset of endocrine tissues are especially vulnerable to increased cAMP-PKA signalling is that CREB is involved in the transcription of specific primary response genes that initiate proliferation in these cells [537]. Growth hormone releasing hormone (GHRH) is produced in the hypophysis and stimulates growth hormone (GH)-producing cells in the pituitary gland, whereas thyroid

stimulating hormone (TSH) is produced in the pituitary gland and stimulates thyroxine (T4)- and triiodothyronine (T3)-producing cells in the thyroid gland. Both GHRH and TSH are ligands of G_{αs}-coupled GPCRs. Specific point mutations of the α_s subunits have been identified which result in abrogation of their intrinsic ATPase function and thus yield constitutive AC activation [538;539]. The mutationally activated forms of G_{αs} are commonly referred to as the *gsp* (Gs protein) oncogene, and occurs with high frequency in pituitary adenomas, thyroid adenomas and thyroid carcinomas [539;540].

PDEs

The possible role of PDEs in cancer has been particularly well studied in haematopoietic malignancies in which the PDE4 family plays the most prominent role in negative regulation of cAMP levels. Due to the anti-proliferative effect observed with cAMP in lymphoid cells, it has been hypothesized that transformed lymphoid cells would benefit from keeping their cAMP levels low by upregulating PDE activity, and that pharmacological inhibition of PDE could be a potential therapeutic strategy. PDE4 inhibition by theophylline or rolipram has been demonstrated to sensitize ALL and B-CLL cells to apoptosis *in vitro* when combined with corticosteroids or chlorambucil respectively. In DLBCL samples, high PDE4B expression has been shown to be correlated with poor prognosis, and it has thus been suggested that consequent suppression of cAMP levels might result in a growth or survival advantage in these tumours. However, this result should be interpreted with caution as *PDE4B* is a cAMP responsive gene, and it is thus possible that the causal relationship could be reversed with augmented PDE4B expression being a consequence of increased cAMP signalling (for an extensive review on PDEs in haematological malignancies, see [541]).

cAMP levels

There have been a few reports on enhanced cAMP levels in primary tumour samples [542;543]. However, these estimations of the cAMP content have been considered inaccurate due to inefficient separation of malignant cells of solid tumours from the surrounding stromal cells [543]. Regarding the effect of increased cAMP signalling in tumour-derived cell lines, the results are highly cell specific, with induction of growth arrest or apoptosis seen in some lymphoid and prostate cancer cell lines [541;544], and increased resistance to apoptosis-inducing agents being observed in some cells of the myeloid lineage [545;546].

PKA

Several lines of evidence indicate that PKAI activity is associated with growth and proliferation, whereas PKAII activity is associated with differentiation and decreased proliferation [547;548]. In keeping with this, the RI α subunit has been found upregulated in a number of cell lines, as well as in primary tumour cells (reviewed in [549;550]). Additionally, there is evidence of an increased RI to RII ratio in human cancer tissues of different origins. Switching of this ratio has been suggested as a novel cancer treatment strategy, with the PKAI downregulator 8-Cl-cAMP as well as antisense RI α being tested in clinical trials [551].

AKAPs

Polymorphic variants of AKAPs 10 and 13 have been found to be associated with increased familial breast cancer risk. As AKAP13 is an upstream effector of Ras homolog gene family member A (RhoA) signalling, it has been suggested that the polymorphic variant might result in constitutive RhoA activation. Regarding AKAP10, the polymorphism has been found to favour binding and activation of RI α , which could possibly contribute to a PKAI-skewed phenotype in these cells.

CREB

Several lines of evidence support a role for CREB in oncogenesis. In acute myeloid leukaemia (AML), CREB is an established proto-oncogene and is found overexpressed in the majority of patients. In clear cell sarcoma (CCS) CREB is translocated to the fusion oncoprotein Ewing's sarcoma protein (EWS)-CREB, and in the process of immortalization of T lymphocytes with HTLV-1, CREB activation has been shown to be essential (reviewed in [552]). In addition, the tumour suppressor activity of LKB1 is in part mediated through inhibition of CREB activity. LKB1 is a serine/threonine kinase mutated in most cases of the inherited intestinal polyposis syndrome Peutz-Jegher's syndrome (PJS), and constitutive activation of CREB has been demonstrated in samples from PJS patients [553].

5 AIMS OF THE STUDY

Previous studies in our laboratory have demonstrated that cAMP can inhibit proliferation of lymphoid cells by inducing both a G1 arrest [554;555] and an intra-S phase arrest [556]. In the latter paper it was also demonstrated that cAMP could profoundly attenuate the effect of S phase-specific cytotoxic drugs. This effect of cAMP could in large part be attributable to its ability to inhibit DNA replication, but prompted us to investigate whether cAMP could additionally exert a direct inhibitory effect on the DNA damage response (DDR).

The overall aim of the present study was thus to explore the relation between cAMP levels and DDR in lymphoid cells, specifically aiming to:

- 1) Unravel the effects of and mechanisms involved in cAMP-mediated regulation of DDRs.
- 2) Elucidate the clinical relevance of cAMP in regulation of DDR by using BCP-ALL as a model system.

6 SUMMARY OF THE PAPERS

PAPER I:

Activation of cAMP signaling inhibits DNA damage-induced apoptosis in BCP-ALL cells through abrogation of p53 accumulation.

The aim of the present study was to explore whether cAMP could inhibit DNA damage-induced cell death in lymphocytes through direct effects on the DDR machinery. The BCP-ALL-derived cell line Reh was used as a model system, and DNA damage was induced by IR or cell cycle phase-independent cytotoxic drugs. We found that cAMP could profoundly inhibit the apoptotic cell death induced by DNA damage, and identified p53 as the most upstream component of the DDR pathway affected. The cAMP-induced reduction of DNA damage-mediated p53 accumulation was shown to be a prerequisite for the inhibitory effect of cAMP on apoptosis. The possible clinical relevance of our findings was underlined by our demonstration that cAMP was equally able to inhibit p53 accumulation and cell death in a small sample of primary BCP-ALL blasts that were treated with IR or DNA-damaging drugs commonly employed in conventional treatment of BCP-ALL.

PAPER II:

Activation of cAMP signalling interferes with stress-induced p53 accumulation in ALL-derived cells by promoting the interaction between p53 and HDM2.

In this paper we aimed to elucidate the mechanisms whereby cAMP prevented the DDR-induced accumulation of p53 observed in paper I. We could show that cAMP inhibited p53 at the level of protein stability, and that this was caused by restoration of the levels of p53 ubiquitination and consequent proteasomal degradation. The activity of the p53-directed E3 ubiquitin ligase HDM2 was demonstrated to be necessary for the inhibitory effect of cAMP on p53 accumulation, and more specifically, cAMP was shown to promote the interaction between HDM2 and p53.

PAPER III:

EBV infection renders B cells resistant to growth inhibition via adenylyl cyclase.

EBV infection is known to cause resistance of B lymphocytes to certain growth inhibitors. The aim of this paper was to examine whether EBV infection would affect the ability of cAMP to act as a growth inhibitor of B lymphocytes, and if so, to investigate the effect of EBV on cAMP-mediated inhibition of cell death induced by S phase-specific cytotoxic drugs. This study showed that although EBV infection rendered cells resistant to the inhibitory effects of the GPCR ligand isoproterenol and the AC activator forskolin, it had no effect on the sensitivity of cells to growth inhibition induced by direct stimulation of PKA. Mechanistically, this was explained by defective AC activation with consequent pronounced inhibition of cAMP induction and PKA activation. The inability of forskolin to inhibit DNA replication and to induce S phase arrest in EBV-infected cells was also shown to render cells insensitive to the normal inhibitory effect of cAMP on cell death induced by the S phase-specific DNA-damaging compound camptothecin.

PAPER IV:

Selective inhibition of cell death in malignant versus normal B cell precursors: Implications for cAMP in development and treatment of BCP-ALL.

Based on our results from the BCP-ALL cell line Reh in papers I and II, we wished to explore whether cAMP-mediated suppression of the tumour suppressor p53 could have implications for development and treatment of BCP-ALL. To address this, we collected primary blasts from nine paediatric BCP-ALL patients and primary CD10⁺ BCPs from seven healthy volunteers. We could show that effects of cAMP on p53 levels and DNA damage-induced cell death previously described in Reh cells were reproduced in the primary BCP-ALL blasts, supporting the clinical relevance of our findings in the Reh cell line. In addition, cAMP provided BCP-ALL blasts with a pronounced protection against spontaneous cell death in culture. In contrast, the viability of normal BCPs was not enhanced by cAMP treatment, implying that the pro-survival effect of cAMP might be a malignancy-associated trait. We found no difference in the levels of total cellular cAMP between BCP-ALL blasts and normal BCPs, indicating that there is no selection for constitutively increased cAMP production during transformation. However, co-culturing BCP-ALL blasts with a BM-derived stromal cell layer could mimic the effect of cAMP on blast survival and p53 levels, suggesting that the leukaemic blasts might be provided with cAMP-mediated protective signals through their

interaction with the stromal cells. Finally, preliminary results suggest that abrogation of the cAMP signalling pathway through inhibition of PKA can augment the cytotoxic effect of DNA-damaging treatment, prompting further investigation into the possible clinical use of PKA inhibitors with the aim to potentiate conventional BCP-ALL treatment.

7 DISCUSSION

7.1 Methodological considerations

7.1.1 Cell systems

A number of different cell systems have been used in this study: continuous cancer cell lines (Reh, EU-3, U2OS, MCF-7), immortalized normal cell lines (EBV-infected B lymphocytes, TK6 and WTK1 cells derived from the spontaneously transformed B lymphoblast cell line WI-L2, iMSC#3 cells which are hTERT immortalized BM-derived mesenchymal stroma cells), as well as primary cultures of cancer cells (BCP-ALL blasts) and normal cells (normal peripheral B and T lymphocytes, normal BCPs, BM-derived stromal cells). The cell systems that were used most extensively will be discussed in more detail below.

7.1.1.1 Cell lines

Since the first human cancer-derived cell line HeLa was established in 1951 (reviewed in [557]), cell lines have been invaluable tools in the study of human cell biology in general and cancer biology in particular. Continuously growing cell lines offer many advantages in cell research. They represent an almost unlimited supply of cells with relatively stable genotypes and phenotypes, thus avoiding the problem of interindividual variation. They also bypass the ethical issues associated with animal and human experiments [558]. However, cancer cell lines may not represent an optimal model for the study of human cancers. Cell lines are not easy to establish, and the cancers that yield successful cell lines tend to be fast growing, high stage, poorly differentiated, and often metastatic; reflecting the need for genetic changes that are often late events in cancer progression [558]. Therefore, although cancer cell lines have been demonstrated to be quite genetically stable and able to recapitulate the histopathology of the original tumour after subcutaneous injections, they cannot automatically be regarded as good models for the clinical spectrum of cancers at their site of origin [558].

Several studies comparing the gene expression profile of cancer cell lines to that of primary normal and cancerous tissues from the same site of origin have been conducted. Expression patterns have been found to cluster according to the tissue of origin [559], but cell lines also tend to share large sets of signatures that can be said to be cell line-specific independent of the

tissue of origin [560]. Common to most of these gene expression studies is the finding that it is the lymphoma and leukaemia cell lines that most accurately model the gene expression pattern of their corresponding primary tumour [561] (reviewed in [562]). Cytogenetic aberrations producing pathophysiologically relevant fusion gene products are common occurrences in haematological malignancies, and it has been shown that key signatures are retained between primary cancer samples and cell lines with similar genetic aberrations [563;564]. These signatures probably reflect regulatory networks of importance for the transforming abilities of the primary genetic changes, and it is thus thought that lymphoma and leukaemia cell lines provide a relatively good model system to study the pathophysiology of their corresponding primary malignancies.

Reh

The Reh cell line was originally derived in 1973 from the peripheral blood of a 15 year old North African girl diagnosed with ALL at first relapse [565;565]. At the time, her disease was diagnosed as a 'non-T, non-B ALL'. However, with the advent of immunophenotypic and cytogenetic classification, Reh was found to represent a BCP-ALL-derived cell line. Immunophenotypic traits include $CD34^-CD10^+CD19^+cyIgM^-sIg^-TdT^+$, providing the basis for its classification as stemming from a B-II (common-B) BCP-ALL [566]. Further, it is one of very few BCP-ALL cell lines harbouring the $t(12;21)(p13;q22)$ translocation resulting in the expression of the *TEL/AML1* fusion gene which is a common cytogenetic aberration occurring in approximately 25% of primary paediatric BCP-ALL specimens [567]. Evidence suggests that the *TEL/AML1* fusion gene represents an initiating mutation that is necessary but insufficient for leukaemia development in this subgroup of BCP-ALL. It is thought to arise predominantly during faetal haematopoiesis at a rate that considerably exceeds that of overt clinical ALL, and the *TEL/AML1*-harbouring clones have been shown to expand and persist for more than a decade in a clinically covert state [568;569]. The exact function of the *TEL/AML1* fusion protein in the leukaemogenic process is not definitely determined. However, *AML1* has been shown to function as a DNA-binding transcriptional activator required for the expression of genes associated with normal haematopoiesis, and evidence suggest that *TEL/AML1* might specifically repress *AML1*-dependent activation, possibly contributing to a differentiation block of early BCPs (reviewed in [569]). Additional genetic abnormalities in Reh include loss of 9p21.3 involving the *p16INK4a/ARF* locus, which is another common occurrence in paediatric BCP-ALL [570].

iMSC#3

iMSC#3 is an immortalised BM-derived human mesenchymal stroma cell line generated by transduction with the pBabe-puro-hTert adenovirus [571]. It has been demonstrated to be non-tumourigenic, have a normal karyotype, and maintain osteogenic and adipogenic potential as well as the ability to support B lymphocyte maturation [572]. We used this cell line to support the viability of primary BCP-ALL blasts in culture. *In vivo*, the bone marrow microenvironment (BMME) provides a far more complex supportive surrounding for normal and malignant BCPs, with osteoblasts, endothelial cells and BM stromal cells supplying them with a multitude of both secreted and cell bound factors. However, the finding that iMSC#3 could affect blast survival to a similar extent as primary BM stromal cell cultures, strongly suggests that this cell line provided the blasts with some of the crucial physiological stimuli normally provided by the BMME.

7.1.1.2 Primary cell cultures

BCP-ALL blasts

Although leukaemic cell lines in general have been shown to provide relatively good models to study leukaemias, and Reh in particular has been shown to share many of the immunophenotypic and genetic traits commonly observed in paediatric BCP-ALL, we considered it important to examine whether we could reproduce the results from Reh cells in primary cancer material. To this end, we initiated a pilot study on primary BCP-ALL blasts isolated from paediatric patients at diagnosis. There are important limitations concerning the quantity and variety of experiments that can be performed on such material. Isolation of cells yields limited material with limited life span, and many useful tools in molecular biology such as transfection are virtually impossible to make use of in primary cells. These cultures therefore primarily served the purpose of confirming the validity of key findings from more elaborate molecular studies performed in Reh.

BCP-ALL blasts were isolated from 1-3 ml of BM aspirate. Isolation of mononuclear cells (MNCs) was performed by density gradient centrifugation, and the proportion of BCP-ALL blasts in this population was evaluated by co-staining of CD10 and CD19 followed by FACS analysis. The yield of MNCs varied between 3×10^6 and 555×10^6 cells, probably reflecting both variations in the blast burden of the patients, and in the quality of the received BM aspirates. The proportion of CD10⁺CD19⁺ cells varied between 34 and 90%. Low relative

blast content was probably due to either partly conserved normal BM function or to admixture of peripheral blood during the sample collection. Contaminating cells were excluded from subsequent cell death measurements by subpopulation analysis of the CD19⁺ cells; however, they were not excluded from immunoblot analysis of p53 or measurement of total cellular cAMP levels. This represents a possible source of error in the interpretation of results from the latter two analyses. The proportion of contaminating cells could have been reduced by proceeding with additional selection procedures on the MNC-population in parallel to what was performed on normal BM samples. However, such selection was not carried out in order to minimize loss of cells.

Normal BCPs

Normal BCPs were isolated from 50-100 ml BM aspirates from healthy voluntary donors. Because normal BCPs account for only a minor portion of the MNCs from normal BM, we enriched for these cells by first depleting CD15⁺ cells to remove CD15⁺CD10⁺ granulocytes before proceeding to positive selection of CD10⁺ cells. The fraction of BCPs in the final population was assessed by CD19 staining and FACS analysis of the CD10-selected population. The final yield after CD10 selection varied between 0.3×10^6 and 6.1×10^6 cells, and the fraction of CD19⁺ cells within this population varied between 68 and 95%. As for BCP-ALL blasts, only the CD19⁺ cells were included in cell death analyses, whereas the total population of selected cells were included in the measurement of total cellular cAMP levels.

It may be questioned whether the population of normal BCPs isolated for our experiments constitutes a true normal counterpart of the BCP-ALL blasts. CD10⁺ BCPs (used in cAMP measurements) encompass cells in all stages from CLP to immature B lymphocytes, and CD10⁺CD19⁺ BCPs (analyzed in cell death experiments) include cells in all stages from pro-B to immature B lymphocytes (Figure 29). We did not conduct further immunophenotypic characterization of the normal BCP material, and can therefore not account for the relative contribution of distinct BCP cell subpopulations in the isolated BCPs. The BCP-ALL blasts used in our study are all classified into either B-II (common B) or B-III (pre-B) subtypes, and thus resemble relatively early stages of normal B lymphocyte development. To avoid additional reduction in cell yield, complicating the performance of assays, we did not perform further sorting of normal BCPs to obtain a cell population representing a more limited span of B lymphocyte development.

In addition, the concern of poor age matching of patient and healthy control groups can be raised. The use of properly matched control groups was not appropriate in our study, because it would have involved young children as donors of considerable volumes of BM. Considering the constraints imposed by practical and ethical considerations mentioned above, we believe that our choice of isolation method for normal BCPs resulted in a cell population representing an acceptable and relevant normal counterpart for BCP-ALL blasts.

Bone marrow-derived stromal cell layers

BM stroma is a term denoting cells anchored in the BM cavity and includes endothelial cells, reticular cells, fibroblasts, adipocytes, and macrophages. They grow readily in culture, forming adherent layers capable of supporting human myelo- and lymphopoiesis [573]. The stromal cell layers were prepared from methylcellulose-separated buffy coat layers of normal BM aspirates. Cells were grown in the presence of corticosteroids which ensure cellular heterogeneity as opposed to stroma grown in the absence of corticosteroids resulting in a morphogenically homogenous monolayer of BM-derived fibroblasts [573]. The cellular composition of the established stromal layers was not determined. The ability of the stromal layers to support blast survival diminished over time, and the cell layers were therefore used in experiments within one week after confluent growth was achieved.

Ethical considerations

There are several ethical concerns to consider when collecting material from patients and voluntary controls. In the case of our study, the patient material was collected concomitantly with diagnostic BM aspiration performed during general anaesthesia, thus representing no additional medical procedure for the children. The volume of donated BM was 1-3 ml, which by the involved paediatricians was considered not to cause any significant additional deterioration of the patient's residual BM function. In spite of this, one should always be sensitive to the additional psychological burden imposed on parents and patients asked to participate in a scientific study during such a turbulent and disturbing phase of their lives. Regarding the healthy donors, they were recruited amongst medical students on a voluntary basis. Aspiration of 50-100 ml of BM was performed from the posterior iliac crest under local anaesthesia. The procedure is experienced as painful but tolerable by volunteers, and is associated with minimal risk of serious complications. The collection of BM material from patients and healthy volunteers was approved by the Regional Ethics Committee of Norway, region Sør-Øst A, and recommended by Competent Authorities.

7.1.2 Modulation of cAMP levels and signalling

7.1.2.1 Adenylyl cyclase activation by forskolin

Forskolin is a diterpene compound with the ability to activate all tmACs [574;575] with the exception of tmAC IX [576;577]. It has no effect on sAC [496;578]. Independently of its effect on ACs, forskolin has been demonstrated to affect voltage-gated K⁺ channels [579] and glucose transporters [580]. Upon addition to cell culture, forskolin induces a rapid and pronounced elevation of cAMP levels. The effect on cAMP levels is transient, but in contrast to G_s-coupled GPCR stimulation in which cAMP return to base-line levels within 1-2 hours, cAMP in forskolin-treated cells does not completely return to preinduction levels (H. K. Blomhoff, unpublished results).

7.1.2.2 cAMP analogues

The cell-permeable synthetic cAMP analogue 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP) was used to exclude the possibility that the observed effects of forskolin could be due to AC-independent signalling. As 8-CPT-cAMP is relatively PDE resistant, its use carries the similar risk to forskolin in inducing a supraphysiological cAMP response both in terms of amplitude and duration. 8-CPT-cAMP is a potent agonist of PKAs; however, it is also a potent EPAC agonist. Therefore, 8-CPT-cAMP is not suitable as a tool to discriminate between effects channelled through these two groups of cAMP effector proteins. To distinguish between PKAs and EPACs as the likely cAMP effector in our experimental system, we made use of the EPAC-specific agonist 8-pCPT-2'-O-Me-cAMP [581], which due to methylation of the 2'-ribose hydroxyl group, is an extremely poor PKA activator.

7.1.3 DNA-damaging treatment

DNA can be damaged in a number of ways in an experimental setting to study pathways that are involved in DDR signalling. The mode of action of the DNA-damaging agents used in the present study is briefly explained below.

7.1.3.1 Ionizing radiation

IR consists of subatomic particles or electromagnetic waves that contain sufficient energy to detach electrons from atoms, thereby ionizing them. The source of IR used in our experiments was ^{137}Cs , yielding high-energy electromagnetic waves termed γ rays. DNA damage by IR can result either from the direct ionization and subsequent deionization of the DNA molecule, or from ionization of surrounding water resulting in highly reactive free radicals that can react with DNA as a secondary event [582]. IR can cause many different types of DNA damage, such as single strand breaks (SSBs), DSBs, various types of base damage and DNA-protein crosslinks [582-584]. However, in terms of damage with lethal consequences for the cell, DNA DSBs are thought to be most important [583;584].

7.1.3.2 Chemotherapeutic drugs

Cell cycle phase-specific action

Camptothecin is a cytotoxic alkaloid extracted from the plant *Camptotheca acuminata*. Its semisynthetic, water-soluble derivative Irinotecan is now in common use in the treatment of colorectal cancer. It is a topoisomerase I inhibitor, binding to the topoisomerase I-DNA complex, thus preventing religation of DNA SSBs in the DNA unwinding process during DNA replication. This results in DNA DSBs that are selectively imposed during the S phase of the cell cycle [585].

Cell cycle phase non-specific action

Doxorubicin and daunorubicin are anthracyclins that act by direct binding via intercalation between base pairs in the DNA helix. Doxorubicin is also a powerful iron chelator, and the iron-doxorubicin complex can bind DNA, producing free radicals that can act directly on the neighbouring DNA molecule. In addition, it can act as an inhibitor of topoisomerase II, thus inhibiting DNA repair. The end result is blockade of DNA and RNA synthesis and fragmentation of DNA [585].

Cyclophosphamide is an alkylating agent of the nitrogen mustard type. It is metabolized and activated in the liver, and acts by alkylation and direct binding to DNA, resulting in DNA crosslinking. In addition, it similarly affects RNA, resulting in inhibition of protein synthesis [585]. Due to the need of metabolic activation, we have used the cyclophosphamide derivative 4-hydroperoxycyclophosphamide (4-OOH-CP). In aqueous solution, this molecule rapidly

generates 4-hydroxycyclophosphamide that further degrades to phosphoramidate mustard and acrolein. These three latter compounds are all active metabolites of cyclophosphamide *in vivo* [585]. In addition, H₂O₂ and OH radicals are produced which might contribute to the cytotoxic effect of 4-OOH-CP *in vitro*.

Cisplatin is a synthetic platinum-based drug which covalently binds DNA, resulting in intra- and inter-strand crosslinks creating cisplatin-DNA adducts and preventing DNA, RNA and protein synthesis [585].

7.1.3.3 The selective use of different DNA-damaging treatments

We had previously shown that cAMP, as a result of its ability to attenuate DNA replication, inhibits cell death induced by S phase-specific cytotoxic drugs [556]. In paper III, we wished to examine this effect of cAMP in EBV-infected B lymphocytes, which are defective in AC activation. We therefore utilized the S phase-specific cytotoxic compound camptothecin.

In paper I, we aimed to study the possible inhibitory effect of cAMP on DDR signalling *per se*. Therefore, we wished to avoid the presumed inhibitory effect of cAMP on the extent of induced DNA damage associated with the use of S phase-specific cytotoxic drugs. To achieve this, we used IR and DNA-damaging chemotherapeutic compounds that act in a cell cycle phase non-specific manner. With the exception of cisplatin, we used drugs that are common constituents of BCP-ALL treatment regimens such as anthracyclins and cyclophosphamide.

In papers I, II and IV, we extensively used IR as a means of introducing DNA DSBs despite the rare use of this treatment modality in BCP-ALL. The reason for this is that IR represented a readily available source of DNA damage that produced highly reproducible results both in the BCP-ALL cell line Reh and in primary BCP-ALL cells. The dose-responses to the different chemotherapeutic drugs were much more variable, especially within primary BCP-ALL samples, representing a challenge when working with limited cell material of limited *in vitro* viability. We therefore chose to perform most of the experiments using IR, and subsequently confirm the generalizability of some of the key results by using clinically relevant chemotherapeutic drugs in selected experiments.

7.1.4 Modulation of p53 levels

7.1.4.1 Knock-down of p53 by transfection

In order to verify our notion that the inhibitory effect of cAMP on cell death was a consequence of the effect of cAMP on p53 levels, we aimed to investigate the effects of cAMP in the absence of p53. This was achieved in part by utilizing the lymphoid isogenic cell lines TK6 and WTK1 which differ in their p53 status. However, as the bulk of our experimental work was performed on Reh cells, we sought to knock-down p53 in this cell line by transfection. Good transfection efficiency is generally difficult to achieve in lymphoid cells. We used the Amaxa Nucleofector technology, an electroporation-based method in which an optimized nucleofector solution and electrical parameters are combined to target plasmid DNA into the cell nucleus to trigger rapid and enhanced gene expression. This method is also suitable for use with small interfering RNAs (siRNAs). The viability of Reh cells after transfection was approximately 80%. For plasmid DNA, a transfection efficiency of 30-35% was achieved as measured by the expression of green fluorescent protein (GFP) 24 hours after transfection with the pMax-GFP control plasmid provided by Amaxa. For siRNA the transfection efficiency was approximately 90% as measured by the incorporation of fluorescein-tagged control siRNA 2 hours after transfection (E. H. Naderi, unpublished results).

The pXJ-E6 plasmid encodes the E6 protein from HPV16, one of the HPV strains associated with high risk of cervical cancer development. E6 recruits the cellular ubiquitin ligase E6AP, facilitating p53 polyubiquitination and proteasomal degradation as described in section 4.5.4.3. Transfection of Reh cells with pXJ-E6 resulted in a substantial but transient knock-down of p53 levels with the maximum effect observed approximately 24 hours after transfection. Transfection with siRNA against p53 yielded slightly better knock-down, with maximum suppression of p53 levels observed after 8 hours. In an effort to circumvent the problem of limited transfection efficiency of Reh cells, we aimed to establish stably transfected Reh cells using either the pXJ-E6 plasmid or an inducible p53 short hairpin RNA (shRNA)-producing plasmid. However, the establishment of such clones expressing sufficient amounts of the relevant plasmid product was not successful.

7.1.4.2 Activation of p53 by nutlin-3

Nutlins belong to a group of *cis*-imidazoline compounds identified in a high-throughput screening of synthetic chemicals capable of inhibiting the p53-HDM2 interaction. One of the enantiomers of nutlin-3, termed nutlin-3a was demonstrated to be the most potent, with an IC₅₀ value of 0.09 μM [444]. Using X-ray crystallography, it has been shown that nutlins bind to the p53-binding site of HDM2 [444]. Nutlin-3 has since its discovery become a valuable molecular tool in cell biology, capable of causing p53 accumulation and activation in the absence of genotoxic stimuli. *In vitro* studies have demonstrated that nutlin-3 is able to induce apoptosis in virtually all the investigated primary haematological malignancies that express wt p53, suggesting that downstream defects of the p53-induced apoptotic pathway are uncommon events in these cancers. This is contrasted to the situation observed in cell lines originating from wt p53-expressing solid tumours in which the cell cycle arrest function appears to be universally preserved, while the apoptotic response vary considerably (reviewed in [586]). Treatment of Reh cells with 10 μM of the racemic mixture of nutlin-3 enantiomers was found to stimulate the accumulation of p53 to a level comparable to that induced by 10 Gy of IR, and lead to apoptotic cell death. The level of nutlin-3-induced death in Reh cells was lower than that observed with 10 Gy of IR, probably reflecting the mere accumulation of p53 induced by nutlin-3 in the absence of DNA damage-induced p53 PTMs and activation of additional signalling pathways.

7.1.5 Measurement of cell death and apoptosis

Recently, a “Guideline for the use and interpretation of assays for monitoring cell death in higher eukaryotes” was published [587], in which the authors review the plethora of methods used in the study of cell death and discuss their strengths and pitfalls. In their conclusion, two fundamental questions that need to be addressed in a hierarchical order are highlighted: 1) are cells truly dead, and if so, 2) by which subroutine did death occur.

7.1.5.1 Cell death

As discussed in section 4.3.4, there is still no clearly defined biochemical event that identifies a ‘point-of-no-return’ in the process of cell death, and consequently morphological definitions still apply [154]. One of these morphological traits easily applicable to *in vitro* studies is the loss of plasma membrane integrity as defined by the incorporation of vital dyes. Propidium

iodide (PI) is such a dye, being impermeant to intact plasma membranes, but penetrating the damaged membranes of necrotic and late apoptotic cells, intercalating DNA and thus staining the cell. In addition, it has the advantage of being a fluorescent molecule, and is therefore compatible with flow cytometry analysis [588]. In our experimental settings studying *in vitro* cell culture systems, PI staining quantitated by flow cytometry has been the method of choice to establish the presence of cell death. One major advantage of using flow cytometry-based methods lies in its detection of changes on a single cell basis. By co-staining procedures, it is therefore possible to perform targeted analyses on defined subpopulations of cells. We made use of this procedure in studies performed on heterogenous cell populations such as isolated BCP-ALL blasts, normal BCPs, and normal peripheral B lymphocytes. By co-staining cells with CD19-FITC (fluorescein isothiocyanate) antibodies and PI, we were able to determine the fraction of PI-positive cells in the CD19⁺ subpopulation.

7.1.5.2 Apoptosis

Having established the appearance of cell death by PI staining, we next aimed to investigate the subroutine by which the cell death occurred. For a long time, it has been recognized that cells of lymphoid origin are especially prone to undergo apoptosis in response to DNA-damaging treatment [589]. Consequently, we concentrated our efforts on providing evidence that components of the intrinsic apoptotic pathway were activated in the cells. To this end, we used several methods based on flow cytometry or immunoblot analysis, the principles of which will be briefly accounted for below.

Changes in mitochondrial membrane potential ($\Delta\psi_m$)

Changes in $\Delta\psi_m$ can be monitored by staining live cells with the fluorochrome 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1). JC-1 is a lipophilic dye bearing a delocalized positive charge. In healthy cells, the negative mitochondrial charge created by an intact $\Delta\psi_m$ attracts JC-1, thereby resulting in its accumulation within the mitochondria and consequent aggregation. In cells which have lost their $\Delta\psi_m$, JC-1 cannot accumulate, but remains in its monomeric form in the cytoplasm. Upon excitation, aggregated JC-1 emits red fluorescence, whereas monomeric JC-1 emits green fluorescence. Loss of $\Delta\psi_m$ can therefore be monitored by flow cytometry detecting a shift from red^{high}/green^{low} to red^{low}/green^{high} [590;591].

Cleavage of caspase-9, caspase-3, and PARP

Apoptosis-associated cleavage of caspase-9 and -3 as well as of the caspase-3 substrate PARP, can be monitored by the use of antibodies able to recognize the cleaved forms of these proteins in conjunction with immunoblot analysis, fluorescence microscopy or flow cytometry.

Annexin V labelling

One of the morphological features of apoptosis is the loss of plasma membrane asymmetry with translocation of phosphatidylserine (PS) from the inner to the outer leaflet of the membrane. The exposure of PS on the cell surface can be detected by the use of Annexin V, a phospholipid-binding protein with high affinity for PS [592;593]. By labelling cells with FITC-conjugated Annexin V, cells with exposed PS can be detected by flow cytometry.

TUNEL

Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is based on the principle that cleavage of DNA during apoptosis yields double-stranded low molecular weight DNA fragments (mono- and oligonucleosomes) as well as single strand breaks in high molecular weight DNA. These breaks can be identified by labelling the free 3'-OH termini with the modified nucleotide fluorescein-dUTP catalyzed by the enzyme TdT [594]. The appearance of fluorescein-tagged DNA breaks can be detected by flow cytometry or fluorescence microscopy.

The specificity of apoptosis detection methods

None of the above mentioned characteristics of apoptosis are truly specific for apoptosis: $\Delta\psi_m$ dissipation can be transient, caspase activation is involved in non-lethal processes, and PS exposure may be reversible (reviewed in [154]). Even TUNEL, which is commonly regarded as one of the more apoptosis-specific assays, has its limitations as necrotic cells can display extensive DNA degradation [595], and the activation of endogenous endonucleases can similarly produce DNA nicks [596]. However, by combining the detection of an established cell death marker such as PI with the detection of several of the above-mentioned markers of apoptosis, the conclusion that cells in a given context undergo apoptosis can be drawn with relative confidence.

7.2 General discussion

7.2.1 Effects of cAMP on the DDR in lymphoid cells

Previous studies in our laboratory described the mechanism by which cAMP induced a transient inhibition of S phase progression by inhibiting DNA replication. Furthermore, it was shown that this effect of cAMP resulted in attenuation of the effect of S phase-specific cytotoxic drugs when the cells were exposed to these drugs during the cAMP-induced S phase arrest [556]. Thus, cell death induced by the topoisomerase I inhibitor camptothecin, the alkylating agent hydroxyurea, and the antimetabolite 5-fluorouracil, all of which are regarded as highly dependent of ongoing DNA replication for their mode of action [585], was inhibited by cAMP. This was in contrast to the inability of cAMP to inhibit cell death induced by the tubulin polymerization inhibitor vinblastine, and the microtubule stabilizer paclitaxel, which are both considered to be G2/M phase-specific [585]. The above study concluded that cAMP inhibited cell death by S phase-specific drugs due to their reduced ability to induce DNA damage during S phase arrest, and did not address whether cAMP could directly affect the cellular response to DNA damage. The study presented in this thesis was initiated to investigate this latter possibility.

To address this question, we made use of IR and DNA-damaging cytotoxic drugs such as anthracyclines, cyclophosphamide and cisplatin that are able to induce DNA damage in cells independent of ongoing DNA replication (see section 7.1.3). We also changed the experimental conditions by exposing cells to cAMP-elevating agents and cytotoxic drugs over a prolonged period of time, exceeding the duration of the transient S phase arrest induced by cAMP. In paper I, we could demonstrate a profound effect of cAMP on cell death induced by all the above-mentioned agents as measured by PI permeability in the BCP-ALL cell line Reh. In addition, the generality of this effect was demonstrated by using other lymphoid cell lines such as TK6 and EU-3, as well as normal peripheral T and B lymphocytes, and primary BCP-ALL blasts.

Although it is known that cells of the lymphoid lineage are prone to apoptotic cell death upon DNA damage [589], we wished to formally demonstrate that the cell death inhibited by cAMP was indeed apoptotic. In paper I, we could demonstrate that IR resulted in loss of $\Delta\psi_m$, cleavage of procaspase-9 and -3, exposure of PS, cleavage of PARP, and apoptosis-related

DNA cleavage as measured by TUNEL. Furthermore, all of these processes were inhibited by increasing intracellular cAMP levels. This strongly supports the notion that DNA damage-induced cell death proceeds through an apoptotic pathway in our cell system, and that cAMP inhibits this process.

7.2.2 Mechanisms involved in cAMP-mediated inhibition of DNA damage-induced apoptosis in lymphoid cells

7.2.2.1 Effects of forskolin are mediated through AC activation and cAMP production

Forskolin has been used extensively throughout this study to induce cAMP levels, but as forskolin has also been shown to exert effects beyond its ability to activate tmACs [579;580], it was important to verify that the observed effects of forskolin could be attributed to elevation of intracellular cAMP levels. In paper I, we confirmed this point by showing that the effect of a low dose of forskolin could be potentiated by the simultaneous inhibition of cAMP degradation by PDEs. In addition, the cAMP analogue 8-CPT-cAMP was found to generally mimic the effects of forskolin throughout our experiments.

In paper III, we demonstrated that forskolin is able to induce AC activity in Reh cells, and that this leads to cAMP production and activation of PKA. We also showed that EBV infection of B lymphocytes leads to an insensitivity of ACs to forskolin treatment, and this was reflected in forskolin's inability to inhibit S phase progression and thus cell death induced by short-term exposure of cells to camptothecin. We also demonstrated that EBV infection renders B lymphocytes insensitive to the inhibitory effect of forskolin on cell death induced by IR or long-term camptothecin treatment, and that this defect can be circumvented by treatment of cells with 8-CPT-cAMP (E. H. Naderi, unpublished results). Taken together, these findings argue in favour of cAMP as the crucial mediator of forskolin's effect on DNA damage-induced apoptosis. The mechanistic background for the insensitivity of ACs to forskolin treatment in EBV infected cells has not been determined. One possible explanation could be provided by the EBV infection-induced overexpression of Epstein-Barr virus-induced receptor 2 (EBI2). EBI2 is an orphan, $G\alpha_i$ -associated GPCR which has been demonstrated to be constitutively activated in EBV-infected cells [597]. Whether sensitivity to forskolin as a growth inhibitor as well as an inhibitor of DNA damage responses can be restored by

inhibition of EBI2 activity in EBV-infected cells is currently under investigation in our laboratory.

7.2.2.2 cAMP regulates the DDR pathway at the level of p53 protein stability via PKA

In order to pinpoint the level at which cAMP exerts its effect on DNA damage-induced apoptosis, we investigated the effect of cAMP on components of the DDR typically involved in the apoptotic signalling induced by DNA DSBs. In paper I, we could show that cAMP did not reduce the IR-induced activating phosphorylations of ATM or Chk2; however, it did have a profound effect on IR-induced p53 protein levels. Because the cAMP-analogue 8-CPT-cAMP acts as an agonist of both PKA and EPAC, we wished to address which of these effector proteins were involved in mediating the effect of cAMP on p53. To do so, we used the EPAC-specific analogue 8-pCPT-2'-O-Me-cAMP, and could show that this compound was unable to affect IR-induced p53 accumulation. This result strongly indicates that the observed effects of cAMP on p53 levels are mediated by PKA. Accumulation of p53 following DNA damage is thought to occur primarily as a result of modulation of its protein stability [279;280], and to a lesser degree through transcriptional and post-transcriptional mechanisms (see section 4.5.2.1). We addressed the mechanisms involved in regulation of p53 in paper II and showed that the steady state levels of *p53* mRNA were unaffected by both IR and cAMP treatment. In contrast, IR was found to profoundly increase the stability of the p53 protein. cAMP treatment antagonized this stabilization, restoring the $T_{1/2}$ of p53 to approximately 30 min.

7.2.2.3 The causal relation between cAMP effects on p53 and apoptosis

In paper I, we demonstrated that in Reh cells exposed to IR, the degree of cAMP-mediated inhibition of p53 accumulation correlated with the degree of cAMP-mediated attenuation of apoptosis. IBMX (an inhibitor of PDEs) or low doses of forskolin produced minor effects on IR-induced p53 accumulation and cell death, whereas the combination of the two, or a single-agent treatment of cells with higher doses of forskolin or 8-CPT-cAMP led to pronounced effects both on p53 levels and cell death. It should be mentioned that most of our immunoblot analyses of p53 levels, including those performed for assessment of p53 protein stability, were carried out on cells that were harvested 4 hours after IR. At this time point, p53 levels are not as efficiently suppressed by 8-CPT-cAMP as by forskolin, which could seem to be in conflict

with the consistent observation of a superior effect of 8-CPT-cAMP on cell death. However, examination of p53 expression (paper II, Figure 1A) shows that 8-CPT-cAMP and forskolin suppresses p53 levels with slightly different kinetics. Whereas 8-CPT-cAMP inhibits expression of p53 less efficiently at early time points, it exerts a slightly more potent inhibitory effect at later time points (from 8 hours post-IR and onwards). There is thus a good correlation between effects on p53 levels and cell death also with regard to forskolin and 8-CPT-cAMP.

Encouraged by the above-mentioned correlation between p53 levels and cell death suppression by cAMP, we aimed to establish a causal relationship between the inhibitory effect of cAMP on IR-induced p53 accumulation and its inhibitory effect on cell death. p53-mediated signalling is generally conceived to play an important role in connecting DDRs to the execution of apoptosis in lymphocytes, as demonstrated by severe apoptotic defects of lymphoid cells in *Trp53* KO mice after exposure to IR [598]. However, because some mitogenically activated or transformed lymphocytes have been shown to display a p53-independent DNA damage-induced apoptosis [598], we considered it important to confirm the role of p53 in DNA damage-mediated cell death in Reh cells. To this end, p53 was knocked down by transient transfection with *HPV E6* or p53 siRNA prior to treatment of cells with forskolin and IR. As shown in paper I, this resulted in partial protection against cell death; however, forskolin retained its ability to inhibit the remaining level of cell death (E. H. Naderi, unpublished results). We believe this probably reflects the heterogeneity of cells produced by transfection due to the incomplete transfection efficiency of Reh cells, as accounted for in section 7.1.4.1. Thus, one fraction of cells is successfully transfected, with efficient knock-down of p53, and these cells consequently display pronounced apoptotic resistance. Another fraction of cells retain sufficient p53 activity to undergo apoptosis in response to IR, and these will also retain their sensitivity to cAMP. Although these transfection experiments supported the notion that p53 is important for DNA damage-induced apoptosis in Reh cells, they did not provide evidence for a causal relationship between cAMP-induced p53 suppression and cell death inhibition.

To address this question, we first investigated the effects of cAMP on the lymphoblast cell line WTK1, a p53-mutated isogenic counterpart of TK6. In paper I, we demonstrated that WTK1 cells were highly resistant to IR-induced cell death compared to TK6 cells. Importantly, the remaining levels of IR-induced cell death observed in WTK1 cells were not

inhibited by cAMP. Furthermore, we found that in Reh cells, cell death induced by cytotoxic compounds that have been demonstrated to work in a p53-independent fashion such as menadione [599;600] and staurosporine [363;369;601], could not be inhibited by cAMP. Finally, in paper II, we showed that nutlin-3 could induce p53 accumulation and cell death in Reh cells. In contrast to the potent ability of forskolin to inhibit IR-induced p53 accumulation and cell death, it only slightly inhibited nutlin-3-induced p53 accumulation and had no inhibitory effect on nutlin-3-induced cell death. Taken together, we believe that our results strongly suggest a causal relationship between the observed cAMP-induced p53 suppression and cell death inhibition.

7.2.2.4 The effect of cAMP on p53 protein stability is HDM2-dependent

As discussed in section 4.5.2.1, the main regulatory mechanism for the stability of the p53 protein is the rate of its proteasomal turnover. The priming of p53 for proteasomal degradation is under the control of several E3 ubiquitin ligases of which HDM2 has retained its position as the main player, in particular with regards to DDR-associated changes in p53 degradation [305;417]. Having demonstrated that cAMP affects p53 accumulation at the level of protein stability, we proceeded in paper II to show that this effect depended on the presence of functional proteasomes. Furthermore, we could demonstrate that cAMP counteracted the IR-induced reduction in p53 ubiquitination. To examine whether the effect of cAMP on DNA damage-induced p53 accumulation was mediated through HDM2, we made use of the specific inhibitor of p53-HDM2 interaction nutlin-3 (see section 7.1.4.2). By exposure of cells to IR in the presence of nutlin-3, we could show that cAMP was no longer able to inhibit IR-induced p53 accumulation, suggesting that the observed effect of cAMP on p53 levels was indeed HDM2-dependent.

7.2.2.5 cAMP promotes p53-HDM2 interaction

When approaching the question of how cAMP could affect HDM2-mediated p53 ubiquitination, we hypothesized three possible mechanisms: 1) augmentation of HDM2 levels, 2) augmentation of p53-HDM2 interaction, and 3) augmentation of HDM2 E3 ubiquitin ligase activity.

Modulation of HDM2 levels

HDM2 expression is tightly regulated by multiple factors both at a transcriptional and post-translational level during the DDR [208;323;327] (see section 4.5.2.1.1). In paper II, we used immunoblot analysis to assess whether cAMP could influence the levels of HDM2. As expected, HDM2 levels were transiently downregulated within 1 hour after IR treatment (E. H. Naderi, unpublished results). However, by 2 hours post-IR, HDM2 expression was elevated above basal levels. By 4 hours, it had increased approximately 2-fold before levelling off. Forskolin did tend to increase IR-induced HDM2 levels by 4 hours, however, by 6 hours, forskolin significantly reduced IR-induced HDM2 levels, and by 8 hours forskolin had no effect on IR-induced HDM2 levels. Because cAMP was found to exert an inhibitory effect on p53 ubiquitination not only at 4 hours post-IR as shown in paper II, but also at 6 and 8 hours (S. Naderi, unpublished results), we do not believe that cAMP-induced alterations of IR-induced HDM2 levels can account for the observed effects of cAMP on p53 ubiquitination.

Modulation of p53-HDM2 interaction

As discussed in section 4.5.2.1.1, modulation of p53-HDM2 interaction is assumed to play an important role in the regulation of p53 levels after DNA damage (reviewed in [305]). In paper II we investigated the effect of cAMP on p53-HDM2 interaction by immunoprecipitation of p53, followed by immunoblot analysis, to assess the amount of HDM2 that co-immunoprecipitated with p53. As expected, we found that IR led to dissociation of p53-HDM2 complexes. However, whereas this dissociation was maintained over time in cells that were treated with IR alone, co-treatment of cells with forskolin led to restoration of p53-HDM2 interaction by 4 and 8 hours. Interestingly, the stimulatory effect of forskolin on p53-HDM2 association was also observed in non-irradiated cells, providing an explanation for the observed repression of basal p53 levels by forskolin (papers I, II, IV).

DNA damage-induced N-terminal p53 phosphorylation has long been in focus as an important inhibitor of p53-HDM2 interaction, but the role of these PTMs has in recent years been increasingly challenged [305;417] (see section 4.5.2.1.1). In paper II, we assessed the effect of IR and forskolin on phosphorylation of p53 on S15, S20 and T18. IR was shown to induce phosphorylation at all these sites as expected; however, forskolin inhibited the IR-induced phosphorylation of p53 only on S20. Given recent evidence suggesting that p53 phosphorylation on T18 but not S15 or S20 inhibits its interaction with HDM2, [192;193;298;299], we believe that the cAMP-mediated modification of p53 N-terminal

phosphorylation is an unlikely mechanism underlying the stimulatory effect of cAMP on p53-HDM2 interaction. After our initial report on the inhibitory effect of cAMP on DNA damage-induced accumulation of p53 and apoptosis in Reh and primary BCP-ALL cells (paper I), similar results were published on the BCP-ALL cell line Nalm-6 [602]. This report claims that cAMP suppresses phosphorylation of p53 on a number of serine residues, including S15 and S20, and suggests that the inhibitory effect of cAMP on p53 levels is attributable to attenuation of p53 phosphorylation at these sites. However, because the levels of phospho-serine p53 were not normalized to the total p53 levels, we do not feel convinced that the cAMP-induced reduction in phospho-serine p53 reported in this paper does more than reflect the reduction in total p53 levels.

As accounted for in section 4.5.2.1.1, acetylation of p53 has also been implicated in the modulation of p53-HDM2 interaction. In addition to reducing the affinity between p53 and HDM2 [307], acetylation can directly contribute to the stabilization of p53 by competing with ubiquitin moieties for available lysines [308-310]. Recent data from our laboratory demonstrates that p53 acetylation is indeed induced by IR in a cAMP-inhibitable manner (S. Naderi and M. M. Kloster, unpublished results). The possible role of these modifications for cAMP-induced restoration of p53-HDM2 interaction and p53 ubiquitination in Reh cells is currently under investigation.

Modulation of HDM2 E3 ubiquitin ligase activity

The enzymatic activity of HDM2 can be affected by various PTMs [316-318] as well as HDM2-interacting proteins [319-322] (see section 4.5.2.1.1). We assessed the ubiquitin ligase activity of HDM2 towards p53 in an *in vitro* ubiquitination assay with HDM2 immunoprecipitates. Although the experiments have proven technically difficult to perform and the results so far have been difficult to interpret, we have no preliminary indications that forskolin treatment enhances HDM2 enzymatic activity towards p53 (S. Naderi, unpublished results).

In conclusion, our results support a mechanism of cAMP action which involves the promotion of p53-HDM2 interaction rather than effects on HDM2 expression levels or enzymatic activity.

7.2.2.6 Is the inhibitory effect of cAMP on cell death induced by S phase-specific and non-S phase-specific DNA-damaging agents mediated by two distinct mechanisms?

The effect of cAMP on cell death induced by DNA-damaging cytotoxic agents has so far in the discussion been regarded as proceeding through two distinct mechanisms of action. In paper III, the result obtained with short-term treatment of cells with the S phase-specific cytotoxic drug camptothecin within the time frame of cAMP-induced S phase arrest, led us to assign the inhibitory effect of cAMP on cell death to its ability to inhibit DNA replication and thus limit the degree of DNA damage inflicted. In papers I, II and IV we exposed cells to a long-term treatment with cAMP-increasing agents in combination with non-S phase-dependent inducers of DNA damage, and attributed the inhibitory effect of cAMP on cell death to its ability to directly inhibit the DDR pathway at the level of p53.

This distinction might be overly simplified. The inhibitory effect of short-term treatment of cells with forskolin on S phase-specific drug-induced cell death can be recapitulated by the DNA polymerase inhibitor aphidicolin (S. Naderi, unpublished results). This strongly supports a role for cAMP in limiting the extent of DNA damage induced by camptothecin or other S phase-specific cytotoxic drugs. However, it is possible that the forskolin-induced S phase arrest does not completely abolish the ability of camptothecin to induce DNA damage, and additional inhibitory effects of cAMP on DNA damage-induced p53 accumulation might therefore contribute to the final inhibition of cell death.

Conversely, it can be speculated that the cAMP-mediated suppression of p53 levels observed in papers I, II and IV, could simply reflect reduced levels of DNA damage as a result of the inhibition of DNA replication by cAMP. However, throughout these studies, we used S phase-independent DNA-damaging agents and therefore assumed that the elevation of intracellular cAMP levels did not affect the degree of DNA damage in a significant way. Nonetheless, although cell cycle phase non-specific chemotherapeutic drugs do not depend on ongoing DNA replication for their mode of action, many are shown to exert maximal cytotoxicity during S phase [585]. Therefore, the use of this class of DNA-damaging agents could be associated with cAMP-induced inhibition of DNA damage levels. Although we have not measured the level of DNA damage in cells in the presence or absence of elevated cAMP, several lines of evidence indicate that possible variations in the extent of DNA damage cannot alone account for the effect of cAMP on p53 accumulation. First, we have used IR extensively

to induce DNA damage. The pattern of sensitivity to DNA damage differ between IR and most cytotoxic drugs, as it is the cells in late G2/M phase that are most radiosensitive whereas cells in late S phase are most radioresistant (reviewed in [603]). Second, if cAMP were to inhibit the level of DNA damage, then short-term elevation of intracellular cAMP levels at the time of infliction of DNA damage would be expected to have an inhibitory effect on cell death. However, we have observed that short-term exposure of cells to forskolin or 8-CPT-cAMP around the time of radiation has minimal effect on the resulting cell death (E. H. Naderi, unpublished results). Third, in paper I we observed no inhibition of IR-induced activation of ATM or Chk2 upon treatment of cells with cAMP-elevating agents, which would have been expected if cAMP had a major inhibitory effect on the level of inflicted DNA damage. Fourth, we have shown that cAMP also inhibits p53 levels in the absence of DNA damage. This is documented in various p53 immunoblots in papers I, II and IV, and is also shown to be statistically significant for BCP-ALL blasts in paper IV. This strongly suggests that cAMP downregulates p53 levels independently of DNA damage. One possible problem with this argument could be the finding that transformed cells have been shown to display constitutive DNA damage due to oncogene-driven replication stress [406]. However, in paper II we show similar suppression of basal p53 levels in phytohaemagglutinin (PHA)-stimulated normal peripheral CD4⁺ T lymphocytes. As oncogene-driven replication stress has been shown to be a tumour specific phenomenon, and not a general feature of mitotically stimulated cells [406], the downregulation of p53 by cAMP in PHA-stimulated T lymphocytes is highly unlikely to be attributable to differences in DNA damage levels.

In conclusion, we believe that cAMP can suppress cell death induced by DNA-damaging treatment through two distinct mechanisms:

- 1) When cells are treated with S phase-specific drugs during the transient cAMP-induced S phase arrest, cAMP limits the extent of DNA damage inflicted.
- 2) cAMP exerts a direct inhibitory effect on the DDR pathway by attenuating the accumulation of p53. This is due to cAMP-induced augmentation of the p53-HDM2 interaction which counteracts DNA damage-associated reduction in p53 ubiquitination and consequent proteasomal degradation.

7.2.3 Clinical implications of the cAMP effect on p53 and apoptosis using BCP-ALL as a model system

Having demonstrated that cAMP can inhibit p53 accumulation and the consequent apoptotic response in various lymphoid cell systems, we wished to examine the possible clinical relevance of our findings. As described in section 4.5.4.1, p53 is inevitably activated during malignant transformation, possibly due to oncogene-induced p14^{ARF} activation [394] or oncogene-induced replication stress with consequent activation of the DDR pathway [406]. Due to its ability to induce apoptosis and senescence in transformed cells, normal p53 function is considered to be a major barrier against malignant development, and is thus assumed to be universally suppressed in cancers [4;355]. This can either be achieved through direct mutational inactivation, through inhibition of wt p53 levels or activity, or through defects in downstream pathways [355]. Most of our experimental work has been performed on Reh, which is a BCP-ALL derived cell line. Wt p53 is almost always preserved in BCP-ALL [604], and furthermore, downstream defects in the p53 pathway appear to be a rare phenomenon in haematological malignancies (reviewed in [586]). This would indicate that suppression of wt p53 levels or activity is likely to be important in the pathogenesis of BCP-ALL. Based on our finding in Reh cells that elevated cAMP levels are able to suppress induction of wt p53 accumulation in an HDM2-dependent manner with consequent suppression of apoptosis, we hypothesized that deregulation of cAMP signalling might be utilized as an anti-tumour-suppressive mechanism by BCP-ALL blasts.

7.2.3.1 The effect of cAMP as a malignancy-associated trait

In order to verify that the observed effects of cAMP in Reh cells were representative of BCP-ALL, we initiated a pilot study on primary BCP-ALL blasts isolated from paediatric patients at initial diagnosis. In papers I and IV, we showed that these primary cancer cells uniformly responded to augmented cAMP signalling in a manner similar to what we had previously observed in Reh: 1) p53 levels were induced upon treatment with IR or DNA-damaging chemotherapeutic drugs commonly used in BCP-ALL treatment, 2) cAMP-elevating agents suppressed both basal and DNA damage-induced p53 levels, and 3) cAMP-elevating agents potently suppressed DNA damage-induced cell death. In addition, cAMP could significantly inhibit spontaneous cell death during *in vitro* culturing of these blasts, an interesting finding that stands in contrast to what we have generally observed in cell lines and in cultured normal

peripheral T and B lymphocytes, in which augmented cAMP signalling has either no effect or a slightly toxic effect.

To investigate whether the protective effect of cAMP signalling was a general feature of BCPs, we isolated normal BCPs from BM aspirates of healthy controls and exposed them to cAMP and IR. Due to the limited BCP cell yield from normal BM, only three of the collected samples were available for such treatment with subsequent cell death analysis. The results presented in paper IV show reproducible results between these three samples: IR yielded limited cell death, and forskolin could neither protect cells from spontaneous nor IR-induced cell death. Indeed, forskolin had a slight toxic effect on BCPs. Although performed on only three samples, the consistency of results is suggestive of a real difference in cAMP-responsiveness between normal BCPs and transformed BCP-ALL blasts. This finding is also supported by a previous report, demonstrating an apoptotic effect of cAMP in CD10⁺ BCPs which was correlated with a decrease in MCL-1 expression [605].

7.2.3.2 Possible implications of cAMP signalling for BCP-ALL development

The observed protective effect of cAMP elevation on blast survival together with its suppressive effect on p53 levels, suggested that augmented intracellular cAMP levels might be selected for during the course of malignant transformation. To test this hypothesis, we first measured total cellular cAMP levels in BCP-ALL blasts and compared it to that of normal BCPs isolated from healthy BM donors (paper IV). We found no indications of elevated cAMP levels in the leukaemic blasts compared to untransformed BCPs. In light of the tight spatiotemporal control of physiological cAMP signalling described in section 4.7.3, the lack of such differences in gross cAMP content might not be unexpected. Nonetheless, given our finding that cAMP exerts an inhibitory effect on p53 expression in BCP-ALL blasts, together with the published studies describing increased cAMP levels in tumour materials [542;543], we found it important to explore the possibility of substantial transformation-related changes in intracellular cAMP concentrations.

Because there was no indication of constitutively upregulated cAMP levels in BCP-ALL blasts, we considered the possibility that cAMP-augmenting signals might be provided to the blasts by the BMME. Similar to solid tumours that are known to exist in interdependence with their surrounding tumour stroma (see section 4.1.3), it is well established that leukaemic

blasts *in vivo* exist in a tight interplay with cells of the BMME, being provided with signals conveyed through secreted factors, cell-cell and cell-matrix interactions [606;607]. Such signalling has also been implicated in the development of treatment resistant subclones (reviewed in [606;607]). To investigate whether interaction with BM stromal cells could recapitulate the observed effects of cAMP on BCP-ALL blasts, we co-cultured the blasts on a confluent layer of a BM-derived stromal cell line or primary stromal cells. In paper IV, we show that such co-culturing can indeed protect BCP-ALL blasts against spontaneous and DNA damage-induced cell death. Furthermore, we demonstrate that this effect is in large part dependent on cell-cell interactions, because much of the protective effect is lost when blasts are physically separated from the stromal cells by porous well inserts. The observation that stromal cells suppress the expression of p53 in BCP-ALL blasts, an event also shown to occur upon elevation of cAMP levels, indicates that stromal cells might utilize cAMP signalling to exert their pro-survival effect on BCP-ALL blasts. Preliminary results with PKA inhibitors suggest that cAMP-induced PKA activity is implicated in the protective signalling provided by stromal cells to BCP-ALL cells.

There is also a possibility that cAMP levels *per se* are not elevated in transformed cells, but that signalling events downstream of cAMP might be aberrantly activated. As described in section 4.7.4.1, constitutive upregulation of RI α and the relative increase in the ratio of PKAI to PKAII has been implicated in malignant transformation (reviewed in [549;550]). In accordance with this, the BCP-ALL cell line Reh has been shown to express only the PKAI isoform [608]. This together with our demonstration that PKA inhibition can potentiate DNA damage-induced cell death in BCP-ALL blasts, warrants further investigation into the relative expression of PKA isoforms in primary BCP-ALL blasts.

The suggestion that the transformation process of BCPs favours the selection of enhanced cAMP signalling to quench the transformation-related activation of p53, should be weighed against the possible negative effect of cAMP on tumour progression due to its growth-inhibitory effect. Indeed, cAMP has been shown to exert an anti-proliferative effect on cells of the lymphoid lineage [554;609;610] including Reh [554;555;611]. Assuming that cAMP also inhibits the proliferation of primary BCP-ALL blasts, it may be suggested that this effect of cAMP might counteract the selection of augmented cAMP signalling as a p53-neutralizing event. However, one could envision a scenario in which the BM normally provides BCPs with stimuli generating a certain level of cAMP sufficient for the execution of various cell

physiological processes, but not enough to compromise their proliferative capacity. Once faced with oncogenic transformation, these same signalling pathways might be taken advantage of by providing sufficient cAMP levels to suppress oncogene-induced p53 accumulation, and thus become part of an essential transformation-related survival pathway.

7.2.3.3 Possible implications of cAMP signalling for BCP-ALL treatment

The possibility that cAMP can be incorporated in a transformation-related survival pathway can potentially be problematic during treatment of the leukaemia, as it would compromise the induction of p53 by DNA-damaging treatment modalities. In support of this, our results presented in papers I and IV clearly demonstrate the ability of cAMP to inhibit chemotherapy- and radiation-induced p53 accumulation and cell death in BCP-ALL blasts. Furthermore, in paper IV, we demonstrate that stromal co-culture can limit radiation-induced p53 accumulation and cell death, an effect that tends to be reversed by PKA inhibitors. These findings suggest a role for PKA inhibitors in the potentiation of conventional BCP-ALL treatment. The observation that the normal BCPs used in our study do not show enhanced survival upon augmentation of cAMP levels, indicates the possible presence of a therapeutic window for such treatment, allowing for the suppression of a survival pathway which is active in the leukaemic blasts but not in their normal counterparts.

The idea of manipulating the cAMP pathway as part of cancer treatment is not new. As briefly reviewed in section 4.7.4.1, deregulation of several of the signalling components of the cAMP pathway have been implicated in cancer development. Regarding reported effects of elevated cAMP levels in experimental settings, the results on spontaneous and DNA damage-induced apoptosis appear to be highly cell type-specific. In the haematopoietic system, transformed cells of the myeloid lineage have in some studies been shown to be sensitive to cAMP-induced apoptosis [612;613], whereas other studies have demonstrated cAMP-mediated protection against both spontaneous and chemotherapy-induced apoptosis [545;546]. These latter reports agree with our findings in lymphoid cells, but whether the mechanisms of action involved are similar is uncertain, as none of the above mentioned papers have examined the regulation of p53 by cAMP signalling.

Regarding transformed lymphoid cells, however, our results seem to generally contrast the published data assigning a pro-apoptotic role for cAMP signalling. PDE inhibitors have been

demonstrated to induce both spontaneous apoptosis and to synergize with chlorambucil in B-CLL [614;615] leading to clinical trials assessing their possible therapeutic potential. Gene expression analysis of clinical DLBCL material has demonstrated overexpression of PDE4B in fatal/refractory cases, and this has been interpreted as an indication that PDE-induced reduction of cAMP levels would be of advantage to the cancer cells, rendering them resistant to chemotherapy [616]. These reports of pro-apoptotic effects of cAMP signalling, in combination with the already established anti-proliferative effect of cAMP, has encouraged speculations into the possible therapeutic use of cAMP-augmenting agents such as PDE inhibitors in various lymphoid cancers [541]. B-CLL and DLBCL are both lymphoid malignancies of a more mature phenotype than BCP-ALL. Therefore, the conclusion that cAMP augmentation might be useful in the treatment of these diseases should not automatically discourage the principle of inhibition of cAMP signalling as a potential therapeutic approach in BCP-ALL.

Regarding cAMP and ALL, one report demonstrates that cAMP elevation can potentiate glucocorticoid-induced cell death in the T lymphoblast cell line CEM as well as in Reh, and consequently, cAMP-inducing therapy was suggested to have possible clinical potential also in ALL [617]. Glucocorticoid-induced apoptosis in lymphocytes has been shown to proceed through a p53-independent pathway [243;372;598], which could explain why cAMP appears to have opposing effects on glucocorticoid- and DNA damage-induced cell death. We have not reproduced the above-mentioned finding that cAMP augments glucocorticoid-induced apoptosis in Reh cells, nor have we examined these effects in the material of primary BCP-ALL blasts. If indeed our suggestion to combine PKA inhibition with conventional BCP-ALL treatment should be translated into clinical use, the possible interference of PKA inhibition with glucocorticoid treatment would be important to clarify, as such effects might necessitate careful attention to the relative timing of PKA inhibition with respect to chemotherapy and glucocorticoid administration.

7.2.3.4 Clinical relevance of cell systems

When hypothesizing potential clinical implications of laboratory-based results, it is important to evaluate the relevance of the cell systems that have been used. Performing experiments on primary cells isolated from patients and healthy volunteers certainly brings research one step closer to the bedside compared to cell line-based assays; however, the impact of the

unphysiological *in vitro* settings as well as the selection of cell material must be critically considered. Regarding the latter point, the cellular origin of BCP-ALL blasts is not definitely determined. Depending on the subtype, both HSCs and early B lineage-committed CD19⁺ progenitor cells have been proposed to be the target for the initial genetic hits that produce the ‘cancer stem cells’ driving the disease (reviewed in [568;569]). These very rare cells then give rise to progeny that are arrested prematurely during their differentiation, implying that the bulk of leukaemic cells found in the patient’s BM display a phenotype which is more differentiated than their transformed cells of origin. As all patients included in our study were diagnosed with either B-II (common-B) or B-III (pre-B) subtypes of BCP-ALL, they shared the CD10⁺CD19⁺ phenotype with the analyzed population of normal BCPs. However, as discussed in section 7.1.1.2, the normal BCP cell population comprises a wider spectrum of BCP stages, including cells with more mature phenotypes, than the ALL blasts. With respect to our speculations into the possible clinical relevance of our findings, there are thus at least two important notes of caution that deserve mentioning. First, the concern that we have studied the progeny population of cancer cells rather than the very few cells that are probably responsible for driving the leukaemia. Secondly, the concern that by comparing the responsiveness of the blasts with a heterogenous and probably more differentiated population of CD10⁺CD19⁺ normal BCPs, we might not be describing a shift in cAMP responsiveness resulting from the malignant transformation, but rather a difference arising as a result of B lymphocyte maturation.

These potential problems regarding the relevance of our cell systems cannot easily be circumvented. The exact nature of ‘cancer stem cells’ for paediatric BCP-ALL is not determined [569;618] and their isolation and study is therefore difficult. Furthermore, even though ‘cancer stem cell’-directed therapy might be appealing in theory, experimental or clinical evidence to prove its superiority over conventional therapy is still pending [619]. With respect to our BCP populations, further isolation procedures could have been performed to enrich for cells at developmental stages matching more closely those present in BCP-ALL samples. Such procedures would have led to additional loss of cell material, and were therefore not carried out.

Consequently, bearing the concerns regarding the clinical transfer value of our *in vitro* findings in mind, we nonetheless conclude that our findings on differences in cAMP sensitivity of primary BCP-ALL blasts and normal BCPs deserve further attention, and that

they lend encouraging support to our hypothesis on the possible role of cAMP in BCP-ALL development and treatment.

8 CONCLUSIONS

In this thesis, we have demonstrated that cAMP is an important modulator of DNA damage responses in lymphoid cells. The data presented show that:

- 1) cAMP inhibits DNA damage-induced apoptosis in a variety of lymphoid cells. The inhibitory effect of cAMP on apoptosis depends on its ability to attenuate p53 accumulation.
- 2) The inhibitory effect of cAMP on p53 accumulation depends on attenuation of p53 protein stabilization as a consequence of its augmented interaction with HDM2, resulting in maintenance of p53 ubiquitination and proteasomal degradation.
- 3) cAMP can potently inhibit both basal and DNA damage-induced p53 levels and cell death in cultured primary BCP-ALL blasts. This is in contrast to the lack of protective effect of cAMP on cell death in normal BCPs. Consequently, we have hypothesized a possible role for cAMP signalling in a transformation-associated survival pathway engaged to quench oncogene-driven p53 activation. Furthermore, we have proposed that such signalling could contribute to resistance against DNA damage-inducing therapy. Our preliminary results suggest that PKA inhibitors can be utilized to potentiate the effect of such agents.

9 FUTURE PERSPECTIVES

In light of the results presented in this thesis, we wish to pursue the following lines of investigation:

- 1) Further characterize the mechanisms involved in cAMP-mediated augmentation of p53-HDM2 interaction. In particular, we intend to explore the possible effect of cAMP on p53 acetylation levels.
- 2) Explore the molecular basis for the observed differential effects of cAMP on BCP-ALL blasts and normal BCPs. In particular, we intend to investigate whether there are differences in expression levels of RI and RII subunits of PKA between these two cell types.
- 3) Validate our hypothesis that the pro-survival effect of BM stromal cells on BCP-ALL blasts is in part mediated through cAMP signalling. As we have indications that cell-cell interactions are of importance, the possible involvement of stroma-bound ligands for AC-associated GPCRs as well as gap junction-mediated cAMP transfer will be investigated.
- 4) Further explore the therapeutic potential of PKA inhibitors. We wish to extend our findings indicating a role for PKA inhibition in potentiation of IR-induced cell death to DNA-damaging chemotherapeutic compounds commonly used in treatment of BCP-ALL. In case of promising *in vitro* results, we aim to test the feasibility of PKA inhibition as an addition to conventional BCP-ALL treatment *in vivo*.

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Errata

The following corrections of the originally submitted manuscript have been approved by the evaluation committee and implemented in the final printed version.

- Pages 7-9: Change the order of references 1 and 2, with corresponding changes in the reference list.
- Page 18: Figure legend of figure 6: add a full stop at the end.
- Page 20: 2nd line: correct "C. elegance" to "C. elegans".
- Page 21: Figure legend of figure 7: remove a double space before "The figure shows...".
- Page 21: Figure legend of figure 7: insert a space to correct "C.elegans" to "C. elegans".
- Page 22: Last sentence: change "revieed by..." to "revieed in...".
- Page 24: First sentence: remove hyphen in "BH-3-only" to "BH3-only".
- Page 35: First sentence: remove a space after the oblique, *i.e.* "kinase/ mitogen" to "kinase/mitogen".
- Page 43: 5th and 6th lines: remove space in "NF- κB" to "NF-κB".
- Page 46: 8th line: remove double space in "targeted by" to "targeted by".
- Page 46: 3rd paragraph, 6th line: remove hyphen in "CBP-/p300" to "CBP/p300".
- Page 49: 4th line: change "or" to "of".
- Pages 51-52: Expand "IR" as "ionizing radiation" at first and not second mention, *i.e.* page 51, 2nd paragraph, 15th line should read: "protected from ionizing radiation (IR)-induced apoptosis", whereas page 52, 1st paragraph, 8th/9th line should read: "... come from whole body IR of mice".
- Page 54: 2nd paragraph, 23rd line: change "INK4A" to "p16^{INK4A}".
- Page 103: 2nd paragraph, last sentence: correct "ovserved" to "observed".
- Page 106: 3rd line in the heading: correct "mechanims" to "mechanisms" with corresponding correction in the index.
- Page 107: 3rd line: remove double space before "Second, if cAMP...".
- Page 108: 1st paragraph, last line: add left-out word to change "tumour-suppressive" to "anti-tumour-suppressive".
- Page 113: 12th line: add left-out word to specify "blasts" as "ALL-blasts".

