The tumour suppressor protein $p14^{ARF}$ regulates cell cycle checkpoint control and induces cell death

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Ich erkläre hiermit, dass ich die vorliegende Arbeit selbständig und nur unter Zuhilfenahme der angegebenen Hilfsmittel angefertigt habe. Die praktischen Arbeiten wurden in der Abteilung Klinische und Molekulare Onkologie an der Robert-Rössle-Klinik, Charité, Campus Berlin-Buch der Arbeitsgruppe Prof. Dr. P. Daniel durchgeführt.

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TO MY FATHER

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P

Zusammenfassung

Das INK4a Gen kodiert für das Suppressorprotein $p14^{ARF}$, welches in fast der Hälfte aller Tumore mutiert ist. Es wurde gezeigt, dass $p14^{ARF}$ eine wichtige Rolle in der Regulation des Zellzyklusarrestes und der Apoptose spielt. Das Ziel dieser Arbeit war es, die Regulation des $p14^{ARF}$-induzierten Zelltodes zu untersuchen. Aus diesem Grund wurde eine MCF-7 p53 wildtyp Brustkrebs Zelllinie verwendet, welche keine Effektor Caspase-3 exprimiert, sowie MCF-7 Zellen die stabil mit Caspase-3 retransfiziert wurden. Die Expression von $p14^{ARF}$ induzierte einen G1 Arrest, sowohl in Caspase-3 defizienten als auch in Caspase-3 exprimierenden MCF-7 Transfektanten, jedoch konnte kein Zelltod detektiert werden. Eine zusätzliche Behandlung mit dem Kinase Inhibitor Koffein führte zu einem Verlust der Zellviabilität in den Caspase-3 profizienten als auch defizienten MCF-7 Zellen. Die MCF-7 Zellen starben dabei aus der G2 Phase des Zellzykluses heraus, unabhängig von der Caspase-3 Expression. In Caspase-3 exprimierenden MCF-7 Zellen, induzierte $p14^{ARF}$, nach Sensibilisierung mit Koffein, Apoptose. Die Vermittlung erfolgte über den mitochondrialen Apoptosesignalweg, detektiert durch den Zusammenbruch des mitochondrialen Membranpotentials, der Cytochrom c Freisetzung und der Caspaseaktivierung. Im Gegensatz dazu, starben Caspase-3 defiziente MCF-7 Zellen durch einen nicht apoptotischen Mechanismus, welcher als Autophagie charakterisiert werden konnte. Die durch Koffein ausgelöste Hemmung der Zellantwort auf DNA-Schäden verminderte die Expression des CDK Inhibitors p21, was zu einer Zelltodinduktion durch $p14^{ARF}$ führte. Der Verlust von p21 führte zu einer Caspase-3 anhängigen $p14^{ARF}$-induzierten Apoptose bzw. zu Autophagy in Abwesenheit einer effizienten Caspaseaktivierung durch den Verlust von Caspase-3. Diese Daten zeigen, dass die Beeinflussung des Zellzyklus entscheidend für die $p14^{ARF}$-induzierte Apoptose ist, welche von der Expression von Caspase-3 abhängig ist. Der Verlust der Caspase-3 führt zu einem Zelltod der durch Autophagie vermittelt wird.

Des Weiteren wurde der Einfluss des anti-apoptotischen Proteins Bcl-2 auf die $p14^{ARF}$-induzierte Apoptose in Kombination mit Koffein in SW480 Zellen untersucht, die stabil Bcl-2 entweder am Endoplasmatischen Retikulum oder an den Mitochondrien exprimieren. Die Ergebnisse zeigten eindeutig, dass die Überexpression von Bcl-2, die $p14^{ARF}$- induzierte Apoptose komplett hemmt, jedoch nur, wenn Bcl-2 an den Mitochondrien lokalisiert war. Dies legt den Schluss nahe, dass nach Sensibilisierung mit Koffein, $p14^{ARF}$ vorzugsweise über die Mitochondrien Apoptose induziert.

Ein weiteres wichtiges Ziel dieser Arbeit war es, neue brauchbare Therapien für bestrahlungsresistente MCF-7 Zellen zu entwickeln. Hier zeigte sich, dass eine Sensitivierung der Zellen für strahlungsinduzierten apoptotischen Zelltod und eine begünstigte Zellzyklusprogression durch $p14^{ARF}$ von Caspase-3 abhängig ist.

%	percent
°C	degree Celsius
A. bidest	aqua bidestillata
AIF	apoptosis inducing factor
Apaf-1	apoptosis associated factor1
APS	ammoniumperoxodisulfate
ARF	alternative reading frame
Bak	Bcl-2 homologous antagonist killer
Bax	Bcl-2 associated protein X
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma / leukemia-2 gene
BSA	Bovine Serum Albumin
C3	caspase-3
Caspase	cysteine aspartate-specific protease
Cdc	Cell division cycle
CDK	Cyclin dependent kinase
CDKI	Cyclin dependent kinase inhibitor
CAPS	3-Cyclohexylamino-1-propansulfon acid
CARD	caspase recruitment domain
CMV	cytomegalovirus
C-Terminus	carboxy-terminus
Cyt c	cytochrome c
Da	Dalton
DD	death domain
DED	death effector domain
Diablo	direct IAP binding protein with low pI
DISC	death inducing signalling complex
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	2´-desoxynukleosidtriphosphate
$\Delta\Psi_m$	mitochondrial membrane potential
DTT	Dithiothreitol
E. coli	Escherichia coli
ECL	enhanced chemoluminescence

EDTA	ethylendiamine-tetraactylacid
ER	endoplasmic reticulum
EtBr	Ethidiumbromide
EtOH	ethanol
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluorescein-isothiocyanat
Fmk	fluoromethylketon
FSC	forward scatter
h	hour
GFP	green fluorescent protein
Gy	Gray
HEPES	2-[4-(2-Hydroxyethyl)-1-piperazinyl]-Ethansulfonic acid
HtrA2	high temperature requirement A2
Hoechst	trihydrochloride trihydrate
IAP	inhibitor of apoptosis protein
IR	irradiation
JC-1	5,5‘,6,6‘tetrachloro1,1‘,3,3‘tetraethylbenzimidazolyl carbocyaniniodide
kDa	kilo Dalton
l	liter
LC3	microtubule-associated protein light chain 3
Mdm2	Mouse double minute-2 Protein
MEF	murine embryonic fibroblasts
Min	minute
µM	micro-molar (µmol/l)
mM	milli-molar (mmol/l)
MOI	multiplicity of infection
MPT (P)	mitochondrial permeability transition (pore)
mTOR	mammalian target of rapamycin
N-Terminus	amino-terminus
PAGE	polyacrylamide gel electrophoresis
PARP	poly (ADP-ribose) Polymerase
PBS	phosphate buffered saline

P_{CMV}	full length “immediate early” promotor of cytomegalovirus
PCR	polymerase chain reaction
PFA	paraformaldehyde
Pfu	plaque forming unit
pH	potentio hydrogenii
PMSF	phenylmethylsulfonylfluoride
PTP	permeability transition pore
Rpm	rounds per minute
RT	room temperature
s	second
SDS	sodium dodecylsulfate
SERCA	sarco (endo) plasmatic reticulum Ca^{2+}-ATPase
Smac	second mitochondrial activator of caspases
SSC	side scatter
tBid	truncated Bid
TEMED	N, N, N‘, N‘-Tetramethylethylendiamin
Tris	Tris-(hydroxymethyl)-aminomethan
U	unit
UV	ultraviolet
V	volt
VDAC	voltage dependent anion channel
XIAP	X-chromosome-linked Inhibitor of Apoptosis Protein

SUMMARY

1. Summary

The tumour suppressor protein $p14^{ARF}$ is encoded by the human INK4a gene locus and it is known that a mutation of this gene is present in almost half of all cancers. It has been shown that $p14^{ARF}$ plays a unique role in the regulation of cell cycle arrest and apoptosis following oncogenic stress. The aim of this thesis was to investigate the regulation of $p14^{ARF}$ induced cell death. To this end, p53 wild type MCF-7 breast carcinoma cells, which have lost the key executioner caspase-3 and MCF-7 cells stably re-transfected with procaspase-3 were employed. Expression of $p14^{ARF}$ induced a dominant G1 arrest in both caspase-3 deficient mock and caspase-3 re-expressing MCF-7 transfectants, but no cell death. Additional checkpoint abrogation by the kinase inhibitor caffeine was accompanied by loss of cell viability in both caspase-3 and mock transfectants. Regardless of the presence of caspase-3, MCF-7 cells died from the G2 phase of the cell cycle. In caspase-3 proficient MCF-7 cells, $p14^{ARF}$ induced apoptosis (type I cell death) after sensitization with caffeine. This was executed via the mitochondrial pathway as evidenced by a breakdown of the mitochondrial membrane potential, cytochrome c release and caspase activation. In contrast, caspase-3 deficient MCF-7 cells died through a non-apoptotic mechanism that was characterized as autophagy. Inhibition of the DNA damage response by caffeine impaired expression of the CDK inhibitor p21 and this was linked to induction of cell death by $p14^{ARF}$. Thus, loss of p21 facilitates $p14^{ARF}$-induced apoptosis in a caspase-3 dependent manner or autophagy in the absence of efficient executioner caspase activation. These data indicate that cell cycle arrest programs interfere with $p14^{ARF}$-induced apoptosis that strictly depends on caspase-3 and that loss of this executioner caspase facilitates induction of autophagy cell death.

The impact of the antiapoptotic Bcl-2 protein on $p14^{ARF}$-induced apoptosis in combination with caffeine was examined using SW480 cells stably overexpressing Bcl-2 at the endoplasmic reticulum or at the mitochondria. Obtained data clearly showed that only when Bcl-2 was localized at the mitochondria apoptosis was completely blocked. These data provide evidence that after sensitization with caffeine, $p14^{ARF}$ predominantly targets the mitochondria to execute cell death.

Another essential objective in this thesis was to propose a new useful tool against radiation resistance MCF-7 cells. Additionally, $p14^{ARF}$ sensitized cells for ionizing irradiation, promoted cell cycle progression, and triggered apoptosis via an entirely caspase-3 dependent pathway.

INTRODUCTION

2. Introduction

2.1 Cell death

2.1.1 Necrosis

Necrosis is a form of cell death referred to as an accidental and uncontrolled cellular event following e.g. thermic, toxic or mitochondrial damage. The process is, however, also initiated when cells, undergoing regulated cell death are not able to activate the energy-dependent apoptotic pathway. The main features of necrosis are due to bio-energetic failures, rapid loss of plasma membrane integrity and swelling of the organelles and the nucleus without chromatin condensation. Release of intracellular components into the extracellular matrix leads to an inflammatory response and can damage the neighbouring cells and tissues (Zong and Thompson, 2006).

2.1.2 Mitotic catastrophe

Mitotic catastrophe is a form of cell death occurring from a deregulated or failed mitosis. It can be accompanied by morphological alterations such as formation of large cells with multiple micronuclei and decondensed chromatin micronucleation (Roninson et al., 2001; Swanson et al., 1995). The combination of checkpoint insufficiency and different types of damage may lead to mitotic catastrophe. Pharmacological inhibition or genetic suppression of several G2 checkpoint genes such as ATM, ATR, Chk1, Chk2 and polo-like kinase (Plk)1, can promote DNA-damage-induced mitotic catastrophe (Bunz et al., 1998; Chen et al., 2003).

2.1.3 Apoptosis (Type I cell death)

The term “programmed cell death”, utilized for the first time in 1964, proposes that cell death during development is not accidental, but follows controlled steps leading to a locally and temporally defined self-destruction (Lockshin, 1964). Following these studies, John Kerr et al., established in 1972 the term “apoptosis” in order to describe the morphological

and biochemical changes leading to controlled cellular suicide. Apoptosis is also known as type I cell death and is an active and tightly regulated process employed to remove unwanted, damaged or aged cells. This physiological mechanism of cell removal has important biological significance in embryonic development, differentiation, tissue homeostasis, and is vital for tissue repair in response to damage (Vaux and Korsmeyer, 1999). Apoptosis involves activation of catabolic enzymes (proteases) in a signalling cascade, leading to the rapid destruction of cellular structures and organelles. The cell membrane remains intact, cellular content is contained and no inflammation occurs. Apoptosis is consisting of cellular shrinkage, blebbing of the plasma membrane, chromatin condensation, which coincides DNA fragmentation and due to proteolysis of structural proteins, culminates in the formation of apoptotic bodies (Wyllie et al., 1984).

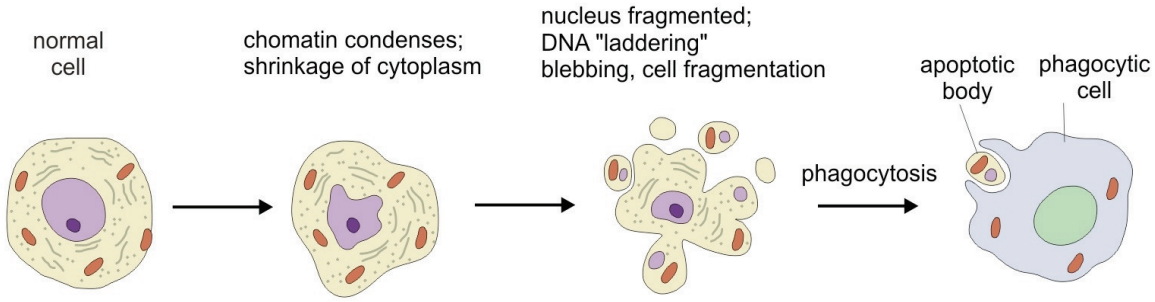

Figure 1. Morphological changes during apoptosis.
Apoptosis is a controlled process consisting of shrinkage of the cell, condensation of the chromatin and membrane blebbing which leads to nuclear fragmentation, formation of apoptotic bodies and finally to phagocytosis (Kerr et al., 1994).

The crucial role of apoptosis is revealed by apoptotic dysfunctions, which cause abnormalities not only during development but also in the pathogenesis of various diseases. Many diseases are associated with either too much or too little cell death. Insufficient cell death shifts the equilibrium of cell homeostasis toward cell proliferation and can therefore lead to cancer (Thompson, 1995).

The apoptotic signalling is divided into two main pathways, based on the mechanism of induction. The intrinsic pathway, which is triggered by different intracellular stress signals mainly depends on mitochondrial changes and formation of the apoptosome reviewed in (Daniel et al., 2003). The extrinsic pathway is activated by extracellular factors that act via the death receptor (DR) mediated signalling cascade (Daniel et al., 2001). Although

different proteins participate in the apoptotic machinery, there is a crosstalk between both apoptosis signalling pathways.

2.2 Apoptotic pathways

2.2.1 The intrinsic mitochondrial pathway

The intrinsic or mitochondrial apoptotic pathway is activated upon various cytotoxic insults such as cytotoxic drugs, DNA damage, viral infections, ionizing radiation and growth factor deprivation. Also, this pathway can be initiated via the endoplasmatic reticulum (ER) or the lysosomal stress pathway. The key step in intrinsic pathway is the release of certain mitochondrial proteins into the cytosol by mitochondrial outer membrane permeabilization (MOMP). This is followed by activation of a caspase cascade eventually leading to apoptotic cell death.

Key regulators of mitochondrial breakdown events are proteins of the Bcl-2 family (Cory and Adams, 2002). They are known to regulate mitochondrial integrity and function by controlling the permeability of the mitochondrial membrane (Krajewski et al., 1993; Tanaka et al., 1993). Different members of the Bcl-2 family can either induce breakdown of the mitochondrial membrane potential or stabilize mitochondrial barrier functions. In response to mitochondrial disruption, cytochrome c is released together with other mitochondrial factors (Green and Reed, 1998; Kluck et al., 1997) and acidification of the cytosol and ion fluxes occur (Daniel et al., 2003; Vaux and Korsmeyer, 1999). Thus, MOMP in turn triggers the formation of a complex consisting of cytochrome c, Apaf-1, ATP and procaspase-9, the so-called apoptosome (Acehan et al., 2002; Yu et al., 2005). Cytochrome c/apoptosome-mediated caspase activation results later in the swelling of the mitochondrial matrix and rupture of the inner and outer mitochondrial membranes (Zamzami and Kroemer, 2001). Apoptosome-activated caspase-9 further activates downstream caspases, such as caspase-3 and -7, which then execute apoptosis (Li et al., 1997; Liu et al., 1996; Zou et al., 1997).

2.2.1.1 Released factors during mitochondrial permeabilization

Several factors are released upon disruption of the mitochondrial membrane, including cytochrome c (Liu et al., 1996), Smac/DIABLO (Du et al., 2000; Verhagen et al., 2000),

AIF (Joza et al., 2001), endonuclease G (EndoG) (Li et al., 2001; Parrish et al., 2001), and HtrA2/Omi (Mizushima et al., 2001) triggering caspase-dependent and independent cell death.

Smac/DIABLO (second mitochondria-derived activator of caspases/direct IAP binding protein with low pI) is one of the proteins, which is released from the mitochondria. It can antagonize IAPs (caspase inhibiting proteins) thereby promoting apoptosis (Du et al., 2000; Verhagen et al., 2000). During apoptosis Smac is released from the mitochondria and relieves IAPs inhibition. Thereby, it reactivates processing of caspases. The serine protease Omi/HtrA2 (high temperature requirement A2), a mitochondrial matrix protein, shows a similar synergistic effect, as it is able to bind and inactivate IAPs, indirectly promoting activation of caspases (Martins et al., 2002; Srinivasula et al., 2003; Suzuki et al., 2001).

Further, apoptosis-inducing factor (AIF) released from the mitochondria, was reported to translocate to the nucleus, initiating chromatin condensation and DNA fragmentation in a caspase independent manner (Daugas et al., 2000; Susin et al., 1999). It was also proposed to contribute to the regulation of apoptotic mitochondrial membrane permeabilization triggering cell death through reactive oxygen species (Joza et al., 2001). However, molecular mechanisms via which AIF induces apoptosis are still discussed.

Endonuclease G is a DNA degrading enzyme, which is important for the repair of mitochondrial DNA and is released from the mitochondria in response to Bid insertion into the mitochondrial membrane during apoptosis (van Loo et al., 2001).

2.2.2 The extrinsic mitochondrial pathway

Death receptors (DRs) are located in the plasma membrane and are responsible for induction and transmission of the extrinsic apoptosis signalling pathway. Activation of death receptors and transmission of extracellular signals into the cytosol occurs upon binding of their respective extracellular death ligands, such as CD95/Fas, tumour necrosis factor alpha (TNF) and TNF-related apoptosis inducing ligand (TRAIL). The DR family is part of the TNF receptor “superfamily” (Bhardwaj and Aggarwal, 2003). Currently, eight death receptors are known: TNF-R1 (DR1/CD120a/p55/p60), Fas (Apo-1/CD95), DR3 (APO-3/LARD/TRAMP), TRAIL-R1 (DR4/APO-2), TRAIL-R2 (DR5/KILLER/TRICK2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor (NGFR) (Ashkenazi and Dixit, 1998; French and Tschopp, 2003; Lavrik et al., 2005).

Death receptors have cysteine-rich repeats in their intracellular domain, termed "death domain" (DD) (Smith et al., 1994). This domain is crucial for the transduction of the apoptotic signal, which is induced by ligand-death receptor binding upon their trimerization. The intracellular DD of the receptor recruits adaptor proteins, such as FADD (Fas-Associated Death Domain) (Daniel et al., 2001; Nagata, 1997). The DD of the receptor interacts with the DD of FADD, whereas the death effector domain (DED) of FADD interacts with the N-terminal tandem DEDs of procaspase-8, leading to the formation of a so-called "death-inducing signalling complex" (DISC) (Kischkel et al., 1995; Muzio et al., 1996). Binding of procaspase-8 to the DISC leads to dimerization, autocatalytic activation, and conversion to the processed heterotetrameric mature form of caspase-8, containing two large subunits (p18) and two small subunits (p10). Procaspase-10 can also bind to the DISC and gets activated by formation of an active heterotetramer (Sprick et al., 2002). Nevertheless, the precise function is still controversial and needs further investigation. In type I cells, the amount of active caspase-8 is sufficient to initiate apoptosis by directly activating the effector caspase-3, -6, -7. However, in type II cells, the amount is too low and mitochondria are used to amplify the death signal via activation of the Bcl-2 family member Bid. Its cleavage by caspase-8 to truncated Bid (tBid) induces the activation of the mitochondrial cell death pathway (Scaffidi et al., 1998). The extrinsic and intrinsic pathways can be connected by the cleavage of Bid to tBid through a caspase-8 dependent mechanism. Truncated Bid can interact with Bax and, by activating it, induces its translocation and insertion into the outer mitochondrial membrane (Luo et al., 1998).

The major regulators at the DISC level are the antiapoptotic c-FLIP (c-FLICE inhibitory proteins), which exist in three splice variants: the long form c-$FLIP_L$, the short form c-$FLIP_S$ and Raji c-$FLIP_R$ (Golks et al., 2005). C-$FLIP_S$ and c-$FLIP_R$ are antiapoptotic proteins due to binding competitively to FADD, thereby blocking activation of caspase-8 (Thome and Tschopp, 2001). C-$FLIP_L$ can exhibit pro- or antiapoptotic functions depending on its expression levels. C-$FLIP_L$ acts as an apoptosis inhibitor only at high expressional levels. In contrast, it was proposed that c-$FLIP_L$ might facilitate procaspase-8 activation through heterodimerization of their respective protease domains and thereby promoting apoptosis (Chang et al., 2002; Yu et al., 2009; Zang et al., 2008).

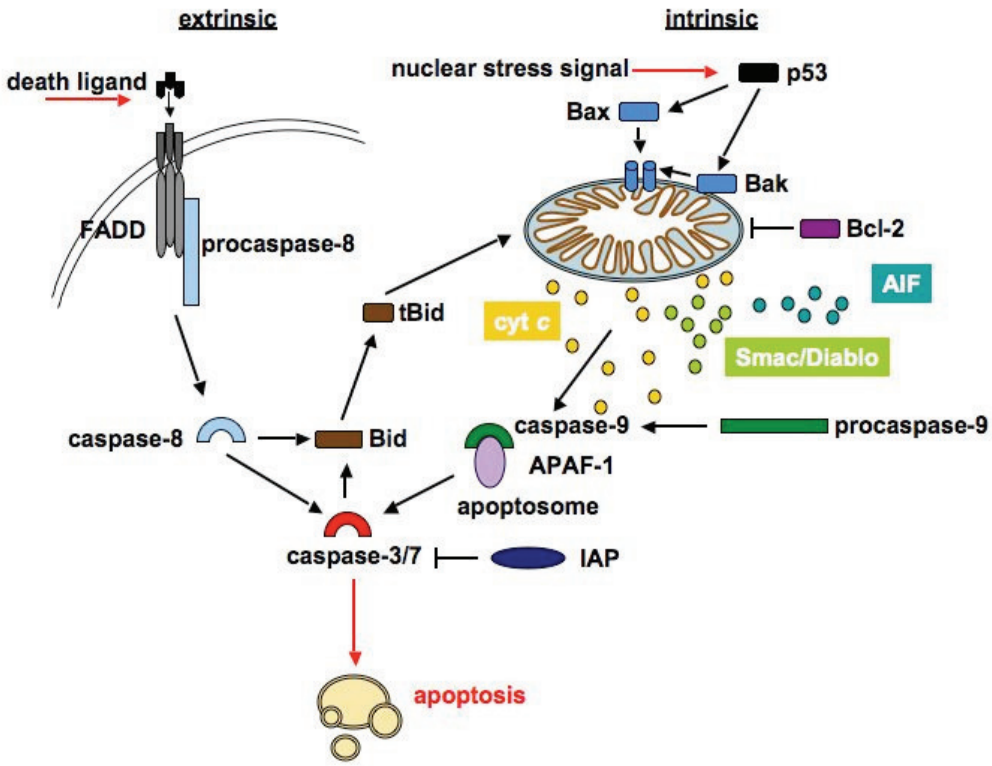

Figure 2: The intrinsic and extrinsic apoptosis pathway.
Right side: *The mitochondrial (intrinsic) apoptotic signalling pathway is initiated upon cellular stress stimuli leading to activation and translocation of proapoptotic members of the Bcl-2 family. Mitochondria are losing their membrane potential triggering the release of cytochrome c and other proapoptotic factors. Released cytochrome c associates with Apaf-1 and procaspase-9 in the presence of dATP to form a cytosolic complex called apoptosome. Activated caspase-9 triggers the caspase cascade network leading to apoptosis.*
Left side: *The death receptor (extrinsic) pathway is activated when extracellular death receptor ligands of the TNF family bind to transmembrane death receptors. Binding of the ligand induces trimerization of the receptor and recruitment of the adaptor protein FADD and caspase-8. Within this complex, caspase-8 is activated and in turn cleaves and activates casapse-3.*
The two pathways can be connected via cleavage of Bid to tBid by a caspase-8 dependent mechanism.

2.2.3 The Bcl-2 family

Bcl-2 (B-cell lymphoma-2) family members are crucial regulators of the intrinsic apoptosis pathway (Cory and Adams, 2002; Daniel et al., 2003). They are divided into two functionally different groups of proteins consisting of anti- and proapoptotic proteins (Cory and Adams, 2002). Prosurvival proteins (Bcl-2, Bcl-x_L, Mcl-1, Bfl-1/A1, Bcl-w) contain four Bcl-2 homology (BH) domains. Proapoptotic Bcl-2 family members can be subdivided

into multi-domain class proteins i.e. Bak, Bax and Bok comprising BH1-3 and those that only contain the BH3-domain and are referred to as BH3-only proteins, including Bim, Bid, Bmf, Puma/Bbc3, Noxa/APR, Bad, Nbk/Bik, and Hrk/DP5 (Youle and Strasser, 2008).

Bcl-2 was the first antiapoptotic gene discovered and was found at the chromosomal breakpoint of a chromosomal translocation present in a B cell lymphoma (Tsujimoto et al., 1984). Antiapoptotic proteins antagonize their proapoptotic relatives, thereby preventing mitochondrial membrane permeabilization (Cory and Adams, 2002). Bcl-2 is localized at the outer mitochondrial membrane but also at the nuclear envelope and the membrane of the ER (Strasser et al., 2000). The prosurvival proteins Bcl-2 and Bcl-x_L have been shown to inhibit the release of cytochrome c from mitochondria and to inhibit apoptosis (Daniel et al., 2003; Kluck et al., 1997). BH3-only proteins have different antiapoptotic proteins as favourite targets. For example, the targets of Bad are Bcl-2 and Bcl-x_L. Nbk/Bik binds preferentially to Bcl-x_L, much less to Bcl-2 and not to Mcl-1. Noxa only binds to Mcl-1 and not to Bcl-2 or Bcl-x_L. The exceptions are Bim and Puma, which bind to all three antiapoptotic proteins. Therefore, Bim or Puma could neutralize all of the antiapoptotic proteins, whereas Bad or Noxa would be partial inducers, since each neutralizes certain antiapoptotic proteins (Chen et al., 2005; Wang et al., 1996).

Proapoptotic proteins are critical executioners of the intrinsic apoptosis pathway. The breakdown of the mitochondrial membrane critically depends on Bax (Bcl-2 associated protein X) (Oltvai et al., 1993) and its homologue Bak (Chittenden et al., 1995). Bax is a monomeric cytosolic protein, which upon activation undergoes an N-terminal conformational change resulting in its translocation, oligomerization and insertion into the outer mitochondrial membrane (Goping et al., 1998). Bak is a permanent integral membrane protein and, similar to Bax, its activation involves an N-terminal conformational change and oligomerization at the outer mitochondrial membrane. One model implies that Bax and Bak are involved in mitochondrial pore formation, which leads to release of the proapoptotic factors. The other hypothesis states that they associate with parts of the permeability transition pore (Cory and Adams, 2002). These oligomers are considered to permeabilize the outer mitochondrial membrane and therefore stimulate cytochrome c release using the voltage-dependent anion channel (VDAC) (Daniel et al., 2003). Either of those two critical proapoptotic proteins is required for mitochondrial membrane permeabilization (Blommaart et al., 1997; Huang et al., 2000; Kihara et al., 2001; Kirisako et al., 1999; Liang et al., 2001; Shintani and Klionsky, 2004; Wei et al., 2001). Cells double deficient for these two proapoptotic proteins are resistant to most intrinsic death pathway stimuli (Lindsten et al.,

2000; Willis and Adams, 2005; Willis et al., 2007). It has become generally accepted that Bax acts on mitochondria to increase the permeability of the outer membrane (Antonsson et al., 1997; Desagher and Martinou, 2000; Eskes et al., 2000).

How Bax and Bak are activated is, however, still controversially discussed. Two different models have been proposed (Chen et al., 2005):

In the first model, a direct activation of Bax/Bak sustains loss of mitochondrial integrity (Certo et al., 2006; Letai et al., 2002; Walensky et al., 2006). According to the direct activation model, certain BH3-only proteins, termed activators (namely Bim, tBid and Puma) could bind and activate Bax/Bak directly, whereas the sensitizer (the remaining BH3-only proteins) could act only by neutralizing antiapoptotic Bcl-2 family members (Chen et al., 2005; Willis and Adams, 2005; Willis et al., 2007). According to this model, a cell undergoes apoptosis when the sensitizers that have been activated or induced via an apoptotic signal neutralize all prosurvival Bcl-2 molecules, thereby releasing the activator proteins.

In the second or indirect activation model, there is no direct activation of Bax or Bak by the BH3-only proteins. All BH3-only proteins compete for binding to the antiapoptotic proteins preventing them from inhibiting Bax and Bak (Willis et al., 2005). As a result, Bax and Bak are liberated and can initiate the apoptotic pathway. In this model, the BH3-only proteins are divided into two categories, that is, the selective ones, which have limited binding partners among the antiapoptotic members, and the more potent inducers of apoptosis, such as Bim, tBid and Puma which can bind and inactivate multiple antiapoptotic members (Cohen, 1997; Daniel et al., 2001; Fadeel et al., 1998).

2.2.4 Caspases

Caspases are cysteinyl aspartate-specific acid proteases that can cleave their substrates at specific aspartate residues. Their catalytic activity depends on cysteine residues reflected by their name. Caspases are synthesized as inactive procaspases i.e. zymogens containing a prodomain and a catalytic domain consisting of a p20 large subunit and a p10 small subunit. They are central players of the apoptotic machinery and are involved in the initiation of a caspase cascade system and execution of the apoptotic program (Cohen, 1997; Earnshaw et al., 1999). Based on an apoptotic signal, the caspase zymogens undergo proteolytic processing to generate two small and two large subunits, which assemble into a

heterotetrameric p20/p10 complex, forming the active caspase (Degterev et al., 2003; Fuentes-Prior and Salvesen, 2004). In apoptotic cells, activated caspases can be detected as cleaved fragments (Alnemri et al., 1996).

The caspase family consists of 15 mammalian members (11 in humans) divided into inflammatory caspases and apoptotic caspases (Li and Yuan, 2008). The inflammatory caspases (caspase-1, caspase-4, caspase-5 and caspase-12,) are involved in cytokine maturation and inflammatory response (Lamkanfi et al., 2002). Polymorphisms in human caspase-12 drastically alter its function. The full length variant of caspase-12 abrogates the inflammatory response due to its inhibitory effect on proinflamatory caspase-1. This function of full length caspase-12 on caspase-1 leads to induction of severe sepsis observed in about 20% of Africans (Hotchkiss and Nicholson, 2006; Saleh et al., 2004; Scott and Saleh, 2007).

Apoptotic caspases can be classified according to their lengths and the structure of their prodomains, which also correspond to their position in the apoptotic signalling cascade (Nicholson, 1999). They can be divided into two groups: initiator caspases containing either DED domains (caspase -8 and -10) or caspase-recruitment domains (CARD) (caspase-2 and -9) and executioner caspases, (caspase-3, -6, and -7) which contain short prodomains (20–30 amino acids) (Li and Yuan, 2008). Initiator caspases possess long prodomains that contain one of the two characteristic protein–protein interaction motifs and are involved in interacting with the upstream adapter molecules. All initiator caspases are activated through autocatalytic intrachain cleavages (Korsmeyer et al., 2000; Luo et al., 1998), while caspase-8 and -9 activation may occur in the absence of proteolytic processing (Degterev et al., 2003).

The apoptosome is a multiprotein complex, which consist of Apaf-1, cytochrome c, and caspase-9 (Costantini et al., 2002; Hu et al., 1998; Li et al., 1997). In the presence of cytochrome c and dATP, Apaf-1 oligomerizes to form the apoptosome complex. Procaspase-9 is recruited to this complex by CARD interactions, which leads to its activation (Porter and Janicke, 1999). DED and CARD domains are responsible for the recruitment of the initiator caspases into the death inducing signalling complexes, resulting in autoactivation of caspases that subsequently initiate cell death. The effector caspases are downstream caspases and they are directly activated and cleaved by apical caspases in the late stage of apoptosis. Caspase-3 is the main downstream effector caspase that cleaves the majority of the cellular substrates such as the nuclear enzyme poly(ADP-ribose) polymerase

(PARP) in cells enduring apoptosis (Le Rhun et al., 1998). PARP is cleaved into a small 24kDa and a large 84kDa fragment during cell death (Koh et al., 2005). The small N-terminal fragment contains the DNA-binding domain that might facilitate the apoptotic process by blocking the access for DNA repair enzymes to the fragmented chromatin. The 89kDa fragment, containing catalytic domains, may be incapable of activation by DNA damage. This suggests a possible mechanism to prevent energy (ATP) depletion, thereby ensuring the energy dependent development of the apoptotic phenotype (Duprez et al., 2009; Fernandes-Alnemri et al., 1994).

The effector caspases with short prodomains mediate downstream apoptosis events by cleaving multiple cellular substrates and are typically processed and activated by upstream caspases (Riedl and Shi, 2004).

Caspase-8 is one of the main caspases of the extrinsic pathway and also has a role in the intrinsic pathway. Formation of the DISC leads to the activation of the initiator caspase-8, which directly triggers the activation of downstream effector caspases-3 and -7 in type I cells. In type II cells with low levels of DISC formation, followed by low levels of active caspase-8, apoptosis signalling requires an additional mitochondria-dependent amplification loop. The extrinsic apoptosis pathway converges with the intrinsic one by the caspase-8 mediated cleavage of the BH3 only protein Bid to generate tBid and subsequently tBid-mediated activation of Bax and release of cytochrome c from the mitochondria (Degterev et al., 2003; Gross et al., 1999).

As caspase-3 and caspase-7 are functionally similar and have similar substrate specificities, cleavage of PARP during apoptosis may occur due to a combination of the action of both caspases (Fernandes-Alnemri et al., 1996). Active caspase-7 contains two subunits, similar to other caspases (MacFarlane et al., 1997). Caspase-7 is activated to its catalytically active large subunit in intact cells undergoing apoptosis (Slee et al., 1999). Several studies imply that caspase-9 is required for the processing of both, effector caspase-3 and -7. It was proposed that the apoptosome complex could directly process procaspase-7 similarly to procaspase-3 (Twiddy et al., 2006).

IAPs are a family of proteins with antiapoptotic functions interacting with the active site of caspases. In humans, six IAP family members have been reported: NAIP (neuronal apoptosis inhibitory protein), c-IAP1/HIAP-2, c-IAP2/HIAP-1, XIAP/hILP, survivin and BRUCE (Deveraux et al., 1998; Roy et al., 1997). They can specifically inhibit the activity of the initiator caspase-9 and effector caspases-3 and -7 and therefore prevent cell death.

Caspase-7 forms a complex with XIAP (X-linked mammalian inhibitor of apoptosis protein), after activation by the apoptosome complex (Deveraux and Reed, 1999). IAPs do not bind or inhibit caspase-8, but they may prevent the proteolytic processing of pro-caspases -3, and -9 and thereby provide protection from cell death (Deveraux et al., 1998).

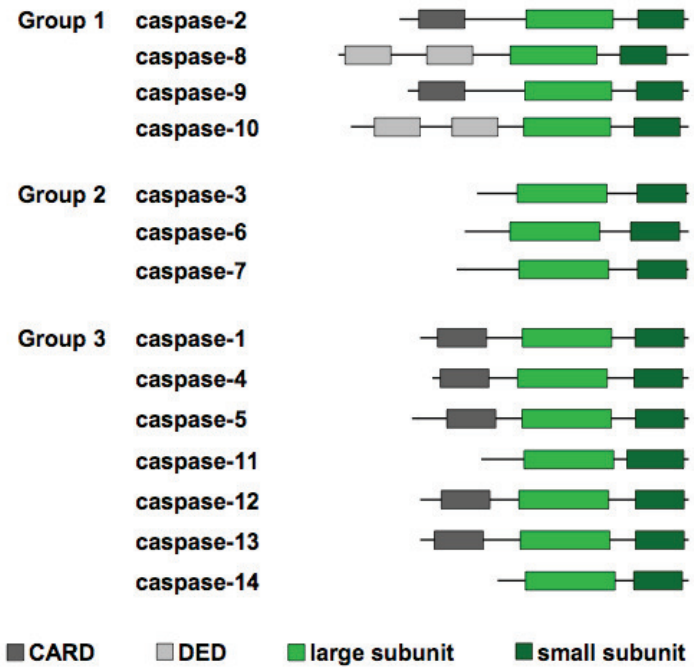

Figure 3. The caspase family.
There are three major groups of caspases, group 1: initiator caspases, group 2: effector caspases and group 3: inflammatory caspases. Caspases are synthesized as inactive procaspases containing a prodomain and a catalytic domain. After proteolytic processing two small and two large subunits, assembled into a heterotetrameric complex, form the active caspase.

2.3 Autophagy (Type II cell death)

The term autophagy has its origin in the Greek words oneself ("auto") and eating ("phagy"). This is the second type of programmed cell death (PCD type II) and is a mechanism of cell self-cannibalism morphologically distinct from apoptosis (Canu et al., 2005; Yu et al., 2004). Under conditions of stress, cells can recycle their own molecules to obtain nutrients, providing ATP, and for sustaining the basic metabolic processes (Lum et al., 2005).

Autophagy refers to the highly regulated and evolutionarily conserved process of turnover and maintenance of cellular components that is required for cellular homeostasis (Mizushima, 2007). First identified in yeasts, many of the corresponding proteins have homologues in higher eukaryotes (Klionsky et al., 2003; Reggiori and Klionsky, 2002). Moreover, autophagy is involved in cellular development and differentiation (Levine and Klionsky, 2004) and may have a protective role against aging (Bergamini et al., 2004). This process involves degradation of proteins and is the only known pathway for degrading organelles (Klionsky and Emr, 2000). Autophagy can be also considered as a housekeeping mechanism involved in cytoplasmic homeostasis because it controls the turnover of peroxisomes, mitochondria and the size of the endoplasmic reticulum. A selective sequestration of these organelles can be observed in various pathophysiological and/or stress situations (Xue et al., 2001). However, the self-digestion not only provides nutrients to preserve essential cellular functions during starvation, but can also help the cell to remove damaged organelles, misfolded proteins and, upon prolonged time of stress, to initiate cell death. Autophagy shares common molecular regulators with apoptosis, such as antiapoptotic members of the Bcl-2 family indicating a possible crosstalk between the two pathways (Liang et al., 1998; Shimizu et al., 2004). It seems that similar death stimuli could induce either apoptosis or autophagy. Autophagy is defined as alternative cell death mechanism in apoptotic-deficient cell systems (Clarke, 1990; Klionsky and Emr, 2000). In most studies, autophagic cell death has been considered by morphological criteria. But it is unclear whether autophagy directly contributes to death or represents a failed effort to preserve cell viability (Pattingre et al., 2005). This type of caspase-independent cell death depends on specific gene functions that distinguish autophagy from non-physiological necrosis, and gives an alternative possibility of killing cells (Yu et al., 2004). Bax/Bak-deficient mouse embryonic fibroblasts (MEFs) do not undergo apoptosis after exposure to a variety of apoptotic stimuli, but rather die in a non-apoptotic fashion demonstrating features of autophagy cell death (Lindsten et al., 2000; Wei et al., 2001).

2.3.1 Mechanism of autophagy induction

2.3.1.1 Autophagy formation

The process of autophagy involves autophagosome formation, which begins with the sequestration of cytoplasmic membranes by an isolated membrane compartment (phagophore). The autophagosome undergoes fusion with a lysosome. This results in the characteristic double membrane morphology of autophagolysosomes that can be observed by use of electron microscopy (Kabeya et al., 2000). The fused compartments assemble in the autophagic body known as autophagolysosome or autolysosome (Dunn, 1994; Kim and Klionsky, 2000). The lumen of this organelle contains acid hydrolases (AH) that are able to degrade essentially any protein, lipid, nucleic acid, and carbohydrate. Degradation of these macromolecules by autolysosomes produces amino acids and other elements needed for biosynthetic pathways by generating energy for protein synthesis and therefore promoting cell survival (Bursch, 2001). Alternatively, removal of the organelles might result in cell death. All steps, including the final degradation of the sequestered cytoplasmic material in the autolysosomes, are ATP-dependent (Blommaart et al., 1997; Kim and Klionsky, 2000; Klionsky and Emr, 2000).

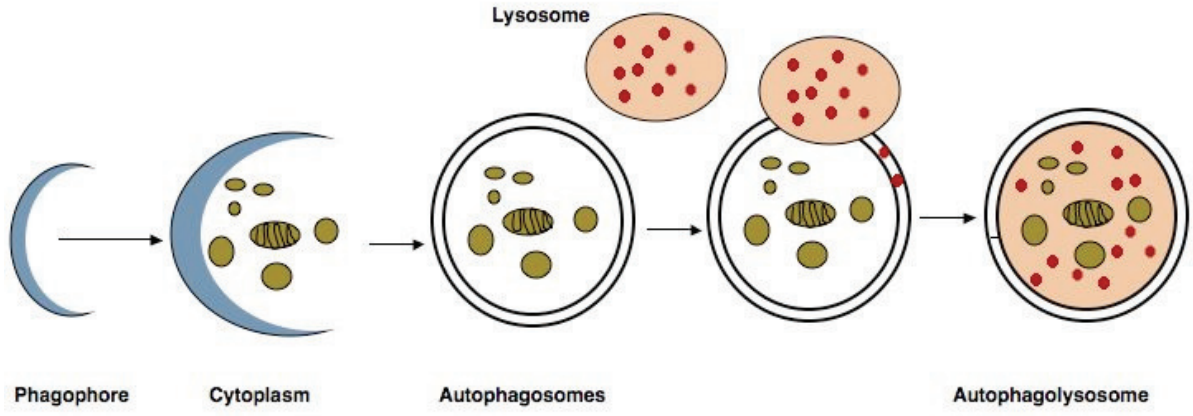

Figure 4. Autophagy formation.
Autophagy first involves the sequestration of a region of the cytosol, containing proteins and/organelles, which are engulfed by a new-formed double-membrane organelle, the autophagosomes (so-called autophagy vacuoles). Autophagosomes undergo maturation via fusion with the acidic lysosomes to create autolysosomes. Within the autolysosomes, the inner membranes as well as the luminal content of the autophagy vacuoles are degraded by lysosomal hydrolases.

2.3.1.2 The autophagy conjugation system

Autophagy-related (ATG) proteins are required for autophagy vesicle formation where two ubiquitin-like conjugation systems play a central role (figure 5). The first consists of the ATG12 system in which ATG12 is conjugated to ATG5 in an ubiquitination-like manner (Mizushima, 2007). The covalent conjugation of ATG12 to ATG5 occurs with the help of the E1-like enzyme ATG7 and the E2-like enzyme ATG10. At this point first ATG7 and then ATG10 are released. The ATG12–ATG5 complex is localized at the expanding phagophore mostly at the outer surface and dissociates from this membrane immediately before or after autophagosome completion (Mizushima et al., 2003). ATG16-binding to this complex (ATG12–ATG5) is necessary for the elongation of the pre-autophagosomal membrane called phagophore, and for the formation of the autophagosomal membrane. This complex finally dissociates upon completion of autophagosome formation, and is not present in the mature autophagosome (George et al., 2000).

The second system involves the conjugation of the microtubule-associated protein light chain 3 (LC3/ATG8) to phosphatidylethanolamine (PE), a lipid molecule (Ichimura et al., 2000). LC3 is synthesized as a cytosolic full-length precursor protein, so-called proLC3, which is converted into LC3I via cleavage by the cysteine protease ATG4, exposing its C-terminal glycine residue (Kirisako et al., 2000). This residue is then conjugated to PE through an amide bond between its C-terminal glycine and the amino group of PE, which is catalysed by ATG7 (an E1-like enzyme) and ATG3 (an E2-like enzyme) (Ichimura et al., 2000). The maturation of the phagophore into the autophagosome is not possible without the cooperation of these two conjugation systems. Further, the formation of the ATG12–ATG5 and LC3/ATG8–PE complex is essential for autophagosome formation (Xie and Klionsky, 2007).

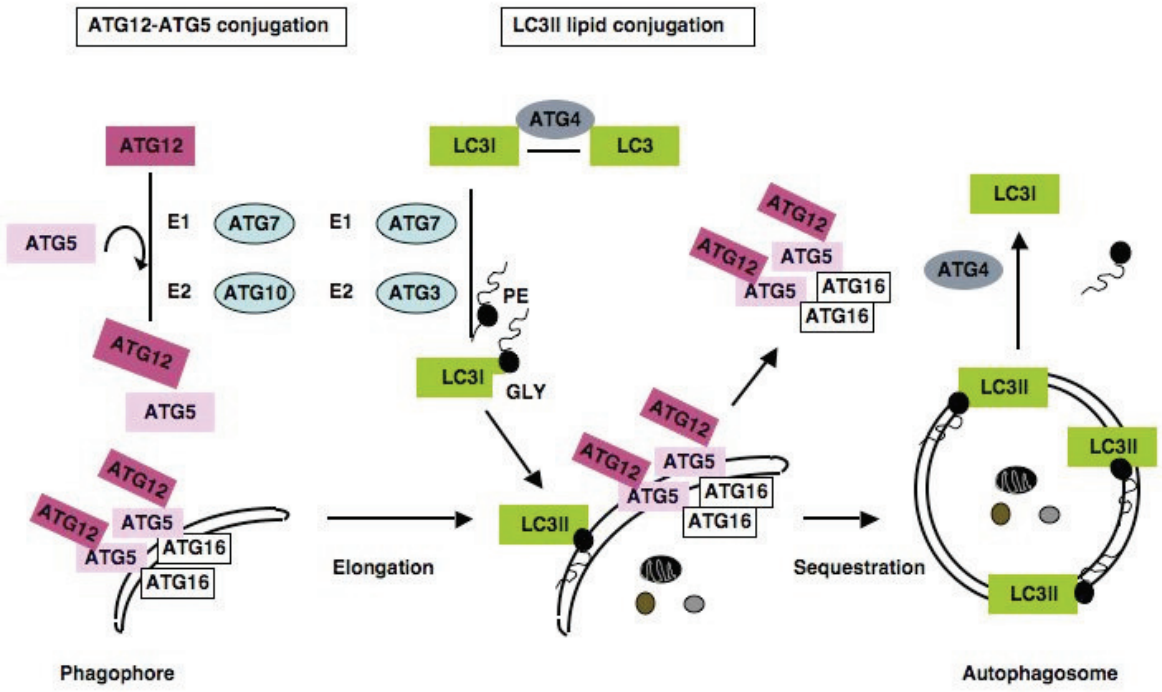

Figure 5. Autophagy conjugation system.
Two ubiquitin-like conjugation systems involved in autophagy vesicle formation are required for degradation of components of the cytoplasm; one involves the attachment of ATG12 to ATG5 and the other the conjugation of phosphatidylethanolamine (PE) to LC3/ATG8.

The amount of LC3II correlates well with the number of autophagosomes (Kabeya et al., 2000). ATG4 can deconjugate PE from LC3II, located in the outer autophagosomal membrane, converting it back to cytoplasmic LC3I (Kabeya et al., 2004). But almost all LC3II is eventually degraded by lysosomal proteases (Tanida et al., 2005). The cycle of conjugation and deconjugation is essential for the normal progression of autophagy (Kabeya et al., 2004; Kirisako et al., 1999). The main function identified so far for the ATG12–ATG5 complex is to promote LC3I lipidation and its correct localization (Fujita et al., 2008). Mutation of any member of the ATG family results in a defect autophagosome formation (Xie and Klionsky, 2007).

If the first system is defective, the second one cannot target the pre-autophagosomal structures as shown in ATG5$^{-/-}$ mice: in fact, in this case LC3II cannot be generated at all (Mizushima et al., 2001). LC3 is a reliable marker for autophagosome formation in mammalian cells, and the relative amount of LC3II reflects the abundance of the autophagosomes with increased electrophoretic mobility on gels compared to LC3I. This formation of autophagosomes can serve as a specific and unique marker for autophagy

induction because its lipidation provides a shift from diffuse to punctuate staining when it is coupled to GFP (Mizushima et al., 2004).

2.3.2 Regulation of autophagy

2.3.2.1 Negative regulation of autophagy

The class I phosphatidylinositol 3-kinase (PI3K) kinase is a negative regulator of autophagy (Petiot et al., 2000). In healthy cells, the mammalian target of rapamycin, mTOR kinase, represents the major negative regulator of autophagy in human cells. It primarily regulates autophagy and is downstream of the nutrient-sensor PI3K mediating the cellular response to nutrients and amino acids (Schmelzle and Hall, 2000). In response to nutrient and growth factor availability, the PI3K/AKT/mTOR axis is activated leading to stimulation of cell proliferation. Thus, upregulation of the proteins of the mTOR pathway triggers inhibition of autophagy in mammalian cells (Blommaart et al., 1997). On the other hand, the inhibition of the mTOR pathway induces transcriptional upregulation of the essential LC3/ATG8 gene of the autophagy vesicle formation (Huang et al., 2000; Kirisako et al., 1999). The negative control of autophagy by mTOR is a generally accepted phenomenon but the underlying mechanism is largely unknown (Shintani and Klionsky, 2004). As a potent inhibitor of autophagy, 3-methyladenine (3-MA) has been found to specifically block the sequestration step of autophagy formation inhibiting class III phosphatidylinositol-kinase activity (Petiot et al., 2000).

2.3.2.2 Positive regulation of autophagy

Beclin-1 was the first identified tumour suppressor protein that functions in lysosomal degradation during autophagy (Klionsky and Emr, 2000). It belongs to the Bcl-2 family and is a member of the BH3-only subgroup (Maiuri et al., 2007; Oberstein et al., 2007). Among all the autophagy genes, Beclin-1 is a key initiator of autophagy in mammalian cells (Cao and Klionsky, 2007) and belongs to a class III PI3K complex participating in autophagosome formation which stimulates the localization of other autophagy related proteins to the preautophagosomal membrane (Kihara et al., 2001). Beclin-1 has been shown to co-localize with the ER, mitochondria and Golgi (Kihara et al., 2001), and when

overexpressed, may stimulate autophagy (Hoyer-Hansen et al., 2005; Liang et al., 1999). Recently, it has been reported that this protein is a novel type of BH3-only protein due to its BH3 domain, but it fails to neutralize the antiapoptotic function of Bcl-2 and to induce apoptosis (Ciechomska et al., 2009). Like other BH3-only proteins, the BH3 domain of Beclin-1 binds to antiapoptotic Bcl-2 family proteins within their hydrophobic groove (Feng et al., 2007; Ku et al., 2008; Oberstein et al., 2007; Sinha et al., 2008).

DRAM (damaged-regulated autophagy modulator) is a target of p53, which in response to stress triggers programmed cell death in a p53-dependent manner. DRAM is a lysosomal trans-membrane protein that controls the initiation of autophagy by an unknown mechanism (Crighton et al., 2006).

Induction of autophagy by p53 has been linked to inhibition of the mTOR pathway, whereas autophagy inhibition by p53 correlates with increased mTOR activity (Feng et al., 2005). Inhibition of p53 degradation prevents autophagy, suggesting that p53 has a role in regulation of autophagy but the exact mechanism remains elusive (Tasdemir et al., 2008).

2.3.3 Crosstalk between autophagy and apoptosis

In response to nutrient deprivation or growth-factor withdrawal, adaptation-autophagy is initiated in order to recycle cellular constituents and provides energy to the cell to avoid cell death. In other cellular settings (e.g. prolonged time of stress or ineffective apoptosis) autophagy constitutes to cell death as an alternative pathway. Destroying large proportions of the cytosol and organelles may cause irreversible cellular damages and consequently the collapse of vital cellular functions leading to cell death.

Not only Beclin-1 represents a potentially important point of convergence of the apoptotic and autophagy pathway. Other BH3-only proteins, such as Bid and Bnip3 are also known to be involved in the communication between these two pathways. Silencing Bid inhibits apoptosis and enhances autophagy, suggesting that Bid may function as a ‘switch’ between the different cell death pathways (Lamparska-Przybysz et al., 2006). Upregulation of Bnip3 was reported to relate to the induction of autophagy in malignant glioma cells (Daido et al., 2004). Moreover, Bnip3 expression has been described to be upregulated in myocytes upon hypoxia resulting in mitochondrial dysfunction and cell death (Regula et al., 2002). In cardiac myocytes, Bnip3 triggers mitochondrial dysfunction, thereby inducing enhanced autophagy, possibly a protective response for the removal of damaged mitochondria.

Nevertheless, its physiological function is still unknown. It is not clear whether Bnip3 directly induces autophagy or whether autophagy is induced as a consequence of mitochondrial damage caused by Bnip3 (Hamacher-Brady et al., 2007). Furthermore, it has been reported that the BH3-only protein Puma may induce mitochondria-selective autophagy and triggers cytochrome c release. In this line, inhibition of autophagy partially rescues the loss of the mitochondrial membrane potential and apoptosis induction (Yee et al., 2009).

2.4 Cell cycle

During their life span, cells go through an individually defined number of cycles. The cell cycle that regulates somatic cell division is precisely regulated to ensure the maintenance of genomic integrity, prevention of mutations, and unwanted cell proliferation. During normal proliferation, cells go through different phases of the cell cycle. The cell cycle is divided into four ordered set of events: GAP-1 stage (G1), Synthesis stage (S), GAP-2 stage (G2) and mitosis stage (M). During stages G1, G2 and S, commonly known as interphase, the cell synthesizes most of the components needed for the new daughter cells, i.e. duplicates the chromosomes and enlarges in size. Mitosis is the final event of the cell cycle where the cell divides the cytoplasm and the duplicated chromosomes, creating two identical daughter cells.

2.4.1 Cyclins and CDKs

Each cell cycle phase is regulated by its own specific key regulator cyclins and **C**yclin-**D**ependent **K**inases (**CDK**), a family of serine/threonine protein kinases that are activated at specific points of the cell cycle (Johnson and Walker, 1999; Li and Blow, 2001; Morgan, 1997). The CDKs, heterodimeric complexes composed of a catalytic kinase subunit and a regulatory cyclin subunit, play critical roles in cell cycle progression control (Meyerson et al., 1992). To date, nine CDK have been identified and, of these, five are active during the cell cycle, i.e. during G1 these are CDK4, CDK6 and CDK2, S (CDK2), G2 and M phase (CDK1). Cyclins bind and activate members of the cyclin-dependent kinase (CDK) family to influence the cell cycle progression. Accumulation and activation of each cyclin fluctuate throughout the different cell cycle phases. Cyclin protein levels rise and fall during the cell

cycle and in this way they periodically activate CDKs (Pines, 1991). Therefore, the entry of each phase of the cell cycle is well controlled by specific, cyclin/CDK-controlled checkpoints. Oscillations in the levels of cyclins, D, A and B are followed by progression through the corresponding cell cycle phase as shown in the model (figure 6).

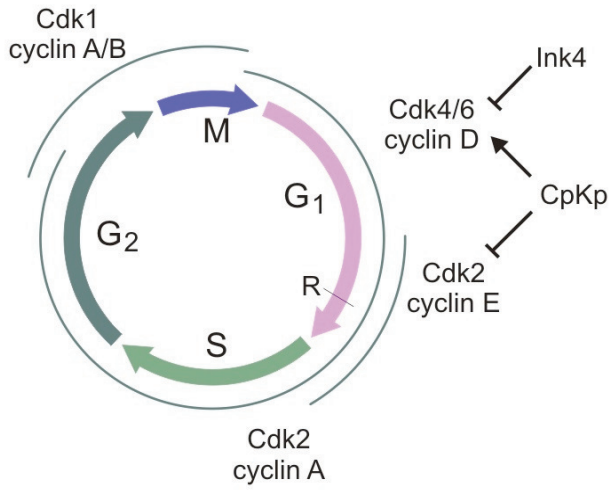

Figure 6. Scheme of the cell cycle.
Presentation of the four different phases of the cell cycle, which are regulated by cyclin-CDK complexes.

Cyclin A binds to CDK2 and this complex is required during S phase (Girard et al., 1991; Walker and Maller, 1991). The three types of D cyclins (cyclin D1, cyclin D2, cyclin D3) bind to CDK4 and to CDK6. CDK/cyclin D complexes are essential for G1 phase of the cell cycle (Sherr, 1994). Cyclin D1 plays a crucial role as regulator of cell cycle progression (Coqueret, 2002; Musgrove, 2006). Overexpression of cyclin D1 has been linked to the development of breast cancer (Hodges et al., 2003; Kenny et al., 1999) as well as to the early onset of cancer and risk of tumour progression and metastasis (Chung, 2004; Guner et al., 2003; Wang et al., 2004; Zhang et al., 1993).

Together with its binding partners CDK4 and CDK6, cyclin D1 forms active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (RB) (Kato et al., 1993; Weinberg, 1995).

When a CDK is active, target proteins become phosphorylated at CDK consensus sites, substrate of CDK4/6-cyclin D, i.e. the product of the retinoblastoma tumour suppressor gene (pRb). During late G1 phase, pRb becomes phosphorylated leading to disruption of the complex histone deacetylase protein (HDAC) and the subsequent release of the transcription factors E2F-1 and DP-1, which positively regulates the transcription of genes whose

products are required for S phase progression, including cyclin A, cyclin E, and Cdc25 (Brehm et al., 1998). Specific inhibition of cyclin D1 in cancer cell lines results in cell cycle arrest (Lee et al., 2000; Zhou et al., 1995).

The major function of the G1-to-S checkpoint in the mammalian systems is the control of genomic stability (Paulovich et al., 1997). G1 arrest following DNA damage occurs in order to restore the integrity of the DNA template and to avoid the replication of damaged DNA (Levine, 1997).

In late G2 and early M phases of the cell cycle, cyclin A forms a complex with CDK1 to promote entry into M phase. Mitotic entry is further regulated by the cyclin B/CDK1 complex (Arellano and Moreno, 1997; King et al., 1994).

Moreover, the cyclin-CDK inhibitor protein p21 has been shown to be both sufficient and required for proper G1 arrest (Rousseau et al., 1999; Waldman et al., 1995) through binding and inhibition of cyclin/CDK complexes (Bunz et al., 1998; Harper et al., 1993).

Cell cycle phases and their corresponding CDKs and cyclins are presented in the table below (figure 7).

CDK	Cyclin	Cell cycle phase
CDK4	Cyclin D1	early G1 ➞ late G1
CDK6	Cyclin D1	early G1 ➞ late G1
CDK2	Cyclin E	late G1 ➞ S
CDK2	Cyclin A	S phase
CDK1	Cyclin A	G2 ➞ mitotic entry
CDK1	Cyclin B	G2 ➞ mitotic entry

Figure 7. Cyclins and CDKs responsible for the indicated cell cycle phases.

2.4.2 Regulation of cyclin-CDK complexes

In mammals, there are three different isoforms of cell division cycle 25 (CDC25) phosphatases (CDC25A, B and C), activating multiple CDK at different phases of the cell cycle (Boutros et al., 2007; Galaktionov and Beach, 1991). CDK/cyclin complexes are controlled by the opposing activities of WEE1/MYT1 kinases and CDC25 phosphatases (Bulavin et al., 2003; Dunphy, 1994). CDK/cyclin complexes are the only known substrates for CDC25 phosphatases (Kristjansdottir and Rudolph, 2004). WEE1/MYT1 kinases inhibit CDK/ cyclin activity by phosphorylating tyrosine 15 and threonine 14 of CDK1 (Malumbres and Barbacid, 2005). CDC25 phosphatases dephosphorylate and activate CDK complexes, which in turn allow catalysis and substrate phosphorylation. When CDK activity is required for progression to the next cell-cycle phase, the phase specific CDC25 phosphatases dephosphorylate these two residues, thereby activating CDK/cyclin complexes. One further activating phosphorylation (Thr161 of CDK1) by the CDK-activating kinase (CAK) is demanded for their complete activation (Kaldis, 1999). CDC25A mainly activates the CDK2/cyclin E and CDK2/cyclin A complexes during the G1/S transition (Blomberg and Hoffmann, 1999; Jinno et al., 1994) but also partially during the G2/M transition (Zhao et al., 2002). CDC25B and CDC25C are primarily necessary for the entry into mitosis (Lammer et al., 1998).

Inhibitors of cyclin/CDKs are modulating the cell cycle by preventing the association of CDKs with their corresponding cyclins. The **in**hibitor of CD**K4 (INK4)** class of CDKIs, notably $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p14^{INK4d}$ can specifically bind to CDK4 and CDK6 and preventing their association with D-type cyclins (Roussel, 1999). These inhibitors inactivate CDK/cyclin complexes at the G1 restriction point, and, to a lesser extent, CDK1/cyclinB complexes (Hengst and Reed, 1998). CDK inhibitors $p21^{waf1}$, $p27^{kip1}$, and $p57^{kip2}$ belong to the second **k**inase **i**nhibitor **p**rotein group **(KIP)** (Sherr and Roberts, 1999). These inhibitors block cyclin/E/CDK and cyclin/A/CDK complexes, although there are many reports illustrating that KIP class proteins can also interact with cyclin D-associated CDKs (Harper et al., 1993). However, all these inhibitory proteins act as fail-safe mechanisms mediating cell cycle arrest to allow the repair of damaged DNA or, if is not possible, to initiate cell death.

2.5 The tumour suppressor protein $p14^{ARF}$

The mammalian INK4a gene locus resides on the human chromosome 9p21 and encodes two structurally and functionally unrelated proteins, $p14^{ARF}$ and $p16^{INK4a}$. The $p16^{INK4a}$ gene utilises exons 1α, 2 and 3 and has a unique promoter (Duro et al., 1995; Mao et al., 1995; Quelle et al., 1995). On the other hand, the INK4a locus also encodes for the $p14^{ARF}$ tumour suppressor protein, which uses the upstream exon 1beta, spliced to exons 2 and 3 (Eischen et al., 1999; Flores et al., 1996). $P14^{ARF}$ is transcribed in an **A**lternate **R**eading **F**rame **(ARF)** from a separate exon 1beta in humans and was originally identified as an alternative transcript of the INK4a/ARF tumour suppressor locus.

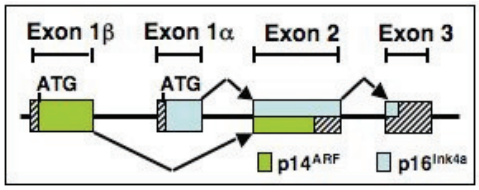

Figure 8. The INK4a–ARF locus and its organization.
The INK4a gene locus encodes two structurally unrelated proteins $p16^{INK4a}$ and $p14^{ARF}$, which are involved in cell cycle regulation. Exons 1α and 1β are transcribed from different promoters.
$P14^{ARF}$ encoding sequences in exons 1β and 2 are indicated in green shading and $p16^{Ink4a}$ coding sequences in exons. Light blue shading represents 1α, 2 and 3. These are spliced to the same acceptor site in exon 2, which is translated in alternative frame (from which the $p14^{ARF}$ gene got its name).

The INK4a locus is often inactivated by deletion or methylation in human melanomas, lymphomas, and other tumours (Inoue et al., 2001; Sharpless and Chin, 2003; Sherr, 2001; Tao and Levine, 1999). It has been extensively studied in the progression of cancer as a tumour suppressor gene (Zhang et al., 1998).

In human tumour cell lines, $p14^{ARF}$ is localized mainly to nucleoli (Korgaonkar et al., 2002; Llanos et al., 2001), and is an active component in p53 activation (Zindy et al., 1998). Healthy cells contain low endogenous levels of $p14^{ARF}$ but the expression is known to be rapidly upregulated upon oncogenic stress including E1A (de Stanchina et al., 1998), c-myc (Bates et al., 1998), E2F (Palmero et al., 1998) Ras (Aslanian et al., 2004), E2F3 (Korgaonkar et al., 2002; Stott et al., 1998) and E2F1 (Bates et al., 1998) $p14^{ARF}$ has the ability to suppress cell growth through multiple p53-dependent or p53-independent pathways (Eymin et al., 2006; Hemmati et al., 2002; Hemmati et al., 2006; Hemmati et al.,

2008; Hemmati et al., 2005; Normand et al., 2005). The discovery of several $p14^{ARF}$ interacting partners and the observation that, aside from oncogenic stimuli, viral, genotoxic, hypoxic and oxidative stresses also activate a $p14^{ARF}$-dependent response, suggest that $p14^{ARF}$ could exert a wider role in maintaining tissue homeostasis and deletion of unwanted or damaged cells (Hemmati et al., 2002).

2.5.1 Interplay of $p14^{ARF}$, p53 and p21

The ability of $p14^{ARF}$ to induce apoptosis regardless of p53 functionality is widely discussed. $P14^{ARF}$ has been shown to have a dual role by inducing either the apoptosis pathway or cell cycle arrest. It was reported that $p14^{ARF}$ expression does not result in extensive apoptosis either in p53 proficient (Gao et al., 2001; Simon et al., 2006) or p53 deficient cells (fibrosarcoma cells) (Magro et al., 2004). It was shown that $p14^{ARF}$ regulates cell cycle arrest and apoptosis in a p53 independent manner. $P14^{ARF}$ is capable of triggering the mitochondrial pathway and executing apoptosis through the caspase cascade network in cells lacking the proapoptotic protein Bax in p53 deficient Du145 cells (Hemmati et al., 2002). In p53 proficient cells, apoptosis proceeds via Bax and Bak (Hemmati et al., 2006). Moreover, previous studies suggested that $p14^{ARF}$-induced G1 arrest is entirely dependent on the presence of functional p53 and its downstream effector p21 (Eymin et al., 2003; Normand et al., 2005). It was shown that the $p14^{ARF}$-induced G2 arrest occurs through a p21 independent pathway in p53-deficient cells (Hemmati et al., 2008; Silva et al., 2003). Furthermore, it was observed that expression of $p14^{ARF}$ arrests cells in G2, suggesting that both p53 and p21 are unnecessary for executing $p14^{ARF}$ induced G2 arrest. However, $p14^{ARF}$ has been recognized as a potent tumour suppressor in vivo (Sherr, 2004). Different types of DNA damage can activate p53, including double-strand breaks of DNA caused by γ-irradiation, resulting in a rapid increase of the p53 level and activation of p53 as a transcription factor (Levine, 1997). P53 targets more than a hundred genes for transcriptional activation or repression by binding to the respecting promoters (Carnero et al., 2000; Wang et al., 2009). 90% of cancer-associated missense mutations in p53 target the DNA binding domain, demonstrating the importance of transactivation in the role of p53 as a tumour suppressor (Levine, 1997). However, $p14^{ARF}$ expression can prevent proliferation of MEFs that lack p53 function, although their arrest occurs much slower than in wild type cells (Kamijo et al., 1998; Midgley et al., 2000).

$P14^{ARF}$ binds to Mdm-2 and inhibits its expression (Barak et al., 1993; Wu et al., 1993). Through an interaction of the N-terminal domain of $p14^{ARF}$ and the C-terminal domain of the p53-antagonist Mdm2, $p14^{ARF}$ blocks the ability of Mdm-2 (Hdm-2 in humans) to tag p53 for degradation (Haupt et al., 1997; Kubbutat et al., 1997). This results in increased accumulation of p53 protein followed by enhanced p53-mediated transcriptional activation. Hdm-2 binding promotes p53 degradation through the ubiquitin-dependent proteasome pathway (Pomerantz et al., 1998; Zhang et al., 1998). Inhibition of Hdm-2 by the tumour suppressor $p14^{ARF}$ is a critical mechanism of p53 activation (Honda and Yasuda, 1999; Moll and Petrenko, 2003; Shangary and Wang, 2008). The role of $p14^{ARF}$ in the p53 pathway is to retain Hdm2 in the nucleolus inhibiting Hdm2 mediated p53 ubiquitination and degradation (Jones et al., 1995; Montes de Oca Luna et al., 1995). Loss of Hdm2 results in embryonic death due to activated p53 (Pomerantz et al., 1998; Zhang et al., 1998). The expression of $p14^{ARF}$ can directly trigger a p53 response, leading to cell cycle arrest and, finally, to cell death. Mdm-2 forms a negative feedback loop that maintains p53 at low levels in the absence of stress (Bertwistle et al., 2004; Kamijo et al., 1997).

The expression of p21 is controlled by the tumour suppressor protein p53 and is mainly dependent on two factors, the stimulus and the type of cell (Rodriguez and Meuth, 2006). P21 is transcriptional target of the tumour suppressor gene p53 (Dotto, 2000). P21 regulates the switch between different cell cycle phases and is a CDK inhibitor, which can interact with almost all CDKs complexes. Whether G1 or G2 arrest is induced by p21 overexpression depends on the cell type and the Rb status and presence or absence of other INK4 or Cip/Kip proteins (Smits et al., 2000). In p53 wild type HCT116 colon carcinoma cells, loss of the p53 induced CDK inhibitor p21 strongly sensitized for $p14^{AEF}$-induced cell death (Hemmati 2005). P21 induced G1 arrest is mediated by direct inhibition of the cyclin/CDK complexes, specific to G1 phase of the cell cycle. G2 arrest induced by p21 has not been fully elucidated. Nevertheless it has been shown that more than the physiological concentrations of p21 are needed to block the CDK1/cyclin B1 complex. P21 is able to inhibit the phosphorylation of CDC2 which is required for its activation (McShea et al., 2000). Furthermore, p21 can induce G2 arrest by binding to a cyclin B interacting protein (CARB) (Suzuki et al., 1998; Weber et al., 2000). Interestingly p21 is also able to bind and inhibit procaspase-3, but cannot block active caspase-3 (Niculescu et al., 1998).

AIM OF THE STUDY

3. Aim of the study

P14^{ARF} plays a central role in the regulation of cellular proliferation. The aim of this thesis was to investigate the regulation of p14^{ARF} induced cell cycle arrest. Checkpoint abrogation overcomes the resistance to p14^{ARF} induced cell cycle arrest, promotes cell cycle progression, and triggers apoptosis or autophagy depending on the presence of caspase-3. Notably, 14^{ARF} as a cell cycle checkpoint regulator gene is very important, providing the possibility to manipulate the cell cycle program, which might be crucial to prevent uncontrolled cancer cell progression. To gain further insight into cell death induced by p14^{ARF}, this tumour suppressor protein was examined as a potential target for cancer therapy. The overall goal of this thesis was to define the pathway of p14^{ARF} in malignant cells.

The specific objectives:

- To investigate the consequences of p14^{ARF} checkpoint regulation as a potential target against tumour proliferation;
- To study the molecular mechanism and functional characteristics of p14^{ARF} in cell death;
- To examine the p14^{ARF} pathway in respect to its function as a cell cycle regulator and cell death inducer, respectively.

MATERIALS AND METHODS

4. Materials and Methods

4.1 Chemicals and reagents

Acrylamid	Biorad, Munich, Germany
Agarose	Biozym, Hessisch Oldendorf, Germany
Ammoniumperoxodisulfate	Sigma, Deisenhofen, Germany
ATP	Promega, Mannheim, Germany
Bacto-Agar	Becton Dickenson, Heidelberg, Germany
Bacto-Trypton	Becton Dickenson, Heidelberg, Germany
Bacto-Yeast Extract	Becton Dickenson, Heidelberg, Germany
Blocking reagent	Roche Diagnostics, Mannheim, Germany
Caffeine	Alexis, Grunberg, Germany
Casein	Roche Mannheim, Germany
Complete protease inhibitor	Roche Diagnostics, Mannheim, Germany
Caspase Inhibitor VI (z-VAD-fmk)	Calbiochem, Bad Soden, Germany
Caspase-3 Inhibitor (z-DEVD-fmk)	Calbiochem, Bad Soden, Germany
Caspase-9 Inhibitor (z-LEHD-fmk)	Calbiochem, Bad Soden, Germany
Caspase-8 Inhibitor (z-IETD-fmk)	Calbiochem, Bad Soden, Germany
Caspase inhibitor (Q-VD-OPh)	Calbiochem, Bad Soden, Germany
DMEM/high Glucose (4.5g/l)	GIBCO Karlsruhe, Germany
Digitonin	Sigma-Aldrich, Taufkirchen, Germany
dNTPs	Roche Diagnostics, Mannheim, Germany
Ethidium bromide	Sigma, Deisenhofen, Germany
FBS	GIBCO, Karlsruhe, Germany
Fluorescent Mounting Media	Dako, Dänemark
Glutamin	GIBCO, Karlsruhe, Germany
JC-1	Molecular Probes, Eugene, USA
HEPES	Sigma, Deisenhofen, Germany
Nocodazol	Sigma, Deisenhofen, Germany
Penicillin/ Streptomycin	GIBCO, Karlsruhe, Germany
Ponceau-Red	Sigma, Deisenhofen, Germany
Propidium iodide	Sigma, Deisenhofen, Germany

Puromycin	Sigma, Deisenhofen, Germany
RPMI 1640	GIBCO, Karlsruhe, Germany
Triton X-100	Sigma, Deisenhofen, Germany
Trypsin-EDTA	Life Technologies, Schwalbach, Germany
3-MA (3-methyladenine)	Sigma, Deisenhofen, Germany

4.2 Kits

ECL (enhanced chemiluminescence) Amersham, Braunschweig, Germany
FuGENE® 6 Transfection Reagent, Roche, Indianapolis, USA
Plasmid Mini-, Midi-, Maxi-Kit QIAGEN, Hilden, Germany
RT-PCR Kit Applied Biosystems, Foster City, USA
TaqMan Universal PCR Master Mix Applied Biosystems, Foster City, USA

4.3 Antibodies

Primary antibodies, species	Catalog number	Company
β-Actin polyclonal, rabbit	#2066	Sigma, Germany
$p14^{ARF}$ (14PO2) monoclonal, mouse	#850	NeoMarkers, USA
Caspase-3 polyclonal, goat	#605	R+D Systems, Germany
Caspase-9 polyclonal, goat	#8301	R+D Systems, Germany
Caspase-8 (12F5) monoclonal, mouse	#804-242	Alexix, GmbH, Germany
PARP polyclonal, rabbit	#G7341	Promega GmbH, USA
Bcl-x_L polyclonal, rabbit	#556361	BD Pharmingen, Germany
Bcl-2 (D5) monoclonal, mouse	#2-486	Novocastra Laboratories, UK
P53 (DO-1) monoclonal, mouse	#554293	BD Pharmingen, Germany
P53 (ser15) polyclonal, rabbit	#9284	Cell Signalling, Germany
Bax polyclonal, rabbit	#554106	BD Pharmingen, Germany
Bak polyclonal, rabbit	#5897	Sigma, Germany
Beclin-1 polyclonal, rabbit	#3738	Cell Signalling, Germany
LC3 polyclonal, rabbit	#100-2331	Novus Biologicals, Germany
Cyclin A polyclonal, rabbit	#751	Santa Cruz Biotechnology, Germany
Cyclin B1 (D-11) monoclonal, mouse	#7393	Santa Cruz Biotechnology, Germany
Cyclin D1 polyclonal, rabbit	#718	Santa Cruz Biotechnology, Germany
Anti-phospho-histone (JBW301) γH2AX (ser 139) monoclonal, mouse	#05636	Millipore, USA
P21 (6B6) monoclonal, mouse	#554228	BD Pharmingen, Germany
Cytochrome c (7H8.2C12) monoclonal, mouse	#556433	BD Pharmingen, Germany
Tom20 (29) monoclonal, mouse	#612278	BD Pharmingen, Germany
Cdk4 polyclonal, rabbit	#260	Santa Cruz Biotechnology, Germany
Cdk6 polyclonal, rabbit	#177	Santa Cruz Biotechnology, Germany

Secondary antibodies	Catalog number	Company
Goat-anti-mouse IgG (H+L)	#1031-05	Southern Biotech, USA
Goat-anti-rabbit IgG (H+L)	#4050-05-147	Southern Biotech, USA
Rabbit-anti-goat IgG (H+L)	#6160-05	Southern Biotech, USA
FITC goat-anti-mouse IgG (H+L)	#115-095-062	Jackson Immuno Research, USA
594 goat-anti-rabbit IgG (H+L)	#A11012	Molecular Probes, Netherlands
488 goat-anti-mouse IgG (H+L)	#A11001	Molecular Probes, Netherlands

4.4 Cell culture

PcDNA3mock-transfected (MCF-7 mock) and pcDNA3caspase-3 transfected (MCF-7 C3) breast cancer MCF-7 cells (Janicke et al., 1998) were grown in Roswell Park Memorial Institute (RPMI) 1640 medium.

MCF-7 EGFP-Bax cells were generated as described earlier (Essmann et al., 2005). Briefly, cDNA of Bax was amplified by PCR. PCR products were digested and cloned as BglII/EcoRI fragments into the corresponding sites of the pEGFP-C2 vector (BD Biosciences Clontech, Heidelberg, Germany) to obtain Bax that carries the EGFP tag at the N-terminus. Stable transfectants were selected by the use of G418 (1 mg/ml), subcloned and EGFP-Bax expression of the clones was determined by fluorescence microscopy. The cells were grown in RPMI 1640 medium.

The expression vector encoding EGFP-LC3 (pEGFP-LC3) was kindly provided by F. Essmann (Institute for Biochemistry, University of Tübingen). This vector was introduced into MCF-7 caspase-3 proficient and MCF-7 caspase-3 deficient cells by using Fugene (FuGENE® 6 Transfection Reagent, Roche, Indianapolis). Stable transfectants were selected by the use of hygromycin, and EGFP-LC3 expression of clones was determined by fluorescence microscopy.

The human colon adenocarcinoma cell line SW480 was purchased from ATCC (Massachusetts, USA) and grown in DMEM high glucose (4.5g/l) medium.

The cervical cancer cell line HeLa was purchased from Tebu-bio, Germany and were grown in DMEM high glucose (4.5g/l) medium. These cells express high-risk human papillomavirus HPV E6, which functionally inactivates p53 (Howley, 2006).

HEK293 were obtained from the American Type Culture Collection. This cell line is a transformed primary human fetal kidney cell line, which constitutively expresses Ad5 E1 (Graham et al., 1977). For this reason, they were used for adenoviral production, amplification and titration.
All media were supplemented with 10% inactivated fetal bovine serum (FBS) (inactivated at 56°C for 30min,), 100 U/ml penicillin and 0.1mg/ml streptomycin. All media and culture reagents were from GIBCO/Invitrogen, Karlsruhe, Germany. Cells were cultured at 37°C with 5% CO2 in a fully humidified atmosphere.

4.5 Cell stocks

For constituting stocks of different cell lines, $1x10^6$ cells in 500µl of medium were supplemented with 500µl of a 1:5 DMSO:FBS mix in cryotubes, frozen for 2h at –20°C, then stored at –80°C and subsequently stowed in liquid nitrogen.

4.6 Adenoviral gene expression

The recombinant, replication deficient adenovirus Ad5-CMV $p14^{ARF}$ (Ad-$p14^{ARF}$) was constructed as described before (Hemmati et al., 2002). Briefly, the following steps were performed:
1) the $p14^{ARF}$ gene was cut out from pBlueIIks-$p14^{ARF}$ (provided by Dr.KG Wiman and Dr U Klangby, Cancer Center Karolinska, Stockholm, Sweden) with the restriction enzymes BamHI/XhoI (New England Biolabs Frankfurt am Main, Germany).
2) $p14^{ARF}$ was inserted into a pcDNA3 vector and placed under the control of a CytoMegaloVirus (CMV) promoter
3) the CMV-$p14^{ARF}$ cassette was excised from pcDNA3 and inserted into the pHVAd2 shuttle vector between the homologues sequences that pHVAd2 shares with the Ad5 vector
4) homologues recombination between pHVAd2 and Ad5 vector was performed in BJ5183 RecBC-sbcB bacteria to allow the transfer of the CMV-$p14^{ARF}$ cassette from the shuttle vector to the Ad5 vector resulting in the adenoviral vector Ad5-CMV $p14^{ARF}$.

4.6.1 Calcium-phosphate transfection

For production of large amounts of recombinant adenovirus, HEK293 cells were transfected with the adenoviral vector by calcium-phosphate precipitation. The HEK293 cell line is a transformed human fetal kidney cell line, which constitutively expresses Ad5 E1 proteins, supplying the missing E1 gene and therefore allowing the replication of the E1 deleted adenovirus (Graham et al., 1977; Shaw et al., 2002). 10µg of PacI digested adenoviral DNA were mixed with 25µl of 2.5M $CaCl_2$ and adjusted with sterile aqua destillata to a total of 250µl. 250µl of transfection buffer were added drop-by-drop to the mix and gently shaken. Upon shaking calcium phosphate crystals are formed that bind DNA. The total of 500µl was incubated for 30min at RT. Different concentrations were added to HEK293 cells incubated in 1ml serum free medium. After 1.5h, 1ml medium with 20% FBS was added to the cells. Once plaques were visible in the cell layer, the cells were harvested and the crude virus lysate (CVL) was used for virus amplification or stored.

Transfection buffer:
8g NaCl
370mg KCl
250mg $Na_2HPO_4x2H_2O$
5g HEPES
in 500ml water
pH 6.75
sterilize by autoclaving

4.6.2 Virus amplification and purification

The CVL retrieved from the calcium-phosphate transfection was used for the amplification of the adenovirus. 50x150cm^2 flasks containing HEK293 cells (80% confluence) were infected with 10 ml mix of serum free medium and CVL. After 1.5h 10 ml medium with 20% FBS was added to the cells. Once the cells showed signs of infection by detaching from the bottom of the flasks, the cells together with the medium were collected and

centrifuged at 1000*g*. Except for 35ml, supernatant was discarded. 35ml CVL containing the pellet was kept at -20°C and used for purification.

As a first step in virus purification, the CVL was thawed and frozen three times. Then, 2.5ml of a 1.25g/cm^3 CsCl solution were poured into an Ultra-Clear centrifuge tube (Beckman Instruments GmbH, Munich, Germany) and 2.5ml of 1.5g/cm^3 CsCl were slowly added beneath. Furthermore, 7ml of CVL from the virus purification was added onto this CsCl gradient. Then, the gradient was centrifuged (Beckmann Optima LE 80-K) at 30000*g* for 2h. After centrifugation, two bands were visible in the gradient. The virus band (the lower one) was harvested using a 1ml syringe and mixed with 1.35g/cm^3 CsCl to a final volume of 3ml. Then, 3ml of 1.35g/cm^3 and subsequently 3ml of 1.25g/cm^3 CsCl were carefully added creating a gradient. This was followed by over night centrifugation at 30000*g* resulting in a purified virus band on the gradient. The virus was collected again with a 1ml syringe. A NAP-25 column (Amersham Buchler, Braunschweig, Germany) was washed 4 times with 5ml of adenovirus suspension buffer. 1ml of the virus, gained from the gradient, was applied onto the column. Next, the column was washed with 1.5ml adenovirus suspension buffer. Finally, 2ml of adenoviral suspension buffer were added to the column and collected into a fresh tube. The purified virus solution was stored as 50µl aliquots at -80°C.

Adenoviral suspension buffer:
135mM NaCl
3mM KCl
1mM $MgCl_2$
100mM Tris/HCl
sterilize by autoclaving
add 10% glycerol

4.6.3 Virus titration

A dilution series starting with 1µl of the adenoviral stock, which was cut in half in each step i.e. a log2 dilution series, were pipeted into a 24well plate using serum free medium for dilution. HEK293 cells were plated into a 24well plate and infected with the pre-prepared dilution series of the virus by using one well per dilution rate in a volume of 500µl. After

1.5h incubation time, 500µl of media containing 20% FBS was added to the cells. Approximately 14 days after infection, the plaques of each well were counted. The titer was calculated based on the plaque formation and the dilution rate.

4.6.4 Verification of replication competent adenovirus (E1a-PCR)

The adenoviral E1a region DNA was tested for unwanted recombination of the virus resulting in E1 proficient replication competent adenovirus. During virus amplification in HEK293 cells, the E1a region could be inserted into the adenoviral vector by homologous recombination. This could enable the virus to replicate and result in a lytic infection making an experimental analysis impossible. To detect E1a DNA adenoviral DNA was amplified for its E1a region by PCR. Detection of the E1a DNA band would indicate that the adenovirus is unusable. For viral DNA purification, 1µl of the purified virus stock was mixed with 20µl of proteinase K-Mix and incubated for 90min at 55°C. Then, 300µl of phenol/chloroform/isoamylalchocol pH 8.0 (ratio 25/24/1) was added to the sample. After vortexing, the sample was centrifuged for 5min at 10000*g*. The upper phase was transferred into a new tube and mixed with 300µl of chloroform. This step was followed again by centrifugation for 5min at 10000*g*. The upper phase containing the DNA was transferred into a new tube and the DNA was acquired by precipitation.
The acquired DNA was tested for E1a by PCR and bands were detected by agarose gel electrophoresis. As a positive control, PCR and gel electrophoresis were performed for the E4 region, which is an integral part of the adenoviral vector.

Proteinase K-Mix: 5mM EDTA
20mM Tris/HCl, pH 8.0
0.2% (v/v) SDS
0.25µg/µl proteinase K

PCR: 20.0 µl (H_2O as negative control)
PCR-Puffer: 1x
$MgCl_2$ 1.5 mM
dNTP: 200 µM
Taq DNA-Polymerase: 1 U

Primers: 500 nM

Program: 94°C 5 min

94°C 30 sec ⎫

58°C 30 sec ⎬ 35 cycles

72°C 30 sec ⎭

72°C 7 min, 4°C hold

E1A sense: 5' – GAG ACA TAT TAT CTG CCA CGG AGG – 3'

anti-sense: 5' – TTG GCA TAG AAA CCG GAC CCA AGG – 3'

(Adesanya et al., 1996)

E4: sense: 5' – GTA GAG TCA TAA TCG TGC ATC AGG – 3'

anti-sense: 5' – TTT ATA TGG TAC CGG GAG GTG GTG – 3'

(Adesanya et al., 1996)

4.6.5 Infection of the cells with adenovirus for transgene expression

$1x10^5$ cells plated in 6-well plates and cultured at 37°C with 5%CO_2. After 24h, these cells were infected with adenoviral vector in RPMI 1640 (MCF-7) or DMEM medium (SW480, HEK293, HeLa) cells. Cells were infected with medium without FBS containing the adenovirus (Ad-lacZ or Ad-$p14^{ARF}$) at the indicated amounts of MOI (multiplicity of infection). After incubation for 1.5h at 37°C, an equal amount of medium with 20% FBS was added to the cells.

4.7 Western blot analysis

4.7.1 SDS-PAGE

SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is a technique to separate proteins and peptides according to their electrophoretic mobility which corresponds to their molecular weight (Laemmli, 1970). SDS, an anionic detergent, denaturates proteins by wrapping the hydrophobic tail around the polypeptide backbone, giving the protein a negative charge proportional to its length. The reducing agent β-mercaptoethanol in the sample buffer cleaves any disulfide bonds between cysteine residues, resulting in a completely unfolded protein. Consequently, the original conformation and charges of the proteins do not have any influence on how they migrate through the gel. The only parameter is their molecular weight. Electrophoresis was carried out with a Bio-Rad Mini-PROTEAN® 3 system. Gels were prepared with 0.75 mm spacers and 15 pockets combs.

4.7.2 Preparation of protein samples and determination of the concentration

$5x10^5$ cells were harvested by trypsinization, washed with ice cold 1xPBS and lysed in an appropriate amount of lysis buffer supplemented with protease inhibitor for 30min on ice. After centrifugation at 10000*g*, 4°C for 15min the supernatant was collected as protein sample.

Lysis buffer	2mM EDTA
	0.1% SDS
	1% Triton-X100
	1mM Na_3VO_4
	1mM beta-glycerolphosphate

1 tablet protease inhibitor cocktail (Complete®, Roche Diagnostics)

For cytochrome c detection, cells were harvested in PBS and centrifuged at 300*g* for 5min, After that, the pellets were resuspend in 20-50µl hypotonic buffer for 3-5 min on ice. Membranes were separated by centrifugation at 10000*g*, 4°C for 15min and the supernatant was used as cytosolic extract. The pellets were lysed in lysis buffer.

hypotonic buffer:	200mM HEPES pH 7.4
	10mM KCl
	2mM $MgCl_2$
	1mM EDTA
	in 100ml
	to 1ml of buffer add prior to use:
	7.5µl 100mg/ml digitonin
	7.5µl 10mM PMSF

The protein concentration was determined using the bicinchoninic acid (BCA) assay (Pierce, Rockford, USA). 10µl of protein were mixed with 200µl of BCA solution (reagent A: reagent B = 50:1) in a 96-well plate and incubated at 37°C for 30min in the dark. The absorption was measured at 620nm. A BSA concentration row served as standard. The protein concentration was calculated based on the standard curve. Samples were mixed with 5x SDS-PAGE sample buffer and boiled for 5 min at 95°C. Equal amounts of protein (25µg) were separated by SDS-PAGE using 14% or 12% separating gels and 5% stacking gels. Electrophoresis was performed at 120 V for the first 5 minutes and continued with 180 V for approximately 1h.

separating gel (14%)	aqua dest.
	1.5M Tris/Hcl, pH 8.8
	acrylamide/bisacrylamide solution (29:1; 40%)
	100µl 10%APS
	100µl 10%SDS
	4µl TEMED
stacking gel (5%)	2.185ml aqua dest.
	380µl 0.5M Tris/HCl, pH6.8
	375µl acrylamide/bisacrylamide solution (29:1; 40%)
	30µl 10%APS
	30µl 10%SDS
	3µl TEMED
5x sample buffer	260 mM Tris/HCl, pH 6.8

	12.5% mercaptoethanol
	20% glycerol
	2% SDS
	0.01% bromophenolblue
5x running buffer	100mM Tris, pH 8.3
	1M glycin
	0.5% SDS

4.7.3 Blotting of proteins onto nitrocellulose membranes

Electrophoretically separated proteins were transferred onto 0.2µm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) by semi-dry blotting using a Bio-Rad Trans-blot SD transfer cell (Bio-Rad, Munich, Germany). Membranes and filter paper (Schleicher & Schuell GB005, Dassel, Germany) were kept in CAPS buffer for 3min prior to use. Three layers of filter followed by the membrane, SDS-gel and again three layers of filters were placed on the anode and finally covered with the cathode. Blotting was performed at 1mA/cm^2 for 1h. Transfer of the proteins was verified with ponceau red staining.

Blotting buffer	10mM CAPS, pH 11
	10% (v/v) methanol
Ponceau solution	0.1% ponceau-S
	in 5% acetic acid

4.7.4 Immunodetection

After blotting, membranes were blocked for 1h in blocking buffer in order to avoid unspecific binding of the antibodies. Furthermore, the membranes were incubated with primary antibodies diluted in blocking buffer for 1h at RT. After washing three times with blocking buffer, the membranes were incubated for one hour with peroxidase-conjugated

secondary antibodies, diluted in blocking buffer. Finally, the membranes were washed three times with PBST and protein bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate system (Pierce, Rockford, USA). This system is based on the chemical reaction of luminol with peroxidase and hydrogen peroxide. The light emission emerging of this reaction is captured on film by autoradiography. The membrane was incubated in the ECL solution for 3min (Luminol:peroxide buffer, 1:1) and exposed to a hyperfilm ECL-film (Amersham Biosciences, Buckinghamshire, England) for an appropriate time.

Blocking buffer	10% casein in 1X PBST
PBST	10X PBS 0.1% Tween-20

4.8 Flow cytometry

FACS (fluorescence-activated cell sorter) is a technology that analyzes single cells according to different characteristics, such as size, granularity and density. Cells floating in a liquid are hit by a laser beam (argon ion laser, excitation at 488nm). For each cell passing, the light absorbance measured (forward scatter, shows the relative size) and reflected (side scatter, represents the relative density and is proportional to the cell granularity) is recorded. Moreover, different fluorescence wave lengths emitted by the cells can be quantified in different channels (FL1=537nm, FL2=597nm, FL3=650nm) of the flow cytometer. Evaluation of the obtained data was displayed by using histograms or x/y-z-parametric scatter plots. They show the fluorescence intensity versus the cell numbers. Flow cytometry analyses were performed with a FACScan cytometer (Becton Dickinson, Heidelberg, Germany) and later evaluated by the use of the CellQuest analysis software.

4.8.1 Measurement of apoptotic cells and cell viability

Apoptotic DNA fragmentation was determined on a single cell basis by measuring the DNA content of individual cells with a logarithmic amplification in the FL-3 channel of a FACScan flow cytometer equipped with the CellQuestPro software as described earlier (Daniel et al., 1999). $1x10^5$ cells were infected with the indicated adenoviral vectors and treated with 1mM caffeine or exposed to IR. At the indicated time points, cells were trypsinized and collected by centrifugation at 300*g* for 5 min, washed once with PBS and fixed in 2% formaldehyde solution for 30 min on ice. Next, cells were incubated in 70% ethanol/PBS. After 30 min on ice, the cells were resuspended in PBS containing 40 mg/ml DNase-free RNase A (Roche Molecular Biochemicals, Mannheim, Germany) for 30 min at 37°C. As a final step, cells were pelleted again and resuspended in PBS containing 50µg/ml propidium iodide (PI) and analyzed in the FL3-channel on a log scale. For cell viability measurements cells were collected by centrifugation and resuspended in 100µl PBS containing 50µg/ml PI, incubated 15min at RT, and analyzed by flow cytometry in the FL3-channel on a log scale.

4.8.2 Measurement of breakdown of the mitochondrial membrane potential

For detection of the breakdown of the mitochondrial membrane potential, cells were incubated with the fluorescent cationic and lipophilic dye JC-1: 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyaniniodide (Molecular Probes, Leiden, The Netherlands). The JC-1 dye accumulates in the matrix of intact mitochondria and has red fluorescence. Upon the onset of apoptosis, the mitochondrial potential collapses and the JC-1 dye can no longer accumulate in the mitochondria, therefore remains in the cytoplasm in a monomeric form and emits green fluorescence. Depolarization of mitochondria is therefore accompanied by increased green fluorescence and is measured in the FL-2 channel of the FACS.

$1x10^5$ cells were seeded in 6-well plates. After 24h, cells were infected with the indicated adenoviral vectors in the presence or absence of 1mM caffeine or IR and incubated with JC-1 at 2.5mg/ml in 500µl PBS for 30 min at 37°C. Cells were then centrifuged at 300*g*, washed twice with ice-cold PBS and resuspended in 200µl PBS. Mitochondrial permeability

transition was subsequently quantified by flow cytometry (Becton Dickinson; Heidelberg) in the FL3-channel gating on cells with decreased red fluorescence. Data are given in percentage of the cells with low mitochondrial membrane potential ($\Delta\Psi_m$).

4.8.3 Detection of caspase activation and inhibition

Caspase activity was measured for each caspase individually by using their specific fluorescent inhibitors (FLICA, Kit assay, Serotec, Oxford, England). These peptides bind covalently to cysteine residues and block the activity of the individual caspase. Caboxyfluorescein is attached to the peptide contributing the fluorescence.
For the measurement, $1x10^5$ cells were harvested 48h after adenoviral infection and resuspended in 500µl PBS with 30µM final concentration of the FITC-VAD-fmk, FITC-DEVD-fmk, or FITC-LEHD-fmk peptide. Samples were incubated for 20min at 37°C and washed 3 times with PBS. Finally, the cells were diluted in 200µl PBS. Increased green fluorescence was measured by FACS in the FL1-channel.
For inhibition of activated caspases, peptides with a specific sequence are used. They are bound by the active site of the corresponding caspase. (For caspase-3, -9, -8 20µM of inhibitor was used. To inhibit all the caspases, 20µM of zVAD-fmk peptide as well as 10µM Q-VD-OPh were used. All inhibitors were obtained from Calbiochem, Bad Soden.

4.8.4 Quantification of mitotic cells by MPM-2 staining

$1x10^5$cells were seeded in 6 well plates. After infection as described before, the cells were harvested by trypsination including floating cells. Cells were centrifuged at 1200rpm for 5 min. Cell pellets were resuspended in 1ml of 1xPBS. After additional centrifugation, the supernatant was removed and cells were fixed with 200µl of ice-cold 70% ethanol and transferred into 96 well round bottom plates. The plates were centrifuged at 300*g* for 5 min and then washed with 1xPBS + 0.05% Tween 20 + 10% Fetal Bovine Serum (PBSTF). After this step, the cells were labelled with the mouse MPM2 antibody (Upstate Biotechnology, USA) with a final concentration of 1µg/ml, diluted in PBSTF and incubated for 1h at 37°C. The cells were washed two times with PBSTF and incubated with the secondary, FITC labelled goat-anti mouse antibody (Jackson Immuno Research, USA) for 1

hour at RT in the dark. The cells were washed 2 times with PBSTF and resuspended in 50µg/ml PI + 40µg/ml RNAse. The cells were measured for increased green fluorescence showing MPM-2 positivity in combination with a 4N (mitotic) DNA content.

4.9 Measurement of cell cycle distribution

$1x10^5$ cells were grown in 6 well plates, cultured over night and infected with adenoviral vectors in serum free medium. After that, cells were trypsinized and centrifuged for 5 min. The cell pellet was washed with PBS and fixed with 70% ethanol and stored at -20°C over night. Thereafter, cells were treated with pepsin, at 37°C for 30 min. After discarding the pepsin, cells were resuspended in 2M HCl and incubated at 37°C for 30 min, and finally washed with borax buffer to neutralize the acid. The cells were pelleted again and resuspended with PBS containing 50µg/ml PI. The cellular DNA content was measured with linear amplification in the FL-2 channel. Data were analyzed with the ModFit software (Verity Software House, Topsham, USA), which calculates the percentages of cells in each phase of the cell cycle.

Pepsin	0.4 mg/ml in 0.1M HCl
Borax buffer	0.1M sodium tetraborate pH 8.5

4.10 Immunofluorescence microscopy

$1x10^5$cells were grown in 6 well plates seeded on sterile round coverslips. After 24h, the cells were infected with adenoviral vectors Ad-lacZ or Ad-$p14^{ARF}$ and treated with 1mM caffeine. After 48h, the cells were washed with PBS and fixed with 3% PFA for 20 min at RT. After that, cells were permeabilized in 0.2% Triton X-100 in PBS for 3 min, and the slides were incubated with blocking solution 30min and incubated overnight with an anti-PARP p85 fragment antibody (Promega, Mannheim, Germany) diluted 1:100. After three washes with PBS, the slides were incubated with the secondary antibody diluted 1:1000 (Alexa Fluor 594–conjugated goat anti–rabbit IgG (Invitrogen) for 1h at RT. For staining of DNA, cells were incubated in 1XPBS + Hoechst (Sigma-Aldrich) at a final concentration of 1µg/ml for 2min. Cells were washed three times with 1xPBS and mounted on slides with fluorescence mounting medium (DakoCytomation). For overview images, specimens were

analyzed with fluorescent microscope at RT using a microscope (Axiovert 200; Carl Zeiss, Inc.) equipped with a 63×/1.4 objective lens (Plan-Apochromat; Carl Zeiss, Inc.) and a digital camera (ORCA ER; Hamamatsu Photonics). Images were obtained by Openlab software (Improvision).

3% PFA	22.5ml aqua dest.
	0.75g paraformaldehyde
	2.5 ml 10X PBS

4.11 Analysis of Bax clustering and LC3 punctuation

Bax oligomerization and LC3 punctuation was determined by the use of a fluorescence microscope. MCF-7 cells, stably expressing EGFP-Bax or EGFP-LC3 were seeded $5x10^4$ on a 16-mm round cover slide (VWR, Darmstadt, Germany) in 12-well plates. Treated cells were analyzed with fluorescent microscope at RT using a microscope (Axiovert 200; Carl Zeiss, Inc.) equipped with a 63×/1.4 objective lens (Plan-Apochromat; Carl Zeiss, Inc.) and a digital camera (ORCA ER; Hamamatsu Photonics). Images were obtained by Openlab software (Improvision).

4.12 Gamma-irradiation

$1x10^5$ cells were seeded in 6-well plates, and after adenoviral transduction, cells were irradiated with 10Gy ionizing irradiation in a Cesium-137 irradiator. Cells were cultured at 37°C and harvested at different time points.

RESULTS

5. Results

5.1 Dose response for overexpression of p14^{ARF}

Resistance of breast cancer tumour cells to chemotherapy or radiotherapy remains a significant challenge for successful cancer therapy. The p14^{ARF} pathway has been thoroughly investigated in order to be employed in the future for effective treatments. P14^{ARF} has been shown to induce apoptotic cell death in prostate cancer cell lines (Hemmati et al., 2002). To characterize cell death signalling by p14^{ARF}, we used MCF-7 breast carcinoma cells that have lost the key executioner caspase-3 and MCF-7 cells that were stably re-expressing procaspase-3. Western blot analyses of untreated MCF-7 cells confirmed caspase-3 deficiency of MCF-7 mock cells in contrast to cell transfectants stably re-expressing procaspase-3, where strong expression was detectable (figure 9). Off note: MCF-7 cells do not express endogenous p14^{ARF} since their INK4a gene is homozygously deleted (Pineau et al., 2003).

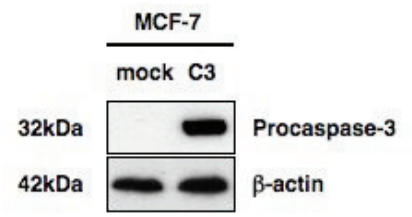

Figure 9. Expression of procaspase-3 in MCF-7 breast carcinoma cells.
Western blot analysis of MCF-7 cells stably re-transfected with procaspase-3. Equal loading was confirmed by β-actin detection.

An adenoviral vector system was employed for transient expression of p14^{ARF} in both cell transfectants. MCF-7 cells proficient or deficient for caspase-3 were transduced with an adenoviral vector containing p14^{ARF} under the control of a CMV promoter. To confirm the functionality of the viral system, both cell transfectants were infected with increasing levels of MOI (multiplicity of infection), ranging from 1 to 100 infectious viral particles of Ad-p14^{ARF} (figure 10A). Western blot analysis after 24h validated increased expression of p14^{ARF} in a dose-dependent manner.

After transduction of MCF-7 cells with Ad-p14^{ARF}, apoptotic cell death was measured in the following experiment. As controls for MCF-7 cells, caspase-3 deficient or proficient cells were cultured in medium or infected with Ad-lacZ and apoptosis was measured after 72h (figure 10B). All control MCF-7 mock transfectants as well as cells infected with 10 MOI of Ad-p14^{ARF} showed an apoptotic rate of 2-5%. However, when the mock cells were infected

with increasing amounts of MOIs of Ad-p14^{ARF} (25, 50 and 100 MOI), apoptotic cell death occurred in less than 10% of the cells. The apoptotic rate was low (4%) in the control MCF-7 cells, which were proficient for caspase-3. Nevertheless, cells demonstrated a low apoptotic rate of 8% after infection with 10 MOI of Ad-p14^{ARF}, which was slightly increased up to 16 % in a dose dependent manner.

Surprisingly, neither MCF-7 caspase-3 deficient nor proficient cells showed prominent signs of p14^{ARF}-induced apoptosis.

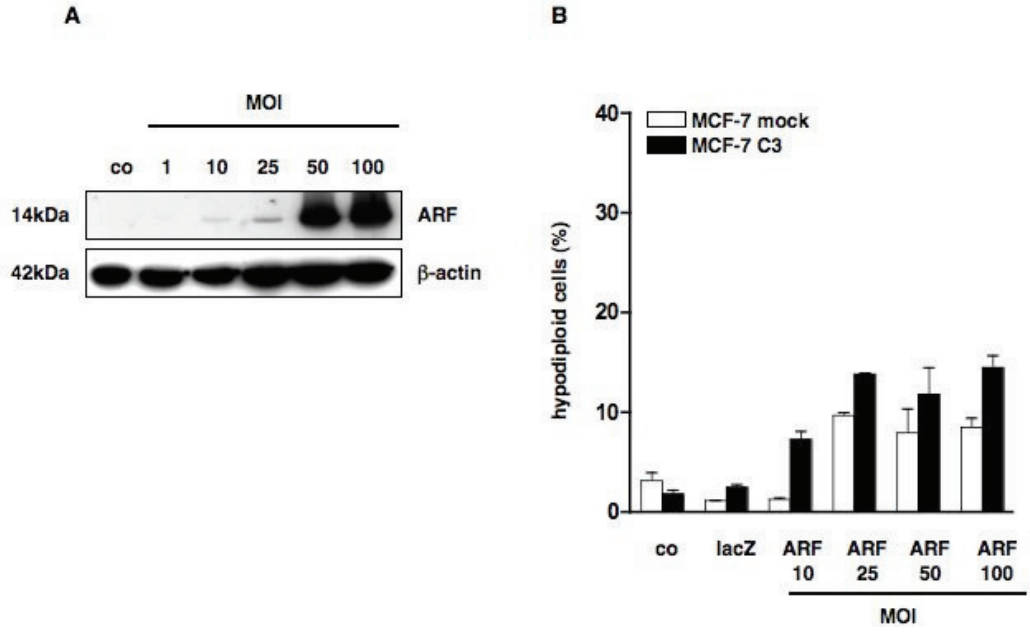

Figure 10. Adenovirus mediated overexpression of p14^{ARF}.
(A) MCF-7 caspase-3 deficient cells were infected with different MOIs of adenovirus as indicated on the Western blot. β-actin was used as a loading control.
(B) MCF-7 cells deficient (white bars) or proficient (black bars) for caspase-3 were infected with 100 MOI of Ad-lacZ (lacZ) or different MOIs of Ad-p14^{ARF} (ARF) as indicated. Determination of cells with hypodiploid DNA content was done by flow cytometric measurement after 72h on a single cell level. Data are means ± SD of triplicates.

5.2 P14^{ARF} localizes to the nucleoli

To verify the localization of p14^{ARF} and to confirm the transfection rate in MCF-7 cells, both cell transfectants were transduced with Ad-p14^{ARF} and stained with a fluorescein-conjugated antibody to p14^{ARF} (figure 11). In addition, mitochondria were stained with Mito Tracker Red thereby allowing the localization of p14^{ARF} as an overlay. Nuclei were stained with Hoechst (visible in blue) showing the absence of chromatin condensation after p14^{ARF}

infections. As can be seen in the microscopic images, the $p14^{ARF}$ protein is clearly localized within the nucleoli, and not co-localized with the mitochondria (figure 11). After infection with 100 MOI of Ad-$p14^{ARF}$ the average transfection rate was 70%, calculated from the reduction in green fluorescence. Therefore, 100 MOI was used as the optimal infection rate in the following experiments for both, MCF-7 mock and caspase-3 cell transfectants.

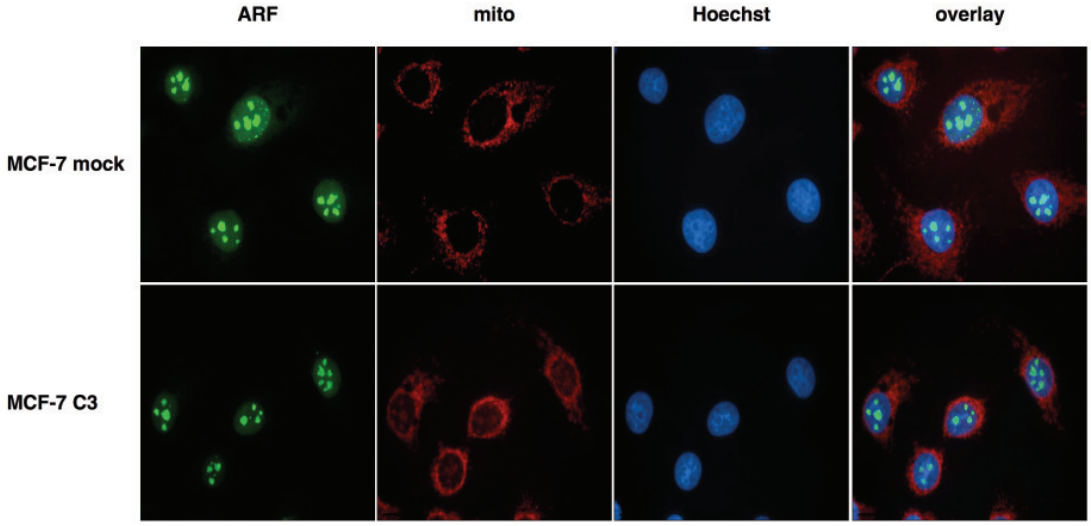

Figure 11. Subcellular $p14^{ARF}$ localization.
Immunofluorescent pictures were taken 24h after transduction with 100 MOI of Ad-$p14^{ARF}$. $P14^{ARF}$ shows nuclear and nucleolar localization. $P14^{ARF}$ was stained in green, mitochondria in red, and nuclei in blue.

5.3 Involvement of $p14^{ARF}$ in cell cycle checkpoint regulation

5.3.1 $P14^{ARF}$ mediates cell cycle arrest via upregulation of p21 in MCF-7 cells

To establish the role of $p14^{ARF}$ in cell cycle arrest and DNA damage, the expression levels of the regulatory proteins were determined by Western blot analysis. Caspase-3 deficient and caspase-3 proficient MCF-7 cells were infected with Ad-lacZ (control virus) or with Ad-$p14^{ARF}$. As shown in figure 12, expression of $p14^{ARF}$ induced an upregulation of p53, a tumour suppressor protein participating in cell cycle arrest and apoptosis, as well as the phosphorylated form of this protein, illustrating its activity. The level of p21 was also elevated upon $p14^{ARF}$ expression indicating transcriptional activity of p53. When DNA is damaged, γH2AX becomes extensively phosphorylated and forms nuclear foci at the damaged site; therefore, this protein was used as a marker for DNA damage. Upregulation

of γH2AX indicated that DNA damage occurred in both, caspase-3 deficient and proficient cells, after $p14^{ARF}$ expression. Cells infected with control virus Ad-lacZ did not show detectable levels of any of the examined proteins.

Furthermore, the expression level of the proapoptotic protein Bax increased after $p14^{ARF}$ expression in both cell transfectants, in contrast to the control cells where its level was barely detectable. Apoptotic cell death was not induced in the caspase-3 proficient cells as no cleavage product of the main executive caspase-3 could be monitored upon $p14^{ARF}$ expression. The levels of proapoptotic Bak and antiapoptotic Bcl-2 did not differ among the cells infected with Ad-lacZ or Ad-$p14^{ARF}$.

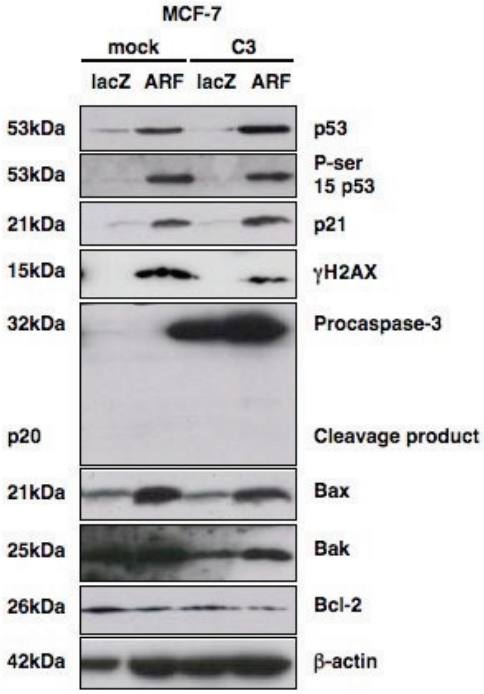

Figure 12. P14^{ARF} signalling pathway and induction of cell cycle arrest in MCF-7 cells.

MCF-7 caspase-3 proficient or deficient cells were transduced with Ad-p14^{ARF} (100 MOI) or Ad-lacZ (100 MOI) and cultured for 48h. Protein expression was determined by appropriate antibody as indicated on the Western blot analysis. Equal loading was confirmed by β-actin detection.

5.3.2 P14^{ARF} induces cell cycle arrest

Each cell cycle phase is regulated by specific key regulatory cyclins and cyclin-dependent kinases. Since they are good markers for the cell cycle changes, their expression level were examined by Western blot analysis (figure 13). Caffeine, an inhibitor of the cell cycle kinases ATM and ATR, has been shown to overcome cell cycle arrest programs and was therefore used in the following experiments to study the functional consequences of p14^{ARF}-induced cell cycle arrest.

As controls, MCF-7 cells, which were either proficient or deficient for caspase-3, were infected with the control virus Ad-lacZ, exposed to caffeine, or were left un-treated, respectively. The expression of p14^{ARF} after transduction with Ad-p14^{ARF} was similar for both cell transfectants. The levels of p53 and p21 were undetectable in all control cells, independent of the presence of caspase-3. However, for both cell transfectants p53 expression was strongly increased after p14^{ARF} expression in the presence or absence of caffeine. In addition, the level of p21 was also elevated after p14^{ARF} expression, however only in the absence of caffeine; this increase was not detected in the presence of caffeine. All control MCF-7 mock or caspase-3 proficient cells demonstrated high levels of cyclin D1 after infection with Ad-p14^{ARF}. However, treatment with caffeine resulted in a downregulation of the cyclin D1 expression level. The same effect was evident for the cyclin dependent kinases CDK4 and CDK6. Furthermore, all control cells expressed cyclin A independently of the presence of caspase-3. However, the expression level of cyclin A was drastically reduced after infection with Ad-p14^{ARF} or the addition of caffeine. Upregulation of cyclin B1 was only detectable after expression of p14^{ARF} and exposure to caffeine in contrast to control cells suggesting that those cells were in the G2/M phase of the cell cycle.

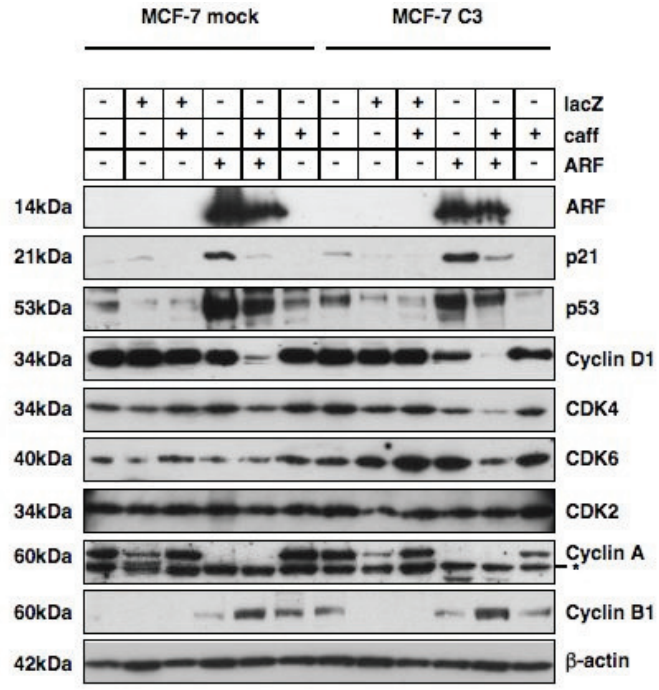

Figure 13. Western blot analysis of cyclins and CDKs after p14^{ARF} expression and treatment with caffeine.
MCF-7 cells deficient and proficient for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ, treated with caffeine (caff) (1mM) and cultured for 48h. Proteins were detected by the appropriate antibodies as indicated on the Western blot. Asterix (*): unspecific band. Equal loading was confirmed by β-actin.

The data obtained in the previous experiment did not clearly differentiate the G2 from the M phase cells. For a more precise analysis of the G2/M accumulation, a specific mitotic marker was used. MPM-2 is a common phosphorylated epitope used for distinguishing proteins that are selectively phosphorylated only during mitosis. After fixation and permeabilization, cells were incubated with a MPM-2 antibody (figure 14). Cells entering mitosis were detected and quantified by flow cytometric analysis. In the untreated controls, approximately 4% of MCF-7 cells deficient or proficient for caspase-3 were stained positively with the MPM-2 antibody indicating a mitotic state. For both cell transfectants, a minor decrease in cell staining was observed when the cells were transduced with the control virus Ad-lacZ or treated with caffeine. However, overexpression of p14^{ARF} induced a decrease in the number of cells staining positively for MPM2, and an even stronger decrease was seen after additional treatment with caffeine indicating mitotic reduction where no cells were entering the M-phase. These results were observed in MCF-7 mock cells as well as caspase-3 transfected cells. Nocodazole, a microtubule-depolymerising agent, was used as a positive control to arrests cells in mitosis. MPM-2 staining was observed in 40% of both

MCF-7 cell transfectants after nocodazole treatment, demonstrating the functionality of this method.

These results demonstrate that $p14^{ARF}$ induces cell cycle arrest in the G1 phase of the cell independently of the presence or absence of caspase-3. This arrest was impaired by the use of caffeine, causing cell cycle progression and mitotic exit. Nevertheless, cells only overexpressing $p14^{ARF}$ and exposed to caffeine are most likely accumulated in G2 phase of the cell cycle since no cells were found in M-phase.

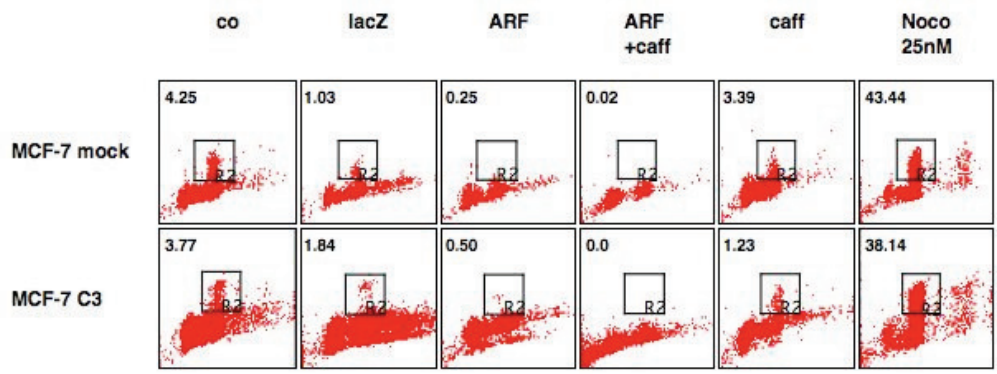

Figure 14. Quantification of MCF-7 cells in mitosis by MPM-2 staining.
MCF-7 cells deficient and proficient for caspase-3 were transduced with Ad-$p14^{ARF}$ or Ad-lacZ and treated with caffeine (1mM) as indicated. Bi-variate analyses of DNA content (PI) and MPM-2 positive staining for identification of 4N (mitotic) cells was performed by flow cytometry after 48h. Nocodazole (Noco) known as a microtubule-depolymerising agent was used to arrest cells in mitosis (100nM/ml) and served as a positive control. Data are means ± SD of triplicates.

Using the cervical cancer cell line HeLa that express high-risk human papillomavirus HPV E6, which functionally inactivates p53 (Howley, 2006), the effect of $p14^{ARF}$ on the cell cycle was further analyzed. Infection with the control virus Ad-lacZ showed 60% of cells in G1 phase and 25% in S phase, respectively (figure 15). Upon $p14^{ARF}$ expression 12% of the cells were found in G1 phase and not more than 3% of the cells were in S phase. HeLa cells infected with the control virus Ad-lacZ showed 18% of cell accumulation in G2 whereas, interestingly, 78% of $p14^{ARF}$ overexpressing cells were accumulated in G2/M phase.

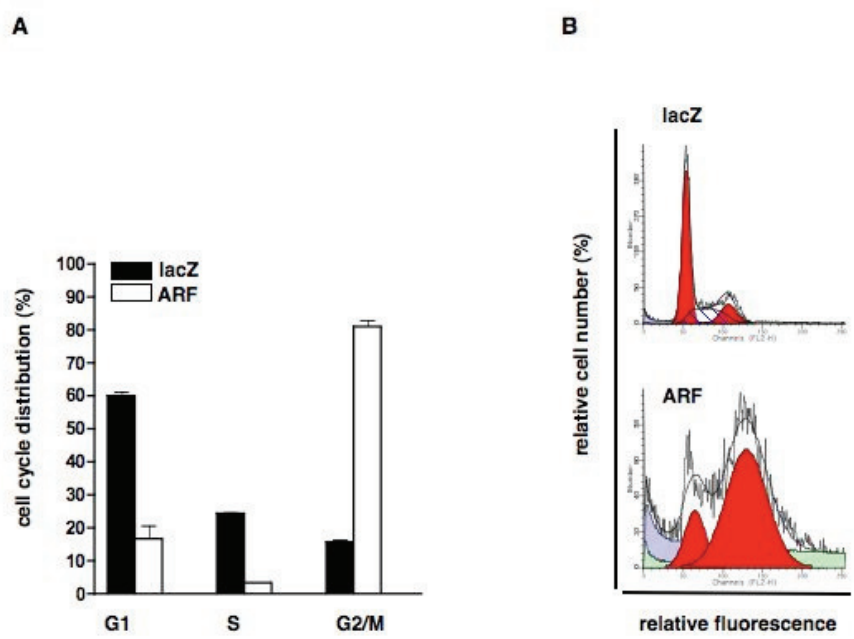

Figure 15. P14^{ARF} induces G2/M phase arrest in HeLa cells.
Cells were infected with 50 MOI of Ad-lacZ or Ad-p14^{ARF} and subjected to cell cycle flow cytometry measurements.
(A) Cell cycle distribution of G1, S, G2/M phases. Data are means ± SD of triplicates.
(B) Representative histograms of the measurements in (A).

Cell cycle arrest analyses in MCF-7 and HeLa cells suggest that p14^{ARF} induces the cell cycle arrest program either by accumulating cells in G1 or G2 which seems to be dependent on the functional p53 status of the cell.

5.4 P14^{ARF} is involved in type I cell death after sensitization with caffeine

5.4.1 Apoptotic response of p14^{ARF} after exposure to caffeine strictly depends on the presence of caspase-3

To determine whether cell cycle abrogation can influence apoptotic cell death in the applied experimental system, MCF-7 mock cells for caspase-3 and MCF-7 procaspase-3 transfectants were transduced with 100 MOI of Ad-14^{ARF} and treated with two different concentration of caffeine (1mM-2mM) (figure 16A,B). As a control, both cell transfectants were not treated, infected with control virus Ad-lacZ or exposed to different concentrations of caffeine. Apoptotic DNA fragmentation was measured after 72h by flow cytometry. For both cell transfectants, 2% to 5% of the medium control as well as cells transduced with Ad-lacZ demonstrated apoptotic death. After treatment with only caffeine, 4% of MCF-7 mock or caspase-3 proficient cells showed apoptosis induction regardless of the used concentration. MCF-7 mock cells transfected with Ad-lacZ and under caffeine treatment remained unaffected in terms of apoptotic cell death independently of the caffeine concentration. After transduction with the control virus Ad-lacZ and additionally treatment with 1mM and 2mM caffeine, respectively, the lower concentration of caffeine did not have any effect on cell death in MCF-7 caspase-3 proficient cells. Nevertheless, 2mM of caffeine caused a slight increase in the apoptotic rate up to 18% in the Ad-lacZ transduced control cultures. However, after overexpression of p14^{ARF} and additional treatment with caffeine MCF-7 caspase-3 transfected cells demonstrated apoptotic cell death that increased in a dose-dependent manner. Namely, 49% of MCF-7 caspase-3 proficient cells displayed apoptotic cell death upon p14^{ARF} expression in combination with 1mM caffeine. This effect was increased even more in the presence of 2mM caffeine where the apoptotic rate was raised to 59%. In contrast, cells deficient for caspase-3 showed not more than 10% apoptotic cells regardless of the caffeine concentration. In respect to these results, a concentration of 1mM caffeine was chosen for further experiments.

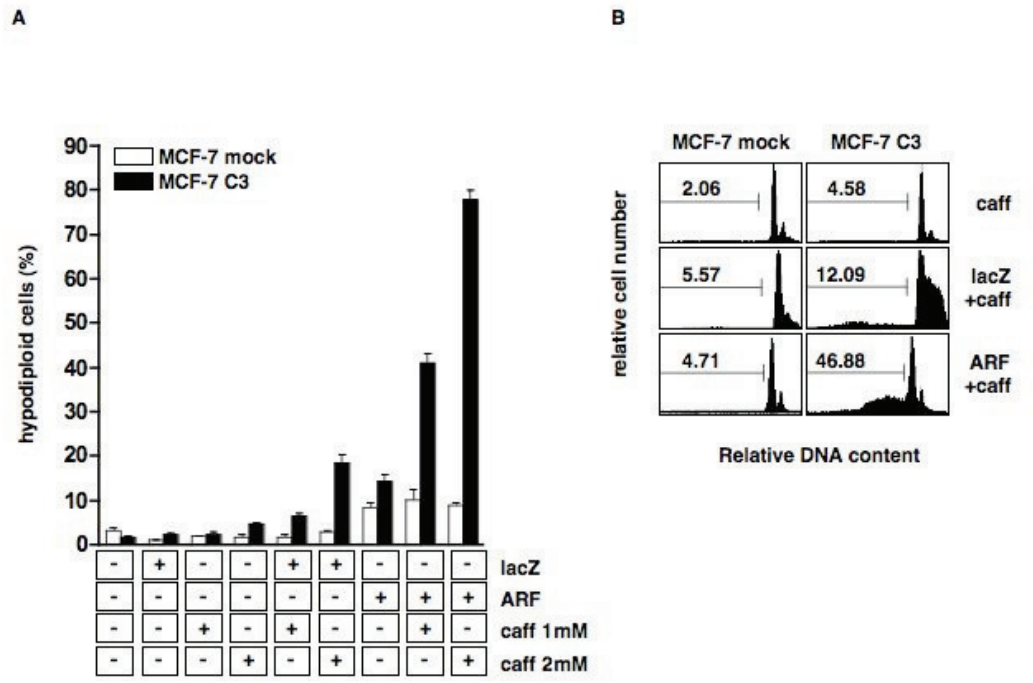

Figure 16. Dose response for treatment with caffeine and apoptosis induction upon p14^{ARF} expression.
(A) Cells were infected with Ad-lacZ or Ad-p14^{ARF} and treated with different concentrations of caffeine (1-2mM). Apoptotic DNA fragmentation was measured by flow cytometry after 72h. MCF-7 cells deficient (white bars) and proficient (black bars) for caspase-3 were analysed. Data are means ± SD of triplicates.
(B) Representative histograms of flow cytometry measurements. Cells were infected with 100 MOI of Ad-lacZ or Ad-p14^{ARF} and additionally treated with or without caffeine (1mM).

In order to provide better evidence for the role of 14^{ARF} in apoptotic cell death, we analyzed the time dependent effect in the same cell system (figure 17). Different time points were chosen, i.e. 48, 72 and 96, respectively. As control, cells were cultured untreated, transduced with Ad-lacZ or additionally treated with caffeine. After 48h, up to 5% of caspase-3 deficient cells demonstrated apoptotic cell death in the controls as well as when these cells were transduced with Ad-14^{ARF} and treated with caffeine. Caspase-3 proficient cells did not show more than 4% apoptotic cell death in all the treatments except when these cells were transduced with p14^{ARF} and exposed to caffeine demonstrating an increase in the apoptotic rate up to 11% (figure 17A). After 72h, neither caspase-3 deficient nor caspase-3 proficient cells showed increased apoptotic cell death when they were left untreated, transduced with Ad-lacZ or additionally treated with caffeine (figure 17B). 12% of the cells showed apoptotic cell death upon p14^{ARF} expression in both cell transfectants. Control cells did not show more than 5% apoptotic cell death for both cell transfectants except of a slight increase in the apoptosis induction of 19% in caspase-3 proficient cells when infected with Ad-lacZ

in combination with caffeine (figure 17C). 17% of the cells demonstrated apoptotic cell death when infected with Ad-p14^{ARF} after 96h. However, after 72h, MCF-7 caspase-3 re-expressing cells showed 49% of apoptotic DNA fragmentation when transduced with Ad-p14^{ARF} and additionally treated with caffeine, increasing to 65% after 96h. Culturing Ad-p14^{ARF} infected MCF-7 mock cells for 96h in the presence of caffeine did not induce more than 10% of apoptosis.

The consequence of sensitization with caffeine for p14^{ARF} resulted in apoptosis starting shortly after 48h that increased in a time dependent manner strictly depending on the presence of caspase-3.

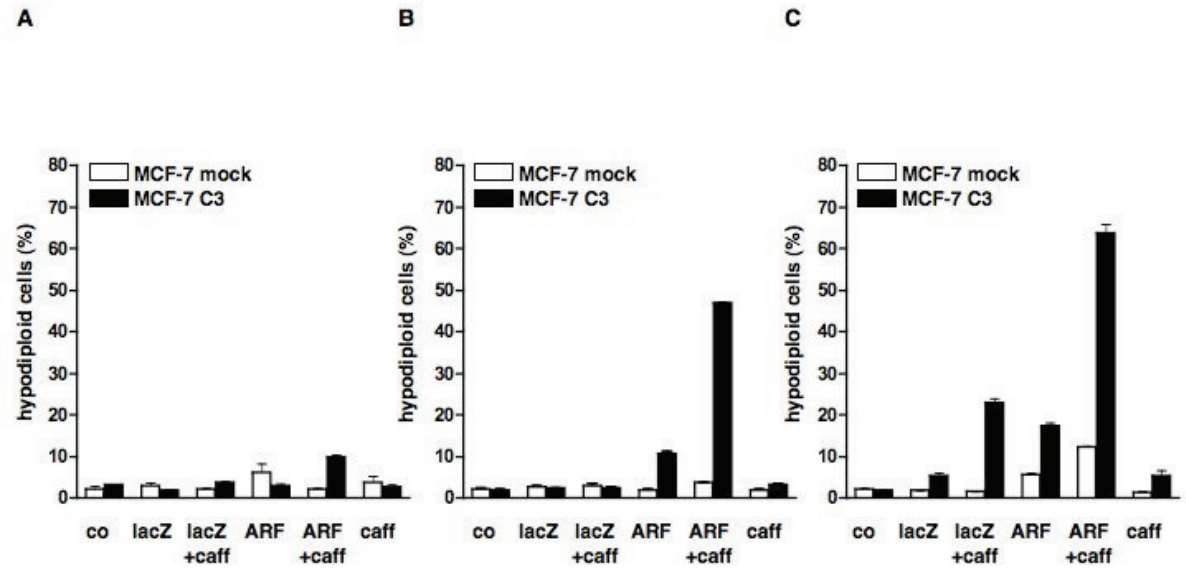

Figure 17. P14^{ARF} induces apoptosis after caffeine treatment that strictly depends on caspase-3.
MCF-7 cells deficient (white bars) and proficient (black bars) for caspase-3 were transduced with Ad-14^{ARF} or Ad-lacZ and treated with caffeine (1mM) as indicated.
(A) DNA fragmentation was measured by flow cytometry after 48h.
(B) DNA fragmentation was measured by flow cytometry after 72h.
(C) DNA fragmentation was measured by flow cytometry after 96h.
Data are means ± SD of triplicates.

Moreover, caspase-3 dependency was also shown in the cervical carcinoma cell line HeLa, which is wild type for caspase-3 (figure 18). HeLa cells transduced with control virus Ad-lacZ regardless of the presence of caffeine showed up to 8% of background apoptosis. Upon p14^{ARF} expression 10% of the cells displayed apoptotic cell death. However, 52% of the cells showed apoptosis induction upon infection with p14^{ARF} and caffeine exposure.

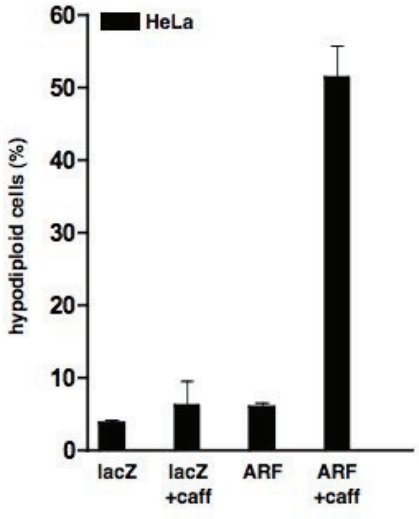

Figure 18. P14^{ARF} overexpression induced apoptosis after caffeine treatment in HeLa cells.
HeLa cells were transduced with 50 MOI Ad-p14^{ARF} or Ad-lacZ and treated with caffeine (1mM). DNA fragmentation was measured by flow cytometry after 72h. Data are means ± SD of triplicates.

The observation made in MCF-7 cells as well in HeLa cell lines leads to the conclusion that p14^{ARF} triggers cell cycle arrest that interferes with apoptosis induction. Thus, apoptosis induction might serve as a fail-save mechanism to eliminate checkpoint deficient tumour cells upon induction of oncogenic stress by p14^{ARF}. The decision of whether the cells are arrested in G1 or G2 is dependent on the p53 status, indicating that in the presence of functional p53 cells are arrested in G1 whereas lack of functional p53 results in G2 arrest upon p14^{ARF} expression. Cells expressing p14^{ARF} in combination with caffeine-mediated abrogation of cell cycle checkpoints would then die through apoptosis, dependent on caspase-3, but independent on the functionality of p53.

5.4.2 Mitochondrial permeabilization upon $p14^{ARF}$ overexpression after exposure to caffeine

Mitochondria play a central role in propagation of cell death signals. Upon apoptotic stimuli, there is release of cytochrome c, which activates caspase-9 via the apoptosome, and subsequently caspase-3. As an early event in apoptosis, mitochondria loose their membrane potential. In order to investigate if mitochondria are involved in $p14^{ARF}$ mediated cell death pathway, MCF-7 cells were incubated with the fluorescent dye JC-1 and analyzed by flow cytometry. Accumulated in the mitochondria of healthy cells as aggregates, has red fluorescence. Upon apoptosis induction, the mitochondrial potential collapses and the JC-1 change its fluorescent emittance from red light to green light. Controls cell transfectants were either cultured in medium, or infected with control virus Ad-lacZ in the presence or absence of caffeine. None of the controls showed breakdown of the mitochondrial membrane potential measured after 48h and 72h, respectively (figure 19). In parallel, cells infected with Ad-$p14^{ARF}$ alone did not show disruption of mitochondria independently of the presence of caspase-3. In contrast, cells re-expressing caspase-3 displayed a high reduction of the mitochondrial membrane potential after infection with Ad-$p14^{ARF}$ and exposure to caffeine, occurring in a time dependent manner. Namely, 46% of the cells showed depolarization of the mitochondria after 48h, increasing up to 72% after 72h. After 48h, 12% of the cells lacking caspase-3 revealed loss of mitochondrial membrane potential upon $p14^{ARF}$ overexpression in combination with caffeine (figure 19A). Interestingly, elevated mitochondrial membrane permeabilization was measured in 40% of those cells after 72h (figure 19C). These data suggest that cells deficient for caspase-3 had mitochondrial dysfunction without demonstrating apoptotic cell death after infection with Ad-$p14^{ARF}$ and incubation with caffeine.

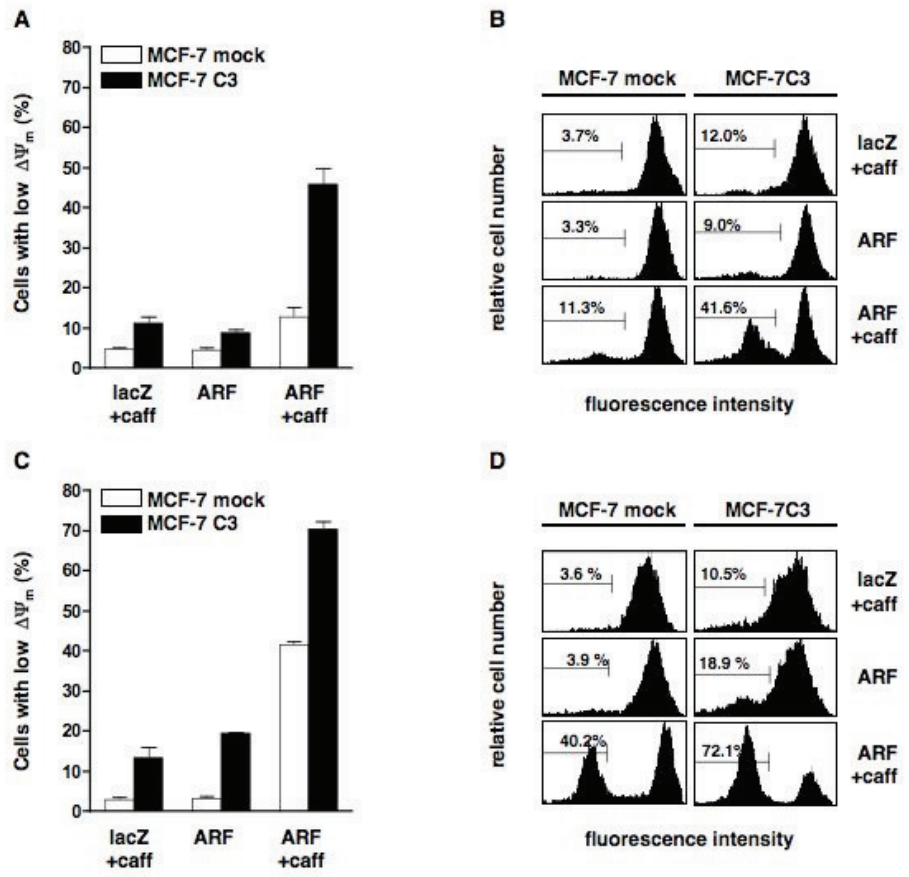

Figure 19. Loss of the mitochondrial membrane potential upon p14^{ARF} overexpression and caffeine treatment.
(A) MCF-7 cells deficient (white bars) and proficient (black bars) for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ. Flow cytometric detection of cells with low mitochondrial membrane potential was performed after 48h by use of the cationic and lipophilic dye JC-1. Data are means ± SD of triplicates of cells with $\Delta\Psi_m$ loss.
(B) Representative histograms of experiment in (A).
(C) MCF-7 cells deficient (white bars) and proficient (black bars) for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ. Flow cytometric detection of cells with low mitochondrial membrane potential was performed after 72h. Data are means ± SD of triplicates of cells with $\Delta\Psi_m$ loss.
(D) Representative histograms of experiment in (C).

In order to analyze if caspases are involved in p14^{ARF} pathway and whether preventing caspase activation can protect the mitochondrial permeabilization, the pan-caspase inhibitor (Q-VD-OPh) was employed. MCF-7 mock and caspase-3 cell transfectants were infected with Ad-lacZ in combination with caffeine and cultured in the presence or absence of the caspase inhibitor, respectively. These control cells did not show loss of the mitochondrial membrane permeability. After 72h, both cell transfectants demonstrated loss of mitochondrial membrane potential upon p14^{ARF} overexpression in combination with caffeine. This effect on mitochondria was blocked after additional exposure to Q-VD-OPh.

Namely, 5% of the caspase-3 deficient cells and 20% of the caspase-3 proficient cells were detected with low mitochondrial membrane potential (figure 20). These data imply that the complete inhibition of caspases can block mitochondrial depolarization regardless of the presence of caspase-3, indicating that caspase activities occur upstream of the mitochondrial depolarisation, which might be a crucial step in $p14^{ARF}$-induced apoptosis.

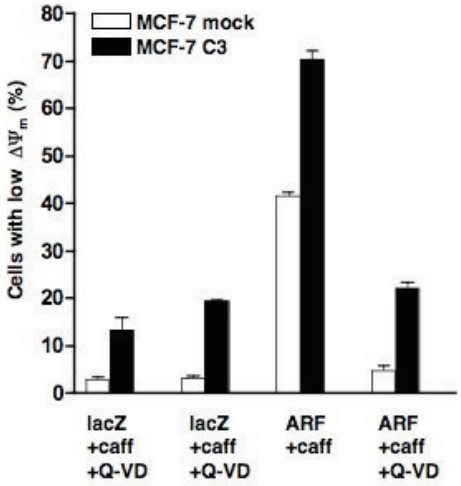

Figure 20. $P14^{ARF}$ expression and caffeine treatment induces loss of the mitochondrial membrane potential, which can be blocked by use of the pan-caspase inhibitor Q-VD-OPh. *MCF-7 cells deficient (white bars) and proficient (black bars) for caspase-3 were transduced with Ad-$p14^{ARF}$ or Ad-lacZ. Flow cytometric detection of cells with low mitochondrial membrane potential was performed after 72h in the presence or absence of the pan-caspase inhibitor Q-VD-OPh (Q-VD) as indicated. Data are means ± SD of triplicates.*

5.4.3 PARP cleavage as evidence for apoptosis

PARP (Poly-(ADP-ribose)-polymerase), an 116kDa protein, is a typical substrate of caspase-3 and is cleaved during apoptosis into an 85kDa and a 32kDa fragment. PARP cleavage is therefore a convenient marker for type I cell death. To confirm that $p14^{ARF}$, in combination with caffeine, induces apoptosis in a caspase-3 dependent manner, cells were stained with a red fluorescent antibody that detects specifically the cleaved form of PARP. Nuclei were stained in blue with Hoechst. MCF-7 cells that have lost the key executioner caspase-3 and MCF-7 cells that were stably re-transfected with procaspase-3 were infected with Ad-$p14^{ARF}$ and treated with caffeine (figure 21). In parallel, cells were infected with control virus Ad-lacZ and cultured with caffeine. After 72h pictures were taken with a fluorescence microscope. Regardless of the treatment, none of caspase-3 deficient cells displayed the cleaved form of PARP. MCF-7 caspase-3 proficient cells demonstrated PARP cleavage only when infected with Ad-$p14^{ARF}$ and treated with caffeine, revealing that caspase-3 is essential in this pathway.

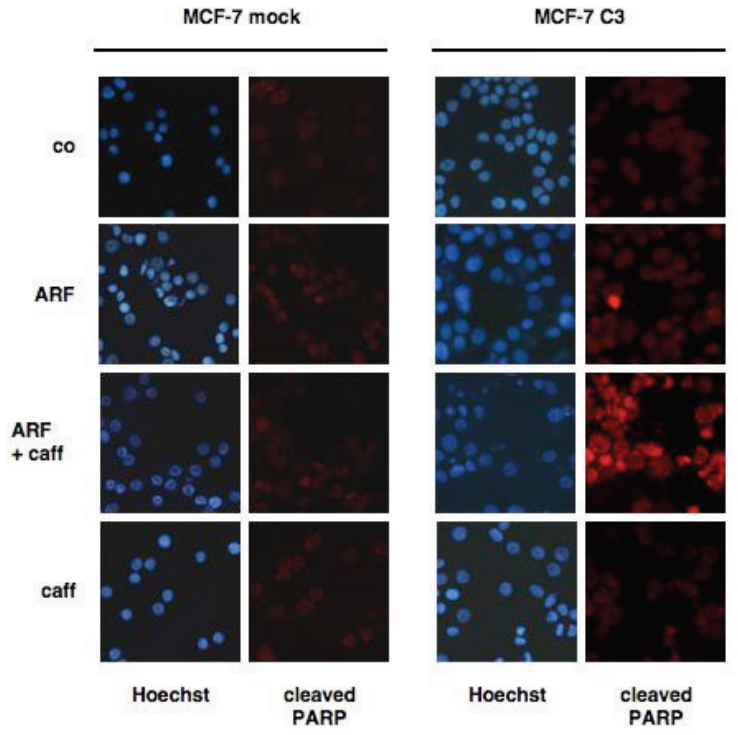

Figure 21. PARP cleavage upon p14^{ARF} overexpression and caffeine treatment.
MCF-7 cells deficient and proficient for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ and treated with caffeine (1mM) as indicated. PARP cleavage was detected as red staining, and nuclei were stained in blue. Representative pictures were taken after 72h.

5.4.4 Activation of caspases upon p14^{ARF} overexpression and exposure to caffeine

The following studies were addressed to investigate involvement of caspases in the intrinsic pathway that are induced by p14^{ARF} after sensitization with caffeine. The activation of two main caspases was investigated, the initiator caspase-9, a LEHDase, and the executioner caspase-3, a DEVDase (figure 22). Both cell transfectants were incubated with cell permeable FITC-labelled tetrapeptide as a substrate for the corresponding activated caspase. The increase of fluorescent light intensity directly correlates to the activation of caspases. As controls, both cell transfectants were not transduced or transduced with the lacZ control adenovirus with or without caffeine or only cultured in the presence of caffeine. These cells showed only a background increase of caspase-9 or caspase3/-7 activity in both cell transfectants. Upon expression of p14^{ARF} 17% of caspase-3 proficient cells and 8% caspase-3 deficient cells showed caspase-9 activation i.e. binding of the fluorescent LEHD peptide. 61% of caspase-3 proficient cells presented elevated levels of caspase-9 activation upon p14^{ARF} expression and caffeine treatment (figure 22A). In contrast, caspase-9 activation was

detected in only 21% of mock transfectants upon the same treatment. When cells were infected with Ad-p14^{ARF} approximately 10% of both cell transfectants showed activation of caspases-3 and -7 (figure 22B). 40% of caspase-3 proficient cells displayed caspase-3/-7 activity in contrast to mock transfectants, which demonstrated only 10% of cells with active caspase-7 upon p14^{ARF} expression combined with caffeine treatment.

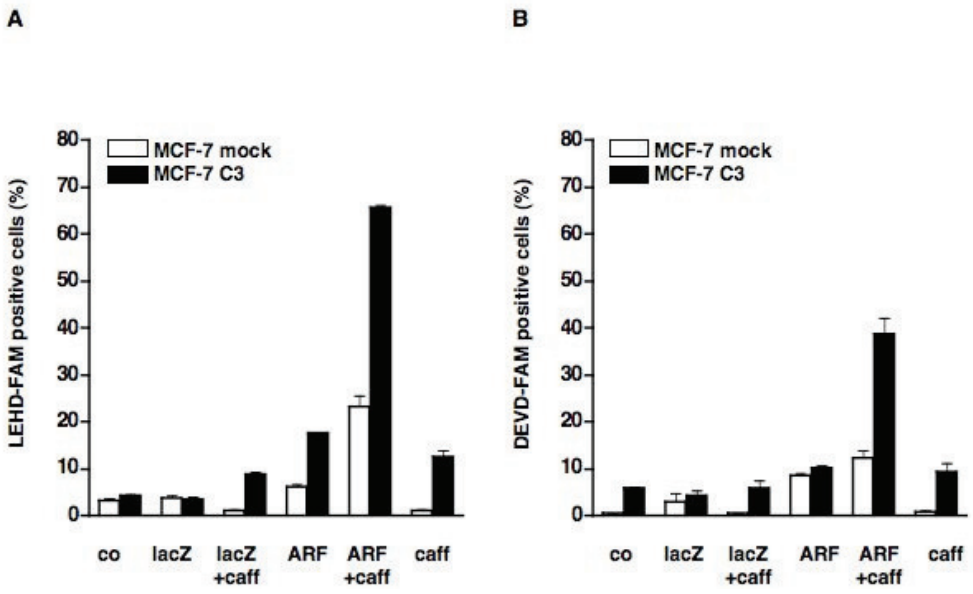

Figure 22. Activation of the initiator caspase-9 (LEHDase) and effector caspase-3 (DEVDase) upon p14^{ARF} expression and caffeine treatment.
MCF-7 cells were transduced with Ad-p14^{ARF} or Ad-lacZ and treated with caffeine (1mM) as indicated.
(A) MCF-7 cells deficient (white bars) or proficient (black bars) for caspase-3 were incubated with a carboxyfluorescein labelled peptide specific for active LEHDases, i.e. caspase-9.
(B) MCF-7 cells deficient (white bars) or proficient (black bars) for caspase-3 were incubated with a carboxyfluorescein labelled peptide specific for active DEVDases, i.e. caspase-3 and -7.
Flow cytometric measurements were performed after 48h. Data are means ± SD of triplicates.

To provide functional evidence for caspase activation and to confirm that caspases are involved in the p14^{ARF} signalling pathway after sensitization with caffeine, MCF-7 caspase-3 proficient cells were incubated with the indicated caspase inhibitors for 72h. Apoptosis was measured in 8% of the non-treated cells, and 9% of apoptotic cells were measured once the cells were additionally treated with corresponding inhibitor. After p14^{ARF} expression, only 10 % of the cells displayed DNA fragmentation in contrast to 45% of the cells upon additional exposure to caffeine (figure 23A*)*. However, after addition of the pan-caspase inhibitor (zVAD-fmk), the apoptotic rate was significantly reduced to 7%. This percentage corresponded to the apoptotic rate of the controls. Similarly, the importance of the initiator

caspase-9 was addressed by using the zLEHD-fmk inhibitor (figure 23B). Upon $p14^{ARF}$ expression and additional treatment with caffeine, 39% of cells presented apoptotic DNA fragmentation. Despite of the irreversible inhibition of caspase-9 by zLEHD-fmk, cells showed only a minor reduction in the apoptotic rate, whether caffeine was present or not. To confirm that caspase-3 is the key effector caspase in $p14^{ARF}$ induced apoptotic cell death cells in the presence of caffeine, the caspase-3 and-7 inhibitor zDEVD-fmk was used (figure 23C). After 72h, measurement of apoptosis revealed 40% of cells with DNA fragmentation upon $p14^{ARF}$ expression and caffeine treatment. Additional treatment with zDEVD-fmk blocked apoptotic cell death resulting in only 18% of dead cells.

These data reveal the caspase-3 dependency in the apoptotic $p14^{ARF}$ pathway, showing that after sensitization with caffeine apoptosis is blocked, almost down to background levels, when this key executioner caspase is inhibited. These data confirms the previous results obtained in caspase-3 deficient MCF-7 cells.

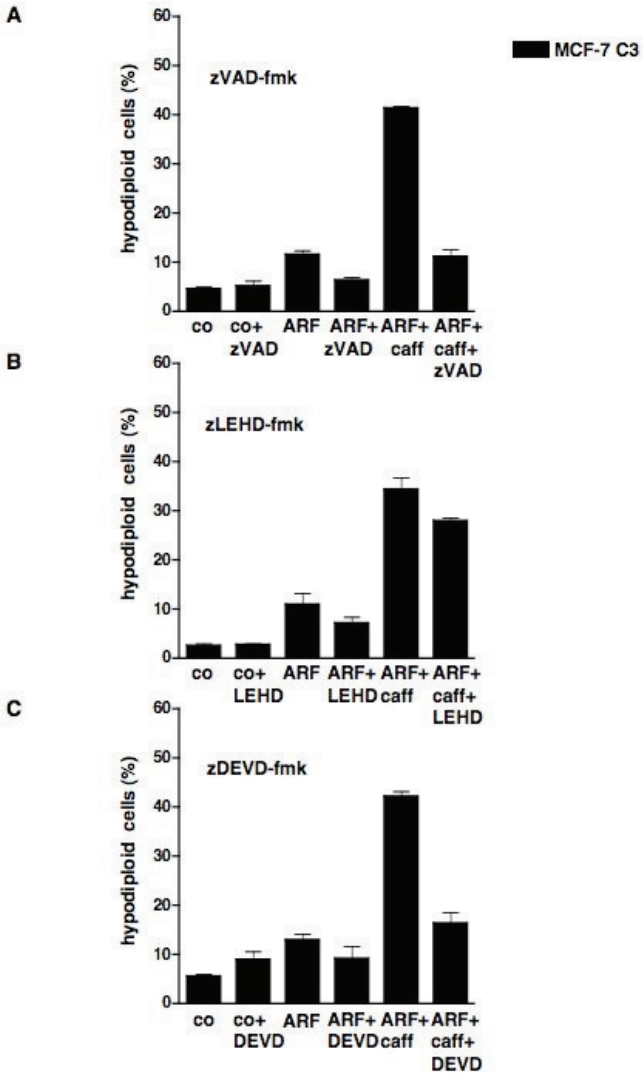

Figure 23. P14^{ARF} induces apoptosis upon caffeine treatment depending on DEVDases.

MCF-7 cells proficient for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ, treated with caffeine (1mM) and cultured for 72h in the presence or absence of indicated caspase inhibitors.

(A) Flow cytometric analysis of cells treated with a broad-spectrum caspase inhibitor zVAD-fmk (zVAD) compared to untreated cells.

(B) Flow cytometric analysis of cells treated with a caspase-9 inhibitor zLEHD-fmk (zLEHD) compared to untreated cells.

(C) Flow cytometric analysis of cells treated with a caspase-3 inhibitor zDEVD-fmk (zDEVD) compared to untreated cells.

Data are means ± SD of triplicates.

5.4.5 The cyclin dependent kinase inhibitor p21 interferes with $p14^{ARF}$ induced apoptosis

In order to investigate apoptosis associated proteins, MCF-7 mock cells and MCF-7 cells that were stably re-transfected with procaspase-3 were infected with Ad-$p14^{ARF}$ and incubated with caffeine as indicated in the legend of figure 24. Corresponding controls consisted of non-treated cells, cells infected with control virus Ad-lacZ cultured in the presence or absence of caffeine and cells treated with caffeine alone. Cells were harvested after 48h and were analyzed on SDS-PAGE followed by Western blot analysis. Both cell transfectants that were transduced with Ad-$p14^{ARF}$ showed similar transgene protein expression. Upon $p14^{ARF}$ expression, the level of the proapoptotic protein Bax was up regulated regardless of the additional caffeine exposure. This was in contrast to control cells were Bax expression was barely detectable in both cell transfectants. However, proapoptotic protein Bak did not show major differences in term of its expression independently of the treatment. The protein level of the antiapoptotic protein Bcl-2 remained unchanged. Neither cytochrome c release from mitochondria into the cytosol nor caspase-9 activation was detectable in the cells lacking caspase-3 upon any kind of treatment.

In control cultures of caspase-3 proficient cells, cytochrome c release was not detectable and the proforms of caspase-3 and -9 were not processed. In contrast these cells underwent apoptosis when transduced with Ad-$p14^{ARF}$ and treated with caffeine as evidenced by cytochrome c release that was associated with the processing of caspase-9, resulting in a reduction of its proform. Under this condition, procaspase-3 was proteolytically processed into its cleavage products.

These data clearly demonstrate that caffeine interferes with apoptotic signalling events upon $p14^{ARF}$ expression resulting in massive apoptosis, thereby strictly depending on the caspase-3.

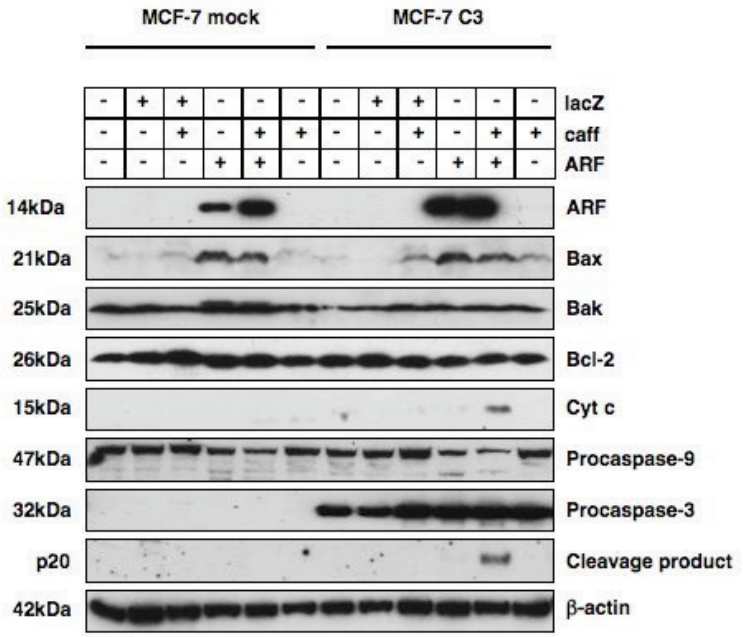

Figure 24. Western blot analysis of apoptosis-related proteins upon p14^{ARF} expression and caffeine treatment.
MCF-7 cells deficient or proficient for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ, treated with caffeine and cultured for 48h. Proteins were detected by the appropriate antibody as indicated. Equal loading was confirmed by β-actin detection.

5.4.6 Bcl-2 targeted to the mitochondria impedes p14^{ARF}-induced apoptosis upon sensitization with caffeine

Bcl-2 is an integral antiapoptotic membrane protein located at the outer membrane of mitochondria, which plays an important role in tumour genesis. This protein is overexpressed in a variety of tumours and causes direct inhibition of apoptosis. Therefore, the following experiments concern the ability of p14^{ARF} to induce apoptosis upon sensitization with caffeine despite Bcl-2 overexpression. For these studies, SW480 cells were employed which were stably overexpressing exogenous Bcl-2. The expressed Bcl-2 was targeted to the mitochondria (Bcl-2-actA) using signal peptides actA of Listeria monocytogenes. To target Bcl-2 to the ER, the rat hepatic ER cytochrome b5 isoform was fused to Bcl-2 (Hacki et al., 2000; Zhu et al., 1996). Western blot analysis confirmed that Bcl2-actA and Bcl-2cb5 were equally expressed in both cell transfectants (figure 25A). Corresponding control cells, SW480neo, were transfected with an empty vector and do not over express Bcl-2. Apoptotic DNA fragmentation as well as loss of cell viability was measured. After infection with control virus and treatment with caffeine, all three cell

transfectants did neither show loss of cell viability as measured by PI staining after 96h nor apoptotic DNA fragmentation after 120h (figure 25B, C). $P14^{ARF}$ expression without caffeine treatment did not influence cell viability and apoptosis of all three cell transfectants either. However, when cells were additionally cultured with caffeine, 40% of SW480neo and 30% of SW480Bcl-2cb5 cells revealed loss of cell viability. In contrast, only 5% of the cells lost cell viability when Bcl-2 was located at the mitochondria. To confirm these results, apoptotic cell death was measured for the same cell settings at 120h post infection. An apoptotic rate of 35% was measured in SW480neo and SW480Bcl-2cb5 cells, whereas only 8% of SW480Bcl-2actA cells demonstrated apoptotic cell death after expression of $p14^{ARF}$ in the presence of caffeine. These data clearly indicate that cells were protected by Bcl-2 when Bcl-2 was targeted to the mitochondria.

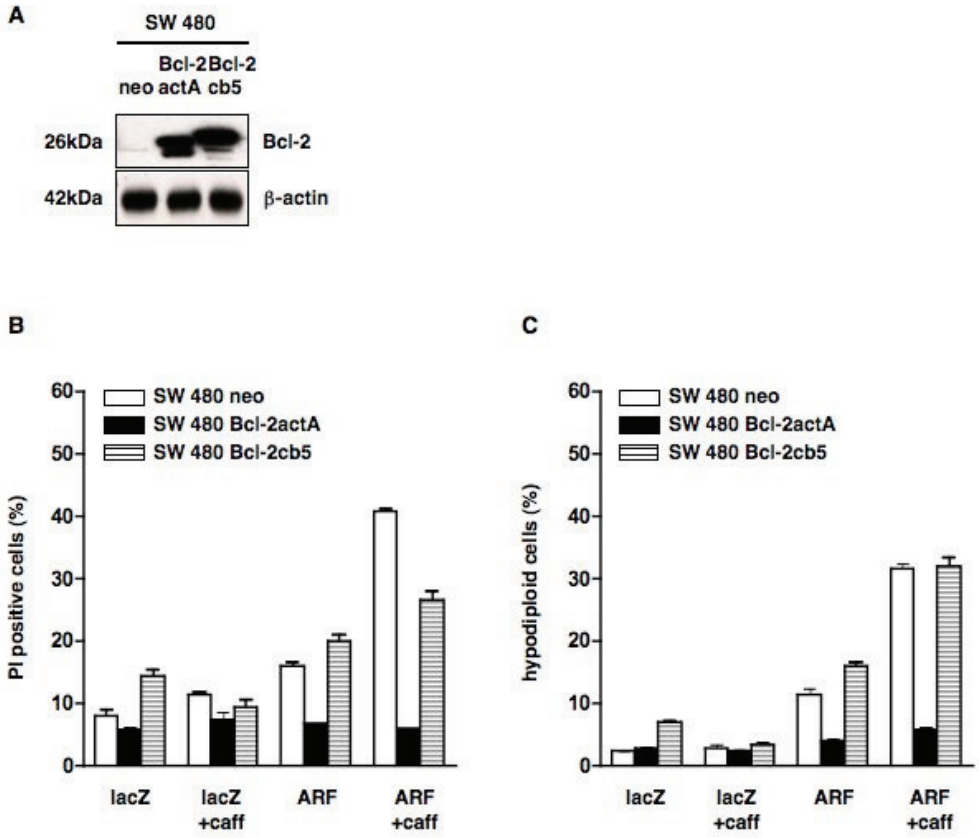

Figure 25. Bcl-2 targeted to the mitochondria can abrogate $p14^{ARF}$-induced apoptotic cell death upon caffeine treatment in SW480 cells.

(A) Western blot analysis of Bcl-2 expression in SW480-Bcl-2neo, SW480-Bcl-2actA, SW480-Bcl2cb-5 cells as indicated. Equal loading was confirmed by β-actin detection.

(B) SW480 cells were transduced with Ad-$p14^{ARF}$ or Ad-lacZ and treated with caffeine (1mM). Loss of cell viability was measured by PI staining after 96h.

(C) Apoptotic cell death was measured by flow cytometry after 120h.

Data are means ± SD of triplicates (B, C).

5.5 Autophagy as a cell death mechanism in the $p14^{ARF}$ signalling pathway

5.5.1.1 Morphological characteristics of the cells based on different types of cell death

The data obtained in MCF-7 cells clearly indicated that $p14^{ARF}$ induces apoptosis after exposure to caffeine thereby strictly depending on caspase-3. Nevertheless, previous results in caspase-3 deficient cells also showed mitochondrial depolarization (figure 19C) but no sign of apoptosis (figure 17). Therefore, the morphological appearances of both cell transfectants were observed after 48h by phase contrast microscopy (figure 26). As a control, both MCF-7 cell transfectants were infected with control virus in combination with caffeine. These cells clearly showed the morphology of healthy cells. Moreover, $p14^{ARF}$ induced significant arrest in cell cycle in both cell populations affecting only cell proliferation. Overexpression of $p14^{ARF}$ resulted in a reduced number of cells without altering the cell morphology. However, after overexpression of $p14^{ARF}$ in combination with caffeine MCF-7 caspase-3 proficient cells displayed cellular changes such as shrinkage, rounding, detachment as well as apoptotic bodies formation acknowledged as typical changes during apoptosis. In contrast, caspase-3 deficient cells did not show any morphological features of apoptosis. Remarkably, these cells displayed the presence of vacuoles that might indicate autophagic vacuole formation. Therefore, to further define these events, the focus further extended to type II cell death, the so-called autophagy. Autophagy represents a distinct cell death phenotype in non-apoptotic dying cells. Presented morphological analyses of cells lacking caspase-3 suggested that extensive autophagy activity could be induced upon overexpression of $p14^{ARF}$ in combination with caffeine.

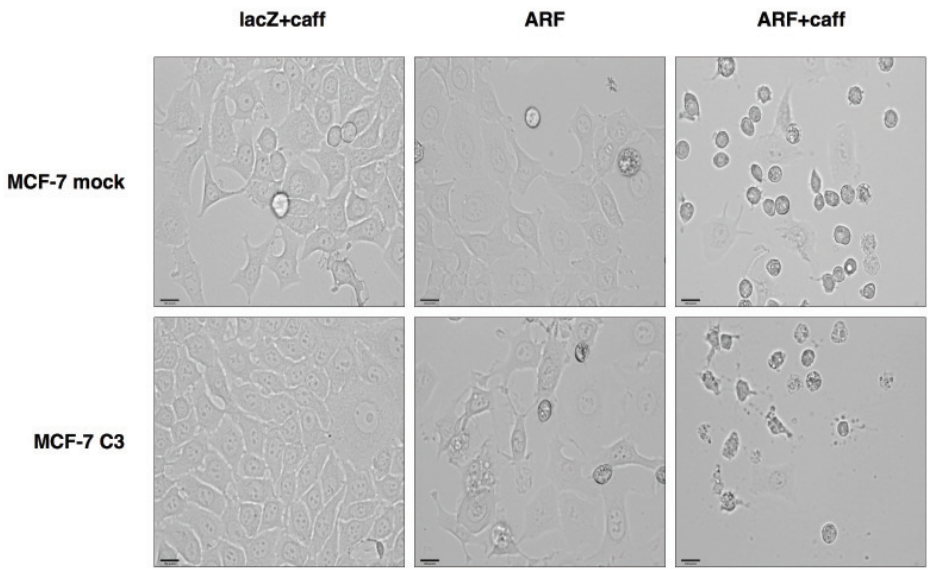

Figure 26. Morphological appearance of MCF-7 cells upon p14^{ARF} expression and caffeine treatment.
MCF-7 cells were transduced with Ad-p14^{ARF} or Ad-lacZ and treated with caffeine (1mM). Pictures were taken after 48h. Upper panel: MCF-7 caspase-3 deficient cells. Lower panel: MCF-7 caspase-3 proficient cells.

5.5.2 Loss of cell viability triggered by p14^{ARF} upon sensitization with caffeine is not dependent on the presence of caspase-3

To examine autophagic cell death, MCF-7 cells proficient and deficient for executioner caspase-3 were stably transfected with a GFP-LC3 fusion protein. Microtubule-associated protein light chain-3 (LC3) is a soluble protein with a molecular mass of 18kDa, which is localized in the cytoplasm. During autophagy, LC3I is conjugated to phosphatidylethanolamine to form an LC3-phosphatidylethanolamine conjugate (LC3II). LC3II is suitable marker for autophagy induction, because its lipidation during autophagosome formation increases electrophoretic mobility on gels compared to LC3I. MCF-7 cells deficient and proficient for caspase-3 stably expressing GFP-LC3 fusion protein, displayed green fluorescence. Green fluorescent protein-LC3 could be used to visualize autophagosomes by fluorescence microscopy presenting a shift from a diffuse to a punctuated GFP-signal. Using these different tools for detection, induction of autophagy by p14^{ARF} after sensitization with caffeine was examined.

In a first attempt, the impact of p14^{ARF} on induction of different types of cell death was studied. Apoptotic cell death was measured after 72h and loss of cell viability after 48h by

flow cytometry. As a control, both cell transfectants were cultured only in medium, or infected with Ad-lacZ control virus in the presence or absence of caffeine. Neither apoptotic cell death nor loss of cell viability was detected in controls for both cell transfectants. After infection with the Ad-p14^{ARF} vector alone, up to 15% of the cells showed apoptotic cell death for both cell transfectants (figure 27A). Loss of cell viability was measured in 16% of caspase-3 deficient cells and in 35% of caspase proficient cells after the same treatment (figure 27B). Moreover, in combination with caffeine, p14^{ARF} induced apoptotic DNA fragmentation in 70% of the caspase-3 proficient cells. In contrast, only 18% of cells lacking caspase-3 showed induction of apoptosis. However, upon p14^{ARF} expression in combination with caffeine, 62% of the MCF-7 caspase-3 proficient cells demonstrated loss of cell viability. A similar effect was observed in the cells deficient for caspase-3 namely, 52% of those cells displayed loss of cell viability after the same treatment. These data verify that each cell transfectant utilizes a different pathway to conduct cell death depending on the caspase-3 status.

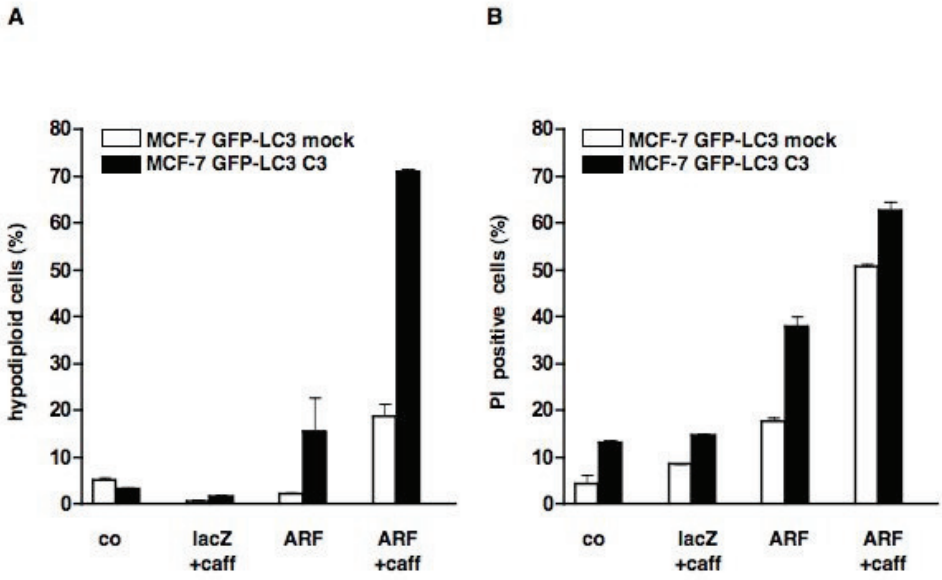

Figure 27. Loss of cell viability in MCF-7 cells deficient and proficient for caspase-3 and stably expressing GFP-LC3.
MCF-7 cells deficient (white bars) and proficient (black bars) for caspase-3 and stably expressing GFP-LC3 were transduced with Ad-p14^{ARF} or Ad-lacZ and treated with caffeine (1mM).
(A) DNA fragmentation was measured by flow cytometry after 72h. Data are means ± SD of triplicates .
(B) Cell viability measurements were performed by flow cytometry after 48h using PI. Data are means ± SD of triplicates.

5.5.3 Detection of autophagy cell death through autophagosome formation

MCF-7 cells deficient and proficient for caspase-3 stably expressing the GFP-LC3 fusion protein, displayed green fluorescence. As controls, cells were infected with Ad-lacZ and treated with caffeine (figure 28). Those cells represented a diffuse staining and cells only expressing $p14^{ARF}$ showed the same pattern. Cells lacking caspase-3 transduced with Ad-$p14^{ARF}$ and treated with caffeine showed a strong increase in the punctuated GFP-signal. This change from a diffuse signal to punctuated dots reflected accumulation of autophagy vesicles, i.e. autophagosomes. Upon the same treatment, MCF-7 caspase-3 re-expressing cells, previously verified as apoptosis-executing cells, did not demonstrate punctuation of LC3-GFP. These results imply that autophagy, as an alternative way of dying, can contribute to cell death in cells deficient for the downstream effector caspase-3.

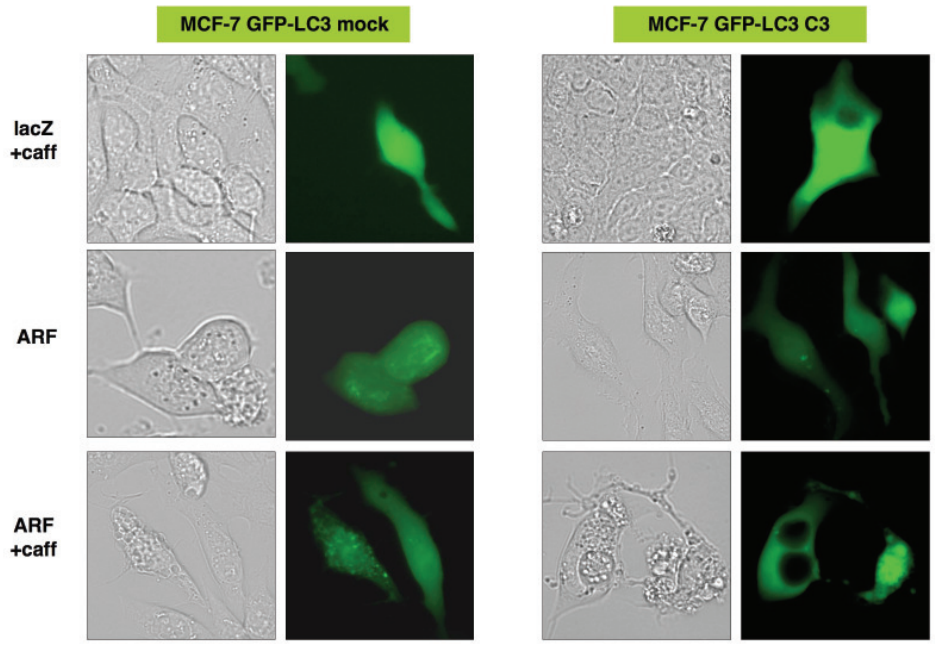

Figure 28. LC3 punctuation in MCF-7 cells lacking caspase-3.
MCF-7 cells deficient and proficient for caspase-3 were stably transfected with LC3-GFP and transduced with Ad-$p14^{ARF}$ or Ad-lacZ and treated with caffeine. Representative pictures were taken after 72h.

5.5.4 Inhibition of autophagy by 3-methyladenine (3-MA)

To confirm induction of autophagy a specific pharmacological inhibitor of autophagy, 3-methyladenine (3-MA) was employed. 3-MA inhibits autophagy at the sequestration stage, inhibiting formation of autophagosomes. MCF-7 GFP-LC3 caspase-3 deficient cells were infected with Ad-lacZ and treated with caffeine. These control cells showed diffuse green staining presenting localization of LC3 in the cytoplasm but no autophagosome formation was observed (figure 29). Infection with Ad-p14^{ARF} in combination with caffeine induced alteration of the LC3 distribution from a diffuse to a punctuated pattern. The same effect occurred in presence of the caspase-3 and -7 inhibitor.
Ad-p14^{ARF} infected cells, which were exposed to caffeine and to pharmacological autophagy inhibitor 3-MA, showed reduced number of autophagosomes.

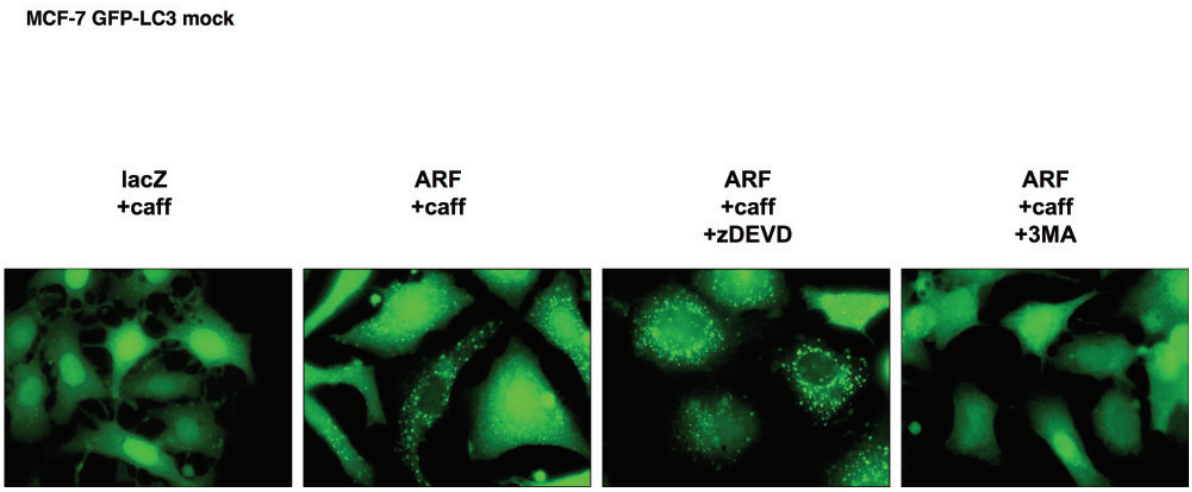

Figure 29. Induction of autophagic vacuole formation is inhibited by 3-MA but not by the caspase-3/-7 inhibitor zDEVD-fmk.
MCF-7 caspase-3 deficient cells were transduced with Ad-p14^{ARF} or Ad-lacZ. Cells were cultured for 72h in the presence of caffeine (1mM), the caspase-3/-7 inhibitor zDEVD-fmk (zDEVD) (20µM) or the autophagy inhibitor 3-methyladenine (3-MA) (5mM) as indicated.

5.6 Crosstalk between apoptosis and autophagy signalling

5.6.1 Western blot analysis of involved apoptotic and autophagy proteins

To investigate the regulation of autophagy in more detail involved proteins were examined by Western blot analyses. Whole cell lysates were collected, electrophoresed and blotted for detection of the indicated proteins after 48h of $p14^{ARF}$ induction. MCF-7 GFP-LC3 caspase-3 proficient and MCF-7 GFP-LC3 caspase-3 deficient cells were infected with Ad-lacZ, Ad-$p14^{ARF}$ and additionally treated with caffeine as shown in figure 30. Cells infected with Ad-$p14^{ARF}$ showed high expression of this protein. As suitable marker for autophagy, LC3 levels were analyzed. During autophagy, lipid conjugation leads to the conversion of the soluble form LC3I (18kDa) into the faster migrating LC3II form (16kDa) as detected by SDS-PAGE. On the Western blot, overexpressed GFP-LC3 protein levels were detected. In caspase-3 deficient cells, in all controls and Ad-$p14^{ARF}$ infected samples only GFP-LC3I, the non-autophagy variant was detectable. In contrast, upon overexpression of $p14^{ARF}$ in combination with caffeine treatment, GFP-LC3II, i.e. the autophagy variant, was identified. In caspase-3 proficient cells, only the non-autophagy form of GFP-LC3I was detected regardless of the treatment. These results confirmed that caspase-3 proficient cells did not undergo autophagy upon $p14^{ARF}$ expression alone or in combination with caffeine.
As shown previously, cells proficient for caspase-3 transduced with Ad-lacZ and co-treated with caffeine or transduced with Ad-$p14^{ARF}$ alone demonstrated high expression of inactive procaspase-3. But only when Ad-$p14^{ARF}$ overexpressing cells were co-exposed with caffeine, activation of caspase-3 was observed as evidenced by detection of its cleavage products. These findings correlate with previous data showing that caffeine sensitized for $p14^{ARF}$-induced apoptosis. For both cell transfectants, the levels of the proapoptotic protein Bax were upregulated upon $p14^{ARF}$ expression with or without caffeine in contrast to control treatment. The levels of the homologous proapoptotic protein Bak remained the same independently of the treatment. Since the members of the Bcl-2 family are critical regulators of apoptosis, the expression levels of the two antiapoptotic proteins Bcl-2 and Bcl-x_L were analyzed. In cells re-expressing caspase-3, $p14^{ARF}$ expression combined with caffeine triggered a strong downregulation of these two proteins as compared to control cells or cells only infected with Ad-$p14^{ARF}$. MCF-7 caspase-3 deficient cells did not show a detectable difference in the expression level of these two proteins when they were treated with Ad-$p14^{ARF}$ in the absence or presence of caffeine as compared to control cells.

Beclin-1, a prominent autophagy related protein is a mediator of autophagy and has a crucial role in autophagy vesicles formation. Both MCF-7 transfectants transduced with Ad-lacZ in combination with caffeine or Ad-p14^{ARF} alone displayed a high expression level of Beclin-1. However, caspase-3 proficient cells showed a significant reduction in the expression of Beclin-1, whereas caspase-3 deficient cells showed stagnant high expression for Beclin-1 upon p14^{ARF} expression in combination with caffeine. Beclin-1 might therefore be a key target of caspase-3, as this study reveals a particular downregulation of Beclin-1, especially since cells lacking caspase-3 did not show such a decrease in Beclin-1 expression.

Caspase-8 is an important amplifier of the apoptotic signal, as it mediates the mitochondrial amplification loop and directly activates caspase-3 (von Haefen et al., 2003). Cells overexpressing p14^{ARF} and exposed to caffeine showed activation of caspase-8 for both caspase-3 proficient and deficient cells. Caspase-8 processing was evidenced by the downregulation of its inactive proform because no cleavage product could be detected. This effect was not seen in control cells as well in p14^{ARF}overexpressing samples. All together, these results indicate that downregulation of procaspase-8 can be seen also in the absence of caspase-3. These studies confirm our initial observation that caspase-3 is required for execution of apoptosis, whereas cells lacking caspase-3 can utilize the alternative autophagy pathway to execute cell death.

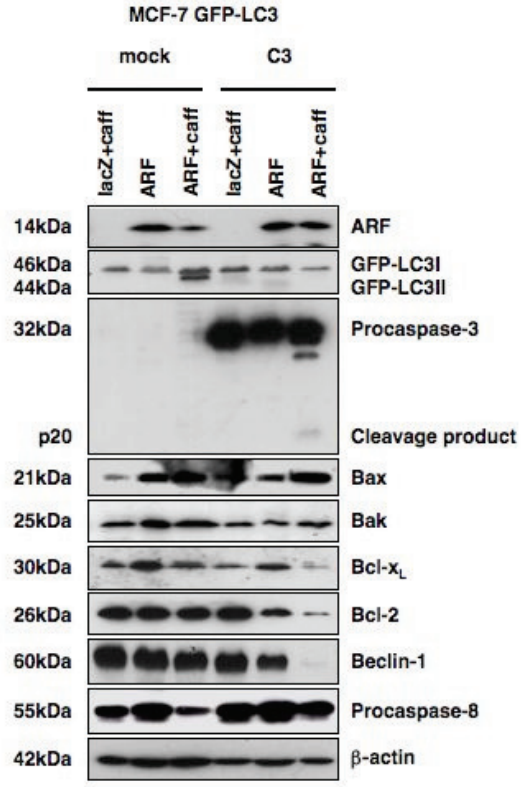

Figure 30. P14^{ARF} induces autophagy after caffeine treatment in the absence of caspase-3.
MCF-7 GFP-LC3 cells deficient and proficient for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ in combination with caffeine for 48h. Expression of indicated proteins was confirmed by immunoblotting. Equal loading was confirmed by β-actin detection.

5.6.2 Caspase dependent cell death

To further elucidate the functional interactions between autophagy and apoptosis, the expression of related proteins was investigated. In order to explore whether processing and activation of caspase-3 is essential for downregulation of Beclin-1, both MCF-7 GFP-LC3 cell transfectants were incubated with the pan-caspase inhibitor 10μM of Q-VD-OPh (figure 31). As previously shown, strong Beclin-1 downregulation was only seen in caspase-3 proficient cells upon $p14^{ARF}$ expression and after exposure to caffeine. However, in the presence of the pan-caspase inhibitor, Western blot analysis revealed that Beclin-1 expression was restored and detectable to a similar extent as in control cells. In caspase-3 deficient cells, the expression level of Beclin-1 did not differ after any kind of treatment, regardless of the presence of Q-VD-OPh compared to controls. Furthermore, Bcl-2 expression was analyzed in the same experiment. Bcl-2 was only downregulated in caspase-3 proficient cells infected with $p14^{ARF}$ and exposed to caffeine, and the pan-caspase inhibitor was able to prevent this downregulation of Bcl-2. In caspase-3 deficient cells, Q-VD-OPh did not alter the expression level of Bcl-2 upon none of the treatments. Likewise, in caspase-3 proficient cells no change of Bcl-2 expression was detected except in cells transduced with Ad-$p14^{ARF}$ in combination with caffeine. There again, Q-VD-OPh counteracted with $p14^{ARF}$ in combination with caffeine to induce decrease in Bcl-2 expression. This indicates that both Beclin-1 and Bcl-2 (presumably also Bcl-x_L) are subjected to caspase-3 proteolysis.

Furthermore, the lipidated form of LC3 could only be detected after $p14^{ARF}$ expression and treatment with caffeine in cells lacking caspase-3. When the same treatment was performed in the presence of pan-caspase inhibitor Q-VD-OPh, the lipidation was completely blocked and no LC3II variant could be detected. Neither caspase-3 deficient nor caspase-3 proficient cells showed LC3 lipidation within controls or when infected with Ad-$p14^{ARF}$ alone, independently of the pan-caspase inhibitor. Caspase-3 proficient cells infected with Ad-$p14^{ARF}$ in combination with caffeine did not show lipidation regardless of the presence of Q-VD-OPh. These data indicate that autophagic cell death does not occur when all caspases are blocked.

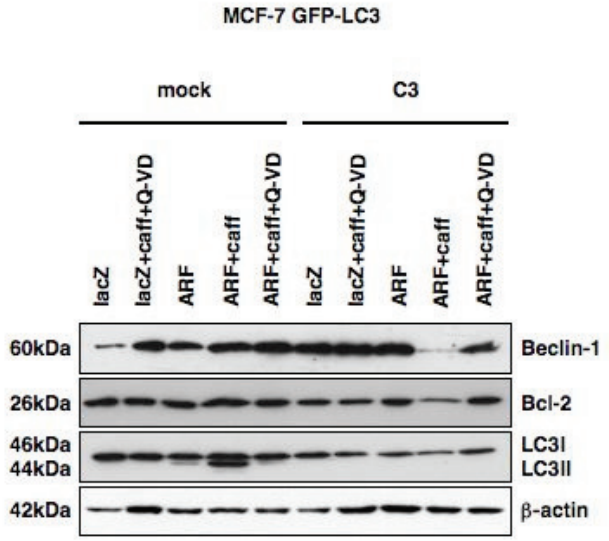

Figure 31. Inhibition of caspases by Q-VD-OPh blocks degradation of Beclin-1 and Bcl-2 in apoptotic cells as well as lipidation of LC3-GFP in autophagic cells.
Western blot analysis of indicated proteins. MCF-7 GFP-LC3 cells deficient and proficient for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ and treated with caffeine. Cells were cultured for 72h in the presence or absence of the broad spectrum caspase inhibitor Q-VD-OPh (Q-VD) (10μM) as indicated. β-actin detection was used as a loading control.

5.6.3 Caspase-8 is involved in different types of cell death

The next experiments addressed the inhibition of caspase-8 in order to investigate if caspase-8 is a part of the autophagy pathway. Apoptotic DNA fragmentation was measured after 72h and cell viability after 48h, by flow cytometry. Neither caspase-3 deficient nor caspase-3 proficient cells showed apoptotic cell death or loss of cell viability when transduced with Ad-lacZ, additionally treated with caffeine, and the caspase-8 inhibitor zIETD-fmk (figure 32A, B). Upon p14^{ARF} expression in combination with caffeine, cells lacking caspase-3 did not display more than 5% apoptotic cell death. A similar effect was seen in the presence of the caspase-8 inhibitor. In contrast, when MCF-7 caspase-3 re-expressing cells were transduced with Ad-p14^{ARF} and additionally treated with caffeine, 50% of the cells demonstrated apoptotic DNA fragmentation. However, presence of the caspase-8 inhibitor decreased the apoptotic rate to 32% (figure 32A). These data provide evidence that caspase-8 plays a role in p14^{ARF}/caffeine induced apoptosis. Interestingly, 58% of caspase-3 deficient and 67% of caspase-3 proficient cells displayed loss of viability upon p14^{ARF} expression and caffeine treatment (figure 32B). Supplementary treatment with the caspase-8 inhibitor did not have any impact on cell viability. Namely, both cell

transfectants demonstrated the same decrease of cell viability whether the inhibitor was present or not.

Regarding apoptosis, inhibition of caspase-8 partially decreased apoptosis induction in caspase-3 proficient cells. Nevertheless cells showed stagnant high loss of cell viability. These data clearly indicate that caspase-8 is important in the $p14^{ARF}$ apoptosis pathway but not required for alternative cell death i.e. autophagic cell death. It therefore can be concluded that cells re-expressing caspase-3 are able to utilize an alternative cell death pathway after blocking of caspase-8.

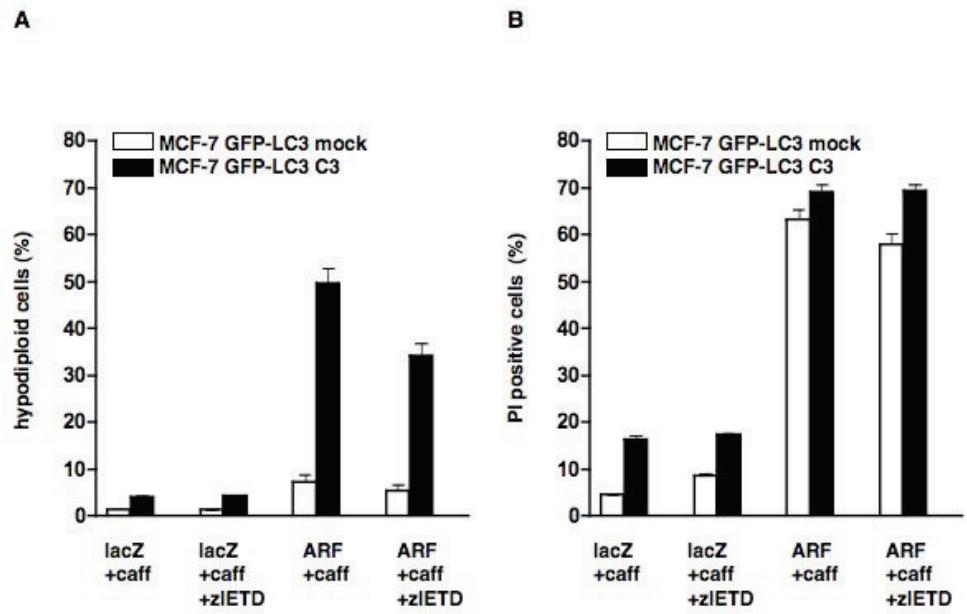

Figure 32. P14^{ARF} induces different types of cell death upon caffeine treatment in dependence on the presence of caspase-3 and caspase-8.

MCF-7 cells deficient and proficient for caspase-3 were transduced with the Ad-p14^{ARF} or Ad-lacZ and treated with caffeine (1mM) and cultured in presence or absence of caspase-8 inhibitor zIETD-fmk (zIETD) as indicated.

(A) Flow cytometry detection of apoptotic DNA fragmentation measured after 72h.

(B) Loss of cell viability was measured after 48h by flow cytometry.

Data are means ± SD of triplicates.

To confirm that cells died via autophagy upon inhibition of caspase-8, the conversion of the autophagy marker LC3 was determined by Western blot analysis (figure 33). As controls, cells were infected with Ad-lacZ, additionally treated with caffeine and the caspase-8 inhibitor zIETD-fmk. Neither caspase-3 deficient nor caspase-3 proficient cells showed the lipidated LC3II autophagy variant in the controls. Upon infection with Ad-$p14^{ARF}$, the cells displayed only the non-autophagy form of LC3 in both cell transfectants. When caffeine was

added, $p14^{ARF}$ triggered induction of autophagy in cells lacking caspase-3. A similar effect was achieved when caspase-8 was additionally blocked with the zIETD-fmk inhibitor. Cells proficient for caspase-3 only showed the non-autophagy variant LC3I upon $p14^{ARF}$ expression and caffeine treatment. Additional inhibition of caspase-8 resulted in the lipidation of LC3, detected as the LC3II autophagy variant. These data show that inhibition of caspase-8 stimulates autophagy as an alternative way to ensure cell death.

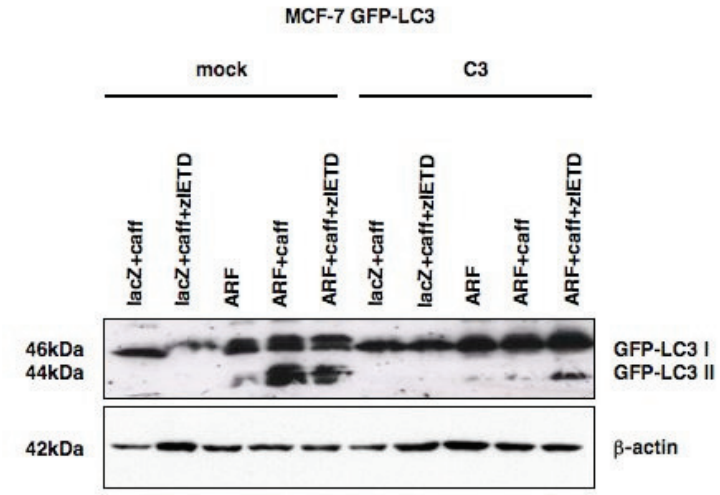

Figure 33. $P14^{ARF}$ and caffeine treatment induce autophagy after inhibition of caspase-8.
MCF-7 cells deficient and proficient for caspase-3 were transduced with Ad-$p14^{ARF}$ or Ad-lacZ. Cells were treated with caffeine (1mM) and cultured for 72h in presence or absence of caspase-8 inhibitor zIETD-fmk (zIETD) as indicated. Equal loading was confirmed by β-actin detection.

In the absence of caspase-3 as well as by the inhibition of caspase-8, autophagic cell death is enforced as confirmed by the high loss of cell viability as well as by LC3II conversion. The next question to address was the role of caspase-7 in autophagy cell death. Loss of cell viability was measured by uptake of PI in MCF-7 caspase-3 deficient cells that are still proficient for caspase-7 (figure 34). As controls, cells were infected with control virus Ad-lacZ and cultured with caffeine in the presence or absence of the caspase-3/-7 inhibitor (zDEVD-fmk). 5% of the control cells demonstrated loss of cell viability. In parallel, cells were infected with Ad-$p14^{ARF}$ and cultured in the presence or absence of the inhibitor. 18% of the cells displayed loss of cell viability whether they were inhibitor-treated or not. Nevertheless, 48% of the cells showed high loss of viability upon $p14^{ARF}$ expression and caffeine treatment independently of the presence of the inhibitor. These results demonstrate that autophagy cell death could not be prevented by inhibition of caspase-7.

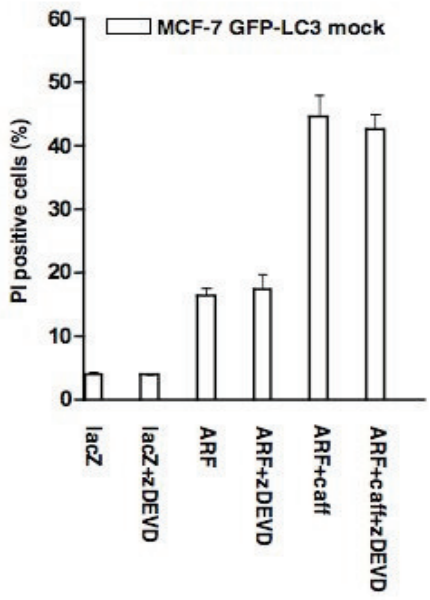

Figure 34. Inhibition of caspase-7 does not impede the induction of autophagy.
MCF-7 caspase-3 deficient cells were transduced with Ad-p14^{ARF} or Ad-lacZ and treated with caffeine. Cells were cultured for 72h in presence or absence of caspase-3 and -7 inhibitor zDEVD-fmk (zDEVD) as indicated. Data are means ± SD of triplicates.

5.7 Irradiation sensitizes Ad-p14^{ARF} induced apoptosis

5.7.1 P14^{ARF} induction of the intrinsic apoptotic pathway after irradiation strictly depends on caspase-3

Previous data showed that the primary response of MCF-7 cells overexpressing p14^{ARF} is to arrest in G1 phase of the cell cycle. Cooperation between p14^{ARF} and irradiation (IR), and consequent cell death has been addressed in the following experiments in order to provide a useful tool against chemotherapeutic resistance in breast tumours. The question if this DNA checkpoint regulator protein could sensitize irradiation resistance was addressed in the following experiments.

As controls, both cell transfectants cells were infected with Ad-lacZ, exposed and not exposed to IR, and cultured with or without the pan-caspase inhibitor zVAD-fmk, respectively (figure 35A, B). A weak background apoptotic cell death up to 9% was measured in all control caspase-3 deficient cells. After infection with Ad-p14^{ARF}, 6% of the cells demonstrated apoptotic death. A similar effect occurred, when p14^{ARF} expressing cells were exposed to IR, namely 8% of the cells died via apoptosis. No change in apoptotic cell death could be determined regardless of the presence of the pan-caspase inhibitor (zVAD-fmk).

Background apoptotic cell death, up to 12% was measured in all control caspase-3 proficient cells. When these were infected only with Ad-p14^{ARF}, 10% of cells displayed apoptotic DNA fragmentation. Interestingly, in p14^{ARF} expressing cells the apoptotic rate was increased up to 32% after additional exposure to IR. Upon blocking of all caspases by the pan-caspase inhibitor the apoptotic rate was reduced to 7% and this percentage correlated with the level of apoptotic cells in controls (figure 35B). This indicates that p14^{ARF} and IR synergize for induction of a caspase-3 dependent apoptosis pathway.

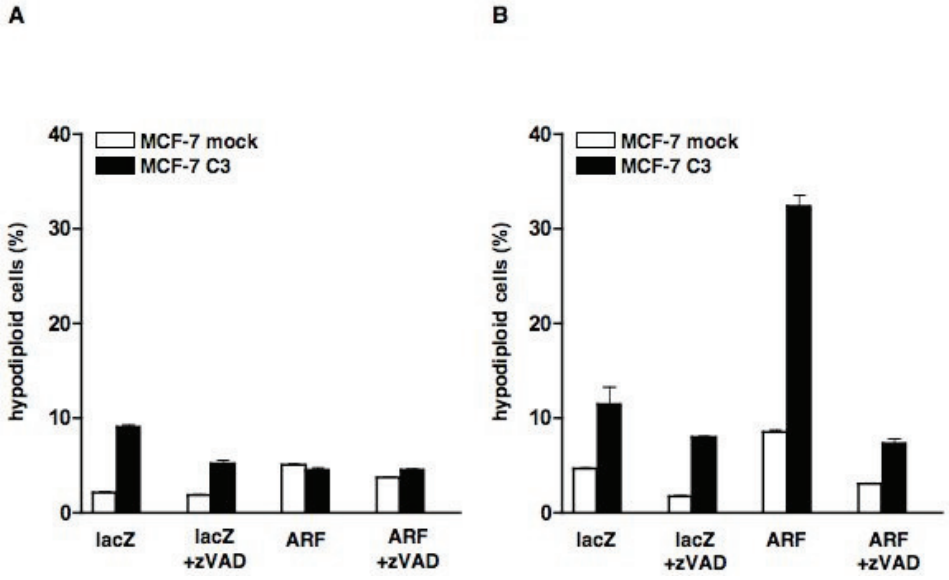

Figure 35. P14^{ARF}-induced apoptosis after irradiation strictly depends on caspase-3. Induction of apoptosis is blocked with the pan-caspase inhibitor zVAD-fmk.
(A) MCF-7 cells deficient for caspase-3 were infected with Ad-p14^{ARF} or Ad-lacZ (white bars) and additionally exposed to 10Gy IR (black bars).
(B) MCF-7 cells proficient for caspase-3 were infected with Ad-p14^{ARF} or Ad-lacZ (white bars) and additionally exposed to 10Gy IR (black bars).
DNA fragmentation was measured after 120h. Data are means ± SD of triplicates.

A hallmark of the intrinsic apoptotic pathway is the depolarization of mitochondria. The initiation of the apoptotic process begins with the permeabilization of the mitochondrial outer membrane.

As controls, both cell transfectants were infected with Ad-lacZ exposed and not exposed to IR, and treated with or without the pan-caspase inhibitor zVAD-fmk, respectively. A background induction of mitochondrial permeability was measured in up to 11% of caspase-3 deficient control cells. Transduction with Ad-p14^{ARF} induced breakdown of the mitochondrial membrane potential in not more than 12% of the cells, independently of

exposure to IR. Similar results were obtained after treatment with the pan-caspase inhibitor (figure 36A).

In caspase-3 proficient cells up to 16% control cultured cells displayed mitochondrial permeabilization as well as upon $p14^{ARF}$ expression (figure 36B). However, 36% of these revealed loss of the mitochondrial membrane potential, when infected with Ad-$p14^{ARF}$ and additionally exposed to IR. In the presence of the pan-caspase inhibitor, apoptotic cell death was measured in only 20% of cells.

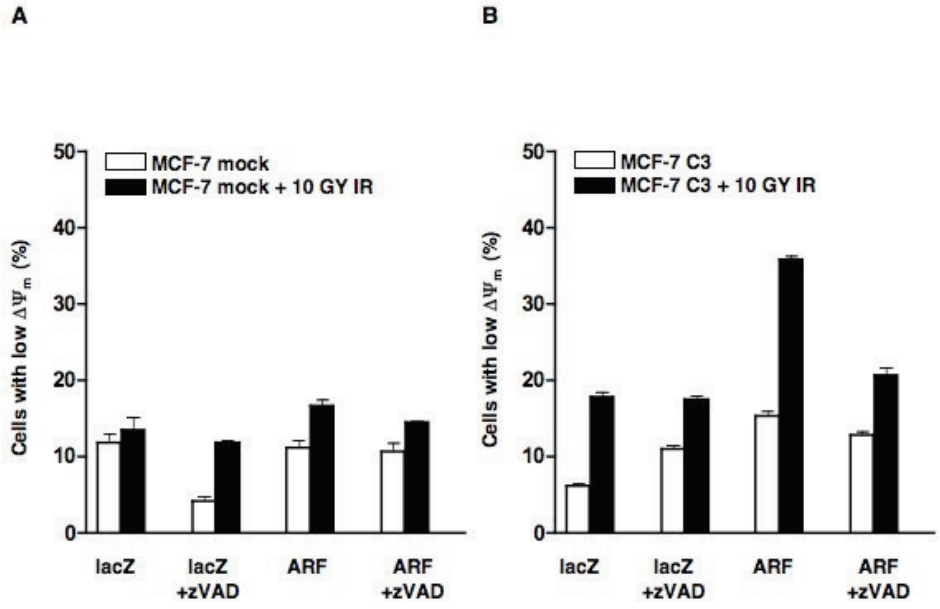

Figure 36. Loss of the mitochondrial membrane potential upon $p14^{ARF}$ expression and irradiation.
Cells were transduced with Ad-$p14^{ARF}$ or Ad-lacZ in the presence or absence of the zVAD-fmk inhibitor and exposed to IR as indicated. Flow cytometric detection of cells with low mitochondrial membrane potential after 96h. Data are means ± SD of triplicates of cells with $\Delta\Psi_m$ loss.
(A) MCF-7 cells deficient for caspase-3 were infected with Ad-$p14^{ARF}$ or Ad-lacZ (white bars) and exposed to 10Gy IR (black bars).
(B) MCF-7 cells proficient for caspase-3 were infected with Ad-$p14^{ARF}$ or Ad-lacZ (white bars) and exposed to 10Gy IR (black bars).

The depolarization of mitochondria i.e. loss of the mitochondrial membrane potential crucially depends on the multi-domain proapoptotic protein Bax. It is a cytosolic protein that undergoes an N-terminal conformational change upon death stimuli and translocates to the outer mitochondrial membrane where it forms oligomers. To verify if irradiation can sensitize $p14^{ARF}$ expressing cells for Bax activation, clustering of Bax was visualized by the use of a GFP-Bax fusion protein stably expressed in MCF-7 caspase-3 proficient cells (figure 37). Ad-lacZ infected cells showed diffuse staining of Bax after exposure to IR

representing its cytosolic localization. Cells infected with Ad-p14^{ARF} showed to a higher extent of Bax clustering compared to Ad-lacZ infected cells. This might be due to the upregulation but still not full activation of Bax upon p14^{ARF} expression. However, after infection with Ad-p14^{ARF} and additional exposure to IR, a dramatic increase of the cells with punctuated pattern of GFP-Bax was observed. These data demonstrate that clustering of proapoptotic Bax occurred after IR sensitization upon p14^{ARF} expression. This leads to mitochondrial permeabilization and finally to induction of apoptotic cell death.

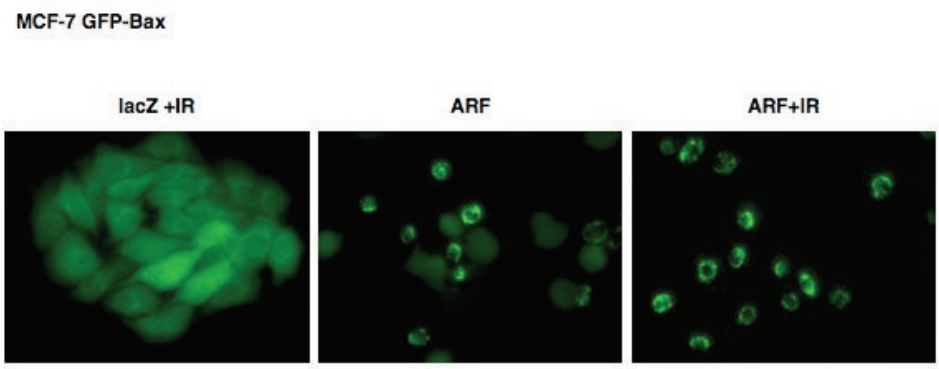

Figure 37. Irradiation sensitises p14^{ARF} expressing cells for Bax activation.
MCF-7 caspase-3 cells stably expressing GFP-Bax were infected with Ad-p14^{ARF} or Ad-lacZ and irradiated at 10Gy. Pictures were taken after 72h.

To identify if caspases play a role in the p14^{ARF} cell death pathway after exposure to irradiation, the activity of the two main caspases was investigated, namely initiator caspase-9 and executioner caspase-3. When MCF-7 caspase-3 deficient cells were infected with control virus, 2% of the cells showed caspase-9 activation. After additional exposure to IR, activation of caspase-9 was seen in 8% of the cells (figure 38A). In 9% of the cells infected with Ad-p14^{ARF}, caspase-9 activation was measured. Additional exposure to IR of p14^{ARF} expressing cells caused a slight increase in cells showing caspase-9 activation up to 20%.
2% of the control caspase-3 re-expressing cells showed caspase-9 activation upon infection with Ad-lacZ. Additional exposure to IR induces activation of caspase-9 in 10% of these cells. P14^{ARF} expressing cells showed caspase-9 activation in 18% of the cells in contrast to cells additionally exposed to IR where 33% of cells displaying activation of caspase-9 were measured.
Upon infection with control virus Ad-lacZ, caspase-7 activation was measured in up to 8% of caspase-3 deficient cells regardless of exposure to IR. 10% of p14^{ARF} expressing cells

displayed caspase -7 activation, which was increased up to 19% after IR (figure 38B). 2% of the control caspase-3 proficient cells displayed activation of caspase-3 and -7 and additional exposure to IR increases their activation up to 10%. Upon $p14^{ARF}$ expression the activation of caspase-7 was measured in 19% of caspase-3 deficient cells. In the presence of IR these cells presented increased activity of caspase-3 and -7, found in up to 38% of the cells. These data indicate that, upon sensitization by IR, $p14^{ARF}$ required the presence of the main executive caspase-3 to ensure full activation of caspases. This may indicate that caspase-3 induces a mitochondrial activation loop to ensure full activation of caspases-9 and -7.

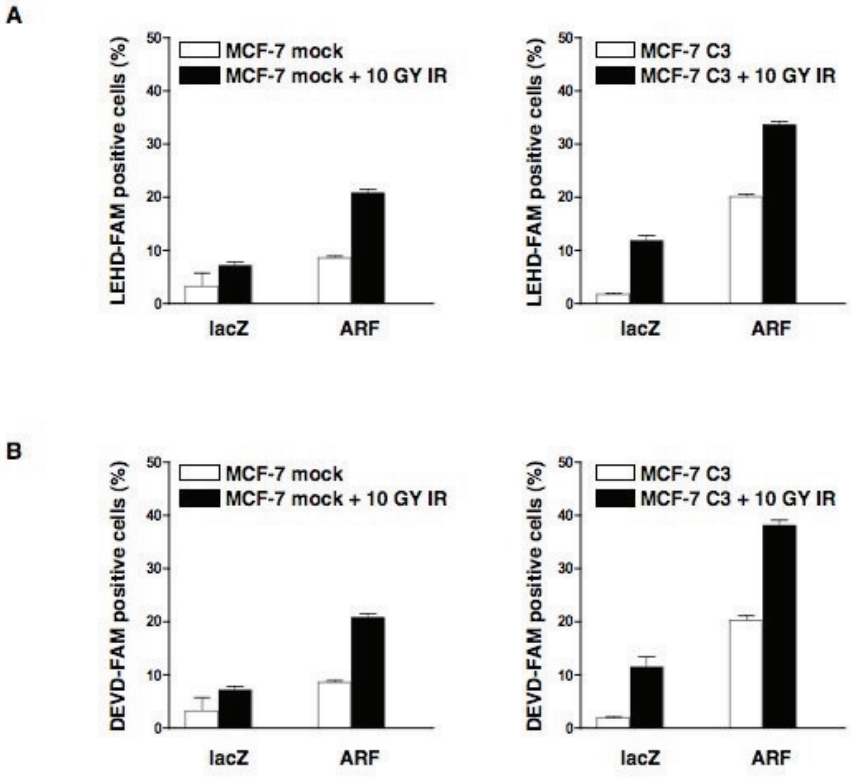

Figure 38. The initiator caspase-9 and the effector caspase-3 are activated after irradiation and $p14^{ARF}$ expression.

MCF-7 cells were transduced with Ad-$p14^{ARF}$ or Ad-lacZ and irradiated with 10Gy as indicated.
(A) After 96h, MCF-7 cells deficient and proficient for caspase-3 were incubated with a carboxyfluorescein labelled peptide specific for active caspase-9 (LEHD-FAM). Non-irradiated cells (white bars) and irradiated cells with 10Gy (black bars) were analysed.
(B) After 96h, MCF-7 cells deficient and proficient for caspase-3 were incubated with a a carboxyfluorescein labelled peptide specific for active for caspase-3 and -7 (DEVD-FAM). Non-irradiated cells (white bars) and irradiated cells with 10Gy (black bars) were analysed.
The activation of the caspases was measured by flow cytometry. Data are means ± SD of triplicates.

To further validate if caspases participate in the $p14^{ARF}$ pathway upon IR, specific caspase-inhibitors were employed. Namely, an inhibitor specific for caspase-3 and -7 (zDEVD-fmk) or for caspase-9 (zLEHD-fmk) was used. Caspase-3 deficient cells did not demonstrate apoptotic cell death after any kind of treatment, independent of exposure to IR (figure 39A). Caspase-3 proficient cells infected with the control virus in the presence or absence of the indicated inhibitor did not showed increased apoptotic rates independent of IR (figure 39B). 19% of the caspase-3 proficient showed apoptotic DNA fragmentation upon infection with Ad-$p14^{ARF}$, which was reduced to 15% in the presence of the caspase-9 inhibitor and to 8% in the presence of the caspase-3 inhibitor. Nevertheless, 39% of these cells showed apoptotic DNA fragmentation after infection with Ad-$p14^{ARF}$ and additional exposure to IR. This percentage of cell death was reduced to 32% in the presence of the caspase-9 inhibitor. However, additional treatment with caspase-3 inhibitor could diminish apoptotic cell death to 18%. These data suggest that caspase-3 is the key effector in $p14^{ARF}$ induced apoptosis cell death upon sensitization by IR.

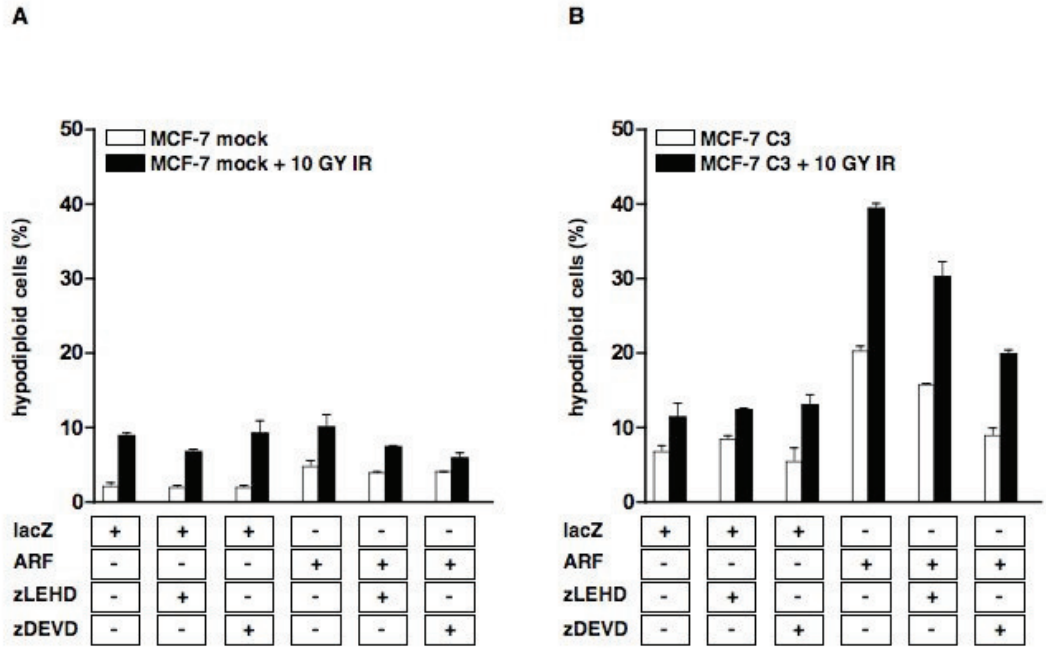

Figure 39. P14^{ARF} induces caspase-3 dependent apoptosis after irradiation.
(A) MCF-7 cells deficient for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ as indicated. Additionally cells were irradiated with 10Gy and cultured for 96h in the presence or absence of the caspase-9 inhibitor zLEHD-fmk (zLEHD) or the caspase-3 and -7 inhibitor zDEVD-fmk (zDEVD) as indicated. Data are means ± SD of triplicates.
(B) MCF-7 cells proficient for caspase-3 were transduced with Ad-p14^{ARF} or Ad-lacZ as indicated. Additionally cells were irradiated with 10Gy and cultured for 96h in the presence or absence of the caspase-9 inhibitor zLEHD-fmk (zLEHD) or the caspase-3 and -7 inhibitor zDEVD-fmk (zDEVD) as indicated. Data are means ± SD of triplicates.

5.7.2 Irradiation abrogates p14^{ARF} induced cell cycle arrest and loss of p21 is important for apoptosis

The next experiment focused on expression levels of proteins involved in cell cycle arrest and apoptosis upon expression of p14^{ARF} and exposure to 10Gy IR (figure 40). As previously described Ad-p14^{ARF} infected cells demonstrate high upregulation of p21, proapoptotic protein Bax as well as DNA damage marker γH2AX in both cell transfectants compared to the cells infected with control virus where these proteins were not detectable. No reduction of the proform and no cleavage product of caspase-3 could be detected as well. After IR, caspase-3 proficient and deficient MCF-7 cells demonstrate high expression of p21 in control cells. Disruption of p21 expression was detectable only in cells additionally overexpressing p14^{ARF}. Upregulation of γH2AX verified that in both caspase-3 deficient and

proficient cells, DNA damage occurred after exposure to IR. Caspase-3 proficient cells after IR and infection with control virus showed only the proform of caspase-3 and no caspase-3 processing. In contrast, upon infection with Ad-p14^{ARF} and additional exposure to IR, cells showed activation of caspase-3 detectable as its cleavage product. Bax was up regulated after IR regardless of the presence of caspase-3 in control cells as well as in Ad-p14^{ARF} infected cells. The level of proapoptotic protein Bak did not differ between both cell transfectants infected with Ad-lacZ or Ad-p14^{ARF}, regardless of exposure to IR. These data reveal that p14^{ARF} sensitizes cells to apoptosis in response to IR and induction of apoptosis depends on the presence of caspase-3. This may be related to the downregulation of p21 in cells expressing p14^{ARF} and exposed to IR.

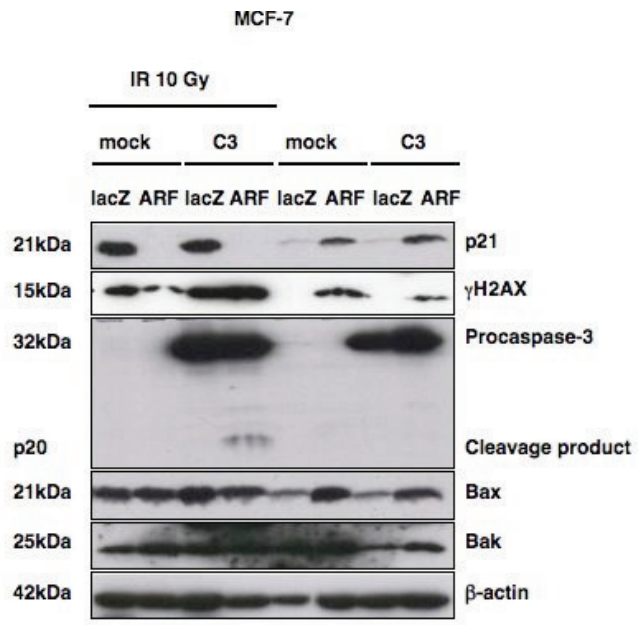

Figure 40. Western blot analysis of apoptosis-related proteins upon p14^{ARF} expression and exposure to IR.
Western blot analysis of MCF-7 cells deficient and proficient for caspase-3 after infection with Ad-lacZ or Ad-p14^{ARF} in the presence or absence of IR as indicated. β-actin detection served as a loading control.

DISCUSSION

6. Discussion

$P14^{ARF}$ is a tumour suppressor protein expressed at very low and undetectable levels in normal tissues. Overexpression or mutational activation of oncogenes induces the transcription of $p14^{ARF}$, which leads to cell cycle arrest, premature senescence or to cell death.

The investigations described here focused on the signalling pathway of $p14^{ARF}$ related to its involvement in cell cycle regulation and induction of different types of cell death.

Data from initial reports imply that the cell cycle arrest programs and apoptosis induction by $p14^{ARF}$ strictly depend on a p53/Mdm-2 (p53/Hdm-2 in humans) signalling axis. Once expressed, $p14^{ARF}$ interferes with the activity of Mdm2, leading to p53 stabilization and the triggering of p53-dependent transcriptional programs. The capacity of $p14^{ARF}$ to interfere with the apoptotic program by preventing the ubiquitination-dependent destruction of p53 via binding to Mdm-2 has been previously described (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998). By inactivating Mdm-2, $p14^{ARF}$ triggers apoptosis via a Bax/Bak-dependent pathway in p53-proficient colon carcinoma HCT116 cells (Hemmati et al., 2006). However, another pathway was proposed indicating that $p14^{ARF}$ also utilises p53 independent signalling events. Different groups investigated cell cycle arrest showing that cells undergo a G2 arrest in the absence of functional p53, suggesting that p53 might be required for G1 checkpoint arrest but dispensable for G2 phase arrest in response to $p14^{ARF}$ induction (Eymin et al., 2003). The ability of $p14^{ARF}$ to induce p53-independent apoptosis was evident in p53-deficient carcinoma cell models e.g. SAOS-2 and HCT116 as well as in DU145 cells (Hemmati et al., 2002). Other p53 family members, such as p73 and p63, can be activated contributing to programmed cell death induction in the absence of p53 (Nozell et al., 2003). In this line, it was reported that p73 is able to replace the function of p53 in adriamycin-treated, p53-deficient breast cancer cells (Vayssade et al., 2005). Nevertheless, the signalling requirements for the induction of both, p53-dependent and p53-independ ent cell cycle arrest or apoptosis by $p14^{ARF}$ remain to be defined in more detail. This is of crucial importance because loss of $p14^{ARF}$ not only mediates resistance to apoptosis, but also is involved in tumour progression and development of drug resistance (Schmitt et al., 1999; Sherr, 2006).

MCF-7 cells were shown to carry a 47-base pair deletion within exon 3 of the *CASP-3* gene that results in failure to express the procaspase-3 zymogen (Janicke et al., 1998). Caspase-3

deficient MCF-7 cells are resistant to drug induced apoptosis, however forced expression of procaspase-3 can overcome acquired chemoresistance, which leads to apoptosis (Radetzki et al., 2002). In contrast, irradiated MCF-7 cells did neither activate the mitochondrial pathway nor the processing of caspase-9 and -3 (Wendt et al., 2006). As a potential target strategy against tumour proliferation, expression of $p14^{ARF}$ was examined in MCF-7 breast carcinoma cells. Data obtained from the present study showed that MCF-7 cells are highly resistant to $p14^{ARF}$-induced apoptosis in contrast to other p53 proficient or deficient carcinoma models. However, investigations of the cell cycle arrest upon $p14^{ARF}$ expression in p53 wild type MCF-7 cells showed evidence for a G1 cell cycle arrest associated with upregulation of p53 as well as its transcriptional target p21. Upregulation of p21 leads to cell cycle arrest, which can be abrogated by caffeine. Caffeine is a derivate of methylxanthin and mediates the inhibition of the cell cycle ATM (for ataxia telangiectasia mutated) and ATR (for ataxia telangiectasia and Rad3-related) kinases which play a central role in DNA damage recognition (Rouse and Jackson, 2002). In MCF-7 cells, caffeine influenced the cell cycle arrest by inducing checkpoint abrogation but did not provoke cell death. The results presented here, show that caffeine leads to strong downregulation of the expression level of p21, which indicates disruption of $p14^{ARF}$ induced p21-mediated cell cycle arrest. Thus, one could speculate that the cells progress in the cell cycle upon caffeine treatment. $P14^{ARF}$ expression and caffeine treatment caused strong downregulation of cyclin D1, a G1 phase cyclin, and corresponding CDK4 and 6 in MCF-7 cells, demonstrating a transition from G1 phase to G2/M. Uncontrolled cell proliferation, a key characteristic of cancer, is often connected with alterations affecting the activation of cyclin D1 that controls the exit from G1 phase into the cell cycle. Unrestrained expression of cyclin D1 may lead to tumour development (Sherr, 1996). Breast carcinoma MCF-7 cells showed an extremely high expression of cyclin D1. Moreover, it was reported that cell proliferation induced by growth factor stimulation was mediated by cyclin D1 (Kerkhoff and Rapp, 1997; Pajalunga et al., 2008). The expression status of cyclin D1 was unchanged upon $p14^{ARF}$ expression compared to non-infected cells regardless of the presence of caspase-3. However, in consequence of exposure to caffeine, $p14^{ARF}$-induced cell cycle arrest led to cell death. This means that loss of checkpoint control results in induction of cell death that proceeds in MCF-7 cells via a caspase-3 dependent apoptosis pathway. The possible transition from G1 arrest was indicated by high upregulation of cyclin B1 known as a G2/M cyclin. To accurately distinguish between G2 and M phase of the cell cycle, cells were stained for the M phase marker MPM2. Cell cycle arrest was able to prevent progression into mitosis. The inability

of arrested cells to enter mitosis was confirmed by the decreased number of cells expressing the MPM2-phosphoepitope. Expression of $p14^{ARF}$ after additional treatment with caffeine induced a dramatic reduction of mitotic cells since caffeine facilitated progress through the cell cycle and cells died upon entering G2 phase. In the absence of caffeine, minor amounts of cells overexpressing $p14^{ARF}$ could be detected in mitotic phase. These results are supported by data obtained monitoring cyclin A expression levels, which reach a maximum during DNA synthesis. Upon $p14^{ARF}$ expression, regardless of the presence of caffeine, strong downregulation of S-phase cyclin A was observed, supporting the observation from ModFit analyses that there are no cells in S phase. Thus, the strong decrease of cyclin A protein levels by $p14^{ARF}$ might be very important to hinder S phase progression and M phase entry. This finding is supported by other reports showing that $p14^{ARF}$ impedes S phase progression and therefore could have a direct role in DNA replication (Yarbrough et al., 2002). Upon $p14^{ARF}$ expression, cell cycle arrest in S, M or G2 phases could be excluded based on the level of downregulation of the corresponding cyclins, leaving G1 as the most likely phase. However, additional treatment with caffeine seems to execute cell death from G2 phase considering the downregulation of G1, M and S phase cyclins. In this perspective, the presented results show that $p14^{ARF}$ expression induced G2 arrest in HeLa cells. In combination with caffeine, an inhibitor of ATM/ATR, cells underwent apoptosis similarly to MCF-7 caspase-3 proficient cells. Likewise, it has been shown that inhibition of ATM/ATR abrogates G2 phase cell cycle arrest (Bode and Dong, 2007). These findings suggest that $p14^{ARF}$ might utilize the ATM/ATR pathway to mediate cell cycle arrest. Additionally, these results revealed that $p14^{ARF}$ induces either G1 or G2 arrest dependent on functional p53. Evidence for a p53 independent cell cycle arrest induction in G1 comes from a study in p21 or p21/14-3-3 sigma knockout cells (Hemmati et al., 2008; Normand et al., 2005). HeLa cells which express mutated p53 showed G2 arrest, whereas MCF-7 cells, which express wild type for p53, were arrested in G1 phase. Therefore, $p14^{ARF}$ mediates either G1 or G2 arrest and thereby prevents cell growth. Abrogation of cell cycle arrest results in cell death.

Furthermore, upon $p14^{ARF}$ expression MCF-7 cells demonstrated high upregulation of the DNA damage marker γH2AX. Upon DNA double strand break (DSB) induction, H2AX, part of the histone complex stabilizing the DNA, is phosphorylated for the efficient recognition and/or repair of DSBs. DNA repair takes place before S phase in order to provide a correct DNA strand for replication. Failure of such treated cells to enter S phase is supported by the strong downregulation of cyclin A, suggesting that $p14^{ARF}$ may have

directly or indirectly induced DNA damage signalling. Similarly, it was reported that the induction of $p14^{ARF}$ in p53-deficient H358 lung carcinoma cells was accompanied by phosphorylated γH2AX implying that $p14^{ARF}$ could trigger DNA damage (Eymin et al., 2006). Previously, it was proposed that $p53^{-/-}$ cells accumulate in G2 following DNA damage (Powell et al., 1995). As already mentioned, HeLa cells were arrested in G2. As these cells express non-functional p53, it can be assumed that $p14^{ARF}$ expression induces DNA damage in these cells. But it is still controversial whether $p14^{ARF}$ either triggers DNA damage or, on the contrary, responds directly to DNA-damage signals.

6.1 $P14^{ARF}$ induces the intrinsic apoptosis pathway

In a next step, the present studies focused on the question whether arrested cells were viable upon $p14^{ARF}$ expression and caffeine treatment. Apoptosis measurements revealed that enforced $p14^{ARF}$ expression could not induce apoptotic DNA fragmentation in MCF-7 cells and moreover, the reconstitution of procaspase-3 expression failed to sensitize for $p14^{ARF}$-induced cell death. Similarly, no loss of cell viability was detected when the cells were transduced with Ad-$p14^{ARF}$. In MCF-7 cells, regardless of the caspase-3 status, $p14^{ARF}$ alone initiates a cell cycle arrest program but does not induce cell death. This was verified in the present MCF-7 system by the finding that p21 was upregulated after $p14^{ARF}$ expression. Previous studies showed that cell cycle arrest induced via upregulation of p21 is linked to the inhibition of cell death following expression of $p14^{ARF}$, however, in p21 inactivated HCT116 cells apoptosis induction by $p14^{ARF}$ was enhanced (Hemmati et al., 2005; Normand et al., 2005). This is in line with data describing that p21 upregulation could inhibit apoptosis downstream of mitochondria by blocking caspase-9 activity (Sohn et al., 2006). Due to sensitization with caffeine, $p14^{ARF}$ expressing cells showed downregulation of p21 indicating that this might be critical for cell death induction. Additionally, cells expressing $p14^{ARF}$ exposed to caffeine revealed massive induction of apoptosis but only in the presence of the main key effector caspase-3. These results confirmed that caspase-3 was essential for the execution of apoptotic cell death, which occurred in a time and dose dependent manner. In the same context, it was previously shown that re-expression of procaspase-3 restores the sensitivity for drug-induced apoptosis in resistant breast carcinoma cells (Friedrich et al., 2001).

The mitochondrial apoptosis signalling pathway relies on the activation of the multi-domain proteins Bax and/or Bak, their oligomerization and insertion into the outer mitochondrial

membrane which leads to release of cytochrome c, caspase activation, PARP cleavage and finally apoptotic DNA fragmentation (Goping et al., 1998). Adenoviral expression of $p14^{ARF}$ led to elevated levels of Bax independently of the presence or absence of caspase-3, however, no apoptosis was detected. These data demonstrate that Bax upregulation is not sufficient to mediate cell death in MCF-7 cells. Irradiation is known to induce DNA damage and therefore can lead to cell death. But irradiated MCF-7 cells did not undergo apoptosis despite of elevated Bax levels. Additionally, high expression levels of γH2HX verified induction of DNA damage provoked by irradiation as well as by $p14^{ARF}$. It may be assumed that DNA damage causes the upregulation of Bax, but was not sufficient to trigger apoptosis. In summary, upregulation of Bax does not necessarily imply its activation since several crucial steps, such as conformational change and oligomerization, are involved in pore formation by activated Bax at the outer mitochondrial membrane.

A main event of the intrinsic apoptosis pathway is the breakdown of the mitochondrial membrane potential. To clarify the mechanism of apoptosis induction by $p14^{ARF}$ in combination with caffeine, mitochondrial permeability shift transition were measured. Mitochondrial permeability shift and the release of cytochrome c in turn trigger the binding of dATP to Apaf-1 and the formation of the apoptosome. This is followed by the activation of caspase-9 that consequently cleaves and activates caspase-3. Interestingly, the mitochondrial membrane was not permeabilized and cytochrome c release did not occur upon $p14^{ARF}$ expression in MCF-7 cells. But $p14^{ARF}$ in combination with caffeine greatly stimulated the breakdown of the mitochondrial membrane potential in the presence of caspase-3. Thus, permeabilization of the mitochondria can be amplified through caspase-3 that involves caspase-8 and the cleavage of Bid in a feedback loop. Western blot experiments revealed that cytochrome c was released into the cytosol upon $p14^{ARF}$ expression in caspase-3 proficient cells sensitized with caffeine. These data therefore provide solid evidence that a persistent loss of mitochondrial membrane potential that is accompanied by cytochrome c release occurs in a caspase-3 dependent manner, suggesting that caspase-3 mediates a feedback loop to amplify the mitochondrial apoptosis signalling.

In contrast to MCF-7 caspase-3 proficient cells, cells deficient for caspase-3 displayed mitochondrial dysfunction i.e. loss of mitochondrial membrane potential without demonstrating apoptotic cell death after infection with Ad-$p14^{ARF}$ and incubation with caffeine. Those cells showed delayed kinetics of the mitochondrial membrane permeability shift but no cytochrome c release. These data suggest that an early and prominent loss of the mitochondrial membrane potential followed by cytochrome c release occurs only when

caspase-3 is activated. Caspase-3 therefore serves as an amplifier of the initial death signal by promoting cytochrome c release. This executioner caspase might therefore act ”upstream“ of the mitochondria to mediate mitochondrial permeability via Bid and a mitochondrial amplification loop since a weak mitochondrial apoptosis signal was observed in MCF-7 cells deficient for caspase-3. However, the presence of post mitochondrial caspase-3 processing is required to trigger a positive feedback loop, which would lead to amplification of the mitochondrial apoptosis signalling and full activation of the caspase network. These data are supported by the report that caspase-3 and -7 are crucial for apoptosis and contribute to some mitochondrial events and further may serve to amplify the initial death signal by helping to promote cytochrome c release (Lakhani et al., 2006; Wieder et al., 2001).

Addition of a pan-caspase inhibitor Q-VD-OPh, prevented permeabilization of mitochondria and therefore completely impaired cell death induction upon $p14^{ARF}$ expression after sensitization with caffeine. The possibility of caspase-dependent disruption of mitochondria during the apoptotic process was observed previously in other studies (Waterhouse et al., 2001). Caspases affect the mitochondria through disruption of the functions of the electron transport chain complex I and II, resulting in loss of mitochondrial membrane potential and generation of reactive oxygen species (ROS) (Ricci et al., 2003). Mitochondria release ROS and inhibition of caspases by the pan-caspase inhibitor can also block ROS production. Therefore, mitochondria might be among the earliest targets of activated caspases during apoptosis and affect the function of the electron transport chain, which might be a crucial factor for the process of caspase-dependent cell death. HeLa cells treated with apoptosis-inducing cytostatic drug actinomycin showed both delayed, mitochondrial permeability shift and delayed cell death after addition of the pan-caspase inhibitor zVAD-fmk (Ricci et al., 2003). The function of caspases therefore seems to be essential for the rapid onset of apoptotic events. As opposed to the current model that caspases are initially activated downstream of the mitochondria, it could be assumed that they also fulfil “upstream” functions such as enhancement of the mitochondrial death signal. However, the relationship between disruption of mitochondrial membrane potential and caspase activation is still controversial. Specifically, the initiator caspase responsible for triggering of caspase-3 in $p14^{ARF}$ induced apoptosis remain to be identified albeit caspase-9/LEHDase have recently been implicated (Hemmati et al., 2006; Hemmati et al., 2008).

Proteins of the Bcl-2 family were shown to regulate mitochondrial outer membrane potential and overexpression of Bcl-2 prevented the mitochondrial disruption and inhibited the release

of cytochrome c reviewed by (Green and Kroemer, 2004). Antiapoptotic members of the Bcl-2 family were able to prevent both caspase-dependent and -independent cell death processes (Okuno et al., 1998). Antiapoptotic Bcl-2 is an integral membrane protein able to insert into the outer mitochondrial membrane, the endoplasmatic reticulum (ER) and the nuclear envelope reviewed by (Strasser et al., 2000). When Bcl-2 was specifically targeted to the mitochondria or to the ER it could prevent apoptosis (Zhu et al., 1996). As mentioned before, $p14^{ARF}$ induced cell cycle arrest whereas in combination with caffeine cells underwent apoptosis. To identify the potential organelle that could be a part of the $p14^{ARF}$ signalling, SW480 cell transfectants, which stably overexpressed Bcl-2 targeted to the mitochondria or to the ER, were employed. Apoptosis induction by $p14^{ARF}$ in SW480 cells in the presence of caffeine indicated that antiapoptotic proteins are involved in this pathway. In control SW480 cells, which did not overexpress Bcl-2, infection with Ad-$p14^{ARF}$ in combination with caffeine induced a high loss of cell viability as well as strong apoptosis induction. Bcl-2 targeted to the mitochondria entirely protected the cells from apoptosis upon $p14^{ARF}$ expression in the presence of caffeine. Namely, the reduced loss of cell viability as well as massively decreased levels of apoptotic DNA fragmentation followed protection of the mitochondria. Furthermore, it was also shown that ER-targeted Bcl-2 interacts with Bax and prevents mitochondrial membrane permeabilization (Ferri and Kroemer, 2001). However, Bcl-2 targeted to the ER was not able to inhibit apoptosis in the and did not have any effect on cell viability in the SW480 cell system. In terms of the apoptotic rate, cells either overexpressing Bcl-2 at the ER or without Bcl-2 overexpression showed similar results suggesting that $p14^{ARF}$ after sensitization with caffeine does not employ the ER stress pathway. The fact that Bcl-2 localized at the mitochondria completely inhibited apoptosis supports the theory that $p14^{ARF}$ utilizes the mitochondrial apoptotic pathway. Additionally, these data provide evidence that, after sensitization with caffeine, $p14^{ARF}$ predominantly targets the mitochondria to execute cell death.

6.2 Activation of caspases upon overexpression of $p14^{ARF}$ and caffeine treatment

Caspases are important members of the apoptotic machinery. Caspase-9 is a key initiator caspase of the intrinsic apoptosis pathway and is recruited by Apaf-1 to the apoptosome to become active by induced proximity, thereby activating the executor caspase-3. The following studies were addressed in order to provide more evidence for caspase activation during apoptosis and cell cycle checkpoint abrogation induced by $p14^{ARF}$. By use of a FITC-

labelled caspase specific peptide substrate, the activity of two main caspases could be monitored. Namely, fluorescent caspase substrate FITC-LEHD-fmk was used to assess caspase-9 activity and FITC-DEVD-fmk to analyze caspase-3 and -7. Upon $p14^{ARF}$ expression and sensitization with caffeine, MCF-7 cells re-expressing caspase-3 illustrated high levels of caspase-9 and caspase-3 activation. Under the same condition, cells deficient for caspase-3 showed a reduced level of caspase-9 activation. Although these cells express caspase-7 that is, like caspase-3 DEVDase, they did not display activity of this caspase measured with FITC-DEVD-fmk. Of note, this indirectly provides evidence that caspase-7 cannot substitute for the lack of caspase-3 during $p14^{ARF}$-induced apoptosis upon sensitization with caffeine. Taken together, these data reveal that caspase-9 and caspase-3 are both involved in the $p14^{ARF}$ apoptotic pathway upon sensitization with caffeine. However, caspase-9 activation depends on the presence of the key executive caspase-3 because loss of caspase-3 impairs loss of mitochondrial permeability as well as cytochrome c release. In MCF-7 caspase-3 deficient cells only a weak activation of caspase-9 was observed. These findings suggest that the presence of caspase-3 processing was necessary for the full activation of the caspase network indicating a positive feedback loop in order to amplify the apoptosis signal.

For further confirmation of the functional relevance of caspase activation in MCF-7 caspase-3 proficient cells in the $p14^{ARF}$ signalling pathway, cells were incubated with different caspase inhibitors that irreversibly bind to the active core of the enzyme. The apoptotic rate was dramatically reduced once the cells infected with Ad-$p14^{ARF}$ were cultured with the pan-caspase inhibitor zVAD-fmk or the caspase-3/-7 inhibitor zDEVD-fmk in the presence of caffeine. It seems that caspase-9 is not crucial for the $p14^{ARF}$ signalling pathway as a much lower inhibition of apoptosis was achieved by the caspase-9 inhibitor zLEHD-fmk. These results were confirmed by Western blot analyses, where processing of procaspase-9 was detected by its reduced proform and processing of procaspase-3 by its cleavage product upon $p14^{ARF}$ expression after sensitization with caffeine. The data obtained by these caspase activity and inhibitory studies suggest that $p14^{ARF}$, upon co-treatment with caffeine, mediates apoptosis in a caspase dependent manner. Especially capase-3 appears to be the most important factor for induction of apoptosis since, in the absence of caspase-3, $p14^{ARF}$ in the combination with caffeine utilizes an alternative pathway to conduct cell death.

Further downstream events in cells undergoing apoptosis are represented by the cleavage of the caspase-3 substrate PARP thereby facilitating DNA fragmentation (Boulares et al.,

1999). PARP cleavage prevents ineffective repair of DNA strand breaks during the apoptotic program (Boulares et al., 1999). PARP is able to estimate the extent of DNA damage in order to decide whether the DNA should be repaired or if the damage is too extensive and cells should undergo apoptosis (Woodhouse and Dianov, 2008). PARP cleavage prevents ATP depletion by polyADP rybosilation thereby ensuring the energy dependent development of the apoptotic phenotype. Immunocytochemical analysis of PARP demonstrated that it was cleaved upon $p14^{ARF}$ expression after additional treatment with caffeine in caspase-3 proficient cells. Hypothetically, the cells shifted through the cell cycle, underwent DNA damage and cleaved PARP and activated caspases supported apoptotic DNA fragmentation. Cleavage of PARP would not occur in cells lacking caspase-3 since its activation was impaired. These results were consistent with the measurements of apoptotic DNA fragmentation, where it was shown that cells proficient for caspase-3 do undergo apoptosis, in contrast to cells lacking caspase-3 where apoptotic cell death was not observed.

6.3 Autophagy as an alternative cell death mechanism

The functional relationship between apoptosis and autophagy is still controversial in the sense that in one instance, autophagy is utilized as a stress adaptation mechanism that avoids cell death, whereas in other cellular settings autophagy represents an alternative pathway to cellular demise (Baehrecke, 2005). Upon apoptotic stimuli, blocking of caspase activation therefore seems to convert the apoptotic phenotype of dying cells to the autophagy type of cell death. Autophagy is therefore able to trigger caspase-independent cell death in the absence of caspase activation (Scarlatti et al., 2009).

The conclusion of the existing results points out that sensitization with caffeine upon $p14^{ARF}$ expression requires the presence of caspase-3 for full activation of the intrinsic apoptotic pathway to execute apoptosis. However, based on the different morphology, observed by phase contrast microscopy, $p14^{ARF}$ expressing MCF-7 cells lacking caspase-3 appeared to form autophagic vacuoles upon exposure to caffeine. These cells were round and detached and vacuoles were visible inside them. These observations led to the assumption that $p14^{ARF}$ is involved in the induction of autophagy. This effect was not detected in caspase-3 proficient cells, which clearly showed the morphology of apoptotic cells upon the same conditions. To clarify the fate of cells deficient for caspase-3 transduced with Ad-$p14^{ARF}$ and exposed to caffeine, cell viability was measured. Obtained data showed high loss of cell viability. Caspase-3 proficient cells also demonstrated strong loss of cell viability under the

same condition. Considering that the apoptotic pathway was already proven to be employed by caspase-3 proficient cells, this result confirmed previous investigations. These findings indicate that each cell transfectant utilizes different pathways to conduct cell death that invariantly occurs as evidenced by the loss of cell viability after sensitization with caffeine upon $p14^{ARF}$ expression.

To clarify whether these morphological features were in fact signs of autophagy, the most prominent autophagic proteins were examined by Western blot analyses and fluorescence microscopy in MCF-7 cells stably expressing GFP-LC3. During autophagy, lipid conjugation leads to the conversion of the soluble LC3I form into the faster migrating LC3II form in the SDS-PAGE. Upon expression of $p14^{ARF}$ in combination with caffeine GFP-LC3II, the autophagic variant, was identified in caspase-3 deficient cells indicating that these cells employed autophagy. The non-autophagic form of GFP-LC3I was detected in caspase-3 proficient cells upon the same conditions and moreover caspase-3 were found to be activated. This illustrates again that in the presence of caspase-3 MCF-7 cells undergo apoptosis. Upon sensitization with caffeine, $p14^{ARF}$ expressing caspase-3 deficient cells showed LC3 lipidation and high loss of cell viability suggesting that constant cellular stress triggered by $p14^{ARF}$ leads to the massive destructive potential of autophagy. In healthy cells, LC3 is a cytosolic protein but upon autophagy stimuli it is translocated to the autophagosomes. Induction of autophagic cell death upon $p14^{ARF}$ expression and caffeine treatment was evidenced by live cell imaging analyses confirming a high increase in the number of caspase-3 deficient cells with a punctuated fluorescent GFP-LC3 pattern. This redistribution from a diffuse to a punctuated pattern reflected accumulation of autophagic vesicles. After the same treatment, caspase-3 re-expressing cells, previously verified as apoptosis executing cells, did not demonstrate punctuation of GFP-LC3. These results imply that autophagy, as an alternative way of dying, can contribute to cell death in cells deficient for the downstream effector caspase-3. Following this assumption the decision for initiation of autophagy would be made downstream of the mitochondrial events. A lesser extent of mitochondrial breakdown would argue for insufficient stimulation of the mitochondria-caspase amplification loop due to caspase-3 deficiency and consequently result in a lack of cytochrome c release. Supposing that cells deficient for caspase-3 undergo autophagy, damaged mitochondria would be degraded by autophagolysosomes. It was reported by other groups that during autophagy the removal of the damaged mitochondria might be essential to trigger cell death. Mitochondria with altered membrane potential are especially sensitive to autophagic sequestration (de Grey, 1997; Reggiori and Klionsky, 2002). Furthermore, it

was shown that mitochondria that undergo permeability transition are digested within autolysosomes (Elmore et al., 2001) and that induction of autophagy is enhanced by mitochondrial permeability transition pore opening (Lemasters et al., 1998). A lesser and delayed mitochondrial membrane shift was observed in MCF-7 cells deficient for caspase-3 in contrast to caspase-3 re-expressing cells. Moreover, in these cells no cytochrome c release could be detected by Western blot analysis. This might be due to a delayed breakdown of mitochondrial membrane potential. Given that all damaged mitochondria are digested within autophagolysosomes, it seems unlikely that cytochrome c would be released under these circumstances into the cytosol. These results indicate that caspase-3 deficient cells undergo autophagy as a form of alternative cell death. Therefore, during prolonged stress an autophagic cell death is capable of destroying major proportions of the organelles leading to a total collapse of the cellular functions.

Confirming the previous findings, autophagosome formation could be successfully blocked by 3-methyadenine (3-MA), a specific pharmacological autophagy inhibitor reassuring that in the absence of caspase-3 cells stimulate the alternative cell death pathway. Taken together, the switch from apoptosis to autophagy, upon prolonged stress due to $p14^{ARF}$ expression and sensitization with caffeine, might serve as a fail-safe mechanism to eliminate damaged and apoptosis-deficient cells.

The most prominent of the autophagic proteins, Beclin-1 mediates autophagy induction and has a crucial role in autophagic vesicle formation. Interestingly, it was recently identified as a BH3-only protein because it contains a BH3 domain (Maiuri et al., 2007; Oberstein et al., 2007). Once upregulated, Beclin-1 does not induce apoptotic cell death indicating that in contrast to other BH3-only proteins, it has no proapoptotic properties and fails to neutralize the antiapoptotic function of Bcl-2 (Ciechomska et al., 2009). Both MCF-7 GFP-LC3 cell transfectants constitutively express high levels of Beclin-1. However, caspase-3 proficient cells illustrated a significant reduction in the expression level of Beclin-1 upon $p14^{ARF}$ expression in combination with caffeine. These data suggest that Beclin-1 might be a key target of caspase-3 since this study demonstrated particular downregulation of Beclin-1 but only when caspase-3 was present. Similar to these findings, it was recently shown that Beclin-1 is cleaved by caspases in TRAIL treated HeLa cells suggesting that Beclin-1 could be a novel caspases-3 substrate in cells undergoing apoptosis (Cho et al., 2009). Equally important, this result verifies that the apoptotic pathway is preferred in case of caspase-3 availability and therefore Beclin-1 is down regulated, not presenting the opportunity for induction of autophagy. In caspase-3 deficient cells, the level of Beclin-1 remained the same

implying indirectly that Beclin-1 was necessary to accomplish autophagy. One can suggest that Beclin-1 represents a potentially important point of convergence of the apoptotic to the autophagy pathway (Boya et al., 2005). Regardless of the caspase status, cells appeared to have started with the apoptotic program, which was interrupted in cells lacking caspase-3. This disturbance of the apoptotic program was also observed in cells deficient for the multidomain proapoptotic proteins Bax/Bak in which apoptosis switched to autophagy (Wei et al., 2001). In cells re-expressing caspase-3, $p14^{ARF}$ expression combined with caffeine triggered a strong downregulation of antiapoptotic Bcl-2 as well as Bcl-x_L leading to apoptotic cell death. Therefore, downregulation of these two antiapoptotic proteins might confirm apoptosis induction, which was shown to be strictly dependent on the activation of caspase-3 upon $p14^{ARF}$ expression and caffeine treatment. Moreover, several studies have demonstrated that upon apoptotic stimuli cleavage of Bcl-2 in MCF-7 cells require activation of caspase-3 (Kirsch et al., 1999). Beclin-1 was identified as an interaction partner of both antiapoptotic Bcl-2 and Bcl-x_L (Liang et al., 1999). In the absence of caspase-3 the levels of Bcl-2, Bcl-x_L and Beclin-1 remained unchanged upon $p14^{ARF}$ expression and exposure to caffeine confirming apoptosis impairment. In response to autophagy-inducing stimuli, Beclin-1 binds to Bcl-2 in a regulated manner (Maiuri et al., 2007; Oberstein et al., 2007). Thus, it could be proposed that the Bcl-2/Beclin-1 complex may act as a rheostat that turns autophagy on or off as required (Pattingre et al., 2005). The interaction between Beclin-1 and Bcl-2 can be competitively disrupted by other BH3 proapoptotic proteins thereby liberating Beclin-1 as this protein has a reduced affinity for Bcl-2 compared to the other BH3-only proteins (Feng et al., 2007). This may explain the survival feature of autophagy as a first reaction to minor cytotoxic insults, whereas prolonged stress stimulates the cell death characteristics of autophagy. However, the role of the interaction between Beclin-1 and Bcl-2/-x_L on autophagy activity is still unclear. As previously discussed, caspase-3 can cleave Beclin-1. Nevertheless inhibition of this caspase by a pan-caspase inhibitor could entirely restore its expression level. Similarly, the pan-caspase inhibitor was also able to prevent downregulation of Bcl-2. Due to the inhibition of the caspases, apoptosis was blocked and therefore the expression of Bcl-2 remained the same as in untreated cells. Additionally, the same broad caspase inhibitor repressed autophagy confirmed by a lack of lipidation of LC3. This phenomenon could be explained by earlier reports demonstrating that the pan-caspase inhibitor blocks other cysteine proteases including cathepsins and calpains (Madden et al., 2007). Calpains belong to the second family of calcium-activated cysteine proteases (Wang, 2000). Like caspases, they are

synthesized as inactive proenzymes and require processing to become active. Caspases facilitate calpain activity through the cleavage of its endogenous inhibitor calpastatin (Porn-Ares et al., 1998; Wang et al., 1998). Calpains directly proteolyse executioner caspases, mediating their activation and have been established to be necessary for autophagy induction. They may modulate one or more key components of the signalling and trafficking networks involved in autophagosome formation (Demarchi et al., 2007; Demarchi et al., 2006). Therefore in analogy to apoptosis, the pan-caspase inhibitor was very potent to suppress autophagic death (Pyo et al., 2005). Inhibition of all caspases could also block cathepsins, relatives of calpains, protecting cells entirely from apoptosis. Therefore, both caspases and cathepsins seem to be involved in apoptosis as well as the autophagic signalling pathway (Scaringi et al., 2004). ATG5, a very important autophagic protein required for the autophagosome-precursor synthesis, also has been reported to be targeted to mitochondria and is a novel calpain substrate. The ATG5 fragment produced by calpain cleavage regulates apoptosis as well as autophagy (Yousefi et al., 2006). ATG5 cleavage to truncated ATG5 (tATG5) is mediated by calcium-dependent calpains leading to the binding to ATG12 (Codogno and Meijer, 2006). Autophagy induction in calpain deficient cells showed no lipidated form of LC3 (Demarchi et al., 2006). Moreover, calpain inhibition by pharmacological agents or knockdown via RNA interference (RNAi) blocked ATG5 cleavage to tATG5 in cells executing cell death (Yousefi et al., 2006). Therefore, the inhibition of calpain activity by a pan-caspase inhibitor might result in suppression of autophagy. In contrast to the inhibition of all the caspases, blocking caspase-8 appeared to contribute to the switch from apoptosis to autophagy. MCF-7 GFP-LC3 caspase-3 proficient cells treated with the caspase-8 inhibitor zIETD-fmk presented to some extent a decrease in the apoptotic rate upon expression of $p14^{ARF}$ in the presence of caffeine. These data suggest that apoptosis was partially blocked in the cells proficient for caspase-3. An interesting aspect of this study came, however, from the observation that inhibition of caspase-8 activated autophagic cell death in the presence of caspase-3. The expression of the LC3II autophagic variant was detected upon $p14^{ARF}$ expression in the presence of caffeine and the caspase-8 inhibitor. Thus, in caspase-3 proficient cells, inhibition of caspase-8 may interrupt the caspase cascade network and switch therefore the apoptotic program to autophagy. Apoptosis induction was abrogated by the inhibition of caspase-8 and interestingly cells were able to utilize the alternative, autophagy pathway displaying a high loss of cell viability regardless of the presence of the inhibitor. In this constellation, the caspase-8 inhibitor diminished induction of apoptosis but did not prevent autophagic cell death in

caspase-3 proficient cells. Similarly, studies in mouse L929 fibroblasts demonstrated high induction of autophagy after caspase-8 inhibition (Yu et al., 2004). Presented data demonstrated that in the absence of caspase-3 and by the inhibition of caspase-8, autophagic cell death occurred, as confirmed by LC3II conversion as well as by the high loss of cell viability. According to these results, it can be proposed that caspase-8 might not be part of the autophagy progression. Inhibition of caspase-8 can convert the induced apoptosis process to autophagy, which leads to the assumption that the often discussed crosstalk between autophagy and apoptosis does exist. When all caspases are inhibited by use of a pan-caspase inhibitor, the induction of autophagy was entirely blocked. Lack of either caspase-3 or caspase-8 function hindered apoptosis and led to autophagy induction upon $p14^{ARF}$ expression in combination with caffeine. Of note, caspase-7 is highly related and structurally similar to caspase-3 (Lakhani et al., 2006). Therefore, caspase-7 that is expressed in MCF-7 cells may theoretically substitute for caspase-3. However, in this case, caspase-7 could not complement for caspase-3 deficiency and therefore was also not able to prevent autophagy cell death. Moreover, distribution of LC3, from a diffuse to a punctuated pattern, confirmed autophagy formation upon inhibition of caspase-7 by DEVD-fmk in the absence of caspase-3. It can be assumed that induction of autophagy is caspase-7 independent since treatment with $p14^{ARF}$ and caffeine with or without caspase-7 inhibition displayed a similar effect in terms of LC3 localization, represented by the increased numbers of autophagosomes.

6.4 Cell death mechanisms induced by $p14^{ARF}$ in combination with irradiation

DNA-damaging agents, such as chemotherapeutic drugs or ionizing radiation, are known to induce apoptosis via the intrinsic, mitochondrial cell death pathway (Belka et al., 2001; Handrick et al., 2009). Of note, MCF-7 breast carcinoma cells, whether they express the key executioner caspase-3 or not, are especially resistant to irradiation-induced apoptosis, although they have a functional p53. In general, irradiation induces apoptosis in cancer cells at higher doses and transient cell cycle arrest or senescence at lower doses. However, this effect was not observed in MCF-7 breast cancer cells, instead a G2 arrest was triggered in these cells (Janicke et al., 2001; Wendt et al., 2006). Thus, both the caspase-3 proficient and deficient MCF-7 cells remained equally resistant to irradiation-induced cell death. Equally to previous reports, in this study irradiation induced a permanent G2-arrest in MCF-7 cells irrespective of caspase-3 proficiency. Although irradiation alone did not provoke an

apoptotic response in caspase-3 proficient cells, additional expression of $p14^{ARF}$ resulted in massive apoptosis. Therefore, the resistance to irradiation appears to result directly from the failure to trigger the intrinsic cell death pathway upon activation of the DNA damage checkpoint. $P14^{ARF}$ was able to restore induction of the mitochondrial apoptotic pathway upon exposure to irradiation but only in the presence of caspase-3. Inhibition of the caspases reduced DNA fragmentation, verifying that apoptotic cell death occurred in a caspase dependent manner following $p14^{ARF}$ expression upon additional exposure to irradiation. Single inhibition of caspase-3 almost entirely decreased induction of apoptosis. Likewise, caspase-3 and -7 activities were strongly initiated in the caspase-3 proficient but only marginally detectable in the caspase-3 deficient MCF-7 cells. Caspase-9 inhibition, however, did not have a major hindering effect on apoptosis in the presence of caspase-3. It was therefore not surprising that in cells lacking caspase-3 the already low apoptotic rate did not change upon caspase-9 inhibition. Only caspase-3 proficient MCF-7 cells showed high induction of caspase-9 enzymatic activity when exposed to irradiation upon expression of $p14^{ARF}$, which leads to the assumption that caspase-9 processing is caspase-3 dependent. Most likely, full caspase-9 processing is achieved by the amplification loop where caspase-8 cleaves Bid to induce strong cytochrome c release from the mitochondria, formation of the apoptosome and consequently, activation of caspase-9.

Mitochondria losing their membrane potential indicate the initiation of the intrinsic apoptosis pathway leading to DNA fragmentation. In this setting, caspases appear to act upstream of the mitochondrial events since their inhibition led to protection from mitochondrial permeabilization. To this end, upregulation and clustering of the pro apoptotic protein GFP-Bax as detected by a punctuated, mitochondrial staining pattern, was detected upon $p14^{ARF}$ in combination with irradiation. The same observation but to a much lesser extent was seen in cells expressing only $p14^{ARF}$. These data suggest that the elevated level of activated Bax might be the response to the extended stress generated by $p14^{ARF}$ in combination with irradiation. The upregulation of γH2AX, in both cell transfectants, additionally pointed to DNA damage in irradiated or $p14^{ARF}$ expressing cells. In normal cell progression, these cells would be arrested in the cell cycle in order to start the repair program. But the downregulation of p21 showed that, after $p14^{ARF}$ expression and irradiation, the cells overcame cell cycle arrest regardless of the caspase-3 status. The significance of the turnover of p21 induced by ionising irradiation might be associated with an abrogation of DNA repair programs leading to cell death. As reported by Wendt et al.

2005, cell cycle arrest and apoptosis are two parallel processes orchestrated, by the level of p21.

Lack of caspase-3 disabled MCF-7 cells to undergo apoptosis regardless of $p14^{ARF}$ expression and irradiation. Based on the results discussed previously, these cells might utilize autophagy as a cell death pathway. This might simply be a protective response i.e. fail safe mechanism to ensure that cells are removed either via apoptosis or autophagy in order to prevent aberrant and unwanted cells from re-entering cell cycle division. Abrogation of checkpoints in the cell division cycle overcomes these protective programs and confers susceptibility for $p14^{ARF}$-induced cell death.

Based on the findings presented throughout this thesis, the following model can be proposed for the $p14^{ARF}$ pathway. Once expressed, $p14^{ARF}$ interferes with the activity of Mdm2, leading to p53 stabilization and triggers p21 upregulation thereby inducing cell cycle arrest. The kinase inhibitor caffeine abrogates cell cycle arrest and therefore enables cells to re-enter the cycle. Following the aberrant disruption of a cell cycle arrest program, $p14^{ARF}$ triggers the permeabilization of the mitochondria, which may involve BH3 only proteins induced by $p14^{ARF}$. The mitochondrial "activation" is the decision point for the type of cell death where caspase-3 plays a crucial role. The cell fate furthermore depends on caspase-8.

In the presence of caspase-3, mitochondrial permeabilization leads to the release of cytochrome c (figure 41A). This in turn triggers the formation of the apoptosome and activation of caspase-9 resulting in the initiation of the executioner caspases network. Caspase-3 or -8 can cleave Bid to tBid, which translocates to the mitochondria. There, it inserts into the mitochondrial membrane increasing permeabilization of mitochondria accompanied by cytochrome c release. This positive feedback loop amplifies the apoptotic signal and can only occur in the presence of caspase-3. All together, these events lead to apoptotic DNA fragmentation and finally to apoptosis. Blocking of caspase-8 partially reduced apoptosis in favour of autophagy. However, when the antiapoptotic protein Bcl-2 or its homologous Bcl-xL prevents the mitochondrial permeabilization, apoptosis is blocked.

In the absence of caspase-3, $p14^{ARF}$ also triggers the mitochondrial shift transition but cytochrome c is not released into the cytosol since this might already occur in the mitochondria that have entered autophagosomes and therefore the amplification loop is blocked (figure 41B). In this case Beclin-1 becomes active and LC3 is lipidated leading to further formation of autophagosomes and, eventually, to autophagic cell death.

However, when all caspases are inhibited, neither of both deadly processes, apoptosis and autophagy, can be conducted.

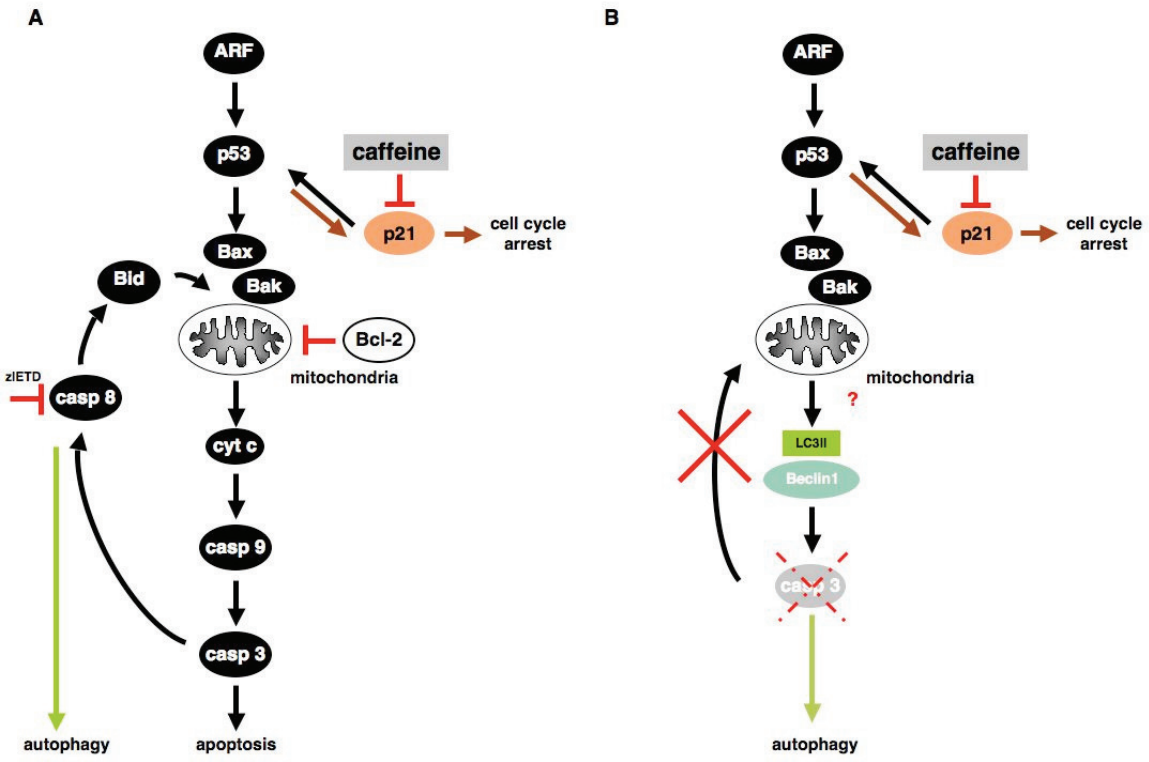

Figure 41. Model for p14^{ARF} pathway

These observations strongly support the idea that p14^{ARF} is involved in the cell death program utilizing both, autophagy and apoptosis pathways. These characteristics of p14^{ARF} make this tumour suppressor protein a potential target in cancer therapy. Facing the problem of irradiation-resistant breast cancer cells, p14^{ARF} could serve as an effective sensitizer for apoptosis. Although autophagy is primarily considered as a cell survival mechanism, it has been shown to function as a tumour suppressor process protecting cells from tumour development (Levine and Kroemer, 2008). Based on the finding that γH2AX was phosphorylated, it can be speculated that the involvement of p14^{ARF} in DNA damage and in the DNA repair program might be the reason for its participation in cell death induction. This could be the cause for the sensitize ability of p14^{ARF} to caffeine or ionising irradiation. However, the role of p14^{ARF} in DNA damage remains to be investigated in more details as well as its function in the DNA repair program. The interplay in between apoptosis and autophagy stimulated by p14^{ARF} could provide a promising tool for cancer therapy.

In this context, p14^{ARF} might have a role as a potential target for cancer therapy in order to diminish or even to kill tumour cells. In combination with caffeine or ionising irradiation, p14^{ARF} showed a massive cell death inducing potency. In contrast, the induction of cell

cycle arrest by $p14^{ARF}$ regulated programs is important for the possibility to manipulate the cell cycle and aberrant, deregulated cellular proliferation, which might be crucial to prevent uncontrolled cancer cell progression. However, abrogation of the checkpoint program leads to different types of cell death opening up a challenging field of research. These results therefore suggest a mechanism, which could provide an opportunity to overcome cell death resistance in malignant cells that may occur in consequence of activation of cell cycle arrest programs.

REFERENCES

7. References

Acehan, D., Jiang, X., Morgan, D.G., Heuser, J.E., Wang, X. and Akey, C.W. (2002) Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. *Mol Cell*, **9**, 423-432.

Adesanya, M.R., Redman, R.S., Baum, B.J. and O'Connell, B.C. (1996) Immediate inflammatory responses to adenovirus-mediated gene transfer in rat salivary glands. *Hum Gene Ther*, **7**, 1085-1093.

Alnemri, E.S., Livingston, D.J., Nicholson, D.W., Salvesen, G., Thornberry, N.A., Wong, W.W. and Yuan, J. (1996) Human ICE/CED-3 protease nomenclature. *Cell*, **87**, 171.

Antonsson, B., Conti, F., Ciavatta, A., Montessuit, S., Lewis, S., Martinou, I., Bernasconi, L., Bernard, A., Mermod, J.J., Mazzei, G., Maundrell, K., Gambale, F., Sadoul, R. and Martinou, J.C. (1997) Inhibition of Bax channel-forming activity by Bcl-2. *Science*, **277**, 370-372.

Arellano, M. and Moreno, S. (1997) Regulation of CDK/cyclin complexes during the cell cycle. *Int J Biochem Cell Biol*, **29**, 559-573.

Ashkenazi, A. and Dixit, V.M. (1998) Death receptors: signaling and modulation. *Science*, **281**, 1305-1308.

Aslanian, A., Iaquinta, P.J., Verona, R. and Lees, J.A. (2004) Repression of the Arf tumor suppressor by E2F3 is required for normal cell cycle kinetics. *Genes Dev*, **18**, 1413-1422.

Baehrecke, E.H. (2005) Autophagy: dual roles in life and death? *Nat Rev Mol Cell Biol*, **6**, 505-510.

Barak, Y., Juven, T., Haffner, R. and Oren, M. (1993) mdm2 expression is induced by wild type p53 activity. *Embo J*, **12**, 461-468.

Bates, S., Phillips, A.C., Clark, P.A., Stott, F., Peters, G., Ludwig, R.L. and Vousden, K.H. (1998) p14ARF links the tumour suppressors RB and p53. *Nature*, **395**, 124-125.

Belka, C., Schmid, B., Marini, P., Durand, E., Rudner, J., Faltin, H., Bamberg, M., Schulze-Osthoff, K. and Budach, W. (2001) Sensitization of resistant lymphoma cells to irradiation-induced apoptosis by the death ligand TRAIL. *Oncogene*, **20**, 2190-2196.

Bergamini, E., Cavallini, G., Donati, A. and Gori, Z. (2004) The role of macroautophagy in the ageing process, anti-ageing intervention and age-associated diseases. *Int J Biochem Cell Biol*, **36**, 2392-2404.

Bertwistle, D., Sugimoto, M. and Sherr, C.J. (2004) Physical and functional interactions of the Arf tumor suppressor protein with nucleophosmin/B23. *Mol Cell Biol*, **24**, 985-996.

Bhardwaj, A. and Aggarwal, B.B. (2003) Receptor-mediated choreography of life and death. *J Clin Immunol*, **23**, 317-332.

Blomberg, I. and Hoffmann, I. (1999) Ectopic expression of Cdc25A accelerates the G(1)/S transition and leads to premature activation of cyclin E- and cyclin A-dependent kinases. *Mol Cell Biol*, **19**, 6183-6194.

Blommaart, E.F., Luiken, J.J. and Meijer, A.J. (1997) Autophagic proteolysis: control and specificity. *Histochem J*, **29**, 365-385.

Bode, A.M. and Dong, Z. (2007) The enigmatic effects of caffeine in cell cycle and cancer. *Cancer Lett*, **247**, 26-39.

Boulares, A.H., Yakovlev, A.G., Ivanova, V., Stoica, B.A., Wang, G., Iyer, S. and Smulson, M. (1999) Role of poly(ADP-ribose) polymerase (PARP) cleavage in apoptosis. Caspase 3-resistant PARP mutant increases rates of apoptosis in transfected cells. *J Biol Chem*, **274**, 22932-22940.

Boutros, R., Lobjois, V. and Ducommun, B. (2007) CDC25 phosphatases in cancer cells: key players? Good targets? *Nat Rev Cancer*, **7**, 495-507.

Boya, P., Gonzalez-Polo, R.A., Casares, N., Perfettini, J.L., Dessen, P., Larochette, N., Metivier, D., Meley, D., Souquere, S., Yoshimori, T., Pierron, G., Codogno, P. and Kroemer, G. (2005) Inhibition of macroautophagy triggers apoptosis. *Mol Cell Biol*, **25**, 1025-1040.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J. and Kouzarides, T. (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*, **391**, 597-601.

Bulavin, D.V., Higashimoto, Y., Demidenko, Z.N., Meek, S., Graves, P., Phillips, C., Zhao, H., Moody, S.A., Appella, E., Piwnica-Worms, H. and Fornace, A.J., Jr. (2003) Dual phosphorylation controls Cdc25 phosphatases and mitotic entry. *Nat Cell Biol*, **5**, 545-551.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W. and Vogelstein, B. (1998) Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science*, **282**, 1497-1501.

Bursch, W. (2001) The autophagosomal-lysosomal compartment in programmed cell death. *Cell Death Differ*, **8**, 569-581.

Canu, N., Tufi, R., Serafino, A.L., Amadoro, G., Ciotti, M.T. and Calissano, P. (2005) Role of the autophagic-lysosomal system on low potassium-induced apoptosis in cultured cerebellar granule cells. *J Neurochem*, **92**, 1228-1242.

Cao, Y. and Klionsky, D.J. (2007) Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. *Cell Res*, **17**, 839-849.

Carnero, A., Hudson, J.D., Price, C.M. and Beach, D.H. (2000) p16INK4A and p19ARF act in overlapping pathways in cellular immortalization. *Nat Cell Biol*, **2**, 148-155.

Certo, M., Del Gaizo Moore, V., Nishino, M., Wei, G., Korsmeyer, S., Armstrong, S.A. and Letai, A. (2006) Mitochondria primed by death signals determine cellular addiction to antiapoptotic BCL-2 family members. *Cancer Cell*, **9**, 351-365.

Chang, D.W., Xing, Z., Pan, Y., Algeciras-Schimnich, A., Barnhart, B.C., Yaish-Ohad, S., Peter, M.E. and Yang, X. (2002) c-FLIP(L) is a dual function regulator for caspase-8 activation and CD95-mediated apoptosis. *Embo J*, **21**, 3704-3714.

Chen, L., Willis, S.N., Wei, A., Smith, B.J., Fletcher, J.I., Hinds, M.G., Colman, P.M., Day, C.L., Adams, J.M. and Huang, D.C. (2005) Differential targeting of prosurvival Bcl-2 proteins by their BH3-only ligands allows complementary apoptotic function. *Mol Cell*, **17**, 393-403.

Chen, Z., Xiao, Z., Chen, J., Ng, S.C., Sowin, T., Sham, H., Rosenberg, S., Fesik, S. and Zhang, H. (2003) Human Chk1 expression is dispensable for somatic cell death and critical for sustaining G2 DNA damage checkpoint. *Mol Cancer Ther*, **2**, 543-548.

Chittenden, T., Flemington, C., Houghton, A.B., Ebb, R.G., Gallo, G.J., Elangovan, B., Chinnadurai, G. and Lutz, R.J. (1995) A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. *Embo J*, **14**, 5589-5596.

Cho, D.H., Jo, Y.K., Hwang, J.J., Lee, Y.M., Roh, S.A. and Kim, J.C. (2009) Caspase-mediated cleavage of ATG6/Beclin-1 links apoptosis to autophagy in HeLa cells. *Cancer Lett*, **274**, 95-100.

Chung, D.C. (2004) Cyclin D1 in human neuroendocrine: tumorigenesis. *Ann N Y Acad Sci*, **1014**, 209-217.

Ciechomska, I.A., Goemans, G.C., Skepper, J.N. and Tolkovsky, A.M. (2009) Bcl-2 complexed with Beclin-1 maintains full anti-apoptotic function. *Oncogene*.

Clarke, P.G. (1990) Developmental cell death: morphological diversity and multiple mechanisms. *Anat Embryol (Berl)*, **181**, 195-213.

Codogno, P. and Meijer, A.J. (2006) Atg5: more than an autophagy factor. *Nat Cell Biol*, **8**, 1045-1047.

Cohen, G.M. (1997) Caspases: the executioners of apoptosis. *Biochem J*, **326 (Pt 1)**, 1-16.

Coqueret, O. (2002) Linking cyclins to transcriptional control. *Gene*, **299**, 35-55.

Cory, S. and Adams, J.M. (2002) The Bcl2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer*, **2**, 647-656.

Costantini, P., Bruey, J.M., Castedo, M., Metivier, D., Loeffler, M., Susin, S.A., Ravagnan, L., Zamzami, N., Garrido, C. and Kroemer, G. (2002) Pre-processed caspase-9 contained in mitochondria participates in apoptosis. *Cell Death Differ*, **9**, 82-88.

Crighton, D., Wilkinson, S., O'Prey, J., Syed, N., Smith, P., Harrison, P.R., Gasco, M., Garrone, O., Crook, T. and Ryan, K.M. (2006) DRAM, a p53-induced modulator of autophagy, is critical for apoptosis. *Cell*, **126**, 121-134.

Daido, S., Kanzawa, T., Yamamoto, A., Takeuchi, H., Kondo, Y. and Kondo, S. (2004) Pivotal role of the cell death factor BNIP3 in ceramide-induced autophagic cell death in malignant glioma cells. *Cancer Res*, **64**, 4286-4293.

Daniel, P.T., Schulze-Osthoff, K., Belka, C. and Guner, D. (2003) Guardians of cell death: the Bcl-2 family proteins. *Essays Biochem*, **39**, 73-88.

Daniel, P.T., Wieder, T., Sturm, I. and Schulze-Osthoff, K. (2001) The kiss of death: promises and failures of death receptors and ligands in cancer therapy. *Leukemia*, **15**, 1022-1032.

Daugas, E., Susin, S.A., Zamzami, N., Ferri, K.F., Irinopoulou, T., Larochette, N., Prevost, M.C., Leber, B., Andrews, D., Penninger, J. and Kroemer, G. (2000) Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. *Faseb J*, **14**, 729-739.

de Grey, A.D. (1997) A proposed refinement of the mitochondrial free radical theory of aging. *Bioessays*, **19**, 161-166.

de Stanchina, E., McCurrach, M.E., Zindy, F., Shieh, S.Y., Ferbeyre, G., Samuelson, A.V., Prives, C., Roussel, M.F., Sherr, C.J. and Lowe, S.W. (1998) E1A signaling to p53 involves the p19(ARF) tumor suppressor. *Genes Dev*, **12**, 2434-2442.

Degterev, A., Boyce, M. and Yuan, J. (2003) A decade of caspases. *Oncogene*, **22**, 8543-8567.

Demarchi, F., Bertoli, C., Copetti, T., Eskelinen, E.L. and Schneider, C. (2007) Calpain as a novel regulator of autophagosome formation. *Autophagy*, **3**, 235-237.

Demarchi, F., Bertoli, C., Copetti, T., Tanida, I., Brancolini, C., Eskelinen, E.L. and Schneider, C. (2006) Calpain is required for macroautophagy in mammalian cells. *J Cell Biol*, **175**, 595-605.

Desagher, S. and Martinou, J.C. (2000) Mitochondria as the central control point of apoptosis. *Trends Cell Biol*, **10**, 369-377.

Deveraux, Q.L. and Reed, J.C. (1999) IAP family proteins--suppressors of apoptosis. *Genes Dev*, **13**, 239-252.

Deveraux, Q.L., Roy, N., Stennicke, H.R., Van Arsdale, T., Zhou, Q., Srinivasula, S.M., Alnemri, E.S., Salvesen, G.S. and Reed, J.C. (1998) IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *Embo J*, **17**, 2215-2223.

Dotto, G.P. (2000) p21(WAF1/Cip1): more than a break to the cell cycle? *Biochim Biophys Acta*, **1471**, M43-56.

Du, C., Fang, M., Li, Y., Li, L. and Wang, X. (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. *Cell*, **102**, 33-42.

Dunn, W.A., Jr. (1994) Autophagy and related mechanisms of lysosome-mediated protein degradation. *Trends Cell Biol*, **4**, 139-143.

Dunphy, W.G. (1994) The decision to enter mitosis. *Trends Cell Biol*, **4**, 202-207.

Duprez, L., Wirawan, E., Berghe, T.V. and Vandenabeele, P. (2009) Major cell death pathways at a glance. *Microbes Infect*.

Duro, D., Bernard, O., Della Valle, V., Berger, R. and Larsen, C.J. (1995) A new type of p16INK4/MTS1 gene transcript expressed in B-cell malignancies. *Oncogene*, **11**, 21-29.

Earnshaw, W.C., Martins, L.M. and Kaufmann, S.H. (1999) Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem*, **68**, 383-424.

Eischen, C.M., Weber, J.D., Roussel, M.F., Sherr, C.J. and Cleveland, J.L. (1999) Disruption of the ARF-Mdm2-p53 tumor suppressor pathway in Myc-induced lymphomagenesis. *Genes Dev*, **13**, 2658-2669.

Elmore, S.P., Qian, T., Grissom, S.F. and Lemasters, J.J. (2001) The mitochondrial permeability transition initiates autophagy in rat hepatocytes. *Faseb J*, **15**, 2286-2287.

Eskes, R., Desagher, S., Antonsson, B. and Martinou, J.C. (2000) Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol*, **20**, 929-935.

Essmann, F., Pohlmann, S., Gillissen, B., Daniel, P.T., Schulze-Osthoff, K. and Janicke, R.U. (2005) Irradiation-induced translocation of p53 to mitochondria in the absence of apoptosis. *J Biol Chem*, **280**, 37169-37177.

Eymin, B., Claverie, P., Salon, C., Leduc, C., Col, E., Brambilla, E., Khochbin, S. and Gazzeri, S. (2006) p14ARF activates a Tip60-dependent and p53-independent ATM/ATR/CHK pathway in response to genotoxic stress. *Mol Cell Biol*, **26**, 4339-4350.

Eymin, B., Leduc, C., Coll, J.L., Brambilla, E. and Gazzeri, S. (2003) p14ARF induces G2 arrest and apoptosis independently of p53 leading to regression of tumours established in nude mice. *Oncogene*, **22**, 1822-1835.

Fadeel, B., Ahlin, A., Henter, J.I., Orrenius, S. and Hampton, M.B. (1998) Involvement of caspases in neutrophil apoptosis: regulation by reactive oxygen species. *Blood*, **92**, 4808-4818.

Feng, W., Huang, S., Wu, H. and Zhang, M. (2007) Molecular basis of Bcl-xL's target recognition versatility revealed by the structure of Bcl-xL in complex with the BH3 domain of Beclin-1. *J Mol Biol*, **372**, 223-235.

Feng, Z., Zhang, H., Levine, A.J. and Jin, S. (2005) The coordinate regulation of the p53 and mTOR pathways in cells. *Proc Natl Acad Sci U S A*, **102**, 8204-8209.

Fernandes-Alnemri, T., Armstrong, R.C., Krebs, J., Srinivasula, S.M., Wang, L., Bullrich, F., Fritz, L.C., Trapani, J.A., Tomaselli, K.J., Litwack, G. and Alnemri, E.S. (1996) In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc Natl Acad Sci U S A*, **93**, 7464-7469.

Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.S. (1994) CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein Ced-3 and mammalian interleukin-1 beta-converting enzyme. *J Biol Chem*, **269**, 30761-30764.

Ferri, K.F. and Kroemer, G. (2001) Organelle-specific initiation of cell death pathways. *Nat Cell Biol*, **3**, E255-263.

Flores, J.F., Walker, G.J., Glendening, J.M., Haluska, F.G., Castresana, J.S., Rubio, M.P., Pastorfide, G.C., Boyer, L.A., Kao, W.H., Bulyk, M.L., Barnhill, R.L., Hayward, N.K., Housman, D.E. and Fountain, J.W. (1996) Loss of the p16INK4a and p15INK4b genes, as well as neighboring 9p21 markers, in sporadic melanoma. *Cancer Res*, **56**, 5023-5032.

French, L.E. and Tschopp, J. (2003) Protein-based therapeutic approaches targeting death receptors. *Cell Death Differ*, **10**, 117-123.

Friedrich, K., Wieder, T., Von Haefen, C., Radetzki, S., Janicke, R., Schulze-Osthoff, K., Dorken, B. and Daniel, P.T. (2001) Overexpression of caspase-3 restores sensitivity for drug-induced apoptosis in breast cancer cell lines with acquired drug resistance. *Oncogene*, **20**, 2749-2760.

Fuentes-Prior, P. and Salvesen, G.S. (2004) The protein structures that shape caspase activity, specificity, activation and inhibition. *Biochem J*, **384**, 201-232.

Fujita, N., Itoh, T., Omori, H., Fukuda, M., Noda, T. and Yoshimori, T. (2008) The Atg16L complex specifies the site of LC3 lipidation for membrane biogenesis in autophagy. *Mol Biol Cell*, **19**, 2092-2100.

Galaktionov, K. and Beach, D. (1991) Specific activation of cdc25 tyrosine phosphatases by B-type cyclins: evidence for multiple roles of mitotic cyclins. *Cell*, **67**, 1181-1194.

Gao, N., Hu, Y.D., Cao, X.Y., Zhou, J. and Cao, S.L. (2001) The exogenous wild-type p14ARF gene induces growth arrest and promotes radiosensitivity in human lung cancer cell lines. *J Cancer Res Clin Oncol*, **127**, 359-367.

George, M.D., Baba, M., Scott, S.V., Mizushima, N., Garrison, B.S., Ohsumi, Y. and Klionsky, D.J. (2000) Apg5p functions in the sequestration step in the cytoplasm-to-vacuole targeting and macroautophagy pathways. *Mol Biol Cell*, **11**, 969-982.

Girard, F., Strausfeld, U., Fernandez, A. and Lamb, N.J. (1991) Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell*, **67**, 1169-1179.

Golks, A., Brenner, D., Fritsch, C., Krammer, P.H. and Lavrik, I.N. (2005) c-FLIPR, a new regulator of death receptor-induced apoptosis. *J Biol Chem*, **280**, 14507-14513.

Goping, I.S., Gross, A., Lavoie, J.N., Nguyen, M., Jemmerson, R., Roth, K., Korsmeyer, S.J. and Shore, G.C. (1998) Regulated targeting of BAX to mitochondria. *J Cell Biol*, **143**, 207-215.

Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*, **36**, 59-74.

Green, D.R. and Kroemer, G. (2004) The pathophysiology of mitochondrial cell death. *Science*, **305**, 626-629.

Green, D.R. and Reed, J.C. (1998) Mitochondria and apoptosis. *Science*, **281**, 1309-1312.

Gross, A., McDonnell, J.M. and Korsmeyer, S.J. (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev*, **13**, 1899-1911.

Guner, D., Sturm, I., Hemmati, P., Hermann, S., Hauptmann, S., Wurm, R., Budach, V., Dorken, B., Lorenz, M. and Daniel, P.T. (2003) Multigene analysis of Rb pathway and apoptosis control in esophageal squamous cell carcinoma identifies patients with good prognosis. *Int J Cancer*, **103**, 445-454.

Hacki, J., Egger, L., Monney, L., Conus, S., Rosse, T., Fellay, I. and Borner, C. (2000) Apoptotic crosstalk between the endoplasmic reticulum and mitochondria controlled by Bcl-2. *Oncogene*, **19**, 2286-2295.

Hamacher-Brady, A., Brady, N.R., Logue, S.E., Sayen, M.R., Jinno, M., Kirshenbaum, L.A., Gottlieb, R.A. and Gustafsson, A.B. (2007) Response to myocardial ischemia/reperfusion injury involves Bnip3 and autophagy. *Cell Death Differ*, **14**, 146-157.

Handrick, R., Ganswindt, U., Faltin, H., Goecke, B., Daniel, P.T., Budach, W., Belka, C. and Jendrossek, V. (2009) Combined action of celecoxib and ionizing radiation in prostate cancer cells is independent of pro-apoptotic Bax. *Radiother Oncol*, **90**, 413-421.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805-816.

Haupt, Y., Maya, R., Kazaz, A. and Oren, M. (1997) Mdm2 promotes the rapid degradation of p53. *Nature*, **387**, 296-299.

Hemmati, P.G., Gillissen, B., von Haefen, C., Wendt, J., Starck, L., Guner, D., Dorken, B. and Daniel, P.T. (2002) Adenovirus-mediated overexpression of p14(ARF) induces p53 and Bax-independent apoptosis. *Oncogene*, **21**, 3149-3161.

Hemmati, P.G., Guner, D., Gillissen, B., Wendt, J., von Haefen, C., Chinnadurai, G., Dorken, B. and Daniel, P.T. (2006) Bak functionally complements for loss of Bax during p14ARF-induced mitochondrial apoptosis in human cancer cells. *Oncogene*, **25**, 6582-6594.

Hemmati, P.G., Normand, G., Gillissen, B., Wendt, J., Dorken, B. and Daniel, P.T. (2008) Cooperative effect of p21Cip1/WAF-1 and 14-3-3sigma on cell cycle arrest and apoptosis induction by p14ARF. *Oncogene*, **27**, 6707-6719.

Hemmati, P.G., Normand, G., Verdoodt, B., von Haefen, C., Hasenjager, A., Guner, D., Wendt, J., Dorken, B. and Daniel, P.T. (2005) Loss of p21 disrupts p14 ARF-induced G1 cell cycle arrest but augments p14 ARF-induced apoptosis in human carcinoma cells. *Oncogene*, **24**, 4114-4128.

Hengst, L. and Reed, S.I. (1998) Inhibitors of the Cip/Kip family. *Curr Top Microbiol Immunol*, **227**, 25-41.

Hodges, L.C., Cook, J.D., Lobenhofer, E.K., Li, L., Bennett, L., Bushel, P.R., Aldaz, C.M., Afshari, C.A. and Walker, C.L. (2003) Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. *Mol Cancer Res*, **1**, 300-311.

Honda, R. and Yasuda, H. (1999) Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. *Embo J*, **18**, 22-27.

Hotchkiss, R.S. and Nicholson, D.W. (2006) Apoptosis and caspases regulate death and inflammation in sepsis. *Nat Rev Immunol*, **6**, 813-822.

Howley, P.M. (2006) Warts, cancer and ubiquitylation: lessons from the papillomaviruses. *Trans Am Clin Climatol Assoc*, **117**, 113-126; discussion 126-117.

Hoyer-Hansen, M., Bastholm, L., Mathiasen, I.S., Elling, F. and Jaattela, M. (2005) Vitamin D analog EB1089 triggers dramatic lysosomal changes and Beclin 1-mediated autophagic cell death. *Cell Death Differ*, **12**, 1297-1309.

Hu, Y., Ding, L., Spencer, D.M. and Nunez, G. (1998) WD-40 repeat region regulates Apaf-1 self-association and procaspase-9 activation. *J Biol Chem*, **273**, 33489-33494.

Huang, W.P., Scott, S.V., Kim, J. and Klionsky, D.J. (2000) The itinerary of a vesicle component, Aut7p/Cvt5p, terminates in the yeast vacuole via the autophagy/Cvt pathways. *J Biol Chem*, **275**, 5845-5851.

Ichimura, Y., Kirisako, T., Takao, T., Satomi, Y., Shimonishi, Y., Ishihara, N., Mizushima, N., Tanida, I., Kominami, E., Ohsumi, M., Noda, T. and Ohsumi, Y. (2000) A ubiquitin-like system mediates protein lipidation. *Nature*, **408**, 488-492.

Inoue, K., Zindy, F., Randle, D.H., Rehg, J.E. and Sherr, C.J. (2001) Dmp1 is haplo-insufficient for tumor suppression and modifies the frequencies of Arf and p53 mutations in Myc-induced lymphomas. *Genes Dev*, **15**, 2934-2939.

Janicke, R.U., Engels, I.H., Dunkern, T., Kaina, B., Schulze-Osthoff, K. and Porter, A.G. (2001) Ionizing radiation but not anticancer drugs causes cell cycle arrest and failure to activate the mitochondrial death pathway in MCF-7 breast carcinoma cells. *Oncogene*, **20**, 5043-5053.

Janicke, R.U., Sprengart, M.L., Wati, M.R. and Porter, A.G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. *J Biol Chem*, **273**, 9357-9360.

Jinno, S., Suto, K., Nagata, A., Igarashi, M., Kanaoka, Y., Nojima, H. and Okayama, H. (1994) Cdc25A is a novel phosphatase functioning early in the cell cycle. *Embo J*, **13**, 1549-1556.

Johnson, D.G. and Walker, C.L. (1999) Cyclins and cell cycle checkpoints. *Annu Rev Pharmacol Toxicol*, **39**, 295-312.

Jones, S.N., Roe, A.E., Donehower, L.A. and Bradley, A. (1995) Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. *Nature*, **378**, 206-208.

Joza, N., Susin, S.A., Daugas, E., Stanford, W.L., Cho, S.K., Li, C.Y., Sasaki, T., Elia, A.J., Cheng, H.Y., Ravagnan, L., Ferri, K.F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y.Y., Mak, T.W., Zuniga-Pflucker, J.C., Kroemer, G. and Penninger, J.M. (2001) Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature*, **410**, 549-554.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. and Yoshimori, T. (2000) LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *Embo J*, **19**, 5720-5728.

Kabeya, Y., Mizushima, N., Yamamoto, A., Oshitani-Okamoto, S., Ohsumi, Y. and Yoshimori, T. (2004) LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. *J Cell Sci*, **117**, 2805-2812.

Kaldis, P. (1999) The cdk-activating kinase (CAK): from yeast to mammals. *Cell Mol Life Sci*, **55**, 284-296.

Kamijo, T., Weber, J.D., Zambetti, G., Zindy, F., Roussel, M.F. and Sherr, C.J. (1998) Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc Natl Acad Sci U S A*, **95**, 8292-8297.

Kamijo, T., Zindy, F., Roussel, M.F., Quelle, D.E., Downing, J.R., Ashmun, R.A., Grosveld, G. and Sherr, C.J. (1997) Tumor suppression at the mouse INK4a locus mediated by the alternative reading frame product p19ARF. *Cell*, **91**, 649-659.

Kato, J., Matsushime, H., Hiebert, S.W., Ewen, M.E. and Sherr, C.J. (1993) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. *Genes Dev*, **7**, 331-342.

Kenny, F.S., Hui, R., Musgrove, E.A., Gee, J.M., Blamey, R.W., Nicholson, R.I., Sutherland, R.L. and Robertson, J.F. (1999) Overexpression of cyclin D1 messenger RNA predicts for poor prognosis in estrogen receptor-positive breast cancer. *Clin Cancer Res*, **5**, 2069-2076.

Kerkhoff, E. and Rapp, U.R. (1997) Induction of cell proliferation in quiescent NIH 3T3 cells by oncogenic c-Raf-1. *Mol Cell Biol*, **17**, 2576-2586.

Kerr, J.F., Winterford, C.M. and Harmon, B.V. (1994) Apoptosis. Its significance in cancer and cancer therapy. *Cancer*, **73**, 2013-2026.

Kihara, A., Kabeya, Y., Ohsumi, Y. and Yoshimori, T. (2001) Beclin-phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep*, **2**, 330-335.

Kim, J. and Klionsky, D.J. (2000) Autophagy, cytoplasm-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. *Annu Rev Biochem*, **69**, 303-342.

King, R.W., Jackson, P.K. and Kirschner, M.W. (1994) Mitosis in transition. *Cell*, **79**, 563-571.

Kirisako, T., Baba, M., Ishihara, N., Miyazawa, K., Ohsumi, M., Yoshimori, T., Noda, T. and Ohsumi, Y. (1999) Formation process of autophagosome is traced with Apg8/Aut7p in yeast. *J Cell Biol*, **147**, 435-446.

Kirisako, T., Ichimura, Y., Okada, H., Kabeya, Y., Mizushima, N., Yoshimori, T., Ohsumi, M., Takao, T., Noda, T. and Ohsumi, Y. (2000) The reversible modification regulates the membrane-binding state of Apg8/Aut7 essential for autophagy and the cytoplasm to vacuole targeting pathway. *J Cell Biol*, **151**, 263-276.

Kirsch, D.G., Doseff, A., Chau, B.N., Lim, D.S., de Souza-Pinto, N.C., Hansford, R., Kastan, M.B., Lazebnik, Y.A. and Hardwick, J.M. (1999) Caspase-3-dependent cleavage of Bcl-2 promotes release of cytochrome c. *J Biol Chem*, **274**, 21155-21161.

Kischkel, F.C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Krammer, P.H. and Peter, M.E. (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *Embo J*, **14**, 5579-5588.

Klionsky, D.J., Cregg, J.M., Dunn, W.A., Jr., Emr, S.D., Sakai, Y., Sandoval, I.V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M. and Ohsumi, Y. (2003) A unified nomenclature for yeast autophagy-related genes. *Dev Cell*, **5**, 539-545.

Klionsky, D.J. and Emr, S.D. (2000) Autophagy as a regulated pathway of cellular degradation. *Science*, **290**, 1717-1721.

Kluck, R.M., Bossy-Wetzel, E., Green, D.R. and Newmeyer, D.D. (1997) The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science*, **275**, 1132-1136.

Koh, D.W., Dawson, T.M. and Dawson, V.L. (2005) Mediation of cell death by poly(ADP-ribose) polymerase-1. *Pharmacol Res*, **52**, 5-14.

Korgaonkar, C., Zhao, L., Modestou, M. and Quelle, D.E. (2002) ARF function does not require p53 stabilization or Mdm2 relocalization. *Mol Cell Biol*, **22**, 196-206.

Korsmeyer, S.J., Wei, M.C., Saito, M., Weiler, S., Oh, K.J. and Schlesinger, P.H. (2000) Pro-apoptotic cascade activates BID, which oligomerizes BAK or BAX into pores that result in the release of cytochrome c. *Cell Death Differ*, **7**, 1166-1173.

Krajewski, S., Tanaka, S., Takayama, S., Schibler, M.J., Fenton, W. and Reed, J.C. (1993) Investigation of the subcellular distribution of the bcl-2 oncoprotein: residence in the nuclear envelope, endoplasmic reticulum, and outer mitochondrial membranes. *Cancer Res*, **53**, 4701-4714.

Kristjansdottir, K. and Rudolph, J. (2004) Cdc25 phosphatases and cancer. *Chem Biol*, **11**, 1043-1051.

Ku, B., Woo, J.S., Liang, C., Lee, K.H., Hong, H.S., E, X., Kim, K.S., Jung, J.U. and Oh, B.H. (2008) Structural and biochemical bases for the inhibition of autophagy and apoptosis by viral BCL-2 of murine gamma-herpesvirus 68. *PLoS Pathog*, **4**, e25.

Kubbutat, M.H., Jones, S.N. and Vousden, K.H. (1997) Regulation of p53 stability by Mdm2. *Nature*, **387**, 299-303.

Laemmli, U. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680-685.

Lakhani, S.A., Masud, A., Kuida, K., Porter, G.A., Jr., Booth, C.J., Mehal, W.Z., Inayat, I. and Flavell, R.A. (2006) Caspases 3 and 7: key mediators of mitochondrial events of apoptosis. *Science*, **311**, 847-851.

Lamkanfi, M., Declercq, W., Kalai, M., Saelens, X. and Vandenabeele, P. (2002) Alice in caspase land. A phylogenetic analysis of caspases from worm to man. *Cell Death Differ*, **9**, 358-361.

Lammer, C., Wagerer, S., Saffrich, R., Mertens, D., Ansorge, W. and Hoffmann, I. (1998) The cdc25B phosphatase is essential for the G2/M phase transition in human cells. *J Cell Sci*, **111 (Pt 16)**, 2445-2453.

Lamparska-Przybysz, M., Gajkowska, B. and Motyl, T. (2006) BID-deficient breast cancer MCF-7 cells as a model for the study of autophagy in cancer therapy. *Autophagy*, **2**, 47-48.

Lavrik, I., Golks, A. and Krammer, P.H. (2005) Death receptor signaling. *J Cell Sci*, **118**, 265-267.

Le Rhun, Y., Kirkland, J.B. and Shah, G.M. (1998) Cellular responses to DNA damage in the absence of Poly(ADP-ribose) polymerase. *Biochem Biophys Res Commun*, **245**, 1-10.

Lee, R.J., Albanese, C., Fu, M., D'Amico, M., Lin, B., Watanabe, G., Haines, G.K., 3rd, Siegel, P.M., Hung, M.C., Yarden, Y., Horowitz, J.M., Muller, W.J. and Pestell, R.G. (2000) Cyclin D1 is required for transformation by activated Neu and is induced through an E2F-dependent signaling pathway. *Mol Cell Biol*, **20**, 672-683.

Lemasters, J.J., Nieminen, A.L., Qian, T., Trost, L.C., Elmore, S.P., Nishimura, Y., Crowe, R.A., Cascio, W.E., Bradham, C.A., Brenner, D.A. and Herman, B. (1998) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta*, **1366**, 177-196.

Letai, A., Bassik, M.C., Walensky, L.D., Sorcinelli, M.D., Weiler, S. and Korsmeyer, S.J. (2002) Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell*, **2**, 183-192.

Levine, A.J. (1997) p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323-331.

Levine, B. and Klionsky, D.J. (2004) Development by self-digestion: molecular mechanisms and biological functions of autophagy. *Dev Cell*, **6**, 463-477.

Levine, B. and Kroemer, G. (2008) Autophagy in the pathogenesis of disease. *Cell*, **132**, 27-42.

Li, A. and Blow, J.J. (2001) The origin of CDK regulation. *Nat Cell Biol*, **3**, E182-184.

Li, J. and Yuan, J. (2008) Caspases in apoptosis and beyond. *Oncogene*, **27**, 6194-6206.

Li, L.Y., Luo, X. and Wang, X. (2001) Endonuclease G is an apoptotic DNase when released from mitochondria. *Nature*, **412**, 95-99.

Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S.M., Ahmad, M., Alnemri, E.S. and Wang, X. (1997) Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*, **91**, 479-489.

Liang, X.H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H. and Levine, B. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*, **402**, 672-676.

Liang, X.H., Kleeman, L.K., Jiang, H.H., Gordon, G., Goldman, J.E., Berry, G., Herman, B. and Levine, B. (1998) Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J Virol*, **72**, 8586-8596.

Liang, X.H., Yu, J., Brown, K. and Levine, B. (2001) Beclin 1 contains a leucine-rich nuclear export signal that is required for its autophagy and tumor suppressor function. *Cancer Res*, **61**, 3443-3449.

Lindsten, T., Ross, A.J., King, A., Zong, W.X., Rathmell, J.C., Shiels, H.A., Ulrich, E., Waymire, K.G., Mahar, P., Frauwirth, K., Chen, Y., Wei, M., Eng, V.M., Adelman, D.M., Simon, M.C., Ma, A., Golden, J.A., Evan, G., Korsmeyer, S.J., MacGregor, G.R. and Thompson, C.B. (2000) The combined functions of proapoptotic Bcl-2

family members bak and bax are essential for normal development of multiple tissues. *Mol Cell*, **6**, 1389-1399.

Liu, X., Kim, C.N., Yang, J., Jemmerson, R. and Wang, X. (1996) Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, **86**, 147-157.

Llanos, S., Clark, P.A., Rowe, J. and Peters, G. (2001) Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus. *Nat Cell Biol*, **3**, 445-452.

Lockshin, R.a.W., CM (1964) Programmed cell death. II. Endocrine potentiation of the breakdown of the intersegmental muscles of silkmoths. *J Insect Physiol*, **10**, 643-649.

Lum, J.J., Bauer, D.E., Kong, M., Harris, M.H., Li, C., Lindsten, T. and Thompson, C.B. (2005) Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell*, **120**, 237-248.

Luo, X., Budihardjo, I., Zou, H., Slaughter, C. and Wang, X. (1998) Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. *Cell*, **94**, 481-490.

MacFarlane, M., Cain, K., Sun, X.M., Alnemri, E.S. and Cohen, G.M. (1997) Processing/activation of at least four interleukin-1beta converting enzyme-like proteases occurs during the execution phase of apoptosis in human monocytic tumor cells. *J Cell Biol*, **137**, 469-479.

Madden, D.T., Egger, L. and Bredesen, D.E. (2007) A calpain-like protease inhibits autophagic cell death. *Autophagy*, **3**, 519-522.

Magro, P.G., Russo, A.J., Li, W.W., Banerjee, D. and Bertino, J.R. (2004) p14ARF expression increases dihydrofolate reductase degradation and paradoxically results in resistance to folate antagonists in cells with nonfunctional p53. *Cancer Res*, **64**, 4338-4345.

Maiuri, M.C., Le Toumelin, G., Criollo, A., Rain, J.C., Gautier, F., Juin, P., Tasdemir, E., Pierron, G., Troulinaki, K., Tavernarakis, N., Hickman, J.A., Geneste, O. and Kroemer, G. (2007) Functional and physical interaction between Bcl-X(L) and a BH3-like domain in Beclin-1. *Embo J*, **26**, 2527-2539.

Malumbres, M. and Barbacid, M. (2005) Mammalian cyclin-dependent kinases. *Trends Biochem Sci*, **30**, 630-641.

Mao, L., Merlo, A., Bedi, G., Shapiro, G.I., Edwards, C.D., Rollins, B.J. and Sidransky, D. (1995) A novel p16INK4A transcript. *Cancer Res*, **55**, 2995-2997.

Martins, L.M., Iaccarino, I., Tenev, T., Gschmeissner, S., Totty, N.F., Lemoine, N.R., Savopoulos, J., Gray, C.W., Creasy, C.L., Dingwall, C. and Downward, J. (2002) The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. *J Biol Chem*, **277**, 439-444.

McShea, A., Samuel, T., Eppel, J.T., Galloway, D.A. and Funk, J.O. (2000) Identification of CIP-1-associated regulator of cyclin B (CARB), a novel p21-binding protein acting in the G2 phase of the cell cycle. *J Biol Chem*, **275**, 23181-23186.

Meyerson, M., Enders, G.H., Wu, C.L., Su, L.K., Gorka, C., Nelson, C., Harlow, E. and Tsai, L.H. (1992) A family of human cdc2-related protein kinases. *Embo J*, **11**, 2909-2917.

Midgley, C.A., Desterro, J.M., Saville, M.K., Howard, S., Sparks, A., Hay, R.T. and Lane, D.P. (2000) An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. *Oncogene*, **19**, 2312-2323.

Mizushima, N. (2007) Autophagy: process and function. *Genes Dev*, **21**, 2861-2873.

Mizushima, N., Kuma, A., Kobayashi, Y., Yamamoto, A., Matsubae, M., Takao, T., Natsume, T., Ohsumi, Y. and Yoshimori, T. (2003) Mouse Apg16L, a novel WD-

repeat protein, targets to the autophagic isolation membrane with the Apg12-Apg5 conjugate. *J Cell Sci*, **116**, 1679-1688.

Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., Tokuhisa, T., Ohsumi, Y. and Yoshimori, T. (2001) Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *J Cell Biol*, **152**, 657-668.

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T. and Ohsumi, Y. (2004) In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol Biol Cell*, **15**, 1101-1111.

Moll, U.M. and Petrenko, O. (2003) The MDM2-p53 interaction. *Mol Cancer Res*, **1**, 1001-1008.

Montes de Oca Luna, R., Wagner, D.S. and Lozano, G. (1995) Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. *Nature*, **378**, 203-206.

Morgan, D.O. (1997) Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annu Rev Cell Dev Biol*, **13**, 261-291.

Musgrove, E.A. (2006) Cyclins: roles in mitogenic signaling and oncogenic transformation. *Growth Factors*, **24**, 13-19.

Muzio, M., Chinnaiyan, A.M., Kischkel, F.C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J.D., Zhang, M., Gentz, R., Mann, M., Krammer, P.H., Peter, M.E. and Dixit, V.M. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death--inducing signaling complex. *Cell*, **85**, 817-827.

Nagata, S. (1997) Apoptosis by death factor. *Cell*, **88**, 355-365.

Nicholson, D.W. (1999) Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ*, **6**, 1028-1042.

Niculescu, A.B., 3rd, Chen, X., Smeets, M., Hengst, L., Prives, C. and Reed, S.I. (1998) Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. *Mol Cell Biol*, **18**, 629-643.

Normand, G., Hemmati, P.G., Verdoodt, B., von Haefen, C., Wendt, J., Guner, D., May, E., Dorken, B. and Daniel, P.T. (2005) p14ARF induces G2 cell cycle arrest in p53- and p21-deficient cells by down-regulating p34cdc2 kinase activity. *J Biol Chem*, **280**, 7118-7130.

Nozell, S., Wu, Y., McNaughton, K., Liu, G., Willis, A., Paik, J.C. and Chen, X. (2003) Characterization of p73 functional domains necessary for transactivation and growth suppression. *Oncogene*, **22**, 4333-4347.

Oberstein, A., Jeffrey, P.D. and Shi, Y. (2007) Crystal structure of the Bcl-XL-Beclin 1 peptide complex: Beclin 1 is a novel BH3-only protein. *J Biol Chem*, **282**, 13123-13132.

Okuno, S., Shimizu, S., Ito, T., Nomura, M., Hamada, E., Tsujimoto, Y. and Matsuda, H. (1998) Bcl-2 prevents caspase-independent cell death. *J Biol Chem*, **273**, 34272-34277.

Oltvai, Z.N., Milliman, C.L. and Korsmeyer, S.J. (1993) Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. *Cell*, **74**, 609-619.

Pajalunga, D., Mazzola, A., Franchitto, A., Puggioni, E. and Crescenzi, M. (2008) The logic and regulation of cell cycle exit and reentry. *Cell Mol Life Sci*, **65**, 8-15.

Palmero, I., Pantoja, C. and Serrano, M. (1998) p19ARF links the tumour suppressor p53 to Ras. *Nature*, **395**, 125-126.

Parrish, J., Li, L., Klotz, K., Ledwich, D., Wang, X. and Xue, D. (2001) Mitochondrial endonuclease G is important for apoptosis in C. elegans. *Nature*, **412**, 90-94.

Pattingre, S., Tassa, A., Qu, X., Garuti, R., Liang, X.H., Mizushima, N., Packer, M., Schneider, M.D. and Levine, B. (2005) Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. *Cell*, **122**, 927-939.

Paulovich, A.G., Toczyski, D.P. and Hartwell, L.H. (1997) When checkpoints fail. *Cell*, **88**, 315-321.

Petiot, A., Ogier-Denis, E., Blommaart, E.F., Meijer, A.J. and Codogno, P. (2000) Distinct classes of phosphatidylinositol 3'-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J Biol Chem*, **275**, 992-998.

Pineau, P., Marchio, A., Cordina, E., Tiollais, P. and Dejean, A. (2003) Homozygous deletions scanning in tumor cell lines detects previously unsuspected loci. *Int J Cancer*, **106**, 216-223.

Pines, J. (1991) Cyclins: wheels within wheels. *Cell Growth Differ*, **2**, 305-310.

Pomerantz, J., Schreiber-Agus, N., Liegeois, N.J., Silverman, A., Alland, L., Chin, L., Potes, J., Chen, K., Orlow, I., Lee, H.W., Cordon-Cardo, C. and DePinho, R.A. (1998) The Ink4a tumor suppressor gene product, p19Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. *Cell*, **92**, 713-723.

Porn-Ares, M.I., Samali, A. and Orrenius, S. (1998) Cleavage of the calpain inhibitor, calpastatin, during apoptosis. *Cell Death Differ*, **5**, 1028-1033.

Porter, A.G. and Janicke, R.U. (1999) Emerging roles of caspase-3 in apoptosis. *Cell Death Differ*, **6**, 99-104.

Powell, S.N., DeFrank, J.S., Connell, P., Eogan, M., Preffer, F., Dombkowski, D., Tang, W. and Friend, S. (1995) Differential sensitivity of p53(-) and p53(+) cells to caffeine-induced radiosensitization and override of G2 delay. *Cancer Res*, **55**, 1643-1648.

Pyo, J.O., Jang, M.H., Kwon, Y.K., Lee, H.J., Jun, J.I., Woo, H.N., Cho, D.H., Choi, B., Lee, H., Kim, J.H., Mizushima, N., Oshumi, Y. and Jung, Y.K. (2005) Essential roles of Atg5 and FADD in autophagic cell death: dissection of autophagic cell death into vacuole formation and cell death. *J Biol Chem*, **280**, 20722-20729.

Quelle, D.E., Zindy, F., Ashmun, R.A. and Sherr, C.J. (1995) Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell*, **83**, 993-1000.

Radetzki, S., Kohne, C.H., von Haefen, C., Gillissen, B., Sturm, I., Dorken, B. and Daniel, P.T. (2002) The apoptosis promoting Bcl-2 homologues Bak and Nbk/Bik overcome drug resistance in Mdr-1-negative and Mdr-1-overexpressing breast cancer cell lines. *Oncogene*, **21**, 227-238.

Reggiori, F. and Klionsky, D.J. (2002) Autophagy in the eukaryotic cell. *Eukaryot Cell*, **1**, 11-21.

Regula, K.M., Ens, K. and Kirshenbaum, L.A. (2002) Inducible expression of BNIP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. *Circ Res*, **91**, 226-231.

Ricci, J.E., Gottlieb, R.A. and Green, D.R. (2003) Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J Cell Biol*, **160**, 65-75.

Riedl, S.J. and Shi, Y. (2004) Molecular mechanisms of caspase regulation during apoptosis. *Nat Rev Mol Cell Biol*, **5**, 897-907.

Rodriguez, R. and Meuth, M. (2006) Chk1 and p21 cooperate to prevent apoptosis during DNA replication fork stress. *Mol Biol Cell*, **17**, 402-412.

Roninson, I.B., Broude, E.V. and Chang, B.D. (2001) If not apoptosis, then what? Treatment-induced senescence and mitotic catastrophe in tumor cells. *Drug Resist Updat*, **4**, 303-313.

Rouse, J. and Jackson, S.P. (2002) Interfaces between the detection, signaling, and repair of DNA damage. *Science*, **297**, 547-551.

Rousseau, D., Cannella, D., Boulaire, J., Fitzgerald, P., Fotedar, A. and Fotedar, R. (1999) Growth inhibition by CDK-cyclin and PCNA binding domains of p21 occurs by distinct mechanisms and is regulated by ubiquitin-proteasome pathway. *Oncogene*, **18**, 4313-4325.

Roussel, M.F. (1999) The INK4 family of cell cycle inhibitors in cancer. *Oncogene*, **18**, 5311-5317.

Roy, N., Deveraux, Q.L., Takahashi, R., Salvesen, G.S. and Reed, J.C. (1997) The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *Embo J*, **16**, 6914-6925.

Saleh, M., Vaillancourt, J.P., Graham, R.K., Huyck, M., Srinivasula, S.M., Alnemri, E.S., Steinberg, M.H., Nolan, V., Baldwin, C.T., Hotchkiss, R.S., Buchman, T.G., Zehnbauer, B.A., Hayden, M.R., Farrer, L.A., Roy, S. and Nicholson, D.W. (2004) Differential modulation of endotoxin responsiveness by human caspase-12 polymorphisms. *Nature*, **429**, 75-79.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K.J., Debatin, K.M., Krammer, P.H. and Peter, M.E. (1998) Two CD95 (APO-1/Fas) signaling pathways. *Embo J*, **17**, 1675-1687.

Scaringi, L., Cornacchione, P., Ayroldi, E., Corazzi, L., Capodicasa, E., Rossi, R. and Marconi, P. (2004) Omeprazole induces apoptosis in jurkat cells. *Int J Immunopathol Pharmacol*, **17**, 331-342.

Scarlatti, F., Granata, R., Meijer, A.J. and Codogno, P. (2009) Does autophagy have a license to kill mammalian cells? *Cell Death Differ*, **16**, 12-20.

Schmelzle, T. and Hall, M.N. (2000) TOR, a central controller of cell growth. *Cell*, **103**, 253-262.

Schmitt, C.A., McCurrach, M.E., de Stanchina, E., Wallace-Brodeur, R.R. and Lowe, S.W. (1999) INK4a/ARF mutations accelerate lymphomagenesis and promote chemoresistance by disabling p53. *Genes Dev*, **13**, 2670-2677.

Scott, A.M. and Saleh, M. (2007) The inflammatory caspases: guardians against infections and sepsis. *Cell Death Differ*, **14**, 23-31.

Shangary, S. and Wang, S. (2008) Targeting the MDM2-p53 interaction for cancer therapy. *Clin Cancer Res*, **14**, 5318-5324.

Sharpless, E. and Chin, L. (2003) The INK4a/ARF locus and melanoma. *Oncogene*, **22**, 3092-3098.

Shaw, G., Morse, S., Ararat, M. and Graham, F.L. (2002) Preferential transformation of human neuronal cells by human adenoviruses and the origin of HEK 293 cells. *Faseb J*, **16**, 869-871.

Sherr, C.J. (1994) G1 phase progression: cycling on cue. *Cell*, **79**, 551-555.

Sherr, C.J. (1996) Cancer cell cycles. *Science*, **274**, 1672-1677.

Sherr, C.J. (2001) The INK4a/ARF network in tumour suppression. *Nat Rev Mol Cell Biol*, **2**, 731-737.

Sherr, C.J. (2004) Principles of tumor suppression. *Cell*, **116**, 235-246.

Sherr, C.J. (2006) Divorcing ARF and p53: an unsettled case. *Nat Rev Cancer*, **6**, 663-673.

Sherr, C.J. and Roberts, J.M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev*, **13**, 1501-1512.

Shimizu, S., Kanaseki, T., Mizushima, N., Mizuta, T., Arakawa-Kobayashi, S., Thompson, C.B. and Tsujimoto, Y. (2004) Role of Bcl-2 family proteins in a non-apoptotic programmed cell death dependent on autophagy genes. *Nat Cell Biol*, **6**, 1221-1228.

Shintani, T. and Klionsky, D.J. (2004) Autophagy in health and disease: a double-edged sword. *Science*, **306**, 990-995.

Silva, J., Silva, J.M., Dominguez, G., Garcia, J.M., Cantos, B., Rodriguez, R., Larrondo, F.J., Provencio, M., Espana, P. and Bonilla, F. (2003) Concomitant expression of p16INK4a and p14ARF in primary breast cancer and analysis of inactivation mechanisms. *J Pathol*, **199**, 289-297.

Simon, M., Voss, D., Park-Simon, T.W., Mahlberg, R. and Koster, G. (2006) Role of p16 and p14ARF in radio- and chemosensitivity of malignant gliomas. *Oncol Rep*, **16**, 127-132.

Sinha, S., Colbert, C.L., Becker, N., Wei, Y. and Levine, B. (2008) Molecular basis of the regulation of Beclin 1-dependent autophagy by the gamma-herpesvirus 68 Bcl-2 homolog M11. *Autophagy*, **4**, 989-997.

Slee, E.A., Harte, M.T., Kluck, R.M., Wolf, B.B., Casiano, C.A., Newmeyer, D.D., Wang, H.G., Reed, J.C., Nicholson, D.W., Alnemri, E.S., Green, D.R. and Martin, S.J. (1999) Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *J Cell Biol*, **144**, 281-292.

Smith, C.A., Farrah, T. and Goodwin, R.G. (1994) The TNF receptor superfamily of cellular and viral proteins: activation, costimulation, and death. *Cell*, **76**, 959-962.

Smits, V.A., Klompmaker, R., Vallenius, T., Rijksen, G., Makela, T.P. and Medema, R.H. (2000) p21 inhibits Thr161 phosphorylation of Cdc2 to enforce the G2 DNA damage checkpoint. *J Biol Chem*, **275**, 30638-30643.

Sohn, D., Essmann, F., Schulze-Osthoff, K. and Janicke, R.U. (2006) p21 blocks irradiation-induced apoptosis downstream of mitochondria by inhibition of cyclin-dependent kinase-mediated caspase-9 activation. *Cancer Res*, **66**, 11254-11262.

Sprick, M.R., Rieser, E., Stahl, H., Grosse-Wilde, A., Weigand, M.A. and Walczak, H. (2002) Caspase-10 is recruited to and activated at the native TRAIL and CD95 death-inducing signalling complexes in a FADD-dependent manner but can not functionally substitute caspase-8. *Embo J*, **21**, 4520-4530.

Srinivasula, S.M., Gupta, S., Datta, P., Zhang, Z., Hegde, R., Cheong, N., Fernandes-Alnemri, T. and Alnemri, E.S. (2003) Inhibitor of apoptosis proteins are substrates for the mitochondrial serine protease Omi/HtrA2. *J Biol Chem*, **278**, 31469-31472.

Stott, F.J., Bates, S., James, M.C., McConnell, B.B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K.H. and Peters, G. (1998) The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. *Embo J*, **17**, 5001-5014.

Strasser, A., O'Connor, L. and Dixit, V.M. (2000) Apoptosis signaling. *Annu Rev Biochem*, **69**, 217-245.

Susin, S.A., Lorenzo, H.K., Zamzami, N., Marzo, I., Snow, B.E., Brothers, G.M., Mangion, J., Jacotot, E., Costantini, P., Loeffler, M., Larochette, N., Goodlett, D.R., Aebersold, R., Siderovski, D.P., Penninger, J.M. and Kroemer, G. (1999) Molecular characterization of mitochondrial apoptosis-inducing factor. *Nature*, **397**, 441-446.

Suzuki, A., Tsutomi, Y., Akahane, K., Araki, T. and Miura, M. (1998) Resistance to Fas-mediated apoptosis: activation of caspase 3 is regulated by cell cycle regulator p21WAF1 and IAP gene family ILP. *Oncogene*, **17**, 931-939.

Suzuki, Y., Imai, Y., Nakayama, H., Takahashi, K., Takio, K. and Takahashi, R. (2001) A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. *Mol Cell*, **8**, 613-621.

Swanson, P.E., Carroll, S.B., Zhang, X.F. and Mackey, M.A. (1995) Spontaneous premature chromosome condensation, micronucleus formation, and non-apoptotic cell death in heated HeLa S3 cells. Ultrastructural observations. *Am J Pathol*, **146**, 963-971.

Tanaka, S., Saito, K. and Reed, J.C. (1993) Structure-function analysis of the Bcl-2 oncoprotein. Addition of a heterologous transmembrane domain to portions of the Bcl-2 beta protein restores function as a regulator of cell survival. *J Biol Chem*, **268**, 10920-10926.

Tanida, I., Minematsu-Ikeguchi, N., Ueno, T. and Kominami, E. (2005) Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. *Autophagy*, **1**, 84-91.

Tao, W. and Levine, A.J. (1999) P19(ARF) stabilizes p53 by blocking nucleo-cytoplasmic shuttling of Mdm2. *Proc Natl Acad Sci U S A*, **96**, 6937-6941.

Tasdemir, E., Maiuri, M.C., Galluzzi, L., Vitale, I., Djavaheri-Mergny, M., D'Amelio, M., Criollo, A., Morselli, E., Zhu, C., Harper, F., Nannmark, U., Samara, C., Pinton, P., Vicencio, J.M., Carnuccio, R., Moll, U.M., Madeo, F., Paterlini-Brechot, P., Rizzuto, R., Szabadkai, G., Pierron, G., Blomgren, K., Tavernarakis, N., Codogno, P., Cecconi, F. and Kroemer, G. (2008) Regulation of autophagy by cytoplasmic p53. *Nat Cell Biol*, **10**, 676-687.

Thome, M. and Tschopp, J. (2001) Regulation of lymphocyte proliferation and death by FLIP. *Nat Rev Immunol*, **1**, 50-58.

Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456-1462.

Tsujimoto, Y., Finger, L.R., Yunis, J., Nowell, P.C. and Croce, C.M. (1984) Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science*, **226**, 1097-1099.

Twiddy, D., Cohen, G.M., Macfarlane, M. and Cain, K. (2006) Caspase-7 is directly activated by the approximately 700-kDa apoptosome complex and is released as a stable XIAP-caspase-7 approximately 200-kDa complex. *J Biol Chem*, **281**, 3876-3888.

van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., Rodriguez, I., Ruiz-Carrillo, A., Vandekerckhove, J., Declercq, W., Beyaert, R. and Vandenabeele, P. (2001) Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death Differ*, **8**, 1136-1142.

Vaux, D.L. and Korsmeyer, S.J. (1999) Cell death in development. *Cell*, **96**, 245-254.

Vayssade, M., Haddada, H., Faridoni-Laurens, L., Tourpin, S., Valent, A., Benard, J. and Ahomadegbe, J.C. (2005) P73 functionally replaces p53 in Adriamycin-treated, p53-deficient breast cancer cells. *Int J Cancer*, **116**, 860-869.

Verhagen, A.M., Ekert, P.G., Pakusch, M., Silke, J., Connolly, L.M., Reid, G.E., Moritz, R.L., Simpson, R.J. and Vaux, D.L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell*, **102**, 43-53.

von Haefen, C., Wieder, T., Essmann, F., Schulze-Osthoff, K., Dorken, B. and Daniel, P.T. (2003) Paclitaxel-induced apoptosis in BJAB cells proceeds via a death receptor-independent, caspases-3/-8-driven mitochondrial amplification loop. *Oncogene*, **22**, 2236-2247.

Waldman, T., Kinzler, K.W. and Vogelstein, B. (1995) p21 is necessary for the p53-mediated G1 arrest in human cancer cells. *Cancer Res*, **55**, 5187-5190.

Walensky, L.D., Pitter, K., Morash, J., Oh, K.J., Barbuto, S., Fisher, J., Smith, E., Verdine, G.L. and Korsmeyer, S.J. (2006) A stapled BID BH3 helix directly binds and activates BAX. *Mol Cell*, **24**, 199-210.

Walker, D.H. and Maller, J.L. (1991) Role for cyclin A in the dependence of mitosis on completion of DNA replication. *Nature*, **354**, 314-317.

Wang, B., Xiao, Z. and Ren, E.C. (2009) Redefining the p53 response element. *Proc Natl Acad Sci U S A*, **106**, 14373-14378.

Wang, C., Li, Z., Fu, M., Bouras, T. and Pestell, R.G. (2004) Signal transduction mediated by cyclin D1: from mitogens to cell proliferation: a molecular target with therapeutic potential. *Cancer Treat Res*, **119**, 217-237.

Wang, K., Yin, X.M., Chao, D.T., Milliman, C.L. and Korsmeyer, S.J. (1996) BID: a novel BH3 domain-only death agonist. *Genes Dev*, **10**, 2859-2869.

Wang, K.K. (2000) Calpain and caspase: can you tell the difference? *Trends Neurosci*, **23**, 20-26.

Wang, K.K., Posmantur, R., Nadimpalli, R., Nath, R., Mohan, P., Nixon, R.A., Talanian, R.V., Keegan, M., Herzog, L. and Allen, H. (1998) Caspase-mediated fragmentation of calpain inhibitor protein calpastatin during apoptosis. *Arch Biochem Biophys*, **356**, 187-196.

Waterhouse, N.J., Goldstein, J.C., von Ahsen, O., Schuler, M., Newmeyer, D.D. and Green, D.R. (2001) Cytochrome c maintains mitochondrial transmembrane potential and ATP generation after outer mitochondrial membrane permeabilization during the apoptotic process. *J Cell Biol*, **153**, 319-328.

Weber, J.D., Jeffers, J.R., Rehg, J.E., Randle, D.H., Lozano, G., Roussel, M.F., Sherr, C.J. and Zambetti, G.P. (2000) p53-independent functions of the p19(ARF) tumor suppressor. *Genes Dev*, **14**, 2358-2365.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. and Korsmeyer, S.J. (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science*, **292**, 727-730.

Weinberg, R.A. (1995) The retinoblastoma protein and cell cycle control. *Cell*, **81**, 323-330.

Wendt, J., Radetzki, S., von Haefen, C., Hemmati, P.G., Guner, D., Schulze-Osthoff, K., Dorken, B. and Daniel, P.T. (2006) Induction of p21CIP/WAF-1 and G2 arrest by ionizing irradiation impedes caspase-3-mediated apoptosis in human carcinoma cells. *Oncogene*, **25**, 972-980.

Wieder, T., Essmann, F., Prokop, A., Schmelz, K., Schulze-Osthoff, K., Beyaert, R., Dorken, B. and Daniel, P.T. (2001) Activation of caspase-8 in drug-induced apoptosis of B-lymphoid cells is independent of CD95/Fas receptor-ligand interaction and occurs downstream of caspase-3. *Blood*, **97**, 1378-1387.

Willis, S.N. and Adams, J.M. (2005) Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol*, **17**, 617-625.

Willis, S.N., Fletcher, J.I., Kaufmann, T., van Delft, M.F., Chen, L., Czabotar, P.E., Ierino, H., Lee, E.F., Fairlie, W.D., Bouillet, P., Strasser, A., Kluck, R.M., Adams, J.M. and Huang, D.C. (2007) Apoptosis initiated when BH3 ligands engage multiple Bcl-2 homologs, not Bax or Bak. *Science*, **315**, 856-859.

Woodhouse, B.C. and Dianov, G.L. (2008) Poly ADP-ribose polymerase-1: an international molecule of mystery. *DNA Repair (Amst)*, **7**, 1077-1086.

Wu, X., Bayle, J.H., Olson, D. and Levine, A.J. (1993) The p53-mdm-2 autoregulatory feedback loop. *Genes Dev*, **7**, 1126-1132.

Wyllie, A.H., Morris, R.G., Smith, A.L. and Dunlop, D. (1984) Chromatin cleavage in apoptosis: association with condensed chromatin morphology and dependence on macromolecular synthesis. *J Pathol*, **142**, 67-77.

Xie, Z. and Klionsky, D.J. (2007) Autophagosome formation: core machinery and adaptations. *Nat Cell Biol*, **9**, 1102-1109.

Xue, L., Fletcher, G.C. and Tolkovsky, A.M. (2001) Mitochondria are selectively eliminated from eukaryotic cells after blockade of caspases during apoptosis. *Curr Biol*, **11**, 361-365.

Yarbrough, W.G., Bessho, M., Zanation, A., Bisi, J.E. and Xiong, Y. (2002) Human tumor suppressor ARF impedes S-phase progression independent of p53. *Cancer Res*, **62**, 1171-1177.

Yee, K.S., Wilkinson, S., James, J., Ryan, K.M. and Vousden, K.H. (2009) PUMA- and Bax-induced autophagy contributes to apoptosis. *Cell Death Differ*.

Youle, R.J. and Strasser, A. (2008) The BCL-2 protein family: opposing activities that mediate cell death. *Nat Rev Mol Cell Biol*, **9**, 47-59.

Yousefi, S., Perozzo, R., Schmid, I., Ziemiecki, A., Schaffner, T., Scapozza, L., Brunner, T. and Simon, H.U. (2006) Calpain-mediated cleavage of Atg5 switches autophagy to apoptosis. *Nat Cell Biol*, **8**, 1124-1132.

Yu, J.W., Jeffrey, P.D. and Shi, Y. (2009) Mechanism of procaspase-8 activation by c-FLIPL. *Proc Natl Acad Sci U S A*, **106**, 8169-8174.

Yu, L., Alva, A., Su, H., Dutt, P., Freundt, E., Welsh, S., Baehrecke, E.H. and Lenardo, M.J. (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science*, **304**, 1500-1502.

Yu, X., Acehan, D., Menetret, J.F., Booth, C.R., Ludtke, S.J., Riedl, S.J., Shi, Y., Wang, X. and Akey, C.W. (2005) A structure of the human apoptosome at 12.8 A resolution provides insights into this cell death platform. *Structure*, **13**, 1725-1735.

Zamzami, N. and Kroemer, G. (2001) The mitochondrion in apoptosis: how Pandora's box opens. *Nat Rev Mol Cell Biol*, **2**, 67-71.

Zang, F., Sun, B., Zhao, X., Niu, R., Zhang, S., Yu, M., Wei, X. and Zhang, L. (2008) Critical role for c-FLIP(L) on Fas resistance in colon carcinoma cell line HT-29. *Cell Biol Int*, **32**, 329-336.

Zhang, Y., Xiong, Y. and Yarbrough, W.G. (1998) ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell*, **92**, 725-734.

Zhang, Y.J., Jiang, W., Chen, C.J., Lee, C.S., Kahn, S.M., Santella, R.M. and Weinstein, I.B. (1993) Amplification and overexpression of cyclin D1 in human hepatocellular carcinoma. *Biochem Biophys Res Commun*, **196**, 1010-1016.

Zhao, H., Watkins, J.L. and Piwnica-Worms, H. (2002) Disruption of the checkpoint kinase 1/cell division cycle 25A pathway abrogates ionizing radiation-induced S and G2 checkpoints. *Proc Natl Acad Sci U S A*, **99**, 14795-14800.

Zhou, P., Jiang, W., Zhang, Y.J., Kahn, S.M., Schieren, I., Santella, R.M. and Weinstein, I.B. (1995) Antisense to cyclin D1 inhibits growth and reverses the transformed phenotype of human esophageal cancer cells. *Oncogene*, **11**, 571-580.

Zhu, W., Cowie, A., Wasfy, G.W., Penn, L.Z., Leber, B. and Andrews, D.W. (1996) Bcl-2 mutants with restricted subcellular location reveal spatially distinct pathways for apoptosis in different cell types. *Embo J*, **15**, 4130-4141.

Zindy, F., Eischen, C.M., Randle, D.H., Kamijo, T., Cleveland, J.L., Sherr, C.J. and Roussel, M.F. (1998) Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. *Genes Dev*, **12**, 2424-2433.

Zong, W.X. and Thompson, C.B. (2006) Necrotic death as a cell fate. *Genes Dev*, **20**, 1-15.

Zou, H., Henzel, W.J., Liu, X., Lutschg, A. and Wang, X. (1997) Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3. *Cell*, **90**, 405-413.

APPENDIX

8. Appendix

8.1 Posters and presentations

Ana Milojkovic, Philipp G. Hemmati, Dilek Güner, Peter T. Daniel (2005). Apoptosis induction by $p14^{ARF}$ is attenuated upon combined loss of Bax and Bak. European students' conference, Berlin, Germany, oral presentation.

Ana Milojkovic, Philipp G. Hemmati, Dilek Güner, Peter T. Daniel (2005).
The role of $p14^{ARF}$ in the intrinsic apoptotic pathway. 13th euroconference on apoptosis, Budapest, Hungary, poster presentation.

Ana Milojkovic, Philipp G. Hemmati, Dilek Güner, Peter T. Daniel (2006).
The effects of $p14^{ARF}$ in apoptosis. European Workshop on Cell Death, Kerkrade, The Netherlands, poster presentation.

Ana Milojkovic, Philipp G. Hemmati, Bernd Gillissen, Jana Wendt, Peter T. Daniel (2006).
DNA damage checkpoint control programs interfere with $p14^{ARF}$-induced apoptosis, 14th euroconference on apoptosis, Sardinia, Italy, poster presentation.

Ana Milojkovic, Philipp G. Hemmati, Bernd Gillissen, Jana Wendt, Peter T. Daniel (2006).
DNA damage checkpoint control programs interfere with $p14^{ARF}$ induced cell death. European students' conference, Berlin, Germany, oral presentation.

Ana Milojkovik, Philipp G. Hemmati, Bernd Gillissen, Jana Wendt, Peter T. Daniel (2007).
$P14^{ARF}$ regulates cell cycle checkpoint control and induces cell death via autophagy in the absence of caspase activation. European students' conference, Berlin, Germany, oral presentation-winner of the session prize.

Ana Milojkovic, Philipp G. Hemmati, Peter T. Daniel (2007).
$P14^{ARF}$ regulate checkpoint control and induce cell death. 15th euroconference on apoptosis, Portoroz, Slovenia, poster presentation.

Ana Milojkovic, Philipp G. Hemmati, Bernd Gillissen, Jana Wendt, Peter T. Daniel (2008). $P14^{ARF}$ is involved in different types of cell death. European students' conference Berlin, Germany, poster presentation.

Ana Milojkovic, Philipp G. Hemmati, Bernd Gillissen, Jana Wendt, Peter T. Daniel (2008). $P14^{ARF}$ induces different types of cell death depending on the presence of caspase-3. European workshop on cell death, Hauenstein, Germany, poster presentation.

Ana Milojkovic, Philipp G. Hemmati, Bernd Gillissen, Jana Wendt, Peter T. Daniel (2009). The tumor suppressor protein $p14^{ARF}$ regulates cell cycle checkpoint control and induces cell death. European students' conference, Berlin, Germany, oral presentation.

8.2 Publications

Philipp G. Hemmati, Annika Müer, Bernd Gillissen, Tim Overkamp, **Ana Milojkovic,** Jana Wendt, Bernd Dörken, Peter T. Daniel (2010). "Systematic genetic dissection of p14(ARF)-mediated mitochondrial cell death signaling reveals a key role for p21(CDKN1) and the BH3-only protein Puma/bbc3". Journal of Molecular Medicine, 1432-1440.

Ana Milojkovic, Philipp G. Hemmati, Bernd Gillissen, Rainer Jänicke, Peter T. Daniel (2010). "Cell cycle checkpoint abrogation sensitizes MCF-7 cells for $p14^{ARF}$ induced apoptosis via an entirely caspase-3 dependent mechanism". in preparation

8.3 Curriculum vitae

For the reasons of data protection, the Curriculm vitae is not published in the online version.