Cancer-related targets sensed by apoptin

Henriëtte Leonore Lanz

The research presented in this thesis was performed at the department of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, The Netherlands.

Cover: Fluorescent microscope picture of human osteasarcoma cells transiently transfected with plasmid encoding flag-apoptin. After fixation cells were stained for apoptin (red), tubulin (green) and DNA (blue).

An electronic version of this dissertation is available at the Leiden University Repository (https://openaccess.leidenuniv.nl)

Printed by Optima, Rotterdam, The Netherlands

Cancer-related targets sensed by apoptin

PROEFSCHRIFT

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus Prof. Mr. P.F. van der Heijden,
volgens besluit van het College voor Promoties
te verdedigen op woensdag 24 oktober 2012
klokke 15.00 uur

door

Henriëtte Leonore Lanz

geboren te Leidschendam in 1983

Promotiecommissie

Promotor	Prof. Dr. M.H.M. Noteborn
Copromotor	Dr. C. Backendorf
Overige leden	Prof. Dr. J. Brouwer
	Prof. Dr. J.-P. Abrahams
	Prof. Dr. M. Tavassoli (King's College, London)
	Prof. Dr. P.C.W. Hogendoorn

Contents

Chapter 1

Thesis Outline

Cancer is one of the most life-threatening diseases worldwide. Many anticancer treatments fail because they are not efficient or reveal too high side effects. Cancer cells develop due to unbalances in cell proliferation, cell cycle regulation and/or cell death. Identification of these aberrant molecular processes will reveal the essential processes leading to tumor formation and allows the generation of potential novel anticancer therapies. Interestingly, a group of viral and cellular proteins has been identified that harbors tumor-selective apoptosis activity. They are named Proteins Killing Tumor Cells (PKTCs).

The aim of this thesis is to search for cellular processes sensed by the original PKTC, apoptin, which distinguish tumor cells from normal cells. Various tumor-related processes that are of importance for the tumor-selective activation of apoptin are reported. They range from proteasome activity and mitotic regulation to protein kinase and phosphatase action. **Chapter 2** introduces our current knowledge on the hallmarks of cancer as well as an overview of the growing family of PKTCs. We argue that PKTCs might recognize the transformed state of a tumor cell via detection of one or more hallmarks of cancer. This biological capability of the various PKTCs might be at the base of the switch from cell growth to cell death.

Chapter 3 describes two independent lines of research revealing that deregulation of the normal function of protein phosphatase 2A (PP2A) is a sufficient step in activating apoptin-induced cell death. Derailed PP2A activity is increasingly linked to oncogenic transformation and especially its involvement during mitosis might be relevant for activation of apoptin. **Chapter 4** shows that in cancer cells apoptin is insensitive to proteasomal degradation, a process that still takes place in normal cells. This cancer-related loss of proteasomal susceptibility appears to be specific for apoptin as it is not found for the tumor suppressor protein p53. In **Chapter 5** the biochemical characterization of the tumor-selective apoptin kinase activity implies the existence in tumor cells of a constitutive endogenous kinase that is present in both the nuclear and cytoplasmic compartments. Weak inaccuracies in mitotic checkpoints are linked to aneuploidy and genetic instability, which are essential for cancer development. In **Chapter 6** it is shown that apoptin likely senses these inaccuracies during cell division and disrupts a cancerous process by inducing mitotic catastrophe and cell death.

All data obtained in this study are discussed in **Chapter 7**. The systematic analysis of both apoptin and other PKTCs reflects various differences between normal and tumor cells. Linking-up the various hallmarks of cancer with the aberrant processes sensed by apoptin and other PKTCs in cancer cells will hopefully contribute to a better understanding of tumor development as well as the generation of efficient and safe anticancer therapies.

Chapter 2

Introduction

Proteins Killing Tumor Cells

The past century life expectancy has steadily been increasing with no limit yet in sight (Oeppen & Vaupel, 2002; Lee, 2011). Cancer risk increases with age due to the nature of tumorigenesis (Anisimov, 2009; Fulop et al., 2011). This implies that a person that gets sufficiently old will get cancer. In spite of the fact that cancer has been threatening human life since ancient antiquity the perfect cure still has not been found. Current therapies lack specificity, killing healthy cells in the process of curing cancer (Pavet et al., 2011). As not to spoil the joys of increased life expectancy with the prospect of suffering from a devastating disease, drugs with high selectivity are duly needed.

Proteins Killing Tumor Cells (PKTCs)

In the past two decades proteins emerged from various origins displaying selective anti-cancer activity. The genes encoding these proteins were dubbed anticancer genes (Grimm & Noteborn, 2010) and the corresponding proteins are referred to as PKTCs: Proteins Killing Tumor Cells (Bruno et al., 2009). In 1997, apoptin opened the field by showing selective apoptosis induction in transformed and malignant cells, while leaving normal cells unharmed (Danen-van Oorschot et al., 1997). Several other proteins with similar characteristics soon followed.

Apoptin, E4orf4 and NS1 are produced by viruses; onconase together with Brevinin-2R are derived from frogs and HAMLET is a milk protein forming a complex with oleic acid Interestingly, the largest subgroup of PKTCs are of human origin (TRAIL, mda-7, Par-4, Noxa and ORCTL3) showing that cancer can be selectively battled with our own weapons.

As cancer cells are derived from our own healthy tissue, it was considered for a long time impossible to target cancer in a way that would not harm healthy cells. Meanwhile each cancer is unique and this diversity made the production of one specific drug seemingly unfeasible. In 2000, the publication on the hallmarks of cancer firmly established tumor cells as distinct from normal cells with a set of common characteristics allowing the selective targeting of malignant tissue (Hanahan & Weinberg, 2000).

PKTCs can recognize the transformed state of a cancer cell, presumably through the recognition of one or more of the hallmarks, and bend the proliferative fate of the tumor towards a fatal ending of undergoing cell death (Argiris et al., 2011).

The Hallmarks of Cancer

Each cancer is unique in its molecular make-up, however, each cancer cell answers to a specific set of capabilities that enabled it to undergo tumorigenesis. These capabilities are named 'the hallmarks of cancer'. They confer upon a cell its tumorigenic potential, while at the same time setting them apart from normal cells to such an extent that they can be specifically targeted (Hanahan & Weinberg, 2000; 2011).

The first hallmark is the ability of cancer cells to *sustain proliferative signaling*. To maintain tissue homeostasis a delicate balance between proliferation, cell senescence and cell death is regulated by various types of signaling. For cancer cells to flourish they have to maintain growth-promoting signals. They can do this through the manufacturing of growth factors themselves or by stimulating neighboring cells to secrete them. Signals are received by growth factor receptors. Upregulation or mutation of these receptors also helps to sustain the proliferative signal (Lemmon & Schlessinger, 2010). Furthermore by constitutively activating the signaling pathways downstream of the growth factor receptors tumor cells can create the required environment for continuous growth (Shaw & Cantley, 2006).

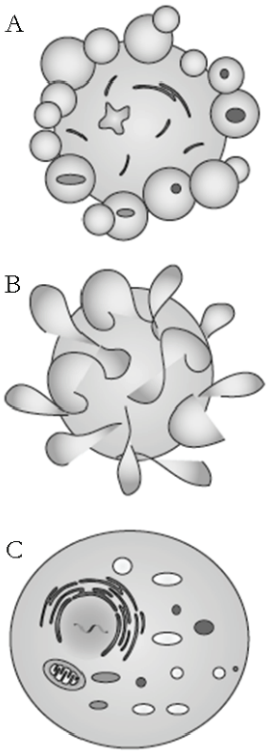
Apart from upholding a continuous growth signal, a cancer cell has to *evade growth suppressors*, which constitutes the second hallmark. Suppressing signals are sent both from outside a cell as well as from the inside. Growth suppression is regulated by surrounding cells to prevent uncontrolled cell proliferation and to avert damaged cells from passing on potential dangerous alterations to progeny. Cancer cells circumvent this block on their generation by the downregulation of tumor suppressor genes such as *RB* and *TP53* (Burkhart & Sage, 2008; Levine et al., 1991).

When tissue homeostasis is threatened by aberrant growth, triggers for programmed cell death are emitted. Activation of tumor suppressors due to cellular defects leads to cell death pathways preventing the formation of malignant cells. For tumorigenesis to proceed, the third hallmark has to be obeyed, *resisting cell death*. This can be achieved by altering the balance between pro-survival and pro-death proteins (Hanahan & Weinberg, 2011; Adams & Cory, 2007) (Box 2.1).

Box 2.1 Various types of cell death

One of the hallmarks of cancer cells is the resistance against cell death signaling. Programmed cell death is important during (embryonic) development and to maintain tissue homeostasis. Several types of cell death have been recognized and all are highly regulated in order to prevent tissue inflammation, excessive cell death and transformation. In 2011 the Nomenclature Committee on Cell Death proposed to no longer identify cell death types by their morphological features, but rather relate them to a subset of biochemical features (Galluzzi et al., 2012).

Apoptosis (a) is the most common and well-described programmed cell death. Rounding-up of the cell, reduction of cellular volume, chromatin condensation, nuclear fragmentation, membrane blebbing and engulfment by phagocytes are the morphological features of apoptosis (Kroemer et al., 2009). It has two distinct routes, intrinsic and extrinsic. Both are dependent on the caspase cascade, the proteases responsible for most of the apoptotic morphology. The extrinsic pathway is triggered by binding of extracellular stress signals to receptors, whereas the intrinsic pathway is regulated by intracellular conditions (Galluzzi et al., 2012). The clearance of the apoptotic cell remnants by phagocytes means that there is no immune response.



apoptosis:
caspase activatioin
pyknosis
nuclear condensation and DNA fragmentation
phosphatidylserine exposure
ROS generation
loss of mitochondrial membrane potential

necrosis:
ROS generation
loss of mitochondrial membrane potential
plasma membrane rupture
protease activation

autophagy:
vacuolization
LC3 lipidation

Apoptosis (a), necrosis (b) and autophagy (c) main cell death characteristics, adapted from Kepp et al. (2011).

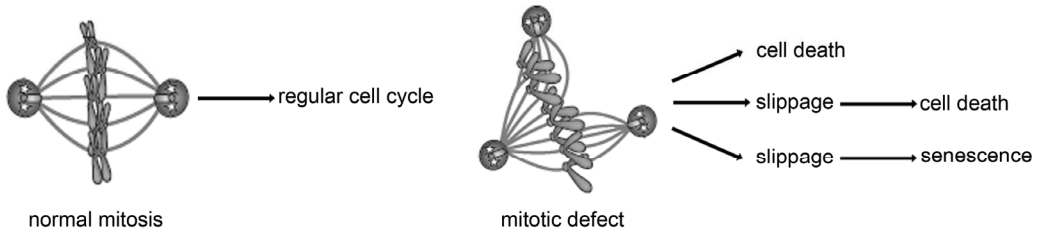
Necrosis (b) is characterized by swelling of both the cell and its organelles, rupture of the plasma membrane and the loss of intracellular contents (Kroemer et al., 2009). For a long time it was considered to be unregulated and as such the opposite of apoptosis. However, regulated necrosis has been shown to happen after various cellular triggers, no longer making it an accidental event. Key-regulators are the RIP kinases that under

regulation of death receptors can opt for necrosis in the absence of a functional caspase route (Declercq et al., 2009). As such necrosis is a backup mechanism when apoptosis is not an option. Furthermore the release of intracellular molecules triggers an innate immune response which in some situations, e.g. viral infection, is more desirable than the neat clean up after apoptosis.

Autophagy (c) is primarily a survival process during starvation. During low nutrient conditions cytoplasmic material is collected in a double membrane vesicle, the autophagosome, which fuses with lysosomes, leading to the degradation of the contents (Denton et al., 2012). Autophagic cell death was coined for a type of cell death that is accompanied by massive cytoplasmic vacuolization and the upregulation of the autophagy markers such as LC3 puncta (Galluzzi et al., 2012). However, it is not surprising that in the situation preceding cell death, autophagy is upregulated as a survival strategy (Denton et al., 2012).

Mitotic catastrophe (d) was originally defined as 'death during mitosis'. During the elucidation of the molecular processes defining mitotic catastrophe it was concluded that it is not a 'pure' cell death mechanism, but rather an oncosuppressive mechanism that results in apoptosis or senescence (Galluzzi et al., 2012). It is characterized by perturbations of the mitotic machinery, starts during M phase, is paralleled by some degree of mitotic arrest and ultimately results in antiproliferative measures (Vitale et al., 2011).

D



Mitotic catastrophe caused by perturbations of the mitotic machinery.

In the past years more cellular types of programmed cell death were biochemically characterized, i.e. anoikis, necroptosis, cornification, entosis, netosis, parthanatos and pyroptosis. Often they are restricted to specific cell types and linked to a very distinct trigger.

In order to become a multi-cellular tumor cancer cells have to overcome the regulatory mechanisms of the environment that could stop their growth, as the previous three hallmarks indicate. There are also internal controls to prevent neoplasia. Cells have a natural build-in replication limit regulated by the telomere length, the ends of the chromosomes that naturally shorten during each cell division. When telomeres have shortened sufficiently after the maximum number of divisions cells go into senescence, a non-proliferative but viable state (Hayflick, 1997). This internal block has to be overcome by cancer cells, *enabling replicative immortality*. The most common way that this happens is by re-activation of telomerase, an enzyme that can lengthen telomeres. Alternative lengthening of

telomeres is a second option and depends on homologous recombination of the telomere repetitive DNA (Cesare & Reddel, 2010).

As any tissue a multi-cellular tumor is in need of sustenance. After reaching a minimal size the regular vasculature of the human body is no longer sufficient to provide the nutrients needed by the budding tumor. In order to feed all the tumor cells new vessels will have to be created in a process referred to as *inducing angiogenesis*. In healthy adults angiogenesis is a rare process that is only transiently switched on in situations as for instance wound healing. During tumorigenesis it has to be turned on permanently, a process referred to as the 'angiogenic switch' (Weis & Cheresh, 2011).

The next hallmark in tumor development is *activating invasion and metastasis*. Reprogramming of the cell through the epithelial-mesenchymal transition (EMT) allows cells to detach from the surrounding cells and extracellular matrix without inducing cell death. With these newfound capabilities the cancer cell is no longer dependent on its original surrounding tissue for survival, a new settlement can be set up in a different part of the body (Valastyan & Weinberg, 2011).

In 2011, the list of hallmarks was extended with two more biological capabilities acquired by cancer cells. Furthermore two characteristics enabling the tumorigenic process were defined (Hanahan & Weinberg, 2011).

Cells have two main pathways for the generation of energy from glucose, the choice depends on the availability of oxygen. Cancer cells reprogram their energy metabolism to a state termed 'aerobic glycolysis' also known as Warburg effect, meaning that even when enough oxygen is present they do not purely rely on oxidative phosphorylation as normal cells would under that circumstance (Koppenol et al., 2011). *Deregulation of cellular energetics* is an emerging hallmark. The switch leaves cancer cells addicted to glycolysis for their ATP production and sensitive to perturbations of this pathway (Pelicano et al., 2006).

The human body is under constant surveillance of the immune system. The immune system is capable of detecting and destroying malignant cells and prevents their outgrowth. However, it also creates a tumor environment that supports development of cancer cells that can *avoid immune destruction*. Tumor cells might have lost their characteristic tumor antigen expression by e.g. the down regulation of their major histocompatibility complex class I proteins presenting the tumor-related antigens to tumor-specific T cells (Schreiber et al., 2011).

Together, the eight hallmarks described above (summarized in figure 2.1) bestow on a normal cell the ability to become a cancer cell. Acquisition of the hallmarks happens due to the so-called enabling characteristics, *genome instability* and *tumor-promoting inflammation*. The immune system can prevent neoplasia, however, inflammation can also contribute to several of the hallmark capabilities by supplying bioactive molecules (Grivennikov et al., 2010).

The biological characteristics described by the hallmarks can in general be achieved through alterations in the genome (Aquilera & Gomez-Gonzalez, 2008). Point mutations, aneuploidy and epigenetic alterations can change the activity of oncogenes and tumor suppressors, allowing a cell to obtain one hallmark at a time (Negrini et al., 2010).

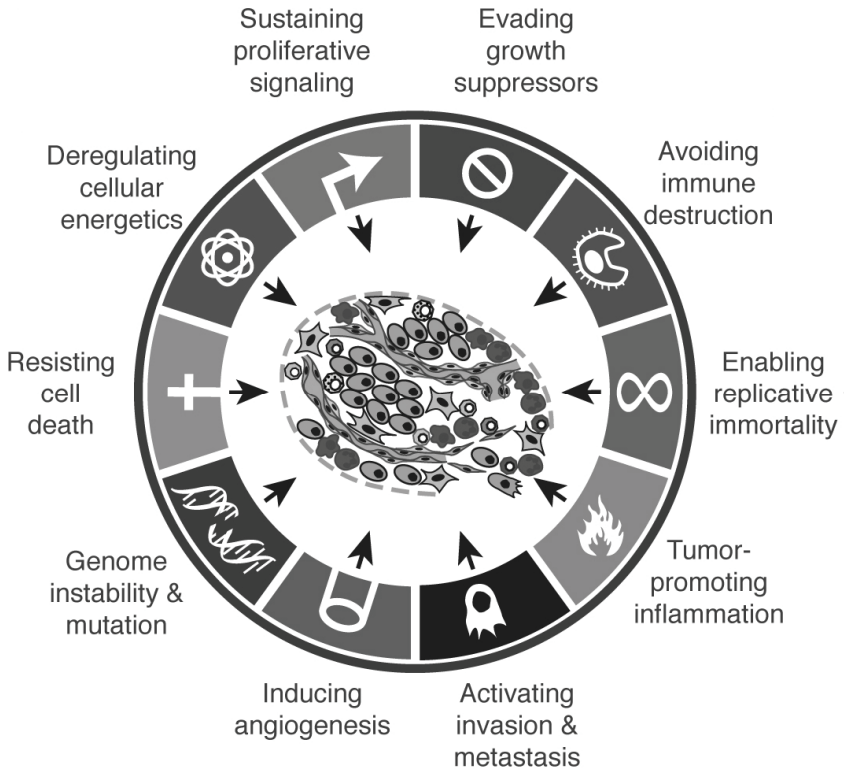


Figure 2.1: The hallmarks of cancer and enabling characteristics adapted from Hanahan & Weinberg, 2011. Schematic representation of the hallmarks of cancer as they, together with the enabling characteristics, create the tumor microenvironment allowing cancer progression to take its course.

Targeting Tumor-Related Processes

Chemical anticancer drugs are designed to target one component in a pathway relevant to one, or a few, of the hallmarks of cancer. While having the desired effect of crippling the cancer by poking at one of its foundations, usually the drug target also has an important

function in normal cells. Synthetic anticancer drugs might as such be too simplistic to take full advantage of the differences between normal, and tumor cells. The PKTCs supplied by nature can act selectively on cancer cells; the question is if they do this by recognizing the transformed state of a tumor through the detection of the hallmarks, and if it is this biological capability that makes that PKTCs can switch cells towards cell death.

PKTCs targeting hallmarks of cancer

To analyze the relationship between the hallmarks of cancer and PKTCs the main characteristics of these proteins, and the cellular characteristics by which they were found to distinguish between normal and tumor cells, will be described below.

apoptin

Apoptin is the Viral Protein 3 (VP3) of Chicken Anemia Virus (CAV). CAV is the causative agent of aplastic anemia in young chickens, leading to high death rates due to depletion of thymocytes. CAV is particularly lethal in combination with Marek's disease, an oncogenic DNA virus pathogen (Noteborn et al., 1991; Osterrieder et al., 2006). In transformed chicken cells CAV causes apoptosis. This effect is attributed to VP3 as expression of this protein alone is sufficient to induce apoptosis. As such VP3 was renamed apoptin (Noteborn et al., 1994).

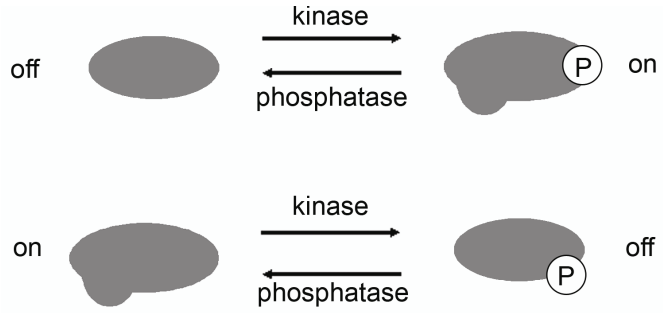
Apoptin can induce cell death in human tumor cells, but is harmless towards healthy human cells, making it the first Protein Killing Tumor Cells (Zhuang et al., 1995; Danen-van Oorschot et al., 1997). Apoptin was shown to induce apoptosis in over 70 cancer cell lines in a p53-independent fashion (Backendorf et al., 2008). Apart from the selective apoptosis induction apoptin shows more differential behavior between normal and tumor cells. In cancer cells apoptin translocates to the nucleus whereas in normal cells it stays in the cytoplasm. The nuclear localization is regulated by a bipartite nuclear localisation sequence (NLS) and the nuclear transport is correlated to the apoptosis induction. However, forced nuclear localization of apoptin in normal cells is not sufficient to trigger cell death (Danen-van Oorschot et al., 2003). Apoptin has a CRM1-recognized nuclear export signal (NES) that allows the apoptin protein to return to the cytoplasm in normal cells (Poon et al., 2005). The inactivity of the NES in tumor cells is attributed to another differential aspect of

apoptin, namely the cancer-specific phosphorylation of threonine 108 (Rohn et al., 2002; Poon et al., 2005) (box 2.2).

Box 2.2 Phophatases and kinases; the cellular on/off switchers

Protein activity can be regulated by phosphorylation, the attachment of a phosphate group from a high energy donor as e.g. ATP to a Ser/Thr or Tyr of the protein. Phosphorylation can switch on or off the activity of a protein based on the modified amino acid and the related (structural) changes (Johnson & Lewis, 2001). Protein kinases are the enzymes responsible for phosphorylating events. Over 500 putative kinases have been identified in the human genome. Due to their crucial role in regulating cellular pathways many kinases are related to disease (Manning et al., 2002). The cellular targets of kinases are often other kinases, leading to phosphorylation cascades. Constitutive activation of a kinase through mutation is a common trigger for several of the hallmarks of cancer, as for example oncogenic casein kinase II and sustainment of proliferative signaling (Hanf et al., 2010). Their central role in (oncogenic) signaling makes kinases a popular target in anti-cancer drug design (Gherardi et al., 2012; Canavese et al., 2012).

Phosphorylation is a reversible process that can be counteracted by phosphatases. Whereas there is a wealth of kinases available, only a small number of phosphatases is at the cell's disposal (estimated at 30 for ser/thr phosphatases and 107 for tyr phosphatases). Instead of shear numbers, phosphatase specificity is regulated by a large number of exchangeable regulatory subunits (Shi, 2009). For example the protein phosphatases 2A (PP2A) consists of three subunits, a scaffold (Aα or Aβ), a catalytic part (Cα or Cβ) and a regulatory subunit determining localization and specificity (B55, B56, PR72 and PR93 families). Over 60 different PP2A compositions are known to be possible (Mumby, 2007). Where kinases are often oncogenes, phosphatases are more likely involved in cancer formation as tumor suppressors. PP2A subunits are targeted by oncogenic viruses and over-expression of PP2A inhibitors support the establishment of several hallmarks of cancer (Westermarck & Hahn, 2008).



Phosphorylation of a protein can either switch the activity on or off. This is a reversible event controlled by the balanced actions of kinases and phosphatases.

In the field a consensus on the kinase responsible for this modification has not yet been reached (for details see **chapter five**). Maddika et al. (2009) reported that aberrant activation of cyclin-dependent kinase 2 (CDK2), complexed with cyclin A, leads to apoptin phosphorylation in the nucleus. This cell cycle regulated complex becomes specifically

activated in tumor cells due to the translocation of Akt from the cytoplasm to the nucleus. Cellular relocalizations of Akt are caused by apoptin itself which interacts with Akt and brings it to the nucleus (Maddika et al., 2007). Phosphoinositide 3-kinase (PI3K) functions upstream of Akt. Apoptin activates PI3K through interaction with one of its subunits (Maddika et al., 2008). The PI3K/Akt pathway is often over-activated in cancer, promoting proliferation and suppressing cell death (Vivanco & Sawyers, 2002). Meanwhile Jiang et al. (2010) showed that down-regulation of protein kinase C isoform β (PKCβ) significantly reduced apoptin phosphorylation in several cell lines. PKCβ positively influences proliferation in cancer cells (Li et al., 2011b).

In normal cells apoptin forms in time granules in the cytoplasm, becomes epitope-shielded and eventually it can no longer be detected (Zhang et al., 2003). The tumor-specific behavior of apoptin can be triggered in normal cells by transformation with the SV40 large and small T antigen (Zhang et al., 2004). At its N-terminus apoptin has a leucine-rich-region which is responsible for many of its protein-protein interactions (Backendorf et al., 2008). One of the most important interaction partners of apoptin is apoptin itself. Apoptin forms globular multimers that induce apoptosis (Leliveld et al., 2003b).

Apoptin binds to DNA directly (Leliveld et al., 2003a). Furthermore it interacts with DEDAF (death effector domain associated factor) (Danen-van Oorschot et al., 2004). DEDAF is a transcriptional repressor that changes conformation upon binding to DNA (Neira et al., 2009). DNA damage leads to p53 stabilization through DEDAF, which is down regulated in tumor cells marking it as tumor suppressor (Chen et al., 2009). Apoptin can be activated in normal cells by DNA damage response (DDR) signaling. Knockdown of key factors in DDR can block apoptin activity in tumor cells (Kucharski et al., 2011). Either directly or indirectly, apoptin seems capable of detecting damaged DNA.

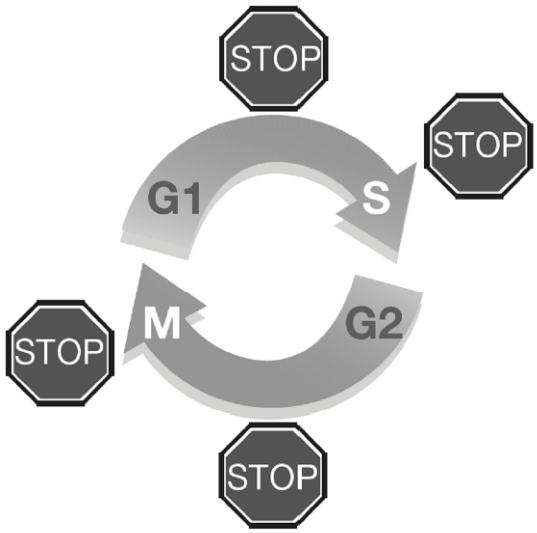
Apoptin can block the cell cycle in G2/M (Box 2.3 and 2.4) through interaction with a subunit of the anaphase promoting complex/cyclosome (APC/C) (Teodoro et al., 2004). APC/C is an E3 ubiquitin ligase charged with the task of maintaining genetic stability (Box 2.5) (Lipkowitz & Weissman, 2011). Through upregulation of ceramide levels and the cytoplasmic translocation of Nur77 apoptin induces apoptosis (Liu et al., 2006; Maddika et al., 2005). The apoptosis induction is p53-independent. Instead, apoptin functions through p53-family member p73, upregulating the pro-death proteins Puma and Noxa (Box 2.6) (Klanrit et al., 2008). Apoptin improves p73 apoptotic signaling by shifting the balance in

favor of the pro-death TAp73. This is mediated by stimulating the ubiquitin ligase PIR2 which targets the anti-apoptotic ΔNp73 isoform for degradation (Taebunpakul et al., 2012).

Box 2.3 The cell cycle

An important facet of life is the ability to create progeny. One cell passes on its information to its two daughter cells during cell division. When the correct signals are present a cell will continue with the circle of life, the cell cycle. There are two main events in the cell cycle, the duplication of the DNA in S phase, and the correct distribution of the two sets of chromosomes over the two daughter cells during M phase. These two phases are separated by two gap phases, G1 and G2 (Alberts et al., 2008). When not enough growth signals are present a cell can withdraw from the cell cycle during G1, into some quiescent state named G0. Once the stop-or-go moment in G1 has been passed a cell is committed to complete the circle.

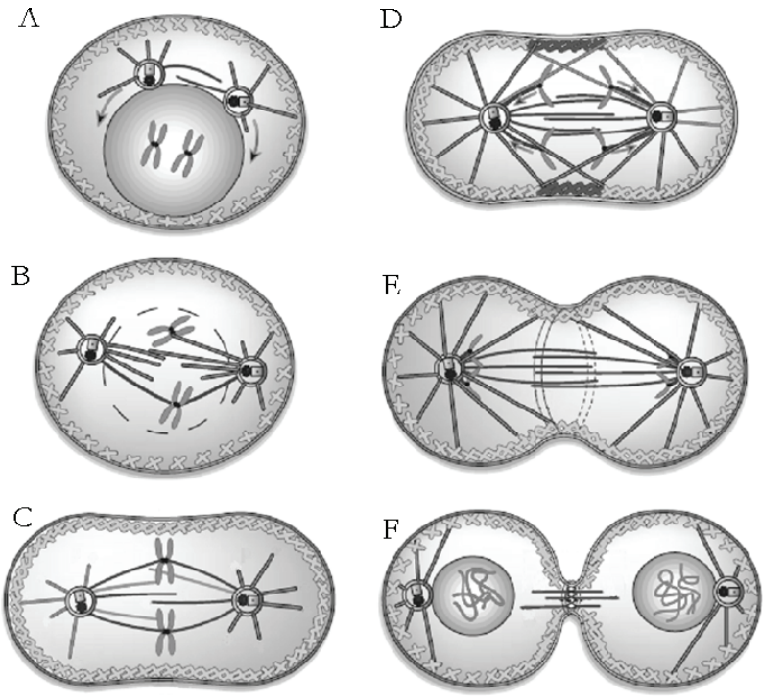
At several points during the cell cycle there are checkpoints to ensure that the cell continues to the next step, only when all appropriate measures have been taken. The first checkpoint is the stop-or-go moment during G1 that assures that the cell is prepared for the duplication of the DNA. Correct copying of the DNA and the repair of DNA damages is closely monitored during the S phase and as long as this is not accurately terminated the progression of the cell cycle is blocked. When blocks of the cell cycle exceed a certain amount of time, survival signaling is switched towards a cell death execution pathway. During G2 all the needs for the physical separation of the two cells during M phase are prepared (box 2.4 mitosis). The G2/M checkpoint is the last hurdle before the start of mitosis. Checkpoints work through the (de)activation of cyclin-dependent kinases (CDKs), which are transiently activated by binding of cyclin. Activity is quickly shut down by degradation of the activating cyclin, making it a very fine-tuned and fast-reacting system. A weakening of the strictness of the checkpoints opens the door for cancer hallmark acquisition (Kastan & Bartek, 2004).



The cell cycle and its checkpoints (adapted from Kastan & Bartek., 2004)

Box 2.4 Mitosis

Mitosis (M phase) is the actual division of one cell into two daughter cells. The faithful distribution of the duplicated DNA is tightly regulated to prevent aneuploidy and genetic instability (Kops et al., 2005). Mitosis can be split into several stages. At the start of M phase, prophase (a), the chromosomes condense. In the cytoplasm the centrosome (the microtubule organizing centre) is duplicated, allowing the formation of two spindles. During prometaphase (b) the nuclear membrane disintegrates and the spindle microtubules can attach to the kinetochores of the chromatids (Alberts et al., 2008). Correct attachment is closely monitored, unattached kinetochores signal to activate the spindle attachment checkpoint (SAC), blocking mitotic progression (Elowe, 2011). During metaphase (c) the chromosomes align and the SAC requirements are met by the attachment of the entire sister chromosomes to the correct spindle. This leads to the meta-to-anaphase transition. The APC/C (anaphase promoting complex/cyclosome) orchestrates this switch that allows the separation of chromosomes towards separate daughter cells during anaphase (d). Telophase (e) marks the moment the two sets of DNA are fully segregated and packaged again within a nuclear envelope. Finally cytokinesis splits the cell in two (f) (Alberts et al., 2008).



The six phases of mitosis adapted from Scholey et al. (2003). a) prophase, b) prometaphase, c) metaphase, d) anaphase, e) telophase, f) cytokinesis.

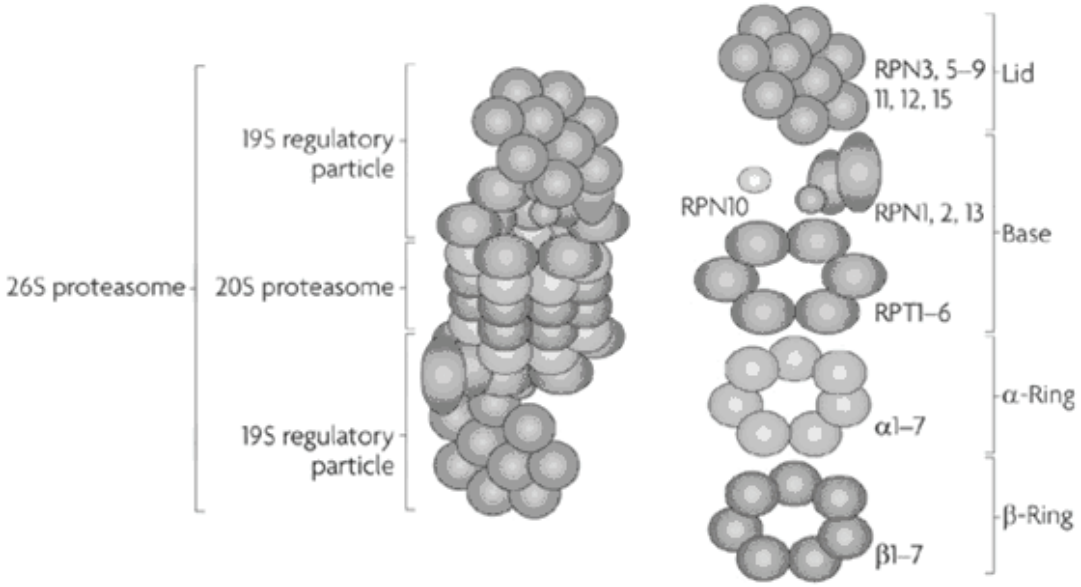
Box 2.5 The ubiquitin-proteasome degradation system

Cellular events are tightly regulated on many levels as e.g. transcription initiation, protein activation and protein degradation. The selective degradation of many proteins is tightly orchestrated by the ubiquitin-proteasome system. When a protein has served its purpose it is tagged for disposal by the covalent attachment of ubiquitin, a 76 amino acid protein. This tagging is performed by three enzymes. E1 binds and activates ubiquitin. E2 is a ubiquitin-carrier protein that temporarily holds on to the activated ubiquitin. Finally the E3 ubiquitin ligase transfers the ubiquitin to the lysine residue of the target protein. There are many E3 families and E3 multisubunit complexes which determine ubiquitination specificity in time and space (Hershko & Ciechanover, 1998).

Ubiquitinated proteins are recognized and degraded by the 26S proteasome. The proteasome is a multiprotein, ATP-dependent protease. It consists of the 20S active core and two 19S regulatory caps. These are themselves composed of several subunits. The caps bind ubiquitinated proteins and 'feed' them to the core. The core is made up of α- and β-rings. From these rings the β1, β2 and β5 subunits have protease activity and can cleave peptides bonds after various amino acids (Murata et al., 2009).

Most cells use the same regular proteasome subunit composition. Hematopoetic cells constitutively express the immunoproteasome. There the β1, β2 and β5 subunits are replaced by β1i β2iand β5i resulting in different substrate specificity and alternative peptides (Huber et al., 2012). The β5t subunit is exclusively expressed in the thymus aiding positive T cell selection (Murata et al., 2007).

Altered protein stability can promote cancer formation and disease development in general. Many of the proteins involved in ubiquitination can be mutated in cancer, supporting roles as oncogenes or tumor suppressor. For example the anaphase promoting complex/cyclosome (APC/C) is an important E3 ligase regulating the progression through mitosis. Changes in this process support aberrant growth (Lipkowitz & Weissman, 2011). Targeting the ubiquitin-proteasome system is a popular approach in cancer therapy. Shifting the balance between pro-survival and pro-death proteins by changing their stability can trigger cell death induction in a semi-cancer-specific way (Hoeller & Dikic, 2009).



26S proteasome subunit composition, adapted from Murata et al. (2009).

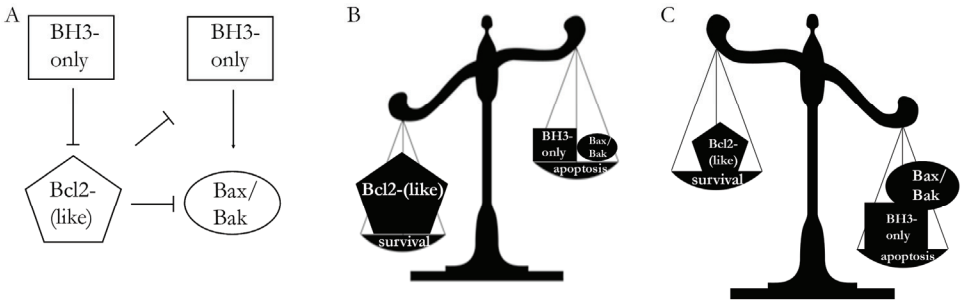
Box 2.6 The Bcl-2 family

The Bcl-2 family of proteins governs the switch between life and death. In the cell death pathways described in box 2.1 there is a moment in the cascade of events that can be considered as a 'point-of-no-return'. Cellular changes in the beginning of cell death are reversible, at some point a modification is made, or a complex formed, that is irreversible. From then on a cell is committed to die. The decision of commitment is made by the Bcl-2 family in the situation of intrinsic apoptosis. Internal stress signals such as DNA damage and low nutrients are sensed by the Bcl-2 family inducing a loss of mitochondrial membrane potential. This leads to leakage of cytochrome C, activation of the apoptosome and subsequent caspase activation (Tait & Green, 2010).

The Bcl-2 family can be roughly split in three groups. The name-giver, Bcl-2 (B-cell lymphoma 2), is a pro-survival protein. Together with Bcl-x_L, Bcl-w, Mcl-1 and A1, Bcl-2 inhibits cell death by associating with the mitochondrial outer membrane, preventing the release of cytochrome and related pro-apoptosis factors. Furthermore they inhibit (indirectly) the pro-death proteins Bax and Bak (Cory & Adams, 2002).

BH3-only clan members only share one domain with the others, the Bcl-2 homology (BH) 3 domain. They are pro-apoptotic and function upstream of the Bcl-2 like proteins. There are at least eight family members, including Noxa and Puma. The BH3-only proteins interpret intercellular signals and when activated they bind and inactivate the pro-survival Bcl-2 family members. Not all BH3-only proteins bind all Bcl-2-like relatives, creating an extra level of mechanistic control. Furthermore, some are controlled by modifications, while others are bound by inhibitors (Cory & Adams, 2002). For example, Puma and Noxa are controlled by the p53 family of DNA damage sensors on a transcriptional level. Puma can inactivate all pro-survival members whereas Noxa only controls Mcl-1 and A1 (Adams & Cory, 2007).

The actual piercing of the mitochondria membrane is done by the third group, composed of the proteins Bax and Bak. Upon activation they undergo a conformational change and oligomerize to form pores in the mitochondrial membrane, allowing pro-death factors to leak out (Westphal et al., 2011). Bax and Bak can be directly activated by BH3-only proteins, or by the removal of the block of Bcl-2 like relatives by the same BH3-only members (a) (Adams & Cory, 2007). In the end it is the balance between active pro-survival and pro-death proteins that determines the outcome, survival or apoptosis (b/c).



The balance between Bcl-2 family members constitutes the switch between life and death. a) BH3-only proteins can directly and indirectly activate Bax and Bak, leading to loss of mitochondrial membrane loss and caspase activation. b/c) It is the balance between the different family members that determines the outcome of the cell's future.

For many years CAV was the only member of the genus *Gyrovirus*. The sequencing of a DNA virus causing apathy and weight-loss in Brazillian chicks led to the discovery of a sequence 40% identical with the CAV genome. The new family member is named Avian gyrovirus 2 (AGV2). AGV2 shows a similar genomic organization as CAV, with an orf2 resembling VP3 (apoptin) of CAV. The leucine-rich-region, NES and bipartite NLS are conserved in the AGV2 version of apoptin (Rijsewijk et al., 2011). A third gyrovirus (GyV3) was detected in human faeces from Chilean children, presumably reflecting chicken consumption (Phan et al., 2012). Apart from the identification of new gyroviruses, the sequencing of CAV derived from several different areas allowed subtyping of the CAV genotypes (Eltahir et al., 2011)

In 2011 a human gyrovirus (HGyV) was isolated from a skin swab. This virus shows similar gene organization as CAV and encodes a homologue of apoptin (Sauvage et al., 2011). HGyV could only be detected in the blood of immune-compromised patients and not of healthy individuals (Maggi et al., 2012). The HGyV apoptin has the same subcellular distribution as CAV-derived apoptin and shows tumor-selective apoptosis induction, resembling the original apoptin (Bullenkamp et al., 2012).

The four VP3 proteins show different levels of sequence similarity. All the confirmed important domains of apoptin however are conserved (Figure 2.2): i.e. the isoleucine-rich region, the bipartite nuclear localization signal, the nuclear export signal and a relevant threonine residue in between the latter are all present in the same order in all four VP3 proteins. As the human gyroviral VP3 has clear tumor-selective apoptosis inducing activity, it is very likely the other two also resemble apoptin in this aspect.

Figure 2.2: Sequence alignment of viral protein 3 of the 4 known gyrovirusses (ClustalW http://www.ebi.ac.uk/Tools/msa/clustalw2/). In color indicated regions are confirmed for apoptin and putative for the other VP3s (references in text). Blue: isoleucine-rich region, Yellow: Nuclear Localisation Sequence, Green: Nuclear Exit Sequence, Red: relevant threonine residue.

E4orf4

E4orf4 is a 14kDa protein encoded by the E4 early transcription unit of adenovirus. Tumor-selective cell death is induced by E4orf4 through mitotic catastrophe resulting in necrosis (Li et al., 2009a). Prior to cell death a G2/M block is triggered which is linked to the interaction of E4orf4 with the APC/C (Kornitzer et al., 2001). As for apoptin, the APC/C links E4orf4 to genome instability which is caused by inaccuracies during mitosis (Weaver & Cleveland, 2005; Lipkowitz & Weissman, 2011).

To acquire cell death inducing activity E4orf4 needs to be phosphorylated by Src kinases (Gingras et al., 2002). Furthermore, E4orf4 disrupts Src signaling in tumor cells promoting cell death induction (Lavoie et al., 2000). The Src family kinases link E4orf4 to several main cancer characteristics such as angiogenesis and invasion (Arai et al., 2012).

E4orf4 interacts with the B55 subunit of the tumor-suppressor protein phosphatase 2A (PP2A). Interaction attenuates PP2A activity, lowering overall dephosphorylation and changing substrates (Li et al., 2009b). PP2A is a key player in PI3-kinase signaling and through that pathway involved in many hallmarks of cancer (proliferative signaling, evading growth suppressors, resisting cell death) (Westermarck & Hahn, 2008).

E4orf4 directs PP2A to the chromatin-remodeling complex of ACF (ATP-dependent chromatin-remodeling factor) with the chromatin-remodeling factor SNF2h (sucrose non fermenting-2h). SNF2h can form chromatin-remodeling complexes with other co-enzymes as for example RSF1 (remodeling and spacing factor 1) and WSTF (Williams-Beurens syndrome transcription factor), all having different chromatin remodeling specificities. The PP2A-E4orf4 complex is thought to remove ACF from its complex, liberating SNF2h to form complexes with other cofactors. This switch in chromatin dynamics is thought to contribute to the cell death induction by E4orf4 (Brestovitsky et al., 2011).

NS1

The third viral PKTC is derived from oncolytic parvoviruses. The autonomous parvovirus MVM (minute virus of mice) and H-1 (natural host is rat) were shown to preferentially lyse transformed cells (Rommelaere & Cornelis, 1991). This cancer-selectivity is attributed to more efficient viral replication in tumor cells (Rommelaere et al., 2010). Furthermore it is related to the non-structural protein NS1 (Geletneky et al., 2005).

NS1 can induce tumor-specific cell death independent of p53 through various pathways including apoptosis (Mincberg et al., 2011), autophagy (Di Piazza et al., 2007) and mitotic catastrophe (Fragkos & Beard, 2011). The human parvovirus B19 NS1 protein can trigger apoptotic cell death in HepG2 cells through DNA damage infliction and a consequent S phase arrest (Kivovich et al., 2012).

The main effector of NS1 is casein kinase II (CKII). Inhibition of CKII protects cells from NS1-induced cell death. NS1 interacts with CKII and modulates its selectivity, e.g. NS1-CKII leads to altered tropomyosin phosphorylation. CKII-dependent cytoskeletal rearrangements and cell death induction are modified by NS1 as well (Nuesch & Rommelaere, 2007). CKII has a significant influence on cell growth and proliferation through its substrates. Upregulated CKII activity is related to cancer development through both the aberrant activation of oncogenes and downregulation of tumor suppressor activity by phosphorylation (Hanif et al., 2010). In addition over-expression of CKII also suppresses apoptosis induction via the extrinsic pathway (Wang et al., 2005).

The oncoselectivity of the whole parvovirus is closely interwoven with the altered immunogenic state of cancer cells. In normal cells parvoviral particles are detected resulting in an interferon response. In contrast, in tumor cells there is a block on the activation of this pathway (Rommelaere et al., 2010). Parvovirus immune modulation goes further; after cell death induction an anti-tumor immune-response is triggered by the release of tumor-associated antigens (Moehler et al., 2005; Grekova et al., 2011).

Onconase

Onconase (ranpirnase) is a ribonuclease isolated from the oocytes of the Northern leopard frog (*Rana pipiens*). It has preferential cytotoxic activity in tumor cells as compared to normal cells, likely due to selective internalization by tumor cells (Lee & Raines, 2008). Onconase is a highly cationic protein (pI>9.5) improving the interaction with the more negatively charged tumor cell membrane (James et al., 1956; Riedl et al., 2011). Treatment with the protein reduces cell proliferation and invasion (Goparaju et al., 2011).

Onconase preferentially degrades tRNAs and miRNAs. Destruction of tRNAs blocks protein synthesis, eventually inducing apoptosis (Iordanov et al., 2000). By cleavage of miRNA precursors onconase reduces the amount of mature miRNAs (Qiao et al., 2012). One of the downstream miRNA targets influenced by onconase is NF-κB (nuclear factor

kappa B) (Goparaju et al., 2011). NF-κB is deregulated in many types of cancer stimulating proliferation (Rayet & Gelinas, 1999); loss of this oncogene likely supports onconase-induced cell death.

Brevinin-2R

Brevinin-2R is a defensin derived from the skin of another frog species, *Rana ridibunda*. This 25-amino acid peptide shows higher cell death induction in several cancer cell lines compared to normal cell lines. Caspase activation is dispensable for the activity that seems to function through the autophagic cell death pathway. Selectivity is attributed to the increased binding of Brevinin-2R to the membrane of cancer cells in a fashion reminiscent of onconase (Ghavami et al., 2008). Tumor cells have a different cellular membrane composition. Especially, phosphatidylserine, sialic acid or heparan sulfate, representing negatively charged membrane components, are differently organised between cancer and normal cells (Riedl et al., 2011). Membrane composition changes further to allow invasion and metastasis (Kier, 1990), a trait that is likely recognized by onconase and Brevinin-2R.

HAMLET

Human alpha-lactalbumin made lethal to tumor (HAMLET) is a protein derived from human milk which is partially unfolded in vitro and forms a complex with oleic acid. Its tumoricidal activity was discovered when it induced apoptosis-like death in human tumor cells during an experiment to show the effect of milk on bacterial attachment to lung carcinoma cells (Hakansson et al., 1995). HAMLET can bind and cross the cellular membrane, accumulating in the nucleus of tumor cells. Cellular membrane traffic is less efficient in untransformed cells, where the protein complex remains in the cytoplasm forming small aggregates (Fischer et al., 2004). Possibly the membrane binding and crossing difference between normal and tumor cells of HAMLET can be attributed to similar changes as those that facilitate onconase and Brevinin-2R entrance as described above. In the nucleus of tumor cells HAMLET binds histones and disrupts the chromatin structure (Duringer et al., 2003).

Morphologically HAMLET induces an apoptosis-like cell death with cytochrome c release, caspase activation, phosphatidylserine exposure and DNA fragmentation. However, neither caspase inhibitors nor over-expression of Bcl-2 could block HAMLET-induced cell

death (Hallgren et al., 2006). Instead the anti-tumor effect of HAMLET was shown to be dependent on components of the autophagy pathway (Aits et al., 2008).

The partially unfolded state is relevant to the activity of HAMLET. Unfolded proteins are normally degraded by the proteasome (see box 2.5 protein degradation). HAMLET is targeted to the 20S proteasome but resists degradation. By binding it inhibits proteasome activity and disrupts the structure (Gustafsson et al., 2009). Alteration of the degradation of cellular proteins might contribute to the lethal effect of HAMLET.

The connection of HAMLET to the hallmarks of cancer became clear with the knockdown of c-Myc. Lack of this oncogene suppressed the cell death induction by HAMLET (Storm et al., 2011). In the same study it was shown that the modified metabolism in cancer cells is a crucial factor in the activity of HAMLET. Over-expression of the transcription factor c-Myc has an effect on proliferative signaling, energy metabolism, angiogenesis, invasion and survival (Hanahan & Weinberg, 2011).

TRAIL

TRAIL is tumor necrosis factor-related apoptosis-inducing ligand (or Apo2 ligand Apo2L) and a member of the tumor necrosis factor (TNF) superfamily of cytokines. TNF family members induce apoptosis by binding to their respective transmembrane receptors. TRAIL is a transmembrane protein that can become extracellularly cleaved, leaving a soluble signaling protein that can form homotrimers (Wang and El-Deiry, 2003). TRAIL binds to death receptors 4 and 5 (DR4 and DR5). Binding leads to oligomerization of the receptors and activation of the death domain, starting the extrinsic apoptosis pathway (Mahalingam et al., 2009). TRAIL specifically induces apoptosis in tumor cells (Walczak et al., 1999). TRAIL protects against tumor development and metastasis (LeBlanc & Ashkenazi, 2003).

The tumorigenic c-Myc pathway positively influences TRAIL-induced apoptosis (Kim et al., 2011). c-Myc upregulates DR5 cell surface levels (Wang et al., 2004) and down regulates the inhibitor of caspase activation FLIP (FLICE inhibitory protein) (Ricci et al., 2004) potentiating the cell death activities of TRAIL. Normal cells can be made sensitive to TRAIL by using oncogenic Ras mutants which increase the recruitment of caspase-8 to the death receptors. Interestingly, immortalizing cells with SV40 early region and telomerase is not enough to trigger TRAIL-mediated cell death (Nesterov et al., 2004).

Not all cancer cells are equally sensitive to TRAIL (Dimberg et al., 2012). A lot of effort is invested in modulating cancer-specific pathways in order to re-sensitize cells to TRAIL, through combination treatment with for example cisplatin, proteasome inhibitors, NF-κB inhibitors and PI3K inhibitors (Johnstone et al., 2008). In cancer cells resistant to TRAIL treatment, TRAIL can actually stimulate tumorigenesis. When the apoptotic signal is not strong enough, e.g. by over-expression of anti-apoptotic proteins, TRAIL-triggered pathways promote proliferation, survival and metastasis by upregulating NF-κB and activating the PI3K-Akt pathway (Johnstone et al., 2008; Chi et al., 2012).

Noxa

Noxa is a BH3-only protein involved in, but not limited to, the p53-regulated apoptosis-induction following cellular stress (see box 2.6). Upon cellular stress the expression of pro-death BH3-only proteins is upregulated, including Noxa and Puma. They bind and thereby inactivate the pro-survival proteins, activating the mitochondrial cell death pathway. In cancer cells Bcl-2 and related pro-survival proteins are regularly upregulated to confer cell death resistance upon the tumor (Adams & Cory, 2007).

Over-expression of Noxa was found to selectively induce apoptosis in tumor cells, whereas Puma over-expression led to cell death in all types of cells (Suzuki et al., 2009). Puma interacts with all pro-survival family members while Noxa only influences a select subgroup (Adams & Cory, 2007). Importantly Noxa interacts with anti-apoptotic protein myeloid cell leukemia-1 (Mcl-1), targeting it for proteasomal degradation (Ploner et al., 2009). Oncogenic c-Myc stimulates tumorigenesis. During the process c-Myc also increases expression levels of Noxa, though not to such an extent that the balance in the Bcl-2 family shifts to apoptosis induction (Nikiforov et al., 2007). It does, however, likely potentiate cancer cells with oncogenic c-Myc to exogenous Noxa-expression.

The adenoviral protein E1A has oncogenic properties. However, over-expression of E1A in cancer activates Noxa to induce apoptosis (Flinterman et al., 2005). Oncogene expression was found to increase Puma levels, which did not automatically lead to cell death induction as Puma is bound and sequestered by pro-survival Mcl-1. Over-expression of Noxa releases Puma, efficiently inducing apoptosis (Nakajima & Tanaka, 2011). It is likely that the tumor-selectivity of Noxa is based on an oncogene-induced accumulation of inactive Puma, sensitizing cancer cells to Noxa.

mda-7/IL-24

Melanoma differentiation associated gene-7 (mda-7) was found during a subtraction hybridization screen and expression was shown to be increased in differentiated melanoma cells (Jiang et al., 1995). Forced expression of mda-7 in human cancer cells induces growth arrest and eventually apoptosis (Su et al., 1998). Based on sequence and functional homology mda-7 is reclassified as IL-24, a member of the IL-10 family of cytokines (Huang et al., 2001).

Ectopically expressed mda-7/IL-24 localizes to the ER where it causes ER stress by binding, and thereby inactivating, glucose-regulated protein 78 (GRP78 or Bip) (Gupta et al., 2006). GRP78/BiP is a chaperone and inhibition results in induction of the Unfolded Protein Response (UPR) (Dash et al., 2010a). Remarkably, GRP78/BiP participates as a cellular membrane receptor in a signaling function in Par-4 mediated apoptosis induction, as described below.

Mda-7/IL-24 can shift the balance between anti- and pro-apoptotic family members of Bcl-2 towards cell death (see box 2.6). Expression of mda-7/IL-24 leads to down regulation of Mcl-1, triggering apoptosis possibly via the same mechanism as Noxa over-expression (Dash et al, 2010b). Apart from stimulating pro-death signaling mda-7/IL-24 triggers apoptosis through various other pathways. Mda-7/IL-24 blocks pro-survival signaling by down regulating Akt expression and increasing cyclin-dependent kinase inhibitors (Valero et al, 2011). Furthermore, mda-7/IL-24 expression leads to increased ceramide accumulation and ROS (reactive oxygen species) production (Dash et al, 2010a).

Although there is a lot of information on the downstream cell death inducing properties of mda-7/IL-24, very little is known about what the tumor processes are that sets it off in cancer cells in the first place. Mda-7/IL-24 can activate the Fas/TRAIL pathway (Ekmekcioglu et al., 2008), possibly using the latter's tumor-specific qualities to enhance its own.

Exogenous mda-7/IL-24 expression can create a bystander killing effect by tumor-selectively increasing endogenous mda-7/IL-24 expression and secretion (Sauane et al., 2008). Furthermore, temporary externally induced mda-7/IL-24 expression can confer cancer immunity upon immune competent mice (Miyahara et al, 2006). Interestingly, the mouse homologue of mda-7/IL-24 was found to have no anti-tumor effects (Nagakawa et al., 2012).

Par-4

The tumor suppressor prostate apoptosis response-4 (Par-4) is down regulated in many cancers by various mechanisms such as promoter methylation and mutations (Shresta-Bhattarai & Rangnekar, 2010). Furthermore Par-4 expression is suppressed by oncogenic Ras and hTERT, aiding tumorigenesis (Sheng et al., 2010). Ectopic expression of Par-4 can block Ras-induced transformation (Qiu et al., 1999). Additionally over-expression of Par-4 selectively induces apoptosis in tumor cells (El-Guendy et al., 2003). The *Par-4* gene is a pro-apoptotic gene induced after calcium-stress or androgen ablation (Sells et al., 1994).

Apart from tumor-specific nuclear translocation needed for apoptosis induction by Par-4, its selectivity is stored in the 'selective for apoptosis induction cancer cells' (SAC) domain. Protein Kinase A (PKA) activates Par-4 by phosphorylating T155 in the SAC domain (Gurumurthy et al., 2005). Par-4 can be phosphorylated on several sites by other kinases. Phosphorylation by Akt renders Par-4 inactive which is needed for cancer cell survival (Goswami et al., 2005). Interestingly active Par-4 inhibits Akt through Protein Kinase C ζ (PKCζ) (Joshi et al., 2008). In the nucleus active Par-4 inhibits NF-κB activity leading to cell death (El-Guendy et al., 2003).

Par-4 is secreted and induces apoptosis through the extrinsic pathway. GRP78/BiP normally resides in the ER. Upon ER stress it translocates to the cellular membrane where it functions as a receptor for Par-4. Par-4 bound to receptor GRP78/BiP recruits and activates caspase 8, mediating caspase 3 apoptosis induction (Burikhanov et al., 2009). GRP78/BiP is over-expressed at the cell surface of tumor cells (Sato et al., 2010).

Elevated levels of PKA and GRP78/BiP in cancer cells confer upon Par-4 its tumor-selective potential (Hebbar et al., 2012). TRAIL and Par-4 can further sensitize cancer cells for one another by inducing ER stress and increasing Par-4 secretion and GRP78/BiP surface expression (Hart & El-Deiry, 2009).

ORCTL3

In 2009 a screen was performed to identify genes with tumor-specific cell death inducing properties; up till then the discovery of the cancer-selectivity of PKTCs had been opportunistic. The organic cation transporter-like 3 (ORCTL3) was found to induce apoptosis in RAS-transformed cells, having no effect on their isogenic counterparts (Irshad et al., 2009). ORCTL3 apoptosis-induction is connected to ER stress and does not need the

cation transporter function of the protein. In several human cancers the expression of the *ORCTL3* gene is repressed (Irshad et al., 2009).

(Pre)clinical trials

None of the PKTCs has yet been approved as anti-cancer treatment. Clinical trials are under way for TRAIL, HAMLET, mda-7, NS1 and onconase. As for preclinical trials, almost all PKTCs have shown anti-neoplastic activity in animal models with no noted negative side effects. No trials or animal model testing have yet been reported with brevinin-2R or ORCTL3.

Apoptin has been shown to successfully inhibit tumor growth in various mouse models. A wide range of delivery methods is under development. Protein therapy in which apoptin is coupled to a modified HIV transduction domain, PTD4, effectively treated various xenografts (Sun et al., 2009). Together with dacarbazine PTD4-apoptin greatly reduced the hematologic side effects of dacarbazine without reducing the antitumor activity (Jin et al., 2011). Other successful in vivo delivery methods are based on viral (Zhang et al., 2012) or bacterial gene delivery (Cao et al., 2010). A fusion construct of E4orf4 with epidermal growth factor (EGF) allows internalization of E4orf4 especially in EGF receptor over-expressing cancer cells. This protein construct was used with no toxic side effect in a nude mice model (Zhou et al., 2009). The oncolytic parvovirus, encoding NS1, is effective against cancer development in several mouse models. The tumor-specific cell death induction, safety and in vitro observed immunomodulation effect are currently being tested in a phase I trial with glioblastoma patients (Rommelaere et al., 2010).

Noxa, expressed through an adenoviral vector, is effective in breast cancer xenografs (Suzuki et al., 2009). Furthermore, stabilization of endogenous Noxa is involved in the anti-cancer effect of several other drugs currently under testing as for example bortezomib (Ohshima-Hosoyama et al., 2011). Par-4 can be secreted and elicits its effect through membrane receptor binding, eliminating the problem of getting the drug into the cell. Par-4/SAC transgenic mice are resistant to the growth of oncogene-induced tumors. Interestingly bone marrow transplantation from these resistant transgenic mice to cancer-susceptible mice confers tumor resistance (Zhao et al., 2011).

HAMLET has been used in two phase I clinical trials, one showing a lasting effect on skin papillomas, and another in which HAMLET caused the shredding of bladder cancer

cells. In both trials no negative effect on healthy cells was observed (Gustafsson et al., 2004; Mossberg et al., 2007). Mda-7/IL-24 delivery through modified adenoviral vectors improves its efficiency in xenografted mice (Dash et al., 2010a). Several phase I trials with adenovirus vector ad.Mda-7 expressing Mda-7 showed efficient specific apoptosis induction in tumor cells with low toxicity. Trials support the bystander effect, triggering surrounding cells to start producing Mda-7/IL-24 (Eager et al., 2008). TRAIL and antibodies against the DR4 and DR5 receptor have been used in a number of clinical trials with differing outcomes. Several mechanisms of TRAIL resistance have been identified. Accurate in advance characterization of cancer types will help to design combination therapies in which the use of TRAIL can add to a positive outcome (Dimberg et al., 2012). Finally, onconase has been enrolled in several phase I and II trials. Promising results in patients who had shown no response to chemotherapy led to the start of phase III trials (Constanzi et al., 2005). Onconase is currently in a phase IIIb confirmatory clinical trial for the treatment of unresectable malignant mesothelioma after an efficacy and safety assessment had been done (Rybak et al., 2009).

Concluding remarks

Cancer is derived from our own cells. This makes it difficult to target tumor cells without harming the normal cells from which they originated. However, the special features acquired by evolving neoplasia can be grouped and denominated. These hallmarks of cancer set the tumor enough apart from healthy tissue to define therapeutic targets. A group of proteins, PKTCs, have been shown to induce programmed cell death pathways selectively in tumor cells without damaging the healthy tissue. There are many different triggers in cancer cells that can activate a PKTC, and the method by which an activated PKTC pushes the tumor cell to commit suicide also varies.

In this thesis we continue the search for features that set tumor cells apart from normal cells, making them vulnerable for apoptin-induced cell death. Various tumor-related processes that are of importance for the tumor-selective activation of apoptin are reported. They range from proteasome activity and mitosis regulation up to protein kinases and phosphatase action. The differences observed among PKTC activation and functioning suggests that they may complement each other during therapy. The overlap in their functioning means that they share one very promising characteristic: the ability to induce cell

death selectively in tumor cells while allowing healthy cells, and organisms, to live long and prosper.

Chapter 3

PP2A inactivation is a crucial step in triggering apoptin-induced tumor-selective cell killing

Henriëtte L Lanz*, Rhyenne ME Zimmerman*, Dong-Jun Peng*,
Ying-Hui Zhang, Astrid AAM Danen-van Oorschot, Shen Qu,
Claude Backendorf and Mathieu HM Noteborn

*contributed equally

Cell Death and Disease (2012) 3, e291

Abstract

Apoptin harbors tumor-selective characteristics making it a potential safe and effective anticancer agent. Apoptin becomes phosphorylated and induces apoptosis in a large panel of human tumor but not normal cells. Here, we used an in vitro oncogenic transformation assay to explore minimal cellular factors required for the activation of apoptin. Flag-apoptin was introduced into normal fibroblasts together with the transforming SV40 LT and ST antigens. We found that nuclear expression of SV40 ST in normal cells was sufficient to induce phosphorylation of apoptin. Mutational analysis showed that mutations disrupting the binding of ST to protein phosphatase 2A (PP2A) counteracted this effect. Knock-down of the ST-interacting PP2A B56γ subunit in normal fibroblasts mimicked the effect of nuclear ST expression, resulting in induction of apoptin phosphorylation. The same effect was observed upon down-regulation of the PP2A B56δ subunit, which is targeted by PKA. Apoptin interacts with the PKA-associating protein BCA3 (AKIP1), and inhibition of PKA in tumor cells by treatment with H89 increased the phosphorylation of apoptin, whereas the PKA activator cAMP partially reduced it. We infer that inactivation of PP2A, in particular of the B56γ and B56δ subunits, is a crucial step in triggering apoptin-induced tumor-selective cell death.

Introduction

Tumor formation occurs due to a complex set of processes roughly based on enhanced survival and limited cell death activities (Wenner, 2010). Remarkably, a set of viral and cellular proteins has been found to selectively induce cell death in tumor cells (Bruno et al., 2009). Among these proteins is the avian virus protein apoptin, which has been shown to induce p53-independent apoptosis in a broad spectrum of human transformed cells (Backendorf et al., 2008). Recent preclinical studies demonstrated the therapeutic potential of apoptin as a safe and efficient anticancer agent (Grimm and Noteborn, 2010). Therefore, it is of interest to study the mechanisms underlying apoptin-induced apoptosis, particularly the 'switch' responsible for its activation during oncogenic transformation.

SV40 T antigens are known to be involved in oncogenic transformation through interference with many cellular processes (Pipas, 2009). Distinct domains on LT that bind and inactivate tumor suppressors p53 and Rb, have long been known to play crucial roles in tumor formation (Hahn et al., 1999). The SV40 ST protein enforces transformation of normal cells via inhibition of protein phosphatase 2A (Sablina et al., 2008; Chen et al., 2007; Cho et al., 2007).

This feature is in accordance with the reduced PP2A levels found in various human tumor cell types (Sablina and Hahn, 2008), and accumulating evidence supporting major tumor suppressive roles for PP2A (Westermarck and Hahn, 2008). Another major cellular regulator implicated in carcinogenesis is protein kinase A (PKA) (Naviglio et al., 2009). Its targets include PP2A (Usui et al., 1998), and for instance, the PKA interacting protein Breast Cancer-associated gene 3 (BCA3) is known to be highly expressed in breast and prostate cancer cells compared to the normal surrounding tissue (Kitching et al., 2003).

In this study, we investigated the minimal steps leading to the activation of apoptin upon malignant transformation. We previously showed that transient expression of the SV40 large and small T antigens in normal human fibroblasts results in activation of apoptin, displaying all three of its characteristic features, namely phosphorylation, nuclear localization, and apoptosis (Zhang et al., 2004). This finding provided us with an elegant tool to analyze which domains of the SV40 large and/or small T antigens are responsible for this activity. In parallel, BCA3 was identified as an apoptin-interacting protein, and the effects of PKA on apoptin phosphorylation were examined. Analysis of both transformation-related pathways revealed that inactivation of PP2A is crucial for the activation of apoptin.

Results

Transient expression of N-terminal SV40 LT136/ST activates apoptin in normal human fibroblasts

Apoptin activity can be induced in normal cells by transient expression of transforming SV40 antigens (Zhang et al., 2004). Hence we examined the minimal SV40 domains responsible for this effect. Expression of the SV40 LT136/ST DNA sequence results, due to alternative splicing, in 2 proteins: LT136, comprising the N-terminal 136 a.a. of LT, and the entire ST protein, consisting of 174 amino acids (Figure 3.1A) (Srinivasan et al., 1997). LT136 contains a DNA J domain, an Rb-binding site and a nuclear localization signal (NLS). ST shares its DNA J domain with LT136, but has a unique C-terminal domain encompassing a PP2A binding site (Pipas, 2009).

In normal human foreskin fibroblasts, co-transfection of the SV40 LT136 and ST proteins with Flag-tagged apoptin resulted in the latter's phosphorylation (Figure 3.1B). Immunofluorescence analysis of F9 cells expressing all three proteins showed that Flag-apoptin became nuclear already 1 day after transfection. Nuclear Flag-apoptin was also shown to be phosphorylated (Figure 3.1C), and 3 and 5 days post transfection, apoptin was shown to induce apoptosis (Figure 3.1D, E). In contrast, F9 fibroblasts expressing apoptin alone contained mainly cytoplasmic Flag-apoptin, which was, as expected, not phosphorylated (Rohn and Noteborn, 2004; Alivisi et al., 2006) and did not induce apoptosis (Figure 3.1C-E).

Our results clearly reveal that transient expression of LT136 and ST proteins in human F9 fibroblasts results in tumor-selective activation of apoptin, providing us with the possibility to explore which domains and respective cellular targets of LT136/ST were responsible for this activity.

Nuclear ST triggers apoptin activation

Next, we examined whether expression of either LT136 or ST protein alone was sufficient to activate apoptin phosphorylation. F9 fibroblasts were co-transfected with plasmids encoding Flag-tagged apoptin and either one of the following: a) pcDNA-LT136, encoding LT136, b) pcDNA-ST, encoding ST, c) pcDNA-LT136/ST, where both LT136 and ST are produced via alternative splicing, or d) pcDNA-neo, as a negative control. In each experiment, activation of apoptin was assayed by its phosphorylation at position T108, assessed by means

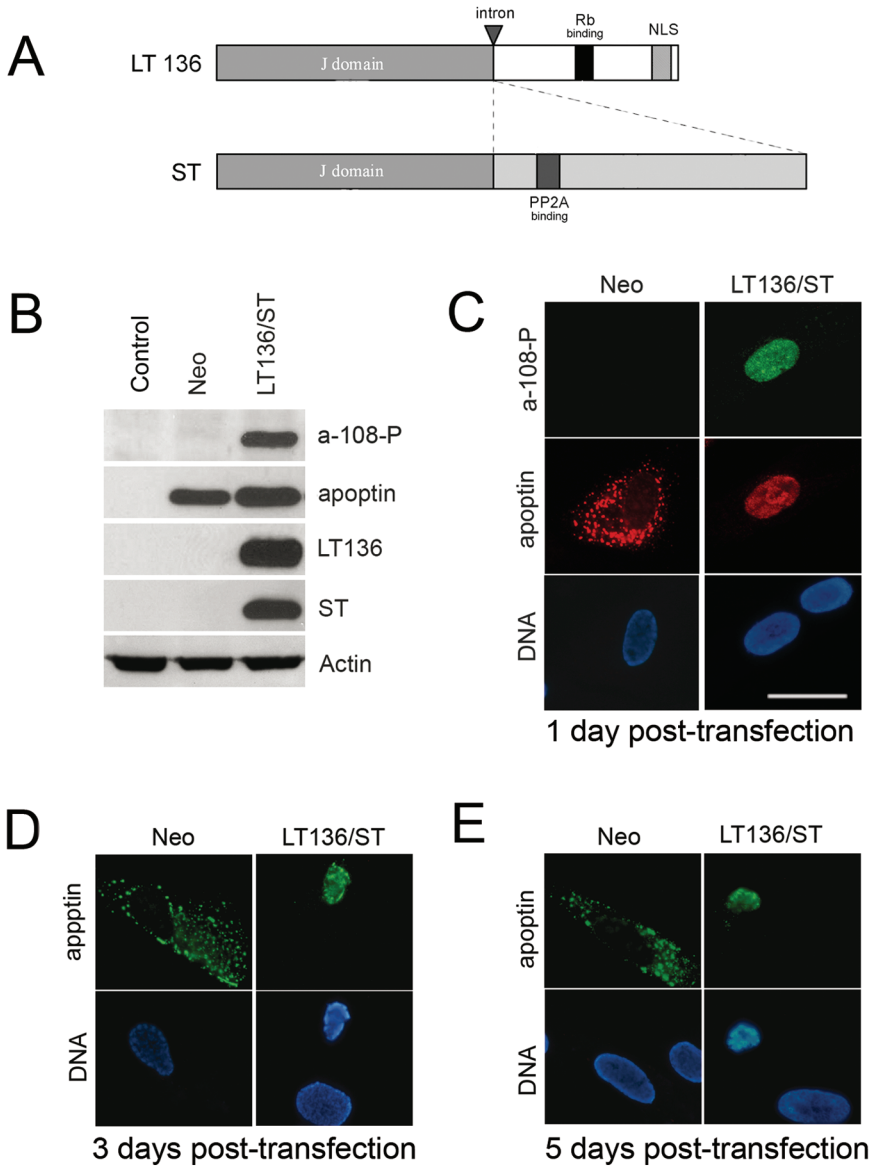


Figure 3.1: Transient expression of N-terminal determinants of SV40 T antigens activates apoptin. (A) Schematic representation of the domains in SV40 LT136/ST. The J domain (a.a. 1-82) is identical in both LT136 and ST. The Rb binding domain and nuclear localization signal (NLS) in LT136 are also shown. ST is expressed by differential splicing and has a unique C-terminus, which contains the PP2A binding domain. (B) Human Fibroblasts (F9) were co-transfected with plasmids pcDNA-Flag-apoptin and pcDNA-LT136/ST (LT136/ST) or pcDNA-neo (neo) by AMAXA nucleofector transfection. Twenty-four hours post-transfection, cells were lysed for Western blot analysis with the indicated antibodies. Mock-transfected F9 cells were used as control. Antibody α-108-P specifically recognizes phospho-apoptin at its Thr108. Flag-apoptin, LT and ST show the respective total protein amounts in the transfected cells. Actin was used as loading control. (C-E). Cells were fixed for immunofluorescence analysis at each given time point after transfection and then stained with the indicated antibodies by indirect immunofluorescence assay. Scale bar = 20 μm.

of Western blotting. Expression of LT136 alone did not trigger apoptin phosphorylation, whereas expression of ST clearly induced apoptin phosphorylation, albeit at a low level (Figure 3.2). Co-transfection of Flag-tagged apoptin with ST fused to an artificial nuclear location signal (NLS-ST) increased the level of apoptin phosphorylation significantly (Figure 3A).

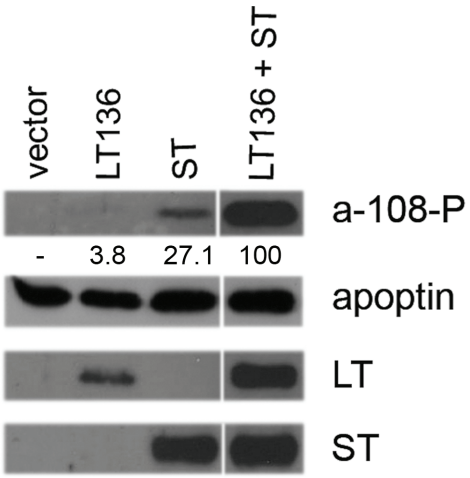


Figure 3.2: Nuclear targeting of SV40 ST activates apoptin. In normal human fibroblasts, apoptin was co-transfected with vector only (Neo), LT136, ST, or both LT136 and ST. Twenty-four hours post-transfection, cells were lysed for Western blot analysis with the antibodies indicated. The relative amount of phosphorylated (as compared to the total amount of apoptin) was quantified and is indicated below the a-108-P panel. The LT136 + ST sample was set at 100.

C103S and P101A point mutations within the PP2A-binding domain of ST disable activation of apoptin

Besides its J domain, shared with LT136, ST contains a unique site for the binding and inactivation of PP2A. This domain has been shown to contribute to cellular transformation (Pipas, 2009). A single amino-acid mutation C103S within the ST protein drastically diminishes the interaction of ST with PP2A (Figure 3.3C) and its transforming capacity (Mungre et al., 1994). Therefore, we studied the effect of the C103S mutation within the PP2A binding site on the activation of apoptin by (nuclear) ST in normal human cells.

F9 cells were analyzed for phosphorylation of Flag-apoptin upon co-expression with ST, NLS-ST or NLS-ST(C103S) protein. Figure 3.3B shows that expression of NLS-ST clearly induced apoptin phosphorylation. Introduction of the NLS-ST(C103S) mutation abolished this induction, although apoptin protein was expressed at a similar level. Similar results were obtained with the NLS-ST-(P101A) mutant (Figure 3.3B). The P101A point mutation within ST is also known to disturb the ST-PP2A interaction and transforming capacity of ST (Mungre et al., 1994). These results suggest that ST interaction with PP2A is

crucial to apoptin activation, and that inactivation of PP2A by ST might be sufficient to activate apoptin.

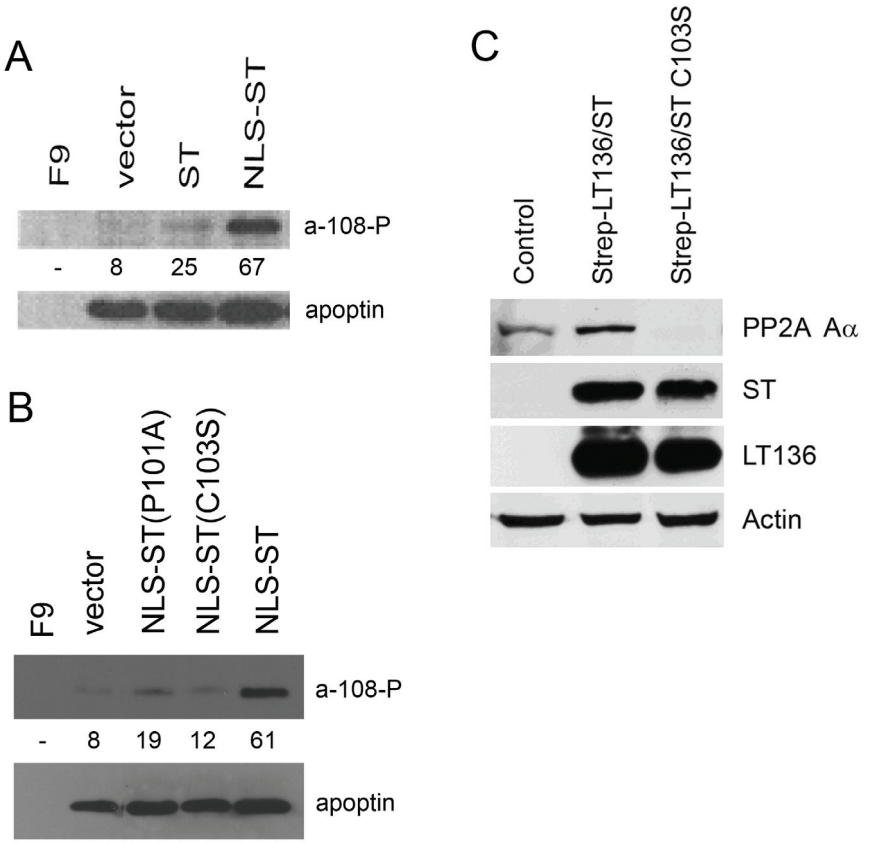


Figure 3.3: Apoptin activation via ST-mediated inhibition of PP2A. (A) pcDNA-Flag-apoptin was co-transfected with plasmids encoding the indicated proteins or vector DNA into F9 primary fibroblasts. Western blot assays were performed with the indicated antibodies at 24h post-transfection. (B) F9 primary cells were co-transfected with pcDNA-Flag-apoptin and plasmids encoding NLS-ST, NLS-ST(P101A), NLS-ST(C103S) or vector DNA as indicated. Western blot assays were performed with the indicated antibodies at 24h post-transfection. In panels A and B the relative percentage of phosphorylated apoptin in the various samples was quantified and is indicated below the a-108-P panel (C) PP2A binding to ST is abolished by C103S mutation. LT136 and ST with or without the C103S mutation (LT136/ST or LT136/ST(C103S) were fused with a Strep-tag at their N-terminus. Cell lysates were prepared at 24h post-transfection for protein-protein interaction assays as indicated in Material and Methods. The final elutions were analyzed by Western Blot with antibodies against PP2A α subunit, LT and ST, respectively. Actin was taken as equal loading control. The first lane (input control) indicated total amount of endogenous proteins in cell lysates.

Knock-down of PP2A B56γ via RNAi activates apoptin phosphorylation in normal human fibroblasts

Two independent studies reported that ST interaction with PP2A resulted in the inhibition of the B56γ regulatory subunit, resulting in cellular transformation (Chen et al., 2007; Cho et al., 2007). Therefore, we examined whether down-regulation of B56γ via RNAi could trigger phosphorylation of apoptin in normal cells. Our shRNA sequence was verified to reduce

ectopic expression of B56γ (Figure 3.4A). Normal F9 fibroblasts co-expressing both apoptin and shRNA directed against B56γ mRNA manifested a clear level of phosphorylated apoptin in comparison to the cells transfected with apoptin and the RNAi control vector (Figure 3.4C). Our data thus indicate that inhibition of the PP2A-B56γ subunit is a crucial and sufficient step for apoptin activation.

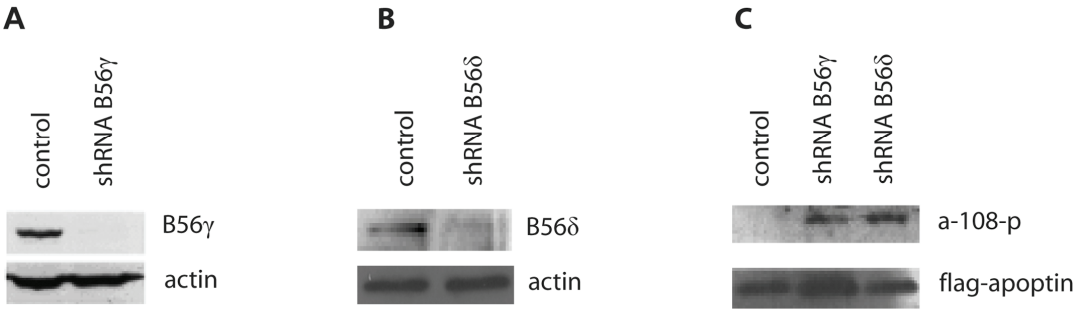


Figure 3.4: Knock-down of PP2A B56γ and B56δ subunits triggers apoptin phosphorylation in normal cells. (A) Down-regulation of PP2A B56γ subunit by shRNA. HeLa cells were co-transfected with pCEP-4HA-B56γ expressing 4HA-tagged B56γ, and either shB56γ or control pSuper vector and lysed 48h post-transfection, followed by Western blotting analysis with the indicated antibodies. (B) Down-regulation of PP2A B56δ subunit by shRNA. F9 cells were transfected with pSuper vector encoding shRNA directed against PP2A B56δ or pSuper vector control; 24h post-transfection, cell lysates were prepared and subsequently analyzed by Western blot. (C) F9 cells were co-transfected with Flag-apoptin and either pSuper vector encoding shRNA directed against PP2A B56γ, δ or control; 24-48h post-transfection, cell lysates were prepared and subsequently analyzed by Western blot.

Overexpression of PKA-interacting protein BCA3 stimulates apoptin activity in tumor cells

Analogous to the enhancing effect of ST on apoptin phosphorylation in normal cells, we observed an enhancement of apoptin phosphorylation in tumor cells by BCA3. BCA3 was identified as an apoptin-interacting protein by means of a yeast two-hybrid assay, and interacts with apoptin in a human cellular background (Figure 3.5A). Co-expression of BCA3 and Flag-apoptin in human Saos-2 tumor cells resulted in a significant increase in the apoptosis activity of apoptin (Figure 3.5B). In fact, as early as 6 hours after transfection, phosphorylated apoptin could readily be detected in Saos-2 cells expressing both apoptin and BCA3, whereas in cells expressing apoptin alone (control), apoptin phosphorylation was not yet visible at this early time-point (Figure 3.5C).

As BCA3 (Westermarck and Hahn, 2008) has been shown to interact with the catalytic subunit of PKA (Sastri et al., 2005), the involvement of PKA in apoptin phosphorylation was investigated. Treatment of Saos-2 cells with H89, a known PKA

inhibitor (Lochner and Moolman, 2006), enhanced apoptin phosphorylation. In contrast, addition of the PKA activator cAMP (Bulun and Simpson, 2008) diminished apoptin phosphorylation (Figure 3.5D). These results suggest that interference with PKA activity favors activation of apoptin. By taking into account the fact that the B56δ subunit of PP2A has been shown to be targeted by PKA (Ahn et al., 2007), we proved that this is also the case in our cellular system. Indeed H89 treatment of Saos-2 cells decreased the level of active serine-phosphorylated B56δ, whereas cAMP enhanced the amount of active B56δ (Figure 3.5E-G).

Down-regulation of PP2A-B56δ subunit activates apoptin phosphorylation in normal cells

The experiments described above indicate that in cancer cells an inverse relation exists between the activity of PP2A-B56δ, as judged by its phosphorylation (Ahn et al., 2007), and the potential of apoptin to become phosphorylated in the same cells. For this reason it was interesting to examine whether inhibition of the expression of the PP2A-B56δ subunit in human F9 cells would positively affect the phosphorylation of apoptin. Down-regulation of B56δ protein expression through RNAi was confirmed in normal F9 fibroblasts (Figure 3.4B). Co-expression of shRNA targeting B56δ mRNA together with apoptin in normal F9 cells clearly resulted in the activation of apoptin phosphorylation, as compared to F9 cells transfected with apoptin and the RNAi control vector (Figure 3.4C).

In conclusion, our results imply that PP2A complexes containing the regulatory subunits B56 δ and γ are essential for maintaining a normal cell environment, as the loss of either one of these subunits results in the activation of apoptin.

Discussion

Activation of tumor-selective apoptosis-inducing proteins is an intriguing phenomenon and revealing the molecular switch behind this process could allow important insights for developing anticancer therapies. Apoptin was the first protein known to harbor apoptosis activity selectively in transformed human cells (Bruno et al., 2009). However, the molecular mechanisms underlying apoptin's tumor-selective activity are largely unknown (Grimm and Noteborn, 2010). Our studies revealed that interference with the normal function of PP2A is

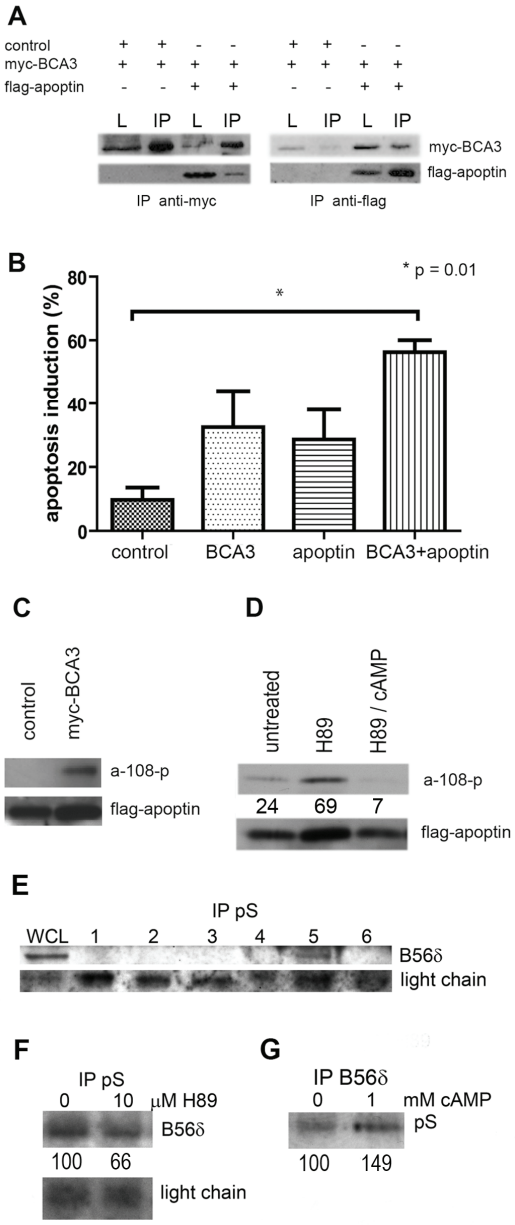


Figure 3.5: BCA3 interacts with apoptin and stimulates its activity. (A) Apoptin interacts with BCA3 in a cellular background. Normal human foreskin fibroblasts were transfected with plasmids encoding myc-tagged BCA3 and Flag-tagged apoptin, or control plasmid in the indicated combinations. Total lysates (L) or protein complexes immunoprecipitated (IP) with antibody against the myc- (left panel) or Flag-tags (right panel) were separated by SDS-PAGE and analyzed by Western blot. (B) Expression of myc-BCA3 together with Flag-apoptin results in increased induction of apoptosis. Human Saos-2 tumor cells were transfected with plasmids encoding Flag-tagged apoptin and myc-tagged BCA3, or vector control in the indicated combinations and grown on glass coverslips. Forty-eight hours post-transfection, slides were fixed and stained with appropriate antibodies for immunofluorescence analysis. pMaxGFP (Amaxa) was used as a negative control. Apoptosis was assessed according to characteristic morphological changes[35] following DAPI staining as shown in figure 1. Data are representative of 3 independent experiments, in which at least 100 cells were scored. (C) Co-expression of myc-BCA3 enhances apoptin phosphorylation. Saos-2 cells transfected with BCA3 and apoptin, or apoptin alone, were lysed 6 hours post-transfection and analyzed for apoptin phosphorylation by Western blot analysis. (D) Inhibition of PKA results in increased apoptin phosphorylation. Saos-2 cells were transfected with Flag-tagged apoptin, and treated with the PKA inhibitor H89 (1h, 10μM) or activator cAMP (30 minutes, 10μM H89, followed by 30 minutes 1mM cAMP) 24hs post-transfection. Cells were then lysed and their contents analyzed by Western blot using indicated antibodies. The relative percentage of phosphorylated apoptin in the various samples was quantified and is indicated below the a-108-P panel (E) Effect of PKA inhibitors/inducers on PP2A B56δ phosphorylation. In a first instance phosphorylated proteins were immuno-precipitated from Saos-2 whole cell lysate (WCL) with six different antibodies directed against phosphoserines, as described (Materials and Methods). Western blot was performed with antibodies against B56δ. The light chain signal shows equal assay conditions. Note that only antibody 5 detects phospho-B56δ.

(F) Saos-2 cells were treated with PKA-inhibitor H89 or mock-treated and immunoprecipitated with the phosphoserine antibody 5 (see panel E). The amount of pulled-down B56δ was determined by Western blot using an antibody directed against B56δ and quantified. The amount of phospho-B56δ in the mock-treated cells was set at 100. (G) Saos-2 cells were treated with PKA-stimulator cAMP or mock-treated and immunoprecipitated with antibodies against B56δ. The amount of phosphorylated B56δ was determined by Western blot using the phospho-serine antibody 5 (panel E) and quantified. The amount of phospho-B56δ in the mock-treated cells was set at 100.

sufficient to activate apoptin in a tumor-selective fashion. This is inferred from two independent lines of research.

In one study, we examined the effect of specific domains within the transforming SV40 proteins LT136 and ST on the activation of apoptin. The C-terminal PP2A-binding and transformation domain of ST (Pipas, 2009; Arroyo and Hahn, 2005), when targeted to the nucleus of normal cells, turned out to be crucial for the tumor-characteristic activation of apoptin. We showed that both C103S and P101A point mutations within the ST PP2A binding site ((Chen et al., 2004) abrogated the phosphorylation of apoptin induced by NLS-ST in normal human fibroblasts. RNA interference studies confirmed that inactivation of the B56γ protein promotes phosphorylation of apoptin in human fibroblasts. We have previously shown (Zhang et al., 2004) that the N-terminal J domain, which is common to both LT and ST (see figure 1), is involved in apoptin activation. Interestingly, since the completion of this work two independent groups have resolved the molecular structure of the SV40 ST complex with PP2A and shown that both the C-terminal PP2A binding domain and the N-terminal J domain are in direct contact with the A subunit of PP2A (Chen et al., 2007; Cho et al., 2007). The structure provides a mechanism how binding of ST to the A subunit of PP2A displaces the B56γ subunit and as such inactivates PP2A. The results described in figure S1 (supplementary information) indicate that also the J domain (either present on LT or ST) needs to be functional in order to activate apoptin in combination with a functional C-terminal PP2A binding site on ST. The data strongly suggest that the J domain of LT can also contribute to the displacement of B56γ and the activation of apoptin in concert with our previous conclusions. However, the contribution of ST, which has not been analyzed in detail in the previous study, is essential for activating apoptin (Figure 2).

A second line of research indicated that interference with the PP2A-B56δ subunit also led to the activation of apoptin. This was achieved by treating human cancer cells with either PKA activator or inhibitor. PKA is known to phosphorylate the B56δ subunit of PP2A leading to increased PP2A activity (Ahn et al., 2007). Ectopic expression of the apoptin- and PKA-interacting protein BCA3, or inhibition of PKA by treatment with H89 both resulted in enhanced phosphorylation and apoptosis activity of apoptin in human cancer cells. Stimulation of PKA by cAMP resulted in enhanced phosphorylation of PP2A-B56δ and a lower level of phosphorylated apoptin in Saos-2 tumor cells. On the contrary, H89 decreased the level of phosphorylated B56δ in tumor cells and enhanced apoptin

phosphorylation. These data indicate that derailed PP2A activity is crucial for activating apoptin in accordance with the data obtained in our SV40 transformation essay. This vision was further highlighted by RNA interference studies showing that inhibition of the expression of PP2A-B56δ activated apoptin even in normal human cells.

Our studies reveal that apoptin senses PP2A inactivation during malignant transformation. Interestingly, the delta and gamma subunits are the only nuclear PP2A-B56 subunits (Chen et al., 2004), and nuclear localization is important for both SV40-T antigen induced cell transformation (Pipas, 2009) and apoptin-induced tumor-selective apoptosis (Backendorf et al., 2008). PP2A complexes containing B56δ domains prevent entry of cells into mitosis upon DNA damage (Visshup and Shenolikar, 2009). B56γ is also known to mediate dephosphorylation and stabilization of the tumor suppressor protein p53 upon DNA damage, inhibiting cellular proliferation and transformation (Li et al., 2007). Evidence has been provided that derailment of B56γ results in aberrancies in functioning of e.g. cell cycle and tumor suppressor proteins resulting in cell transformation (Sablina and Hahn, 2008; Chen et al., 2004). Many of these features are likely due to the fact that PP2A-B56 subunits play an essential role in the stabilization of chromosome-spindle interactions during normal cell division (Foley et al., 2011). In this respect it is interesting to mention that inhibition of Bub1, another mitotic regulator, results in nuclear translocation of apoptin in normal cells (Kucharski et al., 2011). Apparently apoptin can sense aberrant mitosis and the ensuing genetic instability (Pihan and Doxsey, 1999) or DNA damage response signalling (Kucharski et al., 2011).

PP2A plays also a role in the activation of other tumor-selective apoptosis-inducing proteins. Besides apoptin, the adenovirus E4orf4 protein has been demonstrated to selectively induce apoptosis in human cancer cells. Direct interaction of E4orf4 with PP2A regulatory B domains is essential for the tumor-selective apoptosis activity of E4orf4. Interaction of E4orf4 with PP2A-B55 results in down-regulation of myc oncogene expression (Ben-Israel et al., 2008). In addition to SV40 ST, other viral transforming proteins also interact with PP2A, further accentuating its relevance in cellular transformation (Zhao and Elder, 2005).

Further steps within the development of tumorigenic cells seem at least not critical for apoptin's tumor-selective apoptosis characteristics. These conclusions are in accordance with the observations by others and ourselves that apoptin is able to induce apoptosis in a

very broad panel of tumor types (Backendorf et al., 2008; Grimm and Noteborn, 2010; Maddika et al., 2006). If one assumes that tumor cells arise by a wide variety of mechanisms, all the while sharing a limited number of key characteristics (Hanahan and Weinberg, 2011), then apoptin simply needs to recognize one (or a subset) of these characteristics.

In summary, our results show that inactivation of the nuclear PP2A B56 δ and/or γ subunits are sufficient to trigger apoptin's tumor-selective apoptosis activity. PP2A provides a central phosphatase activity affecting many cellular signaling pathways. As derailment of PP2A activity is increasingly linked to oncogenic transformation, the sensing of such a central regulator by apoptin might provide a rationale for its efficient killing of tumor cells arising from a wide range of different origins.

Materials and Methods

Cell culture

Human diploid foreskin F9 fibroblasts, isolated from neonatal foreskin, were obtained in the late 1980's from Dr. M. Ponec (Dept. Dermatology, Leiden University Medical Center). Cells were batch-frozen after careful morphological inspection. At subsequent passages cells were regularly screened for their typical fibroblast-like morphological appearance. All cells used were below passage 15 and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% newborn calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Breda, the Netherlands). The human Saos-2 osteosarcoma and the HeLa cervical carcinoma cell lines were purchased from the American Type Culture Collection (ATCC) and cultured in the same medium as mentioned above. Cultures were regularly tested to ensure the absence of *Mycoplasma* infection. Cell morphology was regularly monitored to control the absence of cross-contamination. The sensitivity to apoptin is characteristic of the various cell types used and is regularly assessed (see below).

Plasmids

The DNA sequence encoding apoptin was synthesized by BaseClear (Leiden, the Netherlands) according to the apoptin sequence use by Danen-Van Oorschot et al. (2004), and cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen). The oligonucleotide fragment encoding the Flag-tag (Invitrogen) was inserted to create the pcDNA-Flag-apoptin plasmid encoding apoptin fused with a Flag-tag at its N-terminus.

Plasmid pRSV-TN136 encoding the first 136 N-terminal amino acids of SV40 LT, including the region coding for SV40 ST, was a kind gift from Dr. J.M. Pipas (Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, USA). From pRSV-TN136, we generated the pcDNA-LT136/ST plasmid, which encodes the LT136 (The N-terminally truncated LT fragment containing the first 1-136 aa) and full length ST. pcDNA-LT136, encoding LT136 only, was constructed by introducing an intron deletion disabling ST expression (Yamashita et al., 1990).

pcDNA expression vectors encoding only ST sequences were derived from pRSV-ST. pcDNA-ST encodes for ST; pcDNA-NLS-ST contains a ST fused to a nuclear localization signal (NLS-ST) and pcDNA-NLS-ST(C103S) and NLS-ST(P101A) mutants encode the N-terminal NLS-ST fusion protein containing respectively the C103S or P101A mutation within the PP2A binding site (Gjoerup et al., 2001).

The sequences encoding the N-terminal 136 aa of LT together with either full-length ST or the C103S ST-mutant were cloned into pEXPR-IBA105 vector to generate pEXPR-IBA105-LT136/ST and pEXPR-IBA105-LT136/STC103S, respectively. These plasmids expressed Strep-tagged LT136/ST or LT136/ST C103S proteins enabling interaction studies with PP2A (see below) (Schmidt and Skerra, 2007). pCEP-4HA-B56γ, encoding 4HA-tagged B56γ, was a kind gift from Dr. M. Mumby (University of Texas, USA).

Apoptin-interacting partners were obtained by yeast two-hybrid screening and verified by immunoprecipitation assays in mammalian cells, as previously described by Danen-Van Oorschot et al. (2004) Positive clones from the yeast two-hybrid screen were digested with XhoI to generate cDNA fragments, and subcloned into pMT2SM-myc to provide the fragments with an in-frame N-terminal myc-tag. The cDNA fragment encoding BCA3, including the myc-tag, was subsequently cloned into pcDNA.

Transfection Methods

We used transfection reagent DOTAP (N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate) (Danen-van Oorschot et al., 2004) or AMAXA nucleofection technology in conjunction with cell type specific NucleofectorTM solution (Lonza, Cologne, Germany) (Rohn et al., 2002) for DNA delivery into cells. When co-transfection or triple-transfection was performed, the ratio of each plasmid was 1:1 or 1:1:1

(in micrograms). In addition to the analysis by Western blot, the cells were seeded on several glass cover slips to allow parallel analysis at the single-cell level by immunofluorescence assay.

Western Blot analysis

Cells were lysed directly in Laemmli buffer (2% SDS, 10% Glycerol, 60mM Tris-Cl [pH6.8], 2% β-mercaptoethanol, 0.002% bromophenol blue). Cell lysates were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis, and electroblotted onto polyvinylidene difluoride membranes (Bio-Rad). Blots were then incubated with antibodies against phosphorylated-apoptin (α-108-P (Zhang et al., 2004)), Flag-apoptin (α-Flag M2, Sigma-Aldrich), SV40 LT (PAb416, Pab419, Calbiochem), SV40 ST (Pab280, Calbiochem), PP2A Aα (C-20, Santa Cruz), PP2A B56γ (α-B56γ, kind gift from Dr. Marc Mumby, Health Science Center, University of Texas, Texas, USA), PP2A B56δ (α-B56δ, Santa Cruz, Heidelberg, Germany), myc-tagged BCA3 (α-myc, BD Biosciences) and actin (α-actin, Santa Cruz). Antibodies directed against phosphorylated serine were 1C8 (1), 4A3 (2), 4A9 (3), 4H4 (4), 7F12 (5) and 16B4 (6); they recognize phospho-serine with various efficiencies depending on the surrounding amino acid motif (Enzo Life Sciences, Antwerp, Belgium). Horseradish peroxidase-conjugated goat antibody against rabbit or mouse immunoglobulin G, or rabbit antibody against goat immunoglobulin G (Sigma-Aldrich) was used as secondary antibody for signal detection by enhanced chemiluminescence. Films were quantified using Quantity One Analysis Software (Biorad).

Protein interaction assays

Detection of a possible interaction of ST or ST-C103S mutant protein with PP2A in human HeLa cells was performed as follows. Twenty-four hours after DNA transfection, cells were washed twice with phosphate buffered saline and harvested in ice-cold mild lysis buffer (50mM Tris [pH 7.5], 5mM EDTA, 250mM NaCl, 0.1% Triton X-100, 5 mM NaF, 1mM Na_3VO_4, 20mM beta-glycerolphosphate, and Protease Inhibitor Cocktail (Roche), followed by incubation on ice for 30 min. The supernatant of the lysates was prepared by centrifugation at 13,000 × g and 4°C for 30 min. Strep-tagged proteins and their interacting proteins were captured using the One-strep kit (IBA, Germany) according to the

manufacturer's protocol, and resolved on sodium dodecyl sulfate-polyacrylamide gel, followed by Western blotting analysis with appropriate antibodies.

RNA interference assay

For human PP2A B56γ, the target sequence was: 5'-GATGAACCAACGTTAGAAG-3'; for the PP2A B56δ two sequences were targeted 1.: 5' GTGTGTCTCTAGCCCCCAT 3' and 2.: 5' GACCATTTTGCATCGCATC 3' (data not shown). The pSUPER vector was designated for shRNA plasmid constructions (Boutros and Ahringer, 2008). The amplification and purification of plasmids were performed as specified by manufacturer's instruction (GeneService, Cambridge, UK). Cells transfected with shRNA plasmids were lysed at 24-48 h after transfection, and then analyzed by Western blot assay as described above.

Immunofluorescence assay

Cells were grown on glass cover slips. At indicated time points after transfection, cover slips were first washed once with phosphate buffered saline, and subsequently fixed with methanol/acetone (50%/50%) for 5-10 min at room temperature. After air-drying, the slides were used for immunocytochemical staining or stored at -20°C for further analysis. Immunocytochemical staining was carried out as described by Danen-Van Oorschot et al. (2004). The following antibodies were used: α-108-P, a rabbit polyclonal antibody recognizing phosphorylated apoptin at T108 and α-Flag, a mouse monoclonal antibody recognizing Flag-apoptin. SV40 proteins were visualized with PAb416, a mouse monoclonal antibody recognizing the epitope residing in amino acids 83-128 of LT and non-reactive with ST or PAb280, a mouse monoclonal antibody against the C terminus of ST. The fluorescein isothiocyanate (FITC) or rhodamine conjugated goat antibodies (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. Nuclei were stained with 2,4-Diamidino-2-phenylindole (DAPI) and apoptosis was assessed according to characteristic morphological changes (Danen-van Oorschot et al., 2004).

PKA inhibition and stimulation

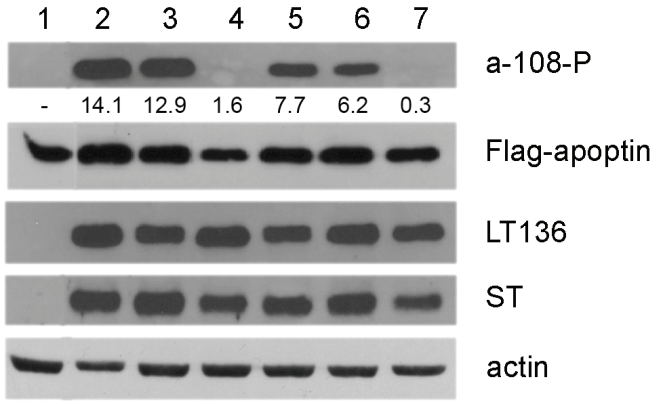
Saos-2 cells were transfected with pcDNA-Flag-apoptin and incubated 24 hours post-transfection with 10μM H89 (Millipore) for 1 hour (PKA inhibition), or 30 minutes with H89 followed by incubation with 1mM cAMP (Sigma) for another 30 minutes (PKA

stimulation). Cells were then lysed in Laemmli buffer and the cell lysates analyzed for apoptin phosphorylation by Western blotting. To determine the phosphorylation status of B56δ, cells were lysed after treatment in mild lysis buffer as described for the protein interaction assay with addition of PhosSTOP (Roche). After 30 minutes of incubation, cell lysate was obtained by 15 minutes of centrifugation at 16100 rcf at 4°C. Whole cell lysate was incubated 1.5 hours with antibody either against B56δ or against phosphorylated serines. μMACS microbeads (Miltenyi Biotech) were added for 1 hour and proteins were pulled down using the μMACS column system according to the manufacturer's protocol. Elution was performed using Laemmli buffer and proteins were resolved on sodium dodecyl sulfate-polyacrylamide gel, followed by Western blotting analysis with appropriate antibodies.

Acknowledgements

We thank dr. James Pipas, University of Pittsburg, and dr. Marc Mumby, University of Texas for their kind gifts and dr. Maarten de Smit, Leiden University for advice. This work was supported by a grant from the Dutch Royal Society of Arts and Sciences (06CDP010).

Supplementary Data



Supplementary figure S3.1: Both J domains on LT136 or ST can contribute to the activation of apoptin. The effect on apoptin phosphorylation of LT136 and ST either expressed by intron splicing (LT/st construct) (lanes 2, 4) or individually transfected on separate plasmids (lanes 3, 5, 6, 7) into normal human fibroblast is presented. The transfected SV40 genes were either wild-type (lanes 2, 3) or mutated (D44N) in the J domain (lanes 4, 7). In lane 5 wild-type LT136 is combined with ST-D44N, whereas in lane 6 wild-type ST is combined with LT136-D44N. The data indicate that when the D44N mutation is present on both genes, apoptin is not activated. Mutation of the J domain on ST, which prevents apoptin phosphorylation (lane 7), can partially be compensated by a functional J domain on LT136 (lane 5). At present the molecular basis for this interference is not understood. The relative percentage of phosphorylated apoptin in the various samples was quantified and is indicated below the a-108-P panel.

Chapter 4

Proteasomal insensitivity of apoptin in tumor cells

Henriëtte L Lanz, Johnny Suijker, Mathieu HM Noteborn and Claude Backendorf

Biochemical and Biophysical Research Communications (2012) 422, 169-173

Abstract

The small viral protein apoptin is capable of inducing apoptosis selectively in human tumor cells. In normal cells apoptin localizes in the cytoplasm where it forms aggregates, becomes epitope-shielded and eventually degraded. By inhibiting the proteasome activity with the chemical inhibitors bortezomib and Ada-Ahx_3L_3VS apoptin levels can be stabilized in normal cells similar to the tumor suppressor p53 protein. In contrast, proteasome inhibition in tumor cells did not affect the apoptin stability while it still stabilized p53 levels. Apparently, apoptin is degraded by proteasomal activity in normal human cells, a process that no longer takes place in tumor cells. This loss of proteasomal susceptibility appears to be specific for apoptin.

Introduction

Apoptin is a small protein produced by Chicken Anemia Virus (CAV) that is capable of inducing apoptosis in human tumor cells while leaving normal cells intact (Danen-van Oorschot et al., 1997). In tumor cells apoptin is phosphorylated on T108 and translocates to the nucleus whereas in normal cells it stays in the cytoplasm in a non-phosphorylated fashion (Rohn et al., 2002; Backendorf et al., 2008). In normal cells, microinjected bacterially expressed MBP-apoptin forms in time large aggregates that after 24 hours can no longer be detected in an immune-fluorescence assay. The protein can still be detected by the use of stringent buffers and Western blot, showing that it becomes epitope-shielded. At later time points the protein could no longer be detected in normal cells and was eventually degraded (Zhang et al., 2003). This particular behavior was not observed in tumor cells.

Zhang et al. (2003) showed that the disappearance of MBP-apoptin was not caused by lysosomal degradation prompting us to investigate the role of the proteasome in the degradation of apoptin in both normal and tumor cells. The proteasome is a multi-subunit protease, which is composed of a proteolytic 20S core with regulatory 19S caps. The proteolytic activity is contained in the β1, β2 and β5 subunits, all with their own substrate specificity (Hershko and Ciechanover, 1998; Murata et al., 2009).

To study the role of the proteasome in the degradation of apoptin we used the proteasome inhibitors bortezomib (Cusack et al., 2001) and Ada-Ahx$_3$L$_3$VS (Kessler et al., 2001) to block proteasomal degradation in cells expressing apoptin. We show that inhibition of the proteasome in normal cells leads to stabilization of apoptin and p53 levels, while in tumor cells both proteasome inhibitors affect p53 protein levels but have no influence on the amount of apoptin protein.

Results

Apoptin is degraded in normal human fibroblasts

In order to analyze a possible role of proteasomal degradation of apoptin we first examined the fate of apoptin levels in normal human fibroblasts ectopically expressing apoptin. After transfection of normal human fibroblasts with plasmid encoding flag-tagged apoptin, cells were fixed at various time-points and stained with antibody against the flag-tag. At 8 hours post transfection a diffuse apoptin distribution is observed throughout the cytoplasm. At later time-points apoptin starts to form granules and eventually can no longer be detected

(Figure 4.1A). Transfected flag-apoptin shows the same cytoplasmic localization as microinjected MBP-apoptin (Zhang et al., 2003). Apoptin protein levels were also analyzed by Western blot. Already 40 hours after transfection a drop in protein can be clearly observed, with hardly any to be detected three days after transfection (Figure 4.1B). This result indicates that apoptin likely gets degraded.

To analyze whether this reduction in protein was due to degradation of the apoptin protein, the proteasome was inhibited with the proteasome inhibitor bortezomib. Stabilization and an actual increase in apoptin protein (1.5x) could be observed after several days (Figure 4.1B/C) suggesting that the decrease in apoptin levels in normal cells is due to proteasomal degradation.

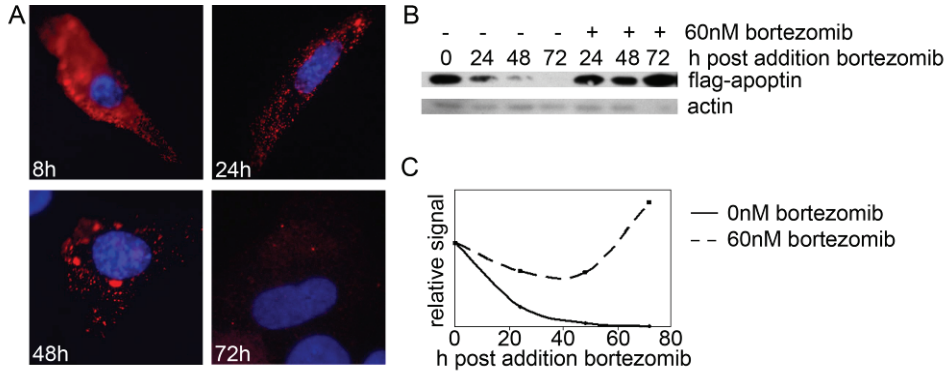


Figure 4. 1: Apoptin is degraded in time in normal cells, a process that can be blocked by bortezomib. Normal human fibroblasts F44 were transfected with plasmid encoding flag-tagged apoptin. (A) Cells were fixed 8, 24, 48 and 72 hours post transfection. Flag-tagged apoptin was stained with a flag-specific antibody conjugated to rhodamine (red) and DNA was detected with Hoechst (blue). Apoptin appears in the cytoplasm as fine particles (8h), gradual clusters (24h), increased aggregates (48h) and eventually becomes degraded (72h). (B) Sixteen hours post transfection 0 or 60nM bortezomib was added to the cells and at three time points after addition cells were lysed. Total apoptin levels were determined by Western blot analysis with anti-flag antibody. (C) The relative amount of apoptin (as compared to t=0) with and without bortezomib was quantified and plotted.

Bortezomib and Ada-Ahx$_3$L$_3$VS inhibit apoptin and p53 degradation equally in normal human fibroblasts

Next, we analyzed whether besides apoptin a protein known to be degraded by the proteasome, i.e., tumor suppressor protein p53, can be stabilized by bortezomib with similar kinetics as apoptin in normal human fibroblasts. To that end, 16 hours after transfection increasing amounts of bortezomib were added to human normal fibroblasts expressing ectopic apoptin and endogenous p53. Twenty-four hours after the addition of bortezomib the cells were lysed and analyzed for apoptin and p53 levels by means of Western blot. A

gradual raise of apoptin protein levels could be observed as the concentration of bortezomib increased (Figure 4.2A, up to ~7.5x increase compared to control). The increase in the amount of apoptin was mimicked by the level of p53 (Figure 4.2B, up to ~7.5x increase compared to control).

As bortezomib mainly inhibits the β5 subunit of the proteasome (Kisselev et al., 2006) we also examined the broad-spectrum proteasome inhibitor Ada-Ahx_3L_3VS (Kessler et al., 2001) in a similar experiment. This led to a stabilization of both apoptin and p53 levels (Figure 4.2), however, not to higher levels than observed with bortezomib (compare Figure 4.2A/B, ~7.5x increase with bortezomib compared to a ~4x increase of flag-apoptin signal with Ada-Ahx_3L_3VS).

Our results based on two different proteasomal inhibitors indicate that apoptin is degraded in normal human cells via the proteasomal pathway.

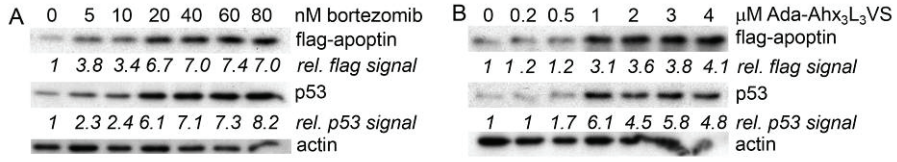


Figure 4.2: Proteasome inhibitors stabilize apoptin and p53 in normal cells. Normal human fibroblasts F44 were transfected with plasmid encoding flag-tagged apoptin. (A) 16 hours post transfection increasing concentrations of bortezomib or (B) Ada-Ahx_3L_3VS were added and 24 hours later cells were lysed. Total levels of apoptin and p53 were determined by Western blot analysis. The relative amounts of apoptin and p53 (relative to the untreated signal) were quantified and are indicated below the corresponding panels.

Bortezomib inhibits phosphorylation-deficient apoptin mutant protein with similar kinetics as p53 in normal human cells

Apoptin induces apoptosis in tumor cells. This complicates the study of proteasomal breakdown in tumor cells. The phosphorylation of apoptin in cancer cells is closely linked to its apoptotic potential. Removal of the phosphorylation site T108 and the adjoining threonines greatly reduces the cell death potential of apoptin (Rohn et al., 2005). Therefore, we used the flag-apoptin(5Ala)106 mutant in which the stretch from position 106 to 110 has been replaced by alanines (Danen-van Oorschot et al., 2004).

Normal human fibroblasts transfected with plasmid encoding this apoptin(5Ala)106 mutant showed high protein expression at early time points, but as for the wild-type apoptin (Figure 4.1B/C), the apoptin(5Ala)106 mutant protein level diminished in time (Figure 4.3A). On the contrary, upon addition of bortezomib the amount of apoptin(5Ala)106 stayed high

(Figure 4.3A). Increasing concentrations of bortezomib resulted in more apoptin(5Ala)106 protein (Figure 3B, almost 3x increase).

The similar kinetics of degradation of the phosphorylation-deficient mutant compared to wild-type apoptin in normal cells makes it an appropriate construct to study the degradation characteristics of apoptin in human tumor cells. P53 stabilization showed similar kinetics as in the wild-type apoptin experiment.

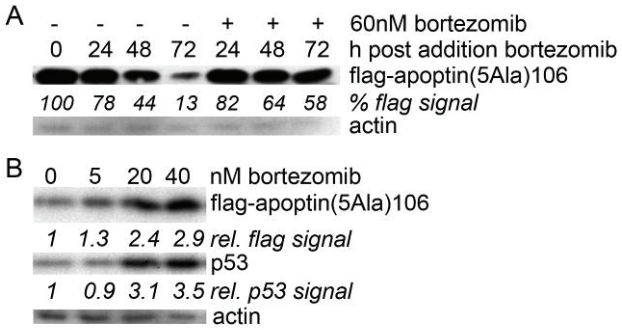


Figure 4.3: Apoptin(5Ala)106 is degraded in normal cells in a similar fashion as p53 and apoptin. Normal human fibroblasts F44 were transfected with plasmid encoding flag-tagged apoptin(5Ala)106 mutant. 16 hours post transfection bortezomib was added. (A) At various time points cells were lysed and total apoptin was determined by Western blot analysis. (B) 24 hours after addition of increasing concentrations of bortezomib total apoptin and p53 were determined by Western blot. The relative amounts of apoptin and p53 (relative to the untreated signal) were quantified and are indicated below the corresponding panels.

Bortezomib and Ada-Ahx$_3$L$_3$VS stabilize p53 but not apoptin(5Ala)106 in human osteosarcoma cells

To examine the behavior of apoptin protein upon a bortezomib or Ada-Ahx$_3$L$_3$VS treatment in tumor cells, human osteosarcoma U2Os cells were used because they express wild-type p53 and are sensitive for apoptin-induced apoptosis (Diller et al., 1990; Zhuang et al., 1995). U2Os cells were transfected with plasmids encoding flag-tagged apoptin(5Ala)106 protein. Sixteen hours after transfection, the p53-positive human osteosarcoma cells were treated with increasing concentrations of bortezomib or Ada-Ahx$_3$L$_3$VS and the cells were lysed 24 hours later.

Increasing amounts of both bortezomib and Ada-Ahx$_3$L$_3$VS led to an increase in total p53 (Figure 4.4A/B). The p53 levels changed similarly as in normal cells as they increased 5x with bortezomib and 4x with Ada-Ahx$_3$L$_3$VS. This shows that the proteasome and the proteasomal inhibitors function in the U2Os tumor cell line. Remarkably, the amount of apoptin(5Ala)106 protein was not influenced by inhibition of the proteasome by increasing the concentrations of both bortezomib and Ada-Ahx$_3$L$_3$VS (Figure 4.4). In accordance with this finding is the observation that the basal level of apoptin protein in the

U2Os cells without addition of inhibitor is 15x higher than in normal cells (Figure 4.4C). Altogether, these results show that apoptin(5Ala)106 can not become degraded by the proteasome in human U2Os tumor cells.

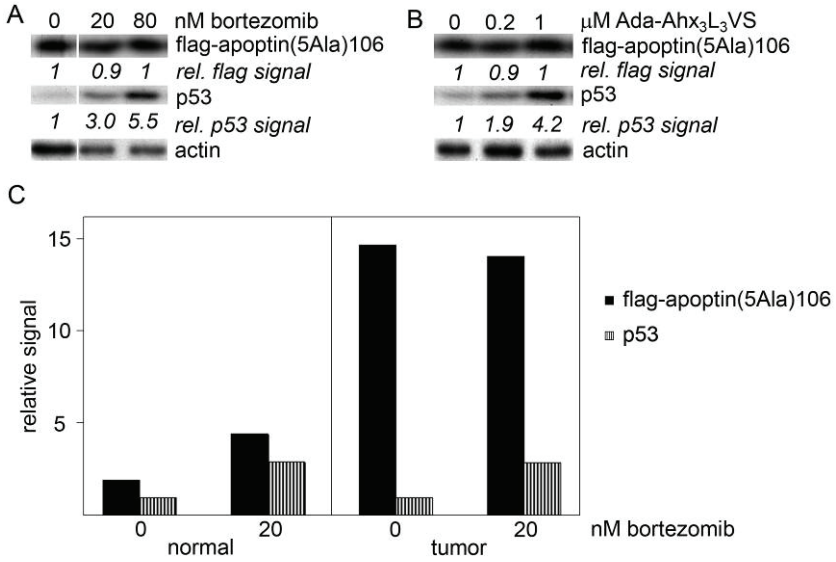


Figure 4.4: Proteasome inhibitors influence p53 but not apoptin(5Ala)106 stability in tumor cells. Human osteosarcoma cells U2Os were transfected with plasmid encoding flag-tagged apoptin(5Ala)106. (A) 16 hours post transfection cells were treated with increasing concentrations of bortezomib or (B) Ada-Ahx_3L_3VS and 24 hours later cells were lysed. Total amounts of apoptin and p53 were determined by Western blot analysis. The relative amounts of apoptin and p53 (relative to the 0nM bortezomib signal) were quantified and are indicated below the corresponding panels. (C) In order to make a direct comparison between the apoptin protein level in normal and tumor cells two representative experiments, one in normal and one in tumor cells, are visualized in the same chart. The relative amounts of apoptin and p53 in fig. 3B and fig. 4A were quantified relative to the untreated p53 levels and are plotted.

Discussion

By inhibiting the proteasome activity with the known proteasome inhibitors bortezomib and Ada-Ahx_3L_3VS, which both clearly positively affected the tumor suppressor p53 protein stability in normal and cancer cells, we revealed that apoptin protein is degraded in normal but not in tumor cells through the process of proteasomal degradation.

Apoptin is a potential therapeutic agent with very distinct behavioral differences between normal and tumor cells. The differential proteasomal degradation of apoptin between normal and tumor cells will likely contribute to its selectivity.

The tumor selective induction of apoptosis is the main feature of apoptin. This characteristic is preceded by the nuclear localization in cancer cells (Backendorf et al., 2008). In tumor cells apoptin is phosphorylated on T108, a modification that is not observed in normal cells (Rohn et al., 2002) To circumvent that apoptin-induced apoptosis interferes

with our protein degradation or stabilization measurements we developed a flag-tagged phosphorylation-deficient apoptin(5Ala)106 mutant that has a greatly reduced cell death inducing property (Rohn et al., 2005). In normal cells this mutant showed the same reaction to inhibition of the proteasome as did wild-type apoptin. In tumor cells, however, both the proteasome inhibitor bortezomib and Ada-Ahx_3L_3VS had no influence on the level of apoptin(5Ala)106, whereas the positive control p53 was clearly stabilized by the inhibition of the proteasome with similar kinetics as in normal cells.

Nuclear translocation is a characteristic of apoptin in tumor cells. P53 can also localize to both the nucleus and cytoplasm and is degraded in both compartments (Yuan et al., 2010). This raises the question if the difference in degradation of apoptin is due to a different proteasomal activity in the nucleus. Although several different catalytic subunit compositions are known between cell types as described above, no distinction has yet been established between the subunit composition of the proteasome in the nucleus and the cytoplasm.

In normal cells the highest levels of apoptin and p53 are reached with the inhibitor bortezomib compared to Ada-Ahx_3L_3VS. Bortezomib has a high affinity for the β5 enzymatic subunit of the proteasome relative to Ada-Ahx_3L_3VS which has an equal effect on all three β subunits. Apparently it is the β5 subunit that plays a major role in both apoptin and p53 degradation.

Although elevated levels of proteasome subunits have been found in some cancers, and proteasome inhibitors are used in the treatment of certain cancer types, a clear molecular difference between tumor and normal proteasome subunits has not yet been reported (Hoeller and Dikic, 2009; Bousquet-Dubouch et al., 2011).

Possibly, it is not a difference in proteasome assembly that leads to the higher stability of apoptin in tumor cells. Some viral proteins are known to modulate (viral) protein stability through the proteasome (Hu et al., 1999). In this respect it is interesting to mention that apoptin influences the anaphase promoting complex or cyclosome (APC/C) complex. Teodoro et al. (2004) showed that interaction of apoptin with the APC1 subunit results in destabilisation of the APC/C complex. The APC/C is an upstream effector of the proteasome that is responsible for correct progression of the cell cycle by targeting cell cycle related proteins for degradation (Garcia-Higuera et al., 2008). APC/C is the main E3 ligase in the nucleus targeting proteins for degradation. Subunits of the APC/C are mutated in

several cancers and are a popular target for viral proteins (Smolders and Teodoro, 2011). It is tempting to hypothesise that by disrupting the APC/C E3 ligase activity apoptin itself creates the situation that enables its stabilization. More research will, however, be needed to fully comprehend the molecular basis of the stable apoptin protein levels in tumor cells.

The tumor-selective apoptosis induction by apoptin makes it a very promising anti-cancer agent. Here, we show a novel characteristic of apoptin. The differential proteasomal sensitivity likely will contribute to the application of apoptin as a safe and efficient therapeutic agent.

Materials & Methods

Cell culture

The normal human diploid foreskin F44 fibroblasts, isolated from neonatal foreskin, were obtained in the late 1980's from Dr. M. Ponec (Dept. Dermatology, Leiden University Medical Center). Cells were batch-frozen after careful morphological inspection. At subsequent passages cells were regularly screened for their typical fibroblast-like morphological appearance. F44 cells were used below passage 15 and cultured in 1:1 Dulbecco's Modified Eagle's Medium : Ham's F12 (DMEM/F12) (PAA, Colbe, Germany) containing 10% fetal calf serum (Thermo Scientific, Geel, Belgium), 100μg/mL penicillin, 100μg/mL streptomycin (Duchefa, Biochemie, Haarlem, The Netherlands) and 2mM glutaMAX (PAA). The human osteosarcoma cell line U2Os was selected as it expresses wild-type p53 (Diller et al., 1990). U2Os cells were purchased from the American Type Culture Collection (ATCC, Wesel, Germany) and cultured in DMEM (PAA) containing 10% newborn calf serum (Thermo Scientific), 100μg/mL penicillin, 100μg/mL streptomycin (Duchefa) and 2mM glutaMAX (PAA). Cells were cultured at 37°C in a humidified 5% CO_2 incubator. Cell morphology was regularly monitored to control the absence of cross-contamination.

Plasmids

The construction and expression of pcDNA3.1(+)flag-apoptin was previously described (Zimmerman et al., 2012). In short, the DNA sequence encoding apoptin was synthesized by Baseclear (Leiden, The Netherlands) according to the apoptin sequence published by

Noteborn et al. (1991) and cloned into the mammalian expression vector pcDNA3.1(+) (Invitrogen, Breda, The Netherlands). The oligonucleotide fragment encoding the flag-tag (Invitrogen) was inserted to create the pcDNA3.1(+)flag-apoptin plasmid encoding apoptin fused with a flag-tag at its N-terminus. The phosphorylation-negative flag-apoptin(5Ala)106 mutant was created as described in Danen-van Oorschot et al. (2004). The flag-tagged apoptin(5Ala)106 mutant plasmid was constructed by replacing the five amino acid stretch from position 106-110 by alanines in the pcDNA3.1(+)flag-apoptin construct background.

Transfection method

Cells were transfected with plasmids by using Amaxa nucleofection (Lonza AG, Cologne, Germany) according to the adapted manufacturer's protocol. 10^6 cells were taken up in 120µL nucleofector buffer (F44: 140mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 5mM KCl, 10mM $MgCl_2$ and U2Os: 90mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 5mM KCl, 10mM $MgCl_2$, 20mM Hepes-KOH pH 7.2), mixed with DNA, transferred to a transfection cuvet (VWR, Amsterdam, The Netherlands) and transfected with program U-20 or X-01 for F44 or U2Os, respectively. After addition of medium, cells were seeded on 6cm dishes.

Inhibitor treatment

The proteasome inhibitors bortezomib and Ada-Ahx_3L_3VS (a kind gift from dr. Bobby Florea, Leiden University, Leiden, the Netherlands) were used to block proteasomal protein degradation (Florea et al., 2010). The proteasome inhibitors were added to cells 16 h after transfection. Increasing concentrations of inhibitors were mixed with medium before addition to cells. After 24 h, unless otherwise indicated, cells were lysed and subjected to Western blot analysis.

Immune fluorescence assay

Cells grown on glass coverslips were first washed with phosphate buffered saline (PBS) at indicated time points after transfection and subsequently fixed at room temperature for 10 min with 1% formaldehyde, 5 min with 100% methanol and 2 min with 80% acetone. After air-drying the slides were used for immunocytochemical staining. For antibody staining, the cells were first incubated with PBS plus 0.05% Tween 20 (PBS-Tween; Sigma Aldrich, Zwijndrecht, The Netherlands) plus 5% normal goat serum (NGS) (Sigma Aldrich) for 1 h.

Next, the cells were incubated with the first antibody (1:150 monoclonal mouse-anti-flag antibody, Sigma Aldrich) in PBS-Tween plus 5% NGS for 2 h, washed with PBS-Tween, and incubated with the second antibody (1:100 rhodamine-conjugated goat-anti-rabbit antibody, Sanbio, Uden, the Netherlands) in PBS-Tween plus 5% NGS for 1 h. After washing with PBS-Tween cells were incubated 15 min with Hoechst 33358 (2μg/mL). Stained sections were mounted using PolyMount Mounting Media (Tebu-Bio, Heerhugowaard, The Netherlands) and analyzed with a fluorescence microscope (Olympus, Zoeterwoude, The Netherlands) with the Cell P software version 2.8 (Olympus).

Western blot analysis

Protein samples were separated on 15% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes (Hybond-P, GE Healthcare, Hoevelaken, the Netherlands). The membranes were blocked with 5% non-fat milk in Tris-HCl-buffered saline containing 0.1% Tween-20 (Sigma Aldrich, Zwijndrecht, the Netherlands) for 1 hour at room temperature. Primary antibodies directed against flag (mouse monoclonal M2, 1:1000, Sigma Aldrich), p53 (mouse monoclonal DO-1, 1:1000, Santa Cruz Biotechnology, Heidelberg, Germany) or actin (goat polyclonal I-19, 1:1000, Santa Cruz Biotechnology) were incubated in blocking buffer for 1 hour at room temperature. Peroxidase-coupled secondary antibodies (1:10.000) were obtained from Jackson ImmunoResearch Laboratories, Suffolk, UK, and membranes were incubated for 1 h at room temperature. Detection was achieved with enhanced chemiluminescence. Films were quantified using Quantity One Analysis Software (Biorad, Veenendaal, the Netherlands).

Acknowledgments

This work was supported by a Grant from the Dutch Royal Society of Arts and Sciences (KNAW) and by the Leiden Institute of Chemistry.

Chapter 5

Development and application of an in vitro apoptin kinase assay

Henriëtte L Lanz, Bogdan I Florea, Mathieu HM Noteborn and Claude Backendorf

Analytical Biochemistry (2012) 421, 68-74

Abstract

Apoptin, a protein derived from chicken anemia virus (CAV), induces apoptosis selectively in human tumor cells as compared to normal cells. This activity depends on phosphorylation and relocation of apoptin to the nucleus of cancer cells. Here, we describe an in vitro kinase assay that allows the biochemical characterization of apoptin-kinase activity in tumor cells. The kinase phosphorylates apoptin in a strictly ATP-dependent fashion and in a broad salt range. The kinase activity is present constitutively in both cytoplasm and nucleus of various human tumor cells. Q-column chromatography showed that both cytoplasmic and nuclear fractions have identical fractionation characteristics, suggesting that the same kinase is present in both cellular compartments. Kinase activity derived from positive Q-column fractions bound to amylose-MBP-apoptin and could be eluted with ATP only in the presence of the co-factor Mg^{2+}. Apparently, unphosphorylated apoptin interacts with the kinase and is released only after phosphorylation has occurred, proving that our assay recognizes the genuine apoptin-kinase. This is further corroborated by the finding that apoptin is phosphorylated in vitro at position Thr-108 and Thr-107, in concert with earlier in vivo observations. Our assay excludes CDK2 and PKC-β, previously nominated by two separate studies as being the genuine apoptin kinase.

Introduction

The chicken anemia virus derived protein apoptin induces apoptosis in tumor cells in vitro and in vivo, but not in normal cells (Backendorf et al., 2008; Noteborn, 2009b). Besides apoptin, a steadily extending group of diverse proteins which display the same remarkable tumor-selective apoptosis activities has been identified (Bruno et al., 2009; Grimm & Noteborn, 2010; Pavet et al., 2011). These proteins are potential therapeutics for the treatment of cancers, as they appear to recognize tumor-related processes resulting in the induction of apoptosis (Noteborn, 2009b; Tavassoli et al., 2005; Maddika et al., 2006).

Apoptin harbors two apoptosis-inducing death-domains which function in an additive manner (Backendorf et al., 2008; Danen-van Oorschot et al., 2003). The N-terminal domain has not been extensively explored but contains a protein-multimerization domain, whereas the C-terminal domain contains a bipartite nuclear localization sequence (NLS, position 82-88 & 111-121), a nuclear export sequence (NES, position 97-105) and a phosphorylation site at Thr-108 (Backendorf et al., 2008). These C-terminal death-domain elements are collectively responsible for the major tumor-selective characteristics of apoptin.

Several studies have indeed established that the nuclear localization and phosphorylation of apoptin are linked to its tumor-selective cell death potential (Rohn et al., 2002; Lee et al., 2007). Poon et al. (2005) have proposed an elegant model for the differential localization of apoptin in normal and cancer cells: the NES is functional in normal cells but inactive in tumor cells, due to phosphorylation of T108, thus trapping the protein selectively in the nucleus of tumor cells. T108 is one of three consecutive threonine residues in the primary structure of apoptin (position 106-108) (Backendorf et al., 2008). Mutational analysis has indicated that a T108A mutation does only partially decrease nuclear translocation of apoptin in cancer cells (Poon et al., 2005; Rohn et al., 2005). For a complete inactivation of the C-terminal death domain of apoptin, mutation of both T108 and T107 is requested (Rohn et al., 2005), suggesting that phosphorylation at T107 can compensate for the loss of the T108 site. Nuclear import via the bipartite NLS is not inhibited by phosphorylation as was shown by using a T108E mutant that mimics constitutive phosphorylation of apoptin Rohn et al., 2002).

The link between phosphorylation of apoptin and its nuclear localization is also clearly displayed in transformation assays using SV40 large T antigen (LT) (Zhang et al., 2004). It was shown that transient co-transfection of apoptin with LT results in both nuclear

localization and phosphorylation of apoptin. However, forced nuclear targeting of native apoptin in normal cells is not sufficient to induce apoptosis, showing that phosphorylation is an essential step in the activation of apoptin (Danen-van Oorschot et al., 2003).

The identity of the tumor-selective kinase responsible for the phosphorylation of apoptin has not yet been univocally determined. As a first approach into this direction we have set-up an in vitro kinase assay to analyze the biochemical characteristics of apoptin-kinase. Our studies reveal that the kinase activity can be readily detected in various human tumor cell lines from different origins. This activity is strictly ATP dependent and can be measured in a broad salt range. It is present constitutively in cancer cells and needs no upstream activation step before isolation. It locates to both cytoplasm and nucleus, binds directly to apoptin and is released after the phosphorylation reaction has occurred.

Recently, some confusion has occurred in the field, as two different candidates for apoptin kinase have been proposed by two separate groups (Maddika et al., 2009; Jiang et al., 2010). Jiang and co-workers inferred that protein kinase C isoforms are responsible for the cell death activity of apoptin, whereas Maddika and colleagues argue that CDK2 is doing so. By using our apoptin kinase assay in combination with antibody depletion, we were able to convincingly show that none of these two kinases can be considered as being the genuine apoptin kinase.

Results

In vitro apoptin is phosphorylated in an ATP-, time-, and concentration-dependent manner

One of the main characteristics of apoptin is its tumor-selective phosphorylation at threonine 108 (Rohn et al., 2002). First, we have examined the kinetics of the apoptin kinase activity in an in vitro system consisting of MBP-apoptin and whole tumor cell lysate derived from human tumorigenic Jurkat cells with addition (or not) of ATP during different time intervals. Addition of ATP showed a time dependent increase in MBP-apoptin phosphorylation by a whole cell lysate as detected by means of Western-blot analysis using the phospho-specific 108P antibody (Figure 5.1A). Variation of the amount of cell lysate shows a kinetic linear range of the kinase activity up till 10μg of the Jurkat cell lysate (Figure 5.1B). In the following experiments we stayed in this linear range in order to quantify and compare the amount of kinase activity in various samples.

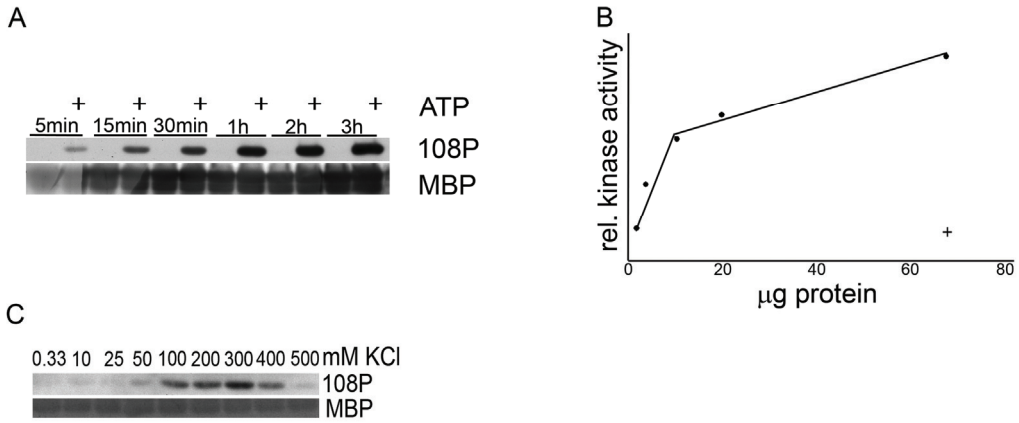


Figure 5.1: ATP and salt-dependent in vitro apoptin kinase assay. Western blot analysis of in vitro kinase assay samples is represented. (A) Jurkat whole cell lysate was incubated with MBP-apoptin in the absence or presence of ATP for different amounts of time. Samples were analyzed with antibody against apoptin phosphorylated on threonine 108 (108P) and antibody against MBP to ensure equal assay conditions. (B) Increasing amounts of Jurkat whole cell lysate were incubated for 30 min with MBP-apoptin and ATP. Phosphorylation was analyzed with 108P antibody and signal intensity was plotted against the amount of protein. (+) indicates background signal when no ATP is used in the assay. The results of one representative experiment are shown. (C) Salt dependency of the in vitro kinase assay: Jurkat whole cell lysate was incubated with MBP-apoptin and ATP and the indicated amounts of KCl. Samples were analyzed with antibody detecting apoptin phosphorylated on threonine 108 (108P) and antibody against MBP detecting total MBP-apoptin in the assay.

Tumor-selective kinase activity of apoptin is salt inducible

It is well known that enzyme activity and structure are influenced by the salt concentration of the buffer (Branden & Tooze, 1991). Therefore, we examined whether increasing KCl concentrations had an effect on the kinase activity in Jurkat whole cell lysates. Western-blot analysis showed that the tumor-selective apoptin kinase activity has a broad salt-dependence ranging from 50 to 400 mM KCl (Figure 5 1C). The fact that the kinase is active within such a broad salt range enables the use of ion exchange chromatography to enrich the kinase activity without inhibiting its activity. A similar activity profile was seen following addition of NaCl (data not shown).

Apoptin kinase activity can be detected in both the cytoplasm and the nucleus

Because nuclear location of apoptin is linked to its phosphorylation (see above) it was interesting to determine the cellular location of the apoptin kinase activity in tumor cells. Therefore we decided to separate cytoplasmic and nuclear fractions from the human tumor cell lines Jurkat, MCF-7, HeLa, U2OS and Saos-2. The cytoplasmic marker MEK-2 and the nuclear marker C23 were used as indicators for assessing the purity of the cytoplasmic and nuclear fractions.

Figure 5.2 clearly shows that the various tumor cytoplasmic and nuclear fractions both can phosphorylate apoptin. The ratio of the cytoplasmic and nuclear apoptin-related kinase activity varies among the different tumor cell types. MCF-7 cells harbor a dominant nuclear activity, whereas Jurkat cells have a higher cytoplasmic activity. The other three analyzed tumor cell types have a more equal cytoplasmic-nuclear distribution of the endogenous tumor-selective kinase activity.

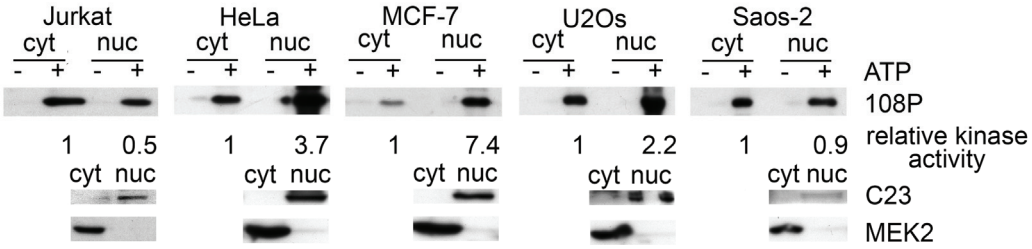


Figure 5.2: Cellular localization of the apoptin kinase activity. Different tumor cells were lysed and both soluble nuclear and cytoplasmic fractions were isolated and tested for kinase activity. Phosphorylation of apoptin was detected by Western blot with the phosho-specific 108P antibody. 108P signal was quantified and the relative kinase activity in cytoplasm or nucleus is indicated (the cytoplasmic activity was set at 1). The absence of cross-contamination between cytoplasmic and nuclear fractions was determined by using MEK2 (cytoplasmic marker) and C23 (nuclear marker) specific antibodies.

Apoptin kinase activity is a unique kinase

To determine whether the nuclear and cytoplasmic fractions contain the same kinase activity we examined Q column fractionation profiles. In Figure 5.3 a fractionation pattern of a Jurkat cytoplasmic lysate is shown. The activity of all fractions was tested in the in vitro kinase assay after samples were diluted at least 5-fold, keeping all the protein-containing fractions within the active salt-concentration range determined above. The kinase activity eluted around 350 mM KCl as a single peak with a parabolic shape (R^2 = 0.994 after polynomial trend-line fitting), suggesting that it represents the activity of a unique kinase (grey curve in Figure 5.3). Fractionation of nuclear extracts of Jurkat cells resulted in an identical kinase activity elution pattern (data not shown), indicating that the same kinase activity is present in both nucleus and cytoplasm.

Apoptin associates directly with the endogenous kinase activity under in vitro conditions

Whole cell lysate (Figure 5.4A) or pooled active cytoplasmic Q-column fractions (Figure 5.4B) derived from Jurkat tumor cells were incubated with MBP-apoptin and fractionated on

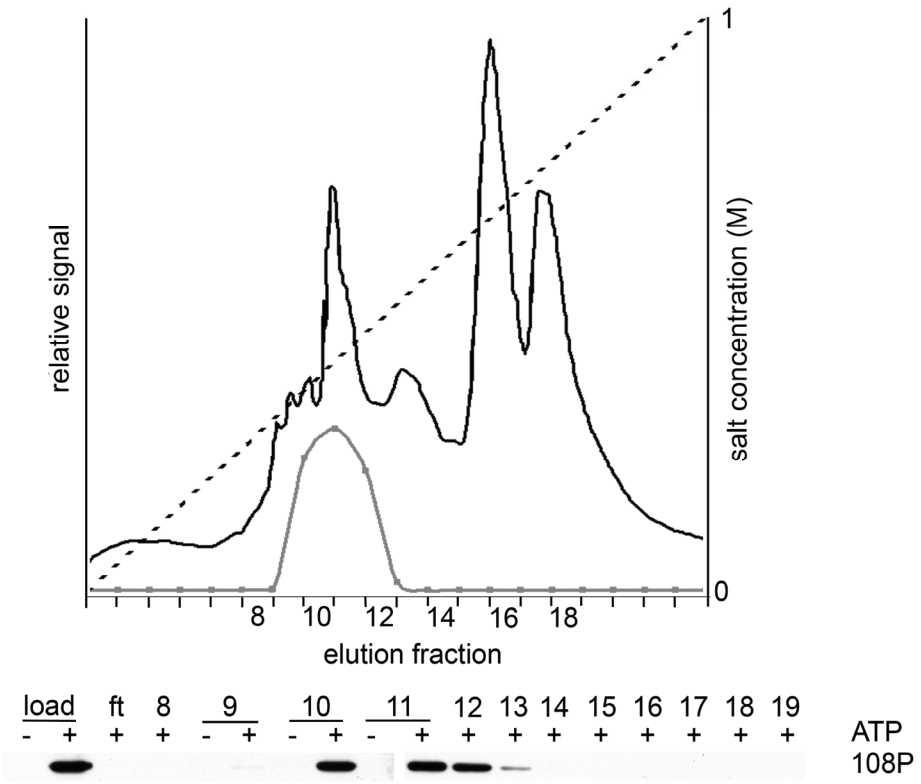


Figure 5.3: Q column purification of apoptin kinase activity. Elution profile of a cytoplasmic fraction from Jurkat cells (UV absorption 280nm; black curve): proteins were loaded on a Q column and fractionated by elution with a 0-1M KCl salt gradient (dotted line). The presence of apoptin kinase in each fraction was checked with the in vitro kinase assay and analyzed on Western blot with the phospho-specific antibody (108P). The activity signal was quantified and the relative signal was plotted (grey line). Fitting a polynomial trend-line gave an R-squared value of 0.994.

an amylose affinity-column. Elution was performed with a buffer containing ATP. In Figure 5.4A no activity was detected in the flow through (ft) and early wash-out (wo) fractions whereas eluted fractions showed clear apoptin kinase activity. In Figure 5.4B more activity was found in the (ft) and (wo) fractions, probably due to overload, but again the kinase was clearly eluted by ATP. Importantly, the kinase could not be eluted with ATP when the cofactor Mg^{2+} was left out of the elution buffer, but it was still eluted with maltose (mw) (Figure 5.4C). Apparently, the interaction between apoptin and the kinase is only abolished after the phosphorylation event took place. To ensure that the phosphorylation observed was the result of the in vitro kinase activity and not due to MBP-apoptin accidentally released from the column, representative fractions were also tested without addition of ATP and MBP-apoptin and shown to be negative (Figure 5.4A/B). Finally, MBP-apoptin could be eluted from the column with maltose (Figure 5.4A/B, mw fractions); in Figure 5.4B these

elution fractions contained phosphorylated MBP-apoptin released from the column (detected in the – samples) and some residual kinase activity, which is identified due to the higher activity in the + samples as compared to the – samples of the mw fractions.

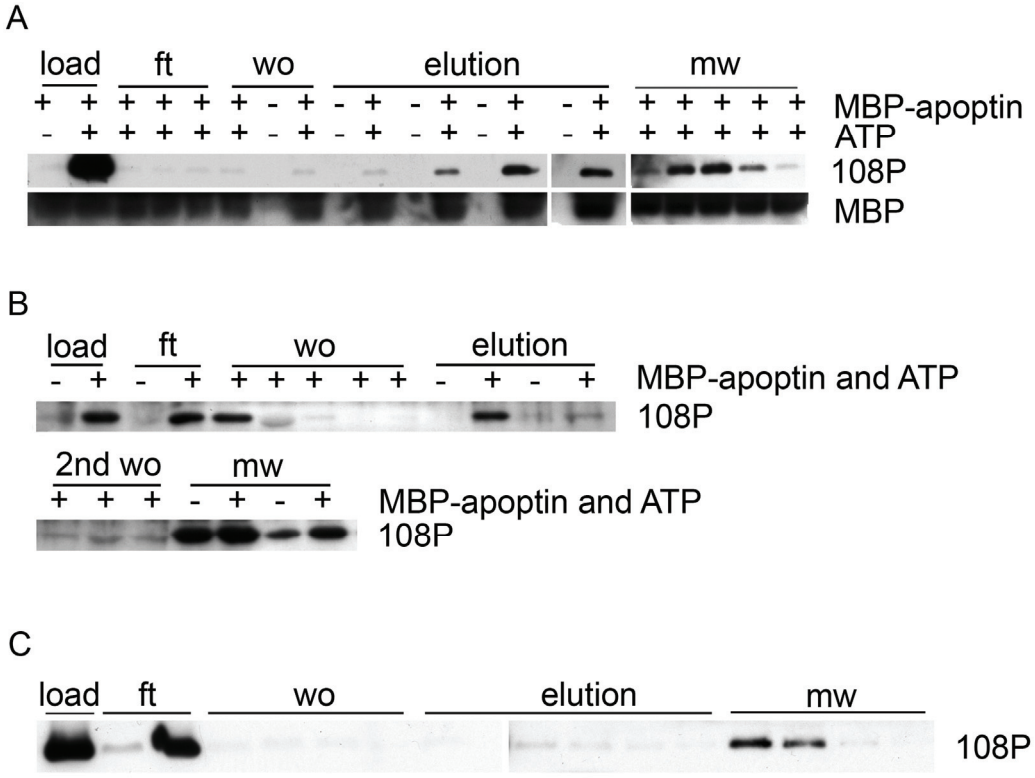


Figure 5.4: Kinase-apoptin interaction/dissociation is phosphorylation-dependent. Jurkat whole cell lysate (A) or pooled active cytoplasmic fractions (B) were incubated with MBP-apoptin and loaded on an amylose column. Following wash-out (wo), proteins were eluted with elution buffer containing ATP and Mg^{2+} (elution). Fractions (+) were tested in vitro for kinase activity and the presence of phosphorylated apoptin was checked by Western blot with the phospho-specific 108P antibody. Selected fractions (-) were tested without addition of ATP and MBP-apoptin. On-column-phosphorylated MBP-apoptin was eluted with maltose (mw). No kinase activity eluted from the column when an elution buffer without Mg^{2+} was used (C). Flow-through fractions are marked (ft).

Apoptin is phosphorylated on threonine 108 as well as on threonine 107

We have mentioned above that mutating Thr-108 to an alanine does not completely abolish the apoptosis induction by the C-terminal death-domain of apoptin, suggesting that phosphorylation of the adjacent threonines at positions Thr-106 and Thr-107 might also occur (Rohn et al., 2005). To confirm apoptin phosphorylation on Thr-108 and potentially on the neighboring threonines, in vitro phosphorylated MBP-apoptin was analyzed by mass

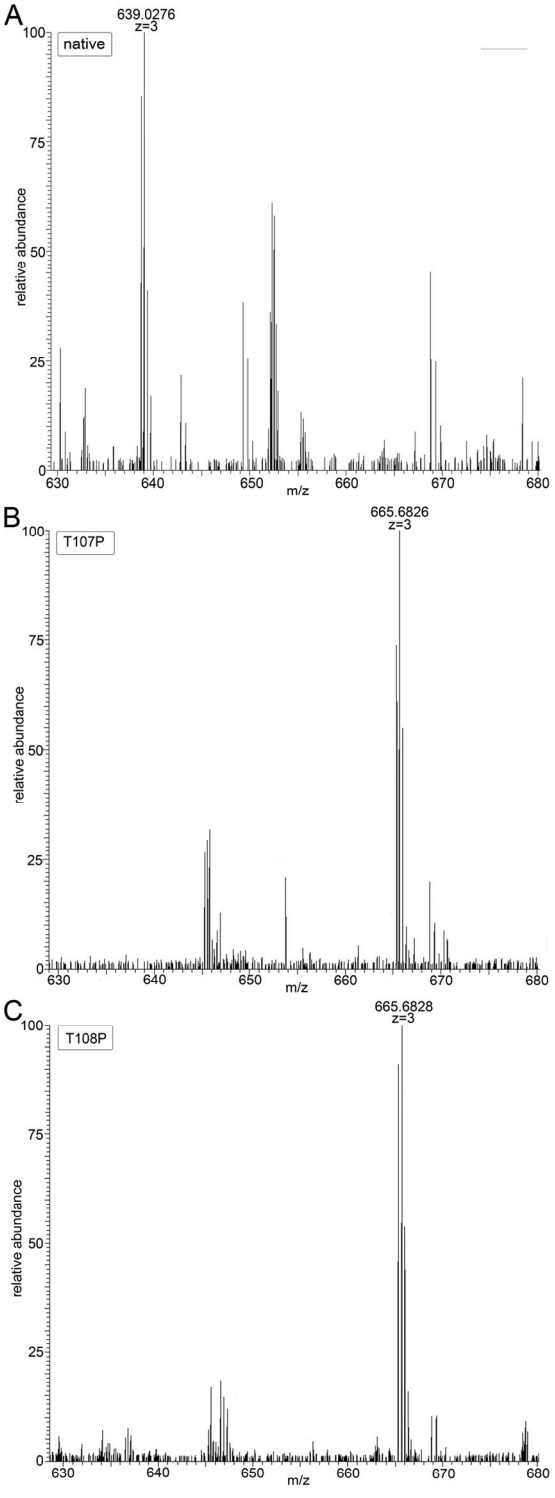


Figure 5.5: Mass spectrometry analysis of phosphorylated MBP-apoptin. MBP-apoptin was phosphorylated in vitro and purified on an amylose column. Phosphorylated protein was digested with trypsin and subjected to mass spectrometry analysis. Detail of three full mass spectrometry scans showing the apoptin peptide VSELKESLITTTPSRPR containing the triple T phosphorylation site eluting at different time points: (A) native peptide, (B) phosphorylation on threonine 107 or on (C) threonine 108. The related mass increments of the y ion in the MS^2 of the fragmented peptide are shown in table 1.

Table 5.1: mass increments of the y ions in the MS^2 of the fragmented peptide
Apoptin 97-113 peptide was selected during the full mass spectrometry scan and further fragmented in the ion trap. Mass increase of the y ion fragments is shown. Bold indicates presence of HPO_3. A: native peptide; B: peptide phosphorylated on T107; C: peptide phosphorylated on T108.

		A	**B**	**C**
Pos	Seq	y	y	y
97	V			
98	S	87.03	87.03	87.03
99	E	129.04	129.04	129.04
100	L	113.08	113.08	113.08
101	K	128.10	128.10	128.10
102	E	129.04	129.04	129.04
103	S	87.03	87.03	87.03
104	L	113.08	113.08	113.08
105	I	113.08	113.08	113.08
106	T	101.05	101.05	101.05
107	T	101.05	**181.01**	101.05
108	T	101.05	101.05	**181.01**
109	P	97.05	97.05	97.05
110	S	87.03	87.03	87.03
111	R	156.10	156.10	156.10
112	P	97.05	97.05	97.057
113	R	175.12	175.12	175.12

spectrometry. Whole cell lysate of human tumor Saos-2 cells was used in the in vitro kinase assay and MBP-apoptin was purified on an amylose column. After tryptic digestion phosphorylated peptides were enriched on a TiO_2 column. The modification on Thr-108 was confirmed (Figure 5.5C and table 1, column C). Additionally, a peptide with an identical m/z value showed a different fragmentation pattern displaying the presence of a phosphate group on Thr-107 (Figure 5.5B and table 1, column B). We did not obtain evidence that Thr-106 was also significantly phosphorylated (data not shown), whereas non-phosphorylated MBP-apoptin peptides were readily observed (Figure 5.5A and table 1, column A).

Exclusion of CDK2 and PKC as genuine apoptin kinases

Recently two separate research groups have respectively proposed CDK2 and PKC-β as the genuine apoptin kinase (Maddika et al., 2009; Jiang et al., 2010). As it is rather unlikely that these kinases which belong to completely different classes of human kinases (Manning et al., 2002) would both be involved in apoptin phosphorylation we have used our in vitro system to analyze the implication of each of these kinases in apoptin phosphorylation. Saos-2 whole cell lysates were depleted either specifically of CDK2 or of all PKC isoforms by antibody pull down. The results in Figure 5.6 clearly show that both for CDK2 (panel A) and PKC (panel B) the kinases could be depleted from the extract but this did not result in a lower apoptin-kinase activity. Apparently neither CDK2 nor PKC-β (nor another member of the PKC kinase family) can be considered as being the genuine apoptin kinase.

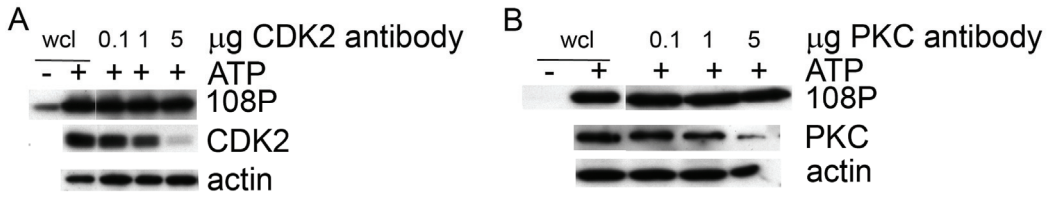


Figure 5.6: Western blot analysis of in vitro kinase assay samples after kinase depletion. Either CDK2 (panel A) or PKC (panel B) were pulled down with increasing amounts of antibody from Saos-2 whole cell lysate (WCL). Cleared lysates were tested for apoptin-kinase activity and the presence of the specific kinase. Actin was used as a loading control.

Discussion

We have set-up a robust kinase assay and identified various biochemical properties of the tumor-selective apoptin kinase activity. Column-fractionation of tumor lysates revealed the existence of a constitutive endogenous kinase activity in tumor cells, which does not need

additional activation steps before isolation. In all analyzed tumor cells the kinase activity was present in both nucleus and cytoplasm, although at various ratios.

The measured kinase activity is strictly dependent on ATP addition and is influenced by the salt concentration of the buffer. The kinase, present in column-chromatography fractions, binds to (MBP)-apoptin and is released upon ATP treatment. Absence of the Mg^{2+} co-factor prevents kinase/apoptin dissociation, illustrating that the kinase releases apoptin only after phosphorylation has occurred. Rohn et al. (2005) have previously reported that preventing phosphorylation at Thr-108 hardly affects apoptosis induction. They showed that both Thr-107 and Thr-108 need to be mutated in order to abolish the apoptotic activity of apoptin (Rohn et al., 2005). The data presented here now prove that the kinase activity that is detected in our in vitro assay has indeed the ability to phosphorylate apoptin on Thr-108 and Thr-107. Both the binding experiment and the observed phosphorylation pattern prove that we are dealing with the genuine apoptin kinase.

Recently, two separate groups proposed two different kinases, CDK2 and PKC-β as candidates for apoptin kinase (Maddika et al., 2009; Jiang et al., 2010). However, depletion of either CDK2 or PKC from whole cell lysates did not affect the phosphorylation of MBP-apoptin in our in vitro kinase assay, casting serious doubt on the role of these two kinases in apoptin phosphorylation. Additionally, other findings in this paper also do not support the models proposed by these two groups. The study of Maddika et al. (2009) predicted an active upstream role of apoptin in the phosphorylation and activation process. In this model, apoptin binds to Akt resulting in transfer of both apoptin and Akt to the nucleus and association with CDK2. CDK2 is then assumed to be activated by Akt and to phosphorylate on its turn apoptin. Our findings contrast with this model. We have demonstrated that a constitutive kinase activity can be isolated from cancer cells that have never been in contact with apoptin. Apart from this feature, the kinase activity is not limited to the nucleus, but can also be extracted from the cytoplasm of tumor cells. This is in line with our previous finding that during SV40 large T transformation of human fibroblasts phosphorylated apoptin can first be observed in the cytoplasm before it is seen in the nucleus at later stages (Zhang et al., 2004). Jiang et al. (2010) showed that phosphorylated apoptin is present in the cytoplasmic membrane of transfected multiple myeloma (MM1.R) cells. They indentified protein kinase C isoform beta (PKC-β) as the kinase that phosphorylates apoptin in MM1.R cells. PKC-β resides in the cytoplasm of tumor cells. Upon transfection with apoptin a small

portion of the kinase appears to transfer to the nucleus. Again this nuclear translocation of the kinase activity requires the cells to have been in contact with apoptin. Both Jiang and Maddika used GFP-tagged apoptin, a construct that has been shown to behave in several aspects differently to native apoptin (Lee et al., 2007; Rohn et al., 2005). For instance, variations in the cellular localization in normal cells have been recorded and should caution against a possible deviant behavior of the recombinant protein in human tumor cells in vivo (Kooistra, 2007). All experiments presented here have been performed in vitro. As a conclusion, by taking into account the data described here, both CDK2 and PKC-β should no longer be regarded as being the genuine apoptin kinase.

Poon et al. (Poon et al., 2005) proposed that phosphorylation of T108 blocks its nuclear export sequence (NES) trapping the protein selectively in the nucleus of tumor cells. Threonine 107 localizes even closer to the NES and as such phosphorylation of this position is equally likely to counteract the nuclear export of apoptin. Nuclear import is not blocked by phosphorylation in the triple T region as was shown by the nuclear import of the T108E mutant in normal cells (Rohn et al., 2002) and by the localization of phosphorylated apoptin in SV40 large T injected fibroblasts first in the cytoplasm and later in the nucleus (Zhang et al., 2004). This shows that the original cellular localization of apoptin phosphorylation has no influence on its later cellular distribution and is in line with our finding that apoptin can be phosphorylated by a kinase activity isolated from different cellular compartments of tumor cells.

The biochemical characterization of the kinase phosphorylating apoptin points to an existing constitutive endogenous kinase activity in tumor cells, which can be present in both the nuclear and cytoplasmic compartments. The endogenous kinase has a broad salt-dependence range and the ability to phosphorylate apoptin on both T107 and T108. These biochemical characteristics need to be taken into account in order to unequivocally identify the genuine kinase responsible for activation of the tumor-selective apoptosis-inducing protein apoptin.

Materials and Methods

Cell culture

All cells were cultured at 37°C in a humidified 5% CO_2 incubator. Jurkat cells were grown in RPMI1640 medium, Saos-2, U2Os, MCF-7 and HeLa cells were cultured in Dulbecco's

modified Eagle's medium. Both culture media were supplemented with 10% fetal calf serum, 100μg/mL penicillin, 100μg/mL streptomycin and 2mM glutaMAX.

Recombinant MBP-apoptin protein

The cloning, expression and biophysical properties of soluble MBP-apoptin have previously been described (Leliveld et al., 2003b). Briefly, the construct pMalTBVP3 encoding an N-terminally MBP-tagged recombinant apoptin protein (MBP-apoptin, 55.8kDa) was expressed in *Escherichia coli*. The MBP gene and the apoptin gene are separated by a flexible hinge region encoding 10 Asn residues and a thrombin cleavage site. The cleared lysate of 250mL bacterial culture was passed over an amylose column (GE Healthcare, Hoevelaken, The Netherlands), equilibrated in 20mM Tris-HCl buffer, pH 7.4, 200mM NaCl, 1mM EDTA and 1mM DTT at 4°C. The column was washed with equilibration buffer and then eluted with 20mM Tris-HCl buffer, pH 7.4, 200mM NaCl, 1mM EDTA, 1mM DTT and 10mM maltose. Eluate was loaded on a Highload 16/60 Superdex 200 gel filtration column (GE Healthcare) equilibrated in PBS, 1mM EDTA. Protein was eluted in PBS, 1mM EDTA. PMSF was directly added to the eluate to a final concentration of 0.5mM to prevent degradation by traces of *E. coli* proteases. Appropriate fractions were pooled and concentrated on an Amicon Ultra centrifugal filter unit (Millipore, Amsterdam, The Netherlands) with a 10,000Da cut-off. Protein solutions were never subjected to freeze/thawing. Stocks were kept at -20°C and a working solution of 4mg/mL at 4°C.

In vitro kinase assay

Cell cultures were suspended in in vitro kinase (IVK) buffer (10mM Tris-HCl pH 7, 10mM $MgCl_2$, 10mM NaF, 100mM KCl, 2mM DTT) containing protease inhibitor cocktail (complete Mini, Roche) and phosphatase inhibitor cocktail (PhosSTOP, Roche, Woerden, The Netherlands) and lysed by freeze-thawing. Protein concentration was determined spectrophotometrically using the A_{230}/A_{260} absorption (Kalb & Bernlohr; 1977). Equal amounts of whole cell lysate (WCL) or equal volumes of column fractions were incubated in the presence of 50μM ATP and 4μg MBP-apoptin at 30°C for 30min unless otherwise indicated. Reactions were stopped by addition of EDTA. Control reactions were performed without addition of ATP. Phosphorylated proteins were subjected to Western blot analysis.

Nuclear-cytoplasmic fractionation

Cellular pellets were suspended in IVK buffer and incubated on ice. After addition of NP40 (final concentration 0.6%) cells were vortexed and centrifuged at 16,100 g for 30 s. Supernatant was collected as a cytoplasmic extract. The pellet was washed, resuspended in IVK buffer containing 400mM NaCl, supplemented with protease and phosphatase inhibitors, and incubated for 15 min at 4°C with regular vortexing. The supernatant, obtained after centrifugation at 16,100g for 15 min, was used as nuclear extract. Equal relative amounts of cytosolic and nuclear extract were used for the in vitro kinase assay and subjected to Western blot analysis.

Western blot analysis

Protein samples were separated on 12.5% SDS-page gels and transferred to polyvinylidene fluoride membranes (Hybond-P; GE Healthcare). The membranes were blocked with 5% non-fat milk in Tris-HCl-buffered saline containing 0.1% Tween-20 for 1 hour at room temperature. Primary antibodies directed against MBP (mouse monoclonal, 1:2000, Zymed, Uden, The Netherlands), MEK-2 (rabbit polyclonal, 1:2000, N-20, Santa Cruz Biotechnology, Heidelberg, FRG), C23 (mouse monoclonal, 1:2000, MS-3, Santa Cruz Biotechnology) were incubated in blocking buffer for 1 hour at room temperature. Membranes treated with primary antibody directed against phosphorylated apoptin (108P) were blocked with 3% BSA in Tris-HCl buffered saline containing 0.1% Tween-20 for 1.5 h at room temperature. Antibody against phosphorylated apoptin was custom made by Eurogentec, Maastricht, The Netherlands and produced as previously described (Danen-van Oorschot et al., 2003). Peroxidase-coupled secondary antibodies (1:10,000) were from Jackson ImmunoResearch Laboratories, Suffolk, UK, and membranes were incubated for 1 h at room temperature. Detection was achieved with enhanced chemiluminescence. Films were quantified using Quantity One Analysis Software (Biorad, Veenendaal, The Netherlands).

Kinase depletion

Cells were freeze-thawed in IVK buffer. WCL was cleared by centrifugation at 16,100g for 5 min. Increasing amounts of an antibody recognizing all PKC isoforms (mouse monoclonal, A-9, Santa Cruz Biotechnology) or a CDK2-specific antibody (rabbit polyclonal, SC-163,

Santa Cruz Biotechnology) were added to the WCL and incubated for 1 hour at 4°C while tumbling. After addition of protein G-conjugated beads (GE Healthcare) lysates were incubated for another hour at 4°C while tumbling. Kinase depleted lysate was obtained after centrifugation at 12,000g for 30 sec by collecting the supernatant. Supernatants were tested in the in vitro kinase assay and subjected to Western blot analysis.

Column purifications

Cells were freeze-thawed in IVK buffer. Cellular extracts were cleared by centrifugation at 16,100g for 5 min and loaded on a resource Q column according to the manufacturer's protocol (GE Healthcare). Proteins were eluted with a KCl gradient. Fractions were diluted at least 5 fold before monitoring kinase activity in IVK buffer in our in-vitro kinase assay. Positive fractions were pooled, diluted in 20mM Tris-HCl pH7.5, 1mM DTT, 100mM KCl and incubated with MBP-apoptin. The sample was loaded on an amylose column. Elution was done with 20mM Tris-HCl pH7.5, 1mM DTT, 100mM KCl, 5mM ATP, 5mM $MgCl_2$.

Mass spectroscopy

In-vitro phosphorylated MBP-apoptin was purified by standard amylose column purification (see above) and precipitated with trichloroacetic acid. Pellets were resuspended in 8M urea, 0.4M ammonium bicarbonate. Prior to digestion, proteins were reduced with 7.5mM DTT and alkylated with 14mM iodoacetamide. The mixture was diluted 3-fold with H_2O before trypsin solution was added to a final concentration of 3μg/mL and incubated overnight at 37°C. Peptides were collected and desalted on stage tips. The TiO_2 chromatography was performed using a triple stage precolumn (Nanoseparations, Nieuwkoop, The Netherlands). Both TiO_2 eluate and flow-through fractions were chromatographically resolved using a 10-55% linear acetonitrile gradient. Peptides were analyzed on a LTQ-Orbitrap mass spectrometer (Thermo-Fischer, Breda, The Netherlands). Full-scan MS spectra were acquired in the Orbitrap and the three most intense ions were selected for MS/MS fragmentation in the linear ion trap. Sequence and phospho-site identification was performed using Mascot Daemon (Matrix Science Inc, London, United Kingdom).

Acknowledgements

Thanks to Dr. Daniel de Geus and Rhyenne Zimmerman for helpful suggestions and experimental set-ups. Prof. Dr. J. Brouwer is acknowledged for constant interest and support. Research was supported by a grant from the Dutch Royal Society of Arts and Sciences (KNAW) and by the Leiden Institute of Chemistry.

Chapter 6

Mitotic catastrophe triggered in human cancer cells by the viral protein apoptin

Henriëtte L. Lanz, Rhyenne M.E. Zimmerman, Jacob Brouwer, Mathieu H.M. Noteborn and Claude Backendorf

Manuscript submitted

Abstract

Mitotic catastrophe is an oncosuppressive mechanism that senses mitotic failure leading to cell death or senescence. As such it protects against aneuploidy and genetic instability and its induction in cancer cells by exogenous agents is currently seen as a promising therapeutic endpoint. Apoptin, a small protein from Chicken Anemia Virus, is known for its ability to selectively induce cell death in human tumor cells. Here we show that apoptin triggers p53-independent abnormal spindle formation in osteosarcoma cells. Approximately 50% of apoptin-positive cells displayed non-bipolar spindles, a ten-fold increase as compared to control cells. Besides, tumor cells expressing apoptin are greatly limited in their progress through anaphase and telophase and a significant drop in mitotic cells past the meta-to-anaphase transition is observed. Time-lapse microscopy showed that mitotic osteosarcoma cells expressing apoptin displayed aberrant mitotic figures, had a prolonged cycling time during mitosis and eventually underwent cell death. We infer that apoptin triggers apoptosis in dividing human tumor cells through induction of mitotic catastrophe.

Mitotic catastrophe is an oncosuppressive mechanism emerging as a promising endpoint in cancer treatment (Galluzzi et al., 2012; Vitale et al., 2011). Originally used as a denomination for 'death during mitosis', mitotic catastrophe reached a tentative consensus definition as a mechanism that senses mitotic failure resulting in an irreversible fate, such as apoptosis, necrosis or senescence. The aberrant mitosis can be caused either by premature or inappropriate entry into mitosis or a failure of related checkpoints in combination with cellular damage (Vitale et al., 2011; Castedo et al., 2004). Mitotic catastrophe is considered an oncosuppressive pathway due to its association with absent or weakened checkpoints (Roninson et al., 2001). It has been argued that the evasion of mitotic catastrophe constitutes one of the gateways to cancer development (Vitale et al., 2011).

Apoptin is a small avian-virus derived protein capable of selectively inducing apoptosis in human tumor cells while having no detrimental effect on healthy normal cells (Backendorf et al., 2008; Argiris et al., 2011). In normal cells it localizes to the cytoplasm where it is efficiently degraded by the proteasome (Lanz et al., 2012b). On the contrary, in transformed cells apoptin is phosphorylated on threonine 108 and localizes to the nucleus (Rohn et al., 2002). Until now apoptin has been shown to induce apoptosis in over 70 human cancer cell lines (Backendorf et al., 2008) and in preclinical mouse model studies apoptin acted as an efficient and safe anticancer therapeutic (Sun et al., 2009; Jin et al., 2011).

Recently, we showed that the tumor-selective behavior of apoptin can be triggered in normal human fibroblasts by down-regulation of the B56γ or B56δ subunits of the tumor-suppressor protein phosphatase 2A (PP2A) (Zimmerman et al., 2012). The B56-PP2A complex is enriched at the centromeres and centrosomes of mitotic cells and responsible for the formation of stable attachments between kinetochores and microtubules (Foley et al., 2011). Inactivation of PP2A by viral oncoproteins contributes to cell transformation (Westermarck & Hahn, 2008) possibly by increasing the frequency of chromosomal instability (Foley et al., 2011). These findings raised the question whether apoptin can sense chromosomal instability and perturb spindle formation during mitosis.

To this end we examined the impact of ectopically expressed apoptin on the mitotic process in human osteosarcoma cells (either Saos-2: p53-null cells (Masuda et al., 1987) or U2Os: p53-wild type cells (Diller et al., 1990). Cells were transiently transfected with plasmid encoding either flag-apoptin or flag-SPRR4, a skin cornification protein (Cabral et al., 2001) that was used as a control and does not induce apoptosis in human cells (see Supplementary

Figure S6.1). Both apoptin-positive and apoptin-negative mitotic cells within the same transfected dish were scored for normal/abnormal bipolar spindle formation. The data demonstrate that apoptin expression caused an increase in abnormal spindle formation in mitotic cells which was not observed in non-transfected cells in the same dish or in SPRR4-transfected cells (Figure 6.1). No difference was observed between Saos-2 (Figure 6.1A-G) and U2Os cells (Figure 6.1H-N). Apparently, the effect exerted by apoptin during mitosis in cancer cells is not dependent on functional p53 while the presence of non-bipolar spindles hints to mitotic catastrophe as the process which is induced (de Bruin & Medema, 2008).

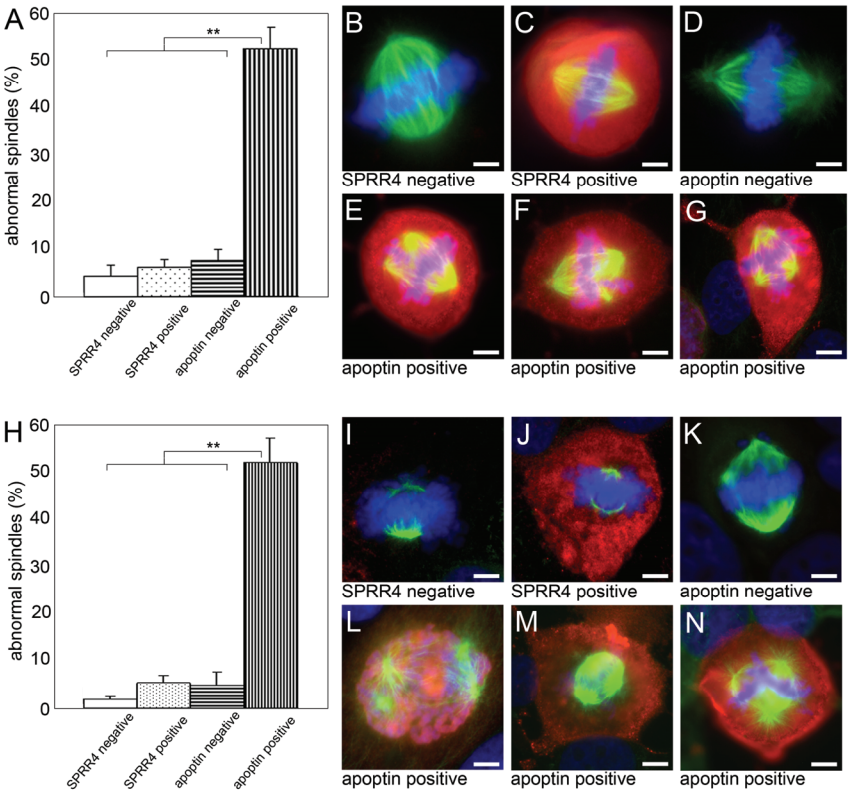


Figure 6.1: Abnormal spindle formation in apoptin-expressing Saos-2 or U2Os osteosarcoma cells. Cells were transiently transfected with plasmid encoding flag-apoptin or flag- SPRR4 and fixed 72 h or 48 h post transfection (Saos-2 and U2Os respectively). Flag-tagged apoptin or flag-tagged SPRR4 were stained with a flag-specific antibody conjugated to rhodamine (red), endogenous tubulin with a FITC-labeled tubulin-specific antibody (green) and DNA with Hoechst (blue). Both transfected and non-transfected cells (in the same dish) were analyzed for spindle formation. Bipolar spindles were scored as normal, all others as abnormal. Data obtained from Saos2 cells are represented in panels A-G and those from U2Os cells in panels H-N. Panels A and H represent the percentages of abnormal spindle formation from 3 separate experiments for each cell line (represented as mean ± SD). Per experiment 100 mitotic cells which were positive or negative for flag-apoptin or flag-SPRR4 were scored. Apoptin-positive mitotic cells contained significantly more abnormal spindles ($p<0.01$) than the three control groups (respectively $p = 0.004$, 0.005 and 0.006 for Saos2 cells and $p = 0.002$, 0.008 and 0.005 for U2Os cells). Representative pictures of normal bipolar spindles in the three control groups: (B, I) non-transfected cells in the SPRR4 experiment; (C, J) SPRR4 expressing cells; (D, K) non-transfected cells in the apoptin experiment. Panels E-G and L-N show representative pictures of abnormal spindles in apoptin-positive cells. Scale bar: 5μm.

Half of the apoptin-positive mitotic cells showed normal spindle formation during metaphase in both Saos-2 and U2Os cells (Figure 6.1A and 1H). To examine whether these bipolar-spindle-positive cells progressed through mitosis in a regular fashion, dividing cells were scored according to their respective phases in mitosis: prophase, metaphase, anaphase, or telophase. Non-transfected cells displayed all four mitotic phases (Figure 6.2A). Regular mitotic figures in apoptin-positive cells were, however, mainly restricted to prophase (Figure 6.2B) and metaphase (Figure 6.2C), while in SPRR4-transfected control cells anaphase (Figure 6.2D) and telophase (Figure 6.2E) cells could readily be found. For quantification purposes mitotic cells were classified in groups of prophase/metaphase or anaphase/telophase. In Saos-2 cells apoptin expression resulted in a six fold drop in anaphase/telophase cells (Figure 6.2F). In U2Os cells apoptin induced a three times decrease in anaphase/telophase mitotic cells (Figure 6.2G). Apparently, even in those cases where spindles appear normal during the metaphase in apoptin-expressing cells, cells are not able to easily progress past the spindle assembly checkpoint into anaphase.

During our analysis apoptotic cells were observed in which tubulin staining indicated that apoptin-induced cell death had occurred during the mitotic cell cycle stage. These include late apoptotic cells with clear spindle remnants (Figure 6.2H), an apoptin-positive mitotic cell with three spindles that has undergone apoptosis during telophase (Figure 6.2I) and finally an apoptin-positive cell that during earlier steps in mitosis would have been classified as normal, but now shows apoptotic blebbing and DNA fragmentation during cytokinesis (Figure 6.2J).

In order to firmly establish that mitosis is disturbed (at various stages) in apoptin-positive cells we performed live-cell-imaging by using fluorescent time-lapse videomicroscopy (Rello-Varona et al., 2010) of U2Os (Figure 6.3A/B/C) or Saos-2 cells (Figure 6.3D) transiently transfected with plasmid encoding mCherry-tagged apoptin (red fluorescence) and GFP-tagged tubulin (green fluorescence). Cells that were only positive for GFP-tubulin divided with similar kinetics as untransfected cells, in approximately 1 hour (Figure 6.3A). An apoptin-expressing cell with three spindles eventually generates daughter cells undergoing apoptosis during cytokinesis (Figure 6.3B). Note that the different phases are prolonged as compared to the cells only expressing GFP-tubulin (compare panel A with panel B). For example prophase lasts 4.5 hours in the apoptin-positive cell compared to 12 minutes in the control, and the entire mitosis took over 11 hours instead of 1 hour. Next an

apoptin-positive cell starts to undergo regular mitosis with the formation of two centrosomes and two spindles (Figure 6.3C). Notice that prophase is delayed to such an extent that an untransfected cell (white circle) completes an entire round of mitosis during the same time. Division is halted during metaphase and after a block of several hours the cell

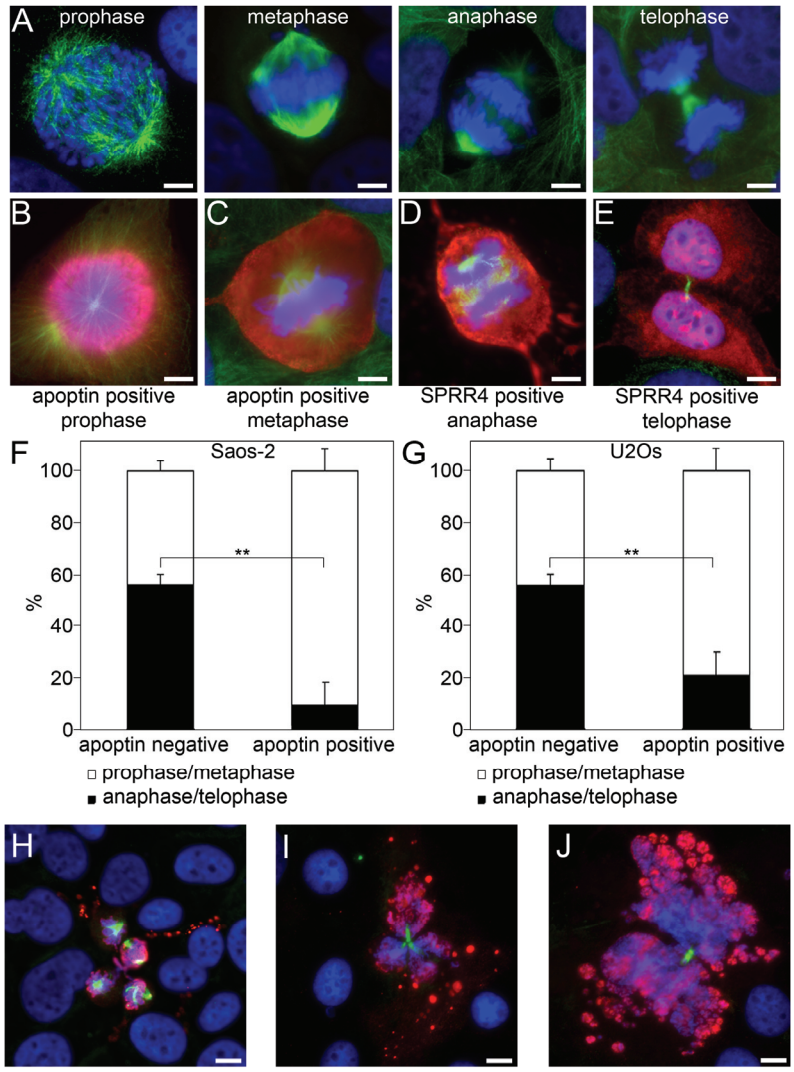


Figure 2 Apoptin inhibits mitotic cells from progressing through anaphase and telophase. U2Os and Saos-2 cells were transiently transfected with plasmid encoding flag-SPRR4 or flag-apoptin and processed for immune fluorescence analysis as described in the legend of figure 1. Both flag-apoptin transfected and untransfected mitotic cells were scored according to their phase in mitosis. Representative images of the four phases in U2Os are in (A) from left to right prophase, metaphase, anaphase and telophase. Apoptin-positive mitotic U2Os cells could be found in prophase (B) and metaphase (C), but were scarce in anaphase and telophase, whereas SPRR4-positive mitotic U2Os cells were readily found in anaphase (D) and telophase/cytokinesis (E). Apoptin transfected mitotic cells were scored as described above, grouped as prophase/metaphase and anaphase/telophase and compared to untransfected cells in the same experiment. The results from 3 separate experiments (represented as means ± SD) in which 100 mitotic cells per group were counted are given. Panel F: Saos-2 cells (** p= 0.0041). Panel G: U2Os cells (** p= 0.0055). Scale bar: 5μm. Panels H-J: Apoptin-positive cells are shown that undergo apoptosis during mitosis: (H) after spindle formation, (I) during abnormal cytokinesis and (J) during normal cytokinesis. Scale bar: 10μm.

becomes apoptotic. Under similar conditions a Saos-2 cell undergoes a round of seemingly normal mitosis, i.e. the cell generates two daughter cells. However, phases are prolonged and in the interphase following division both daughter cells undergo cell death (Figure 6.3D).

Our analysis proves that in apoptin-expressing tumor cells various well defined events occur in a clearly successive manner. These include perturbed spindle formation, slower progression through the various phases of mitosis and eventually induction of cell death. Therefore, the observed behavior of a mitotic cancer cell expressing apoptin conforms to the set of characteristics attributed to mitotic catastrophe by the 2012 Nomenclature Committee on Cell Death: a) it is triggered by perturbations of the mitotic apparatus; b) it is initiated during the M phase of the cell cycle; c) it is paralleled by some degree of mitotic arrest; d) it ultimately triggers cell death or senescence (Galluzzi et al., 2012).

We have recently shown that apoptin can be activated in normal cells by inactivation of the B56γ or δ subunits of PP2A (Zimmerman et al., 2012). The PP2A-B56 complex is essential for the stabilization of kinetochore-microtubule attachments and plays an important role in correct chromosome segregation (Foley et al., 2011). Similarly, knockdown of the spindle assembly checkpoint (SAC) kinase Bub1 can trigger tumor-like behavior of apoptin in normal cells (Kucharski et al., 2011). Apparently apoptin can sense malfunctioning of the SAC. In the present study, both the timing of initiation (at the meta-to-anaphase transition) and the character of the various mitotic perturbations that are observed also pinpoint at an activation of apoptin around the mitotic spindle checkpoint. As many (if not all) human tumor cells have a weakened SAC (Kops et al., 2005) our analysis implies that apoptin is likely able to sense minor inaccuracies of the SAC in tumor cells in a similar way as previously shown in genetically modified normal cells (Zimmerman et al., 2012; Kucharski et al., 2011).

How then can active apoptin induce mitotic instability and cell death? As long as spindles are not correctly organized and not all kinetochores are properly attached, the SAC is activated and prevents progression through mitosis by directly signaling to the anaphase-promoting complex/cyclosome (APC/C) (Musacchio and Salmon, 2007). Inhibition of APC/C by the SAC leads to stabilization of cyclin B1 and prolonged activation of cyclin B1 is associated with mitotic catastrophe (Chan et al., 2009). In this respect it is relevant to mention that increased levels of cyclin B1 have actually been observed following apoptin

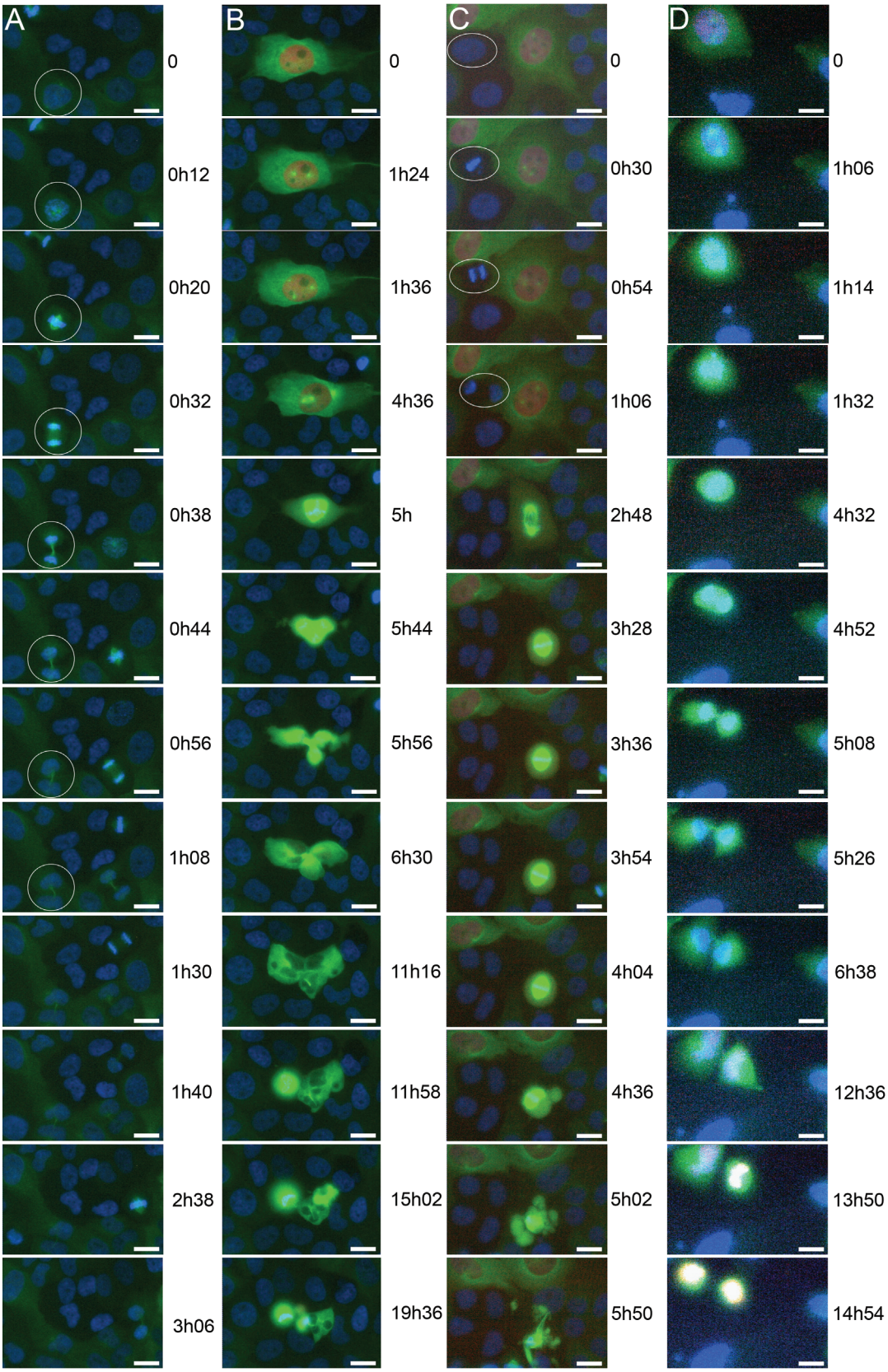

A
0
0h12
0h20
0h32
0h38
0h44
0h56
1h08
1h30
1h40
2h38
3h06
B
0
1h24
1h36
4h36
5h
5h44
5h56
6h30
11h16
11h58
15h02
19h36
C
0
0h30
0h54
1h06
2h48
3h28
3h36
3h54
4h04
4h36
5h02
5h50
D
0
1h06
1h14
1h32
4h32
4h52
5h08
5h26
6h38
12h36
13h50
14h54

Figure 6.3: Dividing apoptin-positive osteosarcoma cells undergo mitotic catastrophe resulting in apoptosis. U2Os and Saos-2 cells were transiently transfected with plasmids encoding GFP-tubulin (green) and mCherry-apoptin (red) and analyzed by time-lapse microscopy. Cells were incubated with Hoechst 33342 DNA stain (blue) prior to picture capturing. Time-lapse images for blue (DNA), green (tubulin) and red (apoptin) signal were recorded for 20 h every 2 min, for U2Os cells from 40 h – 60 h post transfection and Saos-2 cells from 65 h – 85 h post transfection. Time points of recording are indicated next to the respective images. Filmstrip (A) displays several U2Os cells transfected solely with GFP-tubulin and dividing normally (1 h to complete mitosis). One dividing cell is encircled with a white ring. Strip (B): mCherry-apoptin-positive U2Os cell with abnormal spindle formation completing mitosis but undergoes apoptosis during telophase after progression through mitosis has been seriously delayed. (C) mCherry-apoptin-positive U2Os cell with normal spindle formation arrested during metaphase and eventually undergoing apoptosis during the same phase. An untransfected cell completes mitosis with normal kinetics in the same culture (white ring). In strip (D) an mCherry-apoptin-positive Saos-2 cell undergoes seemingly normal but prolonged mitosis and apoptotic cell death occurs after cytokinesis in the two daughter cells. Scale bar: 20μm.

expression in cancer cells (Teodoro et al., 2004). Additionally it has been shown that apoptin is able to associate directly with APC1, a subunit of the APC/C, and that the resulting inactivation of APC/C is sufficient for the induction of apoptosis (Teodoro et al., 2004).

By combining the cellular analysis, which is presented here, with the earlier molecular evidence mentioned above, the following model of apoptin action arises. Apoptin senses a weakened SAC (Zimmerman et al., 2012; Kucharski et al., 2011) and binds directly to APC1 which is dislodged from the APC/C complex (Teodoro et al., 2004). Inhibition of APC/C results in a serious disabling of the mitotic checkpoint control, resulting in blockage of the meta-to-anaphase transition (Figure 6.2F/G) and the appearance of atypical non-bipolar spindles (Figure 6.1). Cell death can be induced either in cells arrested before transition to anaphase, during the progression to cytokinesis or even in daughter cells arising by inaccurate cell division (Figure 6.2H/I/J). The various disturbances of the mitotic process have been recorded by live-imaging and represented as filmstrips (Figure 6.3A/B/C/D).

Weak inaccuracies in mitotic checkpoints are believed to be responsible for aneuploidy and genetic instability (Kops et al., 2005), which are inherent to cancer cells and drive tumor progression (Hanahan & Weinberg, 2011). Apparently, apoptin is able to sense these inaccuracies during cell division and disrupts a cancerous process by triggering mitotic catastrophe and cell death.

Matherials & Methods

Cell culture

The human osteosarcoma cell lines Saos-2 (p53-null) and U2Os (p53 wild type) were purchased from the American Type Culture Collection (ATCC, Wesel, Germany) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) (PAA, Diegem, Netherlands) containing 10% newborn calf serum (Thermo Scientific, Geel, Belgium), 100μg/mL penicillin, 100μg/mL streptomycin (Duchefa, Biochemie, Haarlem, The Netherlands) and 2mM glutaMAX (PAA). Cells were cultured at 37°C in a humidified 5% CO_2 incubator. Cell morphology was regularly monitored to control the absence of cross-contamination.

Plasmids

The construction and expression of pcDNA3.1(+)flag-apoptin was previously described (Zimmerman et al., 2012) and contains the apoptin sequence published by Noteborn and co-workers (1991) fused with a flag-tag (Life Technologies, Bleiswijk, Netherlands) at its N-terminus. Full-length SPRR4 cloned into the pECV25 expression vector was previously described (Vermeij et al., 2011) and provided with the same N-terminal flag-tag as apoptin. SPRR4 was chosen as a control because, in contrast to apoptin, it does not induce apoptosis (supplementary Figure S6.1A) but still shares many biochemical characteristics with apoptin, i.e. protein size, high proline content, isoelectric point, nuclear/cytoplasmic localization (supplementary Figure S6.1B-E) and DNA binding (Leliveld et al., 2003a; Vermeij et al., 2012). Plasmid encoding GFP-tubulin was purchased from Clontech (Mountain View, CA). mCherry was amplified by PCR from pRSET-b-mCherry (Shaner et al., 2004) and introduced into pcDNA3.1(+)apoptin to yield a plasmid encoding apoptin N-terminally fused to mCherry.

Transfections

Cells were transfected with plasmids by using Amaxa nucleofection (Lonza AG, Cologne, Germany) according to an adaptation of the manufacturer's protocol. Briefly, 10^6 cells were taken up in 120μL nucleofector buffer (90mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 5mM KCl, 10mM $MgCl_2$, 20mM Hepes), mixed with DNA, transferred to a transfection cuvet (VWR, Amsterdam, The Netherlands) and transfected with program D-24 or X-01 for Saos-2 or

U2Os, respectively. After addition of medium, cells were either seeded on glass cover slips or in dishes appropriate for time-lapse microscopy.

Immune fluorescence assay

Cells grown on glass cover slips were, at indicated time points after transfection, first washed with phosphate buffered saline (PBS), and subsequently fixed for 10 min with 1% formaldehyde, 5 min with 100% methanol and 2 min with 80% acetone, all at room temperature. After air-drying the slides were used for immunocytochemical staining or stored at -20°C for further analysis. For the antibody staining, the cells were first incubated with PBS, 0.05% Tween 20 (PBS-Tween) (Sigma Aldrich, Zwijndrecht, Netherlands), 5% normal goat serum (NGS) (Sigma Aldrich) for 1 h. Next, the cells were incubated with the first antibody in the same buffer for 2 h, washed with PBS-Tween, and incubated with the second antibody in PBS-Tween, 5% NGS for 1 h. After washing with PBS-Tween cells were incubated 15 min with Hoechst 33358 (2μg/mL). Stained sections were mounted using PolyMount Mounting Media (Tebu-Bio, Heerhugowaard, Netherlands) and analyzed with a fluorescence microscope (Olympus, Zoeterwoude, The Netherlands) with the Cell P software version 2.8 (Olympus). The following antibodies were used: anti-flag (rabbit polyclonal, 1:150, Sigma Aldrich) together with a rhodamine-conjugated goat antibody (1:100, Sanbio, Uden, The Netherlands), and monoclonal mouse-anti-tubulin conjugated with fluorescein isothiocyanate (FITC) (1:100, Sigma Aldrich). Mitotic cells were scored based on their tubulin and DNA staining. Apoptosis was assessed based on characteristic DNA staining (Danen-van Oorschot et al., 1997).

Live-cell imaging

Transfected cells were incubated with 6.25 or 25 ng/mL Hoechst 33342 for Saos-2 or U2Os respectively at the indicated time points after transfection. Live cell imaging was performed on an Olympus IX81 time-lapse microscope equipped with an MT10 lamp (Olympus) and different excitation and emission filters (Olympus). Images were captured every 2 min for 20 h with the appropriate settings for GFP, mCherry and Hoechst. Movies were analyzed with the Cell M software version 3.1 (Olympus). Results are represented as filmstrips (Figure 6.3) but the original movies (avi format) can be requested from the corresponding author.

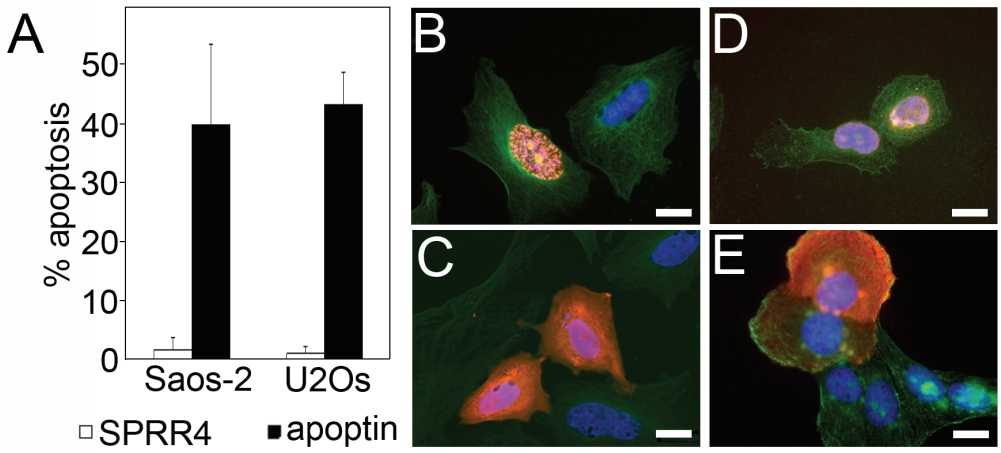
Statistical analysis

Each immune fluorescence assay was performed 3 times. For each experiment 100 mitotic cells were scored per group. Datasets were analyzed with the paired two-tailed student's t-test in which apoptin positive mitotic cells were compared to each of three negative control groups. All data are means ± standard deviation (SD); p values below 0.05 were considered significant (** $p<0.01$).

Acknowledgements

We are grateful to Marcel Schaaf (IBL) for providing the pRSET-b-mCherry plasmid and Hans den Dulk (LIC) for technical support. This work was supported by a grant from the Dutch Royal Society of Arts and Sciences (KNAW) and by the Leiden Institute of Chemistry.

Supplementary Information



Supplementary Figure S6.1: Apoptin but not SPRR4 induces apoptosis in osteosarcoma cell lines. Saos-2 and U2Os cells were transfected with plasmid encoding flag-apoptin or flag-SPRR4 (control) and were fixed 72 h or 48 h post transfection (Saos-2 and U2Os respectively). The percentage of apoptosis (represented as means ± standard deviation) induced by apoptin and SPRR4 in Saos-2 and U2Os cells was scored by aberrant DNA staining[30]. SPRR4 did not induce apoptosis in these cells compared to apoptin (A). Flag-tagged apoptin or flag-tagged SPRR4 were stained with a flag-specific antibody conjugated to rhodamine (red), endogenous tubulin with a FITC-labeled tubulin-specific antibody (green) and DNA with Hoechst (blue). Apoptin localizes to the nucleus of interphase cells (panels B, D) and SPRR4 to both nucleus and cytoplasm (panels C, E). Saos-2 cells (B, C); U2Os cells (C, E). Scale bar: 20μm.

Chapter 7

Summary & Discussion

Proteins Killing Tumor Cells:

Targetting The Hallmarks Of Cancer

Hallmarks of cancer

In 2000, Hanahan and Weinberg revolutionized the way the scientific world thought about cancer. By describing the hallmarks of cancer, they set a framework to discuss and think about the specific contours of tumorigenesis. Though each cancer is different on the molecular level, the same set of alterations is often needed for a cancer cell to become a full blown neoplasia. These alterations are described by eight hallmarks of cancer and two enabling characteristics (Hanahan & Weinberg, 2000; 2011) (see also **chapter two)**.

The hallmarks can be roughly grouped as endogenous and exogenous. Endogenous being those traits that regulate tumorigenesis inside a cell (*sustaining proliferative signaling, evading growth suppressors, enabling replicative immortality and resisting cell death*) and exogenous those that induce changes in the cell's environment to further support the development of the tumor (*inducing angiogenesis, activating invasion & metastasis, deregulating cellular energetics and avoiding immune destruction*). While the hallmarks are the traits acquired by a transformed cell, the enabling characteristics are those features that allow the acquisition of the changes that constitute the hallmarks.

Proteins killing tumor cells

Proteins Killing Tumor Cells (PKTCs) are a group of proteins all shown to have selective cell death inducing activity against tumor cells, having no detrimental effect on healthy tissue. This in contrast to regular anti-cancer drugs, which have many unwanted side effects. The eleven currently known PKTCs have been described in detail in **chapter two**. In short there are various classes: the virus-derived apoptin, E4orf4 and NS1; frog-derived Brevinin-2R and onconase; HAMLET is created in vitro from human breast milk; finally there are the human proteins that under the right conditions can help to battle cancer, TRAIL, noxa, mda-7/IL-24, Par-4 and ORCTL3. All PKTCs tested in (pre)clinical trials show positive outcomes.

As with the hallmarks of cancer, all PKTCs have a common trait, the selective killing of cancer cells, but it is caused by different triggers on a molecular level. The upstream activating characteristics of a cancer cell that activate a PKTC might differ per protein, and the downstream execution of cell death activation is also variable. Here we set out to test the hypothesis that the selectivity of PKTCs is achieved through the recognition of the hallmarks of cancer. This is done for all PKTCs, but for apoptin specifically.

Tumor-related derailment of protein phosphatase 2A activates apoptin

Apoptin behaves differently in normal cells as compared to tumor cells. Not only does it induce apoptosis in cancer cells, it translocates to the nucleus and becomes phosphorylated. These features made it possible to monitor the switch during tumorigenesis that constitutes for apoptin the change from a normal to a tumor cell. In **chapter three**, we examined whether inactivation of two specific B56 subunits of protein phosphatase 2A (PP2A) is sufficient to activate the tumor-selective behavior of apoptin in normal cells. The simian virus 40 antigens large T and small T (SV40 LT and ST) has previously been shown to be oncogenic to normal cells and could activate apoptin (Zhang et al., 2004). Here, we found that cancer-selective activation of apoptin occurs through inactivation of the PP2A-B56γ complex. In addition the observed interaction of apoptin with BCA3/AKIP1 led to a series of experiments to manipulate protein kinase A (PKA). Indeed, BCA3/AKIP1 is an inhibitor of this tumor-related kinase and stimulation of PKA blocked apoptin activation, whereas inhibition of PKA had the opposite effect. Importantly, activation of apoptin could be achieved through inactivation of the PP2A-B56δ complex.

Inactivation of PP2A complexes is a known step in tumorigenesis (Westermarck & Hahn, 2008), and one that is apparently recognized by apoptin. Interestingly, both B56δ and B56γ are nuclear B56 subunits and PP2A-B56 complexes were shown to be involved in the formation of stable attachments between kinetochores and microtubules. Correct attachment deactivates the spindle attachment checkpoint and allows progression through mitosis (Foley et al., 2011). As will be discussed below, apoptin likely senses deregulations during mitosis, which are caused by the disruption of correct spindle attachments due to disturbed PP2A-B56 complex formation.

E4orf4 does not detect irregularities in PP2A complexes, but causes them. By binding to PP2A-B55 E4orf4 lowers the activity of the PP2A complex resulting in hyperphosphorylation (Li et al., 2009b). E4orf4 interaction with PP2A-B55 leads to G_2/M arrest and cell death, possibly through changing the balance in chromatin remodeling complexes. PP2A leads E4orf4 to the ACF-SNF2h complex and by inhibiting ACF, liberates SNF2h to form other complexes. This switch in chromatin remodeling supports the induction of cell death (Brestovitsky et al., 2011). Interestingly apoptin interacts with another factor that can form a chromatin remodeling complex with SNF2h, namely Rsf1 (remodeling

and spacing factor 1) (Danen-van Oorschot, unpublished data). Rsf1 is over-expressed in an increasing number of cancer types and levels are correlated to disease aggressiveness (Li et al., 2012). Over-expression of Rsf1 in normal cells leads to DNA damage signaling and promotes genomic instability (Sheu et al., 2010). DNA damage signaling activates apoptin in normal cells (Kucharski et al., 2011). High levels of Rsf1 in cancer cells potentially facilitate apoptin both in an upstream activating and downstream executive fashion. The DNA damage signaling by Rsf1 could activate apoptin, and by direct binding to Rsf1 apoptin could disrupt the abnormal high levels of Rsf1-SNF2h complex, releasing SNF2h for other complexes in a similar fashion as E4orf4.

Proteasomal degradation

A novel distinguishing feature of apoptin which differs between normal and tumor cells was discovered in **chapter four**. Normal cells efficiently shield apoptin (Zhang et al., 2004) and degrade the protein through the proteasomal degradation pathway. In cancer cells, this clearance does not seem to be functional (Lanz et al., 2012b, **chapter four**). This differential characteristic contributes to apoptin safety in cancer treatment. The precise processes responsible for the differential apoptin stability is unclear: no differences in proteasome composition between normal and tumor cells have yet been reported, however, changes in proteasome subunit modifications can cause differential degradation by nuclear and cytoplasmic proteasomes (Guo et al., 2011). The differential localization and degradation of apoptin could be related. Per analogy, the nuclear localization and stability of the serine hydroxymethyltransferase 1 (SHMT1) is determined by competition between sumoylation and ubiquitination of the protein (Anderson et al., 2012). Apoptin can be sumoylated in tumor cells (Janssen et al., 2007), which might explain of its insensitivity to proteasomal degradation in tumor cells.

Unfolded proteins are degraded by the proteasome. HAMLET, a partially unfolded PKTC, is indeed targeted to the proteasome, but resists degradation and influences proteasomal activity (Gustafsson et al., 2009). Possibly the inhibition of the proteasome leads to changes in protein levels and shifts in the Bcl-2 family the balance in favor of the pro-apoptotic members, leading to cell death (Adams & Cory, 2007). Apoptin has not been shown to disturb proteasome activity directly, however, by interacting with a subunit from the anaphase promoting complex/cyclosome (APC/C) apoptin does influence the activity of

an E3 ligase (Teodoro et al., 2004). The APC/C is the major E3 ligase in the nucleus responsible for the correct timing of degradation of cell cycle regulatory proteins (Garcia-Higuera et al., 2008). Furthermore, apoptin can induce the expression of the E3 ligase PIR2, leading to a shift in the balance between pro-survival ΔNp73 and pro-death TAp73 in favor of the latter (Taebunpakul et al., 2012).

Misfolded proteins in the endoplasmic reticulum (ER) activate the Unfolded Protein Response (UPR). Normally unfolded proteins from the ER are degraded by the ubiquitin-proteasome pathway (Liu & Ye, 2011). Upon activation of the UPR several processes are activated that block translation, activate macroautophagy and change transcription. A cell can sense the duration of the UPR, when the ER stress caused by unfolded proteins persists for too long, cell death processes are activated. Under normal conditions two of the UPR effectors, IRE1α and PERK, are inactive due to binding to GRP78/BiP. During ER stress, GRP78/BiP binds to unfolded proteins, releasing IRE1α and PERK (Hetz, 2012). GRP78/BiP is over-expressed in cancer cells which helps to suppress cell death pathways (Sato et al., 2010). It is a popular target among PKTCs as Par-4, Mda-7/IL-24 and apoptin can interact with GRP78/BiP (Burikhanov et al., 2009; Gupta et al., 2006; Danen-van Oorschot, unpublished results), presumably by activation of the UPR and subsequent cell death induction.

Tumor-selective phosphorylation of apoptin

It has been proposed that the nuclear localization of apoptin depends on its tumor-selective phosphorylation of threonine 108 (Poon et al., 2005). The identity of the kinase responsible for apoptin phosphorylation is still under debate, with both cyclin dependent kinase 2 (CDK2) and protein kinase c isoform β (PKCβ) coined for the job (Maddika et al., 2009; Jiang et al., 2010). In **chapter five**, we showed that neither one of them is required in our in-vitro phosphorylation assay of apoptin (Lanz et al., 2012a). It was also shown that T107 can be phosphorylated, with T108 present. Previously, it was shown that upon mutation of T108 apoptin can still induce apoptosis (Rohn et al., 2005). It could be possible that the T108 of apoptin falls within the consensus sequence of more than one kinase. The Herpes simplex virus 1 protein Us3 can be phosphorylated on serine 147 both by itself and by PKA which influences its localization and activity (Kato et al., 2008). Other PKTCs such as E4orf4 or

NS1 also interact with specific kinase activities such as Src tyrosine kinases and casein kinase II, respectively, enabling their activation (Noteborn, 2009a).

Genomic instability and mitotic catastrophe

More than one hundred years ago, the German scientist Theodor Boveri observed that the equal distribution of duplicated chromosomes among daughter cells is crucial for embryonic development (Holland & Cleveland, 2009). Many cancers have an incorrect number of chromosomes, a situation termed aneuploidy. Furthermore, a lot of tumors vary in their number of chromosomes due to loss or gain during mitosis, a concept named chromosomal instability (Kops et al., 2005). Genomic instability is propagated during mitosis. Incorrect spindle assembly and chromosome segregation lead to unequal distribution. The mitotic checkpoint, or spindle assembly checkpoint (SAC) should arrest cells during mitosis as long as chromosomes are not correctly attached. Many tumor cells have a weakened SAC due to mutations or altered protein levels of components of the checkpoint (Kops et al., 2005). An important target in the mitotic checkpoint is the APC/C. Inhibition of the APC/C by the SAC blocks mitosis in the meta-to-anaphase transition. Apoptin can bind the APC1 subunit of the APC/C and block cell cycle progression (Teodoro et al., 2004; **chapter six**). E4orf4 blocks cell cycle progression also through manipulation of the APC/C (Smolders and Teodoro, 2011).

In healthy cells, one unattached kinetochore is enough to activate the SAC. In tumor cells more dramatic deviations need to occur to block mitosis (Kops et al., 2005). We infer that apoptin senses mitotic abnormalities, for example caused by deregulated PP2A complexes or a weakened SAC. In **chapter six**, we showed that apoptin causes apoptosis through the induction of mitotic catastrophe. In an already chromosomal instable cancer cell background apoptin causes an even bigger mitotic mayhem pushing the tumor towards cell death. Apoptin might sense mitotic inaccuracies through its interaction with Fam96B (Zimmerman et al., 2011). Fam96B is part of a complex that is essential for proper chromosome segregation (Ito et al., 2010). An extra level of DNA inaccuracy sensing is likely done by apoptin through its interaction with DEDAF (Danen-van Oorschot et al., 2004). Furthermore, disruption of chromatin, and increased signaling to the SAC, might be mediated by apoptin through its interaction with Rsf1 as described above. Together with its interacting partners, apoptin apparently senses mitotic inaccuracies or defects, and possibly

manages to activate a weakened SAC by increasing upstream signaling. E4orf4 over-expression in human lung cancer H1299 cells revealed the occurrence of tetraploid and polyploid cells and the appearance of micronuclei, suggesting that E4orf4 induces mitotic catastrophe which eventually leads to cell death (Li et al., 2009a).

Cancer, Hallmarks and PKTCs

A lot of effort in the field of PKTCs is invested in showing the clinical importance and use of the protein in (pre)clinical testing. Alongside there is a lot of investigation into the downstream pathways of PKTCs involved in inducing the cell death, and how this relates to the tumor-selectivity. Our literature review revealed that only rarely investigations are undertaken to specifically identify which tumorigenic changes are needed in a cell in order to activate a PKTC, as for example the SV40 research in the case of apoptin (Zhang et al., 2004; Zimmerman et al., 2012) and the knock down of oncogenic pathways related to HAMLET functionality (Storm et al., 2011). Upstream activating hallmarks are important as they will help identify which cancer types will be susceptible for treatment with a specific PKTC, leading to personalized medicine. In this chapter we have used the cellular components that are recognized by PKTCs (Table 7.1) and are responsible for their selective cell death induction, to link PKTCs to their upstream activating hallmarks of cancer (Table 7.2).

There is a huge diversity in how strictly or broadly a hallmark is described. Some are very well defined, with clear cellular markers. For example, enabling replicative immortality is a hallmark with very clearly defined markers. In most cancers this is achieved by re-activating telomerase, and in some situations by alternative lengthening of telomeres. On the other hand, the sustainment of proliferative signaling can be achieved by modulating one of many cellular pathways. Sometimes it is not so much the hallmark that is recognized by the PKTC as much as the oncogene causing a hallmark(s), e.g. oncogenic RAS-activation of TRAIL (Nesterov et al., 2004). As shown in table 7.1, eight out of ten cancer hallmarks/enabling characteristics are detected by one or more of the different PKTCs.

The first two hallmarks involved in keeping up unscheduled growth are recognized by many of the PKTCs. Both the sustainment of proliferative signaling and evasion of growth suppressors are broadly defined hallmarks, with many of the major signaling pathways involved. They are essential already early in tumorigenesis (Hanahan & Weinberg, 2000). Because these two hallmarks are caused by mutations in crucial components of

important pathways, e.g. oncogenic c-Myc (Whitfield & Soucek, 2012), they are linked to other hallmarks as well. In case of some PKTCs it is not always clear if there is a direct relationship between the marker of the hallmark and activation of the PKTC, or if it is a downstream effect within one specific hallmark.

The resistance of cell death signaling is a hallmark that can activate six PKTCs (apoptin, NS1, TRAIL, Noxa, mda-7 and Par-4). GRP78/BiP is a key component in PKTC recognition of cell death resistance though the effect is different depending on the PKTC. GRP78/BiP is a chaperone that normally resides in the ER. Upon ER stress it locates to the cellular membrane. In cancer cells it is over-expressed on the cell surface (Sato et al., 2010). For instance, Mda-7/IL-24 inactivates GRP78/BiP in the ER triggering the Unfolded Protein Response (Dash et al., 2010a). In contrast, Par-4 activates the cellular membrane bound GRP78/IL-24 leading to caspase activation (Burikhanov et al., 2009). Either way, it is the abnormal amount of GRP78/BiP that is characteristic for tumor cells, that upon further deregulation by a PKTC leads to cell death.

Some hallmarks as the deregulation of cellular energetics and the avoidance of immune destruction seem to be less recognized by PKTCs. However, this might be due to a lack of active research in these areas on PKTC activation. In case of HAMLET is does play an important role (Storm et al., 2011) and it might be informative about the other PKTCs as well. So far there are no reports on the influence of oxidative glycolysis on PKTC activation.

Several crucial oncogenes as Src, c-Myc and RAS (Table 7.1 and references within) link PKTCs to angiogenesis induction. As these oncogenes are involved in the occurance of several hallmarks it is not always clear whether the PKTC recognizes angiogenesis per se.

Parvoviruses, encoding NS1, optimally use the evasion of the immune system by cancer cells to their advantage (Rommelaere et al., 2010). As discussed above, apoptin and E4orf4 might sense irregularities during mitosis, and this genomic unstable background could be one of the triggers for them to induce mitotic catastrophe.

Replicative immortality seems to be a hallmark that none of the PKTCs use to their advantage. Even worse, the re-activation of telomerase, one of the main molecular characteristics of this hallmark, actually negatively influences some of the PKTCs, Par-4 and TRAIL (Sheng et al., 2010; Zhang et al., 2010). In contrast, several therapy-focused experiments with PKTCs use vectors on which the gene of interest is expressed downstream of the telomerase promoter, as this will confer more tumor-specificity on the expression of

the gene (apoptin: Li et al., 2010 and TRAIL: Li et al., 2011a). In this fashion, recognition of this hallmark of cancer can be indirectly added to the spectrum of PKTCs.

The emerging hallmark of tumor-promoting inflammation also seems to go undetected by PKTCs. Not completely surprising as this specific hallmark is completely tumor-environment related, and most of the research with PKTCs is done under tissue culture conditions. The effect of tumor-promoting inflammation can only be studied in vivo and thus far no data on the effect of inflammation on the activation of PKTCs have been reported.

Since the introduction of the concept of 'hallmarks of cancer' in 2000 attempts have been undertaken to expand and (re)define them, with in 2011 an update by the original authors themselves. Luo and colleagues (2009) introduced several cancer-related stresses, to further define the concept of a cancer cell. Metabolic, proteotoxic, mitotic, oxidative and DNA damage stress are cancer-related, but not all incorporated in the hallmarks of cancer.

Through their tumor-selectivity PKTCs can point out those aspects of cancer cells that set them apart from healthy tissue. They do not so much introduce completely new hallmarks as much as point out aspects of hallmarks that thus far went rather unremarked and could prove to be important targets in cancer therapy.

Tumor-selective membrane transport is an import aspect of HAMLET, onconase and Brevinin-2R. An important challenge in medication administration is getting the compound at the site of action. Tumor-specific uptake of medicines would be a worthwhile improvement. Furthermore it points at distinct differences in membrane composition between normal and cancer cells. A so called 'lipogenic switch', i.e. a change in the lipid profile, is reported for many types of cancer (Schug et al., 2012) and could be the reason for distinguishable cellular membranes. Apoptin and Mda-7/IL-24 influence ceramide levels, potentially exercising an effect on this lipogenic switch (Liu et al., 2006; Dash et al., 2010a).

Besides regulation at the level of transcription, homeostasis of protein levels is mainly achieved by the proteasome. Though several PKTCs influence transcription factors, some of them point at an important role for protein degradation in 'cancer protein homeostasis'. Apoptin is degraded in normal, but not in tumor cells by the proteasome (Lanz et al., 2012b). HAMLET achieves its own stability and a deregulation of protein levels through interaction with the proteasome (Gustaffson et al., 2009). Imbalances in protein levels that govern life-or-death decisions potentiate tumor development; PKTC research

shows that they are not only caused by altered transcription, but also by inaccuracies in proteasomal degradation.

If aneuploidy, or genomic instability in general, is a cause, a consequence, or even a cure for tumorigenesis is an ongoing debate (Holland & Cleveland, 2009). However, whichever of the three it is, the cellular processes responsible for mitotic instability also enable apoptin, and E4orf4, to induce tumor-selective apoptosis. PKTCs indeed sense imbalances in crucial cellular processes that are too important to go completely wrong (as that would lead to eminent cell death), but when slightly skewed, can pave the way to tumorigenesis. Whether it is a weakened SAC that creates genomic instability, or abnormal protein levels in the Bcl-2 family, the general theme of PKTC activation seems to be that "what allowed a tumor cell to live now leads to its demise".

The in-depth study of these proteins, that by one way or another manage to kill tumor cells with no harmful side effects, will not only allow us to develop promising cancer therapeutics, but it might also allow us to further nuance and elaborate the hallmarks of cancer.

	Sustaining proliferative signalling 	Evading growth suppressors 	Enabling replicative immortality 	Resisting cell death 	Inducing angiogenesis 
apoptin	Akt (Maddika et al., 2007) PKCβ (Jiang et al., 2009) PP2A/PKA (Zimmerman et al., 2012)	PP2A (Zimmerman et al., 2012)		GRP78/BiP (Noteborn, unpublished results) Akt (Maddika et al., 2007) PP2A (Zimmerman et al., 2012)	
E4orf4					Src (Gingras et al., 2002)
NS1	CKII (Nuesch & Rommelaere, 2007)	CKII (Nuesch & Rommelaere, 2007)		CKII (Nuesch & Rommelaere, 2007)	
onconase					
Brevinin-2R					
HAMLET	c-Myc (Storm et al., 2011)	c-Myc (Storm et al., 2011)			c-Myc (Storm et al., 2011)
TRAIL	c-Myc (Kim et al., 2011) RAS (Nesterov et al., 2004)	c-Myc (Kim et al., 2011)		RAS (Nesterov et al., 2004)	c-Myc (Kim et al., 2011) RAS (Nesterov et al., 2004)
Noxa	c-Myc (Nikiforov et al., 2007)	c-Myc (Nikiforov et al., 2007)		Puma (Nakajima & Tanaka, 2011)	c-Myc (Nikiforov et al., 2007)
mda-7				GRP78/BiP (Gupta et al., 2006)	
Par-4	PKA (Gurumurthy et al., 2005)			GRP78/BiP (Burikhanov et al., 2009)	
ORCTL3					

Table 7.1: The hallmarks of cancer & enabling characteristics that activate Proteins Killing Tumor Cells.

Activating invastion & metastasis 	Deregulating cellular energetics 	Avoiding immune destruction 	Tumor-promoting inflammation 	Genome instability 	
				APC/C (Teodoro et al., 2004) PP2A (Zimmerman et al., 2012) fam96A/B (Tian & Lanz, unpublished results) Rsf-1 (Danen-van Oorschot, unpublished)	apoptin
Src (Gingras et al., 2002)				APC/C (Koenitzer et al., 2001)	E4orf4
		IFN (Rommelaere et al., 2010)			NS1
membrane composition (Lee & Raines, 2008)					onconase
membrane composition (Ghavami et al., 2008)					Brevinin-2R
c-Myc (Storm et al., 2011) membrane composition (Fisher et al., 2004)	glycolysis (Storm et al., 2011)			c-Myc (Storm et al., 2011)	HAMLET
c-Myc (Kim et al., 2011) RAS (Nesterov et al., 2004)	RAS (Nesterov et al., 2004)	RAS (Nesterov et al., 2004)		c-Myc (Kim et al., 2011)	TRAIL
c-Myc (Nikiforov et al., 2007)				c-Myc (Nikiforov et al., 2007)	Noxa
					mda-7
					Par-4
					ORCTL3

Table 7.1 continued.

	Sustaining proliferative signalling 	Evading growth suppressors 	Enabling replicative immortality 	Resisting cell death 	Inducing angiogenesis 
PP2A	Westermark & Hahn, 2008	Westermark & Hahn, 2008		Kong et al., 2004	
CKII	Hanif et al., 2010	Hanif et al., 2010		Wang et al., 2005	
c-Myc	Whitfield & Soucek, 2011	Hanahan & Weinberg, 2011			Baudino et al., 2002
PKA	Almeida & Stratakis, 2011				
Akt	Vivanco & Sawyers, 2002			Vivanco & Sawyers, 2002	
PKCβ	Li et al., 2011b				
RAS	Pylayeva-Gupta et al., 2011			Cor & Der, 2003	Sparmann & Bar-Sag, 2004
GRP78/BiP				Sato et al., 2010	
Puma				Nakajima & Tanaka, 2011	
Src					Arai et al 2012
IFN					
APC/C					
Glycolysis					
Membrane composition					

Table 7.2: The hallmarks of cancer & enabling characteristics and a selection of accomodating genetic markers.

Activating invasion & metastasis 	Deregulating cellular energetics 	Avoiding immune destruction 	Tumor-promoting inflammation 	Genome instability 	
				Foley et al., 2011	PP2A
					CKII
Hanahan & Weinberg, 2011				Vafa et al., 2002	c-Myc
					PKA
					Akt
					PKCβ
Pylayeva-Gupta et al., 2011	Chiaradonna et al., 2006	Pylayeva-Gupta et al., 2011			RAS
					GRP78/BiP
					Puma
Arai et al 2012					Src
		Rommelaere et al., 2010			IFN
				Lipkowitz & Weissmman, 2011	APC/C
	Koppenol et al., 2011				Glycolysis
Kier et al., 1990					Membrane composition

Table 7.2 continued.

References

Adams JM and Cory S (2007) The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 26, 1324-1337.

Ahn J-H, McAvoy T, Rakhilin SV, Nishi A, Greengard P and Naim AC (2007) Protein kinase A activates protein phosphatase 2A by phosphorylation of the B56δ subunit. *Proc Natl Acad Sci USA* 104, 2979-2984.

Aits S, Gustafsson L, Hallgren O, Brest P, Gustafsson M, Trulsson M, Mossberg A-K, Simon H-U, Mograbi B and Svanborg C (2008) HAMLET (human alpha-lactalbumin made lethal to tumor cells) triggers autophagic tumor cell death. *Int J Cancer* 124, 1008-1019.

Alberts B, Johnson A, Lewis J, Raff M, Roberts K and Walter P (2008) Molecular biology of the cell; fifth edition. Garland Science, Taylor & Francis Group, New York, USA.

Almeida MG and Stratakis CA (2011) How does cAMP/protein kinase A signalling lead to tumors in the adrenal cortex and other tissues? *Mol Cell Endocrinol* 336, 162-168.

Alvisi G, Poon IK and Jans DA (2006) Tumor-specific nuclear targeting: promises for anti-cancer therapy. *Drug Resist Update* 9, 40-50.

Anderson DD, Eom JY and Stover PJ (2012) Competition between sumoylation and ubiquitination of serine hydroxymethyltransferase 1 determines its nuclear localization and its accumulation in the nucleus. *J Biol Chem* 287, 4790-4799.

Anisimov VN (2009) Carcinogenesis and aging 20 years after: Escaping horizon. *Mech Ageing Dev* 130, 105-121.
Aquilera A and Gomez-Gonzalez B (2008) Genome instability: a mechanistic view of its causes and consequences. *Nat Rev Genet* 9, 204-217.

Arai R, Tsuda M, Watanabe T, Ose T, Obuse C, Maenaka K, Minami A and Ohba Y (2012) Simultaneous inhibition of Src and Aurora kinases by SU6656 induces therapeutic synergy in human synovial sarcoma growth, invasion and angiogenesis in vivo. *Eur J Cancer* in print.

Argiris K, Panethymaitaki C and Tavassoli M (2011) Naturally occurring, tumor-specific, therapeutic proteins. *Exp Biol Med (Maywood)* 236, 524-536.

Arroyo JD and Hahn WC (2005) Involvement of PP2A in viral and cellular transformation. *Oncogene* 24, 7746-7755.

Backendorf C, Visser AE, de Boer AG, Zimmerman RME, Visser M, Voskamp P, Zhang Y-H and Noteborn MHM (2008) Apoptin: Therapeutic potential of an early sensor of carcinogenic transformation. *Annu Rev Pharmacol Toxicol* 48, 143-169.

Baudino TA, McKay C, Pendeville-Samain H, Nilsson JA, Maclean KH, White EL, Davis AC, Ihle JN and Cleveland JL (2002) c-Myc is essential for vasculogenesis and angiogenesis during development and tumor progression. *Genes Dev* 16, 2530-2543.

Ben-Israel H, Sharf R, Rechavi G and Kleinberger, T (2008) Adenovirus E4orf4 protein downregulates Myc expression through interaction with PP2A-B55 subunit. *J Virol* 82, 9381-9388.

Bousquet-Dubouch MP, Fabre B, Monsarrat B and Burlet-Schiltz O (2011) Proteomics to study the diversity and dynamics of proteasome complexes: from fundamentals to the clinic. *Expert Rev Proteomics* 8, 459-481.

Boutros M and Ahringer J (2008) The art and design of genetic screens: RNA interference. *Nat Rev Genet* 9, 554-566.

Branden C and Tooze J (1991) Introduction to protein structure. Garland Publishing, Inc, New York and London.

Brestovitsky A, Sharf R, Mittelman K and Kleinberger T (2009) The adenovirus E4orf4 protein targets PP2A to the ACF chromatin-remodeling factor and induces cell death through regulation of SNF2h-containing complexes. *Nucleic Acids Res* 39, 6414-6427.

de Bruin EC and Medema JP (2008) Apoptosis and non-apoptotic deaths in cancer development and treatment response. *Cancer Treat Rev* 34, 737-749.

Bruno P, Brinkmann CR, Boulanger MC, Flinterman M, Klanrit P, Landry MC, Portsmouth D, Borst J, Tavassoli M, Noteborn MHM, Backendorf C and Zimmerman RM (2009) Family at last: highlights of the first international meeting on proteins killing tumour cells. *Cell Death Differ* 16, 184-186.

Bullenkamp J, Cole D, Malik F, Alkhatabi H, Kulasekararaj A, Odell EW, Farzaneh F, Gaken J and Tavassoli M (2012) Human gyrovirus apoptin shows a similar subcellular distribution pattern and apoptosis induction as the chicken anaemia virus derived VP3/apoptin. *Cell Death Dis* e296.

Bulun SE and Simpson ER (2008) Aromatase expression in women's cancers. *Adv Exp Med Biol* 630, 112-132.

Burikhanov R, Yanming Z, Goswami A, Qiu S, Schwarze SR and Rangnekar VM (2009) The tumor suppressor Par-4 activates an extrinsic pathway for apoptosis. *Cell* 138, 377-388.

Burkhart DL and Sage J (2008) Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat Rev Cancer* 8, 671-682.

Cabral A, Sayin A, de Winter S, Fischer DF, Pavel S and Backendorf C (2001) SPRR4, a novel cornified envelope precursor: UV-dependent epidermal expression and selective incorporation into fragile envelopes. *J Cell Sci* 114, 3837-3843.

Canavese M, Santo L and Raje N (2012) Cyclin dependent kinases in cancer: Potential for therapeutic intervention. *Cancer Biol Ther* 13, 451-457.

Cao HD, Yang YX, Lu L, Liu SN, Wang PL, Tao XH, Wang LJ and Xiang TX (2010) Attenuated Salmonella typhimurium carrying TRAIL and VP3 genes inhibits the growth of gastric cancer cells in vitro and in vivo. *Tumori* 96, 296-303.

Castedo M., Perfettini JL, Roumier T, Andreau K., Medema R. and Kroemer G (2004) Cell death by mitotic catastrophe: a molecular definition. *Oncogene* 23, 2825-2837.

Cesare AJ and Reddel RR (2010) Alternative lengthening of telomeres: models, mechanisms and implications. *Nat Rev Genet* 11, 319-330.

Chan YW, Chen Y and Poon RY (2009) Generation of an indestructible cycling B1 by caspase-6-dependent cleavage during mitotic catastrophe. *Oncogene* 28, 170-183.

Chen W, Possemato R, Campbell KT, Plattner CA, Pallas DC and Hahn, WC (2004) Identification of specific PP2A complexes involved in human cell transformation. *Cancer Cell* 5, 127-136.

Chen Y, Xu Y, Bao Q, Xing Y, Li Z, Lin Z, Stock JB, Jeffrey PD and Shi Y (2007) Structural and biochemical insights into the regulation of protein phosphatase 2A by small t antigen of SV40. *Nat Struc Mol Biol* 6, 527-534.

Chen D, Zhang J, Li M, Rayburn ER, Wang H and Zhang R (2009) RYBP stabilizes p53 by modulating MDM2. *EMBO Rep* 10, 166-172.

Chi H-C, Chen S-L, Liao C-J, Liao C-H, Tsai M-M, Lin Y-H, Huang Y-H, Yeh C-T, Wu S-M, Tseng Y-H, Chen C-Y, Tsai C-Y, Chung I-H, Chen W-J and Lin K-H (2012) Thyroid hormone receptors promote metastasis of human hepatoma cells via regulation of TRAIL. *Cell Death Diff* in press.

Chiaradonna F, Sacco E, Manzoni R, Giorgio M, Vanoni M and Alberghina L (2006) Ras-dependent carbon metabolism and transformation in mouse fibroblasts. *Oncogene* 25, 5391-5404.

Cho US, Morrone S, Sablina AA, Arroyo JD, Hahn WC and Xu W (2007) Structural basis of PP2A inhibition by small T antigen. *PLoS Biol* 5, e202.

Constanzi J, Sidransky D, Navon A and Goldsweig H (2005) Ribonucleases as a novel pro-apoptotic anticancer strategy: review of the preclinical and clinical data for ranpirnase. *Cancer Invest* 23, 643-650.

Cory S and Adams JM (2002) The CL2 family: regulators of the cellular life-or-death switch. *Nat Rev Cancer* 2, 647-656.

Cox AD and Der CJ (2003) The dark side of Ras: regulation of apoptosis. *Oncogene* 22, 8999-9006.

Cusack Jr JC, Liu R, Houston M, Abendroth K, Elliott PJ, Adams J and Baldwin Jr AS (2001) Enhanced chemosensitivity to CPT-11 with proteasome inhibitor PS-341: implications for systemic nuclear factor-B inhibition. *Cancer Res* 61, 3535–3540.

Danen-van Oorschot AAAM, Fischer DF, Grimbergen JM, Klein B, Zhuang S, Falkenburg JH, Backendorf C, Quax PH, van der Eb AJ and Noteborn MHM (1997) Apoptin induces apoptosis in human transformed and malignant cells but not in normal cells. *Proc Natl Acad Sci USA* 94, 5843-5847.

Danen-van Oorschot AAAM, Zhang Y-H, Leliveld SR, Rohn JL, Seelen MC, Bolk MW, van Zon A, Erkeland SJ, Abrahams J-P, Mumberg D and Noteborn MHM (2003) Importance of nuclear localization of apoptin for tumor-specific induction of apoptosis. *J Biol Chem* 278, 27729-27736.

Danen-van Oorschot AAAM, Voskamp P, Seelen MC, van Miltenburg MH, Bolk MW, Tait SW, Boesen-de Cock JG, Rohn JL, Borst J and Noteborn MHM (2004) Human death effector domain-associated factor interacts with the viral apoptosis agonist Apoptin and exerts tumor-preferential cell killing. *Cell Death Differ* 11, 564-573.

Dash R, Bhutia SK, Azab B, Su Z-Z, Quinn BA, Kegelmen TP, Das SK, Kim K, Lee S-G, Park MA, Yacoub A, Rahmani M, Emdad L, Dmitriev IP, Wang X-Y, Sarkar D, Grant S, Dent P, Curiel DT and Fisher PB (2010a) *mda*-7/IL-24: a unique member of the IL-10 gene family promoting cancer-targeted toxicity. *Cytokine Growth Factor Rev* 21, 381-391.

Dash R, Richards JE, Su Z-Z, bhutia SK, Azab B, Rahmani M, Dasmahapatra G, Yacoub A, Dent P, Dmitriev IP, Curiel DT, Grant S, Pellecchia M, Reed JC, Sarkar D and Fisher PB (2010b) Mechanism by which Mcl-1 regulates cancer-specific apoptosis triggered by mda-7/IL-24, and IL-10-related cytokine. *Cancer Res* 70, 5034-5045.

Declercq W, Vanden Berghe T and Vandenabeele P (2009) RIP kinases at the crossroads of cell death and survival. *Cell* 138, 229-232.

Denton D, Nicolson S and Kumar S (2012) Cell death by autophagy: facts and apparent artefacts. *Cell Death Differ* 19, 87-95.

Di Piazza M, Mader C, Geletneky K, Herrero y Calle M, Weber e, Schlehofer J, Deleu L and Rommelaere J (2007) Cytosolic activation of cathepsins mediates parvovirus H-1-indueced killing of cisplatin and TRAIL-resistant glioma cells. *J Virol* 81, 4186-4198.

Diller L, Kassel J, Nelson CE, Gryka MA, Litwak G, Gebhardt M, Bressac B, Ozturk M, Baker SJ and Vogelstein B (1990) p53 functions as a cell cycle control protein in osteosarcomas. *Mol Cell Biol* 10, 5772-5781.

Dimberg LY, Anderson CK, Camidge R, Behbakht K, Thorburn A and Ford HL (2012) On the TRAIL to successful cancer therapy? Predicting and counteracting resistance against TRAIL-based therapeutics. *Oncogene* in press.

Duringer C, Hamiche A, Gustafsson L, Kimura H and Svanborg C (2003) HAMLET interacts with histones and chromatin in tumor cell nuclei. *J Biol Chem* 278, 42131-42135.

Eager R, Harle L and Nemunaitis J (2008) Ad-MDA-7; INGN 241: a review of preclinical and clinical experience. *Expert Opin Biol Ther* 8, 1633-1643.

Ekmekcioglu S, Mumm JB, Udtha M, Chada S and Grimm EA (2008) Killing of human melanoma cells induced by activation of class I interferon-regulated signaling pathways via MDA-7/IL-24. *Cytokine* 43, 34-44.

El-Guendy N, Zhao Y, Gurumurthy S, Burikhanov R and Rangnekar VM (2003) Identification of a unique core domain of Par-4 sufficient for selective apoptosis induction in cancer cells. *Mol Cell Biol* 23, 5516-5525.

Elower S (2011) Bub1 and BubR1: at the interace between chromosome attachment and the spindle checkpoint. *Mol Cell Biol* 31, 3085-3093.

Eltahir YM, Qian K, Jin W and Qin A (2011) analysis of chicken anemia virus genome: evidence of intersubtype recombination. *Virol J* 8, 512.

Fischer W, Gustafsson L, Mossberg AK, Gronli J, Mork S, Bjerkvig R and Svanborg C (2004) Human alpha-lactalbumin made lethal to tumor cells (HAMLET) kills human glioblastoma cells in brain xenografts by an apoptosis-like mechanism and prolongs survival. *Cancer Res* 64, 2105-2112.

Flinterman M, Guelen L, Ezzati-Nik S, Killick R, Melino G, Tominaga K, Mymryk JS, Gaken J and Tavassoli M (2005) E1A activates transcription of p73 and Noxa to induce apoptosis. *J Biol Chem* 280, 5945-5959.

Florea BI, Verdoes M, Li N, van der Linden WA, Geurink PP, van den Elst H, Hofmann T, de Ru A, van Veelen PA, Tanaka K, Sasaki K, Murata S, den Dulk H, Brouwer J, Ossendorp FA, Kisselev AF and Overkleeft HS (2010) Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit beta5t. *Chem Biol* 17, 795–801.

Foley EA, Maldonado M and Kapoor TM (2011) Formation of stable attachments between kinetochores and microtubules depends on the B56-PP2A phosphatases. *Nat Cell Biol* 13, 1265-1271.

Fragkos M and Beard P (2011) Mitotic catastrophe occurs in the absence of apoptosis in p53-null cells with a defective G1 checkpoint. *PLoS One* 6, e22946.

Fulop T, Larbi A, Kotb R, de Angelis F and Pawelec G (2011) Aging, immunity, and cancer. *Discov Med* 11, 537-550.

Galluzzi L, Vitale I, Abrams JM, Alnemri ES, Baehrecke AH, Blagosklonny MV, Dawson TM, Dawson VL, El-Deiry WS, Fulda S, Gottlieb E, Green DR, Hengartner MO, Kepp O, Knight RA, Kumar S, Lipton SA, Lu X, Madeo F, Malorni W, Mehlen P, Nunez G, Peter ME, Piancentini M, Rubinsztein DC, Shi Y, Simon H-U, Vandenabeele P, White E, Yuan J, Zhivotovsky B, Melino G and Kroemer G (2012) Molecular definitions of cell death subroutines: recommendations of the nomenclature committee on cell death 2012. *Cell Death Differ* 19, 107-120.

Garcia-Higuera I, Manchado E, Dubus P, Canamero M, Mendez J, Moreno S and Malumbres M (2008) Genomic stability and tumour suppression by the APC/C cofactor Cdh1. *Nat Cell Biol* 10, 802–811.

Geletnekey K, Herrero y Calle M, Rommelaere J and Schlehofer JR (2005) Oncolytic potential of rodent parvoviruses for cancer therapy in humans: a brief review. *J Vet Med B* 52, 327-330.

Ghavami S, Asoodeh A, Klonisch T, Halayko AJ, Kadkhoda K, Kroczak TJ, Gibson SB, Booy EP, Naderi-Manesh H and Los M (2008) Brevinin-2R[1] semi-selectively kills cancer cells by a distinct mechanism, which involves the lysosomal-mitochondrial death pathway. *J Cell Mol Med* 12, 1005-1022.

Gherardi E, Birchmeier W, Birchmeier Cand Vande Woude G (2012) Targeting MET in cancer: rationale and progress. *Nat Rev Cancer* 12, 89-103.

Gingras MC, Champagne C, Roy M and Lavoie JN (2002) Cytoplasmic death signal triggered by SRC-mediated phosphorylation of the adenovirus E4orf4 protein. *Mol Cell Biol* 22, 41-56.

Gjoerup O, Zaveri D and Roberts TM (2001) Induction of p53-independent apoptosis by simian 40 small t antigen. *J Virol* 75, 142-155.

Goparaju CM, Blasberg JD, Volinia S, Palatini J, Ivanov S, Donington JS, Croce C, Carbone M, Yang H and Pass HI (2011) Onconase mediated NFKβ downregulation in malignant pleural mesothelioma. *Oncogene* 30, 2767-2777.

Goswami A, Burikhanov R, de Thonel A, Fujita N, Goswami M, Zhao Y, Eriksson JE, Tsuruo T and Rangnekar VM (2005) Binding and phosphorylation of par-4 by akt is essential for cancer cell survival. *Mol Cell* 20, 33-44.

Grekova S, Aprahamian M, Giese N, Schmitt S, Giese T, Falk CS, Daeffler L, Cziepluch C, Rommelaere J and Raykov Z (2011) Immune cells participate in the oncosuppressive activity of parvovirus H-1PV and are activated as a result of their abortive infection with this agent. *Cancer Biol Ther* 10, 1280-1289.

Grimm S and Noteborn MHM (2010) Anticancer genes: inducers of tumour-specific cell death signaling. *Trends Mol Med* 16, 88-96.

Grivennikov SI, Greten FR and Karin M (2010) Immunity, inflammation, and cancer. *Cell* 140, 883-899.

Guo X, Engel JL, Xiao J, Tagliabracci VS, Wang X, Huang L and Dixon JE (2011) UBLCP1 is a 26S proteasome phosphatase that regulates nuclear proteasome activity. *Proc Natl Acad Sci USA* 108, 18649-18654.

Gupta P, Walter MR, Su Z-Z, Lebedeva IV, Emdad L, Randolph A, Valerie K, Sarkar D and Fisher PB (2006) BiP/GRP78 is an intracellular target for MDA-7/IL-24 induction of cancer-specific apoptosis. *Cancer Res* 66, 8182-8191.

Gurumurthy S, Goswami A, Vasudevan KM and Rangnekar VM (2005) Phosphorylation of Par-4 by protein kinase A is critical for apoptosis. *Mol Cell Biol* 25, 1146-1161.

Gustafsson L, Leijonhufvud I, Aronsson A, Mossberg AK and Svanborg C (2004) Treatment of skin papillomas with topical alpha-lactalbumin-oleic acid. *N Engl J Med* 350, 2663-2672.

Gustafsson L, Aits S, Onnerfjord P, Trulsson M, Storm P and Svanborg C (2009) Changes in proteasome structure and function caused by HAMLET in tumor cells. *Plos ONE* 4, e5229.

Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Mary W. Brooks MW and Weinberg RA (1999) Creation of human tumour cells with defined genetic elements. *Nature* 400, 464-468.

Hakansson A, Zhivotovsky B, Orrenius S, Sabharwal H and Svanborg C (1995) Apoptosis induced by a human milk protein. *Proc Natl Acad Sci USA* 92, 8064-8068.

Hallgren O, Gustafsson L, Irjala H, Selivanova G, Orrenius S and Svanborg C (2006) HAMLET triggers apoptosis but tumor cell death is independent of caspases, Bcl-2 and p53. *Apoptosis* 11, 221-233.

Hanahan D and Weinberg RA (2000) The hallmarks of cancer. *Cell* 100, 57-70.

Hanahan D and Weinberg RA (2011) The hallmarks of cancer: the next generation. *Cell* 144, 646-674.

Hanif IM, Hanif IM, Shazib MA, Ahmad KA and Pervaiz S (2010) Casein Kinase II: an attractive target for anti-cancer drug design. *Int J Biochem Cell Biol* 42, 1602-1605.

Hart LS and El-Deiry WS (2009) Cell death: a new Par-4 the TRAIL. *Cell* 138, 220-222.

Hayflick L (1997) Mortality and immortality at the cellular level. A review. *Biochemistry* 62, 1180-1190.

Hebbar N, Wang C and Rangnekar VM (2012) Mechanisms of apoptosis by the tumor suppressor Par-4. *J Cell Physiol* in print.

Hershko A and Ciechanover A (1998) The ubiquitin system. *Annu Rev Biochem* 67, 425-479.

Hetz C (2012) The unfolded protein response: controlling cell fate decisions under ER stress and beyond. *Nat Rev Mol Cell Biol* 13, 89-102.

Hoeller D and Dikic I (2009) Targeting the ubiquitin system in cancer therapy. *Nature* 458, 438-444.

Holland AJ and Cleveland DW (2009) Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nat Rev Mol Cell Biol* 10, 478-487.

Hu Z, Zhang Z, Doo E, Coux O, Goldberg AL and Liang TJ (1999) Hepatitis B virus X protein is both a substrate and a potential inhibitor of the proteasome

Huang EY, Madireddi MT, Gopalkrishnan RV, Leszczyniecka M, Su Z-Z, Lebedeva IV, Kang D-C, Jiang H, Lin JJ, Alexandre D, Chen Y, Vozhilla N, Mei MX, Christiansen KA, Sivo F, Goldstein NI, Mhashilkar AB, Chada S, Huberman E, Pestka S and Fisher PB (2001) Genomic structure, chromosomal localization and expression profile of a novel melanoma differentiation associated (mda-7) gene with cancer specific growth suppressing and apoptosis inducing properties. *Oncogene* 20, 7051-7063.

Huber EM, Basler M, Schwab R, Heinemeyer W, Kirk CJ, Groettrup M and Groll M (2012) Immuno- and constitutive proteasome crystal structures reveal differences in substrate and inhibitor specificity. *Cell* 148, 727-738.

Iordanov MS, Ryabinina OP, Wong J, Dinh T-H, Newton DL, Rybak SM and Magun BE (2000) Molecular determinants of apoptosis induced by the cytotoxic ribonuclease onconase: evidence for cytotoxic mechanisms different from inhibition of protein synthesis. *Cancer Res* 60, 1983-1994.

Irshad S, Mahul-Mellier AL, Kassouf N, Lemarie A and Grimm S (2009) Isolation of ORCTL3 in a novel genetic screen for tumor-specific apoptosis inducers. *Cell Death Diff* 16, 890-898.

Ito S, Tan LJ, Andoh D, Narita T, Seki M, Hirano Y, Narita K, Kuraoka I, Hiraoka Y and Tanaka K (2010) MMXD, a TFIIH-independent XPD-MMS19 protein complex involved in chromosome segregation. *Mol Cell* 39, 632-640.

James AM, Ambrose EJ and Lowick JH (1956) Differences between the electrical charge carried by normal and homologous tumour cells. *Nature* 177, 576-577.

Janssen K, Hofmann TG, Jans DA, Hay RT, Schulze-Osthoff and Fischer U (2007) Apoptin is modified by SUMO conjugation and targeted to promyelocytic leukemia protein nuclear bodies. *Oncogene* 26, 1557-1566.

Jiang H, Lin JJ, Su ZZ, Goldstein NI and Fisher PB (1995) Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene* 11, 2477-2486.

Jiang J, Cole D, Westwood N, Macpherson L, Farzaneh F, Mufti G, Tavassoli M and Gaken J (2010) Crucial roles for protein kinase C isoforms in tumor-specific killing by apoptin. *Cancer Res* 70, 7242-7252.

Jin JL, Gong J, Yin TJ, Lu YH, Xia YY, Di Y, He L, Guo JL, Sun J, Noteborn MHM and Qu S (2011) PTD-apoptin protein and dacarbazine show a synergistic antitumor effecto on B16-F1 melanoma in vitro and in vivo. *Eur J Pharmacol* 654, 17-25.

Johnson LN and Lewis RJ (2001) Structural basis for control by phosphorylation. *Chem Rev.* 101, 2209-2242.

Johnstone RW, Frew A and Smyth MF (2008) The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nat Rev Cancer* 8, 782-798.

Joshi J, Fernandez-Marcos PJ, Galvez A, Amanchy R, Linares JF, Duran A, Pathrose P, Leitges M, Canamero M, Collado M, Salas C, Serrano M, Moscat J and Diaz-Meco MT (2008) Par-4 inhibitis Akt and suppresses Ras-induced lung tumorigenesis. *EMBO J* 27, 2181.

Kalb VF and Bernlohr RW (1977) A new spectrophotometric assay for protein in cell extracts. *Anal Biochem* 82, 362-371.

Kastan MB and Bartek J (2004) Cell-cycle checkpoints and cancer. *Nature* 432, 316-323.

Kato A, Tanaka M, Yamamoto M, Asai R, Sata T, Nishiyama Y and Kawaguchi Y (2008) Identification of a physiological phosphorylation site of the herpes simplex virus 1-encoded protein kinase Us3 which regulates its optimal catalytic activity in vitro and influences its function in infected cells. *J Virol* 82, 6172-6189.

Kepp O, Galluzzi L, Lipinski M, Yuan J and Kroemer G (2011) Cell death assays for drug discovery. *Nat Rev Drug Discov* 10, 221-237.

Kessler BM, Tortorella D, Altun M, Kisselev AF, Fiebiger E, Hekking BG, Ploegh HL and Overkleeft HS (2001) Extended peptide-based inhibitors efficiently target the proteasome and reveal overlapping specificities of the catalytic b-subunits. *Chem Biol* 8, 913–929.

Kier AB (1990) Membrane properties of metastatic and non-metastatic cells cultured from C3H mice injected with LM fibroblasts. *Biochim Biophys Acta* 1022, 365-372.

Kim DY, Kim MJ, Kim HB, Lee JW, Bae JH, Kim DW, Kang CD and Kim SH (2011) Suppression of multidrug resistance by treatment with TRAIL in human ovarian and breast cancer cells with high level of c-Myc. *Biochem Biophys Acta* 1812, 796-805.

Kisselev AF, Callard A and Goldberg AL (2006) Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate. *J Biol Chem* 281, 8582–8590.

Kitching R, Li H, Wong MJ, Kanaganayakam S, Kahn H and Seth A (2003) Characterization of a novel human breast cancer associated gene (BCA3) encoding an alternative spliced proline-rich protein. *Biochim Biophys Acta* 1625, 116-121.

Kivovich V, Gilbert L, Vuento M and Naides SJ (2012) The putative metal coordination motif in the endonuclease domain of human parvovirus B19 NS1 is critical for NS1 induced S phase arrest and DNA damage. *Int J Biol Sci* 8, 79-92.

Klantrit P, Flinterman MB, Odell EW, Melino G, Killick R, Norris JS and Tavassoli M (2008) Specific isoforms of p73 control the induction of cell death induced by the viral proteins, E1A or apoptin. *Cell Cycle* 7, 201-215.

Kong M, Fox CJ, Mu J, Solt L, Xu A, Cinalli RM, Birnbaum MJ, Lindsten T and Thompson CB (2004) The PP2A-associated protein alpha4 is an essential inhibitor of apoptosis. *Science* 306, 695-698.

Kooistra K (2007) Viral elements inducing tumor-related apoptosis. PhD Thesis, Leiden University, Leiden, The Netherlands.

Koppenol WH, Bounds PL and Dang CV (2011) Otto Warburg's contributions to current concepts of cancer metabolism. *Nat Rev Cancer* 11, 325-337.

Kops GJPL, Weaver BAA and Cleveland DW (2005) On the road to cancer: aneuploidy and the mitotic checkpoint. *Nat Rev Cancer* 5, 773-785.

Kornitzer D, Sharf R and Kleinberger T (2001) Adenovirus E4orf4 protein induces PP2A-dependent growth arrest in Saccharomyces cerevisiae and interacts with the anaphase-promoting complex/cyclosome. *J Cell Biol* 154, 331-344.

Kroemer G, Gelluzzi L, Vandenabeele P, Abrams J, Alnemri ES, Baehrecke EH, Blagosklonny MV, El-Deiry WS, Golstein P, Green DR, Hengartner M, Knight RA, Kumar S, Lipton SA, Malorni W, Nunez G, Peter ME, Tschopp J, Yuan J, Piancentini M, Zhivotovsky B and Melino G (2009) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death Differ* 16, 3-11.

Kucharski TJ, Gamache I, Gjoerup O and Teodoro JG (2011) DNA damage response signaling triggers nuclear localization of the chicken anemia virus protein Apoptin. *J Virol* 85, 12638-12649.

Lanz HL, Florea BI, Noteborn MHM and Backendorf C (2012a) Development and application of an in vitro apoptin kinase assay. *Anal Biochem* 421, 68-74.

Lanz HL, Suijker J, Noteborn MHM and Backendorf C (2012b) Proteasomal insensitivity of apoptin in tumor cells. *Biochem Biophys Res Commun* 422, 169-173.

Lavoie JN, Champagne C, Gingras MC and Robert A (2000) Adenovirus E4 open reading frame 4-induced apoptosis involves dysregulation of Src family kinases. *J Cell Biol* 150, 1037-1056.

LeBlanc HN and Ashkenazi A (2003) Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ* 10, 66-75.

Lee YH, Cheng CM, Chang YF, Wang TY and You CY (2007) Apoptin T108 phosphorylation is not required for its tumor-specific nuclear localization but partially affects its apoptotic activity. *Biochem Biophys Res Commun* 354, 391-395.

Lee JE and Raines RT (2008) Ribonucleases as novel chemotherapeutics: the ranpirnase example. *BioDrugs* 22, 53-58.

Lee R (2011) The outlook for population growth. *Science* 333, 569-573.

Leliveld SR, Dame RT, Mommaas MA, Koerten HK, Wyman C, Danen-van Oorschot AA, Rohn JL, Noteborn MHM and Abrahams J-P (2003a) Apoptin protein multimers form distinct higher-order nucleoprotein complexes with DNA. *Nucleic Acids Res* 31, 4805-4813.

Leliveld SR, Zhang Y-H, Rohn JL, Noteborn MHM and Abrahams J-P (2003b) Apoptin induces tumor-specific apoptosis as a globular multimer. *J Biol Chem* 278, 9042-9051.

Lemmon MA and Schlessinger J (2010) Cell signaling by receptor tyrosine kinases. *Cell* 141, 1117-1134.

Levine AJ, Momand J and Finlay CA (1991) The p53 tumour suppressor gene. *Nature* 351, 453-456.

Li H, Cai X, Shouse GP, Piluso LG and Liu X (2007) A specific PP2A regulatory subunit, B56gamma, mediates DNA damage-induced dephosphorylation of p53 at Thr55. *EMBO J* 26, 402–411.

Li S, Szymborski A, Miron MJ, Marcellus R, Binda O, Lavoie JN and Brandton PE (2009a) The adenovirus E4orf4 protein induces growth arrest and mitotic catastrophe in H1299 human lung carcinoma cells. Oncogene 28, 390-400.

Li S, Brignole C, Marcellus R, Thirlwell S, Binda O, McQuoid MJ, Ashby D, Chan H, Zhang Z, Miron M-J, Pallas DC and Branton PE (2009b) The adenovirus E4orf4 protein induces G2/M arrest and cell death by blocking protein phosphatases 2A activity regulated by the B55 subunit. *J Virol* 83, 8340-8352.

Li X, Liu Y, Wen Z, Li C, Lu H, Tian M, Jin K, Sun L, Gao P, Yang E, Xu X, Kan S, Wang Z, Wang Y and Jin N (2010) Potent anti-tumor effects of a dual specific oncolytic adenovirus expressing apoptin in vitro and in vivo. *Mol Cancer* 9, 10.

Li JT, Bian K, Zhang AL, Kim DH, Ashley WW, Nath R, McCutcheon I, Fang B and Murad F (2011a) Targeting different types of human meningioma and glioma cells using a novel adenoviral vector expressing GFP-TRAIL fusion protein from hTERT promoter. *Cancer Cell Int* 11, 35.

Li XY, Li Y, Zhang Y, Wang K, Yuan X, Jin J, Zhang Y, Liu ZZ and Chen XG (2011b) A novel bisindolymaleimide derivative (WK234) inhibits proliferation and induces apoptosis through the protein kinase Cβ pathway, in chronic myelogenous leukemia K562 cells. *Leuk Lymphoma* 52, 1312-1320.

Li Q, Dong Q and Wang E (2012) Rsf-1 is overexpressed in non-small cell lung cancers and regulates cyclinD1 expression and ERK activity. *Biochem Biophys Res Commun* 420, 6-10.

Lipkowitz S and Weissman AM (2011) RINGs of good and evil: RING finger ubiquitin ligases at the crossroads of tumour suppression and oncogenesis. *Nat Rev Cancer* 11, 629-643.

Liu X, Zeidan YH, Elojeimy S, Holman DH, El-Zawahry AM, Guo GW, Bielawska A, Bilawski J, Szulc Z, Rubinchik S, Dong JY, Keane TE, Tavassoli M, Hannun YA and Norris JS (2006) Involvement of sphingolipids in apoptin-induced cell killing. *Mol Ther* 14, 627-636.

Liu Y and Ye Y (2011) Proteostasis regulation at the endoplasmic reticulum: a new perturbation site for targeted cancer therapy. *Cell Res* 21, 867-883.

Lochner A and Moolman JA (2006) The many faces of H89: a review. *Cardiovasc Drug Rev* 24, 261-274.

Luo J, Solimini NL and Elledge SJ (2009) Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell* 136, 823-837.

Maddika S, Booy EP, Johar D, Gibson SB, Ghavami S and Los M (2005) Cancer-specific toxicity of apoptin is independent of death receptors but involves the loss of mitochondrial membrane potential and the release of mitochondrial cell-death mediators by a Nur77-dependent pathway. *J Cell Sci* 118, 4485-4493.

Maddika S, Mendosa FJ, Hauff K, Zamzow CR, Paranjothy T and Los M (2006) Cancer-selective therapy of the future: apoptin and its mechanism of action. *Cancer Biol Ther* 5, 10-19.

Maddika S, Bay GH, Kroczak TJ, Ande SR, Maddika S, Wiechec E, Gibson SB and Los M (2007) Akt is transferred to the nucleus of cells treated with apoptin, and it participates in apoptin-induced cell death. *Cell Prolif* 40, 835-848.

Maddika S, Wiechec E, Ande SR, Poon IK, Fisher U, Wesselborg S, Jans DA, Schulze-Osthoff K and Los M (2008) Interaction with PI3-kinase contrigutes to the cytotoxic activity of apoptin. *Oncogene* 27, 3060-3065.

Maddika S, Panigrahi S, Wiechec E, Wesselborg S, Fischer U, Schulze-Osthoff K and Los M (2009) Unscheduled Akt-triggered activation of cyclin-dependent kinase 2 as a key effector mechanism of apoptin's anticancer toxicity. *Mol Cell Biol* 29, 1235-1248.

Maggi F, Macera L, Focosi D, Vatteroni ML, Boggi U, Antonelli G, Eloit M and Pistello M (2012) Human gyrovirus DNA in human blood, Italy. *Emerg Infect Dis* 18, 956-969.

Mahalingam D, Szegezdi E, Keane M, de Jong S and Samali A (2009) TRAIL receptor signaling and modulation: Are we on the right TRAIL? *Cancer Treat Rev* 35, 280-288.

Manning G, Whyte DB, Martinez R, Hunter R and Sudarsanam S (2002) The protein kinase complement of the human genome. *Science* 298, 1912-1934.

Masuda H, Miller C, Koeffler HP, Battifora H and Cline MJ (1987) Rearrangement of the p53 gene in human osteogenic sarcomas. *Proc Natl Acad Sci USA* 84, 7716-7719.

Mincberg M, Gopas J and Tal J (2011) Minute virus of mice (MVMp) infection and NS1 expression induce p53 independent apoptosis in transformed rat fibroblast cells. *Virology* 412, 233-243.

Miyahara R, Banerjee S, Kawano K, Efferson C, Tsuda N, Miyahara Y, Ionnides CG, Chada S and Ramesh R (2006) melanoma differentiation-associated gene-7 (*mda-7*)/interleukin(*IL)*-24 induces anticancer immunity in a syngeneic murine model. *Cancer Gene Ther* 13, 753-761.

Moehler MH, Zeidler M, Wilsberg V, Cornelis JJ, Woelfel T, Rommelaere J, Galle PR and Heike M (2005) Parvovirus H-1-induced tumor cell death enhances human immune response in vitro via increased phagocytosis, maturation, and cross-presentation by dendritic cells. *Hum Gene Ther* 16, 996-1005.

Mossberg AK, Wullt B, Gustafsson L, Mansson W, Ljunggren E and Svanborg C (2007) Bladder cancers respond to intravesical instillation of HAMLET (human α-lactalbumin made lethal to tumor cells). *Int J Cancer* 121, 1352-1359.

Mumby M (2007) PP2A: unveiling a reluctant tumor suppressor. *Cell* 130, 21-24.

Mungre S, Enderle K, Turk B, Porras A, Wu Y-Q, Mumby MC and Rundell K (1994) Mutations which affect the inhibition of protein phosphatase 2A by simian virus 40 small-t antigen in vitro decrease viral transformation. *J Virol* 68, 1975-1681.

Murata S, Sasaki K, Kishimoto T, Niwa S, Hayashi H, Takahama Y and Tanaka K (2007) Regulation of CD8+ T cell development by thymus-specific proteasomes. *Science* 316, 1349-1353.

Murata S, Yashiroda H and Tanaka K (2009) Molecular mechanisms of proteasome assembly. *Nat Rev Mol Cell Biol* 10, 104-115.

Musacchio A and Salmon ED (2007) The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol* 8, 379-393.

Nagakawa H, Shimozato O, Yu L, Wada A, Kawamura K, Li Q, Chada S, Tada Y, Takiguchi Y, Tatsumi K and Tagawa M (2012) Expression of a murine homolog of apoptosis-inducing human IL-24/MDA-7 in murine tumors fails to induce apoptosis or produce anti-tumor effects. *Cell Immunol* 275, 90-97.

Nakajima W and Tanaka N (2011) Noxa induces apoptosis in oncogene-expressing cells through catch-and-release mechanism operating between Puma and Mcl-1. *Biochem Biophys Res Commun* 413, 643-648.

Naviglio S, Caraglia M, Abbruzzese A, Chiosi E, Di Gesto D, Marra M, Romano M, Sorrentino A, Sorvillo L, Spina A and Illiano G (2009) Protein kinase A as a biological target in cancer therapy. *Expert Opin Ther Targets* 13, 83-92.

Negrini S, Gorgoulis VG and Halazonetis TD (2010) Genomic instability – an evolving hallmark of cancer. *Nat Rev Mol Cell Biol* 11, 220-228.

Neira JL, Roman-Trufero M Contreras LM, Prieto J, Singh G, Barrera FN, Renart ML and Vidal M (2009) The transcriptional repressor RYBP is a natively unfolded protein which folds upon binding to DNA. *Biochemistry* 48, 1348-1360.

Nesterov A, Nikrad M, Johnson T and Kraft AS (2004) Oncogenic Ras sensitizes normal human cells to tumor necrosis factor-alpha-related apoptosis-inducing ligand-induced apoptosis. *Cancer Res* 64, 3922-3927.

Nikiforov MA, Riblett M, Tang WH, Gratchouck V, Zhuang D, Fernandez Y, Verhaegen M, Varambally S, Chinnaiyan AM, Jakubowiak AJ and Soengas MS (2007) Tumor cell-selective regulation of NOXA by c-MYC in response to proteasome inhibition. *Proc natl Acad Sci USA* 104, 19488-19493.

Noteborn MHM, de Boer GF, van Roozelaar DJ, Karreman C, Kranenburg O, Vos JG, Jeurissen SH, Hoeben RC, Zantema A and Koch G (1991) Characterization of cloned chicken anemia virus DNA that contains all elements for the infectious replication cycle. *J Virol* 65, 3131-3139.

Noteborn MHM, Todd D, Verschueren CA, de Gauw HW, Curran WL, Veldkamp S, douglas AJ, McNult MS, van der Eb AJ and Koch G (1994) A single chicken anemia virus protein induces apoptosis. *J Virol* 68, 346-351.

Noteborn MHM (2009a) Apoptosis and cancer closely linked. In Proteins killing tumour cells, Eds Backendorf C, Noteborn MHM and Tavassoli M, Transworld Research Network, pp 1-17

Noteborn MHM (2009b) Proteins selectively killing tumor cells. *Eur J Pharmacol* 625, 165-173.

Nuesch JPF and Rommelaere J (2007) A viral adaptor protein modulating casein kinase II activity induces cytopathic effects in permissive cells. *Proc Natl Acad Sci USA* 104, 12482-12487.

Oeppen J and Vaupel JW (2002) Broken limits to life expectancy. *Science* 296, 1029-1031.

Ohshima-Hosoyama S, Davare MA, Hosoyama T, Nelon LD and Keller C (2011) Bortezomib stabilizes NOXA and triggers ROS-ossociated apoptosis in medulloblastoma. *J Neurooncol* 105, 475-483.

Osterrieder N, Kamil JP, Schumacher D, Tischer BK and Trapp S (2006) Marek's disease virus: from miasma to model. *Nat Rev Microbiol* 4, 283-294.

Pavet V, Portal MM, Moulin JC, Herbrecht R and Gronemeyer H (2011) Towards novel paradigms for cancer therapy. *Oncogene* 30, 1-20.

Pelicano H, Martin DS, Xu R-H and Huang P (2006) Glycolysis inhibition for anticancer treatment. *Oncogene* 25, 4633-4646.

Phan TG, Li L, O'Ryan MG, Cortes H, Mamani N, Bonkoungou IJ, Wang C, Leutenegger CM and Delwart E (2012) A third gyrovirus species in human faeces. *J Gen Virol* 93, 1356-1361.

Pihan GA and Doxsey SJ (1999) The mitotic machinery as a source of genetic instability in cancer. *Semin Cancer Biol.* 4, 289-302.

Pipas J (2009) SV40: Cell transformation and tumorigenesis. *Virology* 384, 294-303.

Ploner C, Kofler R and Villunger A (2009) Noxa: at the tip of the balance between life and death. *Oncogene* 27, S84-S92.

Poon IKH, Oro C, Dias MM, Zhang J and Jans DA (2005) Apoptin nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells. *Cancer Res* 65, 7059-7064.

Pylayeva-Gupta Y, Grabocka E and Bar-Sagi D (2011) RAS oncogenes: weaving a tumorigenic web. *Nat Rev Cancer* 11, 761-774.

Qiao M, Zu LD, He XH, Shen RL, Wang QC and Liu MF (2012) Onconase downregulates microRNA expression through targeting microRNA precursors. *Cell Res* 22, 1199-1202.

Qiu SG, Krishnan S, el-Guendy N and Rangenkar VM (1999) Negative regulation of Par-4 by oncogenic Ras is essential for cellular transformation. *Oncogene* 18, 7115-7123.

Rayet B and Gelinas C (1999) Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* 18, 6938-6947.

Rello-Varona S, Kepp O, Vitale I, Michaud M, Senovilla L, Jemaa M, Joza N, Galluzzi L, Castedo M and Kroemer G (2010) An automated fluorescence videomicroscopy assay for the detection of mitotic catastrophe. *Cell Death Dis* 1, e25.

Ricci MS, Jin Z, Dews M, Yu D, Thomas-Tikhonenko A, Dicker DT and El-Deiry WS (2004) Direct repression of FLIP expression by c-myc is a major determinant of TRAIL sensitivity. *Mol Cell Biol* 24, 8541-8555.

Riedl S, Zweytick D and Lohner K (2011) Membrane-active host defense peptides – challenges and perspectives for the development of novel anticancer drugs. *Chem Phys Lipids* 164, 766-781.

Rijsewijk FAM, Dos Santos HF, Teixeira TF, Cibulski SP, Varela AP, Dezen D, Franco AC and Roehe PM (2011) Discovery of a genome of a distant relative of chicken anemia virus reveals a new member of the genus Gyrovirus. *Arch Virol* 156, 1097-1100.

Rohn JL, Zhang Y-H, Aalbers RI, Otto N, Den Hertog J, henriquez NV, van de Velde CJ, Kuppen PJ, Mumberf D, Donner P and Noteborn MHM (2002) A tumor-specific kinase activity regulates the viral death protein Apoptin. *J Biol Chem* 277, 50820-50827.

Rohn JL and Noteborn MHM (2004) The viral death effector apoptin reveals tumor-specific processes. *Apoptosis* 9, 315-322.

Rohn JL, Zhang Y-H, Leliveld SR, Danen-van Oorschot AAAM, Henriquez NV, Abrahams J-P and Noteborn MHM (2005) Relevance of apoptin's integrity for its functional behavior. *J Virol* 79, 1337-1338.

Rommelaere J and Cornelis JJ (1991) Antineoplastic activity of parvoviruses. *J Virol Methods* 33, 233-251.

Rommelaere J, Geletneky K, Angelova AL, Daeffler L, Dinsart C, Kiprianova I, Schlehofer JR and Raykov Z (2010) Oncolytic parvoviruses as cancer therapeutics. *Cytokine Growth Factor Rev* 21, 185/195.

Roninson IB, Broude EV 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.

Rybak SM, Arndt MA, Schirrmann T, Dubel S and Krauss J (2009) Ribonucleases and immunoRNases as anticancer drugs. *Curr Pharm Des* 15, 2665-2675.

Sablina AA and Hahn WC (2008) SV40 small T antigen and PP2A phosphatase in cell transformation. *Cancer Metastasis Rev* 27, 137-146.

Sablina AA, Hector M, Colpaert N and Hahn WC (2008) SV40 small antigen and PP2A phosphatase in cell transformation. *Cancer Res* 70 10474-10484.

Sastri M, Barraclough DM, Carmichael PT and Taylor SS (2005) A kinase-interacting protein localizes protein kinase A in the nucleus. *Proc Natl Acad Sci USA* 102, 349-354.

Sato M, Yao VJ, Arap W and Pasqualini R (2010) GRP78 signaling hub a receptor for targeted tumor therapy. *Adv Genet* 69, 97-114.

Sauane M, Su Z-Z, Gupta P, Lebedeva IV, Dent P, Sarkar D and Fisher PB (2008) Autocrine regulation of mda-7/IL-24 mediates cacner-specific apoptosis. *Proc natl Acad Sci USA* 105, 9763-9768.

Sauvage V, Cheval J, Foulongne V, Gouilh MA, Pariente K, Manuguerra JC, Richardson J, Dereure O, Lecuit M, Burguiere A, Caro V and Eloit M (2011) Identification of the first human gyrovirus, a virus related to chicken anemia virus. *J Virol* 85, 7948-7950.

Schmidt TGM and Skerra A (2007) The Strep-tag system for one-strep purification and high-affinity detection or capturing of proteins. *Nature Protocols* 2, 1528-1535.

Scholey JM, Brust-Mascher I and Mogilner A (2003) Cell division. *Nature* 422, 746-752.

Schreiber RD, Old LJ and Smyth MJ (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331, 1565-1570.

Schug ZT, Frezza C, Galbraith LC and Gottlieb E (2012) The music of lipids: how lipid composition orchestrates cellular behaviour. *Acta Oncol* 51, 301-310.

Sells SF, Wood DP, Joshi-Barve SS, Muthukumar S, Jacob RJ, Crist SA, Humphreys S and Rangnekar VM (1994) Commonality of the gene programs induced by effectors of apoptosis in androgen-dependent and –independent prostate cells. *Cell Growth Differ* 5, 457-466.

Shaner NC, Campbell RE, Steinback PA, Giepmans BNG, Palmer AE and Tsien RY (2004) Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol* 22, 1567-1572.

Shaw RJ & Cantley LC (2006) Ras, PI(3)K and mTOR signaling controls tumour cell growth. *Nature* 441, 424-430.

Sheng JF, Chen W, Yu Y, Liu J and Tao ZZ (2010) PAR-4 and hTERT expression are negatively correlated after RNA interference targeting hTERT in laryngocarcinoma cells. *Tissue Cell* 42, 365-369.

Sheu JJ, Guan, B, Choi JH, Lin A, Lee CH, Hsiao YT, Wang TL, Tsai FJ and Shih IM (2010) Rsf-1, a chromatin remodelling protein, induces DNA damage and promotes genomic instability. *J Biol Chem* 285, 38260-38269.

Shi Y (2009) Serine/Threonine phosphatases: Mechanism through structure. *Cell* 139, 468-484.

Shrestha-Bhattarai T and Rangnekar VM (2010) Cancer-selective apoptotic effects of extracellular and intracellular Par-4. *Oncogene* 29, 3873-3880.

Smolders L and Teodoro JG (2011) Targeting the anaphase promoting complex: common pathways for viral infection and cancer therapy.

Sparmann A and Bar-Sagi D (2004) Ras-induced interleukin-8 expression plays a critical role in tumor growth and angiogenesis. *Cancer Cell* 6, 447-458.

Srinivasan A, McClellan AJ, Vartikar J, Marks I, Cantalupo P, Li Y, Whyte P, Rundell K, Brodsky JL and Pipas JM (1997) The amino-terminal transforming region of simian virus 40 large T and small t antigens functions as a J domain. *Mol Cell Biol* 17, 4761-4773.

Storm P, Aits S, Puthia MK, Urbano A, Northen T, Powers S, Bowen B, Chao Y, Reindl W, Lee DY, Sullivan NL, Zhang J, Trulsson M, Yang H, Watson JD and Svanborg C (2011) Conserved features of cancer cells define their sensitivity to HAMLET-induced death; c-Myc and glycolysis. *Oncogene* 30, 4765-4779.

Su Z-Z, Madireddi MT, Lin JJ, Young CSH, Kitada S, Reed JC, Goldstein MI and Fisher PB (1998) The cancer growth suppressor gene *mda*-7 selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice. *Proc Natl Acad Sci USA* 95, 14400-144405.

Sun J, Yan Y, Wang XT, Liu XW, Peng DJ, Wang M, Tian J, Zong YQ, Zhang YH, Noteborn MHM and Qu S (2009) PTD4-apoptin protein therapy inhibits tumor growth in vivo. *Int J Cancer* 124, 2973-2981.

Suzuki S, Nakasato M, Shibue T, Koshima I and Taniguchi T (2009) Therapeutic potential of proapoptotic molecule Noxa in the selective elimination of tumor cels. *Cancer Sci* 100, 759-769.

Taebunpakul P, Sayan BS, Flinterman M, Klanrit P, Gaken J, Odell EW, Melino G and Tavassoli M (2012) Apoptin induces apoptosis by changing the equilibrium between the stability of Tap73 and ΔNp73 isoforms through ubiquitin ligase PIR2. *Apoptosis* 17, 762-776.

Tait SWG and Green DR (2010) Mitochondria and cell death: Outer membrane permeabilization and beyond. *Nat Rev Mol Cell Biol* 11, 621-632.

Tavassoli M, Guelen L, Luxon BA and Gäken J (2005) Apoptin: specific killer or tumor cells? *Apoptosis* 10, 717-724.

Teodoro JG, Heilman DW, Parker AE and Green MR (2004) The viral protein apoptin associates with the anaphase-promoting complex to induce G2/M arrest and apoptosis in the absence of p53. *Genes Dev* 18, 1952-1957.

Usui H, Inoue R, Tanabe O, Nishito Y, Shimizu M, Hayashi H, Kagamiyama H and Takeda M (1998) Activation of protein phosphatase 2A by cAMP-dependent protein kinase-catalyzed phosphorylation of the 74-kDa B" (delta) regulatory subunit in vitro and identification of the phosphorylation sites. *FEBS Lett* 430, 312-316.

Vafa O, Wade M, Kern S, Beeche M, Pandita TK, Hampton GM and Wahl GM (2002) c-Myc can induce DNA damage, increase reactive oxygen species, and mitigate p53 function: a mechanism for oncogenes-induced genetic instability. *Mol Cell* 9, 1031-1044.

Valastyan S and Weinberg RA (2011) Tumor metastasis: molecular insights and evolving paradigms. *Cell* 147, 275-292.

Valero V, Wingate H, Chada S, Liu Y, Palalon F, Mills G, Keyomarsi K and Hunt KK (2011) MDA-7 results in downregulation of AKT concomitant with apoptosis and cell cycle arrest in breast cancer cells. *Cancer Gene Ther* 18, 510-519.

Vermeij WP, Alia A and Backendorf C (2011) ROS quenching potential of the epidermal cornified cell envelope. *J Invest Dermatol* 7, 1435-1441.

Vermeij WP, Florea BI, Isenia S, Alia A, Brouwer J and Backendorf C (2012) Proteomic identification of in vivo interactors reveals novel functions of skin cornification proteins. *J Proteome Res* in print.

Virshup DM and Shenolikar S (2009) From promiscuity to precision: protein phosphatases get a makeover. *Mol Cell* 33, 537-545.

Vitale I, Galluzzi L, Castedo M and Kroemer G (2011) Mitotic catastrophe: a mechanism for avoiding genomic instability. *Nat Rev Mol Cell Biol* 12, 385-392.

Vivanco I and Sawyers CL (2002) The phosphatidylinositol3-kinase-akt pathway in human cancer. *Nat Rev Cancer* 2, 489-501.

Walczak H, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC and Lynch DH (1999) Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 5, 157-163.

Wang S and El-Deiry WS (2003) TRAIL and apoptosis induction by TNF-family death receptors. *Oncogene* 22, 8628-8633.

Wang Y, Engels IH, Knee DA, Nasoff M, Deveraux QL and Quon KC (2004) Synthetic lethal targeting of MYC by activation of the DR5 death receptor pathway. *Cancer Cell* 5, 501-512.

Wang G, Ahmad KA and Ahmed K (2005) Modulation of death receptor-mediated apoptosis by CK2. *Mol Cell Biochem* 274, 201-205.

Weaver BA and Cleveland DW (2005) Decoding the links between mitosis, cancer and chemotherapy: The mitotic checkpoint, adaptation and cell death. *Cancer Cell* 8, 7-12.

Weis SM and Cheresh DA (2011) Tumor angiogenesis: molecular pathways and therapeutic targets. *Nat Med* 17, 1359-1370.

Wenner CE (2010) Cell signaling and cancer-possible targets for therapy. *J Cell Physiol* 223, 299-308.

Westermarck J and Hahn WC (2008) Multiple pathways regulated by the tumor suppressor PP2A in transformation. *Trends Mol Med* 14, 152-160.

Westphal D, Dewson G, Czabotar PE and Kluck RM (2011) Molecular biology of Bax and Bak activation and action. *Biochim Biophys Acta* 1813, 521-531.

Whitfield JR and Soucek L (2012) tumor microenvironment: becoming sick of Myc. *Cell Mol Life Sci* 69, 931-934.

Yamashita K, Ikenaka Y, Kakutani T, Kawaharada H and Watanabe K (1990) Comparison of human lymphotoxin gene expression in CHO cells directed by genomic DNA or cDNA sequences. *Agric Biol Chem* 54, 2801-2809.

Yuan J, Luo K, Zhang L, Cheville JC and Lou Z (2010) USP10 regulates p53 localization and stability by deubiquitinating p53. *Cell* 140, 384–396.

Zhang Y-H, Leliveld SR, Kooistra K, Molenaar C, Rohn JL, Tanke HJ, Abrahams J-P and Noteborn MHM (2003) Recombinant Apoptin multimers kill tumor cells but are nontoxic and epitope-shielded in a norml-cell-specific fashion. *Exp Cell Res* 289, 36-46.

Zhang Y-H, Kooistra K, Pietersen A, Rohn JL and Noteborn MHM (2004) Activation of the tumor-specific death effector apoptin and its kinase by an N-terminal determinant of simian virus 40 large T antigen. *J Virol* 78, 9965-9976.

Zhang RG, Zhao JJ, Yang LQ, Yang SM, Wang RQ, Chen WS, Peng GY and Fang DC (2010) RNA interference-mediated hTERT inhibition enhances TRAIL-induced apoptosis in resistant hepatocellular carcinoma cells. *Oncol Rep* 23, 1013-1019.

Zhang KJ, Qian J, Wang SB and Yang Y (2012) Targeting gene-viro-therapy with AFP driving apoptin gene shows potent antitumor effect in hepatocarcinoma. *J Biomed Sci* 19, 20.

Zhao RY and Elder, RT (2005) Viral infections and cell cycle G2/M regulation. *Cell Res* 115, 143-149.

Zhao Y, Burikhanov R, Brandon J, Qiu S, Shelton BJ, Spear B, Bondada S, Bryson S and Rangnekar VM (2011) Systemic Par-4 inhibits non-autochthonous tumor growth. *Cancer Biol Ther* 12, 152-157.

Zhou Y, Chen H, Ma XL, Xie HJ, Wang CL, Zhang SH, Wang X and Huang BR (2009) Fusion protein of adenovirus E4orf4 and human epidermal growth factor inhibits tumor cell growth. *Int J Cancer* 125, 1186-1192.

Zhuang SM, Shvarts A, van Ormondt H, Jochemsen AG, van der Eb AJ and Noteborn MHM (1995) Apoptin, a protein derived from chicken anemia virus, induces p53-independent apoptosis in human osteosarcoma cells. *Cancer Res* 55, 486-489.

Zimmerman RME (2011) Apoptin: Oncogenic transformation and tumor-selective apoptosis. PhD Thesis, Leiden University.

Zimmerman RME, Peng D-J, Lanz HL, Zhang Y-H, Danen-van Oorschot AAAM, Qu S, Backendorf C and Noteborn MHM (2012) PP2A inactivation is a crucial step in triggering apoptin-induced tumor-selective killing. *Cell Death Dis* 3, e291.

Samenvatting

Kanker is een verstoring van de balans tussen het delen en het natuurlijk afsterven van cellen. De transformatie van een gezonde naar een kankercel is een meervoudig proces. Transformatie bestaat uit een opeenstapeling van kleine verstoringen in belangrijke cellulaire processen. Voor elke kanker is het pakket aan verstoringen uniek, alleen het resultaat lijkt gelijk. In 2000 zijn de zogenaamde 'hallmarks of cancer' geïntroduceerd. Deze benoemen de specifieke veranderingen die altijd nodig zijn voor de formatie van een kankercel, onwillekeurig hoe deze zich op moleculair niveau hebben voltrokken. **Hoofdstuk 2** begint met de omschrijving van de huidige 'hallmarks of cancer', waaronder het ontduiken van geprogrammeerde celdood en het immuunsysteem, activering van eindeloos delen en het in staat stellen tot uitzaaiingen.

Een groot struikelblok bij de behandeling van kanker is het gebrek aan selectiviteit binnen de huidige medicatie. Medicijnen die ingrijpen in bepaalde ontregelde processen beïnvloeden ook processen in gezonde cellen. In **hoofdstuk 2** wordt een groep eiwitten geïntroduceerd genaamd 'Proteins Killing Tumor Cells' (PKTCs, eiwitten die kankercellen vermoorden). Expressie van deze eiwitten leidt selectief in tumorcellen tot de inductie van gecontroleerde celdood (apoptose), terwijl normale cellen ongemoeid worden gelaten.

Het doel van dit proefschrift is het ontrafelen van de gedereguleerde cellulaire processen die worden herkend door apoptin, de eerste van de PKTCs. Deze processen differentiëren tussen normale en tumorcellen en vormen een aangrijppunt voor nieuwe therapieën.

Apoptin is een klein eiwit dat wordt gemaakt door een kippenvirus, Chicken Anemia Virus (CAV). Voor kuikens is in het veld besmetting met dit virus, veelal in combinatie met het transformerende Marek's disease virus, dodelijk doordat het apoptose induceert in de thymus (zwezerik). Verantwoordelijk voor de dood van in-vitro getransformeerde Marek's disease virus cellen bleek het derde eiwit van CAV te zijn, apoptin. Apoptin bleek selectief celdood te induceren in menselijke kankercellen. CAV is niet besmettelijk voor mensen. Experimenteel kan het apoptin-eiwit wel tot expressie worden gebracht in menselijke cellen m.b.v. plasmiden, en geproduceerd apoptin-eiwit kan in cellen worden geïntroduceerd.

Apoptin heeft een aantal karakteristieken die verschillend zijn tussen normale en tumorcellen. Meest kenmerkend is dat apoptin onschadelijk is voor normale cellen, maar zeer toxisch voor kankercellen. In gezonde cellen bevindt apoptin zich in het cytoplasma,

terwijl in getransformeerde cellen het apoptin-eiwit zich verplaatst naar de kern van de cel. Wanneer apoptin met een bepaalde techniek tijdelijk tot expressie wordt gebracht in humane cellen valt het op dat het eiwit snel niet meer te detecteren is in normale cellen, terwijl het in tumorcellen lang stabiel blijft. In **hoofdstuk 4** laten wij zien dat dit komt omdat in kankercellen het apoptin-eiwit niet langer wordt afgebroken door het proteasoom.

Verschillende cellulaire situaties vragen om verschillende eiwitcomposities in de cel. Om hier flexibel mee om te gaan worden er continu bepaalde eiwitten aangemaakt, en ook weer afgebroken. Beide processen worden strikt gereguleerd. Alleen eiwitten gelabeld voor destructie worden 'uit het veld' genomen. Het proteasoom is een eiwitcomplex dat gelabelde eiwitten herkent en afbreekt tot kleinere, herbruikbare moleculen. Wanneer wij in normale cellen de werking van het proteasoom blokkeren met verschillende chemische verbindingen blijft de hoeveelheid apoptin stabiel. Dit impliceert dat het apoptin-eiwit normaal gesproken wordt afgebroken door het proteasoom. In tumorcellen heeft het blokkeren van de afbraak van eiwitten geen effect op de hoeveelheid apoptin. Dit is gunstig voor de ontwikkeling van apoptin als medicijn. Belangrijk bij de ontwikkeling van medicatie is dat deze ook weer verwijderd kan worden uit het lichaam. Doordat apoptin van nature instabiel is in normale cellen, en stabiel in tumoren, wordt zijn selectieve effect vergroot. Daarnaast duidt dit verschil in afbraak van apoptin op een algemene verstoring in de regulatie van eiwitniveaus in kankercellen.

Een andere methode die een cel tot zijn beschikking heeft om snel op veranderende situaties te reageren, is door eiwitten aan en uit te schakelen in plaats van te maken en af te breken. Het (de)activeren van eiwitten gebeurt voornamelijk door het koppelen van een fosfaatgroep aan een eiwit, fosforyleren. Apoptin wordt selectief in tumorcellen gefosforyleerd op Threonines 107 en 108 (**hoofdstuk 5**). De negatieve lading van een fosfaatgroep leidt vaak tot een structuurverandering die bijvoorbeeld een katalytisch deel van het eiwit blokkeert. In het geval van apoptin resulteert de fosforylering in kankercellen tot blokkering van het signaal voor transport de kern uit, en uiteindelijk tot het verschil in cellulaire lokalisatie.

Fosforylering van een eiwit wordt uitgevoerd door een kinase-eiwit. In **hoofdstuk 5** karakteriseren wij de kinase die verantwoordelijk is voor de fosforylering van apoptin. In de literatuur zijn twee kandidaten geopperd voor de rol van apoptin-kinase. Wij laten zien dat

beide niet voldoen aan de karakteristieken die door ons zijn bepaald, zoals de lokalisatie van de kinase in zowel het cytoplasma als de kern.

Door transformerende stappen na te bootsen in normale cellen kunnen we achterhalen welke veranderde processen leiden tot activering van apoptin in tumorcellen. In **hoofdstuk 3** leidt ontregeling van de fosfatase 'protein phosphatase 2A (PP2A)' in normale cellen tot activering van apoptin. Fosfatases zijn eiwitten die fosfaatgroepen verwijderen, de tegenhanger van de kinase. Wanneer een fosfatase niet langer correct werkt, blijven er bijvoorbeeld eiwitten actief die dat niet zouden moeten zijn.

Eén van de meest strikt gereguleerde processen in de cel en daardoor zeer gevoelig voor veranderingen die kunnen leiden tot transformatie, is de celdeling. Voordat één cel twee cellen kan worden moet al het DNA zorgvuldig worden gekopieerd. Vervolgens moeten deze kopieën correct worden verdeeld over de twee dochtercellen. Wanneer hier iets in misgaat, leidt dit tot genetische instabiliteit, één van de grootste drijfveren van transformatie. PP2A is betrokken bij de controle tijdens de celdeling (mitose). In kankercellen bevinden zich vaak mutaties in PP2A die kunnen leiden tot fouten in de mitose en een verdere stap in het transformatie proces. **Hoofdstuk 6** laat zien dat apoptin in kankercellen de verdeling van het DNA tijdens de celdeling grof verstoort, en juist in deze fase van de celcyclus celdood (apoptose) induceert via mitotische catastrofe. Waarschijnlijk herkent apoptin het gebrek aan controle tijdens de mitose en grijpt dan in.

In **hoofdstuk 7** vullen we de definities van de 'hallmarks of cancer' aan met de kennis die wordt verkregen uit het onderzoek naar de werking van apoptin en de rest van de PKTCs. Dankzij PKTCs krijgen we meer inzicht in kankerselectieve processen die gemanipuleerd kunnen worden om selectief celdood te induceren. Deze kennis zal een bijdrage leveren aan de ontwikkeling van verbeterde antikanker therapieën.

Curriculum Vitae

Henriëtte Leonore Lanz was born on the 1st of October 1983 in Leidschendam. In 2001, she graduated from the gymnasium at College 't Loo in Voorburg. Following a year of working abroad she started her Life, Science & Technology studies at Leiden University and Delft University of Technology in 2002. After obtaining her Bachelor degree in 2005, she received her Master degree in 2008. During her final master internship at the department of Molecular Genetics of the Leiden Institute of Chemistry under supervision of Prof. M. Noteborn and Dr. R. Zimmerman she was introduced to the research on apoptin. Following a succesful intership she was invited to continue her research in a PhD project entitled 'Cancer-related targets sensed by apoptin' supervised by Prof. M. Noteborn and Dr. C. Backendorf. The results of this project are presented in this thesis. In March 2012 she started working for Explant Biotechnologies BV, a company focussed on the production and analysis of secondary plant metabolities.

List of Publications

Lanz HL, Florea BI, Noteborn MHM and Backendorf C (2012) Development and application of an in vitro apoptin kinase assay. *Anal Biochem* 421, 68-74.

Lanz HL, Suijker J, Noteborn MHM and Backendorf C (2012) Proteasomal insensitivity of apoptin in tumor cells. *Biochem Biophys Res Commun* 422, 169-173.

Lanz HL, Zimmerman RME, Brouwer J, Noteborn MHM and Backendorf C (2012) Mitotic catastrophe triggered in human cancer cells by the viral protein apoptin. Manuscript submitted.

Zimmerman RME*, Peng D-J*, Lanz HL*, Zhang Y-H, Danen-van Oorschot AAAM, Qu S, Backendorf C and Noteborn MHM (2012) PP2A inactivation is a crucial step in triggering apoptin-induced tumor-selective killing. *Cell Death Dis* 3, e291. *contributed equally