The handle http://hdl.handle.net/1887/20021 holds various files of this Leiden University dissertation.

**Author:** Lanz, Henriette Leonore  
**Title:** Cancer-related targets sensed by apoptin  
**Issue Date:** 2012-10-24
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 anticancer 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).
Introduction: Proteins Killing Tumor Cells

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.

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).

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 tumorogenesis 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.](image)

**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 peptide 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-xL, 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).

![Diagram of Bcl-2 family balance](https://example.com/diagram)

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 Brazilian 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 gyroviruses (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.](http://www.ebi.ac.uk/Tools/msa/clustalw2/)
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-Beuren 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 paroviruses. The autonomous parovirus 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 (Minchberg 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
Introduction: Proteins Killing Tumor Cells

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).
Introduction: Proteins Killing Tumor Cells

**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 ORCT13 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 ORCT13.

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 xenografts (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
Chapter 2

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