# Apoptin : oncogenic transformation & tumor-selective apoptosis

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# Chapter 7

## Discussion, outlook and conclusions

## Mechanisms behind the tumor-specific apoptosis inducing protein apoptin: clues from apoptin-interacting proteins

## Abstract

With the discovery of apoptin's unique capability to induce tumor-specific apoptosis, came the promise of the development of a potentially powerful, yet safe and effective, novel concept in anti-cancer therapy. Efforts to uncover the mechanisms underlying the differential behavior of apoptin in normal and transformed cells identified a number of interacting proteins – including Dedaf in tumor cells, and Hippi in normal cells – while a central dogma emerged: in tumor cells, apoptin was postulated to be phosphorylated, move to the nucleus and induce apoptosis, whereas in normal cells, according to the same dogma, apoptin would not be phosphorylated, remained in the cytoplasm and would eventually be eliminated from the cell. Recent research presented in this thesis, has provided important new clues into the workings of this enigmatic protein. This chapter discusses these results against the backdrop of nearly two decades of apoptin research, highlighting the roles of new, key interacting partners of apoptin in the human cell. Novel insights into the pathways underlying apoptin cellular function are discussed, as well as how these could be used to improve cancer therapy.

**The discovery of apoptin and its establishment as a Protein Killing Tumor Cells**

As introduced in **chapter 2**, apoptin is a small, proline-rich, protein encoded by the Chicken Anemia Virus (CAV; figure 7.1). Infection of young chicks with CAV causes anemia and immunodeficiency due to apoptosis of bone marrow, splenic and thymus cells, and apoptin was identified as the agent responsible for the induction of apoptosis (Jeurissen, et al., 1992; Noteborn, et al., 1994; Noteborn and Koch, 1995). Upon over-expression in human tumor cells, apoptin was shown to induce apoptosis in these cells as well (Zhuang, et al., 1995, 1995b), and even more excitingly, it did not do so when overexpressed in normal human cells (Danen-van Oorschot, et al., 1997; Noteborn, et al., 1998).

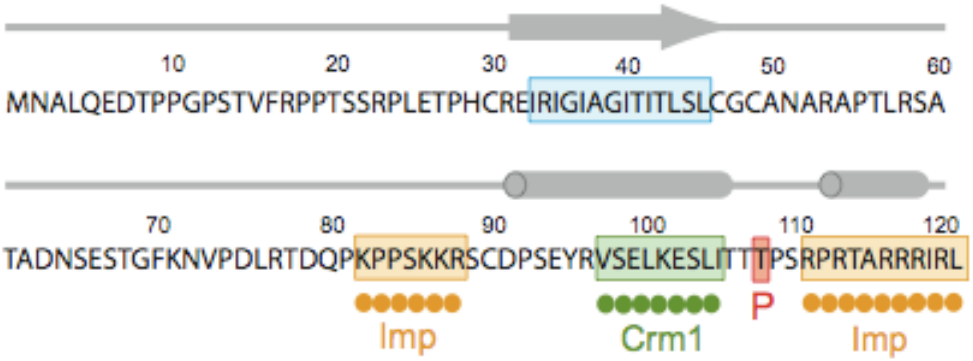


**Figure 7.1** Schematic representation of the apoptin protein. A putative β-strand and two α-helices, as predicted by the PSIPRED software, are indicated. The N-terminal (iso)leucine-rich, multimerization and protein-protein interaction domain is indicated in blue, the bi-partite NLS is indicated in yellow, surrounding the NES in green, and the T108 phosphorylation site in red. Adapted from Backendorf, et al., 2008.

Further comparison of the differential behavior of apoptin in normal and cancer cells revealed that apoptin had a cytoplasmic localization in the former, versus a nuclear localization in the latter (Danen-van Oorschot, et al., 1997). A bipartite nuclear localization signal (Poon, et al., 2005; Danen-van Oorschot, et al., 2003) surrounding a nuclear export signal was identified between amino acids 80-121 (Poon, et al., 2005b). Located precisely in this region, a threonine amino acid at position 108 was identified

as the target of a yet unknown kinase activity, which appeared to be present in tumor cells but lacking in normal cells (Rohn, et al., 2002). Hence, it was hypothesized that apoptin was able to shuttle between the nucleus and cytoplasm, accumulating in the nucleus of tumor cells upon phoshorylation of T108, which would block the nuclear export signal (NES) while leaving the nuclear location signal (NLS) exposed. In normal cells, it would remain in the cytoplasm as a result of the exposed NES, where it would eventually be degraded and eliminated from the cell (Zhang, et al., 2003). Poon et al. (2005b) postulated that the phosphorylation of apoptin would most likely take place in the (tumor) cell nucleus. This was, however, challenged by the fact that phosphorylated apoptin could also be found in the cytoplasm of tumor cells (Zhang, et al., 2004; R. Zimmerman, unpublished results), consistent with recent findings in our laboratory that apoptin kinase activity can be found in both the cytoplasm and nucleus of various cancer cell lines (Lanz, et al., 2012).

Immunoelectron microscopy determined that, within the nucleus, apoptin colocalized with heterochromatin and nucleoli, and apoptin was shown to bind DNA *in vitro* (Leliveld, et al., 2003, 2004). Intriguingly, however, experiments using actinomycin D, an RNA synthesis inhibitor, and two protein synthesis inhibitors (emetine and puromycin), demonstrated that apoptin's cell-killing effects did not require *de novo* transcription or translation (Danen-van Oorschot, et al., 2003). Biochemical analysis further showed that apoptin functioned as a multimeric protein, with each multimer consisting of approximately 20-40 monomeric subunits (Leliveld, et al., 2003). An N-terminal amphiphatic helix (amino acids 29-49) was identified as the multimerization domain; this multimerization domain was also postulated to be the interacting domain with other cellular proteins.

**Apoptin and its interacting proteins**

In an attempt to elucidate the molecular mechanisms involved in a) apoptin's discrimination between normal and human cells, and b) the subsequent induction of apoptosis, human cellular proteins interacting with apoptin

were sought and identified. Below, we recapitulate the most important interactions found in literature, as well as the novel interacting proteins presented in this thesis (see Table 7-1 at the end of this chapter).

*PI3-K and Akt*

Class I phosphatidylinositol 3-kinases (PI3-Ks) play a central role in various regulatory processes, such as cell growth, survival and differentiation. As discussed in **chapter 2**, they are also involved in autophagic cell death, and can function both to limit and promote tumor development. The downstream effectors of PI3K include the Akt kinase (Marte and Downward, 1997; Vanhaesebroeck and Waterfield, 1999), which modulates the function of numerous substrates related to the regulation of cell proliferation, such as cyclin-dependent kinase inhibitors, p21Cip1/Waf1 (Li, et al., 2002), p27kip1, (Fruman, et al., 1998; Song, et al., 2005) and Nur77 (Pekarsky, et al., 2001). Another important function of activated PI3-K/Akt in cells is maintaining cell survival by inhibition of apoptosis, e.g. through Akt phosphorylation of Bad (Coffer, et al., 1998).

Apoptin was shown to interact with the p85 regulatory subunit of PI3-K in both normal and cancer cells, leading to constitutive activation of PI3-K (Maddika, et al., 2008). Inhibition of PI3-K activation either by chemical inhibitors or by genetic approaches severely impaired cell death induced by apoptin. Downstream of PI3-K, Akt was activated and translocated to the nucleus together with apoptin. Direct interaction between apoptin and Akt was documented, and co-expression of nuclear Akt significantly potentiated cell death induced by apoptin (Maddika, et al., 2007). Accordingly, Nur77, one of the targets of activated Akt, was previously shown to translocate from the nucleus to mitochondria during apoptin-induced cell death (Maddika, et al., 2005).

*Hippi*

In **chapter 2**, we briefly alluded to the interaction between apoptin and Hippi. Hippi is the protein interactor and apoptosis co-mediator of

Huntingtin interacting protein 1 (Hip1). Diverse cellular functions have been described for Hippi (also known as estrogen-related receptor beta like 1 or intraflagellar transport 57 homolog). In Huntington's disease, the Huntingtin (Htt) protein undergoes polyglutamine repeat expansion and loses its ability to interact with the endocytic protein Hip1. This allows Hip1/Hippi complexes to form and in turn trigger caspase-8-mediated apoptosis (Gervais, et al., 2002). Moreover, Hippi was found to be a transcriptional regulator of caspase expression: exogenous expression of HIPPI increases expression of caspases-1, -8 and -10 in HeLa and Neuro2A cells and induces apoptosis (Majumder, et al., 2007; 2007b). In addition to this possible caspase-dependent mechanism for pathological cell death in Huntington's disease (Ferrier, 2002), Hippi was also reported to activate the mitochondrial (intrinsic) apoptotic pathway in neuronal cells through Bid cleavage (Majumder, et al., 2006), and to bind other apoptosis-related proteins, including apoptin, and the apoptin-interacting protein Rybp/DEDAF (Stanton, et al., 2007; see below).

Mapping studies indicate that Hippi binds within the self-multimerization domain of apoptin, and apoptin binds to the C-terminal half of Hippi, including its death effector domain-like motif (Cheng, et al., 2003). Subcellular localization studies showed that Hippi and apoptin perfectly colocalized in the cytoplasm of normal human HEL cells, whereas in cancerous HeLa cells most apoptin and Hippi were located separately in the nucleus and cytoplasm, respectively. These results suggested that the interaction with Hippi might play a role in the suppression of apoptin-induced apoptosis in normal cells. However, over-expression of Hippi in HeLa cells did not prevent apoptin nuclear accumulation, nor did it inhibit its apoptotic activity, indicating that there is still another factor involved in determining the cytoplasmic localization of apoptin and inhibition of apoptin-induced apoptosis in normal cells (Cheng, et al., 2003).

*FAM96B (**Chapter 4**)*

Family with sequence similarity 96, member B (FAM96B) is a highly conserved protein, whose function was until recently unknown. Proteins sharing its conserved domain DUF59 serve a variety of roles, including phenylacetic acid degradation, iron-sulfur cluster biosynthesis, nucleotide-binding and chromosome partitioning, and calcium-dependent protein phosphorylation (Finn, et al., 2010).

We found that FAM96B interacts with apoptin in both normal and tumor cells (**chapter 4**). Interestingly, this function was independent of apoptin phosphorylation. Proteomic analysis suggests FAM96B is involved in the establishment of sister chromatid cohesion and subsequent chromosome segregation (**chapter 4**; Ben Arroya, et al., 2008; Ito, et al., 2010). FAM96B associates with various proteins involved in DNA replication and repair, as well as, cytoskeletal components, effectively linking the DNA damage response to sister chromatid cohesion and segregation, and cell cycle regulation. Preliminary results indicate FAM96B is down-regulated in tumor cells (R. Zimmerman, J. Tian and C. Backendorf, unpublished results). Furthermore, studies in yeast (Ben-Aroya, et al., 2008) and preliminary observations in zebrafish (R. Zimmerman, unpublished results) indicate that FAM96B is an essential gene required for normal embryonic development. This implies that ectopic expression of FAM96B reimposes a cellular control mechanism (most likely regarding its function in linking the DNA damage response to control of the mitotic spindle), which is recognized by apoptin: in normal cells, this prevents apoptin-induced apoptosis, whereas in tumor cells, where this control mechanism is lacking, apoptin-induced apoptosis is triggered.

*RYBP/DEDAF and other nucleolar proteins (**chapter 5**)*

One of the proteins found to associate with FAM96B is Rybp/DEDAF (**chapter 4**). Coincidentally, the same protein was also found to interact with both Hippi (Stanton, et al., 2007) and apoptin (Danen-van Oorschot, et al., 2004).

Rybp (RING1 and YY1 binding protein) belongs to the Rybp/Yaf2 family of small, basic, Npl4 zinc finger (NZF)-containing proteins that have been highly conserved throughout evolution. Initially, Rybp was characterized as an interacting partner for proteins involved in transcriptional regulation, such as YY1 and members of the E2F family, as well as with transcriptional co-repressors such as Polycomb group proteins (Bejarano, et al., 2005; Garcia, et al., 1999; Sawa, et al., 2002; Schlisio, et al., 2002; Trimarchi, et al., 2001). Importantly, Rybp is involved in the p53 response to DNA damage, by interacting with MDM2 and decreasing MDM2-mediated p53 ubiquitination, leading to stabilization of p53 and cell-cycle arrest (Chen, et al., 2009).

Beyond this, Rybp has been implicated in the promotion of apoptosis. Rybp binds heterotypically to several DED-containing apoptotic mediators, and accordingly is also known as DEDAF, for death effector domain associated factor (Zheng, et al., 2001). Homophilic interactions of death effector domains (DEDs) are crucial for the signaling pathways of death receptor-mediated apoptosis. Rybp/DEDAF interacts with FADD, procaspase-8, and procaspase-10 in the cytosol, as well as with the DED-containing DNA-binding protein (DEDD) in the nucleus. It can enhance both death receptor- and caspase-10 DED-mediated apoptosis in lymphoma cell lines (Zheng, et al., 2001). At the cell membrane, it augmented the formation of CD95-FADD-caspase-8 complexes and enhanced death receptor- as well as DED-mediated apoptosis. In the nucleus, Rybp/DEDAF caused the DEDD protein to relocalize from subnuclear structures to a diffuse distribution in the nucleoplasm.

As mentioned before, Rybp/DEDAF also interacts with apoptin and has been suggested, like apoptin, to induce apoptosis preferentially in transformed cell lines (Danen-van Oorschot, et al., 2004; Novak and Phillips, 2008). Accordingly, expression of Rybp/DEDAF is decreased in human cancer tissues compared with adjacent normal tissues. Rybp/DEDAF is predominantly nuclear and partially co-localizes with apoptin in intact and

apoptotic tumor cells, but not in normal cells, where it is nuclear while apoptin remains cytoplasmic.

In **chapter 5**, apoptin was demonstrated to interact with chromatin, and possibly nucleolar (R-)chromatin. Indeed, proteins co-purifying with chromatin-associated apoptin complexes included the nucleolar proteins Cdc5L, nucleophosmin and nucleolin, as well as components of the mitotic spindle and the PP2A inhibiting, cell cycle regulatory protein SET1. Like DEDAF, both Cdc5L and nucleophosmin play important roles in the response to DNA damage and maintenance of genomic stability; in addition, Cdc5L and SET1 are involved in the progression of both S-phase and the G2/M transition. Nucleophosmin is also involved in cellular growth through nucleocytoplasmic export of ribosomes, and centrosome duplication and mitotic spindle formation, with nucleolin sharing similar functions. Together with the identification of proteins involved in ribosome biogenesis and RNA metabolism, the data suggest that apoptin orchestrates its apoptotic effects from within the nucleolus, where it senses DNA damage and shuts down the cellular protein factory.

*Rsf-1*

One of the apoptin-interacting proteins found by yeast-two-hybrid assay is Rsf-1. We found that Rsf-1 interacted and colocalized with apoptin in the nucleus of human tumor cells (R. Zimmerman and A. Danen-van Oorschot, unpublished results). RSF is a heterodimer of the PHD-finger containing protein Rsf-1 and the SNF2H ATPase belonging to the ISWI family, and a unique chromatin remodeling factor that can assemble regularly spaced nucleosomal arrays without the aid of additional histone chaperones (Loyola, et al., 2001; 2003). Such nucleosome remodeling is essential for transcriptional activation or repression (Vignali, et al., 2000), DNA replication (Flanagan and Peterson, 1999), and cell cycle progression (Cosma, et al., 1999). Rsf-1 was found to play a role in silent chromatin formation by promoting histone H2Av replacement. H2Av is the *Drosophila* variant of mammalian H2AZ, a histone H2A variant that is essential for

establishing proper chromatin structure in many organisms (Raisner and Madhani, 2006; Greaves, et al., 2007; Rangasamy, et al., 2004), and is involved in the formation of constitutive, as well as, facultative heterochromatin (Greaves, et al., 2006; Sarcinella, et al., 2007). *Drosophila* H2Av mutants exhibit reduced H3 lysine 9 (H3K9) methylation and HP1 binding (Swaminathan, et al., 2005), both of which are required to recruit Suv420h1/2 for the establishment of heterochromatin (Schotta, et al., 2004). In *Drosophila melanogaster*, loss of RSF function reduced the levels of the histone variant H2Av and histone H3-K9 methylation, and suppressed silencing of transcription in a euchromatic region near the centromeric heterochromatin (Hanai, et al., 2008).

RSF was also found to interact with CENP-A chromatin, a histone H3 variant that is crucial to the formation of centromeric chromatin (Perpelescu, et al., 2009). Rsf-1 depletion induced loss of centromeric CENP-A, and purified RSF complex reconstituted and spaced CENP-A nucleosomes *in vitro*, further implicating the involvement of Rsf-1 in this process.

The sequence of the gene for Rsf-1 is located on chromosome 11q13, and includes that for HBXAP, which was postulated to be involved in the transcriptional regulation of the hepatitis B virus (Shamay, et al., 2002). HBXAP actually contains a 252-amino-acid truncation of the amino terminus of Rsf-1, which lacks the ISWI-interaction domain, and therefore is not functional within the RSF complex. Amplification at the 11q13 locus is commonly observed in breast, ovarian, head and neck, oral, and esophageal cancer (Mao, et al., 2006; Schwab, 1998; Shih Ie, et al., 2005; Nakayama, et al., 2007). Patients with Rsf-1 amplification or over-expression had a significantly shorter overall survival than those without (Shih Ie, et al., 2005). *In vitro,* over-expression of the Rsf-1 gene stimulated cell proliferation and transformation of nonneoplastic cells by conferring serum-independent and anchorage-independent growth. Furthermore, Rsf-1 gene knockdown inhibited cell growth in OVCAR3 cells, which harbor Rsf-1 amplification (Shih Ie, et al., 2005). The association of Rsf-1 amplification cq over-

expression with worse survival in (ovarian) cancer patients, combined with the stimulatory effect on cell proliferation *in vitro*, is similar to the properties of known oncogenes, including HER2/neu in breast cancer (Borg, et al., 1990; Tsuda, et al., 1989) and N-myc in neuroblastoma (Rubie, et al., 1997). This suggests that Rsf-1 itself has oncogenic potential; consistent with such a function, Rsf-1 was found to be up-regulated in paclitaxel-resistant ovarian cancer cell lines, and *Rsf-1* gene knockdown sensitized tumor cells to paclitaxel (Choi, et al., 2009). Down-regulation of hSNF2H or disruption of hSNF2H and Rsf-1 interaction also enhanced paclitaxel sensitivity in tumor cells with Rsf-1 up-regulation. On the other hand, ectopic expression of Rsf-1 significantly enhanced paclitaxel resistance in ovarian cancer cells.

Preliminary observations showed that co-expression of Rsf-1 together with apoptin stimulates apoptin-induced apoptosis (R. Zimmerman, unpublished results), corroborating both the oncogenic potential of Rsf-1 and the preference of apoptin for a transformed environment.

*Suv420h1*

Suppressor of variegation 4-20 homolog 1, or Suv420h1, is a SET-domain containing protein, which was found to interact with apoptin in a yeast two-hybrid assay (A. Danen-van Oorschot, unpublished results). SET domains are found in histone methyl transferases (HMTs) (Qian and Zhou, 2006), and also serve as interaction domains with dual-specificity protein phosphatases (dsPTPases) (Cui, et al., 1998). Intriguingly, CAV VP2 has been shown to encode such a dsPTPase (Peters, et al., 2002), and SET domain-dsPTPase interactions appear to be critically important for regulating the growth properties of lymphoid progenitors (De Vivo, et al., 1998), which are among the preferentially targeted cell types during CAV infection (Adair, 2000).

Drosophila Suv4-20 is a mixed product specificity methyltransferase that dimethylates approximately 90% and trimethylates less than 5% of total H4 at lysine 20 in S2 cells (Yang, et al., 2008). Similar to the Drosophila enzyme, human Suv4-20h1/h2 enzymes generate di- and trimethyl H4K20

(Schotta, et al., 2004; 2008; Yang, et al., 2008). Dimethyl H4K20 has been shown to recruit the p53 binding protein 53BP1 to DNA damage foci in the fission yeast *S. pombe*, and is essential for the proper execution of the DNA damage response (Sanders, et al., 2004; Greeson, et al., 2008). Depletion of Suv4-20h1/2 in human HeLa cells impaired the formation of 53BP1 foci (Yang, et al., 2008), suggesting that dimethyl H4K20 is also required for a proper DNA damage response in human cells. The proposed mechanism involves exposure of normally buried H4K20me2 epitopes upon DNA double strand breaks (Yang and Mizzen, 2009; Botuyan, et al., 2006).

Histone H4 lysine 20 trimethylation (H4K20me3) has been implicated in the formation of constitutive heterochromatin, particularly at (peri)centromeric and (sub)telomeric regions (Schotta, et al., 2004; Benetti, et al., 2007; Kourmouli, et al., 2004; Sims, et al., 2006). This requires trimethylation of H3K9 by Suv39h1/2 enzymes and the subsequent binding of HP1 proteins, which function to recruit Suv420h1/2 (Schotta, et al., 2004). Accordingly, trimethylation of H4K20, but not dimethylation, is reduced in Drosophila larvae lacking HP1 (Yang, et al., 2008).

H4K20me3 has been shown to be down-regulated in human cancer (Fraga, et al., 2005; Van Den Broeck, et al., 2008). This might be achieved via downregulation of the Suv420h1/2 enzymes (Van Den Broeck, et al., 2008; Pogribny, et al., 2006; Tryndyak, et al., 2006), but also by alteration of HP1 binding dynamics (Siddiqui, et al., 2007). Loss of H4K20me3 might lead to activation of transcription of previously repressed genes (through alteration of chromatin structure), unstable kinetochore attachment sites, as well as uncapped telomeres (Benetti, et al., 2007a; 2007b). In a normal cell, these effects might lead to cell cycle arrest and/or programmed cell death; however, in a malignant cell, this might only serve to increase genomic instability, contributing to further tumorigenesis. In addition, downregulation of the Suv420h enzymes also impairs DNA damage signaling through loss of H4K20me2, further contributing to genomic instability (Schotta, et al., 2008; Rouse and Jackson, 2002; Zhou and Elledge, 2000).

We found that apoptin interacts with Suv420h1 in human tumor cells, and both proteins seem to cooperate to induce apoptosis in these cells (R. Zimmerman, unpublished results). Preliminary results suggest that Suv420h1 itself was also able to induce tumor-selective apoptosis, as its over-expression induced apoptosis in tumor cells but not in normal cells. Furthermore, we found that Suv420h1 interacted with HP1 beta in both normal and cancer cells (R. Zimmerman and H. Lanz, unpublished results). This concurs with reports that HP1 beta is involved in DNA damage signaling originating from DNA breaks in H3K9me3-containing chromatin (Ayoub, et al., 2008).

*BCA3/AKIP1 (**Chapter 6**)*

Another protein identified as an interacting partner of apoptin in our yeast two-hybrid assay is BCA3 (**chapter 6**). Breast cancer associated gene 3 (BCA3) was identified as a gene that was overexpressed in breast cancer compared to surrounding normal stroma (Kitching, et al., 2003; Leon and Canaves, 2003). It has been shown to interact with a number of proteins, including the catalytic subunit of protein kinase A (hence the alternative name A-kinase interacting protein 1, or AKIP1) (Sastri, et al., 2005), the transcription factor KyoT2 (Qin, et al., 2004), and Rac1 (Yu, et al., 2007).

Under normal cellular conditions, PKA resides in the cytoplasm as an inactive holoenzyme consisting of two regulatory subunits complexed with two catalytic subunits. A-kinase associated proteins, or AKAPs, interact with the regulatory subunits and control PKA activity by anchoring the holoenzyme complexes at specific subcellular localizations. Upon activation by cAMP, the regulatory subunits dissociate, freeing the catalytic subunits. AKIP1 binds to the latter, reportedly facilitating its translocation into the nucleus (Sastri, et al., 2005).

Over-expression of BCA3 appeared to induce apoptosis in human tumor cells, and co-expression with apoptin led to an increase in tumor cell death (**Chapter 6**). The negative effect of BCA3 on cellular proliferation had been

observed previously in osteoclasts (Yu, et al., 2007). In these cells, it was shown to interact with Rac1, a small GTPase that belongs to the Ras superfamily and participates in a wide range of biological processes, including rearrangement of the actin cytoskeleton, cell motility, cell transformation, gene transcription, and cell cycle progression (Michiels and Collard, 1999; Hall, 2005). The interaction of BCA3 with Rac1 attenuated colony stimulating factor-1 (CSF-1) induced cell spreading.

Another interaction partner of BCA3 was identified as TAp73 (Leung and Ngan, 2010). TAp73 is highly similar to p53, and exhibits growth-inhibitory, tumor-suppressive, and proapoptotic functions (Kaghad, et al., 1997; Jost, et al., 1997). TAp73 binds and stabilizes BCA3 in the cervical cancer cell line HeLa (Leung and Ngan, 2010). When coexpressed with TAp73, BCA3 interacts and colocalizes with TAp73 at the mitochondria. Furthermore, BCA3 augments the transactivation activity of TAp73 on Bax promoter, enhancing Bax protein expression. In addition, the expression of BCA3 also increases the sensitivity of TAp73-transfected cells to γ-irradiation–induced apoptosis, inducing activation of caspase-7 and caspase-9. Interestingly, Klanrit et al. (2008) reported that over-expression of TAp73 was able to sensitize p53-negative tumor cells to apoptin-induced cell death.

*APC*

In transformed cells but not in normal cells, apoptin associates with APC1, the largest subunit of the anaphase-promoting complex/cyclosome (APC/C) (Teodoro, et al., 2004) and an essential component of the mitotic checkpoint apparatus (see **chapter 2**). Apoptin expression, or depletion of APC1 by RNA interference, inhibits APC/C function in p53 null cells, resulting in G2/M arrest and apoptosis. Furthermore, apoptin expression in transformed cells induces the formation of nuclear bodies and recruits APC/C to these subnuclear structures (Heilman, et al., 2006); the interaction with PML is, however, not required for induction of apoptosis by apoptin (Janssen, et al., 2007).

Teodoro, et al. (2004) also reported the interaction of apoptin with α-tubulin, β-tubulin and β-actin, suggesting an association with filamentous networks. As subunits of the APC/C, including APC1, have been shown to localize to the active centromere of dicentric chromosomes (Saffery, et al., 2000), they proposed that the observed association of apoptin with α- and β-tubulin might result from interaction between apoptin and the spindle complex. The interaction of apoptin with FAM96B (which controls chromosome segregation, **chapter 4**) and BCA3 (which localizes to the centrosome, **chapter 6**), as well as the identification of tubulin in chromatin-bound apoptin complexes (**chapter 5**), indeed appears to be in agreement with this suggestion.

**Apoptin phosphorylation**

Alas, the list of apoptin interacting proteins lacks one crucial protein: a kinase, which is able to phosphorylate apoptin at T108, and which is only active in transformed and tumor cells. For, though they were found to interact with apoptin, neither PI3K nor PKA were able to phosphorylate it.

Maddika, et al. (2009) described that Cdk2 complexed with cyclin A, but not E, could phosphorylate apoptin at T108, yet could not prove that this phosphorylation was specific to the tumorigenic environment. PKCβ has also been implicated in apoptin phosphorylation (Jiang, et al., 2010), Recently, however, our laboratory showed that that immunoprecipitation of both CDK2 and PKCβ significantly reduced the levels of these proteins in tumor cell lysates, without affecting the level of apoptin phosphorylation by these tumor cell lysates in an *in vitro* kinase assay (Lanz, et al., 2012). Therefore, at the moment no conclusive statement can be drawn about the kinase modifying and activating apoptin.

Zhang, et al. (2004) did find that the apoptin kinase activity could be specifically turned on by transient transformation of normal human fibroblasts expressing apoptin with the SV40 large T antigen. These results were further investigated in **chapter 6**. Here, we demonstrated that upon

expression of the SV40 large T antigen (LT), the small T antigen (ST) was also expressed. In fact, co-expression of the ST was essential to induce apoptin phosphorylation upon cellular transformation by SV40 LT. Analysis of ST functional domains, through stepwise mutational inactivation, proved that the interaction of ST with B56 gamma-containing PP2A was required for apoptin activation. Simultaneously, analysis of the effect of PKA activity on apoptin phosphorylation pointed to its involvement in the regulation of PP2A B56 delta activity. Subsequently, we found that down-regulation of PP2A B56 gamma and B56 delta expression through RNAi in normal cells indeed resulted in apoptin phosphorylation. Hence, the data presented in this thesis argue for an apoptin normal-cell-specific phosphatase next to a tumor-specific kinase, even not excluding the possibility that apoptin might be phosphorylated by more than one kinase, and that its differential activation between normal and tumor cells rather depends on the activity of the tumor suppressor phosphatase PP2A.

**Outlook**

*Synthesis – how might the pieces of the apoptin puzzle fit together?*

The experimental results with the newly discovered apoptin-interacting proteins presented in this thesis, combined with knowledge from previously published analyses, lead to the proposal of the following model for apoptin behavior in the human cell (figure 7.2).

In the normal human cell (figure 7.2A), upon expression of the apoptin protein, apoptin is at first free to move between the nucleus and cytoplasm, owing to its NLS and NES. Apoptin might be phosphorylated, but it is also dephosphorylated by PP2A, reaching an equilibrium which finds it mostly unphosphorylated. Apoptin is mainly located in the cytoplasm, where it interacts with Hippi and accumulates in cytoplasmic granules. The exact nature of these cytoplasmic granules is not known; immune fluorescence analysis has revealed partial co-localization with markers for the lysosome, Golgi, ER and endosomal vesicles as well (R. Zimmerman, unpublished

results). Whichever the mechanism, apoptin does in fact disappear from the normal cell, without inducing cell death.

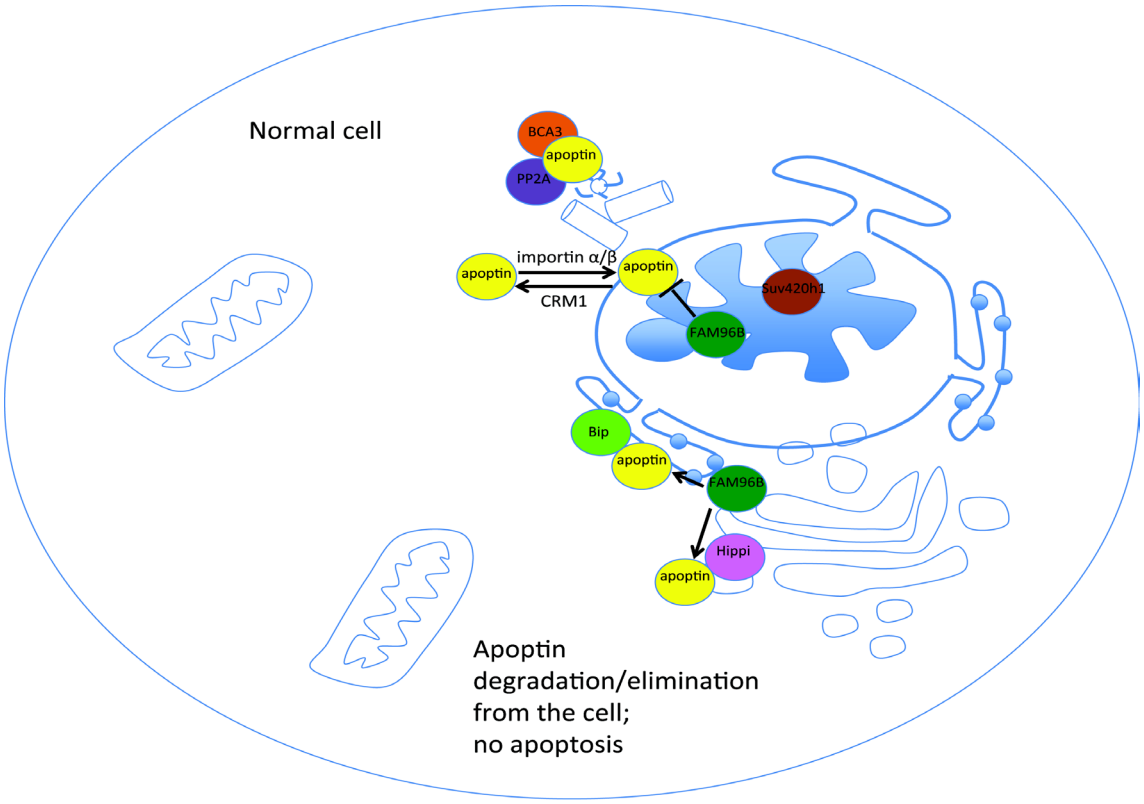


**Figure 7.2** Model of apoptin tumor-specific cell death pathways. **A.** In the normal cell, apoptin phosphorylation is opposed by the action of PP2A B56 gamma- and delta-containing complexes. The presence of FAM96B safeguards the cell cycle, while H4K20 di- and trimethylation function to recruit DNA repair machinery and telomere-capping proteins, respectively, preventing genomic instability. As a result of these actions, apoptin is redirected to the cytoplasm, where it interacts with e.g. Bip in the ER and Hippi in Golgi vesicles, and is eventually eliminated from the cell without inducing apoptosis. See text for further details.

In the transformed cell (figure 7.2B), many cellular processes have gone awry: the activity of (potential) tumor suppressor proteins PP2A, FAM96B and Suv420h1 is derailed, while the expression of (potentially) oncogenic proteins, Rsf-1 and BCA3 has increased. Loss of FAM96B results in a failure to arrest the cell cycle upon DNA damage, whereas loss of H4K20 trimethylation results in telomere uncapping and lack of proper pericentric heterochromatin formation, resulting in inappropriate kinetochore attachment sites. Over-expression of Rsf-1 and BCA3 might also contribute to the formation of aberrant mitotic spindles; altogether, the resulting

genomic instability would constantly signal to the DNA damage response machinery, whereas the ability to arrest the cell cycle and/or induce apoptosis has been lost. Loss of PP2A activity means apoptin phosphorylation is no longer opposed, leading to the accumulation of apoptin in the tumor cell nucleus. Within the nucleus, apoptin localizes to the nucleoli, where it cooperates with Rybp/DEDAF and e.g. nucleophosmin in sensing the many DNA damage signals. Furthermore, apoptin interacts with Rsf-1 at chromosome centromeres, where it inhibits APC1 activity. As a result, the APC cyclosome cannot catalyze chromosome segregation; in addition, interference of apoptin and BCA3 with the mitotic spindle contributes to the resulting mitotic failure and the inevitable cell death following it (figure 7.2 C, D).

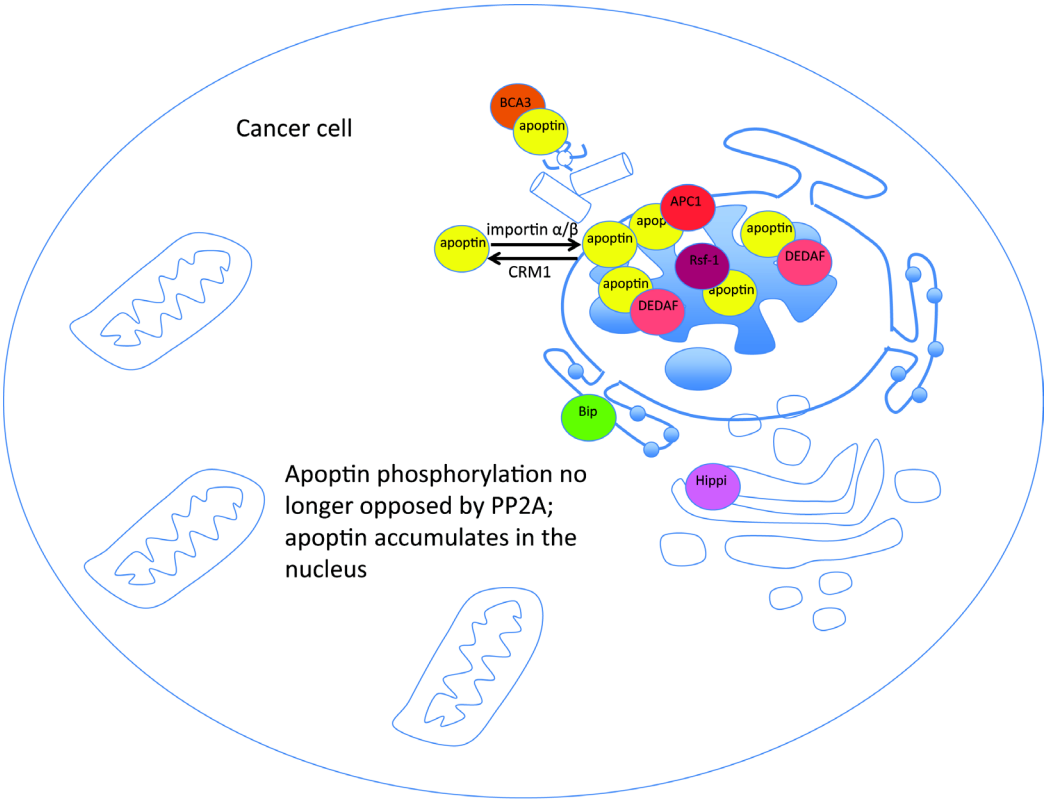


**Figure 7.2** Model of apoptin tumor-specific cell death pathways. **B.** In the cancer cell, apoptin phosphorylation is no longer opposed, and phosphorylated apoptin accumulates in the nucleus, colocalizing with DEDAF and other proteins in the nucleolus, while also colocalizing with BCA3 at the centrosome. See text for further details.

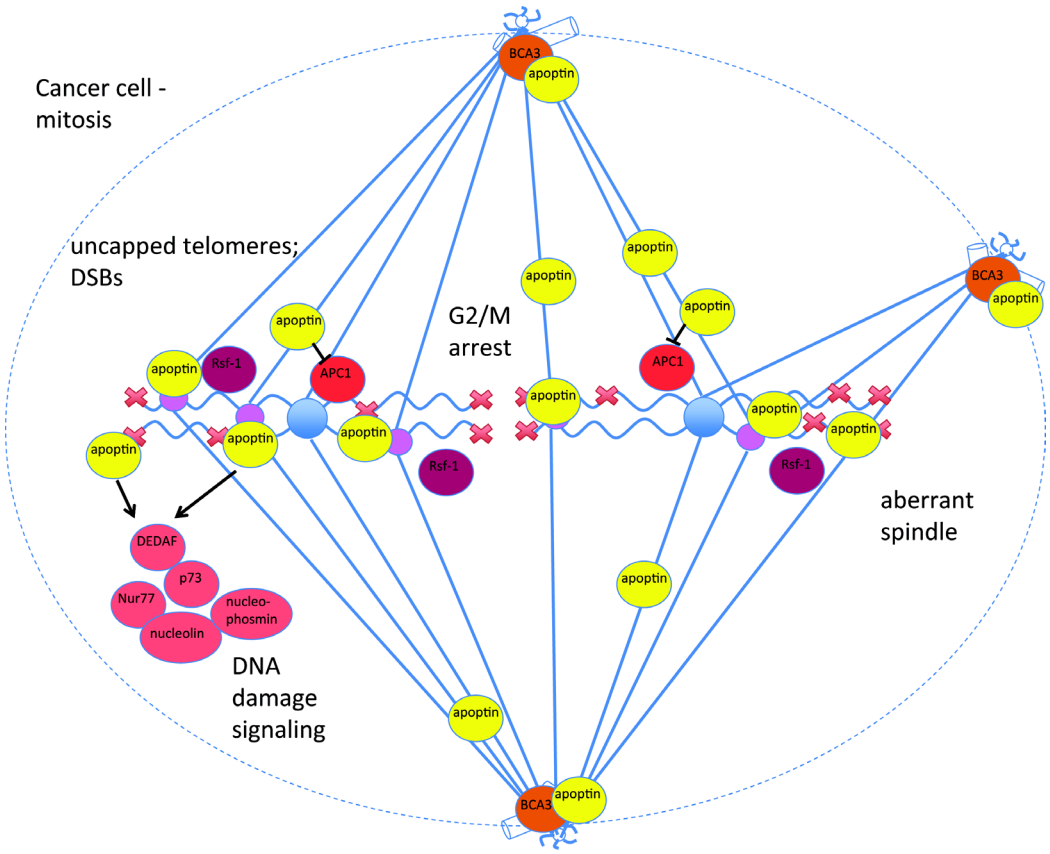


**Figure 7.2** Model of apoptin tumor-specific cell death pathways. **C.** Over-expression of Rsf-1 and BCA3 contribute to the formation of faulty mitotic spindles. Lack of H4K20 methylation means DSBs are no longer repaired, and telomeres become uncapped. Apoptin recognizes the activation of the DNA damage response, and inhibits APC1 activity, halting the anaphase-promoting complex and inducing G2/M arrest. See text for further details.

*How apoptin might distinguish between normal and tumor cells*

From the proposed model, it results that apoptin's differential recognition of normal and transformed cells likely depends on three key properties of malignant cells: (1) sustained cellular proliferation and (2) the lack of execution of programmed cell death in the face of (3) severe DNA damage. Though apoptin has been shown to possess two different death domains, one acting from within the cytoplasm (Danen-van Oorschot, et al., 2003), it seems that the crucial step in blocking apoptin-induced apoptosis in normal cells is inhibiting its nuclear accumulation. Still, even if apoptin is forced to the nucleus of normal cells, it has been confirmed that this is not enough to induce apoptosis (Danen-van Oorschot, et al., 2003) – probably because of the lack of signals that cellular proliferation has gone awry, including silencing of tumor suppressor genes (e.g. FAM96B, PP2A), over-expression of

oncogenes (e.g. Rsf-1, and possibly BCA3), and the lack of DNA damage. It is the combination of these events that appears to tip the equilibrium to the side of apoptin phosphorylation, and hence, activation. Indeed, recent experiments (Kucharski, et al., 2011) demonstrated that induction of DNA damage was sufficient to activate apoptin, resulting in its nuclear translocation and induction of apoptosis in primary cells.

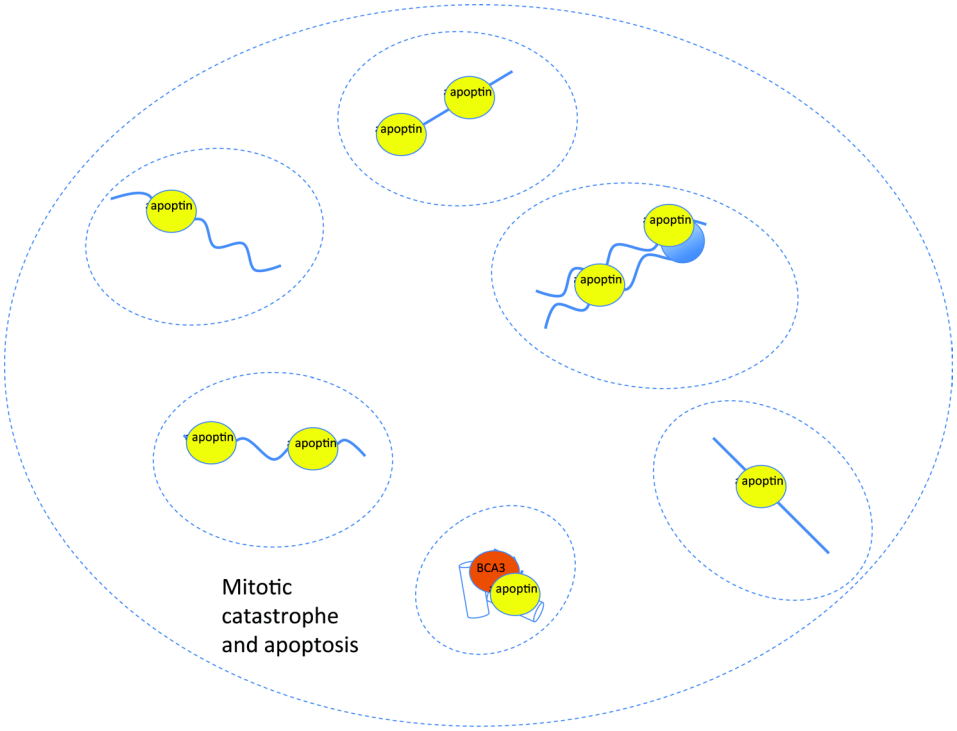


**Figure 7.2** Model of apoptin tumor-specific cell death pathways. **D.** These effects, in addition to apoptin association with the mitotic spindle, lead to mitotic catastrophe and apoptotic cell death. See text for further details.

*Mechanism of apoptin-induced apoptosis in tumor cells*

In the model proposed above, activated apoptin induces cell death through several modes of action: i) it localizes to the nucleoli, where it ia) senses DNA damage, activating DEDAF, among other proteins, to induce apoptosis, and ib) interacts with ribosomal chromatin and constituents, effectively shutting off cellular biosynthesis; ii) it induces mitotic failure through a doubly lethal combination of iia) interaction cq interference with several components of the mitotic spindle; and iib) inhibition of APC-catalyzed segregation of chromosomes. Not only is the cell therefore physically unable to divide,

leading to mitotic catastrophe, but also, even if the cell would manage to survive this event, it would die as a result of the lack of cellular biosynthesis.

The results described in this thesis correlate with the previous findings in literature, stating that apoptin-induced tumor-selective apoptosis does not require *de novo* gene transcription/translation (Danen-van Oorschot, et al., 2003). The same is true for the independence of p53 activity (Zhuang, et al., 1995b), and the paradoxical relation with Bcl-2. As discussed in **chapter 2**, during mitosis, DNA damage activates the ATM/ATR signaling pathways; DNA single strand breaks (SSBs) activate Chk1, which inhibits the action of Cdc25c, leading to G2/M cell cycle arrest – as previously observed for apoptin, and independently of p53. DNA double strand breaks (DSBs) activate Chk2, which activates both p53 and p73; intriguingly, p73 has indeed been shown to be involved in apoptin-induced apoptosis. Furthermore, DNA damage-induced signaling, through an as yet unclear mechanism, leads to the activation of caspase-2, which directly stimulates cytochrome c release, G2/M cell cycle arrest and mitotic catastrophe, independently of either p53 or Bcl-2. In fact, it has been reported that over-expression of Bcl-2 resulted in an enhanced frequency of mitotic catastrophe (see **chapter 2**). This is consistent with reports that over-expression of Bcl-2 does not necessarily impede apoptin-induced apoptosis, and may even have a stimulatory effect (Danen-Van Oorschot, et al., 1999; Danen-Van Oorschot, et al., 1999b).

*Implications for CAV pathogenesis*

One cannot help but wonder how all this applies to the natural function of apoptin in CAV pathogenesis. In young chicks, CAV has been shown to preferentially target the cells of the bone marrow and thymus (Adair, 2000); besides rapid proliferation, these cells are also constantly undergoing rearrangements of their DNA, which could be perceived by apoptin as damaged DNA. In addition, many viral proteins have been shown to localize to the nucleolus (Hiscox, 2007), where they hijack the cell's protein factory and redirect it towards their own multiplication. The subsequent induction

of apoptosis then leads to release of the viral progeny, and, concurrently, to further spreading of the infection, as nearby cells engulf the apoptotic bodies containing newly formed viral particles.

Chickens develop a resistance to CAV-induced disease by two weeks of age; furthermore, experiments in normal human lymphocytes have shown that apoptin is not toxic to these cells. In addition, systemic expression of apoptin in mice does not induce apoptosis in erythroid or lymphogenic cells or their precursors, indicating that apoptin is indeed safe for anti-tumor therapy in humans (Pietersen, et al., 2005; Peng, et al., 2007).

*Implications for anticancer therapies*

A comparison between apoptin and the other PKTC presented in **chapter 3** reveals a number of common strategies, which could be exploited in the design of novel anticancer therapies.

Apoptin (Liu, et al., 2006a, 2006b), TRAIL (Voelkel-Johnson, et al., 2005) and MDA-7/IL-24 (Sauane, et al., 2010) have all been shown to up-regulate ceramide production, indicating that this might be a convenient strategy in killing cancer cells. Autophagy seems to be a common theme, with HAMLET (Aits, et al., 2009), NS1 (Bruno, et al., 2009), and Brevinin-2R (Ghavami, et al., 2008) all employing autophagic cell death to specifically kill tumor cells. As one of the pathways mitigating the metabolic switch (the seventh cancer hallmark), this indeed seems warranted. Perhaps related to this, the adenovirus E4orf4 protein has been demonstrated to kill tumor cells by perturbing the traffic of endosomal vesicles (Landry, et al., 2009). E4orf4-induced cell death relies on Src tyrosine kinases and RhoGTPase-dependent perturbation of actin dynamics (Robert, et al., 2006); similarly, apoptin has also been shown to target the cytoskeleton and mitotic spindle (**chapters 4-6**), and both apoptin (Teodoro, et al., 2004) and E4orf4 (Kornitzer, et al., 2001) have been shown to induce G2/M cell cycle arrest by targeting the APC/C anaphase promoting complex. With respect to the various microtubule-destabilizing agents that are now being used, the APC/C

cyclosome might well represent a novel target for the development of cancer-combating agents (Heilman, et al., 2005).

The interaction between apoptin and Rsf-1 and Suv420h1 (A. Danen-van Oorschot and R. Zimmerman, unpublished results) suggests employing molecules that target epigenetic chromatin modifiers. Besides the inhibitors of histone deacetylases (HDACs), which are already being used, alteration of the activities of histone methylases and chromatin remodeling complexes could either limit genomic instability and thus tumor progression, or, alternatively, promote genomic instability beyond a point compatible with life. The over-expression of Rsf-1 in tumor cells has also made it an attractive target for immunotherapies. In fact, transduction of dendritic cells with plasmid DNA encoding the Rsf-1 gene demonstrably generated specific cytotoxic T lymphocytes against ovarian cancer *in vitro* (Sun, et al., 2010).

In **chapter 5**, we inferred the localization of apoptin in the nucleoli of cancer cells, which in fact are the ribosome factories facilitating the rapid cell growth characteristic of tumor cells. Thus, complementary to the proteasome inhibitors already employed in current anticancer therapies, shutting off excessive ribosomal production might also prove an effective strategy in the fight against cancer.

**Conclusions**

As discussed in **chapter 2**, the tumor-killing potential of the apoptin protein has already been established *in vivo*, as demonstrated by experiments in our laboratory with apoptin-producing adenovirus (Pietersen, et al., 1999), Asor-apoptin (Peng, et al., 2007) and PTD4-apoptin (Sun, et al., 2009; Jin, et al., 2011), all in which apoptin reduced tumor cell mass and prolonged survival of the affected organism, without harming normal cells.

In this thesis, the molecular aspects of apoptin-induced tumor-selective apoptosis have been investigated. The basis for apoptin's tumor-selectivity has been further deciphered, as well as the mechanisms leading to apoptin-

induced apoptosis in malignant cells. These involve the recognition by apoptin of several of the cancer hallmarks, including:

- genomic instability (apoptin senses DNA damage through e.g. Rybp/DEDAF, and telomere uncapping by loss of H4K20 trimethylation).
- insensitivity to inhibitory cell cycle checkpoints (lack of FAM96B, aberrant chromatin and spindle structures through interaction with overexpressed Rsf-1 and BCA3);
- evasion of programmed cell death (apoptin-induced apoptosis is stimulated by over-expression of Bcl-2);
- activation of the metabolic switch (apoptin interacts with PI3K and Akt)

Thus, recognition of malignant transformation by apoptin occurs already in the early stages of transformation, before the acquisition of angiogenic, metastatic and immune evasive potential, and possibly even before activation of telomere maintenance and cellular immortalization. As described above, apoptin also utilizes these cancer hallmarks to attack the tumor cell at various points and bring about cell death.

It seems that apoptin, but also the other PKTC presented in **chapter 3**, employ several different strategies to ensure death of the cancer cell. Considering this, as well as the fact that cancer cells, owing to their propensity to rapidly accumulate genetic changes, are essentially moving targets, it might be naïve to assume that we can win the fight against cancer by employing a single strategy. Thus, rather than trying to devise a magic bullet, we might instead compose a magical cluster bomb, containing subparticles aimed at each of the above-described tumor-specific targets, attacking cancer from every conceivable angle, and attaining cell death, without the harmful side-effects of radiation and conventional chemotherapy.

Table 7-1 **Summary of apoptin-associating proteins.**

| **Protein** | **Cellular function** | **Subcellular localization** | **Expression in cancer** | **Involvement in apoptin activity** | **References** |
|---|---|---|---|---|---|
| Rybp/DEDAF | Transcription regulation; DNA damage response; Apoptosis | Predominantly nuclear | Decreased | Co-expression stimulates apoptosis | *See references in text* |
| Rsf-1 | Chromatin remodeling | Nuclear | Increased | Co-expression stimulates apoptosis | *See references in text* |
| FAM96B | Sister chromatid cohesion; Cell cycle regulation | Nuclear + cytoplasmic | Decreased | Co-expression inhibits apoptosis | *See references in text* |
| Suv420h1 | Histone H4 lysine 20 di- and trimethylation | Nuclear | Possibly decreased | Co-expression stimulates apoptosis | *See references in text* |
| BCA3/AKIP1 | Regulation of transcription and possibly cytoskeleton dynamics | Perinuclear | Increased | Co-expression stimulates apoptosis | *See references in text* |
| Bip/GRP78/HSPA5 | Protein chaperone; mediator of unfolded protein response and ER stress | Endoplasmic reticulum | Increased | Unknown | Sato, et al., 2010; Teodoro, et al., 2004; Danen-van Oorschot and Zimmerman, unpublished results |

Table 7-1 **continued.**

| **Protein** | **Cellular function** | **Subcellular localization** | **Expression in cancer** | **Involvement in apoptin activity** | **References** |
|---|---|---|---|---|---|
| Nmi | Transcription regulation | Primarily cytosolic | Increased | Uknown | Sun, et al., 2002; Zhou, et al., 2000 Danen-van Oorschot and Zimmerman, unpublished results |
| Hippi | Intracellular transport; Apoptosis | Golgi | Unknown | Possibly involved in apoptin elimination from the normal cell | *See references in text* |
| Ppil3 | Protein-folding chaperone catalyzing cis-trans peptidylprolyl somerisation | Nuclear + cytoplasmic | Possibly decreased | Co-expression enhances apoptin cytoplasmic localization in cancer, but has no effect on apoptosis activity | Huo, Yi, & Yang, 2008 |
| APC1 | E3 ubiquitin ligase; subunit of anaphase-promoting complex | Nuclear + cytoplasmic | Unknown | Inhibition by apoptin results in G2/M arrest and apoptosis | *See references in text* |
| PML | Transcription regulation; DNA damage response | Nucleolus; PML bodies | Increased; decreased in advanced stages | Interaction not essential for apoptosis induction | *See references in text* |

Table 7-1 **continued.**

| Protein | Cellular function | Subcellular localization | Expression in cancer | Involvement in apoptin activity | References |
|---|---|---|---|---|---|
| PKAc | Catalytic subunit of cAMP-dependent protein kinase, involved in many intracellular signalling patways, including those stimulating proliferation and survival | Various compartments, dependent on association with AKAPs | Various isoforms up-regulated | Stimulation reduces apoptin phosphorylation | *See references in text* |
| p85 | Regulatory subunit of PI3K, a kinase strongly implicated in tumorigenesis, with functions in proliferation, survival, angiogenesis, autophagy. | Cytoplasmic | Activating gain-of-function mutations | Activation stimulates apoptin-induced apoptosis | *See references in text* |
| Akt | Downstream effector of PI3K signalling | Nuclear + cytoplasmic | Increased | Co-expression of nuclear Akt stimulates apoptin-induced apoptosis | *See references in text* |
| Cdk2/cyclin A | Promotes G1/S transition and replication of DNA and centrosomes in S-phase | Nuclear + cytoplasmic | Unknown | Phosphorylates apoptin directly *in vitro* | *See references in text* |

Table 7-1 **continued.**

| **Protein** | **Cellular function** | **Subcellular localization** | **Expression in cancer** | **Involvement in apoptin activity** | **References** |
|---|---|---|---|---|---|
| PKCβ | Diverse intracellular signaling pathways, including transformation, proliferation, (inhibition of) apoptosis | Nuclear + Cytoplasmic | Unclear, though increased expression is correlated with poorer prognosis | Phosphorylates apoptin directly *in vitro* | *See references in text* |
| PP2A B56γ | Protein phosphatase with important tumor suppressor functions, including regulation of DNA damage response and several stages of cell cycle progression | Nuclear, centromeric | Decreased/inactivated in cancer | Dephosphorylates apoptin directly *in vitro* | *See references in text* |

***AKAPs, A-kinase anchoring proteins.**

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