Apoptin : oncogenic transformation & tumor-selective apoptosis

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Chapter 5

Apoptin interaction with chromatin

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Abbreviations: apoptin, apoptosis inducing protein; ATR, ataxia telangiectasia mutated and Rad3 related; CAV, chicken anemia virus; Cdc5L, cell cycle regulated phosphatase 5L; Cdk, cyclin-dependent kinase; ChIP, chromatin immunoprecipitation; Chk1, checkpoint kinase 1; DBPA, DNA-binding protein A; DDX5, dead box proteins p68; DEDAF, death effector domain-associated factor; DEDD, DED-containing DNA-binding protein; EEF/EIF, translation elongation and initiation factors; hnRNP, heterogenous nuclear ribonuclear proteins; FAM96B, Family with sequence similarity 96, member B; HCMV, human cytomegalovirus; HPV, human papilloma virus; HSV, herpes simplex virus; HTLV, Human T-lymphotropic virus**;** MDM2, mouse double minute 2 protein; MS, mass spectrometry; mTOR, mechanistic target of rapamycin, serine/threonine kinase; Nur77 orphan nuclear

receptor 77; NONO, non-POU domain containing, octamer-binding; NPM, nucleophosmin; PCBP2, poly(rC)-binding protein 2; PP2A, protein phosphatase 2a; pRb, retinoblastoma susceptibility protein; RP, ribosomal protein; RRP1B, ribosomal RNA processing 1 homolog B; SAF-A, scaffold attachment factor A; SARS, Severe Acute Respiratory Syndrome; SV40, simian virus 40; U2AF, U2 snRNP auxiliary factor

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Abstract

The Chicken Anemia Virus-derived protein apoptin has been identified as one of a select number of proteins preferentially exhibiting toxicity towards cancer cells. Coupled to this tumor-selective behavior is a differential subcellular localization: in tumor cells, apoptin-induced apoptosis involves its nuclear translocation, whereas in normal cells, it remains cytoplasmic, where it is degraded without killing the cell. To obtain a comprehensive nuclear interaction map of apoptin in the cancer cell nucleus, and shed light on the mechanisms underlying its tumor cell-killing abilities, we designed a proteomic strategy based on chromatin immunoprecipitation (ChIP) coupled with mass spectrometry. We found that apoptin localizes to chromatin containing proteins relevant for ribosome biogenesis, RNA metabolism, DNA damage response and cell cycle regulation. Our data suggest that in transformed cells, apoptin might induce apoptosis from within the nucleolus, where it senses the activation of the DNA damage response and interferes with cellular protein synthesis.

Introduction

The viral protein apoptin has emerged as a promising new instrument in the development of effective anti-cancer therapies (Backendorf, et al., 2009; Li, et al., 2010; Wagstaff and Jans, 2009). Apoptin has been shown to induce apoptosis specifically in tumor cells, compared to normal tissue (Danen-van Oorschot, et al., 1997; Sun, et al., 2009). Apoptosis induction by apoptin is preceded by its phosphorylation and nuclear accumulation (Danen-van Oorschot, et al., 2003; Maddika, et al., 2009; Rohn, et al., 2002;). *In-vitro* experiments with recombinant MBP-apoptin have demonstrated that apoptin is able to interact with both single- and double-stranded DNA, with little or no sequence specificity (Leliveld, et al., 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). Furthermore, apoptin specifically requires a tumorigenic nucleus: enforced nuclear localization of apoptin in normal human cells by fusion of a general nuclear localization signal to apoptin (Danen-van Oorschot, et al., 2003) or by direct micro-injection of recombinant MBP-apoptin protein in the nucleus (Zhang, 2004a) did not result in apoptosis. In other experiments, apoptin remained in the cytoplasm of normal human cells, and only translocated to the nucleus upon cellular transformation by ectopic expression of the oncogenic SV40 large T antigen (Zhang, 2004b).

In view of these properties, we aimed to further characterize the nuclear activities of apoptin, and how these result in apoptosis induction in cancer cells. We present evidence that chromatin-bound apoptin complexes contain proteins involved in ribosome biogenesis and RNA metabolism, the DNA damage response pathway and regulation of the cell cycle. Taken altogether, our data indicate that

apoptin might coordinate its tumor-specific apoptosis-inducing activity from inside the nucleolus.

Materials & methods

Cell lines, plasmids and transfections

The human SV40 transformed fibroblast cell line VH10/SV40 (Klein, et al., 1990) was grown in DMEM, supplemented with 10% newborn bovine serum (NBS). The human normal diploid skin fibroblast strain F44 (up to passage 15) was grown in 1:1 DMEM/Ham's F12, supplemented with 10% fetal calf serum (FCS), and 2 mM L-glutamine. All culture media were obtained from Invitrogen (Breda, The Netherlands) and contained penicillin and streptomycin.

Plasmid pcDNA-Flag-apoptin containing the DNA sequences encoding Flag-tagged apoptin have been described elsewhere (Zimmerman, et al., 2011a). For transfection experiments, nucleofection technology was used in conjunction with cell type specific Nucleofector solution (AMAXA Biosystems, Cologne, Germany), according to manufacturer instructions.

Immunofluorescence

Cells were grown on glass cover slips. After transfection, the cover slips were first washed once with phosphate buffered saline, and subsequently fixed with methanol/acetone (50%/50%) for 10 min at room temperature. After air-drying, the slides were used for immunocytochemical staining or stored at -20°C for further analysis. Immunocytochemical staining was carried out as described by Danen-Van Oorschot, et al. (1997). Antibody α-Flag (Sigma-Aldrich, Zwijndrecht, Netherlands), a mouse monoclonal antibody against the Flag-tag fused to apoptin, was used to detect the presence and cellular localization of apoptin. The fluorescein isothiocyanate (FITC) or Rhodamine conjugated goat antibodies (Jackson ImmunoResearch

Laboratories, Suffolk, UK) were used as secondary antibodies. At least 100 cells were scored per sample in two independent experiments.

In vivo cross-linking

Twenty-four hours after transfection, SV40-transformed VH10 fibroblasts were subjected to *in vivo* cross-linking and chromatin immunoprecipitation (Fousteri, et al., 2006). All procedures were carried out at 4°C unless otherwise stated. Briefly, 3×10^7 cells were cross-linked with 1% formaldehyde (HCHO) prepared from an 11% stock (0.05 M HEPES (pH 7.8), 0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 11% HCHO) for 16 min. Next, 0.125 M (final concentration) of a glycine solution was added, and the cells were collected by scraping in cold PBS. All buffers used for cell extraction and ChIP contained, in addition to the specified components, 0.1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF, and a mixture of proteinase and phosphatase inhibitors. The cell pellet was resuspended in lysis (CL) buffer (50 mM HEPES (pH 7.8), 0.15 M NaCl, 0.5% NP-40, 0.25% Triton X-100, and 10% glycerol) and rotated for 10 min. After centrifugation (1300 rpm, 5 min), the supernatant was removed. The pellet was washed with buffer consisting of 0.01 M Tris-HCl (pH 8.0), 0.2 M NaCl, 0.5 mM DTT, and resuspended in 1 × RIPA buffer (0.01 M Tris-HCl (pH 8.0), 0.14 M NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.1% SDS). The nuclear suspension was sonicated on ice using a Branson Sonifier 250 (Danburry, CT, USA), yielding fragments between 200 and 1000 bp. The supernatant containing the cross-linked chromatin was collected by centrifugation (13200 rpm, 10 min) and stored in aliquots at -80°C.

Chromatin Immunoprecipitation

For each ChIP reaction, an equal amount of cross-linked chromatin was immunoprecipitated with 0.5–2 μg of the specific anti-Flag antibody or mouse IgG (negative control) in RIPA buffer during o/n incubation. The immunocomplexes were collected by adsorption (3 hr)

to pre-cleared protein G Sepharose beads (Upstate Biotechnology, Inc., Lake Placid, NY, USA) in RIPA buffer containing 0.1 mg/ml sonicated salmon sperm DNA (ssDNA). The beads were next washed twice with 20 vol of RIPA, once with RIPA containing ssDNA, and twice with RIPA containing ssDNA and 0.3 M NaCl. Finally, the beads were washed with 20 vol of LiCl buffer (0.02 M Tris (pH 8.0), 0.25 M LiCl, 0.5% Triton X-100, 0.5% Na-deoxycholate) and the immunocomplexes were resuspended in TE buffer.

Mass Spectrometry

The proteins obtained by ChIP analysis were identified by mass spectrometry (MS) analysis as follows. The ChIP samples were processed by chloroform-methanol precipitation (Wessel and Flugge, 1984), then solubilized in urea and submitted to in-solution proteolytic digestion according to the filter-aided sample preparation (FASP) protocol using a 10K filter, as described by Wiśniewski, et al. (2009). Before proceeding with MS, samples were purified, desalted and concentrated using StageTips (Rappsilber, et al., 2007). The samples were analyzed on a LTQ-orbitrap mass spectrometer (Thermo-Fischer, Breda, the Netherlands) and MS and MSMS was run. Sequence identification was performed using Mascot Daemon (Matrix Science Inc, London, United Kingdom).

Results and Discussion

Apoptin localizes to specific regions of the cancer cell nucleus

We set out to gain a better understanding of the mechanisms behind tumor-selective apoptosis induction by apoptin, starting with its particular nuclear localization. Earlier studies have shown that, in human cancer cells, apoptin forms distinct intranuclear granules, localizing to heterochromatin and nucleoli (Leliveld, et al., 2003). In order to gain further evidence for these findings, we transfected both normal and SV40-transformed human fibroblasts with plasmid DNA

encoding flag-tagged apoptin. Twenty-four hours after transfection, cells were fixed and the subcellular localization of apoptin was determined by immunofluorescence assay. As shown in figure 5.1, apoptin was localized in the cytoplasm of the normal cells, whereas in the transformed cells, apoptin exhibited its characteristic intranuclear localization pattern.

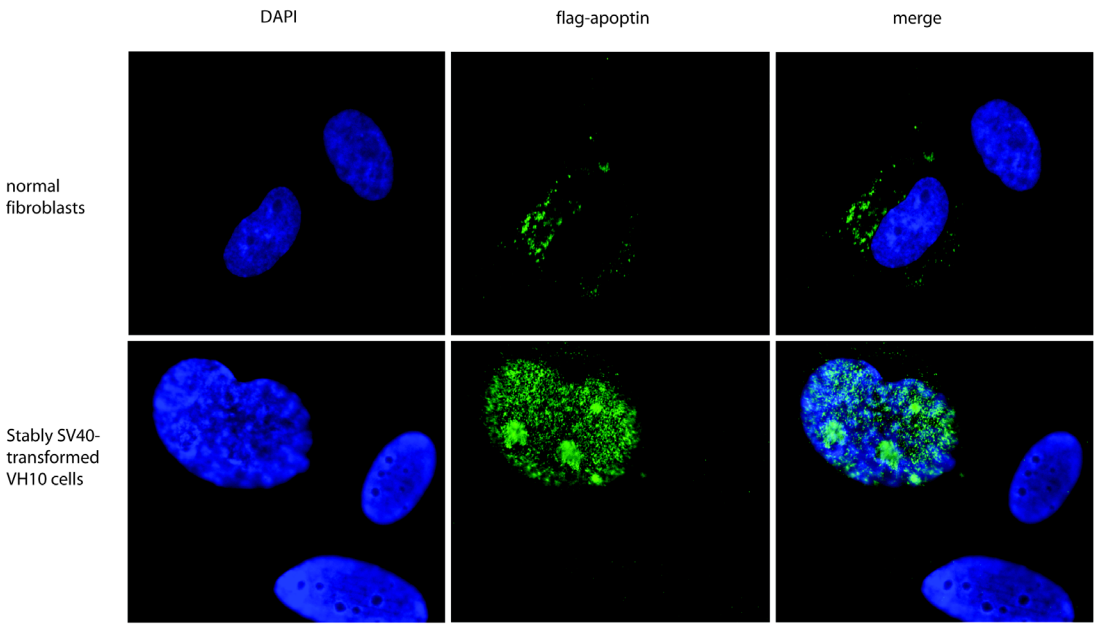

Figure 5.1 Differential localization of apoptin in normal and transformed cells. Normal fibroblasts (upper panel) or stably SV40-transformed fibroblasts (lower panel) were transfected with plasmid DNA encoding flag-tagged apoptin. Twenty-four hours post-transfection, cells were fixed and stained for indirect immune fluorescence analysis as indicated in the Materials & Methods section.

Chromatin Immunoprecipitation identifies the interaction of apoptin with various nuclear proteins

Next, we examined whether apoptin could be found in specific protein complexes interacting with nuclear chromatin. To this end, we designed a proteomic strategy based on chromatin immunoprecipitation (ChIP) coupled with mass spectrometry (Figure 5.2). SV40-immortalized fibroblasts were transfected with flag-apoptin, and chromatin-protein complexes were precipitated using an antibody against the flag-tag. As a negative control, chromatin-protein complexes were incubated with human IgG.

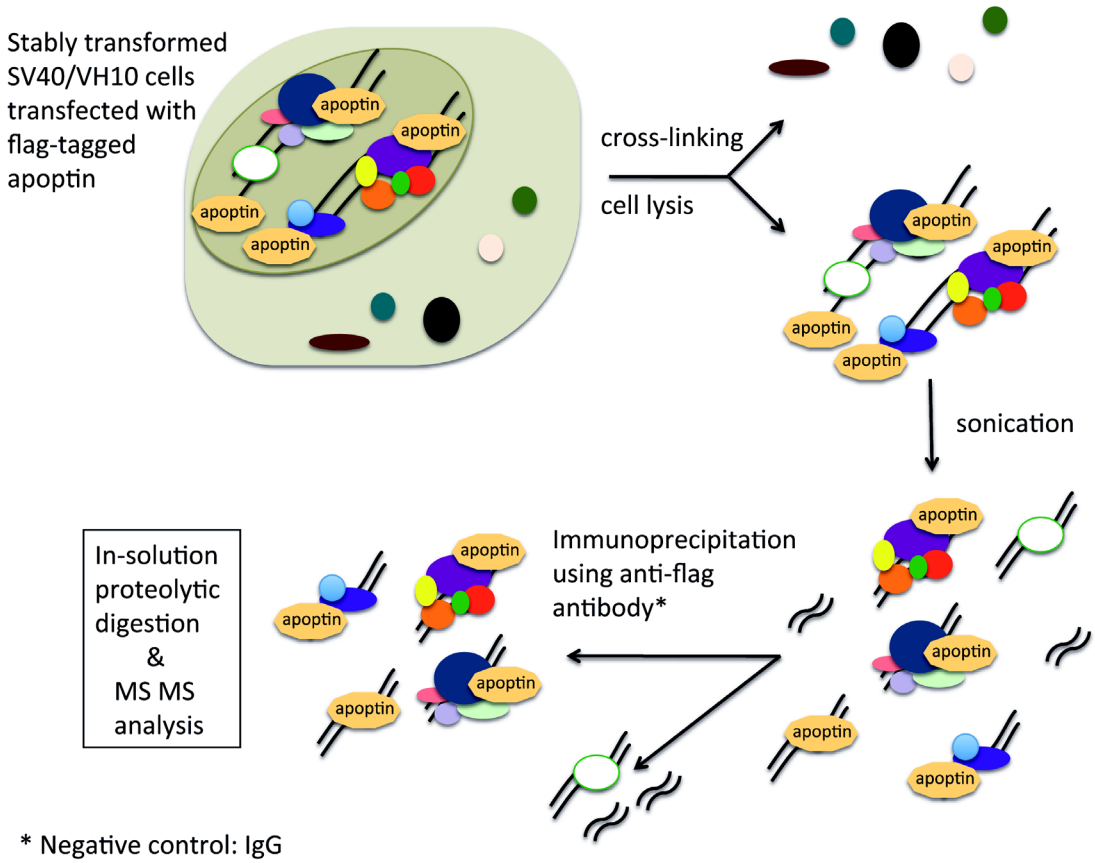

Figure 5.2 Schematic representation of the proteomics approach undertaken in our study to obtain a comprehensive nuclear interaction map of apoptin in the cancer cell nucleus. Stably SV40-transformed fibroblasts were transfected with flag-tagged apoptin. Twenty-four hours post transfection, proteins were cross-linked to chromatin using 1% formaldehyde, after which cells were lysed. Nuclei were pelleted, washed, resuspended, then sonicated to yield chromatin fragments between 200 and 1000 bp. Size of DNA fragments was checked by DNA gel electrophoresis (data not shown). Apoptin-bound complexes were precipitated using an anti-flag antibody* (recognizing the flag-tag fused to apoptin). After in-solution proteolytic digestion, proteins were identified using mass spectrometry analysis. *Alternatively, proteins were incubated with mouse IgG as a negative control.

Several proteins, which were clearly absent in the negative control, were found to co-precipitate with chromatin-associated apoptin complexes (Figure 5.3). Following in-solution digestion, the identity of these proteins was determined by mass spectrometry analysis. Proteins identified in the negative control were considered aspecific to the apoptin-chromatin interaction, and were excluded from further analysis. The list of remaining proteins, specifically present in chromatin-bound apoptin complexes, is presented in Table 5-1.

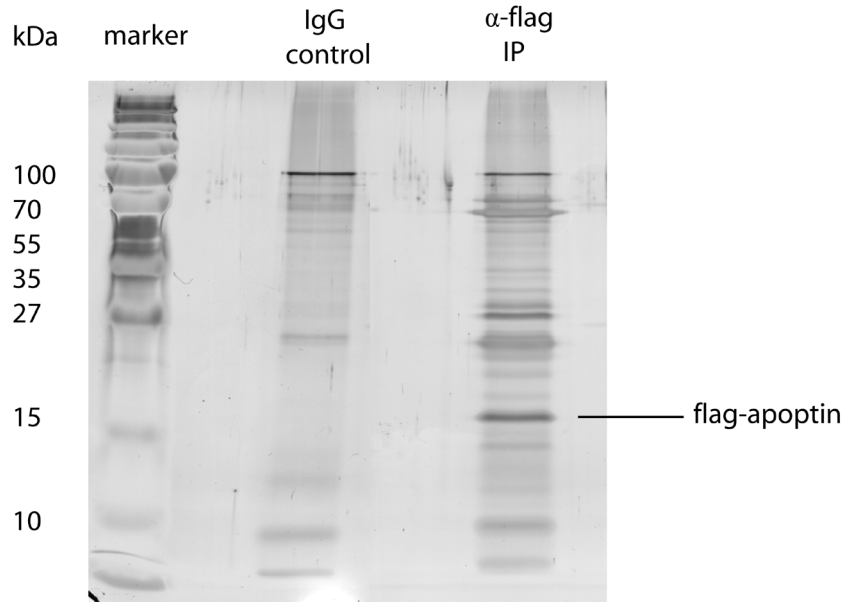

Figure 5.3 Interaction profile of chromatin-bound apoptin complexes, isolated from SV40-transformed cells, 24 hours post transfection with flag-tagged apoptin. Chromatin immunoprecipitation was carried out using flag-antibody (bait) or mouse IgG (control). Specifically interacting proteins were subsequently resolved by SDS-PAGE and silver-stained according to manufacturer protocol (Bio-Rad, Hercules, California, USA). The resulting protein profile obtained with the anti-flag antibody is specific and composed of bands of distinct size and intensity, representing putative proteins interacting with chromatin-bound apoptin. The band belonging to flag-apoptin is indicated (this was determined by western blot analysis, data not shown).

The identification of histone proteins proves that we indeed precipitated chromatin-bound apoptin complexes, while the identification of nucleolar proteins is consistent with the previously observed nucleolar localization of apoptin in cancer cells. Additionally, apoptin has previously been shown to interact with importin beta-1 (Poon, et al., 2005) and tubulin alpha and beta (Teodoro, et al. 2004), confirming the specificity of our analysis. Many of the identified proteins have been demonstrated to harbor cell cycle-related functions and possess either oncogenic or tumor suppressive activities. In addition, several of these proteins have been shown to interact with viral proteins, and are involved in the replication of viral genomes.

Table 5-1 Proteins identified by mass spectrometry analysis of purified apoptin-chromatin immunocomplexes. Proteins unspecifically coprecipitating with the IgG negative control were subtracted from this list.

	Human proteins			
Mass (kDa)	**Protein name**	**Alternative names**	**Function**	**Links to cancer**
14.1 13.9 15.4	histone H2a histone H2B histone H3.2		Nucleosome constituent	
16.5 23 14.9 26.8 30.2 22.1 29.8 15.4 18 18.6 14.8 17.9 14.5 12.6 34.5 29.2	40S ribosomal protein S16 40S ribosomal protein S5 40S ribosomal protein S15a 40S ribosomal protein S3 40S ribosomal protein S3a 40S ribosomal protein S7 40S ribsosomal protein S4, x 40S ribosomal protein S24 60S ribosomal protein L12 60S ribosomal protein L21 60S ribosomal protein L22 60S ribosomal protein L24 60S ribosomal protein L31 60S ribosomal protein L35A 60S acidic ribosomal protein P0 60S ribosomal protein L7		Ribosome constituent	

Table 5-1 continued.

Mass (kDa)	Protein name	Alternative names	Function	Links to cancer
38.9	hnRNP A1		Formation, packaging, processing, and nuclear-cytoplasmic transport of mRNA	Over-expression is associated with non-small cell lung cancer. (Boukakis, et al., 2010)
36.3	hnRNP A/B			
37.5	hnRNP A2/B1			Over-expressed in non-small cell lung cancer (Boukakis, et al., 2010), gastric cancer (Jing, et al., 2011), and hepatocellular carconoma (Cui, et al., 2010).
33.7	hnRNP C1/C2			
46	hnRNP F			Expression is up-regulated in colon cancer (Balasubramani, et al., 2006)
49.5	hnRNP H			
60.7	hnRNP L			
77.7	hnRNP M			
91.2	hnRNP U	SAF-A		
39	PCBP2	hnRNP E2		Down-regulated in oral cancer (Roychoudhury, et al., 2007)
28.4	U2AF		Splicing factor	
54.3	NONO	p54NRB	RNA-binding protein, which plays various roles in the nucleus, including transcriptional regulation and RNA splicing, as well as the DNA damage response	Highly expressed in malignant melanoma (Shiffner, et al., 2011)
16.7	NFAT5		Transcription factor	Activity promotes carcinoma invasion (Jauliac, et al., 2002)
40.1	DBPA	CSDA		Up-regulated in gastric cancer (Wang, et al., 2009)
50.8	EEF1A2		Eukaryotic translation initiation and elongation	Putative oncogenes (Lee and Surh, 2009; Lew, et al., 1992; Tang, et al., 2010).
50.4	EEF1G			
17	EIF5A-1,2			

Table 5-1 continued.

Mass (kDa)	**Protein name**	**Alternative names**	**Function**	**Links to cancer**
76.6 32.7 19.6	Nucleolin Nucleophosmin Nucleophosmin-3	C23 B23, NPM1 NPM3	Nucleolar proteins, involved in the synthesis and maturation of ribosomes, as well as regulation of many other cellular processes, including DNA damage repair, cell cycle and apoptosis. See text for further details.	
33.5	SET	TAF-I	PP2A inhibitor, involved in the regulation of cell cycle progression; see text for further details.	
92.4	CDC5L		Splicing factor and essential regulator of G2/M progression (Bernstein and Coughlin, 1998). Also regulates S-phase checkpoint in response to DNA damage (Zhang, et al., 2009).	
69.6	DDX5	p68	DEAD-box RNA helicase implicated in cellular growth and division; see text for further details.	Overexpressed in prostate cancer (Clark, et al., 2008)
122.9	A26C1		Pro-apoptotic actin isoform (Liu, et al., 2009)	Overexpressed in hepatocellular carcinoma (Chang, et al., 2006)
28.3 50	Filamin A Tubulin alpha, beta		Cytoskeletal proteins	
58.2 98.4	Importin subunit alpha-2 Importin subunit beta 1		Nuclear protein import	
	Viral proteins			
Mass (kDa)	**Protein name**			
13.5 82.4	apoptin SV40 large T antigen			

To facilitate interpretation, we grouped the proteins identified in our apoptin ChIP assay into the following 8 functional groups (Table 5-1, Figure 5.4): 1) Histones; 2) Ribosome constituents and other proteins involved in ribosomal biogenesis; 3) hnRNPs; 4) translation factors; 5) transcription factors; 6) regulatory nucleolar proteins; 7) proteins regulating progression through the cell cycle; 8) cytoskeletal proteins.

Below, we will discuss a select number of these proteins in more detail, highlighting possible novel insights into the mechanisms underlying tumor-selective cell killing by apoptin.

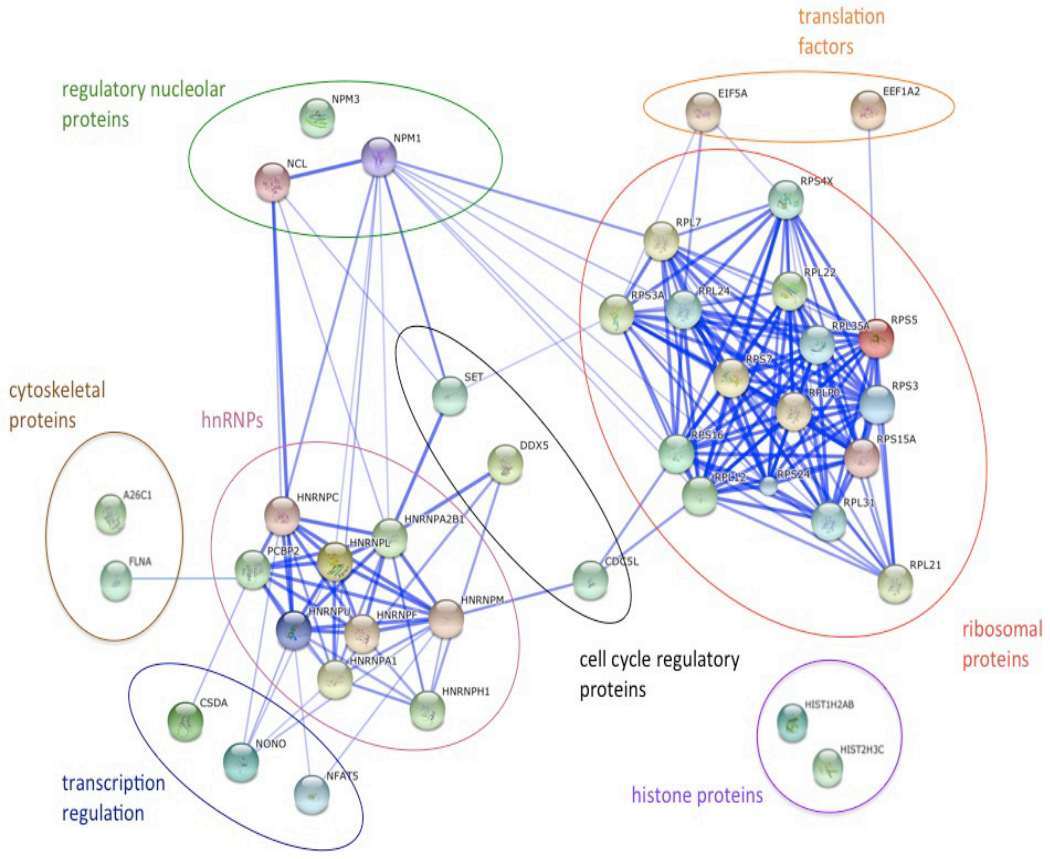

Figure 5-4 The STRING database (Jensen, et al. 2009) was used to identify functional network connectivity among the proteins identified by apoptin chromatin immunoprecipitation. See text for further details.

Apoptin: functions in and out of the nucleolus

Ribosome biogenesis

The nucleolus is generally regarded as a cellular hub for ribosomal biogenesis, vital to cellular proliferation (Montanaro, et al., 2008). The identification of apoptin in chromatin containing ribosomal constituents confirms not only its presence at this important hub, but also its propensity for a transformed environment, given that the presence of multiple, large nucleoli has long been regarded as an immunohistochemical hallmark of cancer cells (Pianese, 1896). In fact, emerging evidence suggests that quantitative and qualitative changes in rRNA synthesis may be among the most important molecular alterations occurring in cancer cells, and several approved anticancer therapeutics are proposed to exert their activity at least in part through interference with this process (Drygin, et al., 2010). For instance, cisplatin, which is traditionally believed to work primarily through the induction of DNA damage, was shown to block rRNA synthesis through inhibition of the rRNA polymerase Pol-I; in contrast, its clinically ineffective analog transplatin, although still being able to damage DNA, had no effect on rRNA synthesis (Jordan and Carmo-Fonseca, 1998). This data imply a role for apoptin interference with ribosome biogenesis as part of its mechanism for inducing apoptosis in cancer cells.

Sensing cellular stress/DNA damage

Recent research has provided evidence that, more than a mere ribosome factory, the nucleolus actively contributes to the regulation of cellular survival and proliferation, playing crucial roles in many fundamental cellular processes, including (regulation of) DNA repair, cell cycle checkpoints in mitosis, and apoptosis (Boisvert, et al., 2007; Boulon, et al., 2010). These pathways are mainly influenced through sequestration of regulators, such as p53, MDM2, and pRB (Tembe and Henderson, 2007). A group of ribosomal proteins (RPs), including

RPL5, RPL11, RPL23 and RPS7, were shown to serve as stress signal transmitters: following stress, they are released from the nucleolus and bind Mdm2, thereby activating p53 (Zhang and Lu, 2009). RPS7 was among the many ribosomal proteins identified in our apoptin chromatin precipitation assay, as was nucleophosmin, a major nucleolar phosphoprotein that has also been shown to act as a nucleolar stress sensor (Yao, et al., 2010). Nucleophosmin associates with all three components of the p14(ARF)-p53-MDM2 cascade, (Bertwistle, et al., 2004; Colombo, et al., 2005; Kurki, et al., 2004), and is further linked to the DNA damage response through its interaction with the checkpoint kinase Chk1 (Chen, et al., 2009). It is also involved in numerous other cellular processes, including ribosome biogenesis (Okuwaki, et al., 2002). Nucleophosmin-regulated ribosome export is a fundamental process in cell growth, and inhibition of nucleophosmin shuttling can block cellular proliferation (Maggi, et al., 2008). It is plausible that the interaction with apoptin achieves just this, thereby contributing to tumor-selective apoptosis induction by apoptin.

Regulation of mitosis

Our assay also identified the nucleophosmin-interacting protein nucleolin, a major nucleolar phosphoprotein with important roles in chromatin structure, rDNA transcription, rRNA maturation, nucleocytoplasmic transport, and ribosome assembly (Ginisty, et al., 1999; Liu and Yung, 1999; Srivastava and Pollard, 1999). Both nucleolin and nucleophosmin are highly expressed in proliferating and cancerous cells (Eichler and Craig, 1994; Pianta, et al., 2010).

Aside from their nucleolar functions, both proteins have important functions in the regulation of mitosis. Nucleolin localizes to the chromosome periphery in mitotic cells, is associated with the spindle poles from prometaphase to anaphase, and is involved in chromosome congression and spindle formation (Ma, et al., 2007), while

nucleophosmin is present at centromeres (Foltz, et al., 2006) and at spindle poles in metaphase cells, and is required for proper chromosome alignment on the equatorial planes during metaphase (Rousselet, 2009). Nucleophosmin is also required for centrosome duplication (Moss and Stefanovsky, 2002; Okuda, et al., 2000), the formation of functional and stable spindles with intact centrosomes and for proper kinetochore-microtubule attachments (Amin, et al., 2008). Apoptin was previously shown to interact with FAM96B, which also localizes to the mitotic spindle and is required for proper sister chromatid cohesion and chromosome segregation (Zimmerman, et al., 2011b; Ito, et al, 2010). The association with nucleolin and nucleophosmin, as well as actin, filamin and tubulin, again places apoptin at the mitotic spindle, further accentuating its important mechanistic function in apoptin-induced apoptosis.

Further evidence comes from the identification of Cdc5L and SET in our assay. Cdc5L is the human homolog of Schizosaccharomyces pombe Cdc5, and like its yeast counterpart, it is an important cell cycle regulator of G2/M transition (Bernstein and Coughlin, 1998). It is also required for progression through the S-phase of the cell cycle, with recent studies describing a function for Cdc5L in the cellular response to DNA damage (Zhang, et al., 2005), demonstrating that it interacts physically with the cell-cycle checkpoint kinase ataxia-telangiectasia and Rad3-related (ATR), and is required for the activation of downstream effectors or mediators of ATR checkpoint function, including Chk1 and Rad17 (Zhang, et al., 2009). SET, a potent inhibitor of the tumor suppressor protein phosphatase 2A (PP2A) (Li, et al., 1996) and an important modulator of chromatin remodeling and condensation, also regulates progression through the cell cycle (Vera, et al., 2007; Leung, et al., 2011). It binds directly to p21CIP1 and reverts the inhibitory effect of the latter protein on cyclin E-Cdk2 kinase activity, thus allowing progression through S-phase (Estanyol, et al., 1999). SET also regulates G2/M transition by

binding to cyclin B and inhibiting cyclin B-Cdk1 activity (Canela, et al., 2003). Hence, through its interactions with nucleophosmin, nucleolin, Cdc5L, and SET, and their roles in the pathways regulating the DNA damage response as well as progression through mitosis, we gain valuable new insights into apoptin's *modus operandi* inside the cancer cell nucle(ol)us.

hnRNPs and other RNA-related proteins (RNPs)

In our chromatin immunoprecipitation experiments apoptin was also found to associate with a large number of heterogeneous nuclear ribonucleoproteins (hnRNPs).

hnRNPs comprise a family of RNA-binding proteins that are principally involved in RNA metabolism (Han, et al., 2010). Within the nucleus, hnRNP proteins participate in RNA splicing, 3'-end processing, transcriptional regulation, and immunoglobulin gene recombination. hnRNP proteins are also involved in nucleo-cytoplasmic transport of mRNAs, mRNA localization, translation and mRNA stability. Recently, evidence has been provided that hnRNPs are also associated with heterochromatin, at least in Drosophila (Piacentini, et al., 2009). hnRNP-U has been found to associate with the heterochromatin protein 1 alpha (HP1 alpha) in both the nucleoplasm and in chromatin (Ameyar-Zazoua, et al., 2009); hnRNP-A2/B1 was shown to associate with DNA-bound proteins (Guha, et al., 2009); and hnRNP-C1/C2 proteins were demonstrated to bind to chromatin in a DNA damage-dependent manner (Wardleworth and Downs, 2005). Additionally, it has been suggested that hnRNP proteins play critical and well-defined roles in carcinogenesis. hnRNP-E1, -A1, and -D have been shown to form a heteromeric complex on telomeric repeats *in vitro* (Ishikawa, et al. 1993), suggesting that they may be involved in the maintenance of telomeric length. This notion is reinforced by the observation that deficient expression of hnRNPs can cause significant telomere shortening and oncogenic transformation

(LaBranche, et al. 1998). A recent study has indeed confirmed that hnRNP-A1 participates in telomere maintenance (Flynn, et al., 2011). hnRNP-A2/B1 is over-expressed in lung, breast, pancreas and liver cancer (Tauler, et al., 2010), and hnRNP-A1 has been identified as a potential biomarker for colorectal cancer (Ma, et al., 2009). Moreover, hnRNP-E1, hnRNP-K, and FUS (or hnRNP-P2) were identified as intricate constituents of spreading initiation centers, which are ribonucleoprotein complexes involved in the initiation of cell spreading during cancer metastasis (de Hoog, et al. 2004). Down-regulation of hnRNP-E1 stimulated cell spreading, consistent with the observation that shRNA-mediated silencing of hnRNP-E1 induces constitutive EMT, another prerequisite for metastatic progression (Chaudhury, et al. 2010; de Hoog, et al. 2004). Reduced hnRNP-E1 expression is also a prerequisite for human papillomavirus (HPV) proliferation and subsequent incidence of cervical carcinoma from cervical dysplasia (Pillai, et al. 2003). A study on the molecular mechanisms by which hnRNP proteins regulate cell proliferation in cancer, showed that hnRNP-A1 and hnRNP-A2 proteins control the alternative splicing of pyruvate kinase mRNA, which facilitates the metabolic shift from oxidative phosphorylation to aerobic glycolysis in cancer (Chen, et al., 2010). Another study demonstrated that hnRNP-F was involved in the regulation of cell proliferation via the mTOR/S6 kinase 2 pathway (Goh, et al., 2010). Finally, importantly, hnRNP-U (SAF-A), is specifically phosphoryated in response to double-stranded DNA breaks (Berglund and Clarke, 2009), and was shown to be a novel spindle regulator with an essential role in kinetochore-microtubule attachment and mitotic spindle organization (Ma, et al., 2011).

The identification of this many RNPs in our assay likely signifies an important association between apoptin and the regulation of RNA metabolism, a conclusion which is further strengthened by the above-described, recently discovered roles of hnRNPs in carcinogenesis.

Viral replication

As briefly alluded to before, several of the proteins identified in our assay have been shown to interact with viral genomes and proteins, and take part in the process of viral replication (see also Table 5-2).

Table 5-2 Proteins involved in viral replication identified by ChIP-MS analysis of chromatin-bound apoptin protein complexes.

Protein	*Relevant to viral replication of*
Nucleolin	Human papilloma virus-16 Herpes simplex virus-1 Cytomegalovirus
DDX5	SARS virus Hepatitis C virus
PCBP2	Hepatitis C virus Dengue virus
hnRNP F	Influenza A virus
hnRNP C	Poliovirus
hnRNP A1	HLTV-1

Nucleolin, for example, binds to the HPV16 genome (Sato, et al., 2009) and has also been suggested to play a role in replication of HSV-1 DNA (Callé, et al., 2008). Furthermore, nucleolin is required for viral DNA synthesis and efficient virus production in human cytomegalovirus (HCMV)-infected cells (Strang, et al., 2010). The RNA helicase DDX5 is required for SARS coronavirus replication (Chen, et al., 2009), and is also involved in the replication of Hepatitis Virus C (HCV) (Goh, et al., 2004). The poly(rC)-binding protein (PCBP2, or hnRNP-E2) directs HCV RNA replication (Wang, et al., 2011), and is also involved in dengue virus replication (Rodenhuis-Zybert, 2010). Similarly, hnRNP-F, hnRNP-C and hnRNP-A1 are involved in the replication of the influenza A virus (Lee, et al., 2010), poliovirus (Brunner, et al., 2010), and HTLV-1, respectively (Kress, et al., 2005). As apoptin is itself a product of the Chicken Anemia Virus (CAV), the question arises whether these interactions might also be relevant for CAV replication. Coincidentally, CAV can only replicate in tumorigenic chicken cell lines, where it is localized in the nucleus, but does not replicate in normal chicken cell cultures (Noteborn, 2004).

Finally, the SV40 large T antigen was also identified in our analysis. Its presence in our experimental system is explained by the fact that this antigen was used to stably transform the cells used in our assay, as described in the Materials and Methods section. The oncogenic transformation potential of SV40 LT relies on its interaction with several tumor suppressors, including p53 and pRb (Ahuja, et al., 2005; Cheng, et al., 2009) and its expression has been shown to swiftly activate the DNA damage response (Boichuk, et al., 2010; Hein, et al., 2009). We have previously demonstrated (Zimmerman, et al., 2011a) that transient transformation of normal cells with SV40 LT is sufficient to activate apoptin, inducing its nuclear translocation, and subsequently, apoptosis. It is, however, the first time that we find an association, either directly or indirectly, between apoptin and SV40 LT.

Conclusion

The results presented here attest that the previously observed nucleolar localization of apoptin is not a random one, and that interaction with nucleolar proteins and R-chromatin is likely essential for apoptin's tumor-selective apoptosis-inducing activity. Importantly, nucleolin and nucleophosmin link apoptin to the DNA damage response; CDC5L and SET1 provide a connection to the regulation of mitosis, while the interactions with tubulin, nucleophosmin and nucleolin also corroborate the association of apoptin with the mitotic spindle apparatus. Furthermore, the interactions with proteins involved in ribosome biogenesis and RNA metabolism suggest a mechanism whereby apoptin shuts down the cellular factory and orchestrates its apoptotic effects from within the nucleolus.

A comparable mechanism has recently been demonstrated for the mouse Polo-like kinase 5, which also localizes to the nucleolus, and induces apoptosis in response to DNA damage (Andrysik, et al., 2010).

Recently, the pRb-regulated E2F family member E2F1 has been shown to induce DNA-damaged mediated apoptosis by transcriptional upregulation of the nucleolar protein RRP1B (Paik, et al., 2010). E2F1 itself is activated by Nur77 (Mu and Chang, 2003), which has been shown to participate in apoptin-induced apoptosis (Maddika, et al., 2005). Furthermore, apoptin has been shown to interact with DEDAF (Danen-van Oorschot, et al., 2004), which associates with DEDD in the nucleolus (Zheng, et al., 2001). It has indeed been reported that DEDD inhibits the Cdk1/cyclin B complex (Arai, et al., 2007), thereby arresting mitosis, and transferring the apoptotic signal to the nucleolus to shut off biosynthesis (Stegh, et al., 1998). Furthermore, apoptin has been shown to localize to the nucleus in response to DNA damage (Kucharski, et al., 2011), and induce G2/M arrest (Teodoro, et al., 2004). We therefore predict that apoptin's tumor-selective nuclear localization functions in recognizing activation of the DNA damage response, interfering with biosynthesis and arresting the mitotic cycle as a result, triggering apoptosis.

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