Apoptin : oncogenic transformation & tumor-selective apoptosis

Zimmerman, R.M.E.

Chapter 4

Cellular partners of the apoptin-interacting protein 3 FAM96B

RME Zimmerman[1], J Sun[1,2], J Tian[1,2], M de Smit[1], AA Danen-van Oorschot[1], M Rotman[1], P Snel[1], P Voskamp[1], MHM Noteborn[1] and C Backendorf[1*]

[1]Department of Molecular Genetics, Leiden Institute for Chemistry, Leiden University, Leiden, the Netherlands. [2]Department of Biochemistry and Molecular Biology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

***Corresponding author:** C Backendorf, Department of Molecular Genetics, Leiden Institute of Chemistry, Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands. Tel: +31 715274409; Fax: +31 715274430; e-mail: backendo@chem.leidenuniv.nl

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Abbreviations: apoptin, apoptosis inducing protein; AIP3, apoptin interacting protein 3; CGI, comparative gene identification; FAM96B, Family with sequence similarity 96, member B; NER, nucleotide excision repair

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Abstract

In tumor cells apoptin is phosphorylated, locates to the nucleus and induces apoptosis in contrast to normal cells. The cellular mechanisms providing this selectivity are largely unknown. Here, we describe the identification of a new interacting partner for apoptin, apoptin interacting protein 3 (AIP3), also known as FAM96B, and examine which processes are targeted by apoptin via its interaction with this protein. We show that FAM96B interacts with apoptin independently of the latter's tumor-selective phosphorylation. Interaction studies including yeast two-hybrid and co-immunoprecipitation experiments demonstrated that FAM96B associates with proteins involved in DNA transcription, replication and repair, as well as components of the actin cytoskeleton. Furthermore, ectopic over-expression of FAM96B alone in cancer cells induced apoptosis. The results indicate that, through its interaction with FAM96B, apoptin is linked to the fundamental cellular processes of DNA repair and cell division. This discovery highlights an important part of the mechanisms responsible for tumor-selective cell death induced by apoptin.

Introduction

Recent years have witnessed the development of a new area of cancer research, focusing on proteins credited with the ability to selectively kill tumor cells (Backendorf, et al., 2009). These proteins are expected to target malignant cells more specifically than the anticancer agents that are presently used (Los, et al., 2009). Also, whereas current therapies mostly result in necrosis of the cancer cells, thus entailing a considerable amount of inflammation, these proteins induce cell death through mechanisms such as apoptosis and autophagy (Bruno, et al., 2009), potentially providing substantial improvement to the treatment of patients.

The chicken-anemia-virus derived protein apoptin has opened the field for this type of tumor-selective research. Apoptin has been shown to efficiently and safely kill tumors in various preclinical models (Grimm & Noteborn, 2010). In tumor cells, apoptin becomes phosphorylated and is located in the nucleus, whereas in normal cells it is unmodified and present in the cytoplasm (Backendorf, et al., 2008). Studies on the underlying mechanisms of apoptin-induced tumor-selective cell death have revealed that apoptin interacts with proteins such as DEDAF (Danen-van Oorschot, et al., 2004) and APC/C1 (Teodoro, et al., 2004). However, the cellular mechanisms underlying the activation of apoptin, and its subsequent execution of cell death are not yet clear (Agriris, et al., 2011).

Here, we describe that apoptin interacts with apoptin-interacting protein 3 (AIP3), previously designated FAM96B. Further analysis revealed that FAM96B itself interacts with a number of cellular proteins, mostly known to be involved in either DNA repair or cytoskeletal organization, offering important insights into the mechanisms behind tumor-selective cell death induced by apoptin.

Materials & methods

Yeast two-hybrid screens

The Matchmaker yeast two-hybrid kit System 3 was purchased from Clontech (Leusden, The Netherlands), and experiments were performed according to manufacturer protocol, with a few slight modifications. Briefly, a cDNA library derived from human keratinocytes was cloned into the leucine-selectable pGAD10yeast expression vector, downstream of the gene encoding the GAL4 activation domain (AD). Full-length apoptin or FAM96B was cloned downstream of the gene encoding the GAL4 DNA-binding domain (BD) in the tryptophane-selectable plasmid pGBKC3 and used as bait in the yeast two-hybrid screen. After proper selection, resulting clones were sequenced by LGTC (Leiden, the Netherlands).

Cells, plasmids & transfections

Positive clones from the apoptin yeast two-hybrid screen were digested with appropriate restriction enzymes to generate cDNA fragments, which were subcloned into pMT2SM-myc, providing the fragments with an in-frame N-terminal myc-tag (9E10) (Gebbink, et al., 1997) The sequence encoding myc-tagged FAM96B was then cloned into the pcDNA 3.1 (+) vector to obtain pcDNA-myc-FAM96B.

Positive clones from the FAM96B yeast two-hybrid screen were digested with appropriate restriction enzymes to generate cDNA fragments, which were subcloned into the pIBA105 vector, providing the fragments with an in-frame N-terminal strep-tag (Schmidt and Skerra, 2007) (IBA GmbH, Mannheim, Germany).

The DNA sequence encoding apoptin was synthesized by Base-Clear (Leiden, the Netherlands), according to the published apoptin sequences (Noteborn, et al., 1991) and cloned into the mammalian expression vector pcDNA. The final construct was checked by sequencing and named pcDNA-apoptin. Next, we generated the pcDNA-Flag-apoptin plasmid encoding apoptin fused with

a Flag-tag at its N-terminus. Subsequently, mutations were introduced to create flag-apoptinT108E, encoding a mutant apoptin mimicking constitutive phosphorylation, and flag-apoptin5Ala106, encoding Flag-apoptin in which amino acids 106-110 were replaced by alanines, resulting in a phosphorylation-negative mutant apoptin.

The human osteosarcoma cell line Saos-2, VH10/SV40 and human foreskin fibroblasts were grown in DMEM, supplemented with 10% newborn bovine serum (NBS), and 2 mM L-glutamine.

For transfection experiments, nucleofection technology was used in conjunction with cell type specific Nucleofector solution (AMAXA Biosystems, Cologne, Germany).

Co-immunoprecipitation and Western blot analysis

For co-immunoprecipitation experiments, cells were lysed 24h after transfection. Cells were washed with cold phosphate buffered saline and harvested in ice-cold mild lysis buffer (50mM Tris (pH 7.5), 5mM EDTA, 250mM NaCl, 0.1% Triton X-100, 5 mM NaF, 1mM Na3VO4, 20mM beta-glycerolphosphate, and Protease Inhibitor Cocktail (Roche, Almere, the Netherlands), followed by incubation on ice for 30 min. The lysate supernatant was prepared by centrifugation at 13,000 × g and 4°C for 30 min, and incubated with indicated antibodies for 90 min, followed by incubation with Protein A/G Microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany). Magnetic immunoprecipitation was performed according to manufacturer protocol. Samples were resolved on sodium dodecyl sulfate-polyacrylamide gel, followed by Western blotting analysis with appropriate antibodies.

Bioinformatics analysis

The STRING database was used to identify functional network connectivity (Jensen, et al., 2009).

Results

Yeast two-hybrid analysis reveals a novel apoptin interacting protein: AIP3/FAM96B

In order to gain more insight into the mechanism by which apoptin induces apoptosis in human tumor cells, we performed a yeast two-hybrid screen using full-length apoptin as bait (Danen-van Oorschot, et al., 2004). One of the interacting proteins identified in this manner was AIP3. This protein of unknown function had previously been designated FAM96B (Finn, et al.,

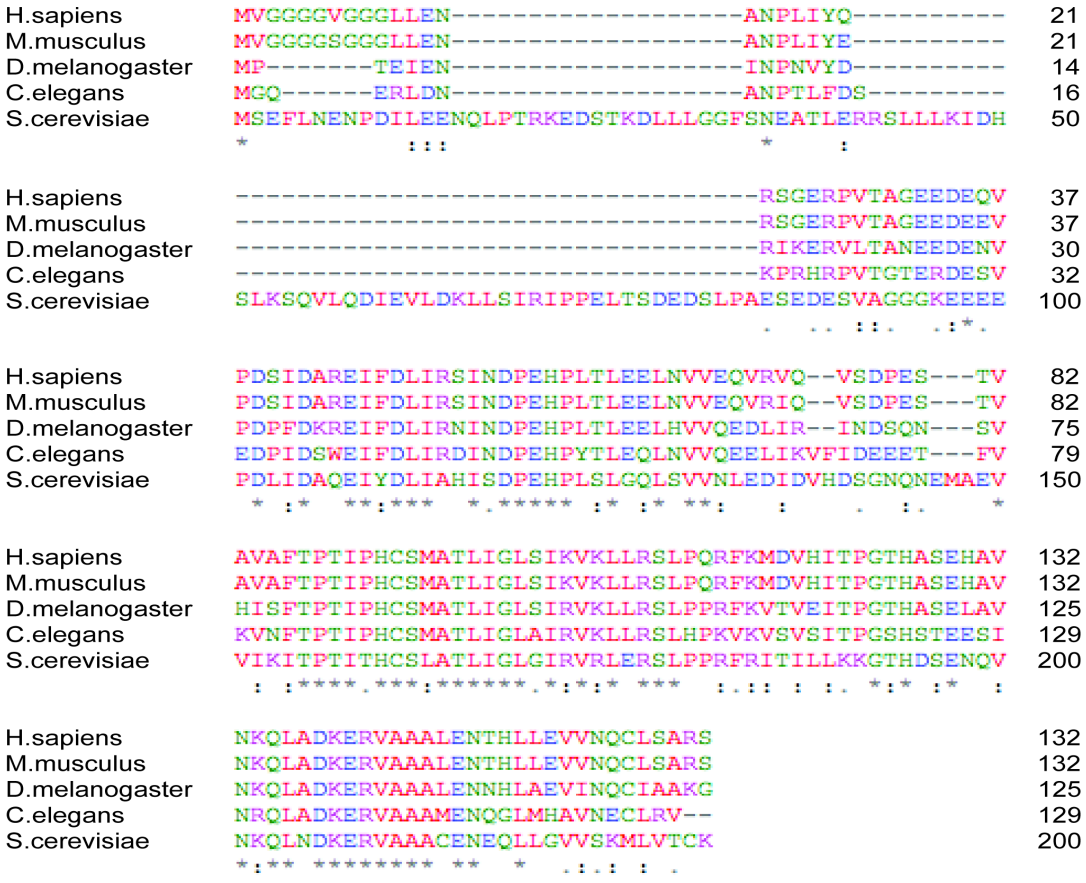

Figure 4.1 The AIP3 (also known as FAM96B) gene is conserved in chimpanzee, dog, cow, mouse, rat, chicken, zebrafish, fruit fly, mosquito, C.elegans, S.pombe, S.cerevisiae, K.lactis, E.gossypii, M.grisea, N.crassa, A.thaliana, and rice. Shown here is a ClustalW alignment of the amino acid sequences of human FAM96B (NP_057146.1) and its orthologs in the mouse Mus musculus (NP_081029.1), Drosophila melanogaster (NP_648416.1), Caenorhabditis elegans (NP_499777.1), and Saccharomyces cerevisiae (NP_011990.1). Small, hydrophobic amino acids are coloured red; acidic amino acids are blue, basic amino acids are magenta, and polar amino acids are shown in green. "*" means that the residues or nucleotides in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed, according to the colours indicated above. "." means that semi-conserved substitutions are observed (Chenna, et al. 2003).

2010). Figure 4.1 shows the alignment of the amino acid sequence of human FAM96B with that of the mouse, *C.elegans*, *Drosophila*, and *S.cerevisiae*, demonstrating the conservation of this protein across a wide range of species.

Apoptin interacts with FAM96B in normal and cancer cells, independently of its phosphorylation status

Next, we examined whether FAM96B associates with phosphorylated and/or non-phosphorylated apoptin in Saos-2 tumor cells and in primary fibroblasts. The cells were co-transfected with plasmids encoding Flag-tagged apoptin, Flag-apoptinT108E mimicking constitutively phosphorylated apoptin, phosphorylation deficient-mutant Flag-apoptin5Ala106 and myc-tagged FAM96B or an empty vector. Co-immunoprecipitation and Western blot analysis were carried out as described in the Materials and Methods section.

Figure 4.2A shows the Western blots of the co-immunoprecipitation analysis of normal and tumor cells expressing Flag-apoptin protein with or without FAM96B. Immunoprecipitation with antibodies against the Flag-tag of apoptin resulted for both cell types in co-immunoprecipitated FAM96B protein product. Similar co-immunoprecipitation results were obtained with lysates derived from normal and tumor cells co-expressing FAM96B and Flag-apoptinT108E or Flag-apoptin5Ala106 mutants (Figures 4.2B and C, respectively). In all cases, myc-tagged FAM96B co-immunoprecipitated with Flag-apoptinT108E or Flag-apoptin5Ala106.

These results indicate that the interaction between FAM96B and apoptin is independent of the phosphorylation status of apoptin, and can occur both in normal and cancer cells.

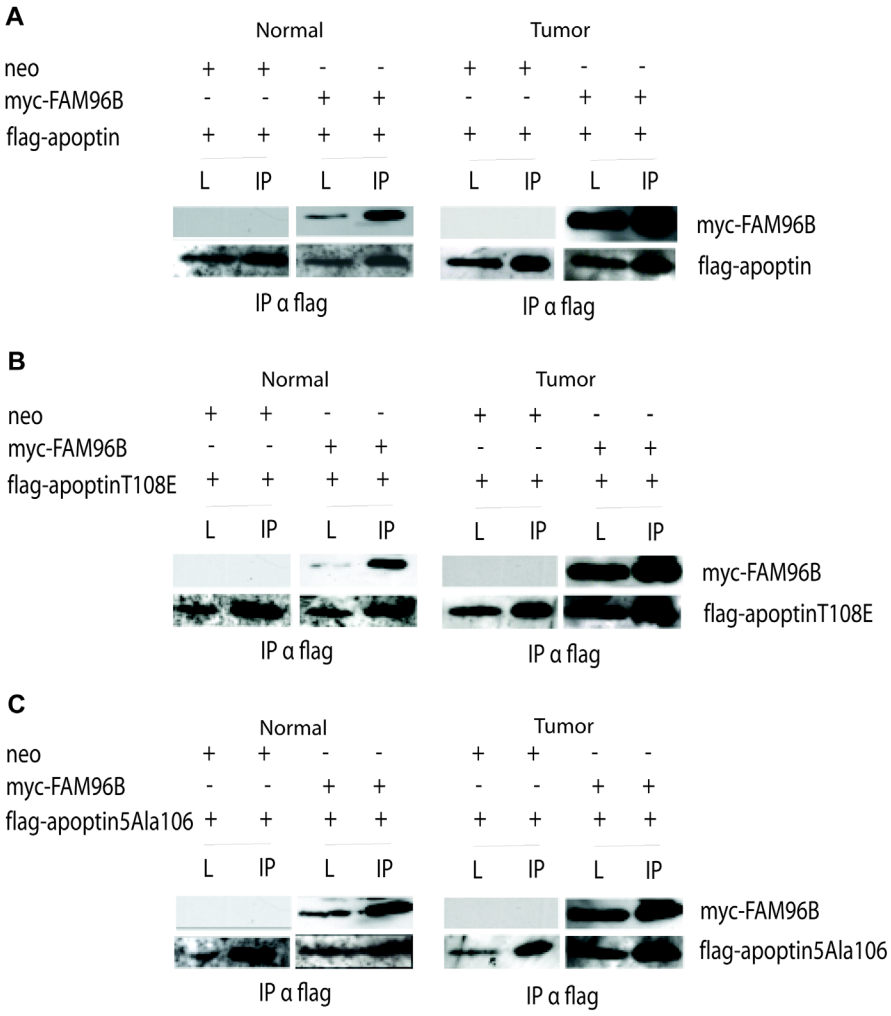

Figure 4.2 FAM96B interacts with apoptin in human cells, independently of apoptin's phosphorylation status. **A.** Cells were transfected with plasmids encoding flag-apoptin and myc-tagged FAM96B, or control plasmid (neo) in the indicated combinations. Total lysates (L) or protein complexes immunoprecipitated with antibody against the flag-tag were separated by SDS-PAGE and analyzed by Western blotting. *Left panel* normal human foreskin fibroblasts; *right panel* human Saos-2 tumor cells. **B.** As **A**, using flag-apoptinT108E, which mimicks constitutively phosphorylated apoptin. **C.** As **A,** using flag-apoptin5Ala106, in which amino acids 106-110 have been replaced with a 5-aa stretch of alanines, thereby abolishing the phosphorylation site at aa108.

Association of FAM96B with DNA repair and the cytoskeleton

A yeast two-hybrid screen was then performed using human FAM96B as bait, with the purpose of understanding the cellular processes involving FAM96B, and how these could be involved in the induction of apoptosis by apoptin. Twenty different proteins were found to associate with FAM96B (Table 4-1). For all but one of these interactants, the association could be confirmed by co-immunoprecipitation in a human cellular background (Figure 4.3).

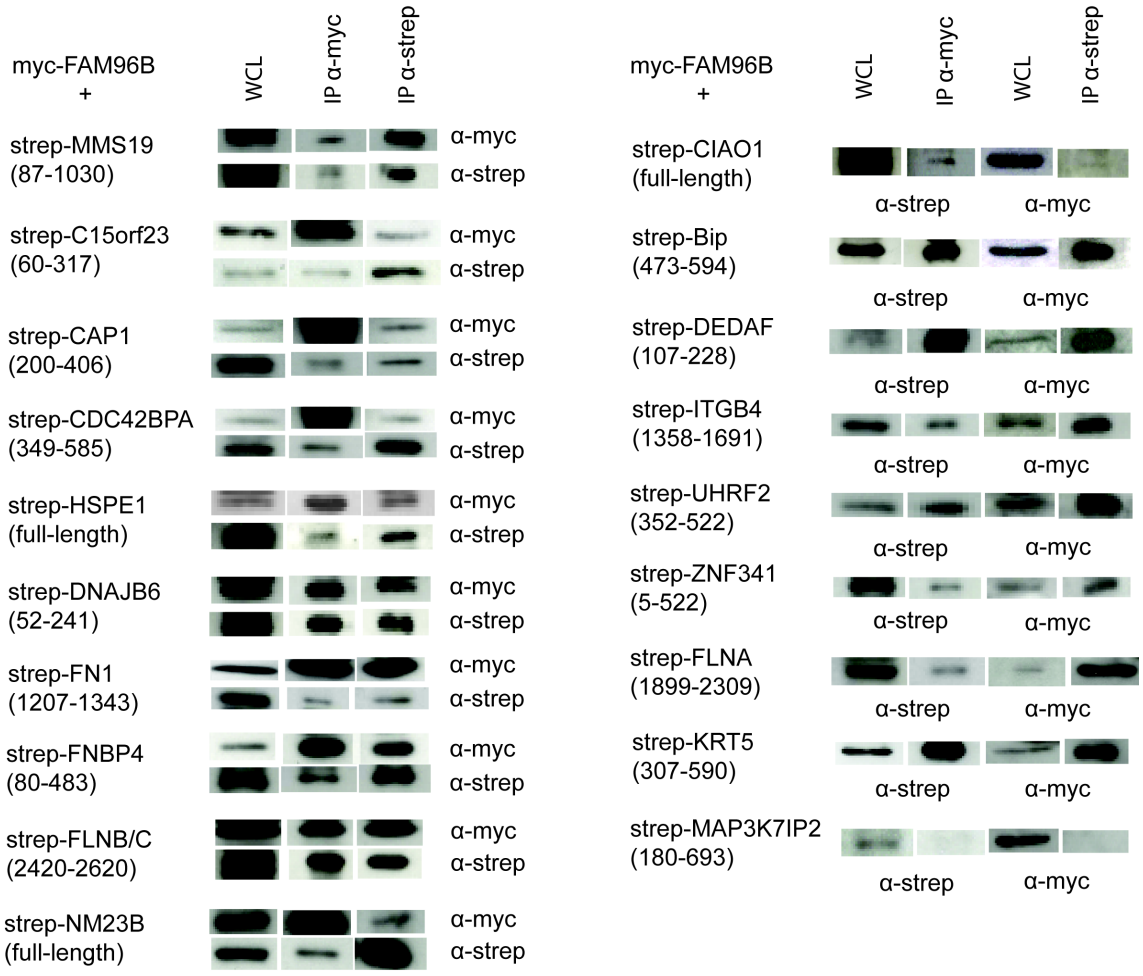

Figure 4.3 Co-immunoprecipitation of FAM96B in human cells with interaction partners identified by yeast two-hybrid. VH10/SV40 cells were transfected with plasmids encoding myc-tagged FAM96B and strep-tagged candidate interaction partners. Twenty-four hours post transfection, cells were lysed and protein complexes immunoprecipitated with antibodies recognizing either the myc- or strep-tag. MAP3K7IP2 was the only candidate partner out of 20 that did not interact with FAM96B in a human cell background.

Bioinformatics analysis of the cellular pathways in which the newly identified FAM96B interactants were involved, revealed the distribution of these partners in two main groups of proteins, namely DNA transcription and repair, and cytoskeleton organization and dynamics, with many linked specifically to chromosome segregation (Table 4-2 and Figure 4.4). Among these proteins, there are clear links to tumor-related processes: the nucleoside diphosphate kinase NM23B, for instance, has been shown to suppress metastasis (Roymans, et al., 2002), while UHRF2 is a putative tumor suppressor gene (Bronner, et al., 2007; Sjöblom, et al., 2006), and CIAO1 is linked to the Wilms tumor suppressor protein 1 (WT1) (Johnstone,

et al,1998). FAM96B was also found to associate with protein chaperones such as BIP (HSPA5) and HSPE1 (Hsp10), both of which have been shown to be differentially expressed in cancer (Zhuang, et al., 2009; Czarnecka, et al., 2006), Interestingly, both BIP and DEDAF, a transcriptional repressor involved in DNA damage-induced apoptosis, has previously also been shown to interact with apoptin, and over-expression of DEDAF in tumor cells has been shown to induce apoptosis (Danen- van Oorschot, et al., 2004; Novak and Phillips, 2008; Sato, et al., 2010).

As depicted in figure 4.4, there are also many links between the FAM96B interactants themselves. From this network of proteins, it emerges that FAM96B might be involved in the regulation of cell division, particularly chromosome segregation, and that this regulation is likely subject to DNA repair pathways.

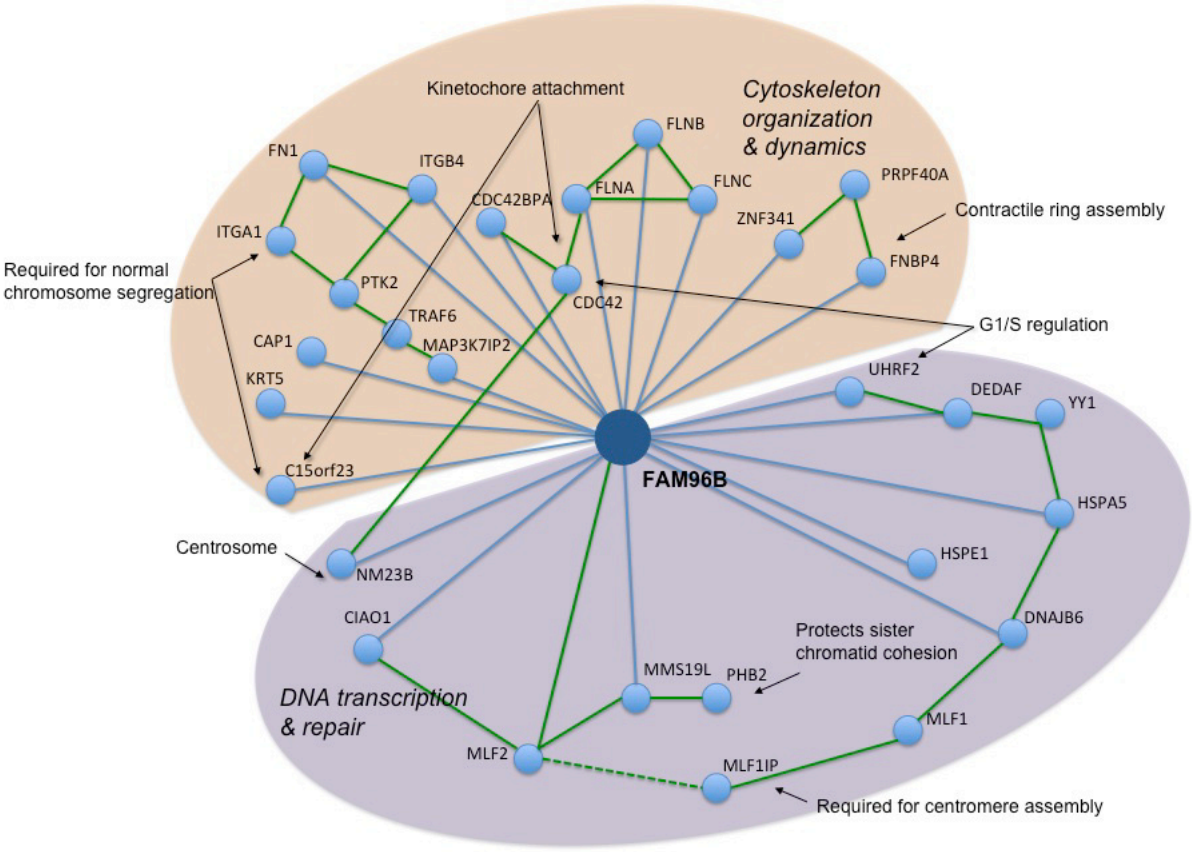

Figure 4.4 Schematic representation of the FAM96B interaction network, as derived from our yeast two-hybrid experiments (blue lines) and literature (green lines). The STRING database was used to identify functional network connectivity (Jensen, et al. 2009).

Table 4-1 Proteins associating with FAM96B. FAM96B interactants were identified through yeast two-hybrid analysis and confirmed as FAM96B partners in a human cellular background. Besides the protein name, the table also lists the proposed function (Funcbase annotation according to Beaver, et al., 2010) and, if applicable, links to cancer.

Protein name	Proposed function	FuncBase annotation	Links to cancer
CIAO1	Cytosolic Fe–S cluster maturation, transcriptional regulation: Depletion of Cia1 impairs the export of the large ribosomal subunit from the nucleus	Positive regulation of cell proliferation	Deletion lethal in S. cerevisiae. Modulates the transactivation activity of Wilms tumor suppressor protein (WT1)
NM23B	Nucleoside diphosphate kinase	Metastasis suppressor	Suppressor of metastasis
MMS19	Nucleotide excision repair and POLII transcription	Nucleotide excision repair	Common variation associated with increased risk of pancreatic cancer. May also function as transcriptional co-activator of estrogen receptor (ER)
DEDAF (RYBP)	PcG protein with transcriptional co-repressor activity Induces cell-cycle arrest and is involved in the p53 response to DNA damage	Transcriptional repressor	Decreased in human cancer tissues
UHRF2 (NIRF)	E3 ubiquitin protein ligase Induces G1 arrest and associates with Cdk2/cyclin E	E3 ubiquitin ligase, cell cycle regulator	Putative tumor suppressor gene
MAP3K7IP2 (TAB2)	MAPK signaling pathway (Links MAP3K7 with TRAF6; also interacts with TRAF2	NF kappa B signaling	
ZNF341	May be involved in transcriptional regulation		
DNAJB6 (MRJ)	DNA J-domain containing molecular chaperone	Regulation of caspase activity, unfolded protein reponse, intermediate filament cytoskeletal reorganization	Expression is lost in breast cancer Over-expression partially reverses mesenchymal phenotype and reduces malignant activity of breast cancer
CAP1	Directly regulates filament dynamics	Cell morphology and motility	Over-expression is involved in aggressive behavior of pancreatic cancer
CDC42BPA	Serine/threonine protein kinase	Actin cytoskeleton reorganization	May act as a downstream effector of CDC42 in cytoskeletal reorganization, contributes to cell invasion
FN1	Cell adhesion and motility	Cell adhesion and migration	
FNBP4	Formin binding protein	Cell cycle checkpoint	

Table 4-1. continued.

ITGB4	Laminin receptor, structural role in the hemi-desmosomes of epithelial cells	Cytokine binding, cell migration	Likely to play a pivotal role in the biology of invasive carcinoma.
FLNA/B/C	Connects cell membrane constituents to the actin cytoskeleton	Actin cytoskeleton	Aberrant FLN's are involved in e.g. acute myelomonoblastic leukaemia
KRT5	Cytokeratin	Cytoskeletal keratin; cell migration	
BIP	Heat shock protein; probably plays a role in the assembly of multimeric protein complexes in the ER	Unfolded protein response	Possibly involved in pathogenesis of renal cell carcinoma, expression appears to correlate with melanoma progression
HSPE1	Mitochondrial heat shock protein	Unfolded protein response, caspase regulation	Expression up-regulated in cancer
C15orf23 (SKAP)	Associates with kinetochores; promotes metaphase-to-anaphase transition		

Table 4-2 FAM96B-associating proteins are grouped according their biological function. Protein names in *italics* are assigned to more than one group, and the asterisk (*) denotes the only protein identified by yeast two-hybrid, for which the interaction could not be confirmed through co-immunoprecipitation in a human cellular background.

DNA transcription & repair	**Cytoskeleton organization & dynamics**
CIAO1 *NM23B* MMS19 DEDAF UHRF2 MAP3K7IP2* ZNF341	*CIAO1* *NM23B* *DNAJB6* C15orf23 CAP1 CDC42BPA FN1 FNBP4 ITGB4 FLNA FLNB FLNC KRT5
Protein chaperone	
BIP HSPE1 *DNAJB6*	

FAM96B induces apoptosis in cancer cells

To investigate this last hypothesis, we analyzed the effect of FAM96B over-expression on cancer cells. As the DNA repair pathways in cancer cells are presumed to be faulty, we expected over-expression of FAM96B in these cells to halt cellular proliferation. To this end, human cancer cells were transfected with plasmids encoding myc-tagged FAM96B and green fluorescent protein (GFP) as a negative control. At twenty-four, forty-eight and seventy-two hours after transfection, cells were fixed and stained with DAPI to determine apoptosis activity. Twenty four hours after transfection, a small percentage of FAM96B-positive cells exhibited morphological features charactristic of apoptosis, steadily increasing up to approximately 40% after 72 hours. In contrast, GFP-expressing cells did not exceed the level of 5% morphologically apoptotic cells. The results clearly demonstrate that expression of FAM96B induced apoptosis in cancer cells, whereas the negative control GFP did not (Figure 4.5).

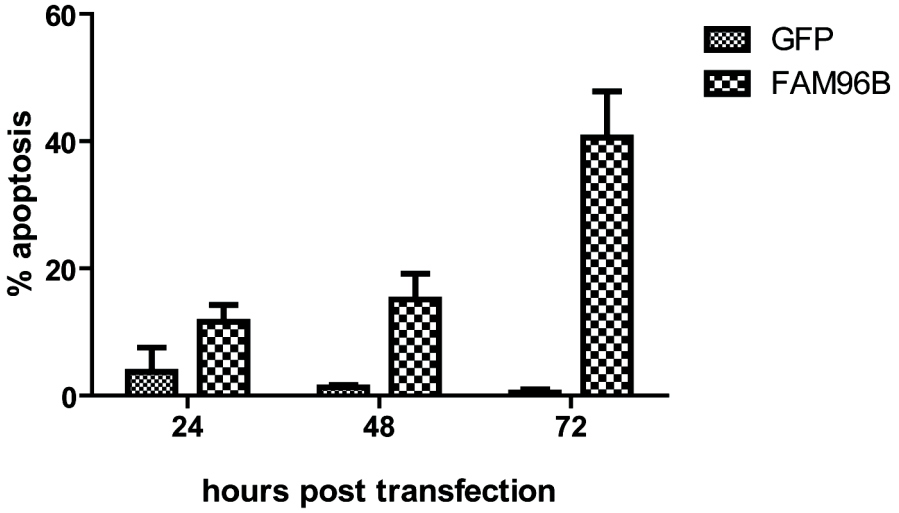

Figure 4.5 Over-expression of FAM96B in apoptosis in human cancer cells. Human Saos-2 tumor cells were transfected with plasmid encoding myc-tagged FAM96B or control and grown on coverslips. Twenty-four, forty-eight, and seventy-two hours post transfection coverslips were fixed and stained with appropriate antibodies for immunofluorescence analysis. Apoptotic cells were scored according to their nuclear morphology as determined by DAPI staining. Data is representative of 3 independent experiments, each in which at least 100 cells were scored.

Discussion

We have identified cellular factors providing insights in the action of the tumor-selective apoptosis inducer apoptin. Our protein-protein interaction studies revealed firstly the association of apoptin with human FAM96B, and secondly of FAM96B with proteins involved in DNA transcription, replication and repair as well as cytoskeleton dynamics. The fact that FAM96B interacts with apoptin in both its phosphorylated and non-phosphorylated state indicates its importance to apoptin's activity: the interaction likely occurs in an already early phase, preceding apoptin's phosphorylation and activation of its tumor-selective apoptosis induction.

Though the exact function of FAM96B cannot yet be established, several clues are provided. FAM96B is highly conserved among eukaryotes, and contains a well conserved domain (DUF59), found in proteins serving various functions, including phenylacetic acid degradation, iron-sulfur cluster biosynthesis, and calcium-dependent protein kinase, but also nucleotide-binding and chromosome partitioning (Finn, et al., 2010). Studies in *S.cerevisiae* suggest that the yeast homolog of FAM96B is an essential protein (Davierwala, et al., 2005), and further analysis even indicates its requirement for the establishment of sister chromatid cohesion (Ben-Aroya, et al., 2008). Recently, Ito and colleagues have reported that a protein complex containing FAM96B is involved in chromosome segregation (Ito, et al., 2010).

Our analyses concur with these findings, indicating a role for FAM96B in linking chromatin-related processes to the cytoskeleton. For example, one of the proteins identified as an FAM96B interactor is filamin A, a cytoskeletal protein, which has recently been shown to interact with the tumor suppressor protein BRCA1 and is required for efficient DNA repair (Velkova, et al., 2010). We found that FAM96B associates with MMS19, which itself is required for nucleotide excision repair (NER) and RNA polymerase II transcription (Kou, et al., 2008). Through MMS19 and DNAJB6, FAM96B is

also linked to PHB2 and MLF1IP, respectively, which have functions in protecting chromatid cohesion (Takata, et al., 2007) and centromere assembly (Foltz, et al., 2006). Another FAM96B-protein interactor, NM23B, has been identified as a constituent of the centrosome (Roymans, et al., 2001), and connects both FAM96B interaction spheres on account of its roles in transcription as well as regulation of cytoskeleton dynamics and suppression of metastasis (Lim, et al., 1998). Importantly, FAM96B-interactor C15orf23, or SKAP, has been shown to promote metaphase-to-anaphase transition by mediating separase activation after spindle assembly checkpoint is satisfied in mitosis (Fang, et al., 2009).

These findings are further supported by the fact that FAM96B is linked to formin, which is essential for contractile ring assembly in animal cells (Glotzer, 2005), as well as Cdc42, which regulates the assembly and organization of the actin cytoskeleton, and is required for kinetochore attachment as well as G1 progression and S phase entry (Hall, 2009). UHRF2, with which FAM96B interacts directly, is also capable of inducing G1 arrest, and associates with CDK2 (Li, et al., 2004). CDK2 complexed with cyclins A and E is essential for DNA replication and G1/S transition, respectively (Sherr, 1994). Intriguingly, Maddika, et al. (2009) reported apoptin phosphorylation and activation by CDK2.

We therefore propose that FAM96B functions in the regulation of cellular proliferation through control of chromosome segregation, and that this regulation is subject to control by DNA repair pathways. Consistent with this proposed function, we also showed that over-expression of FAM96B in cancer cells induces apoptosis. This apoptotic activity is highest 72 hours post transfection, and thus most likely represents a late-phase response, presumably upon failure to activate and/or execute a proper DNA damage response in a tumorigenic environment.

In conclusion, our protein-protein interaction analysis revealed that apoptin interacts with FAM96B, which appears to link the cell division process to the

essential process of DNA repair. Further research is underway, seeking to characterize and more precisely define the role of FAM96B in these processes, and the involvement in apoptin-induced tumor-selective cell death.

Note added in proof:
During preparation of this manuscript, Ito et al. described that FAM96B (named MIP18 by the authors) is part of a multiprotein complex involved in DNA repair and chromosome segregation in human cells (Ito, et al., 2010).

Acknowledgements

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