The role of cad, flash and fam129b in cancer cell survival and apoptosis

Song Chen
Wayne State University,
THE ROLE OF CAD, FLASH AND FAM129B IN CANCER CELL SURVIVAL AND APOPTOSIS

by

SONG CHEN

DISSERTATION

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Approved by:

__________________________  ________________
Advisor                     Date
DEDICATION

I would like to dedicate this dissertation to my family, my wife, Shuowen Gu, my daughter, Serena, especially my father, Yuegui Chen and my mother, Xiulan Xu for their unconditional love and support.
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CHAPTER 1
INTRODUCTION

This dissertation focuses on three proteins that are associated with cell survival and apoptosis in normal and neoplastic cells: 1) CAD, a large multifunctional complex that is invariably elevated in tumor cells, 2) FLASH, a large protein with multiple growth related functions and 3) FAM129B, a newly discovered protein that has been implicated in metastasis of melanoma cells.

The pyrimidine biosynthetic complex CAD

The biosynthesis of pyrimidine nucleotides is essential to all living organisms. The supply of pyrimidines within the cell is maintained by two pathways: the salvage pathway and de novo biosynthesis. The de novo pathway consists of six enzymatic reactions leading to the formation of uridine monophosphate (UMP) from glutamine, bicarbonate, 2 adenosine triphosphate (ATP) molecules, aspartate and 5-phosphoribosyl-1-pyrophosphate (PRPP) (Figure 1). In prokaryotes, these six reactions are catalyzed by six independent enzymes including carbamoyl phosphate synthetase II (CPSase II), aspartate transcarbamoylase, dihydroorotase (DHOase), dihydroorotate dehydrogenase (DHOD), orotate phosphoribosyltransferase (OPRT) and orotidine 5’ monophosphate decarboxylase (OMPDC).

In higher eukaryotes, however, the first three steps in the pathway are catalyzed by a single multifunctional enzyme CAD (Shoaf and Jones 1973; Mori, Ishida et al. 1975; Coleman, Suttle et al. 1977), which has also been investigated in our laboratory for many years. The product of CAD (dihydroorotate) diffuses into the mitochondria, where it is oxidized by the fourth enzyme dihydroorotate dehydrogenase (DHOdehase), which
Figure 1.1 The *de novo* pyrimidine biosynthetic pathway (Evans and Guy 2004). The *de novo* synthesis of pyrimidine nucleotides begins with ATP, glutamine and bicarbonate. The first three steps in the pathway are catalyzed by a trifunctional, cytoplasmic enzyme known as CAD, The fourth enzyme is a mitochondrial enzyme, dihydroorotate dehydrogenase (DHOdehase). The last two steps are cytoplasmic and are catalyzed by the bifunctional enzyme UMP synthase.

The flux through the pyrimidine pathway is regulated by the activity of carbamoyl phosphate synthetase. The activity of carbamoyl phosphate synthetase can be regulated by a number of different mechanisms, including allosteric regulation, phosphorylation and selective degradation. For example, the CPSII activity is allosterically inhibited by the end product of the pathway, UTP and activated by PRPP, a downstream substrate and the initial substrate in the *de novo* purine biosynthetic pathway (Hager and Jones 1967; Levine, Hoogenraad et al. 1971). Phosphorylation by MAPK at Thr456 has been shown to...
affect the allosteric regulation of CAD with the loss of inhibition by UTP, and the increase in activation by PRPP (Graves, Guy et al. 2000). As the cells emerge from S phase, Thr456 is de-phosphorylated by PP1 and Ser1406 is phosphorylated by protein kinase A (Lander, Linton et al.). This then decreases sensitivity to PRPP and enhances UTP inhibition. Thus, the well documented increased pyrimidine biosynthetic activity in tumor cells is due in part to an increase in the intracellular levels of CAD and to sequential phosphorylation and de-phosphorylation by MAP kinase and PKA. There is also a cell cycle dependent translocation to different cellular compartments (see Chapter 3). These cell cycle dependent controls breakdown in breast cancer cells. MAP kinase is elevated in tumor cells, which results in persistent activation of CAD and continuously elevated pyrimidine biosynthesis (Sigoillot, Sigoillot et al. 2004; Kotsis, Masko et al. 2007; Sigoillot, Kotsis et al. 2007).

Until recently, it has been assumed that the effects of CAD on growth were entirely dependent on its role in pyrimidine biosynthesis. However, our lab’s recent evidence suggests that CAD might have novel growth-related functions that are unrelated to the supply of pyrimidine nucleotides. CAD is suggested to be associated with centrosomes.
and interact with FLASH (CHAPTER 3). Both the signaling complexes and the 
interactions with the apoptotic protein, FLASH provide new opportunities for targeting 
this important protein for chemotherapy. Most pyrimidine pathway antimetabolites have 
been designed to mimic the substrates or transition state analogs of specific pathway 
enzymes. However, most have not been tried in clinical trials in part because 
competitive inhibitors are usually not optimal chemotherapeutic agents. Although they 
effectively block the targeted enzyme in vitro, the substrates accumulate in vivo to 
concentrations that effectively out-compete the binding of the drug to the active site. An 
alternative and more promising approach would be targeting the interactions between 
CAD and signaling molecules, centrosomal proteins and components of apoptotic 
pathways.

Hence, the further study will lead to a more comprehensive understanding of the 
role of CAD in cancer cell proliferation and perhaps reveal new insights into other vital 
processes such as: mitosis and apoptosis. This could then ultimately lead to the 
development of new therapeutic approaches in the treatment of proliferative diseases.

**Centrosome structure and function**

The centrosome is primarily known for its role as a major microtubule organizing 
center. As shown in Figure 1.3, each centrosome consists of two centrioles (daughter 
and mother), perpendicular to each other at a distance of approximately 2 µm at their 
proximal end. The centriole is a barrel shaped structure composed of nine triplets of 
microtubules. The duplication of centrioles is semi-conservative and occurs only once in 
each cell cycle, initiating at the transition of G1/S and finishing at the beginning of G2 
phase (Azimzadeh and Bornens 2007). The centrosome plays a particularly important
role in dividing cells by ensuring the proper segregation of chromosomes between the two daughter cells in association with the spindle microtubules. Studies have shown more important roles in a variety of key cell cycle regulating events such as: orchestrating G1/S transition, entry into mitosis, anaphase onset, cytokinesis, and monitoring DNA damage (Schatten 2008).

More than 500 potential centrosomal proteins have been identified by mass

**Figure 1.3. Diagram of centrosome structure** (Doxsey 2001) A pair of centrioles is shown, each with nine-fold symmetry owing to the nine triplet microtubules. Each centriole has pericentriolar material that nucleates microtubules around the ends closest to one another. Only the maternal centriole has two sets of extra appendages, distal and subdistal; the latter seems to anchor microtubules. A series of interconnecting fibres, different from the pericentriolar material (PCM), links the closest ends of the two centrioles.
spectrometry analysis of an isolated centrosome fraction (Andersen, Wilkinson et al. 2003). Because the centrosome is a non-membrane organelle, the isolated centrosome fraction may include cytosolic contaminants, and/or some low abundance critical protein may be removed during the process of isolation. Thereby, current biochemical methods may not detect all the centrosomal proteins. The well characterized centrosome proteins are mainly grouped into three categories based on the protein function, which are: the structural proteins (alpha tubulin, beta tubulin, gamma-tubulin, centrin 2 and 3, AKAP450, pericentrin), the regulatory molecules (cell division protein 2 (Cdc2), Cdk1, PKA type II alpha regulatory chain, serine/threonine-protein kinase Nek2, Casein kinase I, delta and epsilon isoforms, protein phosphatase 2A, protein phosphatase 1 alpha isoform) and the heat shock proteins (heat shock protein Hsp90 and heat shock protein Hsp73) (Schatten 2008).

Interestingly, a few proteins involved in the purine nucleotide de novo biosynthesis pathway were detected in the isolated centrosomes (Andersen, Wilkinson et al. 2003). However, so far no studies confirmed these localizations or potential functions other than the involvement in the purine biosynthesis pathway. In our study, CAD is found to be mainly involved in the pyrimidine biosynthesis pathway, and localized in centrosome by fluorescence microscopy (Chapter 3). Further characterization of CAD and elucidation of the role in centrosome should provide important insights: into the mechanism of CAD involved in cell division, and on understanding centrosome abnormalities and implications in disease.
While new information has been obtained regarding the intracellular location of the pyrimidine biosynthetic complex, the focus of our research shifted when it was discovered that CAD associates with FLASH.

**FLASH, a large protein with multiple growth related functions**

FLASH was discovered in 1999 by Imai et al in a yeast-two hybrid screen of mouse T cell cDNA library (Imai, Kimura et al. 1999). They found that it associates with caspase-8 (FLICE) so that they designated FLASH (FLICE associated huge protein) or CASP8AP2 (caspase-8 associated protein 2). FLASH is a protein with a calculated molecular weight of 220 kD. Structure prediction shows that FLASH contains a predicted coiled-coil domain (Figure 1.4), found typically in centrosomal proteins, which may mediate functionally important protein–protein interactions (Lupas, Van Dyke et al. 1991). FLASH also contains a Death effector domain-recruiting domain (DRD) which interacts with proteins involved in apoptosis (Figure 1.4). In addition, functional studies show that the DED-recruiting domain (DRD) in FLASH can bind to the tandem DEDs of caspase-8 and also to the DED of FADD. FLASH also contains a predicted nuclear-localization signal (NLS) in the C-terminal region and a nuclear exclusion signal (NES)-like sequence in the central region of FLASH (LFEKLKKILL) which indicates that it could be translocated into the nucleus.

**Figure 1.4 Diagram of the predicted domain organization of FLASH.** Boxes, predicted globular regions; lines, predicted non-globular domains; CC, coiled-coil. Regions of alleged similarity to the apoptotic ATPases (CED-4) and DED domains (DRD, or DED-related domains) are indicated by broken lines. Modified from (Koonin, Aravind et al. 1999)
Since then, 4 more proteins have been reported to interact with FLASH by yeast two-hybrid screen: glucocorticoid receptor-interacting protein 1 (GRIP1), mineralocorticoid receptor, PML nuclear body component Sp100 and transcription factor c-Myb (Kino and Chrousos 2003; Obradovic, Tirard et al. 2004; Milovic-Holm, Kriehhoff et al. 2007; Alm-Kristiansen, Saether et al. 2008). It should be noted that all of the identified FLASH clones in yeast two-hybrid analyses are C-terminal portions of FLASH containing the DED-recruiting domain (DRD). No studies report the function of the N-terminal part of FLASH containing the coiled-coil domain. Using the nuclear receptor binding domain of the glucocorticoid receptor-interacting protein 1 (GRIP1) as bait, FLASH was identified as a binding partner of GRIP1 in a yeast two-hybrid screening of a human Jurkat cell cDNA library (Kino and Chrousos 2003). The C-terminal part (1709-1982) of FLASH was shown to specifically interact with the nuclear receptor binding domain of GRIP1. In the study, the authors proposed that FLASH could translocate from the cytosol into the nucleus to inhibit the transcription activity of the glucocorticoid receptor in response to tumor necrosis factor α (TNFα).

FLASH was found primarily in the cytoplasmic fraction by western blotting, indicating that FLASH is a cytoplasmic protein (Imai, Kimura et al. 1999). However, in 2006, this localization was challenged by the Barcaroli group (Barcaroli, Bongiorno-Borbone et al. 2006). They found that FLASH was not localized in the cytoplasm, but clearly localized in Cajal bodies which are important sites for S-phase progression. In addition, FLASH was shown to promote histone gene transcription in the S-phase of the cell cycle and depletion of FLASH resulted in S phase block (Barcaroli, Bongiorno-Borbone et al. 2006; Barcaroli, Dinsdale et al. 2006).
On the contrary, another independent group recently reconfirmed the role of FLASH involved in CD95-mediated apoptosis, but through a previously unrecognized nuclear pathway (Milovic-Holm, Krieghoff et al. 2007). They suggested that endogenous FLASH is not localized in Cajal bodies but localized in PML nuclear bodies. Moreover, FLASH could translocate from the PML bodies into cytosol and co-localize with caspase-8 in mitochondria of HT1080 cells. Knock-down of FLASH in HT1080 cells by siRNA resulted in substantial apoptosis inhibition. Most recently, the third group found that FLASH co-localizes with c-Myb in nuclear speckles and is only partly co-localized with PML nuclear bodies and Cajal bodies (Milovic-Holm, Krieghoff et al. 2007). This observation reconciles the previous two contradicting results, indicating that FLASH localization may be cell cycle and cell type dependent. FLASH was also suggested to be a promising prognostic marker in childhood acute lymphoblastic leukemia. High levels of FLASH expression were correlated with a great propensity of leukemic lymphoblasts to undergo apoptosis (Flotho, Coustan-Smith et al. 2006). On the other hand, knockdown of FLASH using siRNA resulted in cell death immediately after cell entry into mitosis, indicating that FLASH is essential for the cell division, as well as apoptosis (Kittler, Putz et al. 2004).

Several other FLASH functions have been discovered including cell cycle progression, histone biosynthesis, and transcriptional control. However, the role of FLASH in promoting apoptosis is controversial. In our study, we found that contrary to previous reports, FLASH suppresses apoptosis (CHAPTER 4). In this project, further studies of the interaction between FLASH and CAD may contribute to a better
understanding of these two important multifunctional proteins in apoptotic and non-apoptotic signaling pathways.
Apoptosis and a putative role of CAD in TNF signaling pathway

Apoptosis is a normal physiological cell suicide program that plays important roles during the development of multi-cellular organisms and in the maintenance of tissue homeostasis. It is characterized by a variety of morphological changes, including plasma membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Elmore 2007).

The induction of apoptosis occurs by two major distinct pathways: the intrinsic or mitochondrial pathway and the extrinsic pathway. In the extrinsic pathway, apoptosis is induced by tumor necrosis factor TNF receptor, a superfamily of death receptors, such as TNFα receptors and Fas/APO-1 (CD95) (Ashkenazi and Dixit 1998). Following the binding of a member of TNF receptor family to its receptor, a membrane receptor complex is induced, and procaspase-8 is recruited to the death inducing signaling complex (DISC) by its DEDs (Sartorius, Schmitz et al. 2001), as shown in Figure 1.5. When bound to the DISC, several procaspase-8 molecules are in close proximity to each other and therefore are assumed to activate each other by auto-proteolysis (Denault and Salvesen 2002). Active caspase-8 then activates downstream effector caspases such as caspase-3, caspase-6 and caspase-7, cleaving various substrates including cytokeratins, the plasma membrane cytoskeletal proteins, nuclear proteins and others, which ultimately cause the morphological and biochemical changes seen in apoptotic cells (Caulin, Salvesen et al. 1997; Schutte, Henfling et al. 2004). The intrinsic pathway is triggered by the release of proteins such as cytochrome c from mitochondria to the cytosol, which is regulated by a family of proteins called the Bcl-2 family (Danial and Korsmeyer 2004). Once cytochrome c is released into the cytosol, it
binds the adaptor apoptotic protease activating factor-1 (APAF1), forming the apoptosome. The apoptosome then recruits and activates caspase 9, which, in turn, activates the downstream effector caspases, including caspase 3, 6 and 7, leading to apoptosis (Henry-Mowatt, Dive et al. 2004).

Figure 1.5 CAD and FLASH in signaling pathway of TNF-R1

TNF binds to TNFR1, and the trimerized receptor recruits TRADD via interactions between death domains. The death domain of TRADD then recruits FADD in one pathway to activate caspase-8. In another pathway, RIP binds to TRADD and transduces an apoptotic signal through the death domain. Modified from: http://en.wikipedia.org/wiki/Tumor_necrosis_factor-alpha

During the apoptosis pathway, CAD was found to be cleaved by caspase-3 to decrease the cellular CPSII activity (Huang, Kozlowski et al. 2002). However, the role of CAD in apoptosis remains to be investigated. In human mammary epithelial cells,
keratin 18 has been shown to interact with human TNF receptor type 1 (TNFR1)–associated death domain protein (TRADD) and attenuates the TNF induced apoptosis by binding to TRADD (Inada, Izawa et al. 2001). CAD has also been found to interact with TRADD by proteomic analysis of human cell lysates in which the TNF alpha/NF-B pathway was activated using tandem affinity purification combined with liquid-chromatography tandem mass spectrometry (Bouwmeester, Bauch et al. 2004). On the other hand, my immunofluorescence microscopy studies indicated that p-T456CAD may co-localize with keratin in MCF-7 cells (Chapter 3). The interaction of CAD with FLASH and TRADD (Figure 1.5), further indicates that CAD functions as a component of the signaling pathway. In addition, indirect immunofluorescence suggested that pThr456CAD is localized in PML-nuclear bodies or splicing speckles. Interestingly, TRADD is an important component in death receptor signaling complex and has also been identified in nuclear bodies such as PML nuclear bodies (Morgan, Thorburn et al. 2002; Milovic-Holm, Krieghoff et al. 2007), which are involved in apoptosis signaling (Krieghoff-Henning and Hofmann 2008). All of these findings imply that CAD may play important roles in apoptosis signaling pathway.

**FAM129B, a newly discovered protein, implicated in metastasis of melanoma cells**

In the course of these studies, an extensive panel of antibodies directed against signaling proteins was used. The localization of phosphorylated proteins was examined to see if any of these proteins co-localizes with CAD. In addition, Western blot analysis of apoptotic tumor cell lysates using these antibodies was performed. One of the antibodies was directed against an unusual protein identified in the human genome.
project called FAM129B. We found that like FLASH and CAD, this protein was gradually degraded during apoptosis. Nothing was published regarding this protein but it was a member of a family of poorly understood proteins that were involved in cancer. Soon after we had begun work on this protein, a paper was published showing that the protein is phosphorylated by MAP kinase and promotes the invasion of melanoma cancer cells (Old, Shabb et al. 2009). The mechanism of action is unknown. Since apoptosis plays an indispensible role in cancer cell invasion and FAM129B, we set out to find out if this protein also influenced apoptosis. We found that it also suppresses apoptosis (Chapter 5).

The FAM129B gene

The gene FAM129B comprises about 73 kbases of the chromosome 9 (9q34.13) in Homo sapiens, and spans 49 kbases of chromosome 2 in Mus musculus (mouse) according to NCBI database. The FAM129B gene is conserved in chimpanzee, dog, cow, mouse, rat, and zebrafish. Human FAM129B transcript variant 1, 4007 bp mRNA (NCBI Reference Sequence: NM_022833.2) encodes the 746 residue FAM129B isoform 1 (NP_073744.2) and FAM129B transcript variant 2, 3818 bp mRNA (NM_001035534.1) encodes the 733 residue FAM129B isoform 2 (NP_001030611.1). The short and long isoforms, differ by 16 amino acids at the amino end of the polypeptide (Figure 1.7).

There is no report about the regulation of the transcription of FAM129B in normal cellular processes. According to the NCBI EST expression database, the expression of FAM129B transcripts is found in most tumor tissues including breast (mammary gland)
tumor, cervical tumor, prostate cancer, lung tumor and skin tumor and is much higher than in normal tissue (Figure 1.6).

<table>
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<td>171</td>
</tr>
<tr>
<td>breast (mammary gland) tumor</td>
<td>137</td>
</tr>
<tr>
<td>cervical tumor</td>
<td>407</td>
</tr>
<tr>
<td>chondrosarcoma</td>
<td>446</td>
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<td>colorectal tumor</td>
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<tr>
<td>esophageal tumor</td>
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<tr>
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<td>63</td>
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<tr>
<td>ovarian tumor</td>
<td>65</td>
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<tr>
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<tr>
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<td>15</td>
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<tr>
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<tr>
<td>retinoblastoma</td>
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<tr>
<td>skin tumor</td>
<td>272</td>
</tr>
<tr>
<td>soft tissue/muscle tissue tumor</td>
<td>135</td>
</tr>
<tr>
<td>uterine tumor</td>
<td>221</td>
</tr>
</tbody>
</table>

**Figure 1.6 FAM129B gene expression patterns in tumor and normal tissues as inferred from EST counts and the cDNA library sources:** The number represents EST transcripts per million.

FAM129B isoform 1: 746aa

MGDVLSHTLDDARRQHIAEKTGKILTEFLQFYEDQYGVALFNSMRHEIEGTGLPQAQLLWRKVPLDERIVFSGNLFQHQEDSKKWRRNFSLVPHNYGLVLYENKAAYERQVPPRAVINSAGYKILTSVDQYLEIGNSLPGGTAKSGSAPILKCPTQFPLILWHPYARHYYFCM
MTEAEQDKWQAVLQDCIRHCNNGIPEDSKVEGPFTDAIRMYRQSKELYGTWEMLCGNEVQILSNLVMEELGPLKAELGPRLKGPKQERQRQWIQISDAVYHMVYEQAKARFEELSKVQQPAMQAVIRTDMDQITSKEHLASKIRAFILPKAECVRNVHQPYIPSILEALMVPTSQGFTEVRDVFKEVTDMNLNVINEGGIDKLGEYMEKLSRLAYHPLLMQSCYEKMESLRLDGLQQRFDVDSTSTSVFKQRAQIHMREQMDNAVYTFETLLLQELGKPTKEELCKSIQRVLERVLKKYDYSSSSVRKRFFREALLSISIPFLKLANDTCKSELPRFQELIFEDFARFILVENTYEEVLQTVMKDILQAKEAAVQRKHNLRYRDSMVMHNSDPNLHLLAEAGAPIGWEEYSNNGGGGSPSTPSTPESATLSEKRRRAKQVVSVQDEEVGLPFEASPPASPDGVTEIRGLLAQGLRPESSPPAGPLLNGAPGESQPKA peptide
SPPASPLQHLLPGKAVDLPQPKPDPSETEGEQVSSPSSHPALHTTEDSAGVQTEF

FAM129B isoform 2: 733aa

MGWMGEKTGKILTEFLQFYEDQYGVALFNSMRHEIEGTGLPQAQLLWRKVPLDERIVFSGNLFQHQEDSKKWRRNFSLVPHNYGLVLYENKAAYERQVPPRAVINSAGYKILTSVDQYLEIGNSLPGGTAKSGSAPILKCPTQFPLILWHPYARHYYFCCMTEAEQDKWQAVLQDCIRHCNNGIPEDSKVEGPFTDAIRMYRQSKELYGTWEMLCGNEVQILSNLVMEELGPLKAELGPRLKGPKQERQRQWIQISDAVYHMVYEQAKARFEELSKVQQVQPMQAVIRTDMDQITSKEHLASKIRAFILPKAECVRNVHQPYIPSILEALMVPTSQGFTEVRDVFKEVTDMNLNVINEGGIDKLGEYMEKLSRLAYHPLLMQSCYEKMESLRLDGLQQRFDVDSTSTSVFKQRAQIHMREQMDNAVYTFETLLLQELGKPTKEELCKSIQRVLERVLKKYDYSSSSVRKRFFREALLSISIPFLKLANDTCKSELPRFQELIFEDFARFILVENTYEEVLQTVMKDILQAKEAAVQRKHNLRYRDSMVMHNSDPNLHLLAEAGAPIGWEEYSNNGGGGSPSTPSTPESATLSEKRRRAKQVVSVQDEEVGLPFEASPPASPDGVTEIRGLLAQGLRPESSPPAGPLLNGAPGESQPKA peptide
SPPASPLQHLLPGKAVDLPQPKPDPSETEGEQVSSPSSHPALHTTEDSAGVQTEF

Figure 1.7 FAM129B isoform protein sequence
FAM129B Domain Structure

FAM129 has a PH domain and a proline rich region (Figure 1.8). The PH domain was originally identified in pleckstrin, the major substrate of protein kinase C in platelets in 1993 (Haslam, Koide et al. 1993). PH domain consists of approximately 100-120 residues and is found in a variety of organisms from yeast to humans. PH domains are found in about 252 human proteins and it is the 11th most abundant domain in the human genome (Lander, Linton et al. 2001). Although, PH domains are best known for their ability to bind phosphoinositides and to target their host proteins to cellular membranes (Lemmon and Ferguson 2000), their functions appear to be very diverse, including cellular signalling, cytoskeleton organization, membrane trafficking and phospholipid modification (Lemmon 2004; Chang, Kim et al. 2005).

Figure 1.8 Diagram of FAM129B structure domain

FAM129B, an 83 kDa polypeptide (residues 1 – 746), has a PH domain near the amino end (residues 69 – 192) and a proline rich region (residues 628 – 730) near the carboxyl end. Four Erk1/2 phosphorylation sites have been identified corresponding to Ser628, Ser633, Ser679 and Ser683 (Old, Shabb et al. 2009).

The proline rich domain contains a motif with the amino acid sequence of PXXPPP-XP, which was proposed to interact with SH3 domain involved in signal transduction and cytoskeleton regulation (Williamson 1994). There are two portions of amino acid
sequences: 639-PESPPPASP-647 and 663-PESPPPAGP-671 in the c-terminal of FAM129B, representing the classical proline rich domain sequence. Src-homology 3 (SH3 domain) is a small protein domain of about 60 amino acid residues. SH3 is present in a large number of proteins involved in signal transduction, cell polarization and membrane-cytoskeleton interactions (Musacchio, Gibson et al. 1992). A more detailed overview of FAM129B will be discussed in Chapter 5.

**Purpose of the study**

**Part I - Identification of CAD functions unrelated to pyrimidine biosynthesis**

The first part of this study, discussed in Chapter 3, investigates the functions of the multifunctional protein, CAD that are unrelated to pyrimidine biosynthesis. The initial hypothesis is based on the data that exogenous uridine did not rescue the phenotype resulting from CAD knockdown by siRNA in MCF-7 cells. In this chapter, I mainly focused on the characterization of the interaction of CAD with FLASH, a previous reported pro-apoptotic protein, providing hints about the physiological function of the interaction.

To investigate the potential CAD function in cell cycle and proliferation, the cell-cycle dependent localization of total CAD and phosphorylated CAD was revealed using different antibodies in a variety of mammalian cells. The dynamic localization of CAD during the cell cycle provides the evidence that CAD performs multifunctional roles during cell growth and cell division.

**Part II - Investigate the role of FLASH in cell apoptosis.**

To further study the role of FLASH and CAD in the apoptosis pathway, we first tried siRNA knockdown of FLASH to test whether apoptosis was blocked as previous
literature suggested (Chapter 4). On the contrary, FLASH knockdown was found to sensitize cell apoptosis. We then aimed to find the mechanism that could explain the possible discrepancy in results. We further suggest a possible mechanism: the knockdown of FLASH could down-regulate the anti-apoptotic proteins: MCL-1 and cFLIPshort, which is dependent on p53 pathway (Chapter 4).

Part III- Identification of a novel anti-apoptotic protein, FAM129B in cancer cells

In Chapter 5, I include our publication, “FAM129B/MINERVA, a novel adherens junction-associated protein, suppresses apoptosis in HeLa cells” (Chen, Evans et al. 2011). I present a preliminary identification of FAM129B as a novel adherent junction associated protein. Previous studies have shown that FAM129B could promote melanoma cell invasion using *in vitro* three dimensional gel matrix models, however, there is little known regarding to the mechanism. In this study, I performed indirect immunofluorescence using a specific antibody against endogenous FAM129B protein in HeLa cells. I found that FAM129B is distributed in the cytosol, but it localizes in the cell-cell junction whenever two cells contact each other. My study suggests that FAM129B could be a novel adherent junction associated protein. Then, I tried four different siRNAs to knockdown the FAM129B protein level by more than 90% in HeLa cells to examine if FAM129B knockdown affects cell growth and apoptosis. My results show that the knockdown of FAM129B in HeLa cells did not induce significant cell death and growth inhibition. However, when I tried inducing HeLa cells apoptosis using TNFalpha, the FAM129B depleted cells showed much more rapid activation of apoptosis pathway compared to control cells.
Part IV - Mechanistic Studies of FAM129B

In chapter 5, I present a preliminary identification of FAM129B as a novel adherent junction associated protein and in chapter 6, I attempt to understand the possible mechanism for how FAM129B suppresses apoptosis and how it promotes cancer cell invasion. I tried to identify potential FAM129B binding partners by co-immunoprecipitation followed by mass spectrometry analysis of the immunoprecipitated complex. In addition, I performed western blot screen of FAM129B expression in a variety of cancer cells. The findings suggest that FAM129B might be an important oncogenic protein in other epithelial cancer cells. I chose breast cancer cells as a model to perform a preliminary study on FAM129B’s function in invasion. These data will be described in Chapter 6.

In addition, Chapter 6 reports the new finding that FAM129B interacts with KEAP1, which provides one possible mechanism to explain how FAM129B play roles in cancer cell apoptosis. A proteomic study identified FAM129B as a KEAP1 interaction partner in an immunocomplex. However, the interaction was not confirmed by other independent methods. In addition, there is no reported functional study of this interaction. The interaction was confirmed by co-immunoprecipitation. Introductory background on KEAP1 physiological function is provided in Chapter 6. The interaction appeared to be very stable in vitro. Possible functions of the interaction between keap1 and FAM129B are discussed in Chapter 6.

Finally, a summary of future work that should be done to further understand FAM129B’s role in cancer cell apoptosis and metastasis is also discussed in chapter 6.
CHAPTER 2

MATERIAL AND METHODS

PCR and Cloning

All plasmids used in the studies were constructed either by the classic cutting and ligation approach or by Invitrogen’s Gateway technology depending on the expression vectors employed. Gateway technology provides a fast and highly efficient way to clone genes into multiple expression vectors for functional analysis and protein expression. In general, the gene of interest was first cloned into Invitrogen's Entry vectors. The insert from the Entry clone can then be recombined efficiently via an attL-attR (LR) reaction into a variety of Destination vectors. Briefly, the interested genes are amplified by the polymerase chain reaction (PCR) using the forward primers with four bases, CACC, in the beginning. The PCR product is then purified to ensure that only a single specific band is present. The purified product is inserted into an Entry clone by the complementary GTGG overhang on the linear Entry clone and topoisomerase I, which binds the duplex DNA. All Entry clones confer kanamycin resistance for selection. All the Destination clones confer ampicillin resistance for selection. Flanking the insert are an attL1 site on the 5’ end and an attL2 site on the 3’ end. The Destination clones contain the ccdB gene which is toxic to most E.Coli strains. This gene is flanked by an attB1 site on the 5’ end and an attB2 site on the 3’ end. Therefore, when the Entry clone is added to the Destination clone in the presence of LR clonase, the gene of interest is transferred from the Entry clone into the Destination clone (to create an expression vector) and the ccdB gene is transferred from the Destination clone to the Entry clone.
(to create a donor vector). It is important to remember that Destination Vectors carrying the ccdB gene cannot be propagated in E. coli DH5α, top 10 and most E. coli strains, because the CcdB protein will kill most of the strains. All Destination vectors were propagated in the E.coli strain DB3.1 (Invitrogen) with ampicillin antibiotic selection. All Entry vectors are propagated in the E.coli strain TOP10F’ (Invitrogen) under kanamycin selection (Campeau, Ruhl et al. 2009).

For cloning of FAM129B, the complete coding region of human FAM129B was amplified from the pOBT7 clone (Invitrogen, MGC clone 5456246). This cDNA clone included two introns that were excised using PCR based deletion. Full length of FAM129B was PCR amplified using primers: FAM-EGFPN1-XhoI Forward: TTA CTC GAG CCG CCA TGG GGG ACG TGC TGT CCA CGC ACC T ; FAM-EGFPN1-HindIII Reverse: GCC GAA GCT TGA ACT CAG TC T GCA CCC CTG CAC TGT CCT C. The PCR product and the vector pEGFP-N1 (Clontech) were cleaved with XhoI and, purified and then digested with HindIII and gel purified. The vector and insert were ligated and transformed into DH5α cells.

FAM129B was also cloned into pEGFP-C3 vector to generate a fluorescent fusion protein. The PCR product was then amplified using primers that included an Xho1 site: 5’CAC CCT CGA GGG GGA CGT GCT GTC CAC GCA CCT GGA CG3’ and a Kpn1 site: 5’GGC GGT ACC CTA GAA CTC AGT CTG CAC CCC TGC ACT G3’. The digested product was then ligated into the Xho1 and Kpn1 sites of the pEGFP-C3 vector (Clontech) to generate the recombinant FAM129B expression plasmid with GFP-fused to the amino end. All amplifications were verified by agarose gel electrophoresis,
and the correct product was cut from the gel and purified using the Freeze and Squeeze gel purification kit (Bio-Rad).

**Plasmids purification and characterization**

The plasmids were transformed into DH5α subcloning efficiency bacteria (Invitrogen) and plated on LB (Luria Broth) plates with the appropriate selective antibiotics. The transformed single colony was inoculated into liquid LB medium with the appropriate selective agent and grown overnight. The plasmids were purified from overnight using a MiniPrep purification kit (Invitrogen). For amplification, the plasmids were transformed into DH5α subcloning efficiency bacteria (Invitrogen). To obtain a greater yield of the plasmids, individual clones were cultured overnight in a volume of 100-250 ml with the appropriate antibiotics and were purified using the Plasmid Maxi kit (Qiagen) following the procedure of the manufacturer. Aliquots were frozen at -20°C as freeze/thaw cycles may decrease the level of supercoiling, thus the efficiency of transfection. The DNA concentrations were determined by measuring the absorbance at 260nm. Purity of DNA was determined by calculating the ratio $A_{260nm}/A_{280nm}$. The plasmids were verified by restriction analysis (restriction enzymes from Invitrogen and New England Biolabs) and by sequencing (WSU DNA sequencing core).

**Agarose gel electrophoresis**

The HOEFER mini DNA electrophoresis apparatus was used for electrophoresis of DNA samples. 0.8% or 1% Agarose gel was made by dissolving 0.8 or 1g UltraPure Agarose (Invitrogen) in 100 ml 1xTAE buffer (40mM Tris base, 0.1% acetic acid, 1mM EDTA, pH 8.0) and heated for 1-2 min in a microwave until boiling. The boiled agarose was cooled down to about 50°C and 10 ul of SYBR® Safe DNA gel stain (S33102,
Invitrogen) was added. The agarose solution was then poured into a gel casting apparatus and a 1.5mm 10-well comb was inserted for formation of the loading well. The gel was then allowed to solidify at room temperature and placed in the tank filled with TAE buffer. Samples were prepared by mixing the DNA with 1/5 the volume of BlueJuice™ Gel Loading Buffer (10X) (65% (w/v) sucrose, 10 mM Tris-HCl, pH 7.5, 10 mm EDTA, 0.3% (w/v) Bromophenol blue (10816-015,Invitrogen) prior to loading. The samples were loaded into the wells, along with a DNA standards, 1 Kb plus, or high molecular weight DNA Markers (Invitrogen). The agarose gels were run at 90-100V for one hour, or until the bromophenol blue dye ran approximately 3/4 of the length of the gel. The DNA fragments were then viewed and photographed using a UV-transilluminator.

**Preparative electrophoresis**

Alternatively, the DNA fragments of interest were recovered by excising the band from the gel with a scalpel and put into a Freeze N squeeze DNA gel extraction spin columns (Catalog:732-6165, Bio-RAD). After freezing the gel for 30 min at -20°C, the cartridge was immediately centrifuged at maximum speed, 13000rpm for 2 min. The recovered buffer contained the DNA fragment of interest. The DNA fragment was purified using the PureLink™ PCR Purification Kit (Cat no: K3100-01, Invitrogen) and recovered in DNA-RNA free water.

**Cell culture**

HEK-293 cell line is suggested to be a neuronal lineage cell line (Shaw, Morse et al. 2002), which is transformed by sheared adenovirus 5 DNA during the original human embryonic kidney culture (Graham, Smiley et al. 1977). This cell line is widely
used for transient gene expression studies due to its extreme transfectability by a variety of methods such as: calcium phosphate based method (Ca-Pi), polyethylenimine-based method (PEI) (Baldi, Muller et al. 2005), and Lipofectamine 2000 (Invitrogen).

The 293FT Cell Line (Invitrogen) is derived from a highly transfectable human embryonic kidney cell that is transformed with the SV40 large T antigen, which allows very high levels of protein to be expressed from vectors containing the SV40 origin of replication. Hence, the 293FT Cell line is ideal for generating high-titer lentivirus (Invitrogen).

The HeLa cell line was derived from human cervical cancer cells, which was reported to contain human papilloma virus 18 (HPV-18) sequences (Scheffner, Munger et al. 1991). The wild-type p53 protein in HeLa cells is rapidly degraded through E6 protein mediated proteasome degradation pathway (Scheffner, Werness et al. 1990).

The MCF10A cells were originally obtained from a female patient with fibrocystic breast disease (Soule, Maloney et al. 1990). These cells are spontaneously immortalized breast epithelial cells. However, the cells retain the characteristics of normal breast epithelium including hormone/growth factor-dependent growth, lack of anchorage-independent growth and lack of tumori-genicity in nude mice. The cell line is used as an apoptosis model in the current study since it was reported to be highly susceptible to Fas-mediated apoptosis compared to other tumorigenic breast cancer cell lines (Starcevic, Elferink et al. 2001).

MCF7, a malignant cell line derived from a pleural effusion from a 69-year-old Caucasian woman with breast adenocarcinoma in 1973 (Brooks, Locke et al. 1973). MCF-7 cells are highly tumorigenic in nude mice in the presence of exogenous estrogen.
These cells have been proven to be a useful model for breast cancer in numerous studies.

MDA-MB-231 cells are triple-negative breast cancer cells, which do not express estrogen receptor (ER), progesterone receptor (PR) or Her2/neu. MAPK is constitutively activated in MDA-MB-231 cells (Lev, Kim et al. 2004).

CHO-K1 cell line (Puck et al., 1958) is an adherent epithelial line derived from Chinese hamster (Cricetulus griseus) ovary. Urd A cell line (Patterson 1977) is a mutant cell line derived from CHO K1 cell line. These cells require exogenous uridine to survive. CHO-K1 cells were treated with ethyl methanesulfonate (EMS) to induce mutagenesis. Cell lines were then selected based on absolute requirement for exogenously added pyrimidines for growth. The Urd-A cell line was later shown to lack the ATCase activity (Patterson et al., 1992), as a result of a mutation in the CAD gene. The amino-terminal part of CAD containing the CPSase II and DHOase activities was expressed but not the ATCase part.

All cell lines were maintained in T75 flasks at 37°C with 5% CO2 in a humidified environment and the 12 ml medium which was changed every 2-3 days. The culture medium was removed and 3 ml trypsin (0.05% Trypsin, Invitrogen) was added to detach the cells from the flasks. The cells were incubated with trypsin, for 5 min at 37°C (MCF10A cells required 10-15 min incubations with 0.25% trypsin). The detached cells were then homogenized by pipetting in 3 ml of medium, which allows trypsin inhibitors present in the serum to stop the digestion. The adequate fraction of cells was then transferred to a new flask and the volume was adjusted to about 12 ml in 75cm2 flask. For experiments requiring a precise number of cells, the cells were counted using a
hemocytometer and the appropriate number of cells were transferred to a flask or plated.

**Freezing and Thawing cultured cells**

This method was used to maintain the low passage batches of cells for long term storage. Exponentially growing cells (80-90% confluence in a 75 cm² flask, were treated with 3 ml trypsin (0.25%) in a 37 °C incubator for 2-3 minutes. Then, 3 ml of growth medium with 10 % FBS were added to inhibit trypsin activity. The mixture was then transferred to a 15 ml sterile tube and centrifuged at 200g for 5 min at room temperature to pellet cells. The cell pellet was re-suspended in 5 ml freezing medium (normal growth medium with 10% (v/v) DMSO. Then 1ml of aliquots containing about 1x10^6 cells were placed in cryogenic foam boxes at -80°C overnight and then immersed in liquid nitrogen. Cells were recovered from the nitrogen cell bank by rapid thawing in a 37°C water bath, and then immediately re-suspended in the appropriate culture medium and transferred to a 75 cm² tissue culture flask.

**Isolation of soluble protein fractions**

Cells grown on 100mm plate were washed twice with PBS (Invitrogen) supplemented with 0.2 mM PMSF (Sigma) and 0.5-1ml lysis buffer consisting of 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 0.2 mM PMSF supplemented with a 1X cocktail of phosphatase and protease inhibitors (Sigma) was added and incubated on ice for 10 minutes. The buffer was collected from the growing surface by scrapping. The cells suspension was vortexed at maximum speed for one min and the tubes were subjected to six 10 sec pulses of sonication in a water/ice bath.
The cell lysate was centrifuged for 15 min at maximum speed. The supernatant was designated the soluble fraction.

**Protein determination**

The Lowry protein assay was used for the quantitative colorimetric determination of total protein quantity in dilute aqueous solutions. A standard curve of 0 to 100 µg of BSA in 200 µl water in glass tubes was prepared from 1 mg/ml BSA stock solution. The samples (5, 7.5 and 10 ul) were diluted in water to 200 ul in glass tubes. Alkaline copper reagent (800 ul; 0.02% Na/K tartrate, 0.01% CuSO$_4$·5H$_2$O, 1.96% Na$_2$CO$_3$ in 0.1 N NaOH) freshly prepared from stock solutions (2% Na/K tartrate, 1% CuSO$_4$·5H$_2$O and 2% Na$_2$CO$_3$ in 0.1 N NaOH) was added to each sample. After vortexing, the tubes were incubated at room temperature for 15-30 min. Folin and Cioclateu’s phenol reagent (Sigma) was diluted 1:1 with water and 100 ul of the dilution were added to each tube. After vortexing, the tubes were incubated for 30 min at room temperature. The optical density (OD) was measured at 750 nm and the plot OD$_{750nm}$ as a function of concentration in BSA was plotted. The concentration in protein of the samples was determined using the standard curve and the mean of the three tests per sample.

**Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)**

The Laemmli system, which uses a discontinuous buffer system, is the most common electrophoresis protocol for SDS-denatured proteins (Laemmli 1970). The Mighty small II Gel SE250 electrophoresis unit from HOEFER consisting of 10 X 8 cm gel slabs was used. The acrylamide percentage in the running gel varied from 7% to 12% depending on the size of the proteins separated (7% gels were typical for CAD detection), and the percentage of cross-linker bis-acrylamide was 2.7%. The acrylamide
stock solution was 30% (w/v) acrylamide (Invitrogen) and 0.8% (w/v) N, N-methylene-bis-acrylamide. The gels were polymerized by addition of 0.03% (v/v) N, N, N', N'-tetramethylenediamine (TEMED; Invitrogen) and 0.03% (w/v) ammonium persulfate (APS; Sigma). The electrophoresis buffer contained 0.192 M glycine, 0.025 M Tris-HCl, pH 8.3 and 0.1% SDS. Proteins (1-5 ug) or protein samples (25-100 ug) were boiled for 3 min at 100°C in 1 X sample buffer (5 X sample buffer: 315 mM Tris-Cl pH 6.3, 10% SDS, 50% glycerol, 0.63% bromophenol blue and 25% beta-mercaptoethanol) and loaded on the gels. The electrophoresis was carried out at a constant voltage of 180 V. When the dye front reaches the bottom of the gel, the electrophoresis unit is disassembled. The gel is subjected to immunoblotting procedure or is stained in 0.05% Coomassie Brilliant Blue R reagent in 45% (v/v) methanol, 9% (v/v) acetic acid in distilled water for 15-30 min, and destained in 30% (v/v) methanol, 7.5% (v/v) acetic acid in distilled water. The gels were dried or wrapped in saran film and scanned directly using a Hewlett Packard ScanJet 4 c scanner and the staining intensity was quantitated using the UNSCAN-IT software (Silk Scientific Corp.).

**Western blotting**

Samples were resolved by SDS-PAGE using either 3-8% Tris-acetate or 4-12% Bis-tris NuPAGE gels (Invitrogen). The protein was then transferred to 0.45 µm pore-size nitrocellulose membrane (Bio-Rad) using a HOEFER transfer apparatus. Briefly, the paper blotters (BioRad) and a piece of nitrocellulose (BioRad) were pre-soaked in transfer buffer. A sandwich was assembled (foam sponge, paper blotter, gel, nitrocellulose membrane-paper blotter, foam sponge) taking care to exclude air bubbles. The sandwich was placed in a transfer tank filled to the required mark with chilled
transfer buffer, with the nitrocellulose membrane facing the anode. Transfer was carried out at a constant intensity of 400 mA for 90 min. Following transfer, the unit was disassembled. To quickly check the protein bands on the western blot, the nitrocellulose membrane was occasionally subjected to a rapid and reversible staining using Ponceau S solution (P7170 - 0.1 % (w/v) in 5% acetic acid, Sigma), a procedure that reveals all the proteins on the membrane. The membrane was then washed with 1x TBST and blocked using a 5% milk solution in 1x TBST (10mM Tris, pH 7.4, 150mM NaCl, 0.1% Tween 20) at room temperature for one hour and incubated in primary antibody diluted with 1% BSA with TBST. Incubation was carried with gentle shaking, overnight in the cold room. The membrane was then washed three times for 5 min each time by gentle shaking in TBST at room temperature. It was then incubated with a suitable secondary antibody coupled to the Horseradish Peroxidase (HRP) diluted in TBST-milk for 1 hour at room temperature with gentle shaking. The membrane was then washed three times in TBST as described above. The membrane was then incubated with a mixture of the SuperSignal West Pico Chemiluminescent Substrate (ECL; about 1 ml per mini gel 7x7 cm²) (Cat no.34080, Pierec) for 1 min at room temperature without shaking. The membranes were then placed in a transparent plastic film (Ziploc bag) and were taped into an X-Ray exposure cassette. The signals were obtained by exposing the membranes to UltraCruz™ Autoradiography Film, Blue, sc-201697 (Santa Cruz) from 20 seconds to 10 minutes depending on the intensity of the signals. The films were then developed by putting them successively in a 1X Developer and a 1X Fixer bath prepared from the 5X stock solution diluted in distilled water (5X stock Developer-replenisher, Cat#190-0984 and 5X stock Fixer-replenisher, Cat#190-
31

2485, Eastman Kodak Co.). The resulting immunoblots were scanned with a HP scanner and the software UNSCAN-IT (Silk Scientific) was used to quantify the signal intensity.

**Co-immunoprecipitation**

HEK293 cells grown in 35mm or 100mm plate were transfected with the expression constructs using Lipofectamine 2000. Cells were lysed 24 hours after transfection in lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 1× proteinase inhibitor mixture (Roche)]. Whole cell extracts were immunoprecipitated with the indicated antibodies in lysis buffer at 4 °C for 3 h and further incubated with Protein G PLUS agarose beads (Santa Cruz), for 1 h in cold room at 4 °C. After 5 washes with binding buffer, the bound proteins were eluted by boiling in a 1× SDS loading buffer and were subjected to the Western blotting for the detection of the associated proteins.

**RNAi Transfections**

All the siRNAs were purchased either from Invitrogen or from Santa Cruz. The lyophilized Invitrogen Stealth siRNAs (20 nmol) were dissolved in 1ml DNA-RNAase free water to a concentration of 20uM. The lyophilized siRNAs (3.3 nmol) from Santa cruz were dissolved in 330ul DNA-RNAase free water to give a 10uM stock solution. All the siRNAs were transfected into living cells with Lipofectamine™ RNAiMAX (13778-075, Invitrogen). Cells were grown on 6 well plates in 2 ml medium without antibiotics until they reached 20-30% confluence. Cells were also transfected with RNAi negative controls (Invitrogen Stealth RNAi controls with differing GC percentages) and positive control (BLOCK-iT™ Alexa Fluor® Red Fluorescent Control, 14750-100, Invitrogen) that
serves to monitor the transfection efficiency by fluorescence microscopy. In one tube, 3 μL (24 nM final) of 20 μM RNAi was gently mixed into 250μl Opti-MEM I reduced serum medium (31985070, Invitrogen). In a second tube, 3 μL lipofectamine RNAiMAX was gently mixed into 250μl Opti-MEM I reduced serum medium and incubated for not more than 5 minutes in room temperature. The RNAiMAX solution was then gently mixed into the first tube (containing the experimental RNAi or control RNAi) and it was incubated for 20 minutes at room temperature. This entire solution was then added dropwisely to the 2 ml of medium covering the plated cells. The plate was then placed in a humidified incubator at 37° C and 5% CO₂. After 4-6 hours, the medium was aspirated and replaced with complete medium. 72 hours post-transfection, the total cell lysates were extracted. The protein knockdown effect was assessed by western blot with corresponding antibody.

**Plasmid Transfections**

Cell were seeded in 6-well plates or in a 100mm plate 24 hours prior to transfection to make sure that the cells are 80-90% confluence prior to transfection. These cells were then transiently transfected with Lipofectamine 2000 reagent (Invitrogen) using 2ug DNA for the 6-well plate and 10ug for the 10cm plate according to the manufacturer’s protocol. After 4-6 hours, the medium was removed from the cells and replaced with complete medium. The cells were then allowed to recover 24 hours before extraction of lysates or observation by fluorescence microscope.

**Fluorescence microscopy**

For visualizing the fluorescence proteins in the live cells, the cells grown in 35mm plates were transfected with plasmids expressing fluorescence tagged proteins. After
24 hours, the DNA or nucleus in the transfected cells were optionally labeled using the cell-permeable compounds Hoechst 33342 (bisbenzimidine trihydrochloride; Sigma) for 15 min at 37°C. The cells were washed with fresh medium and immediately analyzed under water immersion lenses directly in the medium under Zeiss Apotome microscope.

For indirect immunofluorescence microscopy using antibodies, cells were plated in 6-well plates containing squared glass coverslips (VWR® Micro Cover Glasses, Square, No.1) and grown as described above for 24-48 hours at 37°C prior to treatments. The cells were washed once with PBS pH7.4. The cells were then fixed with either 10% formalin (Sigma) containing 3.7% formaldehyde in Phosphate Buffered Saline pH7.4 (Invitrogen) for 15 min and then permeabilized by 0.2% Triton X-100 (Sigma) in PBS, for 15 min. Alternatively, for cytoskeleton protein staining or using mouse monoclonal antibodies, the cells were fixed with cold methanol in -20°C without further permeabilization. The cells were rinsed and washed two times with PBS for 5 min and then blocked with 1% Bovine Serum Albumin in PBS pH7.4 to reduce non-specific protein bindings for 1 hour in room temperature. Then, the cover slip was taken out from the well and placed on parafilm. 250 ul diluted primary antibody solution in 1% BSA-PBS was added directly to the coverslip on the parafilm. Incubation without shaking was carried out for 2 hours. In the next steps, exposure to light was minimized because the fluorophores coupled to the secondary antibodies are light sensitive. After three 5-10 min washes with PBS with gentle shaking in the 6 well plate, the secondary antibodies (Invitrogen), Chicken anti-rabbit IgG (1:4000 dilution) conjugated to either Alexa Fluor 488 (green fluorescent) or Alexa Fluor 594 (red fluorescent) and chicken anti-mouse IgG (1:3000 dilution) conjugated to Alexa Fluor 488 diluted in 1% BSA-PBS
were added onto the coverslip and incubated for 1 hour in room temperature. The cells were rinsed and washed three times 5-10 min with PBS with gentle shaking. The coverslips were drained and mounted on glass slides with one drop of ProLong® Gold antifade reagent with DAPI (P36935, Invitrogen). The slides were allowed to dry at room temperature overnight in the dark (the slides were covered with foil and placed in a drawer). Indirect immunofluorescence was analyzed using 63x oil immersion lenses under a Zeiss Apotome microscope or a Leica TCS SP5 Laser Scanning Confocal Microscope (Wayne State University Core Imaging Facility).
CHAPTER 3
CHARACTERIZATION OF THE INTERACTION BETWEEN CAD AND FLASH AND THEIR ROLES IN CELL MITOSIS AND APOPTOSIS

INTRODUCTION

CAD is a multifunctional protein that initiates and regulates mammalian de novo pyrimidine biosynthesis. The phosphorylation of CAD at Thr-456 by mitogen-activated protein (MAP) kinase induces the activation of the pathway required for cell proliferation (Graves, Guy et al. 2000). Although most of the CAD in the cell is cytosolic, cell fractionation and fluorescence microscopy showed that activated Thr(P)-456 CAD was primarily localized within the nucleus (Sigoillot, Kotsis et al. 2005). Fluorescence microscopy has recently revealed that CAD is translocated from the cytoplasm into the nucleus just before S phase (Sigoillot, Evans et al. 2002). Subcellular localization studies showed that P-Thr456 CAD is also associated with the centrosome during mitosis in a variety of cell lines. CAD clearly co-localizes with gamma tubulin, the centrosome component responsible for the initiation of microtubule growth. Indirect fluorescence also suggests that Thr456 phosphorylated CAD is strongly associated with the cytoskeleton in MCF-7 cancer cells. Moreover, if the expression of CAD is knocked down using the siRNA in MCF-7 cells, cell growth is arrested and cannot be rescued by feeding the cells exogenous pyrimidines. This observation strongly suggests another functional role for CAD beyond providing the pyrimidine nucleotides needed for cell growth and maintenance. In addition, a yeast two-hybrid system screen identified a new CAD interacting protein FLASH, first identified as a pro-apoptotic protein involved
in Fas-induced apoptosis (Imai, Kimura et al. 1999). These discoveries and other evidence support the hypothesis that CAD has other, entirely unexpected roles in cell division and apoptosis which are unrelated to pyrimidine biosynthesis.

The regulation of CAD by MAPK and PKA has been extensively studied (Kotsis, Masko et al. 2007; Sigoillot, Kotsis et al. 2007). However, little is known about the function of CAD beyond the pyrimidine biosynthesis. It is critical to further investigate those unexpected roles for understanding how CAD is regulated in tumorigenic cell proliferation and apoptosis. The overall aim of this project is to investigate these other roles for CAD in the cells besides initiating and regulating pyrimidine biosynthesis, in the hope of providing insights into the design of new therapeutic drugs. Based on these observations, I proposed three specific aims in this chapter.

Aim 1: Characterize the interaction between CAD and the novel interaction partner FLASH

Aim 2: Determine the roles of CAD and its interacting protein, FLASH in centrosome organization, duplication and cell division

Aim 3: Investigate the roles of CAD and FLASH during apoptosis
METHODS

CAD knockdown

MCF-7 Cells were plated on 10cm plates and grown 30-50% confluence. Cells were then transfected with 3 different CAD siRNA with scrambled low GC*, 36% and med GC* 48% RNAi as negative control using RNAi Lipofectamine RNAiMAX (invitrogen), following the manufacture’s protocol. The siRNAs used are as follows: CAD-HSS101299-1: UUCAAGAGAAUUUGCUCCUCCUC; CAD-HSS101300-2: UGUCUGAGAAAGCCACACUUGGC; CAD HSS101301-3: AACUGUGUCAAUCUGUUUCACUGCU. Total protein was extracted from the cells 72 hours after transfections. Protein concentration was determined by the Lowry method using BSA as standard. The equal amount of the total protein extract (10ug per lane) was analyzed by western blotting with anti-CAD antibody. The Anti-GAPDH was used as control to verify the equal amount of loading protein.

Flow cytometry and cell cycle analysis

The cells were grown on 10cm plates and treated with 1mM PALA and 30uM uridine. The cell cycle was analyzed by flow cytometry. Briefly, 1 × 10^6 cells were harvested and washed in PBS, then fixed in 70% ethanol for 30 minutes at 4°C. After washing in cold PBS, cells were resuspended in 1 mL of PBS solution with 50 μg of propidium iodide and 100 μg of RNase A for 30 minutes at 37°C. Samples were then analyzed for their DNA content by FACSCalibur (Wayne state FACS core facility).

Immunoprecipitation
HEK-293 cells were transfected with HA-FLASH expression constructs using Lipofectamine 2000. Cells were lysed 24 h after transfection in lysis buffer [50 mM Tris, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, and 1× proteinase inhibitor mixture (Pierce)]. After a pre-clearing step using Protein G PLUS agarose beads (Santa Cruz), 500ul of protein lysates (1mg) were incubated overnight with 4ug of either rabbit IgG (sc-2027; Santa Cruz) or antibodies raised against total CAD, or rabbit anti-FLASH (Bethyl). 50 ul Protein G PLUS agarose beads were added to the sample which was then incubated for two hours at 4°C on an end-over end shaker to capture the immuno-complex. After 5 washes with lysis buffer, the bound proteins were eluted by boiling in a 1× SDS loading buffer and were subjected to the western blotting using anti-CAD and anti-FLASH antibodies.

Cloning of Cherry-CAD and stable Urd-A cell line construction

Full-length of human CAD DNA was amplified by PCR from pENTR-CAD plasmid using the primers: BspE1 site: 5’CACCTCCGGAATGGCGGCCCTAGTGTTGGAG 3’; Apal 5’AACGGGCCCTTCTAGAAACGGCCCAGCAGCCG3’ and then cloned into pmCherry-C1 vector (Clontech). Urd-A cells deficient in full length CAD, derived from chinese hamster ovary cells, are unable to grow in medium without the supply of exogenous uridine. Cherry-CAD was transfected into Urd-A using Lipofectamine 2000 (Invitrogen). The transfected cells were selected in medium supplemented with 1mg/ml geneticin and grown in medium without uridine.
RESULTS

CAD may have other functions unrelated to pyrimidine synthesis

The increased pyrimidine biosynthesis is correlated with increased CAD activity. To confirm that inhibiting CAD activity could arrest cell growth, I tried treating the MC7 breast cancer cells with PALA, a potent competitive inhibitor of the aspartate transcarbamylase activity of CAD. MCF-7 cells were grown in DMEM/F12 with 5% Dialyzed FBS (26400-036, Invitrogen) in 50-60% confluence and treated with 1mM PALA with and without 30uM uridine for 24 h. The untreated and treated cells alone were used as controls. After 24 h, all the cells except the PALA treated ones reached confluence. This indicates that PALA effectively inhibits MCF-7 cell growth. The cells were then treated with trypsin and subjected to flow cytometry analysis for DNA content. The results showed that 74% (Figure 3.1C) of PALA treated cells were arrested in S phase. However, only 33% of PALA treated cells, in the presence of uridine, were in S phase, which is very similar to the control untreated cells (Figure 3.1A). As shown in Figure 3.1B, uridine treatment did not affect the cell cycle. These data suggest that exogenous uridine could rescue the S phase arrest caused by CAD activity inhibition in MCF-7 cells.

In addition to treatment with PALA, pyrimidine biosynthesis could also be blocked by direct depletion of endogenous CAD protein using siRNA. MCF-7 cells were transfected with 3 different siRNAs against CAD and a scramble control RNAi for 72 hours. After that, the total cell lysates were extracted and subjected to western blot using anti-CAD antibody with GAPDH as loading control. As shown in Figure 3.2A,
siRNA-CAD-2 could knock down almost 90% CAD protein. I decided to use siRNA – CAD-2 for further experiments.

To determine whether depletion of CAD from the cells has the same effect on cell growth as uridine rescues the inhibition of its catalytic activity, I transfected MCF-7 cells with siRNA-CAD-2. After 6 hours, the medium was replaced with fresh growth medium containing DFBS with and without 30uM uridine. The total cell number was determined using a hemocytometer after 48 h, 72h and 96h transfection. The cell number in the plate transfected with siRNA-CAD at 96 hours was about half of the scrambled RNAi transfected cells (control). Surprisingly, the number of the cells transfected with siRNA-CAD grown in the medium with uridine was also much less than those of cells transfected with scramble control RNAi with the addition of uridine (Figure 3.2B). These results clearly demonstrate that exogenous uridine did not rescue the cells’ growth when CAD protein was depleted by siRNA.
Figure 3.1 Flow cytometry analysis of MCF-7 cells treated with PALA and uridine.

MCF-7 cells were grown in DMEM/F12 with 5% dialyzed FBS and were treated with 1mM PALA with and without 30 uM uridine for 24 hours. The cells were treated with trypsin, washed with cold PBS, and fixed with 70% ethanol. The fixed cells 1x10^6 were stained in 1ml PBS with 50ug/ml propidium idodide and subjected to flow cytometry analysis for DNA content according to Material and Methods.
Figure 3.2 Exogenous uridine does not rescue the cell growth when endogenous CAD is depleted by siRNA

A) MCF-7 Cells were plated in 10cm plates and grown to 30-50% confluence. They were then transfected with CAD siRNA using scrambled low GC* 36% and med GC* 48% RNAi as negative control by RNAi Lipofectamine RNAiMAX (invitrogen), according to the manufacture’s protocol. Total protein was extracted from the cells 72 hours after transfections. Protein concentration was determined by Lowry method using BSA as standard. The equal amount of the total protein extract (10ug per lane) was analyzed by western blotting with anti-CAD antibody. The GAPDH antibodies were used as control to verify the equal amount of protein had been loaded. B) MCF-7 cells were transfected with siRNA-CAD-2 and scramble RNA and grown in medium with and without addition of 30uM uridine respectively for indicated times. The cells were then trypsinized and counted using a hemocytometer and their viability was assessed by trypan blue staining.
Cell cycle dependent localization of CAD

Localization of p-Thr456CAD in cytoskeleton and nuclear body in MCF-7 cells

To understand these potential other functions of CAD, I was first interested in confirming the cell cycle dependent localization of CAD shown previously observed by Sigoillot et al in our lab (Sigoillot, Berkowski et al. 2003). I first repeated the immunofluorescence microscopy using the antibody specifically directed against phosphorylated CAD on Thr456 in MCF-7 and MCF10A cells. There are significant differences in the immuno-staining of the MCF-10A and MCF-7 cell lines. Immunofluorescence using the p-Thr456CAD antibody in MCF-7 cell showed that the cytoplasmic phosphorylated Thr456CAD was strongly associated with cytoskeleton structure (Figure 3.3A). In addition, the staining of p-Thr456CAD in MCF7 tumorigenic cells was more clearly defined in mitosis (Figure 3.4) and sometimes the centrosome in interphase was also labeled by p-Thr456CAD (data not shown). The overall p-Thr456CAD staining in MCF-7 was strong throughout the cell cycle, even in G1 phase. In contrast, under the same experimental conditions, immunofluorescence in MCF-10A cells showed strong staining only in mitotic cells. The staining was almost undetectable in interphase MCF10A cells. This could be due to a stronger phosphorylation level on the Thr456 of CAD in the tumorigenic line due to the increased MAP Kinase activity in MCF-7 (Sigoillot, Sigoillot et al. 2004). Immunofluorescence using anti-keratin-18 showed that the P-Thr456 CAD in MCF-7 appears to partially co-localize with keratin (Figure 3.3C). In addition, strong dot-like staining is also present in MCF-7 cell with 10 to 30 dot number in each nucleus. In general, PML nuclear bodies (Ching, Dellaire et al. 2005) and nuclear splicing speckles (Zimber, Nguyen et al. 2004) are the two major
Figure 3.3 Cytoskeleton and nuclear dots localization of p-Thr456CAD in MCF-7 cells

AB) pThr456 CAD is localized in the cytoskeleton in MCF-7 but in the nucleus in interphase of MCF10A cells. Non-synchronized, exponentially growing MCF-7 and MCF10A cells, were cultured in 6 well plates on a glass coverslip. The cells were fixed by incubation in 3.7% formaldehyde in PBS for 15 minutes followed by permeabilization with 0.2% Triton X-100 in PBS. They are then subjected to the indirect immunofluorescence procedure as described in Chapter 2 (Material and Methods). C) MCF-7 cells were fixed with 3.7% formaldehyde in PBS for 15 minutes, and then co-stained with mouse anti-cytokeratin 18 (green) and rabbit anti-pThr456 CAD antibody (red). DNA was stained with Hoechst 33342 (Hoechst; blue). D) MCF-7 cells were fixed and co-stained with rabbit anti-pThr456CAD and mouse anti-SC35 antibody (green).
classes of nuclear bodies with the 10 to 30 dots and diameter from 0.5-2 μm. To confirm the specific localization in nuclear bodies, I performed immunofluorescence in MCF-7 cells using PML nuclear bodies marker, PML antibodies or splicing speckle marker, sc-35 antibody with pThr456 CAD antibody. The results showed clear co-localization of pThr456 CAD with sc-35 (Figure 3.3D). This suggests that CAD could be involved in pre-mRNA splicing or gene transcription.

**Localization of pThr456 CAD in centrosome by indirect immunofluorescence microscopy**

In order to confirm that CAD is a bona fide centrosomal protein, I have done extensive indirect immunofluorescence microscopy using anti-pThr456CAD, anti-γtubulin antibodies on a variety of cell lines including human normal and cancer cell lines (HEK293, MCF10A, MCF-7, HeLa and PC3), hamster cell lines including BHK23, CHO-K1 and Urd-A. Using rabbit-anti pThr456CAD phosphor-specific antibody, one or two strong dot staining patterns were consistently observed during mitosis in all of these cell lines. The representative staining in MCF10A is shown in Figure 3.3B. Co-immunostaining using rabbit anti-pThr456CAD with mouse anti-γ tubulin (14C11: sc-53777-Santa cruz) in MCF-7 cells showed that phosphorylated CAD is localized in centrosome. Representative pictures from metaphase to telophase staining during cell division are shown in Figure 3.4. To exclude the potential staining artifact, I tried two different fixation methods. One was to fix the cells in -20°C methanol for 10 minutes. The other was to fix the cells in 3.7% formaldehyde with PBS for 15 minutes at room temperature. In both cases, the pThr456CAD and γ tubulin co-localization in
centrosome in mitotic cells was clearly observed. This indicates that the specificity of the labeling is independent on the fixation method.

To rule out the non-specific staining by rabbit anti-Thr456 CAD antibody, another commercially available antibody, goat anti-thr456 CAD antibody, from Santa cruz was also tested. Co-localization with γ tubulin was also detected using goat anti-Thr456CAD antibody and mouse anti-γ tubulin antibody (data not shown). Finally, the strong centrosome staining of pThr456CAD in mitotic cells also occurred in all the other cell lines tested above.

![Image of immunofluorescence microscopy](image)

**Figure 3.4 Localization of pThr456 CAD in centrosome during mitosis by indirect immunofluorescence microscopy**

MCF-7 cells were fixed with cold methanol and stained with rabbit anti-pThr456CAD (red) and mouse anti-gamma tubulin (green). The representative staining in different phases of mitosis is shown.
Localization of pThr1037 CAD and pSer1406 CAD in centrosome and midbody

To further investigate the cell cycle dependent localization of CAD, I also tried immunofluorescence microscopy using other two antibodies made by our lab, rabbit anti-pThr1037CAD and rabbit anti-pSer1406CAD antibodies which recognize the phosphorylated autophosphorylation site and the phosphorylation of the serine residue that down-regulates pyrimidine biosynthesis, respectively. Non-synchronized HeLa cells were fixed with cold methanol and co-stained with antibodies directed against gamma tubulin and beta tubulin and with pThr1037CAD and pSer1406CAD antibodies. The different phase cell of the cell cycle (interphase, prophase, metaphase, anaphase and telophase) could be easily distinguished according to the number and position of centrosomes together with the DNA staining. Staining using Thr1037 CAD antibody was weakly distributed throughout both in cytosol and nucleus. Although p-Thr1037CAD localization on centrosome in mitotic cells was barely detected (data not shown), strong co-localization of pThr1037CAD with gamma tubulin in centrosome was observed in interphase cells (Figure 3.5A). pSer1406 CAD staining in centrosome was also observed during late mitosis. Strikingly, Figure 3.5B showed clear localization of pSer1406CAD in midbody during cytokinesis.
Figure 3.5 Localization of pThr1037 CAD and pSer1406 CAD in centrosome and midbody

Non-synchronized HeLa cells were fixed with cold methanol and co-stained by gamma tubulin (green) with pThr1037CAD (red) and beta tubulin (green) with pSer1406CAD antibodies (red).
Co-Localization of Total CAD and Cherry-CAD with γ-tubulin in Centrosome by Fluorescence Microscopy

All of the above evidence indicates that CAD is localized in the centrosome throughout cell cycle. To confirm this localization, I performed immunofluorescence using antibody directly against total CAD. Again, the results showed clear co-localization with gamma tubulin in non-mitotic cells (Figure 3.7A). Immunofluorescence using anti-DHOase and anti-ATCase antibodies also detected that CAD was co-localized with gamma tubulin in the centrosome (data not shown). Although the indirect immunofluorescence results are informative, another more direct approach is needed to verify the localization in vivo. To this end, I have constructed two fluorescence fusion proteins, Cherry-CAD and GFP-gamma tubulin. Stable Urd-A cell lines expressing red fluorescence tag Cherry-CAD protein were obtained (Figure 3.6). Fluorescence microscopy showed that the protein is highly fluorescent and its localization is similar to that observed by indirect immunofluorescence microscopy of wild type CAD. ATCase assay also indicated that the stable cell line can highly express the functional CAD. In addition, Western blots showed that the recombinant protein had the expected size of 270kDa. I also constructed GFP-gamma tubulin, which can specifically target centrosome (Figure 3.7B). To further investigate the intracellular localization and identify the CAD domains that associate with centrosome, I have also created Cherry-ATCase and Cherry-DHOase-Linker-ATCase stable Urd-A cell lines. Interestingly, Cherry-ATCase seems to be partially localized in the nucleus to a much greater extent than the full length protein (Figure 3.6). GFP-γ tubulin was co-transfected into Urd-A cells with the Cherry-CAD plasmid. After 24 hours post-transfection, the cells
expressing the GFP-γ tubulin showed a dot-like localization of the protein (Figure 3.7). Remarkably, Zeiss ApoTome fluorescence microscopy using 40X dipping lens showed that cherry-CAD could co-localize with GFP-γ tubulin in live mitotic cells (Figure 3.7B). Although more experiments need to be performed to confirm the localization in interphase cells, the in vivo centrosomal localization is consistent with indirect immunofluorescence results.

**Figure 3.6 Stable cell line construction expressing Cherry CAD and its truncation mutants**

Urd-A cells are grown in 6 well plate and were transfected with 2 ug corresponding Cherry-CAD mutants using lipofectamine 2000. 24 hours later, the cells were split into new plate with 1:5 dilution. The transfected cells were screened using 1mg/ml geneticin.
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Figure 3.7 Co-Localization of Total CAD and Cherry-CAD with γ tubulin in Centrosome by Fluorescence Microscopy

A) HeLa cells were fixed with cold methanol and co-stained with rabbit anti-CAD (red) and mouse anti-gamma tubulin (green) antibodies. Nucleus was stained with Hoechst 33342 (blue).

B) Urd-A cells grown in 35mm plates were co-transfected with GFP-gamma tubulin and Cherry-CAD plasmids for 24 hours. The live cells were subjected to Zeiss ApoTome fluorescence microscopy using 40X dipping lens. The representative pictures are shown for mitotic cells expressing both Cherry-CAD and GFP-gamma tubulin.
Depletion of CAD and Thr456 CAD may cause mitotic defects

All of the above evidence suggests a possible role of CAD and P-T456 CAD in the centrosome structure and hence its role in cell division. To investigate if CAD is involved in the centrosome duplication and cell division, I first used siRNA to deplete the endogenous CAD, considering our highly effective siRNA CAD knockdown, to observe if there was any abnormal mitosis by immuno-staining of gamma tubulin and the nucleus. My preliminary results showed that the depletion of CAD in MCF-7 cells caused some mitotic defects or abnormal centrosome structure with bi-nucleus which I did not find in control cells (Figure 3.8). Furthermore, because pThr456 CAD is concentrated in the centrosome during mitosis, then the delivery of the phosphor-specific antibody might bind to pThr456 CAD in the centrosome, or the binding might dissociate the pThr456 CAD from centrosome which may lead abrogation of centrosome function and ultimately mitotic defect. To test this hypothesis, it is reasonable to avoid antibody delivery that would block the cells in S phase. Ideally, the antibody should be delivered in the very late S phase or prophase. MCF-7 cells were synchronized in S phase by 2mM thymidine for 16 hours, and then released for 4 hours. Then, pThr456CAD antibody was delivered into cells growing in medium without serum for 3 hours using BioPORTER® QuikEase™ Protein Delivery Kit (sigma) following the manufacture’s protocol. 6 hours later, the medium was changed to normal growth medium. 12 hour later, the cells were fixed and subjected to immunofluorescence. The fluorescence showed that the delivered antibody was not dispersed in the cytosol, but concentrated around the nuclear envelope. The results suggest that the delivered antibody maintained the ability to recognize CAD. Cells treated with rabbit IgG were used as
controls. Compared to control, delivery of pThr456CAD antibody also resulted in serious mitotic aberrations with multi-spindle staining (data not show).

Figure 3.8 Representative staining of aberrant gamma tubulin and nucleus upon knockdown of CAD
Characterization of the interaction between CAD and the novel interaction partner

FLASH

To extend our understanding of this unexpected function of CAD in cell proliferation and mitosis. A yeast two-hybrid screen was performed by Dr. Finley’s lab to identify novel interacting partners of CAD using full-length CAD as a bait. One clone encoding of C-terminal of human FLASH (or CASP8AP2; NM_012115) was isolated. According to the two-hybrid results, the first ~1330 amino acids (Figure 3.9A) of CASP8AP2 are dispensable for its interaction with CAD. This indicates that the interaction domain is within the C-terminal ~630 amino acids of FLASH.

Co-Immunoprecipitation to confirm the interaction in mammalian cells

To further confirm whether CAD and FLASH interact in mammalian cells, co-immunoprecipitation was performed in the human HEK293 cell line. Previously, it was shown that FLASH expression level is low in Jurkat cells and endogenous protein could not be immunoprecipitated using anti-FLASH antibody (Choi, Kim et al. 2001). Consistent with this observation, my preliminary immunoprecipitation results using the anti-FLASH antibody M-300 (Santa Cruz) also showed that endogenous FLASH might not be effectively immunoprecipitated with the available antibodies. Therefore, co-immunoprecipitation was performed using FLASH-overexpressing cells. HA epitope-tagged FLASH (a kind gift from Dr. Vincenzo De Laurenzi) was transiently transfected into in HEK293 cells for 24 hours. The total lysate was then subjected to immunoprecipitation by anti-CAD antibodies followed by western blot analysis using anti-HA or anti-FLASH. The reverse immunoprecipitation was performed using anti-
FLASH and anti-HA antibody. The results showed that FLASH could be pulled down by anti-CAD antibody but not the control IgG (Figure 3.9), a result consistent with the results of the yeast two hybrid screen.
Figure 3.9 Characterization of the interaction between CAD and FLASH

A) Diagram of isolated FLASH domain interacting with CAD in yeast two-hybrid.

B) Co-immunoprecipitation of CAD and FLASH. HEK293 cells were transfected with HA-FLASH for 24 hours and then the total lysate was subjected to immunoprecipitation by anti-CAD and anti-FLASH antibody.

C) Urd-A cells grown in coverslip in 6 well plate were transfected with GFP-FLASH plasmid using lipofectamine 2000. After 24 hours transfection, the cells were fixed with 3.7% formaldehyde and subjected to fluorescence microscopy. The representative overexpressed GFP-FLASH localization was shown here.

D) Urd-A cells were co-transfected with Cherry-CAD and GFP-FLASH. Representative co-localization was shown here.
Co-localization of CAD and FLASH

To study the physiological function of the interaction between FLASH and CAD, I first wanted to confirm FLASH intracellular localization. I performed immunofluorescence microscopy using anti-FLASH antibody in a variety of human cell lines. However, the results were inconsistent with previous proposed FLASH localization which indicated that FLASH was cytosolic. Unexpectedly, I found that FLASH was always weakly stained in cytoplasm and that a small number of strong staining dots could be detected in the nucleus. Double staining using rabbit-anti-FLASH antibody and mouse anti-γ tubulin showed that FLASH co-localized with γ tubulin in centrosome in all of the human cells tested such as CHO-K1, HeLa and MCF10A cells. The representative pictures in MCF10A are shown in Figure 3.10B. However, the co-localization could not be detected throughout the cell cycle. FLASH co-localizes with γ tubulin only in interphase as shown in Figure 3.10B. Moreover, FLASH also partially co-localizes with coilin (Figure 3.10A), which is consistent with the previous report that FLASH partially colocalizes with Cajal bodies (Barcaroli, Dinsdale et al. 2006). Co-immunostaining of FLASH with PML also clearly showed that FLASH did not co-localize with PML nuclear body which will be further discussed in chapter 4.

Next, I performed fluorescence microscopy analysis of GFP-FLASH localization in transfected Urd-A cells. Even though, most of GFP-FLASH is found in nuclear bodies, overexpressed GFP-FLASH also showed distributed nucleus and cytosol localization in a small portion of transfected cells, as shown in Figure 3.9C. Although a more detailed analysis and addition data are needed, it appears so far that there is strong co-localization of cherry-CAD and GFP-FLASH in those cells where GFP-FLASH is in both
nucleus and cytosol (Figure 3.9D). Together, these results confirm the interaction between CAD and FLASH in cells.

**Figure 3.10  FLASH is partially co-localized with coilin and gamma tubulin**

MCF10A cells were grown on coverslip in 6 well plate for 24 hours. Then, the cells were fixed with methanol and co-stained with rabbit anti-FLASH with mouse anti-coilin (green) (A). Cells in panel B were treated by the same procedure and co-stained with rabbit anti-FLASH and gamma tubulin (green).
Knockdown of both CAD and FLASH results in inhibition of cell proliferation

To study the physiological function of CAD and FLASH interaction, I first wanted to compare the phenotypic differences between CAD and FLASH knockdown cells. HeLa cells grown in medium with and without addition of uridine were transfected with siRNA against CAD, FLASH and the scrambled control RNAi. Western blot showed that CAD and FLASH were effectively knocked down by more than 80% after 72 hours and 24 hours siRNA transfection, respectively. Similar to the results of observed upon CAD knockdown in MCF-7 cells, HeLa cells transfected with siRNA-CAD showed decreased growth rate compared to control cells transfected with scramble RNAi (Figure 3.11A). Strikingly, following FLASH knockdown, HeLa cells grew much more slowly and the cell number started to decrease after 72 hours, indicating that FLASH knockdown alone might induce HeLa cell apoptosis. An observation is direct contradiction to the pro-apoptotic role assigned to FLASH (1). In addition, knockdown FLASH in HeLa cells results in flattened cell morphology and enlarged cell size (Figure 3.11B). Immunofluorescence microscopy further showed that most of the enlarged cells contain multi-centrosomes and multi-nucleus, or large nucleus (Figure 3.11C).
Figure 3.11 Both CAD and FLASH Knockdown results in inhibition of cell proliferation
**CAD is degraded during Fas-mediated apoptosis**

Western blot showed that the full length of CAD is gradually degraded when HT1080 cells were induced into apoptosis by anti-CD95 antibody. Concomitant with this decline, there was a clear increase of two immunoreactive bands that seemed to be proteolytic products of the CAD protein (Figure 3.12). One band, possibly a doublet, with a molecular mass of about 100 kDa appeared first within 4 h of anti-CD95 antibody treatment, whereas the other band in about 40kDa occurred in about 16 hours treatment. CAD is cleaved in the proteasome inhibitor of mg132, indicated that CAD cleavage might be caspase-dependent during apoptosis, which is consistent with previous study by Graves and colleagues (Huang, Kozlowski et al. 2002).
Figure 3.12 CAD cleavage during apoptosis induced with anti-CD95 antibody

HT1080 cells were treated with 1μg/ml anti-CD95 in the presence of 10μM proteasome inhibitor Mg132 for indicated times. The total lysates were subjected to western blot using anti-total CAD antibody.
DISCUSSION

In this chapter, I first confirmed that exogenous uridine, the end product of pyrimidine biosynthesis pathway, can rescue the growth inhibition and cell cycle arrest resulted from CAD activity inhibited by PALA in MCF-7 cells (Figure 3.1). In addition, uridine alone did not affect MCF-7 cell growth and cell cycle. In contrast, when CAD protein was depleted by siRNA, the addition of uridine did not rescue the cell growth (Figure 3.2B). This suggests that CAD has novel growth related functions that are unrelated to pyrimidine biosynthesis. Therefore, direct evidence has been shown that the multifunctional protein, CAD, has other unexpected functions in cell proliferation. It is intriguing to decipher the significance of these novel CAD functions.

Previously, Sigoillot et al. in our lab suggested that pThr456CAD is associated with the centrosome during mitosis but not during interphase. CAD has also been found to interact with several other proteins localized in centrosome such as HSP90, a conserved centrosomal protein essential for centrosome function, integrity and mitotic cell cycle progression (Lange, Bachi et al. 2000). To investigate the function of CAD in centrosome, I first wanted to verify the centrosomal localization of CAD using different antibodies in a variety of cancer cell lines including MCF-7 and HeLa cells. Consistent with previous findings, immunofluorescence microscopy using rabbit anti-pThr456 CAD antibody showed that pThr456 CAD is co-localized with γ-tubulin in mitotic MCF-7 cells (Figure 3.4). Next, I wanted to know if total CAD is also localized in centrosome. Immunofluorescence microscopy using antibody directly against full length CAD indicated that CAD also co-localized with γ-tubulin (Figure 3.7A). Two other antibodies specifically against DHOase or ATCase domain of CAD also revealed the similar
centrosomal staining but not the corresponding IgG control (data not shown). While many proteins are found in association with the centrosome, centrosome adventitious association of CAD is unlikely because multiple antibodies and overexpressed Cherry-CAD also show centrosomal staining.

It is known that most of the centrosomal proteins contain a coiled-coil domain. However, structure prediction failed to predict a coiled coil domain in CAD. Hence, the key question comes, how is CAD recruited to centrosome? My results have thus confirmed the yeast two hybrid results indicating that CAD interacts with FLASH. Since FLASH does have a coil-coil domain, the presence of FLASH in centrosome would suggest the intriguing hypothesis that FLASH anchors CAD to this organelle. Immunofluorescence using rabbit anti-FLASH(M-300) from Santa Cruz revealed that, while FLASH is localized in nuclear bodies, there was also one dot containing FLASH that co-localized with gamma tubulin in centrosome in interphase MCF10A cells (Figure 3.10B). While this observation is consistent with the mechanism in which centrosomal FLASH recruits CAD, additional experiments are needed to prove this hypothesis. Recently, many centrosome proteins were identified in my mass spectrometry analysis of a FLASH co-immunoprecipitation complex (data not shown) using another commercial (Bethyl) rabbit antibody. However, centrosome localization of FLASH is elusive due to lack of other independent antibodies suitable for immunofluorescence and identification of centrosomal localization domain of FLASH. siRNA knockdown technique might help to validate the specific localization of FLASH in the few dots. One experiment that may be revealing would be to determine if the presence of CAD in the centrosome is abolished by FLASH knockdown. Nevertheless, it is difficult to get rid of
all non-specific dots staining especially without a highly specific antibody for immunofluorescence. Further work to revalidate the FLASH centrosome localization is essential for understanding the physiological function of the interaction between CAD and FLASH in centrosome.

In addition to its centrosomal localization, CAD was found to be present in the isolated mammalian midbody by tandem mass spectrometry (Skop, Liu et al. 2004). My data also show that pSer1406 CAD is clearly localized in midbody (Figure 3.5B). The phosphorylation of Ser1406 of CAD is responsible for the down-regulation of pyrimidine biosynthesis and would be expected to be a major species present during mitosis and cytokinesis. This further suggests that CAD may play important role in cytokinesis during cell division. Consistent with this postulated role, preliminary siRNA CAD knockdown results showed that depletion of CAD leads to formation of bi-nucleus cells and abnormal centrosome.

FLASH was initially reported to function as a pro-apoptotic protein so that our investigation of the interaction between CAD and FLASH was primarily focused on apoptosis pathway. Western blot analysis reveals that CAD is gradually degraded during Fas-induced apoptosis in HT1080 cells. The fact that many of the proteins are shown to be associated with CAD, including TRADD, MAP37K7, MAP3K71P2, Rad9 (Lindsey-Boltz, Wauson et al. 2004) which are involved in Fas signaling, and now FLASH, highly suggests that CAD may play a role in apoptosis. Given the observation that CAD expression is appreciably up-regulated in actively proliferating cells and that the protein is indispensable for cell growth, one would expect it to be anti-apoptotic. Like other anti-apoptotic proteins, CAD was cleaved during apoptosis. One possibility is that
the cells do not require the synthesis of pyrimidine nucleotides once the cells are committed to undergo apoptosis. Moreover, cleavage of CAD during apoptosis would also be expected to block its anti-apoptotic function. We also found that siRNA knockdown of CAD in HT1080 cells for 72 hours can also increase the sensitivity of the cells to Fas-mediated apoptosis (data not shown). However, we don’t know if this is due to the observed S phase block that occurs upon CAD depletion (data not shown) or because CAD is somehow involved in the regulation of Fas apoptosis pathway. In an attempt to test this hypothesis, we attempted to overexpress CAD in MCF10A cells with low CAD level to test if overexpression of CAD could block or decrease MCF10 cells apoptosis induced by Fas. The cells are very sensitive to Fas-mediated apoptosis. However, due to the extreme difficulty in transfecting plasmids into MCF10A cells, the experiment was not successful.

As described in Chapter 4, FLASH knockdown alone did not induce apoptosis in HT1080 cells. However, here I have shown that knockdown of FLASH in HeLa cells can induce cell death as indicated by decreased total live cell number in 72 hours siRNA posttransfection. Even though, there are considerable differences between the two cell lines, the big difference I can notice is that HT1080 cells contain wild-type p53, while HeLa cells do not contain functional p53 due to the rapid degradation mediated by E6 gene in HPV (Hoppe-Seyler and Butz 1993). A p53-dependent S-phase checkpoint helps to protect cells from DNA damage induced apoptosis, which might account for the discrepancy when FLASH is knocked down in different cell lines.

Other studies have shown that FLASH is involved in a myriad of cellular processes (Chapter 1). The preliminary data reported here indicate that FLASH
localizes to centrosome. Thus, in addition to S phase progression and the many other functions assigned to FLASH, it may also be involved in cell division. In that case, knockdown of FLASH might be expected to lead to abnormal mitosis. Our data (Figure 3.11C) that knockdown of FLASH resulted in bi-nuclear and large nuclear cells further support this hypothesis.

Yeast two hybrid identified that CAD could interact with C-terminal domain of FLASH containing the Death effector domain recruiting domain (DRD) which binds to caspase 8 in the Fas mediated apoptosis. My hypothesis is that when the apoptotic signal is received, caspase-8 is recruited to the DISC complex but is not activated since FLASH is associated with CAD. There is an equilibrium between FLASH binding to CAD and FLASH binding to caspase-8. FLASH-caspase-8 activates caspase-3 which cleaves CAD, so it cannot bind to FLASH. The release of FLASH results in full activation of caspase-8, caspase-3 and a commitment to apoptosis. To test the hypothesis, I used immunofluorescence microscopy to look at the co-localization of CAD and FLASH when apoptosis is induced. However, I did not observe the dynamic translocation of FLASH from nucleus to cytosol. Once the cells underwent apoptosis and the DNA began to fragment, no clear FLASH containing nuclear dots could be detected (data not shown). Instead, strong non-specific staining was observed throughout the cells, hindering the further experiments. Co-immunoprecipitation was performed on the cells treated with anti-Fas antibody to check if CAD, FLASH and caspase 8 could form complex during apoptosis. However, even though the FLASH could pull down CAD, I failed to detect the interaction of FLASH with caspase 8 as previously reported.
In summary, in this chapter, I have confirmed that FLASH interacts with CAD. Knockdown of CAD and FLASH show almost same phenotypes: inhibition of cell growth, abnormal cell division, S phases arrest and increased sensitivity to apoptosis. While several intriguing observations have been made, additional experiments will be required to decipher the role of CAD and FLASH and the interaction of these two large proteins in cell growth and survival. However, it seems likely that CAD may co-operate with FLASH in cell proliferation and the apoptosis pathway.
ABSTRACT

FLASH (FLICE-associated huge protein or CASP8AP2) is a large multifunctional protein that is involved in many cellular processes associated with cell death and survival. It has been reported to promote apoptosis, but we show here that depletion of FLASH in HT1080 cells by siRNA interference can also accelerate the process. As shown previously, depletion of FLASH halts growth by down-regulating histone biosynthesis and arrests the cell cycle in S-phase. FLASH knockdown followed by stimulating the cells with Fas ligand or anti-Fas antibodies was found to be associated with a more rapid cleavage of PARP, accelerated activation of caspase-8 and the executioner caspase-3 and rapid progression to cellular disintegration. As is the case for most anti-apoptotic proteins, FLASH was degraded soon after the onset of apoptosis. Depletion of FLASH also resulted in the reduced intracellular levels of the anti-apoptotic proteins, MCL-1 and the short isoform of cFLIP. FLASH knockdown in HT1080 mutant cells defective in p53 did not significantly accelerate Fas mediated apoptosis indicating that the effect was dependent on functional p53. Collectively, these results suggest that under some circumstances, FLASH suppresses apoptosis.
INTRODUCTION

FLASH (CASP8AP2) is a large multifunctional protein that has been implicated in many different cellular processes including apoptosis, histone mRNA processing, S-phase progression, NF-kB activation and the regulation of transcription. In 1999, Imai et al. (Imai, Kimura et al. 1999) discovered a 220 kDa protein, which they designated FLICE associated huge protein or FLASH, since it associates with caspase-8 and promotes Fas induced apoptosis. There are two major apoptotic pathways. The binding of ligands to the FAS receptor, a member of the TNF family of plasma membrane receptors, triggers the assembly of the death inducing signaling complex (DISC) (Figure 4.1). Imai et al. (Imai, Kimura et al. 1999) showed that in 293T cells, FLASH associates with the adaptor protein, FADD, recruiting caspase-8 to the activated DISC. Oligomerization of FLASH results in the proteolytic cleavage and activation of caspase-8. Caspase-8 in turn activates other caspases including the executioner protease, caspase-3.
Figure 4.1. The role of FLASH in the apoptotic pathways. In the extrinsic pathway, the Fas ligand (FasL) binds to the Fas receptor and triggers the assembly of the DISC complex. FLASH binds pro-caspase 8 and translocates to the DISC complex where it associates with FADD. Active caspase-8 is formed at the DISC by proteolytic cleavage. The active caspase then cleaves and activates the executioner protease, caspase-3. c-FLIP short is also part of the DISC and inhibits the activation of caspase-8. Caspase-3 is also activated in the intrinsic or mitochondrial pathway triggered by a variety of apoptotic signals that culminate in the formation of pores that allow the release of cytochrome c. Cytochrome c associates with Apaf-1 forming the apoptosome which recruits and activates pro-caspase 9, which in turn activates pro-caspase 3. The translocation of FLASH from the nucleus to the mitochondria is thought to be one of the signals that initiate the mitochondrial apoptotic pathway. The extrinsic and intrinsic
pathways are linked by Bid, a cytoplasmic proapoptotic protein that is cleaved by caspase-8 generated at the DISC complex. Once cleaved, the truncated Bid (tBid) migrates to the mitochondria where it interacts with Bax and Bad, proteins that promote mitochondrial permeability and cytochrome c release. FLASH also binds to the histone gene locus where it participates in processing the histone mRNA that is necessary for S-phase progression. FLASH is also a coactivator of c-Myb which controls the expression of several proteins that play a role in proliferation, including the anti-apoptotic protein, BCL-2. P53 down regulates the expression of BCL-2 and another pro-apoptotic protein, MCL-1.
In the intrinsic or mitochondrial apoptotic pathway several intra- and extracellular apoptotic signals induce the release of proteins from the mitochondria including cytochrome c (Figure 1). Cytochrome c associates with the apoptotic protease activating factor 1 (APAF-1) to form the apoptosome. The recruitment of pro-caspase-9 molecules to the apoptosome promotes its proteolytic activation which leads to the activation of the downstream executioner, caspase-3. The mitochondrial apoptotic pathway also serves to amplify the apoptotic response triggered by the activation of the Fas receptor (Kuwana, Smith et al. 1998).

The response to stimulation of the Fas receptor differs according to cell type (Scaffidi, Fulda et al. 1998) Type I cells such as SKW6.4 and H9 cells quickly assemble large amounts of DISC upon binding of the Fas ligand with the rapid activation of caspase 8 and caspase 3. Very little DISC is formed upon stimulation of Type II cells such as CEM and Jurkat cells. However, sufficient caspase-8 is activated to cleave the cytoplasmic protein, Bid. Truncated Bid, tBid, relocates to the mitochondria where it binds to Bak/Bax which together with Bad promote the formation of mitochondrial pores and the release of cytochrome C. The loss of the mitochondrial membrane potential occurs prior to the activation of caspase-3 and caspase-8. Thus, the mitochondrial pathway is indispensable for type II cells to undergo apoptosis.

FLASH was originally thought (Imai, Kimura et al. 1999) to be exclusively a cytoplasmic protein but more recent studies showed that it is primarily nuclear and that it is localized within a variety of discrete nuclear bodies. FLASH was identified (Barcaroli, Dinsdale et al. 2006) as an indispensable component of Cajal bodies, small nuclear organelles involved in numerous cell functions. RNA interference showed that
depletion of FLASH resulted in disruption of Cajal body structure and relocation of its components. In other studies, FLASH was found (Krieghoff, Milovic-Holm et al. 2007; Krieghoff-Henning and Hofmann 2008) to be primarily localized in promyelocytic leukemia nuclear bodies which are involved in apoptosis, the regulation of senescence and tumor suppression. FLASH associates with Sp100 (Krieghoff, Milovic-Holm et al. 2007; Milovic-Holm, Krieghoff et al. 2007), an essential PML component. Although PMLs are distinct nuclear bodies, they are often found in association with Cajal bodies and other nuclear organelles. Immunofluorescence microscopy (Barcaroli, Bongiorno-Borbone et al. 2006) showed that FLASH was 100% coincident with NPAT, the nuclear protein localized near histone locus bodies (Ma, Van Tine et al. 2000) on chromosome 6 and 12. HLBs are often associated with but are not identical to the coilin containing Cajal bodies, although the two organelles co-localize during the S phase of the cell cycle. These authors (Barcaroli, Bongiorno-Borbone et al. 2006; Bongiorno-Borbone, De Cola et al. 2008) did not find that FLASH in other nuclear bodies such as nuclear speckles or PML bodies.

In 2007, Milovic-Holm et al. (Milovic-Holm, Krieghoff et al. 2007) made the intriguing observation that activation of the Fas receptor triggers the translocation of FLASH from the PML nuclear bodies to the cytoplasm, where it associates with caspase-8 at the mitochondrial surface, thereby activating the mitochondrial apoptotic pathway. Leptomycin B, an inhibitor of Crm1-dependent nuclear export, blocked egress of FLASH from the nucleus and prevented mitochondrial damage. Caspase-8 was nevertheless still activated, albeit to a lesser extent, presumably at the DISC assembly. FLASH depletion by siRNA interference followed by induction of the Fas receptor with
anti-Fas antibodies for 7 hours resulted in a 57% decrease in apoptosis. A recent study (Tanaka and Kamitani 2010) may provide insight into the translocation mechanism. FLASH was shown to form a complex with Ro52, an E3 ubiquitin ligase that moves along cytoplasmic microtubular networks. Simultaneous overexpression of Ro52 and FLASH induces the relocation of another apoptotic protein, DAXX, from the nucleus to the cytoplasm.

Barcaroli et al. (Barcaroli, Bongiorno-Borbone et al. 2006; Bongiorno-Borbone, De Cola et al. 2008) discovered that depletion of FLASH by RNA interference abolished histone biosynthesis and induced cell cycle arrest in S phase. It was subsequently shown (Yang, Burch et al. 2009) that FLASH is necessary for proper processing of the 3'-end of the histone pre-mRNA. FLASH also plays a significant role in the transcriptional regulation of histone genes. The FLASH binding partner, NPAT(p220), is an activator of histone gene transcription (Ma, Van Tine et al. 2000; Zhao, Kennedy et al. 2000) under the control of cyclin E/Cdk2 kinase. Moreover, CHIP assays demonstrated that FLASH interacts with histone gene promoter sequences (Barcaroli, Bongiorno-Borbone et al. 2006). Depletion of FLASH by RNA interference results in suppression of histone synthesis and cell cycle arrest in S phase (Barcaroli, Bongiorno-Borbone et al. 2006). The interaction of FLASH with the arsenite resistance protein, Ars2, a protein involved in the formation of microRNA, was shown (Kiriyama, Kobayashi et al. 2009) to be indispensable for cell cycle progression. Similar results (De Cola, Bongiorno-Borbone et al. 2011) were observed during embryogenesis where FLASH cooperates with the transcription factor p73 to regulate histone gene transcription and
cell cycle progression. These authors also found that FLASH knockout is lethal in embryonic mice.

FLASH is also a co-activator of c-Myb, a transcription factor normally associated with growth and survival. Both proteins colocalize at active transcription loci (Alm-Kristiansen, Saether et al. 2008; Alm-Kristiansen, Lorenzo et al. 2011). The enhancement of transcriptional activity by FLASH is comparable with that obtained with the c-Myb co-activator, P300. The E3 SUMO-protein ligase, PIAS1, was also found to interact with FLASH and enhance its transcriptional activity and the expression of genes under control of c-Myb (Alm-Kristiansen, Lorenzo et al. 2011). In some instances, FLASH was found to repress transcription. It binds to and inhibits the activity of the p160 nuclear receptor coactivator (GRIP 1) thus suppressing the expression of the glucocorticoid receptor (Kino and Chrousos 2003) in human colon carcinoma cells. In contrast, it enhances transactivation of both the glucocorticoid and mineralocorticoid receptors in mouse hippocampal cells but had only a small repressive effect in neuroblastoma cells (Obradovic, Tirard et al. 2004). FLASH also modulates the activity of the transcription factor NFκB via a TRAF-2 dependent pathway (Choi, Kim et al. 2001; Jun, Chung et al. 2005) Depletion of FLASH by RNA interference abolishes the activation of NFκB, while overexpression of FLASH activates its activity in a dose dependent manner.

Thus, FLASH is involved in several pathways related to cell death, growth and survival. Those studies (Imai, Kimura et al. 1999; Milovic-Holm, Kriehoff et al. 2007) that specifically examined its role in cell death, suggest that it promotes apoptosis. We report here that under certain circumstances, it can also effectively suppress apoptosis.
Materials and Methods

Antibodies and Reagents - Antibodies used for this study were rabbit anti-FLASH (Bethyl Laboratories, Montgomery, TX), mouse anti-caspase-8 (9746), rabbit anti-caspase-3 (9662), rabbit anti-cleaved caspase-3 (9664), rabbit anti-poly (ADP-ribose) polymerase, PARP (9542), rabbit anti-cleaved PARP(5625), rabbit anti-MCL-1 (5453) (Cell Signaling, Beverly, MA); mouse monoclonal β-tubulin (sc-5274), mouse monoclonal anti-p53 (sc-126), rabbit anti-FLASH M300 (sc-9088), mouse anti-NPAT (sc-136007) and mouse anti-PML (sc-966), mouse anti-FLIPS/L (sc-5276), rabbit anti-IKKα (sc7607) (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-histone H3 (21137) (Signalway Antibody, Pearland, TX), rabbit anti-HDAC1(10197-1-AP), rabbit anti-coilin (10967-1-AP) and rabbit anti-p21(10240-1-AP) (Proteintech Group, Inc). A panel of caspase inhibitors (FMKSP01) and recombinant human Fas Ligand/TNF9SF (126-FL-010) were purchased from R&D (Minneapolis, MN). MG132, cycloheximide (CHX), actinomycin D were from Sigma and adriamycin was from Santa Cruz. Staurosporine (STS) was from Invitrogen (Carlsbad, CA).

Cell Culture and Induction of Apoptosis – HT1080 cells (wild type p53) and HT1080-6TG (mutant p53) (a gift of Dr Eric J Stanbridge, Department of Microbiology and Molecular Genetics, University of California, Irvine, CA) and HeLa cells (ATCC) were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, in 5% CO₂ at 37°C. The MCF10A cell line, obtained from Drs. Santner and Pauley (Karmanos Cancer Institute, Wayne State University, Detroit, MI), was cultured according to the original publications (Soule, Maloney et al. 1990; Basolo, Elliott et al. 1991). For the induction of apoptosis, the cells were incubated with 100
ng/ml of the recombinant Fas ligand (R&D, Minneapolis, MN) or 1 µg/ml of the agonist mouse monoclonal anti-human CD95 (Fas) antibody (Invitrogen, AHS9552) for the indicated periods of time at in 5% CO₂ at 37°C. Alternatively, apoptosis was induced by incubating the cells with 1 µM staurosporine. The progression through apoptosis was monitored by measuring the activation of the caspases and the cleavage of PARP. The distribution of cells in different phases of the cell cycle was measured using a Becton-Dickinson FACScan cytofluorometer at the Wayne State University, Karmanos Cancer Institute, Flow Cytometry Facility.

**SiRNA Interference** - HT1080 cells were grown in 6 well plates to 20-30% confluence. Cells were transfected with siRNA directed against the FLASH mRNA and, as a negative control, with a scrambled siRNA, using RNAi Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. The oligonucleotides used for these studies were purchased from Invitrogen (Carlsbad, CA).

1) FLASH Stealth RNAi™ siRNA HSS115171: GAAACAGAAUGAACCAAAGACUGAU;
2) FLASH Stealth RNAi™ siRNA HSS115172: GAAAGCUGAGAGUGGUCCAAAUGAA;
3) FLASH Stealth RNAi™ siRNA HSS115173: CCUGUGGUAAUGGAUGUAUUACAAA.

To assess the extent to which the expression of FLASH was suppressed, cell extracts were isolated at various times following transfection and the cell lysate was analyzed by Western blotting. Equivalent amounts of total protein were analyzed as determined by the Lowry method using bovine serum albumin (BSA) as a standard. Immunoblotting of β-tubulin or β-actin was used to verify that equal amounts of total protein had been loaded on the gel. The same procedure was used to suppress p53 expression using a
commercial siRNA of proprietary sequence (Santa Cruz, sc-29435) and siRNA against coilin was purchased from Invitrogen (HSS112012).

**Western blot Analysis** – Total cell extracts were prepared in a lysis buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 0.2 mM PMSF supplemented with a 1X cocktail of phosphatase and protease inhibitors (Sigma). Protein samples were heated at 95°C for 10 min and separated by SDS-PAGE using 4-12% gradient gel and transferred to nitrocellulose membrane. Western blots were developed using the Western Lighting Plus-ECL reagent (NEL104001EA, Perkin Elmer, Waltham, MA).

**Cell Fractionation** - Cytoplasmic and nuclear fractions were isolated using the Qproteome Nuclear Protein Kit (Qiagen) according to the manufacturer's protocol. The purity of the fractions was confirmed by Western Blotting using anti-PARP, anti-HDAC1 (nuclear markers) antibodies and anti-β-tubulin (cytoplasmic marker) antibody.

**Immunofluorescence microscopy** - Cells grown on cover slips in 6-well plates were fixed with cold methanol at -20°C for 10 min and then blocked with 3% BSA in PBS for 1 h at room temperature. Cells were incubated with the primary antibody, rabbit anti-FLASH M-300, mouse anti-NPAT monoclonal antibody or mouse anti-PML monoclonal antibody, alone or in combination, overnight at 4°C. The cells were then incubated with chicken anti-rabbit IgG antibody conjugated with Alexa Fluor 594 (1:2,000) and a chicken anti-mouse IgG antibody conjugated with Alexa Fluor 488 (1:2,000) (Molecular Probes, Invitrogen, Carlsbad, CA) as secondary antibodies for 1 h at room temperature. After extensive washing with PBS, the cells were counterstained with Hoechst 33342, mounted and visualized using a Leica TCS SP5 Laser Scanning Confocal Microscope.
(Karmanos Cancer Institute Imaging and Cytometry Resources Core Facility). The images were analyzed using the Leica LAS AF Imaging software.

RESULTS

Intracellular localization of FLASH

In agreement with previous studies (Milovic-Holm, Kriehoff et al. 2007; Bongiorno-Borbone, De Cola et al. 2008; Kriehoff-Henning and Hofmann 2008), cell fractionation showed that FLASH was localized exclusively in the nuclear extract (Nuc) in the absence of apoptotic signals (Figure 4.2A). The purity of the cell fractions was assessed by Western blotting of the nuclear marker proteins, poly ADP-ribose polymerase (PARP), the nuclear protein ataxia-telangiectasia locus (NPAT) and histone deacetylase I (HDAC1) and the cytoplasmic marker β-tubulin. There was little or no cross contamination of nuclear and cytosolic fractions.
Figure 4.2. FLASH was found in the nucleus co-localized with NPAT. (A) HT1080 cells (5 X 10^6) were fractionated into cytoplasmic (Cyt) and nuclear (Nuc) fractions (Materials and Methods). The fractions were analyzed by immunoblotting using antibodies directed against FLASH, NPAT, PARP, HDAC1 and β-tubulin. (B) Immunofluorescence co-localization (Materials and Methods) of FLASH and PML or NPAT. HT1080 cells were fixed with cold methanol for 10 minutes, blocked, and incubated with rabbit anti-FLASH and mouse anti-PML antibodies or mouse anti-NPAT antibodies at 4°C overnight. Cells were then washed 3 times and incubated at room temperature for 1 hour with a 1/2000 dilution of the secondary antibodies, Alexa Fluor 594–conjugated anti-rabbit IgG (red) and an Alexa Fluor 488–conjugated anti-mouse IgG antibody (green). The cells were also stained with Hoechst 33342 (blue).
Within the nucleus, FLASH has variously been reported to be associated with Cajal bodies (Barcaroli, Dinsdale et al. 2006), PML bodies (Promyelocytic leukemia nuclear bodies) (Milovic-Holm, Krieghoff et al. 2007; Krieghoff-Henning and Hofmann 2008) and in histone gene clusters (Barcaroli, Bongiorno-Borbone et al. 2006). In HT1080 cells, immunofluorescence microscopy showed (Figure 4.2B) that FLASH was concentrated in a relatively small number of discrete foci within the nucleus. In agreement with the cell fractionation results, no FLASH could be detected in the cytoplasmic compartment. When the cells were co-stained with antibodies directed against PML, there was little colocalization of FLASH and PML. In contrast, staining the cells with antibodies directed against, NPAT, a major component of the histone cluster loci, there was 100% overlap. Each of the 2-4 NPAT histone gene loci also contained FLASH, although there were additional non-overlapping FLASH foci present in other nuclear bodies.

**siRNA silencing of FLASH gene expression**

Three oligonucleotides complimentary to different regions of the FLASH mRNA were used to silence the expression of FLASH. All three reduced the intracellular concentration of FLASH by at least 90% as determined by Western blotting of whole cell extracts (Figure 4.3A). Cells subjected to RNA interference with each siRNA exhibited the same phenotype with no detectable off target effects. Immunofluorescence microscopy (Figure 4.3B) showed that a comparable percentage of the cells lacked FLASH. In contrast, there was no effect on the number or distribution of nuclear PML bodies, as detected with anti-PML antibodies, when the expression of FLASH was suppressed.
Figure 4.3. siRNA silencing of FLASH expression. (A) HT1080 cells were transfected with FLASH siRNA and scrambled siRNA (Control) (Materials and Methods). After 72 hours, the extracts of the transfected cells were analyzed by immunoblotting using FLASH antibodies and as a loading control, β-tubulin antibodies. (B) HT1080 cells were transfected with either a scrambled siRNA (left, Control) or a specific siRNA directed against FLASH (right). The cells were fixed with cold methanol for 10 minutes after 72 hours transfection, blocked, and incubated with rabbit anti-FLASH and mouse anti-PML at 4 °C overnight. After washing three times, the cells were incubated with the secondary antibodies as described in the legend to Figure 3. The cell nucleus was stained with Hoechst 33342. (C) Flow cytometry analysis showed that
FLASH knockdown cells were blocked in S phase. HT1080 cells were transfected with siRNA against FLASH or scrambled RNAi for 72 hours. The cells were trypsinized, washed with cold PBS, fixed with 70% ethanol, treated with RNase A and stained with 50 µg/ml propidium iodide. The DNA content was analyzed using a Becton-Dickinson FACScan cytofluorometer.

**FLASH is necessary for cell cycle progression in HT1080 cells.**

As reported previously (Barcaroli, Bongiorno-Borbone et al. 2006) in other cell lines, depletion of FLASH also caused cell cycle arrest in S phase (Figure 4.3C) in HT1080 cells, presumably due to the reduction of histone gene expression. In cells transfected with the scrambled siRNA control for 72 hours, 69% were found in G0-G1 and 16% in S phase. In contrast, 10% are in G0-G1 phase and 60% of the cells are in S phase in cells transfected with FLASH siRNA. Thus, the cells can progress through the G1/S check point, but cannot exit S phase. Similarly, FLASH knockdown resulted in S-phase arrest in HeLa, MCF10A and MCF-7 cells (data not shown).

**Silencing FLASH gene expression accelerates the onset of apoptosis.**

RNA interference of FLASH expression did not induce apoptosis in HT1080 cells in the absence of apoptotic signals at all times tested up to 72 hours. However, apoptosis in the FLASH depleted cells proceeded much more rapidly when the Fas receptor was activated as compared to the cells transfected with control siRNA. A time course over six hours following stimulation of the Fas receptor (Figure 4.4A) clearly showed that both caspase 8 and the executioner caspase, caspase 3, are activated by proteolytic cleavage much more rapidly than the control cells. Similar results were obtained for caspase 8 when the receptor was activated by the recombinant human Fas
ligand (Figure 4.4B). Quantification the bands on the gel (Figure 4.4B) indicated that after three hours, there was an approximately eleven fold increase in the activation of caspase 8 in cells depleted of FLASH. After six hours, there was still an approximately three fold higher caspase-8 activity when FLASH was knocked down. There appeared to be a slight depletion of coilin six hours post stimulation of the receptor when FLASH expression was suppressed, an observation that may suggest that a fraction of the FLASH may be associated with coilin containing Cajal bodies. The significantly more rapid degradation of the anti-apoptotic protein, poly ADP-ribose polymerase (PARP), a hallmark of the early stages of apoptosis, in cells lacking FLASH was a further indication that FLASH depleted cells are more sensitive to Fas mediated apoptosis (Figure 4.4C). Immunofluorescence micrographs of cells stained with antibodies directed against cleaved caspase-3 (Figure 4.4D) were taken 6 hours following stimulation of HT1080 cells with Fas antibodies. The micrographs showed that caspase-3 activation had progressed more rapidly in cells depleted of FLASH. After 16 hours, most of the cells lacking FLASH had either died or were in advanced stages of apoptosis (Figure 4.4E). Similar results were obtained for MCF-10A cells (data not shown).
Figure 4.4 Effect of FLASH knockdown on apoptotic progression.

(A) HT1080 cells transfected with control siRNA or with siRNA directed against FLASH were stimulated with mouse anti-Fas antibody (1 µg/ml) following the standard protocol (Materials and Methods) for the indicated times. The cell lysates were subjected to
western blotting using anti-FLASH, anti-caspase 8, anti-cleaved caspase 3 and as a loading control, anti-β-tubulin antibodies. (B) HT1080 cells were transfected with two different FLASH siRNAs (FLASH-1 and FLASH-2) and the scrambled siRNA (Control) for 48 hours and then treated with 100 ng/ml FasL for the indicated times. The cell lysates were subjected to immunoblotting using FLASH, caspase 8, coilin and Fas antibodies. The developed blot was scanned to determine the relative levels of active caspase-8 shown in the bar graph. (C) A time course showing the progression of apoptosis by immunoblotting of PARP and PARP cleavage products in control and FLASH knockdown cells following the procedure outlined in panel B. (D) Immunofluorescence assay of caspase-3 activation (Materials and Methods) in HT1080 cells transfected with FLASH or control siRNA for 48 hours with additional 6 hours treatment with 100 ng/ml FasL. (E) Light micrographs of HT1080 cells transfected with FLASH and control siRNA for 72 hours and then stimulated with FasL for 16 hours.
The Intracellular level of FLASH decreases during apoptosis

The proteasome inhibitor MG132, significantly augments the rate at which many cell types progress through apoptosis perhaps as a consequence of blocking the proteasomal degradation of pro-apoptotic proteins (Adams 2004). The accelerated cleavage of caspase-8 indicated that MG132 had the same effect on HT1080 cells (Figure 4.5A). As expected, the intracellular level of P21, a protein which turns over very rapidly, significantly increased confirming that proteasomal degradation had been inhibited.

The intracellular level of FLASH, like the anti-apoptotic protein, PARP, rapidly decreased during apoptosis in cells stimulated by FasL and MG132 (Figure 4.5B). The extensive cleavage of pro-caspase-3 served as a marker of apoptosis. However, the degradation of FLASH was completely arrested by the potent caspases 3, 8 and 10 inhibitors (Figure 4.5B), indicating that FLASH, like PARP, was degraded by caspases once apoptosis was underway. Similar results were obtained when apoptosis was initiated with staurosporine (Figure 4.5C). The time course following exposure to staurosporine showed that FLASH and PARP, as well as the Cajal body component, coilin, rapidly disappeared from the cell extract as apoptosis progressed. In this experiment, IKK and β-actin served as negative controls. FLASH was also degraded during apoptosis resulting from exposure to UV light (Figure 4.5D). DNA damage was confirmed by immunoblotting of phospho-p95/NBS1, a protein that is part of a complex that is phosphorylated by ATM in response to DNA breaks (Lim, Kim et al. 2000).

FLASH mRNA (Figure 4.5F) was found to be relatively stable with a half-life of approximately 6 hours as indicated by exposing the cells to the transcriptional inhibitor,
actinomycin D. In contrast, exposure of the cells to cycloheximide, an inhibitor of protein synthesis (Figure 4.5E) suggests that the FLASH protein turns over rapidly (half-life 2-3 hours) raising the possibility that down-regulation of FLASH levels could be due to proteasomal degradation. However, since in the presence of MG132, FLASH degradation was blocked by the specific caspase inhibitors (Figure 4.5B), it is more likely that FLASH is degraded by caspases during apoptosis.
Figure 4.5 The Intracellular level of FLASH decreases during apoptosis.

(A) The proteasome inhibitor MG132 potentiates caspase 8 activation induced by FasL in HT1080 cells. HT1080 cells were treated with the indicated concentration of FasL with or without 10 µM MG132 for 4 hours. The activation of caspase-8 was monitored by immunoblotting of the total cell lysates using caspase 8 antibodies. β-tubulin served as a loading control and p21, a protein with a short half-life, was a control showing that MG132 effectively blocks proteasomal activity. (B) FLASH was down-regulated following induction of apoptosis. HT1080 cells were either pretreated with the vehicle (DMSO) or caspase 3, 8 and 10 inhibitors for 30 minutes and then induced into apoptosis by exposure to 100 ng/ml FasL and 10 µM MG132 for 4 hours. The relative intracellular levels of FLASH, PARP, intact and cleaved, and caspase-3 were determined by immunoblotting. β-tubulin served as a loading control. (C) FLASH was also downregulated following induction of apoptosis in HeLa cells by exposure to 1 µM staurosporine for the indicated times. The cell lysates were analyzed by immunoblotting of FLASH, caspase-9, IKK, coilin and β-actin. (D) Apoptosis was induced by exposure to UV light (Materials and Methods). The cells were harvested 12 hours and 24 hours following a 5 minute UV exposure. The relative levels of FLASH, phospho-p95/NBS, an indicator of DNA damage, P21 and β-tubulin were determined by immunoblotting. (E) Protein synthesis was blocked by incubating HT1080 cells with 50 µg/ml cycloheximide (CHX) for the indicated times and the relative level of FLASH, coilin, β-actin and p53 was determined by immunoblotting. (F) The relative levels of the same proteins as in panel (E) were determined by immunoblotting following inhibition of RNA transcription by exposure of HT1080 cells to 1 µg/ml actinomycin D for the indicated times.
Anti-apoptotic proteins were down regulated when FLASH was depleted.

The effect of silencing FLASH gene expression on several proteins implicated in apoptosis was assessed in HT1080 and MCF-10A cells. Both coilin siRNA and scrambled siRNA served as controls and three different FLASH siRNAs were tested. None of the proteins tested were affected by coilin siRNA or scrambled siRNA. Histone H3 levels were clearly reduced in FLASH depleted cells consistent with the result showing that FLASH knockdown causes cell cycle arrest (Figure 4.6A) by down regulating the synthesis of histones (Barcaroli, Bongiorno-Borbone et al. 2006). FLASH knockdown significantly reduced the intracellular levels of two anti-apoptotic proteins, MCL1 and the short isoform of c-FLIP in both HT1080 (Figure 4.6A) and MCF10A (Figure 4.6B) cells. No significant differences were found for two other anti-apoptotic proteins, the long isoform of c-FLIP or BCL-2 in either HT1080 or MCF-10A cells.
Figure 4.6 Effect of FLASH knockdown on the level of anti-apoptotic proteins.

(A) HT1080 cells were transfected with 3 different FLASH siRNAs for 72 hours. Coilin and the scrambled siRNA served as controls. The intracellular level of FLASH, coilin, MCL-1, histone H3 and the long and short isoforms of cFLIP, cFLIP (L) and cFLIP (S), respectively, were determined by immunoblotting using the corresponding antibodies. β-tubulin served as a loading control. (B) Following the same protocol, MCF-10A cells were transfected with siRNA directed against FLASH or with control siRNA. Cell extracts were prepared 72 hours following transfection and the cell lysates were subjected to immunoblotting using antibodies directed against the indicated proteins.
**Suppression of apoptosis by FLASH is p53 dependent.**

RNA interference was used to reduce the level of p53 in HT1080 cells by approximately 80%. As expected, p53 knockdown (Figure 4.7A, lane 1) did not induce significant apoptosis judging from the observation that PARP was not degraded. Similarly, depleting the cells of FLASH or both FLASH and p53 (lanes 3 and 4) did not result in apoptosis. However, FLASH knock down cells with wild type 53 rapidly progressed through apoptosis following stimulation with the Fas ligand (lane 5). In contrast, stimulation of cells in which both FLASH and p53 were knocked down did not undergo apoptosis (lane 6), suggesting that a functional p53 is essential.

To confirm the involvement of p53 in promoting apoptosis upon FLASH knockdown, the expression of the anti-apoptotic protein MCL-1 was assessed in mutant HT1080 cells, 6TG-p53, which over express inactive p53. As shown previously (Yun, Lee et al. 2000), exposure of the cells to adriamycin, which produces double stranded breaks in DNA, results in up-regulation of p53 and as a result the up-regulation of its target, P21 (Figure 4.7B). These results confirm that although 6TG-p53 cells overproduce p53, the protein lacks transcriptional activity.

Stimulation of the Fas receptor in FLASH depleted wild type cells with a functional p53 gene resulted in down-regulation of MCL-1 (Figure 4.6 and 4.7B). However, in the 6TG-p53 cells, transfection with FLASH siRNA did not alter the intracellular level of MCL-1. Collectively, these results indicate that the suppression of apoptosis by FLASH is dependent on transcriptionally active, p53.
A

| siControl  | - | + | - | - | - | - |
| siP53      | + | - | - | + | - | + |
| siFLASH    | - | - | + | + | + | + |
| FasL       | - | - | - | - | + | + |

P53
PARP
cleaved PARP
β-actin

B

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P53
P21
γ-H2A.X
β-actin

C

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**Figure 4.7 The effect of FLASH knockdown on apoptosis was dependent on p53.**

(A) HT1080 cells were transfected with scrambled siRNA (siControl), FLASH siRNA (siFLASH) and p53 siRNA (siP53) or co-transfected with both FLASH siRNA and p53 siRNA for 48 hours. Apoptosis was then induced by incubation with 100 ng/ml FasL for an additional 4 hours. Immunoblotting using p53 antibodies showed that p53 was effectively knocked down with siP53 in the presence and absence of siFLASH. Upon stimulation with the FasL, the increase in apoptosis in cells lacking FLASH was abolished in cells depleted of both FLASH and p53. (B) The effect of DNA damage incurred by exposure to adriamycin on the relative intracellular level of p53 and p21. Two isogenic cell lines, HT1080 (wildtype p53) and HT1080-6TG (p53 mutant), were treated with 200 ng/ml adriamycin for the indicated times. The intracellular level of FLASH, p53 and p21 was determined by immunoblotting. The level of p-Histone H2A.X (Ser139) was used to monitor the progressive DNA damage induced by adriamycin treatment. β-actin served as a loading control. (C) The wild type HT1080 and HT1080-6TG cells (p53 mutant) were transfected, as in panel B, with siRNA against FLASH and the scrambled siRNA (Con) for 72 hours. The intracellular levels of FLASH and MCL-1 were determined by immunoblotting. β-tubulin served as a loading control.
DISCUSSION

In accord with previous studies (Bongiorno-Borbone, De Cola et al. 2008), we find that FLASH is a nuclear protein localized within nuclear bodies, primarily but not exclusively within histone gene clusters, where it co-localizes with NPAT. A fraction of FLASH was associated with other nuclear bodies although colocalization with PLM bodies appeared to be minimal. We also found that depletion of FLASH lead to a decrease in histone H3 in the cell and arrest in the S phase of the cell cycle.

An unanticipated result of this study was that FLASH was found to be anti-apoptotic, whereas previous work indicated that it promotes apoptosis. Imai et al (Imai, Kimura et al. 1999) observed that over expression of FLASH resulted in an approximately 25% increase in apoptosis as judged by the altered morphology of the cells. It may be significant that our studies were conducted with the Type II cells in which activation of the mitochondrial pathway is paramount (Scaffidi, Fulda et al. 1998).

Similarly, Milovic-Holm et al. (Milovic-Holm, Krieghoff et al. 2007) found that FLASH was pro-apoptotic although the mechanism proposed was quite different than that suggested by Imai et al. (Imai, Kimura et al. 1999). They found that depletion of FLASH by siRNA interference followed by induction of the Fas receptor with anti-Fas antibody for 7 hours resulted in an approximately 57% decrease in apoptosis. They proposed that activation of the receptor resulted in translocation of FLASH from nuclear bodies to the mitochondria where it activates caspase-8. These authors also conducted their studies with HT1080 cells, the same cells we used in this study. However, there may be differences in strain, P53 status, growth conditions, and methods of induction or antibody titer that could account for the differences in the results. In assessing the
effect of FLASH knockdown on apoptosis, these authors (Milovic-Holm, Kriehoff et al. 2007) induced with the anti-Fas antibody but with far lower concentrations than we employed in our studies (0.025 µg/ml versus 1 µg/ml). Although differences in antibody titer cannot be ruled out, it is perhaps significant that these authors found that the suppression of apoptosis resulting from FLASH depletion was significantly impaired at higher concentrations of the Fas antibody.

The evidence presented here that FLASH, can also suppress apoptosis is compelling. The acceleration of FasL induced apoptosis by FLASH depletion was observed with three different siRNAs that targeted different regions of the FLASH mRNA. Transfection with FLASH siRNA did not induce apoptosis unless the Fas receptor was stimulated but growth was arrested in S phase. However, when FLASH was depleted, activation of the Fas receptor resulted in 1) more rapid activation of caspase 8 and caspase 3, 2) rapid degradation of PARP, 3) nuclear disintegration and DNA fragmentation and 4) the characteristic morphological changes of the cell. Moreover, like most anti-apoptotic proteins, FLASH was rapidly degraded once apoptosis has been irreversibly initiated. The current study is not the only report indicating that FLASH suppresses apoptosis. A siRNA screen identified 37 proteins essential for cell division (Kittler, Putz et al. 2004). FLASH is one of six proteins that when knocked down results in rapid cell death upon entry into mitosis.

There is precedence for apoptotic proteins playing a dual role. For example, DAXX, a nuclear protein that, like FLASH, is involved in both apoptosis and repression of gene expression has been variously reported to be both pro- and anti-apoptotic. Over expression of Daxx promotes Fas induced apoptosis by direct interaction with the
Fas receptor (Yang, Khosravi-Far et al. 1997) or via a nuclear pathway (Zhong, Salomoni et al. 2000) suggesting that it is pro-apoptotic. The opposite conclusion was drawn from studies of Daxx-knockout embryos and embryonic stem cell lines (Michaelson, Bader et al. 1999) and by siRNA suppression of Daxx expression (Chen and Chen 2003; Michaelson and Leder 2003) These latter studies showed that depletion of Daxx resulted in an increased sensitivity to Fas mediated or stress induced apoptosis, suggesting an anti-apoptotic function. Whether Daxx promotes or suppresses apoptosis may be dependent on its modification by other signaling pathways. It was recently shown (Chang, Naik et al. 2011) that phosphorylation of Daxx by CK2 kinase promotes the binding of SUMO-1 and stress-induced apoptosis by down-regulation of anti-apoptotic regulatory proteins.

The involvement of FLASH in the activation of caspase-8 at the DISC (Imai, Kimura et al. 1999) and at the mitochondria (Milovic-Holm, Krieghoff et al. 2007) is pro-apoptotic, but there are other functions ascribed to FLASH that would be expected to protect against entry into apoptosis.

Stimulation of TNF-α receptor elicits two opposing effects, apoptosis and activation of the anti-apoptotic transcription factor, NFkB (Dixit and Mak 2002). Suppression of FLASH expression has been shown to abolish TNF-α induced activation of NFkB via a TRAF2 dependent pathway in HEK293 cells (Jun, Chung et al. 2005), an effect that would be expected to stimulate apoptosis. While the relationship between FLASH and Fas signaling has not been investigated, it has been shown that the stimulation of the Fas receptor also activates NFkB in human bladder carcinoma T24 and Jurkat cells (Ponton, Clement et al. 1996) and in SK-Hep1 hepatocellular carcinoma cells (Okano,
Shiraki et al. 2003), so it is likely that FLASH has a comparable role in the FAS receptor signaling that would lead to suppression of apoptosis.

Another FLASH function consistent with an anti-apoptotic role is that it serves as an important coactivator of cMyb (Alm-Kristiansen, Saether et al. 2008) (Alm-Kristiansen, Lorenzo et al. 2011) a transcription factor generally considered to promote growth and survival. c-Myb is a major target of glycogen synthase kinase 3β (GSK3β). Inhibition of GSK3β results in ubiquitin mediated degradation of cMyb and the induction of apoptosis by inhibiting the expression of BCL2 and survivin (Zhou, Zhang et al. 2011). Survivin, a caspase inhibitor (Tamm, Wang et al. 1998), may not be relevant since it is expressed primarily during G2 and FLASH knockdown arrest cell cycle progression in S phase (Altieri 2003). BCL-2 is a potent inhibitor of apoptosis that blocks the function of the death inducing protein Bak at the mitochondrial membrane. However, we did not find a significant decrease in the BCL-2 levels when FLASH was depleted suggesting that it also does not play a role in the suppression of apoptosis by FLASH.

In agreement with Barcaroli et al (Barcaroli, Bongiorno-Borbone et al. 2006), we found that FLASH colocalizes with NPAT in histone gene clusters. Moreover, the intracellular level of histone H3 is dramatically reduced upon FLASH depletion and the cells accumulate in S phase. Normally, cell cycle arrest in S-phase is not sufficient to induce apoptosis. However, the failure to assemble functional nucleosomes to protect the newly synthesized DNA may accelerate the onset of apoptosis when the cells are stimulated by FasL.
Suppression of apoptosis by FLASH was found to be dependent on the transcription factor, p53. In cells depleted of endogenous p53 by siRNA interference, knock down of FLASH had an appreciably smaller stimulatory effect on apoptosis when the Fas receptor was activated (Figure 4.5B). Similarly, in HT1080 cells harboring a mutant p53, FLASH knockdown did not significantly enhance the apoptotic response. P53 is known to regulate the expression of several pro- and anti-apoptotic proteins (Pietrzak and Puzianowska-Kuznicka 2008). Previous studies suggested that P53 down-regulates the expression of the anti-apoptotic protein, MCL-1 up to 30-fold (Pietrzak and Puzianowska-Kuznicka 2008). In this study, depletion of FLASH resulted in significant reduction in the level of MCL-1 in the cell. The lower concentration of MCL-1 would be expected to relieve its inhibitory effect on the formation of the mitochondrial channel and promote apoptosis. The precise role that FLASH may play in down-regulation of MCL-1 remains to be determined.

The other anti-apoptotic protein that we found down regulated by FLASH depletion is the short isoform of c-FLIP (cellular FLICE inhibitory protein) which acts directly at the Fas death-inducing DISC complex inhibiting caspase-8 activation and Fas receptor mediated apoptosis (Short 2010). In hepatocellular carcinoma cells, c-FLIP is the major regulator of cell death and survival. In addition to inhibiting caspase activation, c-FLIP is a potent inhibitor of apoptosis by inhibiting the activation of NFκB (Kavuri, Geserick et al. 2011). The function of the long isoform c-FLIP has been controversial, but recent studies indicate that it can be either pro- or anti-apoptotic depending on the circumstances (Fricker, Beaudouin et al. 2010; Short 2010). A plausible hypothesis is
that the association of FLASH with the DISC complex may modulate the activity of c-FLIP.

While there are several functional connections between FLASH and various key factors in the apoptotic pathways, the mechanism by which FLASH suppresses apoptosis remains to be deciphered. Of particular interest, is the identification of the switch that determines whether FLASH functions to promote or suppress apoptosis. Very little is known regarding the regulation of FLASH function, however, FLASH has been shown to interact with the SUMO-conjugation enzyme, Ubc9 (Alm-Kristiansen, Norman et al. 2009). Sumoylation attenuates the transcriptional activity of FLASH as measured by the Gal4 tethering assay. Given the size and complexity of FLASH, other regulatory mechanisms are likely to be discovered.

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**Abbreviations** – Ad, adriamycin; CHX, cycloheximide; CASP8AP2, caspase 8 associated protein 2 (FLASH); γ-H2A.X, phosphorylated histone 2A (Ser139) encoded by one of the H2A genes; HDAC1, histone deacetylase 1; NPAT, nuclear protein, ataxia-telangiectasia locus; PARP, poly (ADP-ribose) polymerase; P-p95/NBS1, phosphorylated form of Nijmegen breakage syndrome protein, an indicator of DNA double stranded breaks; STS, staurosporine; TNFα, tissue necrosis factor alpha;
CHAPTER 5

FAM129B/MINERVA, A NOVEL ADHERENS JUNCTION ASSOCIATED PROTEIN, SUPPRESSES APOPTOSIS IN HELa CELLS

Abstract

A recent proteomics study identified FAM129B or MINERVA as a target of the MAP kinase (Erk1/2) signaling cascade in human melanoma cells. Phosphorylation of the protein was found to promote cell invasion and the dissociation of the protein from the cell-cell junctions. Suppression of apoptosis during metastasis is a prerequisite for the survival and spread of cancer cells. During apoptosis, the adherens junctions are disassembled as the dying cell retracts and new contacts are formed between normal neighboring cells. In this study, we show that FAM129B was cytosolic in exponentially growing HeLa cells but was translocated to the adherens junctions where it colocalized with β-catenin whenever contact between two or more cells was established. Silencing the FAM129B gene expression by specific siRNAs did not induce apoptosis or inhibit the growth of HeLa cells. However, when apoptosis was induced by exposure to TNFα/cycloheximide or other apoptotic signaling molecules, the onset of apoptosis was accelerated 3-4-fold when FAM129B was depleted. Annexin V binding, the inactivation of the DNA repair enzyme, poly ADP-ribose polymerase, and the activation of the caspases occurred more rapidly in the cells lacking FAM129B. The rapid induction of apoptosis in FAM129B knock down cells was reversed by co-transfection with recombinant FAM129B indicating that its effect on apoptosis was specific. As apoptosis proceeded, FAM129B was degraded and disappeared from the plasma membrane. Thus, one crucial facet of the mechanism by which FAM129B promotes cancer cell invasion is likely to be the suppression of apoptosis.
**Introduction**

FAM129B or MINERVA is a member of a small family of proteins that include Niban (FAM129A) and Niban-like protein 2 (FAM129C). The function of these homologous proteins is not well understood. FAM129A is an endoplasmic reticulum stress-induced protein that is up-regulated in renal and thyroid cancer (Majima, Kajino et al. 2000; Adachi, Majima et al. 2004; Matsumoto, Fujii et al. 2006), while FAM129C is a B-cell membrane protein that is overexpressed in chronic lymphocytic leukemia (Boyd, Adam et al. 2003). FAM129B has a predicted molecular mass of 83 kDa and includes two distinctive regions (Fig. 1). The PH or pleckstrin homology domain found near the amino end of the polypeptide of FAM129B is also present in FAM129C, while FAM129A has only a truncated PH domain. Near the carboxyl end of the FAM129B polypeptide chain is a region rich in proline shown in a recent proteomics study to be phosphorylated at six serine residues (Old, Shabb et al. 2009). The phosphorylation of four of these serines is catalyzed by the B-RAF/MKK/ERK (MAP kinase) signaling cascade. In melanoma cells, FAM129B was found dispersed throughout the cytoplasm when the MAP kinase cascade was active. However, exposure to the MKK inhibitor, UO126, which effectively shuts down the cascade, resulted in the migration of FAM129B to the cell membrane. A well established *in vitro* assay for invasion (Smalley, Haass et al. 2006), in which cells are grown in a three dimensional collagen matrix, was used to show that shRNA mediated knockdown of FAM129B had no effect on growth. However, the invasion into the collagen matrix was blocked, suggesting that FAM129B plays a critical role in cancer cell invasion. Mutants in which the serine residues targeted by MAP kinase were replaced with alanine were less invasive, while
transfection of a wild type clone overexpressing FAM129B enhanced the invasiveness of the melanoma cells. The authors concluded that MAP kinase-dependent phosphorylation of FAM129B controls melanoma cell invasion and proposed that the protein be renamed MINERVA (Melanoma IN-VAision by ERK). There are no other published studies of FAM129B thus far.

Apoptosis plays a crucial role in cancer progression and invasion (Lowe and Lin 2000). During metastasis, the cells are subjected to numerous challenges in escaping the site of the primary tumor, traversing the circulatory system and invading the distal cells, that would normally induce apoptosis (Townson, Naumov et al. 2003). As a consequence, metastasis is a very inefficient process because very few metastatic cells survive to colonize other tissues (Mehlen and Puisieux 2006). Thus, the survival of the cancer cell depends on the suppression of apoptosis. Many cancer related genes can disrupt apoptosis. The gene Bcl-2 does not promote cell cycle progression or cell proliferation but instead prevents induction of apoptosis (Thompson 1995). The expression of Bcl-2 has been shown to be associated with a poor prognosis in prostatic cancer, colon cancer, and neuroblastoma (McDonnell, Troncoso et al. 1992; Hague, Moorghen et al. 1994). Moreover, there is a high frequency of apoptosis in tumors that spontaneously regress and in tumors treated with chemotherapeutic agents (Kerr, Winterford et al. 1994; Lowe and Lin 2000). The efficacy of many anticancer agents is related to their ability to promote apoptosis (Schmitt and Lowe 1999). Moreover, drug resistance in melanoma is most likely the result of dysregulation leading to suppression of apoptosis, although other mechanisms may be involved as well (Grossman and Altieri 2001).
Disruption of the adherens cell junctions is an early event in apoptosis (Brancolini, Lazarevic et al. 1997). Adherens junctions are protein complexes at the plasma membrane responsible for establishing cell-cell adhesion (Takeichi 1995; Drees, Pokutta et al. 2005; Gumbiner 2005; Pokutta, Drees et al. 2008; Green, Getsios et al. 2010). The adherens junction complex includes a transmembrane receptor, cadherin and the associated components on the cytosolic face of the membrane, α-catenin, β-catenin and P120 catenin that mediate the interaction of cadherin with the underlying actin cytoskeleton. During apoptosis, the adherens junction proteins are cleaved and the junction is lost. At this stage of apoptosis, the actin cytoskeleton retracts and new junctions are formed between neighboring, robust cells to fill the gap created by the shrinkage of the dying cell (Suzanne and Steller 2009).

This study was undertaken to explore the role of FAM129B/MINERVA in apoptosis.

Material and Methods

Antibodies and Reagents—Antibodies used for this study were rabbit anti-FAM129B (5122), rabbit anti-caspase-3 (9662), rabbit anti-cleaved caspase-3 (9664), rabbit anti-poly ADP-ribose polymerase (PARP), (9542), rabbit anti-caspase-9 antibody (9502), mouse monoclonal anti-cdk6 (3136), mouse anti-caspase-8 (9746) (Cell Signaling, Beverly, MA); rabbit anti-cdk2 (21111), rabbit anti-Akt (21054) (Signalway Antibody, Pearland, TX), mouse monoclonal antibody anti-β-catenin (610154) (BD Transduction Laboratories), mouse monoclonal antibody anti-p53 (sc-126), mouse monoclonal anti-GFP (sc-9996), mouse monoclonal β-tubulin (sc-5274) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The general caspase inhibitor (Z-VAD-FMK) was purchased from R&D (Minneapolis, MN), MG132, Cycloheximide (CHX) was from Sigma and
staurosporine (STS), recombinant human tumor necrosis factor-α (TNF-α) were purchased from Invitrogen (Carlsbad, CA). The compounds UO126 (catalog U120), EGF, wortmannin (W1628) and phorbol 12 myristate 13 acetate (PMA) were from Sigma and bisindolylmaleimide (SC-24004) and the general Akt inhibitor (SC-221226) were from Santa Cruz.

Cell Culture and Induction of Apoptosis – HeLa cells were used in this study because they are a well-established system to study apoptosis. HT1080 cells (a gift of Dr. Avraham Raz, Wayne State University) which can be induced to undergo apoptosis without the addition of CHX were used in some protein degradation studies. Both cell lines were cultured in DMEM containing 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, in 5% CO₂ at 37°C. NIH3T3 cells were cultured in the same media except that 10% bovine calf serum replaced the fetal bovine serum. For the induction of apoptosis, HeLa cells were incubated with 10 ng/ml TNFα and 10 µg/ml CHX for the indicated periods of time at 37°C, 5% CO₂. Alternatively, HeLa cells were induced into apoptosis by incubation with 1 µM STS for the indicated time. HT1080 cells were induced by incubation with 1 µg/ml of mouse monoclonal anti-human CD95 antibody (Invitrogen, AHS9552) with or without 10 µM MG132. The progression through apoptosis was monitored using an Annexin V-FITC/PI staining kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The samples were analyzed using a Becton-Dickinson FACScan cytofluorometer at the Wayne State University, Karmanos Cancer Institute, Flow Cytometry Facility.

FAM129B Knock Down – HeLa cells were grown in 6 well plates to 20-30% confluence. Cells were transfected with siRNA directed against the FAM129B mRNA
and, as a negative control, with a scrambled siRNA, using RNAi Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. The oligonucleotides used for these studies were commercially available: 1) SC-92820-A: CUGUGGGAUGAUCUCAGAU (Santa Cruz Biotechnology, Santa Cruz, CA); 2) FAM129B Stealth RNAi™ siRNA HSS127849 (Invitrogen, Carlsbad, CA) UCACGGACAUAGAACCUGAACGUCAU; 3. SC-92820-B: CAUCAUCCUCCUGAUAA (Santa Cruz Biotechnology, Santa Cruz, CA); 4) FAM129B Stealth RNAi™ siRNA HSS185602 (Invitrogen, Carlsbad, CA) CAGUAUGGCGUGGCUCUCAACA. To assess the extent to which the expression of FAM129B was suppressed, cell extracts were isolated at various times following transfection and the cell lysate was analyzed by Western blotting. Equivalent amounts of total protein were analyzed as determined by the Lowry method using BSA as a standard. Immunoblotting of β-tubulin was used to verify that equal amounts of total protein had been loaded on the gel.

Figure 5.1. FAM129B mRNA and polypeptide. FAM129B, an 83 kDa polypeptide (residues 1 – 746), has a PH domain near the amino end (residues 69 – 192) and a
proline rich region (residues 628 – 730) near the carboxyl end. Four Erk1/2 phosphorylation sites have been identified (5) corresponding to Ser628, Ser633, Ser679 and Ser683. The diagram also shows the FAM129B mRNA and the location where four siRNAs bind (see Experimental Procedures). All four oligonucleotides effectively silenced FAM129B expression, but oligonucleotide HSS185602 exhibited some off target effects and was not used in these studies.

**Western blot Analysis** - Whole-cell extracts were prepared in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, 1% Triton X-100, 10% glycerol, 0.2 mM PMSF supplemented with a 1X cocktail of phosphatase and protease inhibitors (Sigma). Protein samples were heated at 95°C for 10 min and analyzed by SDS-PAGE on a 4-12% gradient gel. Western blots were developed using the Western Lighting Plus-ECL reagent (NEL104001EA, Perkin Elmer, Waltham, MA). The resulting immunoblots were scanned with a HP Scanjet 4c and the software UNSCAN-IT (Silk Scientific) was used to quantify the signal intensities.

**Cell Fractionation** - Cytoplasmic and nuclear fractions were prepared using the Qproteome Nuclear Protein Kit (Qiagen) according to the manufacturer's protocol. The purity of the fractions was assessed by Western Blotting of nuclear (PARP) and cytoplasmic (β-tubulin) marker proteins.

**Caspase Assays** - The intracellular levels and activation of caspase-8 and caspase-3 were followed by Western blotting using antibodies specific for the pro-enzymes and activated species. Caspase 3 activity was measured using the EnzChek Caspase-3 Assay Kit II (Molecular Probes, Invitrogen, Carlsbad, CA). Briefly, 50 µL of the
supernatant was added to an individual well of a 96-well microfluorescent plate and incubated with or without 1 µL of Ac-DEVD-CHO inhibitor for 10 min at room temperature. After incubation, 50 µL of the 2X working substrate (5 µM Z-DEVD-R110) were added to each well and further incubated for 30 min. Fluorescence was measured at 485 nm excitation and 538 nm emission using a Germini XS spectrofluorometer (Molecular Devices, Union City, CA). Caspase 3 activity was expressed as arbitrary units of fluorescence.

**Immunofluorescence microscopy** - HeLa cells grown on cover slips in 6-well plates were fixed with 3.7% formaldehyde for 10 min at room temperature and then permeabilized with 0.2% Triton X-100 in PBS for 15 min at room temperature. The permeabilized cells were blocked using 3% bovine serum albumin in PBS for 1 h at room temperature. Cells were incubated with the primary antibody, rabbit anti-FAM129B and/or mouse anti-β-catenin monoclonal antibody, overnight at 4°C. The cells were then incubated with chicken anti-rabbit IgG antibody conjugated with Alexa Fluor 594 (1:2,000) and a chicken anti-mouse IgG antibody conjugated with Alexa Fluor 488 (1:2,000) (Molecular Probes, Invitrogen, Carlsbad, CA) as secondary antibodies for 1 h at room temperature. After extensively washing with PBS, the cells were counterstained with Hoechst 33342, mounted and visualized using a Leica TCS SP5 Laser Scanning Confocal Microscope (Wayne State University Core Imaging Facility). The images were analyzed using the Leica LAS AF Imaging software.

**Cloning of FAM129B** - The complete coding region of human FAM129B was amplified from the pOBT7 clone (Invitrogen, MGC clone 5456246). This cDNA clone included two introns that were excised using PCR based deletion. The PCR product was then
amplified using primers that included an Xho1 site: 5’CAC CCT CGA GGG GGA CGT GCT GTC CAC GCA CCT GGA CG3’ and a Kpn1 site: 5’GGC GGT ACC CTA GAA CTC AGT CTG CAC CCC TGC ACT G3’. The digested product was then ligated into the Xho1 and Kpn1 sites of the pEGFP-C3 vector (Clontech) to generate the recombinant FAM129B expression plasmid with GFP-fused to the amino end. The fidelity of the construct was verified by nucleotide sequencing. Cotransfection of siRNA and the FAM129B construct was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

**RESULTS**

Since cancer progression and invasion depend on the suppression of apoptosis, the potential role of FAM129B in programmed cell death was investigated.

Intracellular localization of FAM129B - HeLa cells were fractionated into nuclear and cytoplasmic fractions and analyzed by immunoblotting (Figure. 5.2). In exponentially growing HeLa cells, FAM129B was localized exclusively in the cytoplasmic compartment (Figure. 5.2A) along with the cytoplasmic marker protein, β-tubulin. FAM129B was not detected in the nuclear fraction, although the nuclear marker PARP was found only in this fraction. Similar results were observed for the mouse embryonic fibroblast cell line NIH3T3 (Figure.5.2B).

These results were confirmed by immunofluorescence microscopy. FAM129B was found to be dispersed throughout the cytoplasm and was completely excluded from the nucleus in exponentially growing HeLa cells where there was little cell-cell contact.
(Figure.5.2C). In confluent HeLa cell cultures, FAM129B migrated to the plasma membrane and appeared to be localized at the cell junctions (Figure.5.2D). Interestingly, localization at the cell membrane was observed during telophase (Figure.5.2E). The localization of FAM129B at the cell-cell junction was confirmed by fluorescence microscopy colocalization of FAM129B and β-catenin, a protein that is part of the adherens junction, the complex present at the cell-cell contacts (Nelson and Nusse 2004). The yellow pixels concentrated at the cell membrane in the merged image (Figure.5.2F) indicate that FAM129B (red fluorescence) and β-catenin (green fluorescence) partially colocalize.
Figure 5.2  Intracellular localization of FAM129B in exponential and confluent cell cultures.  Panel A:  HeLa cells (5 X 10^6) were harvested during the late exponential growth phase and the cell extracts were fractionated into cytoplasmic (Cyt) and nuclear (Nuc) fractions (Experimental Procedures).  The fractions were analyzed by immunoblotting using antibodies directed against FAM129B, PARP, p53, CDK6 and CDK2.  Panel B:  The same procedure was used to analyze NIH3T3 cell fractions.  Panel C:  Immunofluorescence microscopy of exponentially growing HeLa cells stained with Hoechst 33342 DNA stain and with antibodies directed against rabbit FAM129B and the secondary antibody, chicken anti-rabbit IgG Alexa Fluor 594.  The same protocol was followed for the cells in Panel D:  confluent HeLa cells and Panel E:  an exponentially growing HeLa culture showing some mitotic cells.  Panel F:  immunofluorescence co-localization (Experimental Procedures) of FAM129B and β-catenin.  The confluent cells were co-stained with rabbit antibodies directed against FAM129B and mouse antibodies directed against β-catenin.  The secondary antibodies were Alexa Fluor 594–conjugated anti-rabbit IgG and an Alexa Fluor 488–conjugated anti-mouse IgG antibody.  The cells were also stained with Hoechst 33342.
Several inhibitors and activators of signaling molecules were tested in an attempt to alter the intracellular location of FAM129B. UO126 (10 µM), EGF (100 ng/ml), the Erk1/2 pathway inhibitor and activator, respectively, a general Akt inhibitor (10 µM), the PKC inhibitor, bisindolylmaleimide (4 µM), the PKC activator phorbol 12 myristate 13 acetate (PMA, 100 nM) and the PI 3-kinase inhibitor, wortmannin (1 µM). Cells were incubated for 4 h with each of these compounds, but none promoted either the membrane association or dissociation of FAM129B.

**Knockdown of FAM129B in HeLa cells** - The expression of the FAM129B gene was silenced in HeLa cells using siRNA technology. Four oligonucleotides corresponding to several coding and non-coding regions of the human FAM129B gene (Figure. 5.1) were tested. All four oligonucleotides effectively knocked down FAM129B gene expression, whereas no effect was observed with control oligonucleotides with a scrambled sequence (Figure. 5.3). Oligonucleotide SC-92820-A, targeted to the 3’ non-coding sequence, which suppressed the expression of the gene by greater than 95% within 72 h, was selected for these studies (Figure. 5.3A, 5.3C). Similarly, immunofluorescence microscopy confirmed that FAM129B was depleted from the transfected cells (Figure. 5.3B).
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B

Control

FAM129B

Merge

C

Control  siRNA  +  -  +  -  +  -

FAM129B siRNA -  +  -  +  -  +

24h  24h  48h  48h  72h  72h

FAM129B

β-tubulin
Figure 5.3 siRNA silencing of FAM129B expression. Panel A: FAM129B knockdown with two different siRNAs, SC-92820-A (siRNA-1) and HSS127849 (siRNA-2) (see Figure 1 and Experimental Procedures). The controls (Cont) for each siRNA had a similar length and nucleotide composition but the sequence was scrambled. Extracts of the siRNA transfected cells were analyzed by immunoblotting using FAM129B antibodies and as a loading control, β-tubulin antibodies. The same blot was developed for a total of 1 min and for 5 min. Panel B: Indirect immunofluorescence confirmed the specific localization of endogenous FAM129B at the cell junction in confluent HeLa cells. HeLa cells were transfected with either a control scrambled siRNA (left, Control) or a specific siRNA directed against FAM129B (right). Cells were fixed 72 h post-transfection, blocked, and incubated with a 1:100 dilution of rabbit anti-FAM129B antibody at 4 °C overnight. Cells were then washed 3 times and incubated at room temperature for 1 h with a 1/2000 dilution of chicken anti-rabbit IgG antibody coupled with Alexa Fluor 594. The cell nucleus was stained with Hoechst 33342. Panel C: The time course for FAM129B knockdown using siRNA (SC-92820-A). The cells were transfected with (SC-92820-A) or with the control siRNA. β-tubulin served as a loading control.
FAM129B knockdown accelerated the onset of apoptosis - Silencing the expression of FAM129B did not induce apoptosis nor did it significantly affect cell growth. However, when apoptosis was induced by TNFα, a ligand that activates the extrinsic apoptotic pathway, and CHX (Aggarwal 2003), the cells entered apoptosis more rapidly than control cells that were transfected with a scrambled siRNA. The initiation and progression of apoptosis was monitored by an annexin V assay (Figure. 5.4A). Annexin V, in the presence of calcium ions selectively binds to phosphatidylserine, a lipid displayed on the cell surface during the early stages of apoptosis (Aggarwal 2003). FACS analysis (Figure. 5.4BC) indicated that after 4 h of exposure to TNFα/CHX, the percentage of apoptotic cells was 3-fold higher in cells in which FAM129B had been depleted. Immunofluorescence microscopy showed extensive fragmentation of nuclear DNA in cells transfected with FAM129B siRNA after induction by TNFα/CHX (Figure. 5.4D). In contrast, the DNA appeared intact in untransfected cells in the same population.
Figure 5.4 Effect of FAM129B knockdown on apoptotic progression. Panel A: HeLa cells transfected with control siRNA or with siRNA directed against FAM129B were exposed to TNFα/CHX following the standard protocol (*Experimental Procedures*). Fluorescence microscopy of cells stained with Hoechst 33342 and FITC-conjugated Annexin V was carried out immediately after the addition of TNFα/CHX (0 h) and at 4 h post-exposure. The green FITC fluorescence bound to the cell surface provides an indication of the progression through apoptosis. Panel B: an Annexin V apoptosis assay (Bossy-Wetzel and Green 2000) (*Experimental Procedures*) of cells transfected with the scrambled siRNA control (si Control) or with FAM129B siRNA (si FAM129B). Samples were analyzed by FACS at the indicated times after induction of apoptosis by exposure to TNFα/CHX. FACS data is presented in a two dimensional dot display showing the fluorescence intensity of cells with bound Annexin-V (x axis) plotted versus the fluorescence intensity of cells dyed with the non-vital stain, propidium iodide (y axis). The lower right quadrant represents the early apoptotic cells expressed as a percentage of the total cell population, whereas the upper right quadrant represents cells in late apoptosis or necrosis. Panel C: The percentage of apoptotic cells transfected with scrambled siRNA (0) or FAM129B siRNA (●) plotted against the time of exposure to TNFα/CHX. Panel D: HeLa cells in which FAM129B expression was silenced were incubated with TNFα/CHX for 2 h, fixed and then immunostained with anti-FAM129B antibodies (red) and Hoechst 33342 (blue). The field shown in the figure was selected because it gives a comparison between untransfected cells and cells in which FAM129B was silenced. Those cells in which FAM129B had been effectively knocked down as indicated by the lack of red staining exhibited extensive degradation of nuclear DNA
(arrows), whereas the DNA appeared intact in the untransfected cells that still expressed FAM129B.

The effect of FAM129B knockdown was mediated by a caspase-dependent pathway - In the extrinsic pathway, the binding of TNFα to its receptor results initially in the activation of caspase-8. Activation is a consequence of the cleavage of pro-caspase-8 into 41 kDa and 43 kDa proteolytic fragments and subsequently to 18 kDa and 10 kDa fragments that associate to form the heterotetrameric, active caspase-8. In HeLa cells transfected with the control siRNA (Figure. 5.5A), the 41/43 kDa intermediates began to accumulate after 4 h exposure to TNFα/CHX. The active 18 kDa species was not yet detectable. Note that the 10 kDa subunit is not recognized by this antibody. In contrast, significant amounts of the intermediates were visible after 2 h in cells transfected with FAM129B siRNA. After 4 h exposure, significant amounts of the 18 kDa subunit of the active species had accumulated.
Figure 5.5  Activation of caspases upon TNFα/CHX exposure. Apoptosis was induced by TNFα/CHX for the indicated times (0, 2 and 4 h) in HeLa cells in which FAM129B was knocked down by exposure for 72 h with siRNA (SC-92820-A) and in control cultures transfected with scrambled siRNA (control). Panel A. The cell extracts were analyzed by Western blotting using antibodies directed against FAM129B and the mouse monoclonal antibody 1C12 that recognizes full length human pro-caspase-8, the 41 Kd and 43 Kd proteolytic intermediates and the 18 Kd subunit of the activated caspase.
caspase. Panel B. Western blotting of cell extracts using an antibody directed against intact and cleaved PARP and an antibody recognizing the 17 Kd and 12 Kd subunits of activated caspase-3. The cell extracts in Panels A and B were also immunoblotted with β-tubulin antibodies.

The next step in the extrinsic pathway is the activation of the executioner caspase-3, the proteolytic enzyme primarily responsible for degradation of the proteins that occurs during apoptosis (Wajant 2002). Caspase-3, a 35 kDa protein, is cleaved into 17 kDa and 12 kDa fragments that associate forming a heterotetramer. Following exposure to TNFα/CHX for 2 h, HeLa cells transfected with control siRNA, had barely detectable levels of active caspase-3 (Figure. 5.5B). However, caspase-3 was rapidly activated in FAM129B knockdown cells. In agreement with this result, immunofluorescence microscopy (Figure. 5.6A) showed that the extent of caspase-3 activation was much greater following TNFα/CHX induction of FAM129B depleted cells. This result was confirmed by directly assaying the caspase-3 activity in control and knockdown cells (Figure. 5.6B). Caspase-3 activity was 4.4 fold higher in cells in which the FAM129B gene had been silenced.

A major caspase-3 target is PARP, an anti-apoptotic protein involved in DNA repair (Satoh and Lindahl 1992; Simbulan-Rosenthal, Rosenthal et al. 1999). PARP is inactivated during apoptosis by cleavage of the 116 kDa active enzyme rendering it unable to repair the massive DNA damage. Again, PARP cleavage occurred much more rapidly following TNFα/CHX stimulation of FAM129B depleted cells.
Figure 5.6  Caspase-3 activity following FAM129B knockdown. Panel A: HeLa cells were treated with either FAM129B siRNA or with the scrambled siRNA control for 72 h. The cells were then induced to undergo apoptosis by exposure to TNFα (10 ng/ml) and CHX (10 µg/ml) for 2 h. The fixed cells were incubated for 2 h at room temperature with rabbit antibodies recognizing only activated caspase-3. The secondary antibody was Alexa Fluor 594–conjugated anti-rabbit IgG. Panel B: HeLa cells (1x10^6) were either treated with FAM129B siRNA or with the scrambled siRNA control for 72 h.
cells were then induced to undergo apoptosis as described in panel A for the indicated times. The cells were then harvested, lysed and assayed for caspase-3 activity using a synthetic substrate Z-DEVD-R110 (*Experimental Procedures*). The unfilled and grey shaded bars correspond to the control and FAM129B knockdown cells, respectively.
FAM129B was degraded during apoptosis - The intracellular levels of FAM129B decreased as TNFα/CHX induced apoptosis proceeded (Figure. 5.7A). However, the interpretation of this experiment was complicated because CHX inhibits protein synthesis. The inhibitor promotes efficient TNFα induction of apoptosis by blocking the synthesis of anti-apoptotic proteins (Aggarwal 2003). Thus, the intracellular levels of FAM129B were also monitored in HeLa cells induced to apoptosis by STS, which activates the intrinsic apoptotic pathway (Antonsson and Persson 2009). Under the conditions used in these experiments (Figure. 5.7B), STS-induced apoptosis proceeded more rapidly than that observed for TNFα/CHX induction (Figure. 5.7A). The decrease in the intracellular level of FAM129B, β-catenin, PARP and Akt and the extent of proteolytic activation of caspase 8 and caspase 9 were monitored as a function of time following exposure to STS (Figure. 5.7BC). After a lag time of approximately 1 h during which time the caspases began to be activated, PARP disappeared rapidly. The intracellular level of PARP was reduced by 50% after approximately 3 h. The rate of degradation of FAM129B and β-catenin was approximately the same with a half time of 4.1 h. Half the Akt was degraded after 5 h. All four proteins were barely detected 12 h after induction of apoptosis.
Figure 5.7  FAM129B levels as apoptosis progresses. Panel A: HeLa cells (1 X 10⁶) were exposed to TNFα/CHX and the intracellular levels of endogenous FAM129B and PARP were assessed by immunoblotting with the corresponding antibodies at the indicated times. β-tubulin served as a loading control. Panel B: Apoptosis was induced with STS as described in the Experimental Procedures. Cells were harvested at the indicated times and the relative levels of PARP, β-catenin, FAM129B, Akt as well as caspase-8 and caspase-9 and their cleavage products were assessed by immunoblotting. Panel C: The relative levels of the proteins, FAM129B (●), PARP (○), Akt (□) and β-catenin (△) was determined by scanning the Western blot in Panel B and the results were expressed as a percentage of the level at the time apoptosis was induced.
The potent proteasome inhibitor, Mg132 strongly potentiates apoptosis induced by antibodies directed against CD95 (Kim, Kim et al. 2003). In HT1080 cells, increased apoptosis in the presence of the inhibitor (Figure. 5.8) was indicated by significantly higher levels of cleaved PARP and activated caspase-8 and caspase-3 (compare lanes 2 and 4). The degradation of FAM129B was also accelerated by Mg132 indicating that its reduced intracellular level was not a consequence of proteolysis mediated by the proteasome. However, a broad spectrum caspase inhibitor blocked the activation of caspase-8 and caspase-3 and the degradation of both FAM129B and PARP (lane 5).
Figure 5.8 Effect of Mg132 and a general caspase inhibitor on FAM129B degradation. HT1080 cells were incubated in the presence (+) or absence (-) of anti-CD95 and Mg132 for 4 h. The relative intracellular levels of FAM129B and full length and cleaved PARP, caspase 8 and caspase 3 were determined by immunoblotting. β-tubulin served as a loading control. The effect of a general caspase inhibitor (Caspase Inh), Z-VAD-FMK, that targets all caspases, on the level of these proteins was also tested following induction of apoptosis with both anti-CD95 and Mg132.

Recombinant FAM129B reversed the effect of endogenous FAM129B knockdown - The results thus far indicated that FAM129B knockdown accelerates the onset of
apoptosis. To confirm that the result was specifically due to the depletion of intracellular FAM129B, the coding region was cloned and expressed in HeLa cells as a fusion protein with EGFP appended to the amino end of the polypeptide (Experimental Procedures). The full length protein was expressed at moderately high levels in HeLa cells. Immunoblotting (Figure. 5.9) using anti-GFP antibody showed that the recombinant protein had the expected molecular mass of 110 kDa. Fluorescence microscopy (not shown) demonstrated that the intracellular localization of the recombinant protein mimicked the behavior of endogenous FAM129B. The siRNA used to suppress the endogenous FAM129B (Figure. 5.1, SC-92820-A) was targeted to the 3’ untranslated region and therefore cannot silence the expression of the recombinant FAM129B gene. To determine if recombinant FAM129B could rescue the siRNA knockdown effect, HeLa cells were co-transfected with siRNA (SC-92820-A) and EGFP-FAM129B or the EGFP vector for 48 h, followed by TNFα/CHX treatment for an additional 4 h. HeLa cells co-transfected with the EGFP-vector and the siRNA entered apoptosis more rapidly as indicated by increased cleavage of caspase-3 and PARP (Figure. 5.9, lane 3). However, these effects were partially reversed in cells that were co-transfected with EGFP-FAM129B as indicated by the suppression of PARP and caspase-3 cleavage (Figure. 5.9, lane 4).
Figure 5.9 Recombinant FAM129B restores the resistance to apoptosis. HeLa cells were co-transfected with FAM129B siRNA, the EGFP vector or recombinant EGFP-FAM129B for 48 h using Lipofectamine 2000. Apoptosis was then induced by incubation with TNFα/CHX for an additional 4 h. Immunoblotting using EGFP specific antibodies showed that recombinant EGFP-FAM129B (lanes 2 and 4) and as a control, the EGFP protein (EGFP vector) (lanes 1 and 3) were both highly expressed in cells treated with TNFα/CHX and in untreated cells. Expression of the recombinant protein
had no effect on the level of PARP or caspase-3 in the untreated controls (lane 2) compared to cells transfected with only the vector (lane 1). Following induction of apoptosis, the cells transfected with EGFP vector (lane 3) exhibited increased PARP cleavage and caspase-3 activation, whereas these effects were diminished in cells transfected with recombinant FAM129B (lane 4). β-tubulin served as a loading control.
DISCUSSION

MINERVA or FAM129B was recently shown by Old and colleagues (Old, Shabb et al. 2009) to be implicated in cell invasion in human melanoma cells in a process controlled by the Erk1/2 signaling cascade. Cancer progression and metastasis requires the suppression of apoptosis to allow the aberrant cells to survive and proliferate in blood and distal tissues.

Silencing FAM129B gene expression did not induce apoptosis or affect the growth rate of HeLa cells. Rather, FAM129B knockdown accelerated the response to apoptotic signals. The Annexin V assay indicated that apoptosis induced by TNFα/CHX proceeds 3-4 fold more rapidly in the FAM129B knockdown cells. This result is significant because if FAM129B knockdown simply induced apoptosis, it would be difficult to sort out whether FAM129B directly affects the apoptotic pathways as opposed to some other crucial function that leads to apoptosis as a secondary consequence. There are several additional observations that support the notion that FAM129B directly affects apoptosis, 1) knockdown with three different oligonucleotides gave an identical phenotype making off target effects unlikely, 2) FAM129B knockdown accelerated apoptosis induced by three different stimuli, TNFα/CHX and anti-CD95 which activate the extrinsic pathway and STS, an intrinsic pathway activator, as indicated by an increased rate of PARP cleavage and caspase activation (only TNFα/CHX data shown), 3) rescue experiments showing that the elevated rate of apoptosis can be partially reversed in cells in which the endogenous protein has been depleted by transient transfection with recombinant FAM129B.

The adherens junction must be dismantled with the onset of apoptosis (Suzanne
and Steller 2009). The cytoplasmic domain of cadherin is cleaved by caspase 3, while the extracellular domain that links the cells together is cleaved by a metalloprotease that is also activated during apoptosis (Steinhusen, Weiske et al. 2001). In addition, there is a caspase 3 dependent proteolytic cleavage of \( \beta \)-catenin (Brancolini, Lazarevic et al. 1997). Once cleaved, \( \beta \)-catenin and cadherin are no longer linked and the junction is lost. The actin cytoskeleton retracts and the cell shrinks and eventually disintegrates.

In agreement with the studies of Old et al. (Old, Shabb et al. 2009), immunofluorescence microscopy indicated that FAM129B can be localized in the cytosol or associated with the cell junctions. In melanoma cells, FAM129B was found dispersed throughout the cytoplasm when the MAP kinase (Erk1/2) cascade was active. However, exposure to the MKK inhibitor, UO126, which effectively shuts down the cascade, resulted in the migration of FAM129B to the cell junctions. We also found FAM129B to be cytosolic in exponentially growing HeLa cells (Figure. 5.2C). However, as the cells became confluent, FAM129B was exclusively localized to the cell junctions (Figure. 5.2D). As observed previously (Old, Shabb et al. 2009) in melanoma cells, FAM129B at the junctions of HeLa cells co-localized with \( \beta \)-catenin (Figure. 5.2F) indicating that it is near the adherens junctions.

In rapidly growing cells, the MAP kinase activity is generally elevated so that the cytosolic location of FAM129B observed in exponentially growing HeLa cells is in accord with the previous study. However, migration to the cell junctions may be dependent on factors other than MAP kinase phosphorylation in HeLa cells since incubation of exponentially growing cultures with the MKK inhibitor, UO126, did not result in the relocation of FAM129B to the cell membrane (data not shown). Similarly,
the activation of the MAP kinase cascade by EGF did not alter the FAM129B intracellular location. Several other inhibitors and activators of signaling pathways also had no effect on the association of FAM129B with the cell junction. Rather, FAM129B was invariably observed at the cell-cell junctions whenever two cells were in contact in both confluent and pre-confluent HeLa cell cultures.

The intracellular dynamics and distribution of FAM129B appeared to closely parallel that of β-catenin. FAM129B and β-catenin were localized together at cell-cell contacts, both disappeared as the junction disassembles and both were degraded at approximately the same rate. In this sense, FAM129B behaves as a normal component of the adherens junction. The only exception is that FAM129B, unlike β-catenin, which also acts as a transcriptional regulator, was not observed in the nucleus. The Wnt signaling pathway controls the fate of β-catenin once it is released from the adherens junction (Nelson and Nusse 2004). Phosphorylation of β-catenin by glycogen synthase kinase 3 (GSK-3), a key component of the pathway (Cohen and Frame 2001), prevents it from entering the nucleus to activate the expression of Wnt pathway target genes. Instead β-catenin undergoes proteolytic degradation. Akt phosphorylation inhibits GSK-3 (Cohen and Frame 2001), so Akt inhibitors would be expected to increase GSK-3 phosphorylation of β-catenin and promote its degradation. In contrast, the Akt inhibitor, had no effect on FAM129B, although more extensive studies are required to conclusively rule out the role of the Wnt pathway in FAM129B turnover. However, there is no evidence thus far that FAM129B has a role in transcriptional regulation.

We also observed that FAM129B was localized in the plasma cell membrane during mitosis. The localization was not confined to the area of contact between the
nascent daughter cells but rather was dispersed throughout the entire plasma membrane (Figure. 5.2E). This unexpected result suggests that FAM129B may play a role in mitosis or cytokinesis, perhaps in the reorganization of the cytoskeleton.

The signal that triggers the relocation of FAM129B to the cell junction in HeLa cells is unknown. One possibility is that FAM129B has an intrinsic affinity for the adherens junction where it binds once the junction is assembled. Perhaps more likely, there may be specific signals that trigger its relocation. In addition to the four serines that are phosphorylated by MAP kinase, two other phosphoserines have been found in the proline rich region near the carboxyl end of FAM129B (Old, Shabb et al. 2009). Experiments are planned to identify the kinase responsible for these modifications and to determine whether their phosphorylation alters the intracellular localization of FAM129B.

Studies are also underway to determine whether FAM129B is physically associated with cadherin or any of the catenins in order to confirm that it is localized in the adherens junction and to establish whether its location there is related to its antiapoptotic function. One attractive hypothesis is that the FAM129B suppresses apoptosis by stabilizing the junction perhaps by protecting the components of the complex from proteolytic degradation. Experiments are underway to investigate this phenomenon.

FAM129B was degraded during apoptosis without the accumulation of smaller proteolytic fragments. Perhaps the epitope, a short sequence near the carboxyl end of the peptide, was present on a small fragment that was not visible in the blot and that the larger fragments thus eluded detection. Alternatively, following an initial cleavage, the
protein may be rapidly degraded to small fragments. Anti-apoptotic proteins and signaling molecules (Widmann, Gibson et al. 1998) are among the early, specific caspase targets. One of the proteins first cleaved is PARP which abolishes the ability of the enzyme to repair the DNA damage occurring in apoptotic cells (Nicholson and Thornberry 1997). The phosphatidylinositol 3-phosphate-regulated protein kinase, Akt, which has been shown to antagonize apoptosis (Franke, Kaplan et al. 1997), was degraded somewhat more slowly by caspases than PARP in accord with previous reports (Widmann, Gibson et al. 1998). The rate of degradation of FAM129B and β-catenin was intermediate between PARP and Akt. It is likely that FAM129B degradation is also mediated by caspases. Proteolysis by the proteasome can be ruled out since degradation proceeded rapidly in the presence of the proteasome inhibitor, Mg132. FAM129B degradation was blocked by a broad spectrum caspase inhibitor although we cannot be sure whether this effect was due to inhibition of the protease or simply the arrest of the apoptotic program in general.

There is evidence that the cytoplasmic domain of cadherin also suppresses apoptosis (Steinhusen, Weiske et al. 2001). The rapid cleavage of FAM129B and cadherin that occurred during apoptosis may be a mechanism that ensures that the antiapoptotic function of these proteins is eliminated once programmed cell death is initiated.

One crucial facet of the mechanism through which FAM129B promotes cancer cell invasion is likely to be the suppression of apoptosis. The protein has some of the hallmarks of a signaling molecule. There is PH domain, found in many signaling molecules, near the amino end of the FAM129B polypeptide. One of the functions of
the PH domain is to dock proteins to the plasma membrane. This domain is also found in FAM129C, the other family member that binds to membrane surfaces. The proline rich region at the carboxyl terminus is a potential target of SH3 domains, a module found in proteins involved in many signaling cascades. The detailed mechanism through which FAM129B exerts its effects on apoptosis and cell invasion is currently under investigation.

Metastasis is the result of the disruption of the precise balance between proliferation and apoptosis (Hoffman and Liebermann 1994). It has been estimated that up to 90% of metastatic tumor cells are lost by apoptosis (Wyllie, Kerr et al. 1980; Mehlen and Puisieux 2006). Thus, chemotherapeutic approaches that can tip the balance in favor of apoptosis would be expected to be effective in combating proliferative disorders.
CHAPTER 6
MECHANISTIC STUDIES OF FAM129B

RESULTS

We have established that FAM129B suppresses apoptosis and others have found that it increases the invasiveness of melanoma cells. However, there is no information concerning the mechanism of either process. The following experiments were designed to further characterize the protein in cancer cells and to begin to elucidate the mechanism.

Knockdown of FAM129B might inhibit NF-κB activation

It has been suggested that TNF-α alone did not induce apoptosis in most cell types due to the activation of NF-κB, which further activates downstream anti-apoptotic protein expression (Wang, Mayo et al. 1998). Therefore, most cell types require inhibition of gene expression, such as the protein synthesis inhibitor cycloheximide (CHX). Since knockdown of FAM129B significantly sensitizes HeLa cells apoptosis induced by TNF-α, I first wanted to see whether NF-κB pathway was affected by FAM129B knockdown. Interestingly, western blot showed that knockdown of FAM12B using two different siRNA resulted in decreased phosphorylated p65 NF-κB level in Ser536, indicating the inhibition of NF-κB transcription activity. On the other hand, the knockdown of FAM129b did not down-regulate total NF-κB p65 and Bcl-2 anti-apoptotic protein level. However, these data need to be further repeated. Other anti-apoptotic proteins such as MCL-1, cFLIP, also need to be confirmed by western blot.
Figure 6.1 Knockdown of FAM129B might inhibit NF-κB activation

HeLa cells were transfected with two different siRNA against FAM129B for 72 hours. The total cell lysates were subjected to western blot analysis using indicated antibodies. Beta actin served as loading control.

Identification of FAM129B interacting protein, KEAP1

In order to further investigate the mechanism, I tried to find if there were any known proteins involved in the apoptosis pathway interacting with FAM129B. This was done by mass spectrometry analysis of co-immunoprecipitated complex of GFP-FAM129B. Although a few potential candidates were identified, among them, no proteins are reported to be related to apoptosis. However, I found that one protein, called KEAP1 (kelch-like ECH-associated protein 1) is shown to interact with FAM129B in NCBI
Keap1 was first identified (Itoh, Wakabayashi et al. 1999) to be an inhibitor of antioxidant responsive element nrf2 protein in a two-hybrid screen. It is a cysteine-rich protein that acts as a substrate adaptor for E3 ligase Cul3. FAM129B was initially identified in the KEAP1 pull-down complex by mass spectrometry (Sowa, Bennett et al. 2009). I further confirmed the interaction by co-immunoprecipitation. As shown in Figure 6.2B, overexpressed GFP-FAM129B can successfully pull down KEAP1, but not the control GFP vector. KEAP1 was reported to interact with a number of proteins including nrf2, IKKbeta and p62 (Lee, Kuo et al. 2009) (Komatsu, Kurokawa et al. 2010). Interestingly, a conserved motif (ETGE) required to interact with KEAP1 was also found in FAM129B (Figure 6.2A). In order to confirm if FAM129B interacts with KEAP1 through the same motif, I mutated GFP-FAM129B (ETGE) wild type to GFP-FAM129B (ETAA) by site-directed mutagenesis. Further co-immunoprecipitation showed that only wild type GFP-FAM129B could effectively pull down KEAP1 in transfected HEK293 lysate but not in GFP-FAM129B mutant transfected cell lysate. This indicates the ETGE motif in FAM129B is important for its interaction with KEAP1.

**FAM129B expression is correlated with breast cancer invasive ability**

To study FAM129B in cancer cell invasion, western blots were performed to test FAM129B protein expression in a large number of cell lines including the breast cancer cell line, the prostate cancer cell line, HEK293 and the leukemia cell line K562. FAM129B was found highly expressed in epithelial cancer cell lines. The representative western blot indicated the role of FAM129B in metastasis of breast cancer cells. More strikingly, the highest FAM129B expression was detected in invasive MDA231 cell lines.
compared to tumorigenic MCF-7 cell lines. Hence, it is likely that FAM129B could be used as a potential marker for invasive tumor derived from normal epithelial cells.

**Figure 6.2 FAM129B associates with KEAP1**
A) Diagram shows that FAM129B contains conserved motif (ETGE) interacting with KEAP1. B) Co-immunoprecipitation shows that GFP-FAM129B pulls down KEAP1 but not GFP vector. IKKbeta was used as control.
Figure 6.3 FAM129B expression is correlated with breast cancer invasive ability

Breast cancer cell lysates were subjected to western blot analysis using the indicated antibody. Vimentin and p120 were used as markers for invasive cell lines. Src was used as a control.

Knockdown of FAM129B in MDA231 cells reduces cell invasive ability

Since FAM129B is found to be highly expressed in invasive MDA231 cells, I chose this cell line to confirm if FAM129B is also involved in breast cancer invasion. SiRNA against FAM129B was tested in MDA231 cells. The results showed that effective FAM129B knockdown occurred 48 hours after siRNA transfection and maintained in more than 96 hours siRNA posttransfection. Hence, MDA231 cells transfected with scrambled control RNAi and siRNA against FAM129B were trypsinized 48 hours posttransfection and the same amount of cells were seeded into BD Matrigel Invasion
Chambers (354480 BD Biosciences). The cells invading through matrigel were counted 24 hours later according to manufacturer’s protocol. The results showed that there is

Figure 6.4 Knockdown of FAM129B significantly reduces the MDA231 cell invading through the Matrigel

MDA231 cells transfected with scramble control RNAi and siRNA against FAM129B were trypsinized 48 hours posttransfection and the same amount of cells were seeded into BD Matrigel Invasion Chambers (354480 BD Biosciences). 24 hours later, the cells invading through matrigel were counted according to manufacturer’s protocol.
more than 5 fold decrease of FAM129B depleted cells invading through the Matrigel compared to control cells. However, this needs to be further repeated.

**PMA induces FAM129B translocation into plasma membrane in MDA231 cells.**

Previously, immunofluorescence microscopy showed that FAM129B is clearly in all of the mitotic cell plasma membrane in HeLa cells (Chapter 5). I wanted to know if the localization is consistent in different cancer cells. The immunofluorescence showed that in all the cells I have tested, FAM129B localizes in plasma membrane in all mitotic cells (data not shown). MDA231 cells with were treated with various inhibitors and activators of MAPK, AKT and PKC pathway. Phorbol 12-myristate 13-acetate (PMA), a PKC kinase activator, affects a variety of cell processes, including proliferation and differentiation. In most cell types, PMA enhances proliferation by a protein kinase C (PKC) - dependent mechanism. Previous work has shown PMA treatment of MDA231 cells inhibits EGF-induced cell spreading. Of five PKC isoforms identified in MDA231 cell line, PMA treatment only induced PKCalpha translocation from the cytosol to the membrane, which is correlated with the occurrence of the rounded morphology (Gauthier, Torretto et al. 2003). Similarly, when the MDA231 cell line was treated with PKC activator, PMA, there was translocation of FAM129B from the cytoplasm or junctions to the plasma membrane. This suggests that plasma membrane association of the FAM129B might be regulated by PKC kinase. This phenomenon did not occur in MDA231 cells treated with U0126 (Figure 6.5)
Figure 6.5 PMA induces FAM129B translocation to plasma membrane

MDA231 cells were grown in coverslips in 6 well plate for 24 hours. Then the cells were treated with the corresponding inhibitors, 10uM U0126 and 10nM PMA for 4 hours. Untreated cells were used as control. The cells were fixed and stained with FAM129B antibody.

Construction of FAM129B knockdown and overexpression stable cell line

MDA231 and MDA435 cells were seeded in 12 well plates to make sure the cell confluence did not reach more than 80% confluence before transfection the next day. MDA231 and MDA435 cells were infected with FAM129B shRNA (h) Lentiviral Particles from Santa Cruz (sc-92820-V, Santa Cruz). 20 ul of Control shRNA Lentiviral Particles-A (sc-108080, Santa Cruz) was added into the 1ml growth medium as control. After screening with 2ug/ml puromycin, stable MDA231 shcontrol and shRNA-FAM129B
cells pool were collected and frozen for further analysis. Western blot showed that FAM129B was knocked down by more than 90% in both cell lines. In addition, pLenti-FAM129B-V5 virus was made from 293FT cells. Human HMLE cells were infected with the pLenti-FAM129B-V5 virus and control pLenti-LacZ-V5 virus. After screening with 10μg/ml blasticidin, the stable clones were pooled and subjected to western blot analysis.

**DISCUSSION**

**Effect of FAM129B on the TNF/NFκB Pathway**

TNFα induces NFκB activation because the inhibitor IκB is degraded, releasing the active transcription factor, NFκB. Activated NFκB translocates to the nucleus and promotes gene expression. Aberrant activation of NF-κB is frequently observed in many cancers. Hence, inhibition of NFκB activation and its downstream gene expression could sensitize cancer cell apoptosis induced by TNFα. FAM129B knockdown affected phosphor-p65 level in HeLa cells indicated that FAM129B might play roles in activation of NFκB. It has been well characterized that IKKβ is the upstream of NFκB (Scheidereit 2006). IKKβ has been reported to induce tumor development in part through NF-κB activation. IKKs may be regulated by various mechanisms. Recently, IKKβ was reported to be associated with KEAP1, Kelch-like ECH-associated protein 1, which binds IKKβ and promotes its degradation by ubiquination and thus prevents the activation of IκB (Lee, Kuo et al. 2009). Keap1 is an adaptor protein for the E3 ubiquitin ligase complex formed by CUL3 and RBXI. It has been shown to target NRF2 a transcription factor that induces the antioxidant response,
for ubiquitination and degradation by the proteasome (Itoh, Wakabayashi et al. 1999). Another independent study reported that NF-κB signaling pathway was suppressed by KEAP1 regulation of IKKβ activity through autophagic degradation and inhibition of phosphorylation (Kim, You et al. 2010).

We found that FAM129B interacts strongly with KEAP1 through the same motif as IKKβ, as shown by co-immunoprecipitation (Figure 6.3). One hypothesis is that FAM129B competes with IKKβ for binding to Keap1, thus preventing its ubiquitination and degradation. The pathway is therefore activated and anti-apoptotic proteins are synthesized. In addition, we found that when the endogenous protein level of FAM129B is high, KEAP is low in a variety of cell lines. However, our preliminary data did not confirm that the knock down of FAM129B has any significant effect on the KEAP1 or IKKβ protein levels. Contrary to literature, overexpression of Keap1 has no significant effect on the levels of IKKβ. In addition, no obvious effect on the levels of NRF2 levels occurred with FAM129b knockdown. Nevertheless, this does not exclude the possibility that FAM129B regulates NF-κB and NRF2 by affect their phosphorylation and nucleus localization. Further analysis of the degree of phosphorylation of NFκB and its localization with and without FAM129B knockdown will provide more insight about the mechanism.

**FAM129B expression level in invasive and non-invasive cell lines**

If FAM129B is important for cell invasion, then one might expect that the intracellular concentration of the protein would be elevated in metastatic cell lines. Western blotting of FAM129B from MCF10A derived from normal breast tissue, MCF7
malignant but non-metastatic and MDA231, a highly invasive cell line supported this expectation. We have confirmed the previous observations made in melanoma cell lines. Knock down of FAM129B in MDA231 cells decreases the invasiveness of the cell 5-fold in an *in vitro* invasion assay. With Dr. Guojun Wu at Karmanos, this study was extended to 9 different cancer cell lines, 5 metastatic and 4 non-metastatic lines. Again, FAM129B was elevated in all of the metastatic cell lines. The same cell lines were analyzed by rtPCR, however, the results are not straightforward. Both the short and long isoforms, differ by 16 amino acids at the amino end of the polypeptide. The longer isoform, isoform 2, was expressed at higher levels in the metastatic cells, but the difference in the mRNA levels is much less than that seen with the protein (data not shown). This observation further indicates that FAM129B protein level is post-translationally regulated.

Thus far, all of the experiments here and elsewhere on FAM129B have been conducted with cancer cell lines grown in culture. We are in collaboration with Dr. Wu to determine whether FAM129B promotes cell invasion in the mouse model system that he has devised. Hence, I have attempted to construct the stable cell lines which express shRNA specifically targeting FAM129B in breast cancer cell lines, MDA231 and MDA435 cell lines with high level of FAM129B. The knock down of FAM129B in both the cell lines was verified by western blot (data not shown). These in vivo experiments will ultimately lead to the confirmation that FAM129B might be an important drug target.

**Role of FAM129B in the Adherens Junction**
Adherens junctions are protein complexes at the plasma membrane responsible for establishing cell-cell adhesion. They play an important role in both apoptosis and cell invasion. During apoptosis, the adherens junctions proteins are cleaved and the junction is lost (Herren, Levkau et al. 1998). At this stage of apoptosis, the actin cytoskeleton retracts and new junctions are formed between neighboring, robust cells to fill the gap created by the shrinkage of the dying cell. Perhaps, FAM129B stabilizes the adherens junction. During metastasis, the adherens junction must also be disrupted to allow the cells to escape from the site of primary tumor. In this case, FAM129B might be playing a role in destabilizing the adherens junction. To reconcile these apparently contradictory interpretations, experiments are being conducted to determine whether and, if so, when FAM129B is localized at the adherens junctions and its effect on the integrity of the assembly. It seems likely that the loci at which FAM129B functions is different for the two different effects. Keap1 has recently been observed to be localized to the adherens junctions. When Keap1 is overexpressed, the formation of the intact actin cytoskeletal organization at the cell-cell contact sites and the recruitment of E-cadherin and β-catenin to the adherens junctions were inhibited (Velichkova, Guttman et al. 2002).

Co-immunoprecipitation studies were conducted to determine whether FAM129B interacts with any of the proteins known to be present in the adherens junction. The components of the adherens junction were solubilized with Triton X-100 following established protocol. FAM129 was immunoprecipitated with FAM129B specific antibodies. The immunoprecipitation did not bring down β-catenin or the P120 catenin. Conversely, immunoprecipitation of β-catenin or P120 did not precipitate FAM129B.
This indicates that the interaction between FAM129B with β-catenin or the P120 catenin might be weak in the lysate condition. In-vivo cross-linking immunoprecipitation might help reveal the interaction. Experiments with E and N cadherin are planned. Since α-catenin is the one that interacts with actin and the interaction is dynamic, so it might be perturbed by FAM129B. Knockdown of FAM129B had no effect on β-catenin or P120. A few interesting questions remain to be answered. Does FAM129B interact with actin? Does FAM129B stabilize or destabilize the adherens junction? What is the relationship between FAM129B and Keap1 at the adherens junction? We are planning to answer these questions using an in vitro assay that measure the shear required to disrupt cell-cell interactions.

**MAPK phosphorylation of FAM129B and its localization**

Previous study indicated that phosphorylation of FAM129BB by MAPK might affect its localization (Old, Shabb et al. 2009). We have constructed the GFP-FAM129B Ser683Ala mutants expressing a mutant FAM129B protein that could not be phosphorylated in response to the MAPK pathway activation, and Ser683Asp mutants that mimic phosphorylated form of FAM129B. However, the localization of both phosphorylated mimic mutants and dead mutants showed the fluorescence is localized in cell-cell junction when they were transfected into HEK293 cells. Furthermore, the intracellular localization FAM129B did not change following EGF stimulation. These results suggest that phosphorylation might be not involved in the FAM129B membrane localization. Instead, preliminary results show that the GFP-PH domain can target the junction. Finally, to better understand FAM129B’s function in plasma membrane, we need to further confirm if PH domain alone can target plasma membrane regardless of
cell-cell contact. It is also interesting to identify which PIPs or IPs the PH domain of FAM129B interacts with.

**FUTURE DIRECTIONS**

1. **To further confirm the interaction of FAM129B and KEAP1 and determine the biological function of the interaction**

2. **To further confirm the GFP-PH domain localization and test the mechanism of how FAM129B is recruited into plasma membrane**

   GFP-PH-FAM129B will be transfected into HEK293, HeLa, MDA231 cells to confirm if PH domain alone could specifically target plasma membrane or cell junctions. The GFP-C-terminal FAM129B will be used as negative control and GFP-full length FAM129B will be used as positive control. If the specific localization of GFP-PH-in plasma membrane is observed, the constructed GST-PH domain will be purified, and then it will be used to identify its lipid-binding specificities using PIP Array™ membranes (P-23748, Invitrogen). Only 10% of PH domain actually target membranes. The PH domain of FAM129B will be one more verified domain. According to the lipid binding specificity, we can further deduce the function of FAM129B. We can also test if this PH domain has dominant a negative effect that can compete with endogenous FAM129B.

3. **To reconfirm if knockdown of FAM129B affects the cell growth rate**

   Previous transient knockdown of FAM129B in HeLa, MDA231 did not significantly affect cell growth. To further investigate the mechanism of how FAM129B affects cancer cell invasion and apoptosis, it is essential to first confirm if long term stable knockdown of FAM129B affects cell growth. The cell growth curve analysis, MTT
assays and Brdu incorporation will be performed to confirm the possibility. If stable knockdown does decrease the cell growth rate, then growth

4. **To reconfirm if the stable knockdown cells show decreased invasive ability in vitro**

With the stable knockdown cells, it is convenient to repeat if knockdown cells show decreased invasive ability in vitro by matrigel analysis. If the results are repeatable, then it is promising to test if FAM129B is involved in breast cancer metastasis by in vivo model.

5. **Test if any of the metastasis related genes are changed in FAM129B overexpressed stable cell line**

In collaborating with Dr.Wu’s lab in Karmanos, we have constructed the stable FAM129B overexpressed human normal breast cell line (HMLE). Western blot analysis will be performed to test if there are any changes in the protein expression level of N-cadherin, E-cadherin, vimentin, a-catenin and beta catenin, which will reveal the possible mechanism.
REFERENCES


Antonsson, A. and J. L. Persson (2009). "Induction of apoptosis by staurosporine involves the inhibition of expression of the major cell cycle proteins at the G(2)/m
checkpoint accompanied by alterations in Erk and Akt kinase activities."


Chang, J. S., S. K. Kim, et al. (2005). "Pleckstrin homology domains of phospholipase C-gamma1 directly interact with beta-tubulin for activation of phospholipase C-


papillomavirus types 16 and 18 promotes the degradation of p53." Cell 63(6):
1129-1136.

and transcription." Oncogene 25(51): 6685-6705.

137.


by human adenoviruses and the origin of HEK 293 cells." The FASEB journal:
official publication of the Federation of American Societies for Experimental
Biology 16(8): 869-871.

Shoaf, W. T. and M. E. Jones (1973). "Uridylic acid synthesis in Ehrlich ascites
carcinoma. Properties, subcellular distribution, and nature of enzyme complexes


Sigoillot , F., Sigoillot, S, et al. (2004). "Breakdown of the regulatory control of
pyrimidine biosynthesis in human breast cancer cells." Int. J. Cancer 109: 491-
498.


ABSTRACT

THE ROLE OF CAD, FLASH AND FAM129B IN CANCER CELL SURVIVAL AND APOPTOSIS

by

SONG CHEN

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Advisor: Dr. David Evans

Major: Biochemistry and Molecular Biology

Degree: Doctor of Philosophy

Apoptosis is a normal process in the human body. However, apoptosis is desregulated in cancer cells. Most cancer cells gain resistance to apoptosis, leading to uncontrolled proliferation. In this dissertation, we identified three proteins, associated with apoptosis pathway. 1) CAD, a large multifunctional complex that is invariably elevated in tumor cells, 2) FLASH, a large protein with multiple growth related functions and 3) FAM129B. We demonstrate that CAD could interact with FLASH by using yeast two hybrid, co-immunoprecipitation and fluorescence microscopy. In addition, functional analysis using siRNA technology further indicated that CAD could co-operate with FLASH and play roles in multiple cellular processes such as cell mitosis, S phase progression and apoptosis. Moreover, we identified FAM129B, a newly discovered protein that has been implicated in metastasis of melanoma cells. My study was focused on identification of this protein in apoptosis pathway. We first demonstrated that this protein is a novel cell cell junction associated protein. Knockdown of this protein could sensitize cells to apoptotic stimuli and induce rapid apoptosis. Consistent with
previous studies, we also provided evidence to show that FAM129B plays important roles in breast cancer metastasis.
AUTOBIOGRAPHICAL STATEMENT

SONG CHEN

Education:

2006-2011 Ph.D  Major: Biochemistry and Molecular Biology; Minor: Cancer Biology
Wayne State University School of Medicine, Detroit, MI USA

1997-2001 B.S  Major: Biotechnology, Soochow University, Suzhou, CHINA

Publications and Presentations:


