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Review

Roles of the Major Apoptotic Nuclease-DNA Fragmentation Factor-in Biology and Disease

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Abstract. It has now been more than ten years since the discovery of the major apoptotic nuclease, DNA fragmentation factor (DFF), also known as caspase-activated DNase (CAD). Here we review the recent literature that has uncovered new insight into DFF's regulation, and both its positive and negative roles in human disease. Cells from mice deficient in DFF still undergo apoptotic death without significant cell-autonomous DNA degradation. Their corpses' genomes are subsequently degraded by lysosomal DNase II after phagocytosis. However, DFF-deficient mice are more susceptible to cancer. Indeed, several different cancers in humans are associated with defects in DFF expression and it has been proposed that DFF is a p53-independent tumor suppressor. Negative aspects of DFF expression include contributing to susceptibility to acquire systemic lupus erythematosus, to chromosomal translocations that result in mixed lineage leukemias, and in the possible spreading of oncogenes and HIV due to horizontal gene transfer.

Keywords. Apoptotic nuclease, CAD, DNA degradation, chromatin, chromosome translocations, *MLL,* tumor suppression, autoimmunity.

Introduction

Programmed cell death, or apoptosis, is an essential fundamental process for both development and maintenance of tissue homeostasis [reviewed in refs. 1, 2]. Kerr et al. [3] first recognized this phenomenon in a pioneering publication with profound insight, for as stated in the first sentence of the summary of this article, "The term apoptosis is proposed for a hitherto little recognized mechanism of controlled cell deletion, which appears to play a complementary but opposite role to mitosis in the regulation of animal cell populations". Cells undergoing apoptosis exhibit cytoplasmic morphological changes, membrane blebbing, DNA fragmentation, chromatin condensation, nuclear breakdown, assembly of membrane-enclosed vesicles termed apoptotic bodies, and eventual subjection to phagocytosis [reviewed in ref. 4]. It is striking that every day an estimated ten-billion cells in the human body undergo apoptosis and efficient phagocytic clearance [5]. Furthermore, defects in apoptosis have been associated with a number of disease states, including AIDS, autoimmunity, ischemic strokes, neoplasia, and neurodegeneration [reviewed in ref. 6].

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Two major apoptotic pathways exist: the mitochondrial pathway and the cell surface death-receptor pathway [reviewed in refs. 7, 8]. Multiple apoptotic stimuli trigger the activation of proteases called caspases, which in turn initiate and execute the apoptotic program [reviewed in ref. 9]. Such stimuli include exposure to ionizing radiation, or to various drugs that target DNA, serum starvation, or the activation of cell-surface death receptors. Several of the key gene products involved in apoptosis were discovered largely from the results of genetic experiments conducted in *Caenorhabditis elegans.* For this work Horvitz was awarded the Nobel Prize in Physiology and Medicine in 2002, together with Brenner and Sulston, who studied other aspects of *C. elegans* developmental biology [10].

One of the hallmarks of the terminal stages of apoptosis is genomic DNA breakdown, which was first recognized in 1980 by Wyllie [11]. Such DNA breakdown occurs in two steps: first, initial cleavage into 50- to 300-kb long genomic fragments, believed to reflect the higher-order organization of the chromatin into chromosomal loop domains [12] and, next, fragmentation down to oligonucleosomal sized segments [11]. Both of these cleavage events are catalyzed by the major apoptotic nuclease, DNA fragmentation factor (DFF) [13–15], also known as caspase-activated DNase (CAD) [16], which is the subject of this review [see ref. 17 for a review on the discovery and mechanism of action of this nuclease]. It is interesting to note historically that this latter DNA fragmentation pattern in dying cells was reported in the literature [18] four years prior to the realization that the eukaryotic genome is packaged into nucleosomes [19], and two years prior to the coining of the term "apoptosis" [3]. Nucleosome structure, from an evolutionary point of view, serves crucial roles in living cells for genome packaging and regulation, but it also prepares the genomes of dying cells to be processed by DFF into "bite-size" pieces for the efficient clearance of their DNA by phagocytosis. However, cell death can occur in the absence of DFF without significant cell-autonomous DNA degradation [20]. The resulting apoptotic corpses display an "eat-me" signal on their surface, which is phosphatidylserine [21]. Macrophages secrete milk fat globule EGF Factor 8 (MFG-E8), which binds to the exposed phosphatidylserine and serves as a bridge between the apoptotic cells and the macrophages [21]. Lysosomal nucleases of these phagocytic cells degrade the DNA of the engulfed apoptotic corpses, largely replacing the function of DFF in a non-cell autonomous fashion. These lysosomal nucleases, which have been called "waste-management nucleases" [22], are exemplified by DNase II of mammalian macrophages [23, 24] and Nuc-1, its homolog in *C. elegans* [25]. However, cell-autonomous apoptotic DNA fragmentation mediated by DFF has functional significance [previously reviewed in refs. 5, 26, 27], and will be highlighted in this review at the levels of biology and disease processes. (See refs. 28, 29 for DFF's importance in chromatin structure research).

DFF was discovered a decade ago thanks to the successful establishment of a biochemical *in vitro* system in which DNA fragmentation in normal cell nuclei could be triggered by the addition of cytoplasmic extracts from normal cells and activated caspase-3. Wang and co-workers used this system to purify a heterodimer composed of 40- and 45-kD subunits, which they termed DFF40 and DFF45, and demonstrated that DFF45 possesses two caspase-3 cleavage sites; yet they did not identify which subunit corresponded to the caspase-3 activatable nuclease [13]. In 1998 Nagata and co-workers used a similar assay to purify the mouse homologues of human DFF40 and DFF45, which they renamed CAD and ICAD respectively for caspase-activated DNase and inhibitor of caspase-activated DNase. These investigators demonstrated clearly that the DFF40/CAD subunit was a latent endonuclease that was inhibited by the DFF45/ICAD subunit [16, 30]. Subsequently human DFF40 was cloned by three groups later that year, who each demonstrated that the encoded protein corresponded to the nuclease [14, 31, 32]. DFF40/CAD (hereafter termed DFF40) is a magnesium dependent endonuclease that cuts DNA to yield 5'-phosphate residues and 3'-hydroxyl groups that are substrates for terminal deoxynucleotide transferase, which is the foundation of the apoptotic TUNEL assay [33]. It is both a deoxyribose-specific and double-strand specific enzyme [34] that generates exclusively double strand breaks (primarily blunt ends) [33]. In addition, the nuclease exhibits an extraordinary preference for cleaving the internucleosomal linker regions in chromatin [28, 33]. This preference possibly stems from the scissors-like structure of the DFF40 active site [35] and from the stimulatory effects of histone H1 and HMGB1, major chromatin proteins interacting with linker DNA (see below). Chromatin condensation, which temporally correlates with DNA breakdown, is another hallmark of the terminal stages of apoptosis [reviewed in ref. 4]. Importantly, activation of DFF leads to advanced peripheral chromatin condensation [36] and such apoptotic nuclear morphology could be mimicked in nuclei isolated from normal healthy cells upon DNA cleavage by DFF, as well as other nucleases, under physiological ionic strengths and independent of ATP utilization [14, 37]. Interestingly, such nuclease-induced chromatin condensation *in vitro* can be inhibited by factors that stabilize compo-

nents of the "nucleoskeleton" or disrupt native nucleosomal DNA wrapping. Therefore, the ability of chromatin fragments with intact nucleosomes to form large clumps of condensed chromatin during apoptosis requires the apparent disassembly of internal nuclear structures that may normally constrain chromosome subdomains in non-apoptotic cells [37].

Pathways of DFF activation and its subunit structure.

Because of the lethal function of the nuclease, multiple fail-safe steps exist to protect non-apoptotic cells from accidental or uncontrolled activation of DFF40. First of all, in order to generate a potentially activatable nuclease, DFF40 must be co-expressed with its inhibitor subunit, DFF45/ICAD (hereafter termed DF45), which also acts as a molecular chaperone to correctly fold DFF40 (expression of DFF40 by itself leads only to inactive enzyme) [14, 16, 31]. The chaperone associates with DFF40 and in turn serves as an inhibitor to block its homo-oligomerization, which is required for nuclease activation [35, 38, 39]. Importantly, there is a vast stoichiometric excess of free DFF45 subunits in normal cells [39]. In addition, a 35-kD splicing variant of DFF45 (DFF35/ICAD-S) also exists, which can serve as an inhibitor but not as a chaperone of DFF40 [16, 40, 41]. Significantly, these free proteins have the ability to inhibit activated DFF40 [39], suggesting that they may serve as fail-safe guardians for reversing the hypothetical accidental activation of DFF40. Although DFF was initially purified from cytoplasmic extracts [13, 16], the nuclease actually resides in the nucleus of normal healthy cells [14, 42–44]; the presence of DFF in cytoplasmic extracts results from artifactual "leakage" of nuclear proteins during cell fractionation [39, 42]. Both DFF40 and DFF45 each possess their own nuclear localization sequence (NLS) located in their C-termini [42–44] and these two separate NLSs synergize for targeting DFF to the nucleus [45]. DFF35 resides in the cytoplasm because of a spliced-out NLS in its C-terminus [16, 43]. Consequently, complexes formed between DFF40 and DFF35 reside predominantly in cytoplasm [46]. Interestingly, alternative splicing of DFF45 gene transcripts depends on the ASF/SF2 splicing factor, and depletion of ASF/SF2 results in increased levels of DFF35 and inhibition of apoptotic DNA fragmentation [47]. The major activator of the DFF40 nuclease is caspase-3, which cuts at two specific sites within DFF45 [14, 16, 31]. However, DFF40 nuclease also can be activated when DFF45 is cut at similar or identical sites by caspase-7 [38], granzyme B [48], granzyme M [49], or even by tobacco etch virus protease (TEVP) when DFF45's caspase-3 cleavage sites have been genetically engineered [29, 41]. Importantly, although inactive procaspase-3 is a cytoplasmic protein, caspase-3 is actively transported to the nucleus in a process that depends on its proteolytic activation [50]. Cleavage of DFF45 results in homo-oligomerization of DFF40 [35, 38, 39], followed by its effective binding to DNA [51]. However, Meiss and coworkers have shown that DFF40 in association with DFF45 can also bind to DNA and be caspase-activated in a DNA-bound state [52].

Precise understanding of the mechanism of DFF regulation requires specific knowledge of the subunit structures of DFF species, in both their latent and nuclease active states. Interactions between DFF40 and intact DFF45 are mediated by homologous N-terminal domains called CIDE-N that are present in both subunits [53]. Size-exclusion chromatography analysis of native DFF40-DFF45 complexes purified from cytoplasmic extracts of non-apoptotic HeLa cells revealed their predominant presence in a fraction corresponding to about 100 kD, which suggested a heterodimeric structure [13]. Very similar gel filtration results were obtained for the masses of the corresponding mouse and Jurkat-cell human DFF species [16, 31]. This heterodimeric structure has been confirmed for bacterially expressed recombinant DFF, by means of size-exclusion chromatography [35, 38], native gel electrophoresis, glutaraldehyde crosslinking and atomic force microscopy [39]. More recent analysis of the subunit structure of endogenous DFF has lead to a broader viewpoint. Lukacs and coworkers showed the presence of endogenous DFF purified from HeLa cells in size-exclusion chromatography fractions corresponding to molecular masses from 100- to 200-kD, predominantly about 180 kD, contrasting with the earlier results from other laboratories using nearly identical techniques. More convincingly, in experiments with recombinant epitope-tagged proteins, they proved the existence in cells of complexes that consisted of two DFF40 and two DFF45 molecules [54]. Though the exact structure of such complexes – heterotetramer or dimer of heterodimers – is not clear at the moment, these may apparently facilitate the formation of homo-oligomers of DFF40 after DFF45 cleavage and disassociation. Thus, it seems possible that DFF40-DFF45 complexes may exist in a dynamic equilibrium between dimeric and tetrameric structures, with the heterodimer being the most stable unit, particularly after biochemical fractionation of cellular extracts. Generation of the active nuclease after DFF45 cleavage involves the formation of DFF40 homo-oligomers [35, 38, 39]. The crystal structure of activated DFF40 clearly reveals that a homodimer is the basic minimum unit structure

Table 1. Proteins that regulate DFF.

Protein	Effect on DFF	Ref
Importin α/β (protein involved in nuclear import)	The DFF40:DFF45 complex binds to the importin α/β heterodimer, which is essential for the nuclear import of DFF; NLSs localized in C-termini of both DFF40 and DFF45 are required for efficient binding to importin and nuclear import.	45
Histone H1 (major chromatin protein)	C-terminal domain of histone H1 binds DFF40 and activates the nuclease due to stimulating DNA binding.	51
	Phosphorylation state of histone H1 affects neither the activity of DFF40 *in vitro* nor chromatin fragmentation *in vivo*.	59
HMGB1 (major non-histone chromatin protein)	HMGB1 stimulates DNA cleavage by DFF40 due to interactions of HMG-boxes with DNA; no direct interactions exist with DFF *in vitro*.	61
Topoisomerase II (chromatin protein, DNA-relaxing enzyme)	Topoisomerase II binds DFF40 and stimulates DNA cleavage by DFF40 *in vitro*.	15, 60
Histone H2A.X (core histone variant)	Histone H2A.X binds DFF40 *in vitro*; phosphorylation of H2A.X is required for DFF-mediated chromatin cleavage in UV-treated apoptotic cells.	62
HSP70 (major molecular chaperone and stress protein)	HSP70 binds to activated DFF40 (but not to the DFF40:DFF45 complex), and stimulates/stabilizes the activity of the nuclease (peptide-binding domain of HSP70 is required).	58
Nucleophosmin/B23 (oncoprotein activated by PI3P)	Nuclear complex of B23-PI3P binds to activated DFF40 (but not to the DFF40:DFF45 complex) and inhibits the activity of the nuclease.	63
Ebp1 (proliferation-associated, ErbB3-binding protein)	Nuclear complex of phosphorylated Ebp1 (by PKC) and phosphorylated Akt binds to DFF40 (either activated or in a complex with DFF45) and inhibits the activity of the nuclease.	64
CIIA (protein interacting with ASK1, apoptosis signal regulating kinase 1)	CIIA binds to the DFF40:DFF45 complex and inhibits the nuclease without affecting the cleavage of DFF45.	65

of the enzymatically active nuclease, with a DNA binding surface and active site in a deep cleft formed by the two subunits [35]. However, questions about further oligomerization of activated DFF40 and its functional importance remain open. Formation of large homo-oligomers, with tetramers being the smallest multimer unit, has been reported for bacterially expressed recombinant protein using size-exclusion chromatography, native gel electrophoresis and atomic force microscopy [38, 39]. Similarly, endogenous DFF40 nuclease resulting from *in vitro* caspase-activation of DFF purified from Jurkat or HL60 cells separate as very large complexes when analyzed by size-exclusion chromatography or native gel electrophoresis, respectively [31, 39]. In contrast, when proteins isolated from apoptotic cells were analyzed by size-exclusion chromatography, epitope-tagged DFF40 separated as 110–190 kD complexes, consistent with DFF40 forming homo-oligomers no larger than tetramers [54]. It is difficult to explain the reasons for these differences in results, without invoking technical variations in sample handling methods. Nevertheless, it is possible that the formation of excessively large homo-oligomers of DFF40 with reduced activity may happen at the very late stages of apoptotic cell nuclear breakdown so as to protect neighbouring cells against possible damage from released nuclease. To explain possible mechanisms that may contribute to formation of large homo-oligomers of DFF40, it is significant to note that DFF40 has ten cysteine residues that are capable of forming intermolecular disulfide bonds [14], and that the CIDE-N domains of DFF40 can form amyloid-like fibrils *in vitro* [55]. Although DFF45 also possesses a CIDE-N domain, free DFF45, either endogenous or recombinant, exists as a monomer [38, 39, 54]; however, there is one report indicating the possibility of oligomerization of a recombinant epitope-tagged DFF45 [56].

Effectors that regulate DFF

In addition to DFF45, the specific chaperone and inhibitor of DFF40 nuclease activity, several other proteins have been identified that are involved in regulating DFF. These proteins, listed in Table 1, can be divided into three functional groups: (1) factors mediating the proper folding and cellular localization of the nuclease; (2) activators of the nuclease; and (3) inhibitors of the nuclease. During translation, the general molecular chaperones HSC70 and HSP40 participate in the assembly of the functional DFF heterodimer [57]. Furthermore, HSP70 can bind to activated DFF40, and stimulate and stabilize the activity of the nuclease [58]. It has been shown

recently that nuclear import of DFF depends on the importin α/β heterodimer. Efficient binding to the importin heterodimer and nuclear import of DFF requires the presence of the NLS localized in the C-termini of both DFF40 and DFF45 [45]. Three major chromatin proteins were the first factors identified that stimulate DNA cleavage by DFF: histone H1, HMGB1/2 and topoisomerase II [14, 15, 33, 38]. The C-terminal domain (CTD) of histone H1 binds to DFF40 and activates the nuclease due to stimulating its ability to bind to DNA [51]. Different variants of histone H1 similarly stimulate the activity of DFF [51], and the phosphorylation state of histone H1 does not affect chromatin cleavage by the nuclease [59]. Topoisomerase II also directly interacts with DFF40 [60]. In contrast to histone H1 and topoisomerase II, there is no direct interaction of HMGB1 with the nuclease; HMGB1 stimulates DNA cleavage by DFF40 due to interactions of its HMG-boxes with the DNA substrate [61]. The activities of all three chromosomal proteins mentioned above apparently contribute to the specificity of chromatin cleavage by DFF: histone H1 and HMGB1 – major proteins that bind linker DNA – may contribute to the preferential internucleosomal chromatin cleavage that results in "DNA laddering", while topoisomerase II may be responsible for targeting the 50- to 300-kb cleavage events of chromosomal loop domains that occur during the initial stages of apoptotic chromosomal DNA fragmentation. Interestingly, DFF40-mediated apoptotic chromatin cleavage in response to UV irradiation requires phosphorylation of histone H2A.X. Furthermore, histone H2A.X binds to DFF40 *in vitro* [62]. Whether this phospho-H2AX requirement regulates caspase-3 mediated cleavage of DFF45 or stimulates DNA cleavage due to recruiting the nuclease to chromatin remains to be determined.

Several nuclear proteins have been identified that inhibit DFF. The complex of nucleophosmin/B23 with PI3P inhibits the nuclease due to its binding to activated DFF40 (but not to the DFF heterodimer) [63]. The complex of Ebp1 and AKT also inhibits the nuclease due to its binding to activated DFF40 (but it can also bind to the DFF heterodimer) [64]. In contrast, CIIA binds to the DFF heterodimer, yet it inhibits the nuclease without affecting caspase-mediated cleavage of DFF45 [65]. All these interactions have functional importance *in vivo* and can suppress chromatin fragmentation in cells that have initiated apoptosis.

The nucleolytic activity of DFF40 after its disassociation from the DFF45 inhibitor can be affected by non-protein factors as well. The nuclease is inhibited by several poly-anions, like heparin, polyglutamic acid [66], poly(ADP-ribose) [67] and nucleic acids that are not its substrates (single stranded DNA, RNA and DNA-RNA heteroduplexes) [34, 66]. Such poly-anions, which are thought to bind to the positively charged surface formed by the α4 helices of DFF40 and impair its ability to bind to double-stranded DNA, are competitive inhibitors of the nuclease [66, 68]. Knowing that RNA, which inhibits DFF40 *in vitro*, is the major cellular poly-anion, one could postulate that apoptotic chromatin breakdown might be coordinated with RNA degradation *in vivo*, and DFF activity would be kept in check until RNA levels fall below some inhibitory threshold. Hypothetically, RNA might suppress DFF40 activity to avoid premature apoptotic DNA breakdown that would inhibit the induction of genes encoding pro-apoptotic factors, and certain apoptotic RNases could co-ordinate the proper timing of RNA and DNA breakdown. However, degradation of RNA does not precede DNA cleavage in cells undergoing apoptosis, and thus the integrity of cellular RNA unlikely affects the activity of DFF *in vivo* [34].

Low-molecular-weight compounds that affect the activity of DFF are much less studied at present. Among them is curcumin, a plant polyphenol. T cells treated with curcumin undergo apoptosis but exhibit an unusual nuclear morphology and no DNA laddering. Although curcumin initiates the activation of caspase-3 and cleavage of DFF45, it inhibits DFF40, probably due to its binding to the active center of the nuclease [69]. Ethidium bromide is another "small" compound that inhibits the activity of DFF and reduces DNA laddering in cells undergoing apoptosis. In this case, however, the inhibitory effect apparently results from intercalation of ethidium into the target DNA substrate, resulting in the perturbation of its structure [Widlak & Garrard, unpublished results]. Certainly, identification of other small molecules that can bind to DFF species and modulate their activities may be potentially beneficial to therapies of various DFF-related pathological conditions.

DFF as a tumor suppressor

It is generally accepted that there is increased risk for cancer when there is a reduced efficiency in the apoptotic elimination of cells with genome damage or malfunctions in regulation of cell proliferation. At the same time, many anticancer strategies are based on the induction of apoptosis in malignant cells and defects in apoptotic pathways frequently reduce benefits from such therapies. Consequently, many anti-apoptotic factors function as oncoproteins, which is exemplified by Bcl-2, originally found as an oncoprotein activated in B-cell lymphomas [70, 71].

Table 2. Reports on the existence of tumor-specific DFF gene mutations or abnormalities in DFF expression in human cancer.

Type of cancer	Observations	Ref
Colon cancer	Higher expression of DFF45 in some cancer cell lines compared to normal colonocytes.	76
Endometrial cancer	Higher expression of DFF45 in atypical hyperplasia and endometrial carcinoma compared to normal endometrium.	77
Esophageal squamous cell carinoma	Low expression of DFF45 correlates with poor outcome.	78
Glioblastoma	The proportion of DFF35/DFF45 is higher in glioblastomas compared to normal brain.	79
Hepatoma	The presence of deletions (resulting from unequal crossing over between Alu elements) and aberrantly spliced transcripts of the DFF40 gene.	80, 81
Neuroblastoma	Frequent deletion of the DFF45 gene in neuroblastoma cell lines.	82, 83
	The presence of rare allele polymorphisms in the DFF45 gene or alternative variants of DFF45 transcripts.	84, 85
	Low expression of DFF45 correlates with poor outcome.	86
	No evidence for tumor-specific mutations in the DFF40 gene.	87
Renal cell carcinoma	Lack of DFF40 expression.	88
Serous ovarian cancer	Increased level of DFF45 in more advanced carcinomas with poor outcome.	89

On the other hand, pro-apoptotic factors function as tumor suppressors. Among them are pro-apoptotic members of the Bcl-2 protein family [reviewed in ref. 72] and p53, the best known tumor suppressor that is defective in the majority of human cancers [reviewed in ref. 73]. Since DFF is involved in the terminal stages of apoptosis, and removal of cells after cell death initiation can occur in the absence of the nuclease [20], one might expect that defects in DFF would have no impact on tumorogenesis. Importantly, however, many investigations have now disproved this presumption. In response to ionizing radiation, DFF-deficient cells exhibit significant increases in mutations, chromosomal instability and survival relative to wild type controls [74]. These observations suggest that DFF contributes to genomic stability by controlling the removal of cells with DNA damage. This proposal is further supported by the observations that the absence of DFF results in an increased frequency of cellular transformation of mouse embryonic fibroblasts and enhanced susceptibility to radiation induced carcinogenesis in DFF40-null mice [74]. Increased rates of radiation-induced chromosome instability and increased survival of irradiated DFF-deficient cells took place irrespectively of their p53 status [75]. Results from this model study are in complete agreement with data collected from human cancers. There are numerous papers in the literature reporting the existence of tumor-specific mutations in DFF genes or abnormalities in the expression levels of DFF in human tumors. Many types of human cancers exhibit either mutations in the DFF45 gene, or abnormally increased or decreased levels of DFF45. In addition, mutations in the DFF40 gene or lack of DFF40 expression also have been observed. Examples of such tumor-specific mutations or abnormalities in the DFF genes in human cancer are shown in Table 2. Given the complex mechanisms of DFF regulation, further detailed investigations will be necessary to identify how all these abnormalities affect DFF species' functions in the various tumors. The data indicate collectively that DFF plays an important role in maintaining genomic stability and suppressing tumor development, and indeed functions as a p53-independent tumor suppressor.

Given that DFF plays an important anti-oncogenic role, it is not surprising that factors that inhibit DFF function are oncoproteins. Nucleophosmin/B23 is an example of such an oncoprotein, which is overexpressed in proliferating and cancer cells, whose aberrant fusion forms are detected in lymphomas. Its nuclear complexes with PI3P bind to activated DFF40, which contributes to NGF-induced PI3P-dependent inhibition of apoptosis [63]. Another proliferation-related protein that inhibits DFF is Ebp1, a protein involved in NGF-induced AKT-dependent inhibition of apoptosis [64]. c-Myc and N-Myc oncoproteins are transcription factors that bind to E-box motifs present in the promoter of the human DFF45 gene. Importantly, both c-Myc and N-Myc oncoproteins activate expression of DFF45 [90], which might further contribute to the involvement of DFF in oncogenesis. It is noteworthy that the potency of DFF to destroy cancer cells has been already tested in experimental therapy. A DFF40:GnRH (gonadotropin releasing hormone) fusion protein has been implemented successfully in experimental, targeted therapy of adenocarcinomas *in vitro* and in a mouse xenograft model [91].

DFF in therapy-related acute myelogenous leukemia (AML)

AML and acute lymphoblastic leukemia in children are associated with translocations between the *MLL* gene breakpoint cluster region (BCR) located at 11q23 and more than 30 other chromosomal loci [92, 93]. In 5–12% of the cases these translocations accompany drug-related anti-tumor therapies with agents that target topoisomerase II (topo II), most notably, etoposide, which is one of the gold standards as an anti-tumor drug [94]. Significantly, experiments with cultured cells treated with etoposide have shown that breaks can be induced at the *MLL* BCR *in vitro* [94–96], but the occurrence of such breaks and AML-characteristic translocations are dependent on caspase activation and any number of other apoptotic triggers, and not directly related to topo II [97–101]. Moreover, these apoptotic triggers result in the activation of DFF. Direct proof that DFF may be the major culprit mediating cleavage at the *MLL* BCR has been obtained using mouse embryonic fibroblasts from wild type and DFF null mice; no *MLL* BCR cleavage was observed after etoposide-induced activation of apoptosis in the absence of DFF [102]. Nevertheless, topo II, which is believed to be localized at the bases of 50- to 300-kb chromosomal loop domains [12], is known to bind to and activate DFF40 [15, 33, 60] and may be responsible for targeting DFF40 cleavage at the *MLL* BCR.

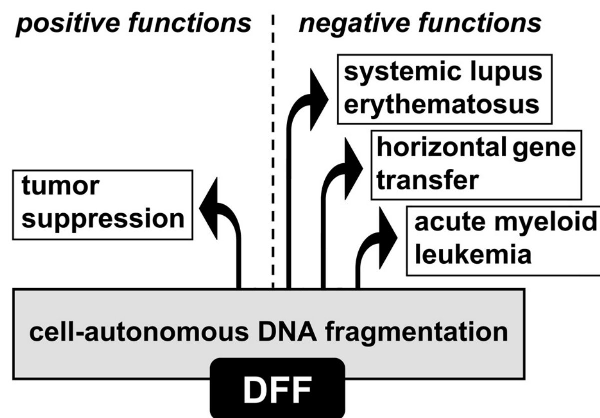

Figure 1. Potential involvement of DFF-mediated cell-autonomous apoptotic DNA fragmentation in different pathological processes.

DFF and susceptibility to systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease in which affected individuals produce antibodies against their own histones, DNA, a variety of nuclear non-histone proteins, and their kidney glomerulus [103]. Mice whose cells are unable to undergo apoptosis because of mutations in their death receptor signaling pathway develop SLE [reviewed in ref. 5]. Interestingly, mice with null mutations in the gene encoding MFG-E8, the molecule that bridges apoptotic cells to macrophages to trigger phagocytosis [21], also develop SLE [5]. It is thought that the unengulfed apoptotic cells undergo secondary necrosis, which leads to the release of their cellular contents, thereby promoting an autoimmune response. Mice engineered to delete their DNase II alleles after birth develop rheumatoid polyarthritis [reviewed in ref. 104]. Interestingly, this is because their macrophages, whose lysozomes are packed full of nucleosome-sized DNA, release TNFα, which stimulates synovial cells in their joints to express pro-inflamatory cytokines and chemokines.

So what about DFF and SLE? First of all, as previously mentioned, cells of DFF null mice still undergo apoptotic cell death and resulting adult mice exhibit no major phenotypes [20]. Furthermore, when DFF null mice are challenged with specific antigens, they mount a normal immune response [105]. In addition, DFF null mice do not develop SLE. By contrast however, in a pristine-induced animal model for SLE, DFF null mice produce significantly lower levels of antibodies to chromatin, small nuclear ribonucleoprotein particles and to other nuclear components, when compared to control wild type mice, but produce normal levels of autoantibodies to cytoplasmic components [105]. It can be concluded from these results that DFF may contribute to the susceptibility to acquire SLE. Cells from DFF null mice exhibit reduced apoptotic chromatin condensation and do not generate apoptotic bodies [106], which are processes thought to be important in concentrating material for phagocytosis by dendritic cells for eventual lupus auto-antigen presentation [107].

DFF in horizontal gene transfer

Horizontal gene transfer is defined as the movement of a DNA segment from a donor cell to a recipient cell where the DNA segment becomes stably integrated into the recipient cell's genome. Several investigations have shown that this can occur when donor cell apoptotic bodies are engulfed by phagocytic recipient cells [108–110]. The process can have important consequences because dying tumor cells can transfer their oncogenes to recipient cells and lead to the spreading of malignant transformation [109, 111]. In addition, HIV infected CD4+ T cells can transfer viral DNA to fibroblasts and dendritic cells through the

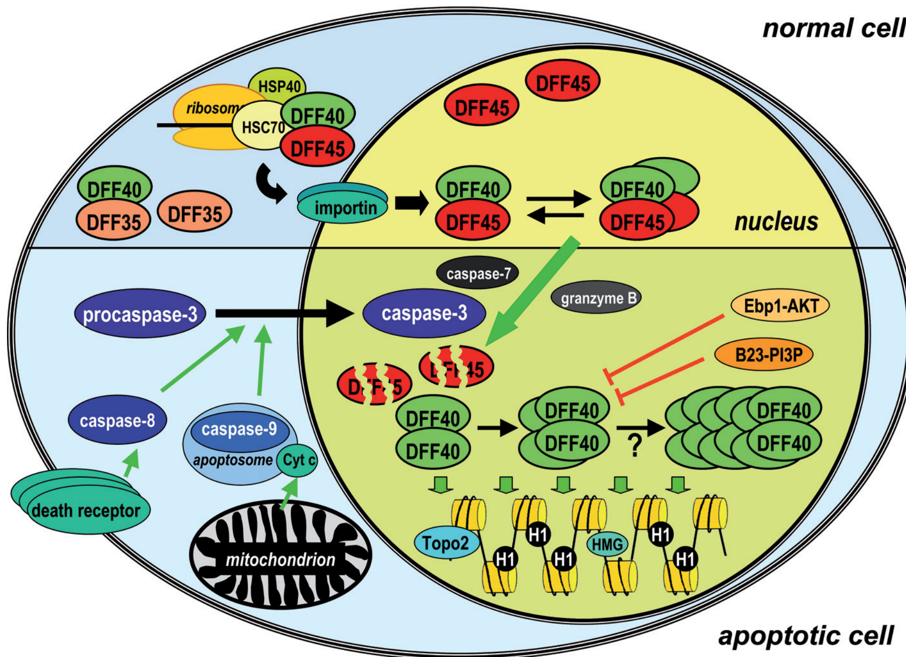

Figure 2. Subunit structures and intracellular localization of DFF species in a normal healthy cell and in a cell undergoing apoptosis (above and below the horizontal line, respectively). Shown schematically are the proteins involved in regulation of DFF and its nucleosomal substrate.

uptake of apoptotic bodies [112]. However, uptake of donor cell DNA normally leads to a p53- or p21-dependent cell cycle arrest. Thus, horizontal gene transfer efficiently occurs only when p53- or p21-deficient cells are used as recipients [111]. Knowing that frequent malfunctions of the p53 pathway occur in tumor cells, this phenomenon might contribute to the spreading of genetic instability within cancer cell populations. Interestingly, such p53-dependent cell cycle arrest does not occur if DFF activation is inhibited in donor apoptotic cells when they are engulfed by DNase II null recipient cells [113]. Thus, there appears to be a causal relationship between DFF-mediated apoptotic DNA breakdown in donor cells, DNase II-mediated DNA breakdown in recipient cells, and p53 activation. In fact, the crucial nuclease that triggers p53-dependent cell cycle arrest is DNase II in the recipient cell, not DFF in the donor cell [113]. Surprisingly, significant horizontal gene transfer occurs in DNase II null recipient cells even when the p53 pathway is functional. Furthermore, the frequency of horizontal gene transfer to p53 null recipient cells is reduced some 4- to 6-fold when DFF activation is inhibited in apoptotic donor cells [110].

Conclusion and future perspectives

In this review one topic we have highlighted is the importance of DFF in human diseases. As summarized in Figure 1, DFF plays a positive role as a tumor suppressor, and negative roles for its involvement in lupus, horizontal gene transfer and AML. Perhaps in the distant future, drugs may be developed that can regulate the expression or correct for an imbalance of DFF subunits in normal individuals who have a predisposed increased risk for cancer because of genetic defects (see Table 2). By contrast, drugs that specifically target and activate DFF in tumor cells would be desirable. One might also imagine that drugs that block DFF action during etoposide treatment of patients with leukemia would be very beneficial, as would drugs that inhibit DFF activity in lupus patients, and drugs that prevent HIV spreading to cells lacking HIV receptors in AIDS patients.

In this review we have also discussed the complexity of the DFF activation pathways and their multiple sites of regulation. As summarized schematically in Figure 2, the inhibitor and chaperone subunit DFF45 is in stoichiometric excess of DFF heterodimers in the normal cell nucleus after their transport from the cytoplasm mediated by their NLSs through importin α/β heterodimers. DFF45, along with HSP40 and HSC70, are necessary for the appropriate folding of DFF40 upon its synthesis on cytoplasmic ribosomes to create a potentially activatable nuclease. Apoptotic triggers working through the cell surface death receptors, or through the mitochondria, result in the activation of initiator caspases-8 or -9 and lead to the activation of the executioner caspases-3 and -7 in the apoptotic cell. These caspases or granzymes in the nucleus are essential for DFF activation upon DFF45 cleavage. Resulting DFF40 homo-oligomers, activated and targeted further by topoisomerase II, histone

H1, and HMGB1, cleave first at the bases of chromosomal loop domains and subsequently at the nucleosomal linkers. The activated enzyme, however, can be inhibited by Ebp1-AKT or B23-PI3P (or free DFF45), and perhaps by DFF40s propensity for the formation of supramolecular homopolymers. We have also discussed non-protein inhibitors of the nuclease (not shown in Fig. 2).

While we have already pointed out that DFF null mice exhibit no major defects in apoptotic cell death because of DNase II activity in phagocytic cells [23], cells from DFF null animals do exhibit several defects in their behavior in response to apoptotic triggers: they display reduced chromatin condensation, do not form apoptotic bodies, have reduced intranuclear cytoplasmic invaginations and lower levels of nuclear fragmentation [106]. Surprisingly, DFF null mice exhibit enhanced spatial learning and memory with longer memory retention compared to wild type controls; indeed, these null mice possess an increase in the number of granule cells in the dentate gyrus [114–116]. Most importantly, DFF null mice are at increased risk to develop cancer and have reduced genomic stability [74, 75].

Finally, it is worth mentioning that DFF is not ubiquitously present among eukaryotes; for example, DFF is absent from *Saccharomyces cerevisiae* and *C. elegans.* Interestingly, studies by Eckhart et al. [117] reveal that DFF orthologs exist in *Nematostella vectensis*, a representative of the primitive metazoan clade cnidarians, and in various vertebrates and insects, but not in representatives of urochordates, echinoderms, and nematodes. The domains mediating the interaction between DFF45 and DFF40 orthologs, a caspase cleavage site in the DFF45 ortholog, and the amino acid residues critical for endonuclease activity of the DFF40 ortholog are conserved in *Nematostella.* These findings suggest that DFF has been a part of the primordial apoptosis system of the eumetazoan common ancestor and that the ancient cell death machinery has degenerated in several evolutionary lineages, including the one leading to the prototypical apoptosis model *C. elegans* [see ref. 118 for a review of the apoptotic nucleases important in this Nematode].

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