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Death effector domain-containing proteins DEDD and FLAME-3 form nuclear complexes with the TFIIIC102 subunit of human transcription factor IIIC

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Abstract

Death effector domain-containing proteins are involved in important cellular processes such as death-receptor induced apoptosis, NF-κB activation and ERK activation. Here we report the identification of a novel nuclear DED-containing protein, FLAME-3. FLAME-3 shares significant sequence (46.6% identical) and structural homology to another DED-containing protein, DEDD. FLAME-3 interacts with DEDD and c-FLIP (FLAME-1) but not with the other DED-containing proteins FADD, caspase-8 or caspase-10. FLAME-3 translocates to, and sequesters c-FLIP in the nucleus upon overexpression in human cell lines. Using the yeast two-hybrid system to identify DEDD-interacting proteins, the TFIIIC102 subunit of human transcription factor TFIIIC was identified as a DEDD- and FLAME-3-specific interacting protein. Co-expression of either DEDD or FLAME-3 with hTFIIIC102 in MCF-7 cells induces the translocation from the cytoplasm and sequestration of hTFIIIC102 in the nucleus, indicating that DEDD and FLAME-3 form strong heterocomplexes with hTFIIIC102 and might be important regulators of the activity of the hTFIIIC transcriptional complex. Consistent with this, overexpression of DEDD or FLAME-3 in 293 cells inhibited the expression of a luciferase-reporter gene under the control of the NF-κB promoter. Our data provide the first direct evidence for the involvement of DED-containing proteins in the regulation of components of the general transcription machinery in the nucleus.

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Abbreviations: DED, death effector domain; EST, expressed sequence tags; CTD, C-terminal domain

Introduction

Death effector domain (DED)-containing proteins are important components of the cell death receptor-signaling pathway.[1,2] Ligation of the death receptors, such as TNF, CD95 and TRAIL receptors, results in the recruitment of the adaptor molecule FADD/Mort-1 through homotypic interactions of its C-terminal death domain with the cytoplasmic death domain of the death receptors.[1,3,4] FADD, which also contains an N-terminal DED, transduces the activation signal from the death receptors to the DED-containing precursors (procaspases) of the initiator caspases 8 and 10, by direct interaction of its DED with their corresponding DEDs.[1,5] The association of FADD with procaspase-8 and procaspase-10 results in their auto-processing/activation and the release of the mature caspases 8 and 10 from the membrane associated death receptor signaling complex into the cytoplasm.[5–7] The mature caspase-8 and -10 could then cleave several important cellular substrates such as Bid and the effector caspases 3, 6 and 7 resulting in initiation of the caspase cascade and apoptosis.[8,9] Thus, the DED regions of the adaptor molecule FADD and the initiator caspases 8 and 10 represent the link between the death receptors and the downstream cell death machinery.

Although some members of the family of DED-containing proteins, such as FADD, caspase-8 and caspase-10, are agonists of the death receptor pathway, yet others, such as the viral and cellular FLIP proteins, are dominant negative inhibitors or antagonists to this pathway.[10] The viral and cellular FLIP proteins inhibit the death receptor pathway by binding to the DED regions of FADD and/or caspases 8 and 10 preventing the formation of a productive death receptor signaling complex.[10] c-FLIP-deficient mice do not survive,[11] suggesting that the anti-apoptotic activity of c-FLIP plays an important role in embryonic development.

Beside regulation of apoptosis, other important functions for DED-containing proteins have been reported. Several studies have shown that the DED protein PEA-15 is involved in the regulation of integrin activation, ERK activation, phospholipase D expression and glucose transport.[12–15] Another DED protein, DEDD, was reported to bind to DNA, inhibit RNA polymerase I activity *in vivo* and inhibit transcription in an *in vitro* transcription assay.[16,17] DED-containing proteins FADD, cFLIP, Caspase-8 and vFLIP can also activate NF-κB pathway through their DEDs.[18–20] These findings indicate that DED-proteins are involved in diverse cellular processes ranging from cell survival to cell death.

In order to identify novel cellular DED-containing and DED-interacting proteins, we searched the National Center for Biotechnology Information GenBank expressed-sequence tags (EST) database for sequences encoding uncharacterized DED-containing proteins related to DEDD. We report here the cloning and functional characterization of a previously uncharacterized DED-containing protein designated FLAME-3, which has high homology to DEDD. Using the yeast two-hybrid system we identified hTFIIIC102 subunit of TFIIIC transcription factor as DEDD- and FLAME-3- interacting protein. Consistent with this result we found that DEDD and FLAME-3 can sequester TFIIIC102 in the nuclei of transfected cells. Our results suggest that the interaction of DEDD and FLAME-3 with this subunit may regulate transcription of class III genes.

Results

Cloning, sequence analysis and tissue distribution of FLAME-3

Death effector domain (DED)-containing proteins play important roles in apoptosis and cellular signaling.[1,10,21] To identify new DED-containing proteins, we searched the entire public GenBank™ EST database for sequence homologus to previously known DED-containing proteins, using the tBLASTN program. One human EST clone (AA090354) encoding a partial open reading frame with significant homology to the C-terminal domain of DEDD protein was identified. Based on its sequence, PCR primers were generated and 5′-RACE was performed with a human PBL library. This resulted in cloning of the entire open reading frame of this new protein, which was subsequently named FLAME-3 based on its overall sequence and structural homology with DEDD/FLAME-2. The human FLAME-3 (Figure 1A) is 46.6% identical to DEDD and its domain structure is very similar to that of DEDD (Figure 1B). FLAME-3 has an N-terminal death effector domain (DED, residues 25–102), which is very similar to the DED domains present in other DED proteins such as DEDD, FADD, caspase-8, caspase-10 and c-FLIP (FLAME-1) (Figure 1C). Following the DED, FLAME-3 contains three central nuclear localization sequences (NLSs, residues 104–109, 131–134 and 153–174) and a C-terminal domain (CTD, residues 175–326), which has no significant homology to any known protein except DEDD.

The mouse FLAME-3 was also identified by searching the GenBank™ EST database and a cDNA encoding its entire open reading frame was obtained by overlapping PCR. The human and mouse FLAME-3 proteins share 91% identity with each other (Figure 1A). Interestingly, most of the amino acid substitutions in the human and mouse sequences are present in the linker region, which contain the NLSs between the DED and CTD. The lack of significant substitutions in the sequences of the DED and CTD suggests that the overall structures of these domains are critical for the function of FLAME-3.

A BLAST search of the NCBI high-throughput genome sequences revealed that the human FLAME-3 gene is located on chromosome 19.

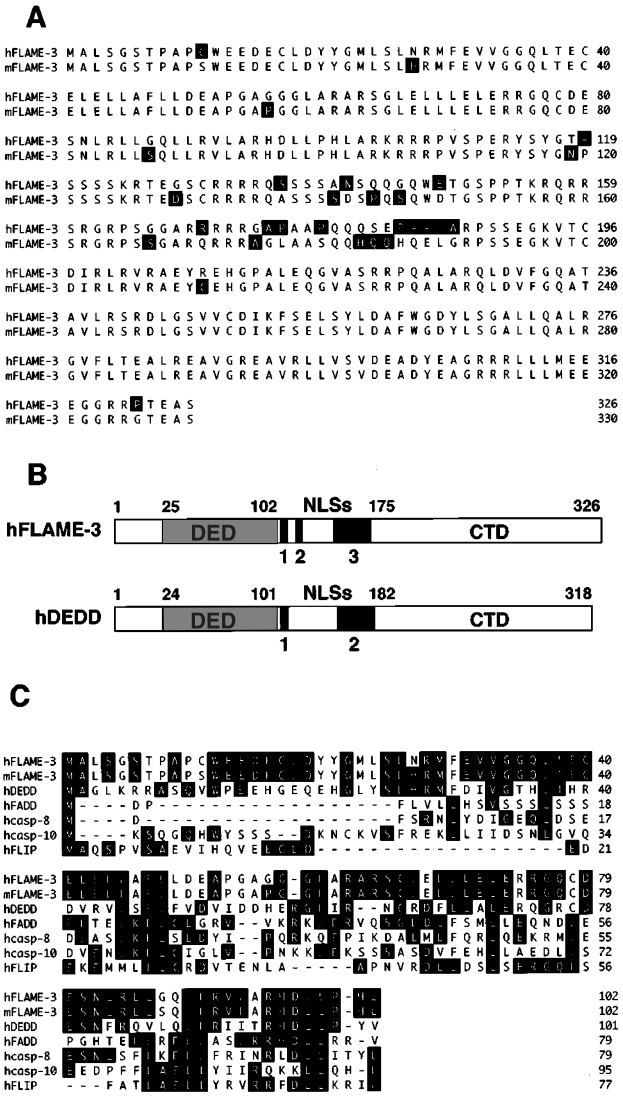

Figure 1 Sequence alignment and domain structure of FLAME-3. (**A**) Co-linear alignment of the predicted amino acid sequences of human and mouse FLAME-3 proteins. Non-identical residues in the two sequences are shaded black. (**B**) Bar diagrams representing the domain structure of human FLAME-3 and DEDD proteins. The three NLSs in FLAME-3 (residues 104–109, 131–134 and 153–174) and two NLSs in DEDD (residues 104–107 and 164–181) are indicated. (**C**) Amino acid sequence alignment of the DED of FLAME-3 with DEDs found in human DEDD, FADD, caspase-8, caspase-10 and c-FLIP. Black shading indicates identical residues

Northern blot analysis of multiple human tissues detected two FLAME-3 mRNA species of 1.6 and 2.0 kilobase pairs (kb) in all tissues examined (Figure 2A). These two mRNA species could result from the use of alternative polyadenylation sites and/or alternative splicing of the FLAME-3 pre-mRNA. The highest level of expression of the FLAME-3 mRNA was found in peripheral blood leukocytes, spleen and testes and lower expression levels were detected in all other tissues tested. The high expression of FLAME-3 mRNA in peripheral blood leukocytes, spleen and testes is reminiscent of that of the DEDD mRNA,[22] suggesting that the two genes are under

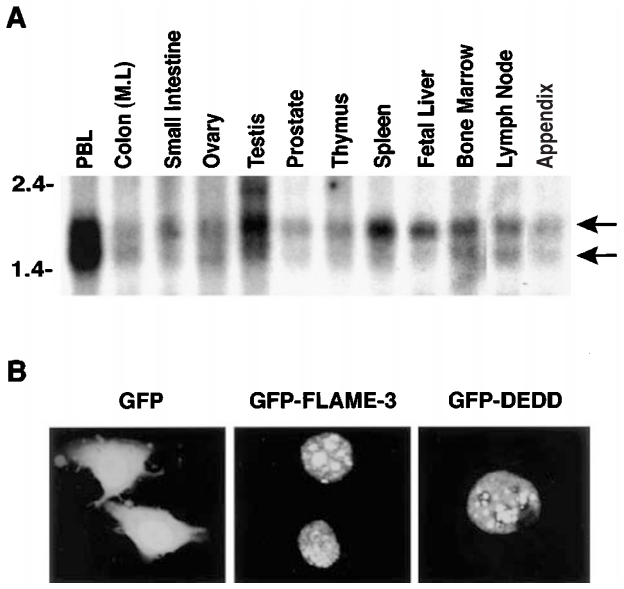

Figure 2 Tissue distribution and subcellular localization of FLAME-3. (**A**) Tissue distribution pattern of the human FLAME-3 mRNA. The Northern blot analysis was performed as described under Materials and Methods. The two major FLAME-3 mRNAs are indicated by arrows. PBL, peripheral blood leukocytes. (**B**) Subcellular localization of GFP–DEDD and GFP–FLAME-3. MCF-7 cells were transfected with 1 lg of expression constructs encoding GFP, GFP–DEDD or GFP–FLAME-3 and 24 h later the cells were fixed and examined under a confocal laser scanning microscope

similar transcriptional regulation and their functions are important in these tissues.

DEDD and FLAME-3 are nuclear proteins

Given that DEDD and FLAME-3 contain typical NLSs between the DED and CTD regions, we tested whether transfection of cells with constructs encoding GFP-tagged DEDD or FLAME-3 would result in targeting of these proteins to the nucleus. As shown in Figure 2B both GFP–DEDD and GFP–FLAME-3 exclusively localized to the nuclei of the transfected cells. Of note, the two proteins accumulated in subnuclear structures resembling nucleoli, suggesting that these proteins may have a role in nucleolar functions.

Interactions of FLAME-3 with other DED-containing proteins

To investigate whether FLAME-3 can interact with other DED-containing molecules, which are involved in the death receptors pathway, we performed co-immunoprecipitation experiments. We co-transfected Flag-tagged FLAME-3 with different T7-tagged DED-containing molecules as indicated in Figure 3A. These experiments showed that FLAME-3 interacts with c-FLIP/FLAME-1 and DEDD, and weakly with caspase-8, but not with caspase-10 or the death receptors adaptor FADD.

To confirm these results we performed *in vitro* GST pull-down experiments with GST-tagged DEDD or FLAME-3 and different ^{35}S-labeled DED-containing proteins. Consistent with the co-immunoprecipitation results, neither GST–DEDD nor GST–FLAME-3 fusion proteins did interact with FADD or caspase-10 (Figure 3B). However, both fusion proteins interacted with c-FLIP and weakly with caspase-8. The *in vitro* interaction experiments also revealed that DEDD and FLAME-3 proteins could homodimerize and heterodimerized with each other as evident from the ability of the GST–DEDD and GST–FLAME-3 to precipitate the ^{35}S-labeled DEDD and FLAME-3 proteins (Figure 3B).

The ability of FLAME-3 to co-immunoprecipitate c-FLIP from cellular extracts expressing the two proteins prompted us to test if the two proteins can indeed interact with each other *in vivo*. To do this we transfected MCF-7 cells with constructs encoding a GFP-tagged c-FLIP and a Flag-tagged FLAME-3 and then visualized the transfected cells by microscopy to see whether FLAME-3 could sequester c-FLIP in the nucleus. Given that FLAME-3 is a nuclear protein and c-FLIP is a cytoplasmic protein we expected to see the GFP-tagged c-FLIP in the nucleus if the two proteins interact with each other *in vivo*. As expected, co-expression of GFP–c-FLIP with FLAME-3 changed its cytoplasmic localization into nucleolar-like structures (Figure 3C). These results indicated that the two proteins do indeed interact *in vivo* with each other.

Taken together these data suggest that FLAME-3, and perhaps DEDD, function distal to components of the death receptor pathway but could interact with and influence the subcellular localization of c-FLIP when overexpressed in mammalian cells.

Identification of TFIIIC102 as DEDD-interacting protein

The exclusive localization of DEDD and FLAME-3 in the nucleus suggests that these proteins could play important roles in nuclear functions. To study the potential role of DEDD proteins in the nucleus we decided to use the yeast two-hybrid system to identify proteins that interact with the full length DEDD. A yeast expression vector was constructed by fusing the cDNAs of the Lex A DNA binding domain to the N-terminus of DEDD in-frame. This bait plasmid was co-transformed into yeast cells with a human brain cDNA library fused to a cDNA cassette encoding the SV40 nuclear localization sequence, the acid Blob B42, and the hemagglutinin epitope tag under the control of the yeast GAL-1 inducible promotor. Out of the 11 positive clones obtained, two clones exhibited strong blue β-Gal staining when co-transformed into yeast cells with the DEDD-bait plasmid but not with the empty bait plasmid (data not shown). Sequence analysis of the two clones revealed that they encode a short isoform of the TFIIIC102 subunit of human TFIIIC transcription factor. The TFIIIC102 short isoform (TFIIIC 102-s) is 413-amino acid long as opposed to the 886-amino acid long full-length human TFIIIC102 (hTFIIIC102) protein (Figure 4A and ref [23]). The short isoform is identical to the full length hTFIIIC102 in the first 406 N-terminal amino acid sequence, and differs only in the last seven C-terminal amino acid sequence from the full-length hTFIIIC102. The mRNA encoding the short isoform of hTFIIIC102 contains an independent stop codon and an unmatched untranslated 3′ region with poly A tail (Genbank accession # AF465407)

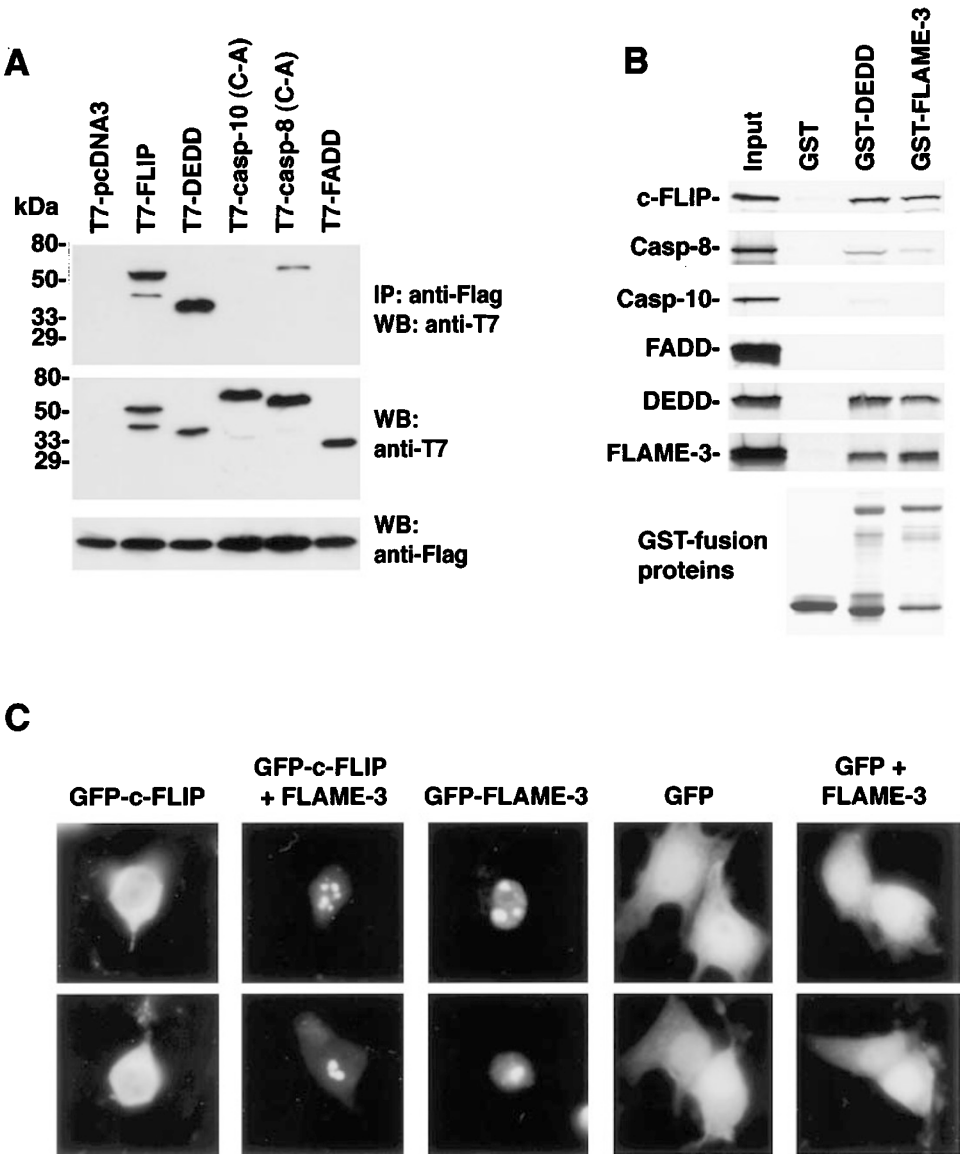

Figure 3 Interactions of FLAME-3 with other DED-containing proteins. (**A**) 293T cells were co-transfected with expression constructs encoding Flag-tagged FLAME-3 and the indicated T7-tagged molecules. Twenty-four hours after transfection, cells were lysed, and the lysates immunoprecipitated with Flag antibody. The immunoprecipitates were immunoblotted with anti T7 antibody (upper panel). The total cell lysates were also immunoblotted with either T7 (middle panel) or anti-Flag (lower panel) antibodies. (**B**) *In vitro* interaction of DEDD and FLAME-3 with other DED-containing proteins. GST, GST–DEDD or GST–FLAME-3 proteins bound to gluthatione-Sepharose beads were incubated with *in vitro* translated ^{35}S-labeled c-FLIP, caspase-8, caspase-10, FADD, DEDD or FLAME-3. The interactions were analyzed by SDS-PAGE and autoradiography. A Coomassie stained gel of the GST-fusion proteins is shown at the bottom. (**C**) Overexpression of FLAME-3 changes the subcellular localization of c-FLIP. MCF-7 cells were transfected with constructs encoding GFP-c-FLIP, GFP-c-FLIP plus T7-FLAME-3, GFP–FLAME-3, GFP or GFP plus T7-FLAME-3 as indicated. After 24 h of transfection, cells were fixed and viewed (100×) using a fluorescence microscope. Data are representative of at least three individual experiments (n=3)

which appears to be the result of the use of alternative splicing and polyadenylation sites in the hTFIIIC102 pre-mRNA.

To rule out that the short isoform is a cloning artifact, we generated sense and antisense primers, where the sense primer corresponds to an identical sequence in the two hTFIIIC102 isoforms and the antisense primer corresponds to a region specific only to the short isoform 3′ untranslated region. The two primers were used to perform a PCR on a human Jurkat cDNA library as a template. The generated PCR products were cloned and sequenced and found to be identical to the cDNA of the hTFIIIC102 short isoform isolated from the human Brain cDNA library by the yeast two-hybrid method.

Since there was no published information on the tissue distribution of hTFIIIC102,[23] we performed Northern blot analysis of multiple human tissues and cancer cell lines using a probe from the coding region of the hTFIIIC102. Figure 4B shows three major transcripts of different sizes (~3.2, 2.5 and 1.2) expressed mainly in the testes, heart and skeletal muscle. The heart contains predominantly the

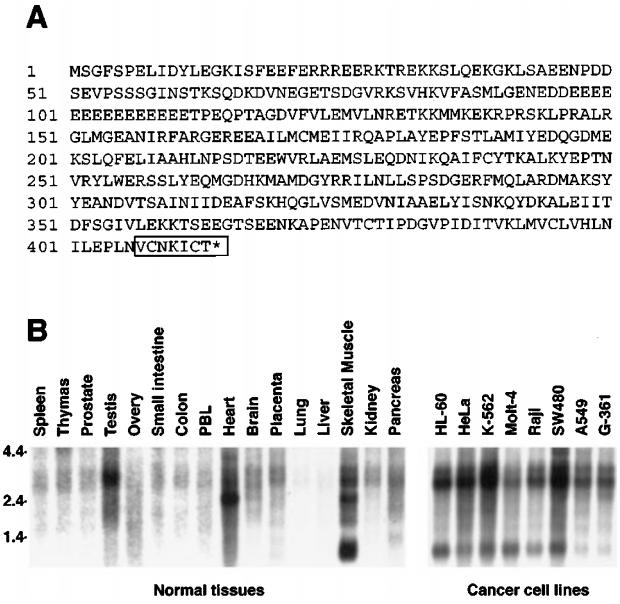

Figure 4 Amino acid sequence of TFIIIC102-s and tissue distribution of hTFIIIC102. (**A**) Predicted amino acid sequence of the short isoform of hTFIIIC102 identified as a DEDD-interacting protein by the yeast two-hybrid method. The last seven residues that do not correspond to residues in the full length hTFIIIC102 are boxed. (**B**) Tissue distribution pattern of the human hTFIIIC102 mRNA in human normal tissues and tumor cell lines. The Northern blot analysis was performed as described under Materials and Methods. HL-60, Promyelocytic leukemia; HeLa, HeLa cell S3; K-562, chronic myelogenous leukemia; MOLT-4, lymphoblastic leukemia; Raji, Burkitt's lymphoma; SW480, colorectal adenocarcinoma; A549, lung carcinoma; G361, melanoma. Numbers on the left indicate kilobases. PBL, peripheral blood leukocyte

2.5 kb transcript, and skeletal muscle has an additional 1.2 kb major transcript. In testis, only the 3.2 kb transcript is observed. These varied transcript sizes indicate tissue specific expression of different isoforms of the protein. The cancer cell lines invariably contain three major transcripts corresponding to 3.5, 3.2 and 1.2 kb. Of note, the 3.2 kb transcript is upregulated in most of the tumor cell lines, suggesting possible involvement in transformation.

DEDD and FLAME-3 interact with hTFIIIC102 *in vitro* and *in vivo*

We next investigated the interaction of DEDD and FLAME-3 with hTFIIIC102 and hTFIIIC102 short isoform by co-immunoprecipitation and GST-pull down experiments. As expected, both hTFIIIC102 and hTFIIIC102 short isoform were precipitated by DEDD and FLAME-3 proteins (Figure 5A,B), indicating that not only DEDD but also FLAME-3 could interact with hTFIIIC102 and its short isoform.

To examine the interactions of DEDD proteins with hTFIIIC102 *in vivo* we transfected MCF7-Fas cells with constructs encoding GFP-tagged hTFIIIC102 or hTFIIIC102 short isoform, together with constructs encoding Flag-tagged DEDD or FLAME-3. The transfected cells were then monitored by fluorescent confocal laser scanning microscopy (Figure 5C). Both GFP-hTFIIIC102 and GFP-hTFIIIC102 short isoform were largely in the cytoplasm. However, in the presence of co-expressed DEDD or

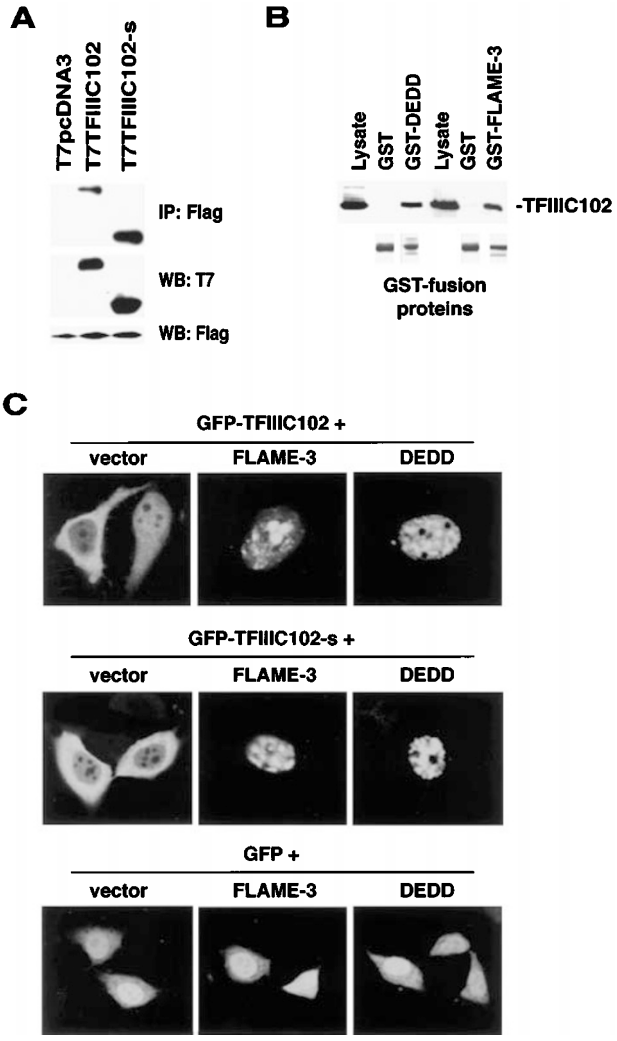

Figure 5 *In vivo* and *in vitro* interaction of DEDD and FLAME-3 proteins with hTFIIIC102. (**A**) 293T cells were co-transfected with expression constructs encoding Flag-tagged FLAME-3 and T7-tagged TFIIIC102 or TFIIIC102-s. Twenty-four hours after transfection, cells were lysed, and the lysates immunoprecipitated with Flag antibody. The immunoprecipitates were immunoblotted with anti T7 antibody (upper panel). The total cell lysates were also immunoblotted with either T7 (middle panel) or anti-Flag (lower panel) antibodies. (**B**) 293T cells were transfected with an expression plasmid encoding T7-tagged TFIIIC102 and 36 h after transfection, cells were lysed, and the lysates precipitated with either GST, GST – DEDD or GST – FLAME-3. The pellets and corresponding cellular lysates were analysed on SDS – PAGE and immunodetected with horse-radish peroxidase-conjugated T7 antibody. A Coomassie stained gel of the GST-fusion proteins is shown at the bottom. (**C**) Overexpression of DEDD or FLAME-3 sequesters TFIIIC102 and TFIIIC102-s in the nucleus. MCF-7 cells were transfected with constructs encoding GFP-TFIIIC102 (upper panels), GFP-TFIIIC102-s (middle panel) or GFP (Lower panel) together with empty vector (left panels) or constructs encoding T7-tagged DEDD (right panels) or FLAME-3 (middle panels). After 24 h of transfection, cells were fixed and viewed using a confocal laser scanning microscope. Data are representative of at least three individual experiments (n=3)

FLAME-3 both GFP-hTFIIIC102 and GFP-hTFIIIC102 short isoform were sequestered in the nucleus in neucleoli-like structures similar to those observed with GFP – DEDD or GFP – FLAME-3. These observations provide clear evi-

dence that the DEDD proteins interact with hTFIIIC102 in cells and may regulate its transport from the cytoplasm to the nucleus under physiological circumstances.

Overexpression of DEDD and FLAME-3 inhibits NF-κB-regulated gene expression

hTFIIIC is a multi-subunit general transcription complex that mediates transcription of class III genes.[23–25] hTFIIIC directly recognizes promoters for tRNA and virus-associated RNA genes or promoter-TFIIIA complexes and subsequently recruits TFIIIB and RNA polymerase III to this complex.[24,25] Given that DEDD and FLAME-3 can bind and sequester TFIIIC102 in the nucleus, we asked whether their overexpression in human cell lines could interfere with the general transcription machinery. To test this hypothesis, we utilized the NF-κB-luciferase reporter plasmid to measure the level of luciferase activity in cells treated with TNF-α in the presence or absence of overexpressed DEDD or FLAME-3. As shown in Figure 6, TNF-α treatment of the vector transfected control cells resulted in fivefold induction of luciferase activity compared to twofold in the DEDD or FLAME-3-expressing cells. These results indicate that overexpression of DEDD and FLAME-3 inhibits the expression of NF-κB-regulated genes, perhaps by sequestering TFIIIC102, resulting in a decrease in the activity of the TFIIIC complex. Overexpression of DEDD or FLAME-3 was also able to inhibit yeast GAL-4-induced luciferase activity in 293 cells, as determined with a GAL-4-luciferase reporter plasmid (data not shown). Combined, our data indicate that overexpression of DEDD or FLAME-3 inhibits the general transcription machinery.

The ability of DEDD and FLAME-3 to inhibit NF-κB-regulated gene expression might be responsible for the weak apoptotic activity observed in two different studies with overexpressed DEDD and DEDD-2/FLAME-3.[16,26] However, in our hands we were unable to detect significant apoptosis induction or caspase activation by the overexpressed FLAME-3 compared to other apoptotic proteins such as FADD or caspase-8 (Figure 7A,B). The FLAME-3-expressing cells had only 10% more apoptosis than the control vector-transfected cells 72 h after transfection. FLAME-3 and DEDD were also unable to significantly potentiate cell death by Fas, TRAIL or TNF (Figure 7C). This indicates that the primary function of the DEDD proteins might not be the regulation of apoptosis *per se* but most probably they are involved in the regulation of general transcription. This is consistent with a recent finding that DEDD inhibits RNA polymerase I dependent transcription and its weak apoptotic activity is dependent on its localization to the nucleus.[17] Targeting of the DED region of DEDD-2/FLAME-3 was also found to be necessary for its weak pro-apoptotic activity.[26] Taken together, these ob-

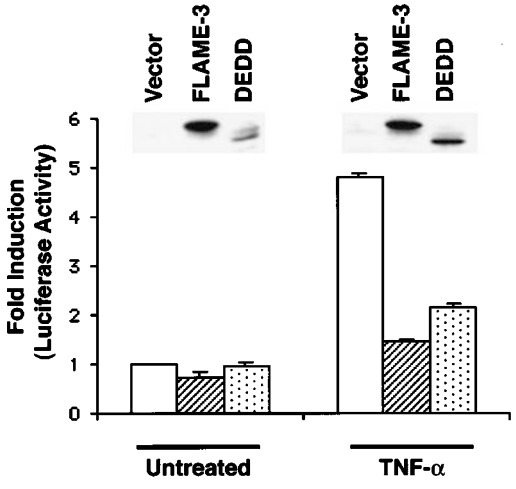

Figure 6 FLAME-3 and DEDD inhibit NF-κB-regulated transcription. A, 293 cells were transfected with 5× NF-κB-Luciferase reporter together with either empty vector or expression constructs for Flag–DEDD or Flag–FLAME-3. Twenty-four hours after transfection, cells were either left untreated or incubated with TNF-α for 5 h. Cells were then collected and lysed, and the luciferase activity in the cell lysates was determined. pRSC–LacZ was included in all transfection reactions to normalize the transfection efficiency. The data represents three different experiments

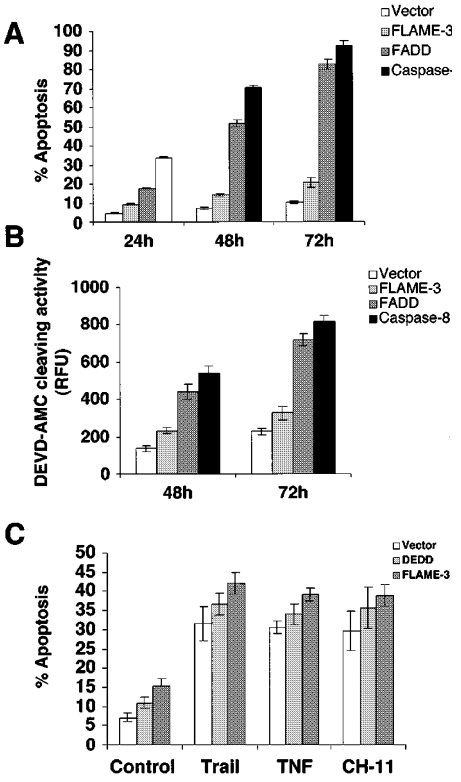

Figure 7 (**A**) Apoptotic activity of FLAME-3 compared with FADD and caspase-8. MCF-7-Fas cells were transfected with empty vector or expression constructs encoding FLAME-3, FADD or caspase-8 together with a GFP-reporter plasmid. The percentages of apoptotic cells were determined after 24, 48 and 72 h after transfection as described in the Materials and Methods. (**B**) 293 cells were transfected with empty vector or expression constructs encoding FLAME-3, FADD or caspase-8 and the cells were lysed 48 and 72 h after transfection. The DEVD–AMC cleaving activities of the cellular extracts were determined with the fluorogenic substrate DEVD-AMC (50 μM) by fluorometry. (**C**) MCF-7-Fas cells were transfected with the indicated expression constructs (vector, DEDD, FLAME-3). Twenty-four hours after transfection the cells were left untreated (control) or treated with TRAIL, TNF-α or the agonist anti-Fas antibody CH-11 as indicated for 5 h. The percentage of round apoptotic cells was determined as described under Materials and Methods

servations suggest that the overexpressed DEDD or FLAME-3 proteins could induce apoptosis from within the nucleus perhaps by sequestering important components of the general transcription machinery such as hTFIIIC102. Therefore, we believe that the inhibition of the RNA polymerase I-[17] and RNA polymerase III- (a component of the TFIIIC complex) dependent transcription by the overexpressed DEDD or FLAME-3 proteins might be responsible for the observed inhibition of the NF-κB-regulated gene expression (Figure 6). This is expected to have serious consequences on cell survival and could ultimately lead to cell death.

Discussion

We have identified and characterized a novel nuclear DED-containing protein, FLAME-3, and its interacting proteins cFLIP and hTFIIIC102. FLAME-3 contains an N-terminal DED region, three central NLSs and a C-terminal domain highly related to that of DEDD. Based on Northern blot analysis, FLAME-3 mRNA is expressed mainly in PBL, spleen and testis, a distribution that is very similar to that of DEDD. Of note, FLAME-3 can heterodimerize with DEDD and thus these proteins could function as heterodimers in these tissues. FLAME-3 and DEDD can interact with c-FLIP and to a lesser extent with caspase-8, but not with FADD or caspase-10, suggesting that the DEDD and FLAME-3 proteins function distal to the death receptor-signaling pathway. Given that overexpression of DEDD or FLAME-3 did not have a significant potentiation effect on death receptor induced apoptosis, the significance of the interaction with c-FLIP, which is an antagonist to the death receptor pathway, is not clear at present.

DEDD and FLAME-3 were found to interact with the hTFIIIC102 subunit of the TFIIIC transcription factor by the yeast two hybrid analysis, *in vitro* GST-pull down and *in vivo*. Given the nuclear localization of the DEDD proteins, this finding suggests that these proteins could be important regulators of the TFIIIC transcriptional complex. hTFIIIC102 is an important component of the TFIIIC transcription machinery.[23–25] hTFIIIC102 is one of the five hitherto identified subunits of transcription factor hTFIIIC2 which, in association with TFIIIB, is responsible for the initiation of RNA polymerase III mediated transcription of group III genes in eukaryotes. The hTFIIIC102 is a 886 amino acid long protein with distinct domains. The N-terminal region of hTFIIIC102 has nine TPR motifs and these domains have been shown to interact with the hTFIIIB90, TBP and hTFIIIC63.[23] Thus the TFIIIC102 subunit is important for recruitment of RNA polymerase III via TFIIIB interaction and for stable complex formation.

Previous studies have shown that TFIIIC is located in both the cytoplasm and nucleus.[27,28] Endogenous DEDD has also been found to be mainly localized to the cytoplasm and is translocated to the nucleus upon stimulation of CD95/Fas.[16] Our finding that co-expression of hTFIIIC102 with either DEDD or FLAME-3 appears to induce translocation of hTFIIIC102 to the nucleus, suggests that the DEDD proteins may regulate hTFIIIC102 transport across the nuclear membrane under normal physiological circumstances. Thus, the DEDD and FLAME-3 proteins could function as chaperones for the hTFIIIC102 to translocate it across the nuclear membrane into the nucleus to be recruited to the TFIIIC transcription complex.

The ability of DEDD proteins to inhibit transcription (our data and ref [16,17]) when overexpressed in mammalian cells, suggest that their interaction with and sequestration of hTFIIIC102 could be the reason for this inhibition. This could also explain the inhibition of NF-κB regulated gene expression by the overexpressed DEDD and FLAME-3 proteins. This inhibition might also be the reason for their observed weak apoptotic activity. It is noteworthy to mention that there are examples of association of apoptosis related proteins with transcription factors.[29] BAG-1, an antiapoptotic protein, interacts with a number of nuclear hormone receptors including receptors for glucocorticoid, estrogen and thyroid hormone.[29] BAG-1 can regulate retinoid activities through its interaction with RAR and suggests that elevated levels of BAG-1 could potentially contribute to retinoid resistance in cancer cells.[29] Thus, it would be interesting in the future to fully characterize the biological significance of the interaction between the DEDD and FLAME-3 proteins and hTFIIIC102, and whether any of these proteins might be de-regulated in cancer cells.

Materials and Methods

Identification and cloning of FLAME-3

ESTs encoding human and mouse FLAME-3 were identified by searching the National Center for Biotechnology Information GenBank expressed-sequence tags (EST) database, for sequences homologous to DEDD. The EST clones (human Genbank accession # AA090354) and (mouse Genbank accession # AA511237) were found to have significant homology to DEDD, but were truncated at the 5′ regions. These clones were obtained from the IMAGE consortium, and their entire nucleotide sequences were determined by automated sequencing. The human and mouse FLAME-3 5′-coding regions were obtained by RACE, and their full-length open reading frames were generated by overlapping PCR.

Expression vectors

Constructs encoding full length DEDD, FLAME-3, TFIIIC102 and TFIIIC102-s were generated by PCR using modified complementary PCR adapter-primers. Flag- and T7- epitope tagging was done by cloning the PCR generated cDNAs of the respective genes in-frame into pFLAG CMV-2 (IBI Kodak), and pcDNA3 (Invitrogen) with T7-tag vectors, respectively. Plasmids encoding GFP fusions were constructed using pEGFP-N1 and pEGF-C1 (CLONTECH). The expression constructs for T7-tagged Flame-1/cFLIP, caspase-8, caspase-10 and FADD were described previously.[30,31] Full length DEDD and FLAME-3 were overexpressed in *E. coli* strain DH5α as N-terminally GST-fusion proteins using a pGEX vector (Pharmacia).

Northern blot analysis

Tissue distribution analysis of human FLAME-3 and TFIIIC102 mRNA was performed by Northern blot analysis on normal and tumor MTN Blots (purchased from CLONTECH). Each lane contains 2 μg of

poly(A)$^+$ RNA from specific tissues or cell lines. The blots were probed with a radiolabeled riboprobe prepared from a full-length human FLAME-3 or TFIIIC102 cDNA template and then subjected to autoradiography.

Cell culture

293T and MCF7 cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide in either Dulbecco's modified Eagle's medium (DMEM)/F12 or RPMI1640 (Life Technologies, Inc) containing 10% fetal bovine serum, 200 mg/ml penicillin and 100 mg/ml streptomycin sulfate.

Transfection, immunoprecipitation, and Western blot analysis

Transient transfection of 293T cells was performed as described previously.[32] In brief, cells were transfected with the indicated Flag-tagged and T7-tagged expression plasmids (8 μg/10 cm plate) using the LipofectAMINE (Life Technologies, Inc.). Cell lysates were prepared in lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% NP-40 and freshly added 1 mM PMSF) 24–30 h after transfection, and immunoprecipitated with anti-FLAG mAb M5. The precipitates were resolved by SDS polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-T7 antibody, to monitor the expression level of the Flag- and T7-tagged proteins. Western blot analyses were performed on total cellular extracts with either anti-Flag M5 or anti-T7 antibodies.

Fluorescence microscopy

MCF-7-Fas cells were grown on glass coverslips in 12 well plates. Cells were transfected 24 h later, with constructs encoding GFP fusion proteins. Twenty-four hours after transfection, the cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature. After washing twice with PBS, the coverslips were mounted on a glass slide and the fluorescence of GFP was detected by Fluorescent or confocal microscopy using excitation wavelength of 488 nm and detection wavelength of 522 nm.

Yeast two-hybrid screening

The human brain cDNA matchmaker libraries in pJG4-5 (*TRP1*, 2 μm, Ap^r) were purchased from Clontech. The yeast two-hybrid screens were performed according to the manufacturer's procedures. In brief, the full-length DEDD open reading frame was cloned in pEG202 (*HIS3*, 2 μm, Ap^r) in-frame with Lex A expression sequence. The PSH18-34 LacZ reporter plasmid (*URA3*, 2 μm, Ap^r) was used for the interaction assays. LEU 2-selection yeast strain EGY-48 (*MAT*α, *trp1*, *his3*, *ura3*, *6ops-LEU2*) was used as the host in the interaction assays. The three vector primary transformants were plated onto Gal/Raff/CM, -Ura, -His, -Trp, -Leu plates and incubated for 5 days at 30°C. All positive growing colonies were further screened on X-gal plates to test for β-galactosidase expression. The cDNA inserts in the positive library clones were sequenced by using vector specific primer. The interacting clones were further confirmed by *in vitro* and *in vivo* interaction studies.

In vitro interaction assay

The GST–DEDD and GST–FLAME-3 fusion proteins were expressed in bacteria and then bound to glutathione-Sepharose beads. ^{35}S-labeled DED-containing proteins were generated by *in vitro* translation with TNT reticulocyte lysates (promega). T7-hTFIIIC102 was generated by transfecting an expression construct encoding T7-hTFIIIC102 into 293T cells. Extracts were prepared, 24 h later, from the transfected cells in a GST-binding buffer (20 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 100 mM NaCl, 1% Triton and freshly added 1 mM DTT and 1 mM PMSF). The ^{35}S-labeled lysates or the 293 extracts were pre-cleared by mixing with glutathione-Sepharose beads and subjected to centrifugation. The supernatants were incubated with GST–DEDD or GST–FLAME-3 immobilized on glutathione Sepharose beads for 2 h at 4°C. After extensive washing, the interacting proteins were recovered by boiling the beads in SDS sample buffer and analyzed on 12% SDS–PAGE. The ^{35}S-labeled proteins were visualized by autoradiography, while the T7-tagged hTFIIIC102 was visualized by immunoblotting with anti-T7.

NF-κB reporter gene assay

For transient reporter gene assay, 293T cells were seeded in 12-well tissue culture plates, and the following day the cells were transfected with 5XκB-luciferase reporter and pRSC–LacZ plasmids and expression plasmids for DEDD or FLAME-3 using the LipofectAMINE^{TM} method as per the manufacturer's instruction. Twenty-four hours after transfection, cells were treated with hTNF-α for 5 h prior to harvesting, and then harvested and subjected to luciferase assay as described in Lin *et al*.[33] To normalize for transfection efficiency, all lysates were assayed for β-galactosidase activity. Luciferase activity was determined using luciferase assay system (Promega) and a FB15 luminometer (Zylux Corporation, USA). Data represent the average of at least three different individual experiments.

Apoptosis assays

The ability of FLAME-3 to induce apoptosis was assayed by transfecting human MCF-7-Fas cells (1×10^5 cells/well) in 6-well plates with 0.4 μg of pEGFP-N1 reporter plasmid (Clontech), 1.0 μg of empty vector or constructs encoding FLAME-3, FADD or procaspases-8 using the LipofectAMINE^{TM} method. Cells were stained with propidium iodide and DAPI stains. Normal and apoptotic GFP-expressing cells were counted after 24, 48 and 72 h after transfection using fluorescence microscopy. The percentage of apoptotic cells in each experiment was expressed as the mean percentage of apoptotic cells as a fraction of the total number of GFP expressing cells. The ability of DEDD or FLAME-3 to potentiate TRAIL-, TNF-α-, or Fas-induced apoptosis was assayed by transfecting human MCF-7 cells (1×10^5 cells/well) in 6-well plates with 1.2 μg of empty vector, or DEDD or FLAME-3 expression construct plus 0.3 μg pEGFP-N1 plasmid as above. Twenty-four hours after transfection cells were treated with TNF (10 ng/ml), TRAIL (1 μg/ml) or CH-11 antibody (100 ng/ml) for 5 h and the percentages of GFP-positive, DAPI-positive apoptotic cells were determined as above.

Accession numbers

The nucleotide sequence(s) reported here has been submitted to the GenBank^{TM}/EBI Data Bank (accession numbers AF457575, AF457576 and AF465407).

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