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Review

Death Effector Domain-Containing Proteins

M. Gudur Valmiki[a,b] and J. W. Ramos[b,*]

[a] Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, 1955 East-West Road, Honolulu, Hawaii 96822 (USA)
[b] Department of Natural Products and Cancer Biology, Cancer Research Center of Hawaii, University of Hawaii at Manoa, 651 Ilalo Street, Honolulu, Hawaii 96813 (USA), Fax: +1-808-587-0742,
e-mail: jramos@crch.hawaii.edu

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Abstract. Death effector domains (DEDs) are protein-protein interaction structures that are found in proteins that regulate a variety of signal transduction pathways. DEDs are a part of the larger family of Death Domain structures that have been primarily described in the control of programmed cell death. The seven standard DED-containing proteins are fas associated death domain protein (FADD), Caspase-8 and 10, cellular FLICE-like inhibitory protein (c-FLIP), death effector domain containing DNA binding (DEDD), DEDD2 and phosphoprotein enriched in astrocytes 15-Kda (PEA-15). These proteins are particularly associated with the regulation of apoptosis and proliferation mediated by the tumor necrosis factor α (TNFα) receptor family. Consequently DED-containing proteins are reported to regulate transcription, migration, and proliferation, in addition to both pro and anti-apoptotic functions. Moreover, DED proteins are essential in embryonic development and homeostasis of the immune system. Here we focus on the role of DED-containing proteins in development and the pathologies arising from abnormal expression of these proteins.

Keywords. Death effector domain, apoptosis, proliferation, signal transduction, migration, caspase, FADD.

Introduction

Death effector domains (DEDs) were originally described as protein-protein interaction domains involved in death receptor initiated programmed cell death or apoptosis. The DED has no enzymatic activity but rather acts as a binding partner for other DEDs, thereby promoting the formation of protein complexes. In these pathways ligation of a member of the tumor necrosis factor receptor (TNFR) family (eg. Fas or TNFR1) by its cognate ligand induces the recruitment of the linker protein FADD which mediates the aggregation of DED-containing proteases called caspases (caspase-8 or -10). Aggregation leads to the activation of the caspases by proteolytic cleavage, mediated by the caspases themselves. The fully activated caspases then target the executioner caspases, such as caspase-3, that in turn mediate cell death by proteolysis of multiple substrates [1, 2]. It was subsequently found that some DED-containing proteins such as c-FLIP and PEA-15 inhibit apoptosis by binding to FADD or caspase-8 and thereby blocking recruitment of the caspases into aggregates [3–5]. Since these initial observations, it has become clear that DED family proteins are also important regulators of cell proliferation [6], transcription [7, 8] and migration [9, 10]. In these functions the DED also

* Corresponding author.

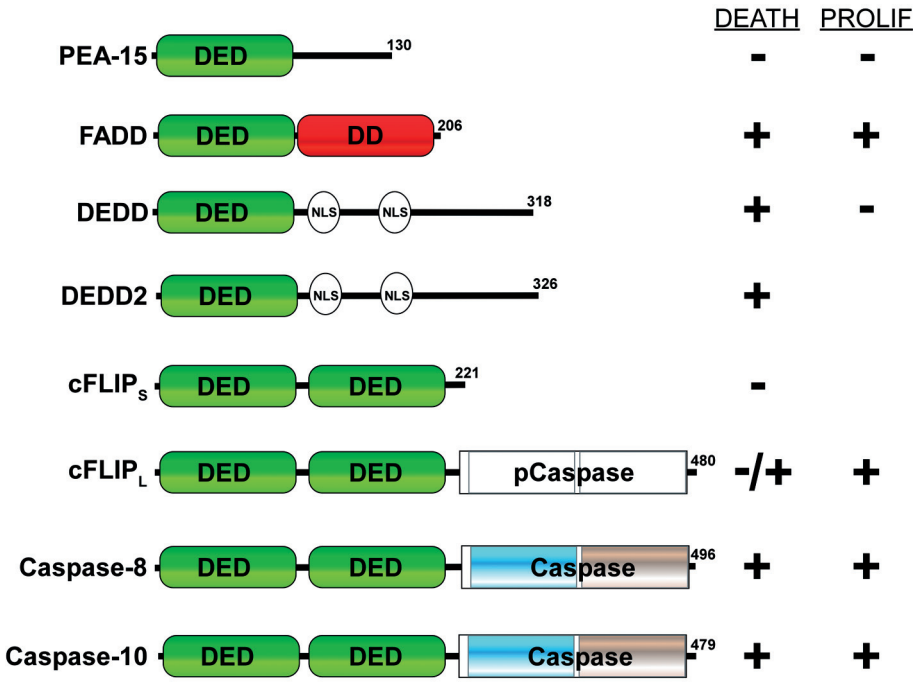

Figure 1. Death effector domain containing proteins. DED family proteins are depicted using domain diagrams. The ability of the protein to activate or inhibit apoptosis and proliferation is indicated. DED is the death effector domain; DD is the death domain, NLS is the nuclear localization sequence. Caspase and pseudocaspase (pCaspase) domains are shown. The length in amino acids is also listed.

appears to work by mediating the formation of protein complexes. In addition, the DED family (Fig. 1) has been expanded beyond the seven proteins containing traditional DEDs to include related proteins containing variant or pseudo-DEDs such as HIPPI, HIP, BAR, Bap31, FLASH and Dap3 [11]. DED proteins therefore constitute a multifunctional group of signal transduction proteins that regulate life and death decisions in the cell. Given the tremendous increase in the available information concerning the mechanism of action and biological importance of DED proteins, our intention is to give a broad overview of the current state of the field. It is our hope that in so doing, we will affirm the multifunctional nature of these proteins in cellular pathways beyond programmed cell death.

Death effector domain structure

The ligated Fas receptor in complex with the linker protein FADD and the recruited, aggregated procaspase-8/10 constitute the death initiating signaling complex (DISC). The formation of the DISC is facilitated through homotypic domain interactions mediated by death domains (DDs) and DEDs [11, 12]. These domains belong to the DD super family comprising the DD, the DED, the Caspase Recruitment Domain (CARD) and the pyrin domain families [13, 14]. Although the sequence similarity of the DD super family is very low (5–25 %), these domains share a common structure consisting of an amphipathic, antiparallel bundle of six α helices in a greek key conformation [15] (Fig. 2). In spite of their common structure, these domains differ in that CARD [16] and DD [17] homotypic interactions are mediated by electrostatic patches, whereas DED–DED interactions appear to be of a predominantly hydrophobic nature [18].

A classification method has been proposed for DED proteins based on the high diversity of the α3 helix. In this system, Class I DED proteins are those that contain the basic amino acid motif KRKLH in the α3 helix. Members of class I share high sequence similarity with the DED of FADD (Fas-associated death domain) and possess basic residues in the α3 helix (residues 33–35 in FADD). The DED of FADD, both DEDs of caspase-8, the N-terminal DED of caspase-10 and the C-terminal DED of c-FLIP belong to this class. Interestingly, functional assays using mutants of FADD revealed that the α3 basic region (KRKLH) influences binding and recruitment of caspase-8 and c-FLIP to the DISC [19]. In Class II DEDs these basic amino acids are absent and α3 is comparatively short [19]. Class II DEDs include the C-terminal DED of caspase-10, the N-terminal DED of c-FLIP, the DED of PEA-15 and most of the viral DEDs (Fig. 2).

Many DEDs possess a characteristic hydrophobic surface formed by conserved residues adjacent to a phenylalanine in the α2 helix (F25 in hFADD) and including regions in the α3 helix [18]. This hydrophobic patch mediates many homotypic DED interactions such as the binding of caspase-8 and c-FLIP to FADD [20, 21]. Interestingly, PEA-15 completely

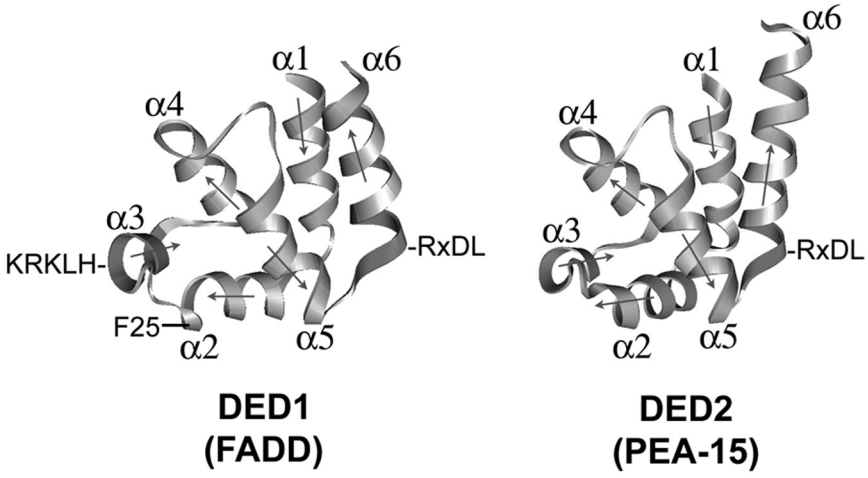

Figure 2. Death effector domain structure. Shown are ribbon diagrams of the structure of the FADD-DED (Class I DED) and the PEA-15-DED (Class II DED). Both DEDs are depicted in the same orientation and each of the 6 α-helices is labeled. The highly conserved RxDL motif in α-helix 6 and the Class I-DED specific KRKLH interaction surface in α-helix-3 are indicated. Structures are derived from data in the NCBI structure database (see also [24, 165]).

lacks this sequence and therefore presumably interacts with FADD through an alternative mechanism. Most DEDs also contain an electrostatic surface in the α6 helix [19, 22]. In particular, the RxDL sequence (position 78–81; Fig. 2) in α6 is highly conserved among DEDs. The RxDL motif is essential for the ability of the molluscum contagiosum virus (MCV) protein MC159 to block apoptosis [22] and for PEA-15 to interact with the extracellular signal-regulated kinase-mitogen-activated protein (ERK MAP) kinase [7]. In addition this sequence has been proposed to mediate dimerization of some DED proteins such as FADD [23].

Caspases-8 and 10 along with c-FLIP and v-FLIP contain two DEDs in tandem. The first structures of these tandem DED repeats have now been solved and have provided a few surprises. The tandem DEDs of the v-FLIP, MC159, form a stiff, barbell-like structure in which the two DEDs are tightly associated by hydrophobic interactions mediated in part by the conserved phenylalanine-leucine (FL) charge patch in DED1 [21, 25]. While the structure of DED2 is highly related to that of the DEDs from FADD and PEA-15 (Fig. 2), the structure of DED1 is highly divergent. The DED1 is missing the equivalent helix 3 which is instead replaced by a short loop. Moreover, DED1 contains two additional helices with one at the N terminus (α0) and the other at the C terminus (α7). Indeed only about half of the residues of DED1 align topologically with those of the MC159 DED2 and the DEDs of FADD and PEA-15 [21, 25]. Fas DD, FADD and MC159 assemble cooperatively as a ternary complex (as do Fas DD, FADD and Caspase 8). The structure of the tandem DEDs of caspase-8 is predicted to be similar to that of MC159 [21]. Using mutational analysis, Yang and colleagues showed that while the conserved hydrophobic patch of the caspase-8 DED1 (containing the FL sequence) interacts with its DED2, the corresponding region in the caspase-8 DED2 binds to FADD to mediate recruitment of the caspase to the DISC. In contrast, MC159 lacks this conserved hydrophobic sequence in the DED2. Instead, MC159 binds to a hydrophobic patch in FADD (RxDL) via a conserved charge triad that includes the RxDL motif from both MC159 DEDs. In the absence of MC159, FADD self-associates. This is required for FADD mediated apoptosis [20, 23, 26]. There is a discrepancy in the literature as to whether FADD self association is mediated by F25 [20] or the RxDL motif [23]. It appears that mutations in the RxDL sequence render the mutant DED unstable as measured by NMR [20]. Thus it may be that the sequences adjacent to F25 may indeed mediate this interaction. This remains to be clarified. Nevertheless, there is agreement that the binding of MC159 to FADD prevents FADD self-association and thereby prevents higher order oligomerization and subsequent activation of caspase-8 in the DISC. This explains how MC159 reduces the size of signaling protein oligomeric transduction structures (SPOTS) during Fas activation and is consistent with the observation that it blocks the formation of FADD death effector filaments (DEFs) [22]. Thus, MC159 does not prevent caspase-8 recruitment to the DISC, but rather prevents the FADD-mediated higher order oligomerization. In contrast other FLIPS, including the c-FLIPs and the v-FLIP E8 (from equine herpesvirus-2), contain the conserved hydrophobic patch and thus compete with caspase-8 for recruitment to the DISC [25, 27].

DED-containing proteins are found only in vertebrates [28]. The closest functional relatives in a non-vertebrate are the drosophila caspase DREDD (death-related CED-3/Nedd2 like protein) and adaptor dFADD. However these do not contain

true DEDs, but rather have pseudo DEDs. Moreover, some DEDs are highly conserved between different species. For example, the DEDD protein and PEA-15 are nearly identical in sequence between human, mouse, and rat. Indeed, this high level of identity for PEA-15 extends to fish (at least three species including Zebrafish) and frog (both *X. laevis* and *X. tropicalis*). Given these observations, it is tempting to speculate that the DED may have evolved fairly recently from an earlier domain such as that in dFADD [29]. This corresponds well to the development in vertebrates of highly developed immune, vascular and nervous systems where DED proteins appear to have their primary function. However, it should also be noted that DED proteins appear to be part of the 20% of genes deleted in birds [30].

Death effector domain family proteins in apoptosis

The function of DED proteins in apoptosis has been extensively reviewed elsewhere [31, 32] and so will only be covered briefly here. Apoptosis is mediated by the aggregation of the various component proteins, due to the homotypic binding of the structurally similar DD, DED, CARD, and pyrin domains. As described above, binding of death receptors such as Fas/CD-95, tumor necrosis factor related apoptosis initiating ligand (TRAIL) and TNFα to their ligands results in recruitment of the adaptor protein FADD. FADD is non-enzymatic and consists of an N-terminal DD and a C-terminal DED (Fig. 1). In the case of Fas, FADD recruitment is mediated by the interaction of the DD and portions of the DED of FADD with the DD of the cytoplasmic tail of trimeric Fas [33]. FADD is similarly recruited to the activated TNF-R1 complex (and the death receptor 3 (DR3) or TRAIL1 and 2) through the interaction of its DD with that of the tumor-necrosis factor receptor-1-associated death domain protein (TRADD) [34, 35]. Once recruited to the death receptor, FADD self-associates to induce the formation of higher order complexes required for activation of caspases and apoptosis [21, 23, 26]. This self-association and higher order structure can be visualized by high-resolution confocal microscopy as SPOTS consisting of ligated Fas and recruited FADD [36]. FADD appears to be essential for all death receptor activation of caspases. The DED of FADD binds the DED2 domain of the initiator procaspases-8 or -10 [25, 37]. The resultant plasma membrane complex is called the DISC and initiates the apoptotic cascade (Fig. 3) [15]. Caspases are cysteine proteases that exist as inactive zymogens, or procaspases, that are activated by cleavage at specific sequences (for reviews see [1, 37, 38]). Aggregation or induced proximity of caspase-8 causes it to autocatalytically process itself at two sites (REQD and VETD) thus removing the DEDs and releasing two large (p20) and two small (p10) domains that form homodimers. These homodimers constitute the active caspase. Caspase-8 and -10 are closely related proteins that can activate apoptosis downstream of FADD by cleavage and activation of the executioner caspase-3. Caspase-3 then causes apoptosis by proteolytic degradation of several cellular proteins such as gelsolin and caspase-activated DNAse (CAD). Whether caspase-10 can replace caspase-8 is unclear, however caspase-10 can in some cases act independently of caspase-8 to initiate apoptosis by the same mechanism [39]. Caspase-8 has alternatively been shown to activate apoptosis by cleaving Bid, a Bcl2 family member, to form truncated Bid (tBid). This activated tBid translocates to the mitochondrial membrane where it joins Bax and Bak to form a pore, thereby promoting the release of cytochrome C into the cytoplasm and subsequent activation of intrinsic or mitochondrial-mediated apoptosis which involves the aggregation and auto-activation of Capase 9 (which contains CARD structures in place of the DED domains of Caspase 8) [31]. These data are summarized in Figure 3.

An alternative mechanism for DED protein induction of apoptosis has been described for DEDD and DEDD2. DEDD and DEDD2 both contain nuclear localization signals and are targeted to the nucleolus where they activate apoptosis [40, 41]. The mechanism by which they activate apoptosis has been attributed to several effects. Nuclear localization of DEDD induces apoptosis in part by causing activation of nuclear caspase-6 and inhibition of RNA polymerase I-dependent transcription [42]. The activation of caspase-6 is dependent on nuclear translocation of DEDD as mutants of DED lacking the NLS signals do not activate caspase-6 or apoptosis. Nuclear DEDD activation of apoptosis is also potentiated by DEDD binding to DED-associated factor (DEDAF) [43]. A second mechanism by which DEDD activates apoptosis involves binding to caspase-3 in the cytosol. Both mono and diubiquitinated forms of DEDD are predominantly cytoplasmic and bind the intermediate filament network (K8/18) and active caspase-3. In this way DEDD acts as a scaffold to enhance caspase-3 cleavage of K18 and degradation of the intermediate filament network [44]. Finally DEDD and DEDD2 are also reported to activate apoptosis by binding and activating caspases-8 and -10. Significantly, this report further suggests that caspase-8 translocates with DEDD into the nucleus [45]. DEDD and DEDD2 remain perhaps the most inscrutable of the DED

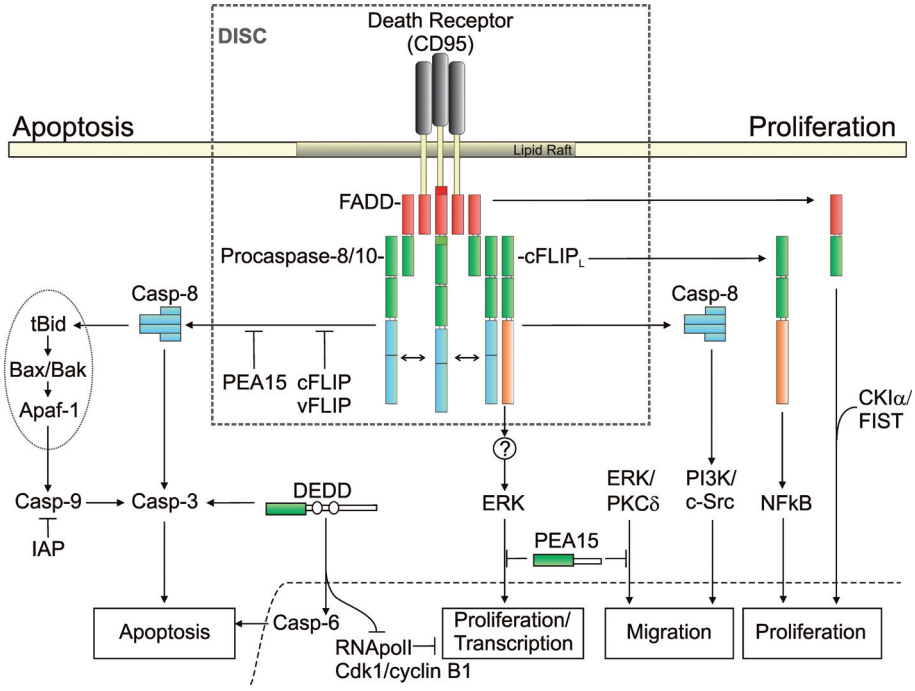

Figure 3. The activation of apoptosis and proliferation by death receptors. DED-containing proteins function in several signal transduction pathways and can thereby regulate apoptosis, proliferation, transcription, and migration. Some of the better characterized pathways are indicated. In this case, an activated CD95 death receptor is shown for simplicity. Other death receptor pathways are similar. DEDs are shown in green, DDs are shown in red, and caspase domains are shown in blue. Activation or inhibition is indicated. The protein complex that constitutes the death initiating signaling complex is defined in the dashed box. See the text for further details.

proteins with respect to apoptosis given the many mechanisms reported.

Two DED protein family members, c-FLIP and PEA-15, prevent apoptosis by blocking FADD recruitment and aggregation of caspase-8 or-10. The FLIPs were first shown to function in this way. There are both viral FLIPs (v-FLIPs, see below) and cellular FLIPs (c-FLIPs). c-FLIPs have several isoforms, all of which contain two DEDs (Fig. 1). The short form, c-FLIP$_S$, has essentially just the two DEDs (like the v-FLIPs), while the long form, c-FLIP$_L$, has both the DEDs and an inactive caspase domain. Both forms of FLIP can block death receptor activated apoptosis when overexpressed [46, 47], as do v-FLIPs [48]. However, c-FLIP$_L$ also activates apoptosis by activating caspases [49]. In this case, c-FLIP$_L$ at physiological levels appears in the DISC where it enhances the enzymatic activity of the caspases and thereby promotes caspase activation. Finally c-FLIP expression is upregulated by the nuclear factor κB (NF-κB). This is one mechanism by which NF-κB signaling promotes cell survival [50].

PEA-15 contains only a single DED, but like c-FLIP can block apoptosis when overexpressed in some cells [5]. Indeed, astrocytes from PEA-15 null mice are more sensitive to TNFα killing than wild type controls, and sensitivity to TNFα is reduced by exogenous expression of PEA-15 in the knockout cells [4]. Like c-FLIP, PEA-15 appears to work by binding to FADD and preventing caspase recruitment to the DISC. PEA-15 binding to FADD and recruitment to the DISC requires phosphorylation at two serines (S104 and S116) [51, 52]. These serines are phosphorylated by the protein kinase Cα (PKCα) and the protein kinase B (Akt) or calcium/calmodulin kinase II (CamKII) respectively [53–55]. Thus, PEA-15 might serve as a point of signal integration whereby various growth factor pathways might promote cell survival.

In general, pro-apoptotic DEDs (from FADD and caspase-8) can self-oligomerize, whereas anti-apoptotic DEDs (eg. c-FLIP and PEA-15) have thus far not been reported to oligermerize in this manner [56]. Sequence comparison of pro- and anti-apoptotic DEDs does not reveal obvious differences in the conservation of key hydrophobic residues. When overexpressed, some DEDs, in particular pro-apoptotic DEDs (Caspase-8 and FADD), oligomerize into higher order filaments called death effector filaments (DEFs), which have been described as cage-like structures around the nucleus that initiate apoptosis [56]. DEFs resemble structures formed in the cytoskeleton but do not appear to require cytoskeletal proteins. The mechanism by which DEFs induce death is thought to involve the aggregation of caspase-8. Interestingly, the full-length procaspase-8 protein contains two DED domains but does not aggregate into filaments. This suggests that there may be major conformational differences between the DED fragment of caspase-8 and the whole procaspase form. Finally, overexpression of anti-apoptotic v-FLIP MC159 prevents DEF formation and apoptosis, whereas overexpression of the anti-apoptotic protein Bcl2 does not [56]. Again, MC159 appears to block DEF formation and apoptosis by preventing self-

aggregation of FADD [23, 25]. These observations indicate that DEFs work in a manner analogous to the formation of the DISC to activate apoptosis.

Death effector domain-containing proteins in proliferation

FADD, caspase-8, c-FLIP, DEDD and PEA-15 have been implicated in proliferation in various ways. The first indication that DED family proteins are involved in the regulation of proliferation came from characterization of various transgenic mice that are defective in FADD or caspase-8 signaling. Mice lacking FADD or expressing a dominant-negative FADD (consisting of just the DD called FADDdd) both show reduced thymic cellularity and inhibition of T-cell development. These mice also have defects in T cell receptor (TCR) activation-induced proliferation of T cells due to impaired $G_1 \rightarrow S$ transition [57–60]. Furthermore, FADD null T cells have increased levels of p21 and decreased levels of cyclin D2, as well as increased activity of cyclin dependent kinases when compared to wild type cells [59]. This suggests that some of the differences in the FADD null cells are caused by changes in the expression and activity of cell cycle regulating proteins. In addition to T cells, FADD is also involved in the proliferation of fibroblasts in a calcium-dependent fashion [60]. Thus FADD may be a general regulator of proliferation.

Similarly, caspase-8 activity is also required for T cell proliferation [61] and T cell receptor activation of NF-κB [62]. For example, caspase inhibitors block TCR-induced proliferation and interleukine-2 (IL-2) production and there is rapid cleavage of caspase-8, but not caspase-3, after activating TCRs [63, 64]. Moreover, patients with mutations in caspase 8 have impaired T cell proliferation and reduced IL-2 production [65]. This is similar to the phenotype of caspase-8 deficient mice [66]. Note that caspase-8 deficient T cells differ from FADD deficient T cells in that FADD T-cells still produce IL-2 [63, 66]. These results indicate that even though FADD and Caspase-8 deficiencies have similar phenotypes, the molecular mechanisms involved may be somewhat different. The mechanism by which caspase-8 activates lymphoproliferation but not apoptosis upon TCR activation is becoming clearer. During TCR activation, procaspase-8 associates with the paracaspase MALT1 and the adaptor protein Bcl10 and this association is required for the activation of NF-κB [62]. MALT1 controls caspase-8 activation by directly associating with caspase-8 [67]. The paracaspase domain of MALT1 activates procaspase-8 in a protease-independent manner, so that procaspase-8 undergoes only limited autoproteolytic activity. This yields a form of caspase-8 that can efficiently cleave c-$FLIP_L$ but not caspase-3. In this way, caspase-8 is directed to activation of c-FLIP and NF-κB activated proliferation as opposed to apoptosis.

Finally, conditional deletion of c-FLIP in mouse T cells also produces a decrease in TCR-activated proliferation and IL-2 production [68]. The reduced TCR stimulation of proliferation in the c-FLIP deficient T cells has been linked to impaired NF-κB signaling [69]. In effector T cells, c-FLIP activates caspase-8 which then cleaves c-$FLIP_L$ to yield p43FLIP that can recruit the receptor-interacting protein (RIP) and thereby activate NF-κB. In comparison, FADD is not required for TCR activation of NF-κB [70]. FADD, caspase-8, and c-FLIP are thus all required for TCR-induced T cell proliferation and in some cases this is independent of NF-κB activation. The mechanism for this NF-κB-independent proliferation remains to be further elucidated. One possibility is that it is mediated by c-FLIP binding to Raf kinase which is upstream of ERK in the ERK MAP kinase pathway [8]. In T cells, c-FLIP can bind c-Raf and activate ERK downstream of Fas/CD95 ligation. Moreover, CD95 can co-stimulate TCR activation of proliferation [8, 61, 71]. Thus FLIP-mediated recruitment of Raf may be an important step in activating ERK and ERK-mediated proliferation of T cells.

In mice, FADD is phosphorylated at serine 191 (serine 194 in human) in its C-terminus [72]. This site is conserved and both phosphorylated and unphosphorylated FADD can be recruited to the DISC. FADD is predominately phosphorylated at this site during the G2/M phase of the cell cycle and remains unphosphorylated during G1 [73]. Replacement of serine 191 with aspartic acid results in a similar phenotype to that of T cells lacking FADD [73–76]. These findings suggest that the phosphorylation of FADD regulates its role in cell cycle progression. Two distinct kinases that phosphorylate FADD have been identified. A Fas-interacting 130-kD kinase that induces FADD phosphorylation and inhibits Fas mediated Jun NH2-terminal kinase activation has been identified and designated as Fas-interacting serine/threonine kinase/homeodomain- interacting protein kinase (FIST/HIPK3) [77]. It was separately demonstrated that phosphorylation of FADD can also be mediated by casein kinase Iα (CKIα) [72]. The FADD-CKIα association regulates the subcellular localization of FADD, and phosphorylated FADD was found to co-localize with CKIα on the spindle poles in metaphase. Inhibition of CKIα diminishes FADD phosphorylation, prevents taxol arrest of mitosis, and blocks mitogen-induced proliferation of mouse splenocytes [72]. These results indicate that

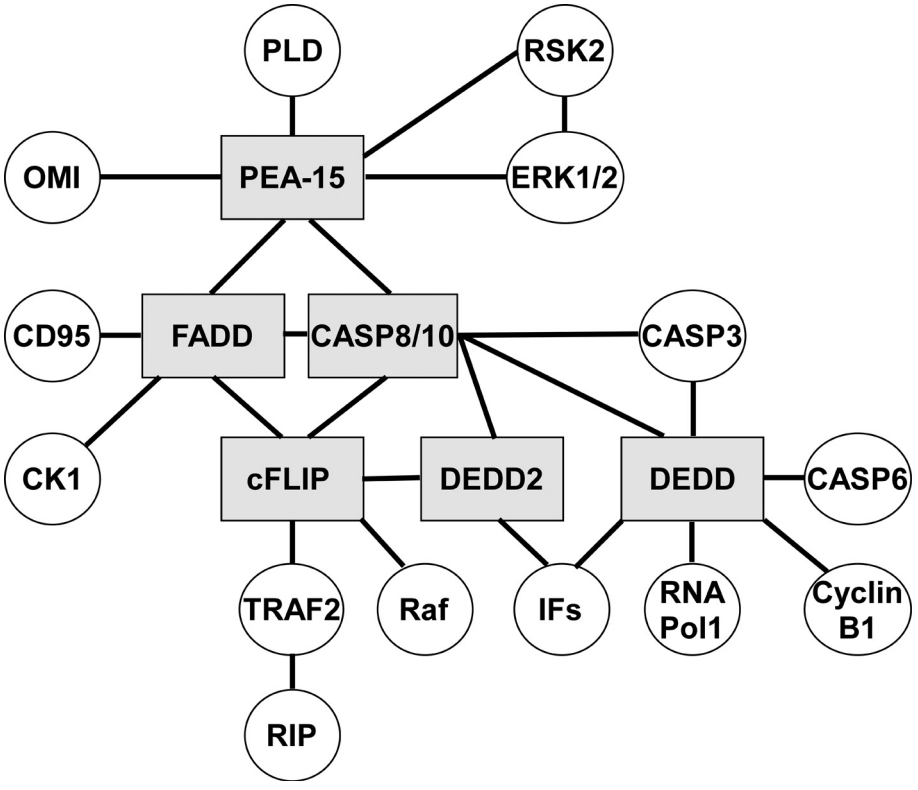

Figure 4. Death effector domain protein interaction map. DED proteins have been shown to interact with a diverse array of proteins, including proteins with and without DEDs. Depicted is a selection of the known binding partners for DED-containing proteins. Proteins with a DED are shown as shaded boxes. All other proteins are depicted in ovals. Lines connect the proteins that interact. PLD, Phospholipase D; IFs, intermediate filaments; Omi, Omi/HtrA2 (a serine protease).

phosphorylation of FADD mediates its cell cycle control during mitosis.

A more direct mechanism for DED family regulation of the cell cycle has recently been identified for DEDD. DEDD binds to and inhibits mitotic Cdk1/cyclin B1 through direct interaction with cyclin B1 [78]. This limits mitotic progression and appears to lengthen the G_2/M phase. Indeed, DEDD null embryonic fibroblasts have increased activity of nuclear cdk1/cyclin B1, reduced G_2/M phase duration, and accelerated mitotic progression in comparison to wild type controls [78]. Interestingly, DEDD also influences cell size. The DED of DEDD is not required for binding to cyclin B1 but is nevertheless indispensable for the effects on cell size [79]. It will be important to determine if other DED family proteins are involved in the DEDD/cdk1/cyclin B1 complex.

FADD, caspase-8, c-FLIP and Fas are also involved in epidermal growth factor (EGF)-stimulated proliferation and cell cycle control in hepatocytes [80], and caspases likewise regulate the osteoblast cell cycle [81]. Whereas FADD, caspase-8 and c-$FLIP_L$ all stimulate cell cycle progression and proliferation, PEA-15 appears to impair cell cycle progression. PEA-15 null astrocytes proliferate significantly more than wild type controls [7]. This is caused in part by increased ERK translocation to the nucleus. Indeed, PEA-15 binds ERK and prevents ERK accumulation in the nucleus and thus limits ERK activated transcription of cell cycle proteins [7]. PEA-15 also binds the ERK substrate ribosomal S6 kinase 2 (RSK2) and serves as a scaffold to enhance ERK activation of RSK2 [82, 83]. RSK2 is a kinase that regulates proliferation through phosphorylation of substrates such as the cyclin-dependent kinase inhibitor $p27^{kip1}$ and GSK3α [84]. Thus some of the effects of PEA-15 on proliferation may be due to changes in RSK2 activation.

These results indicate that DED-containing proteins not only participate in apoptosis but also regulate proliferation and cell cycle progression. Moreover, they associate with proteins that modulate these pathways (Fig. 4). Which pathway is activated may be regulated by receptor internalization. Recent reports show that recruitment of the DISC components and subsequent activation of apoptosis requires TNFR1 and CD95 receptors to be in an endosomal compartment [85–87]. Endosomal uptake of these receptors occurs in large lipid rafts and is mediated by clathrin-internalization. If CD95 remains at the cell surface (e.g. due to chemical inhibition of endocytosis) then CD95 ligation instead activates the ERK and NF-κB pathways. How receptor internalization is regulated and thereby determines the signaling activated by receptor ligation remains to be further elucidated.

Death effector domain family proteins in adhesion and migration

The first indication that DED proteins might contribute to the regulation of cell adhesion came when PEA-15 was shown to block H-Ras signaling to integrins in fibroblasts [88]. Activated H-Ras can shut down integrin ligand binding and therefore cell adhesion to matrix proteins like fibronectin by a mechanism dependent on ERK MAP kinase [89]. Enforced expression of PEA-15 blocks the ERK signal and thereby promotes full integrin ligand binding (integrin activation). Since migration in the extracellular matrix requires integrin activity, these results suggested PEA-15 might be able to influence migration and tumor metastasis. PEA-15 has since been reported to inhibit migration of both astrocytes [90] and breast cancer cells [91]. However the proposed mechanism for these respective functions is significantly different. PEA-15 is suggested to block astrocyte migration by altering PKCδ activity, while in breast cancer cells, the effect of PEA-15 is suggested to be through its binding to ERK1/2. PEA-15 expression down-regulated an active 40kDa fragment of PKCδ in astrocytes. Moreover, PEA-15 -/- astrocytes show increased migration and increased expression of the 40kDa fragment of PKCδ. Finally, inhibitors of PKCδ block PEA-15 -/- astrocyte migration, while inhibitors of ERK, Akt, and Cam Kinase II have no effect [90]. In contrast, PEA-15 inhibition of breast cancer tumor cell invasion is entirely dependent on PEA-15 binding to ERK and prevention of ERK nuclear translocation [91]. PEA-15 expression was shown to be reduced in invading astrocytomas and in metastatic breast cancer cells. Moreover, enforced expression of PEA-15 in invasive breast cancer cells reduces invasion while knock-down of PEA-15 in tumor cells expressing PEA-15 enhances invasion. Thus, PEA-15 inhibition of migration is important in inhibiting metastasis and suggests PEA-15 is a suppressor of metastasis. Currently, the two mechanisms by which PEA-15 can influence migration appear to be distinct. PEA-15 may therefore affect migration in more than one way, dependent on the cells being examined. The precise mechanism by which PEA-15 influences migration remains to be elucidated in more detail.

Caspase-8 is also known to modulate migration but unlike PEA-15, caspase-8 promotes cell motility [92]. Several recent papers have addressed the role of caspase-8 in cell adhesion and migration. Using caspase-8 knock-out mouse embryo fibroblasts it was reported that caspase-8 promotes cell motility. In addition, caspase-8 increases calpain activity as well as Rac GTP loading and the assembly of lammelopodia [92]. It was therefore proposed that caspase-8 regulated motility by directly regulating calpain-2 activation. The mechanism by which caspase-8 regulates calpain is currently only hypothetical.

Caspase-8 is phosphorylated at tyrosine 380 by Src and this prevents caspase-8 auto-cleavage at D374/D384 and thereby blocks Fas ligand (FasL)-induced apoptosis [93]. Src can be activated by both the epidermal growth factor receptor and integrin adhesion [93, 94]. Two groups have investigated the contribution of Src phosphorylation to caspase-8 function in migration. In one report, a non-cleavable form of caspase-8 is able to fulfill all the functions of the caspase in adhesion and migration even without the active site cysteine [95]. The phosphorylated, non-cleavable caspase-8 associates with the p85α subunit of PI3 kinase and this phosphorylation-dependent association is required for caspase-8 to promote cell motility. Moreover, binding of caspase-8 to p85α contributes to the activation of Rac but not Akt. Alternatively, phosphorylation of caspase-8 at tyrosine 380 can enhance caspase recruitment to the periphery of migrating and spreading cells and thereby enhance caspase-8 mediated migration [96]. The recruitment to the lammelopodia is proposed to be mediated by binding to Src itself. In a separate study, re-expression of caspase-8 in neuroblastoma cells lacking endogenous caspase-8 activates cell adhesion to extracellular matrix and adhesion-dependent ERK activation [97]. In this report, caspase-8 is also recruited to the plasma membrane through binding to Src. How recruitment of caspase-8 leads to enhanced adhesion and migration is not known. However one possibility is that only plasma membrane localized caspase-8 can activate calpain.

c-FLIP is also reported to promote cell migration. Here c-FLIP is reported to promote the motility of HeLa cells by activating focal adhesion kinase (FAK) and ERK and also by increasing matrix metalloproteinase-9 (MMP-9) expression [98]. In these experiments the pseudocaspase domain of c-FLIP$_L$ is required for the effect, but the precise mechanism of c-FLIP activation of ERK and FAK is not examined. One possibility is that c-FLIP works in part through activation of caspase-8 as has been described in NF-κB activation; however this provides no clear mechanism for how these proteins induce the multiple changes attributed to them in cell migration.

The observation that caspase-8 and c-FLIP can promote cell migration of tumor cells as well as fibroblasts is consistent with the findings that the CD95 ligand will induce the migration and invasion of many apoptosis resistant tumor cells [99]. Again the mechanism is unclear. CD95L was shown to work at least in part through activation of NF-kB, ERK and urokinase plasminogen activator [99]. It seems likely

that there may be at least some common mechanisms for the regulation of migration by these DED family members. Given that CD95L, caspase-8, and c-FLIP activity can contribute to cell migration and metastasis and that PEA-15 appears to inhibit migration and metastasis, it is crucial to get a better understanding of the molecular mechanism(s) involved. Understanding how DED proteins regulate migration may suggest novel anti-metastasis cancer therapies.

Death effector domain proteins in embryonic development

DED-containing proteins play important roles in development in addition to the essential functions in T cell differentiation, homeostasis and activation described above. Knock-out studies indicate that the DED proteins FADD, caspase-8, and c-FLIP have similar defects in mouse embryo development and in all cases lead to death at embryonic days 10–12. These knockout mice have many abnormalities during development and die in part from circulatory failure [100–102]. The first signs of abnormality appear in the extra-embryonic tissue of the yolk sac. By embryonic day 10.5 the yolk sac vasculature begins to form inappropriately and defects in the formation of the heart and neural tube appear [103]. In caspase-8 null mice, the number of hematopoietic precursors is decreased and macrophage differentiation is affected. Caspase inhibitors can likewise inhibit macrophage differentiation and activation [104, 105]. Deletion of caspase-8 in bone-marrow cells results in arrest of hemopoietic progenitor cell function, and in cells of the myelomonocytic lineage, its deletion leads to arrest of macrophage differentiation and to cell death [106]. None of these phenotypes has a correlate in a death receptor knock-out thus far and they are more easily interpreted as arising from differences in cellular proliferation and differentiation rather than from defects in apoptosis. There are however some developmental functions that can be attributed to defects in apoptosis. For example, c-FLIP null embryonic fibroblasts are highly sensitive to FasL or TNF-induced apoptosis, in contrast to FADD and caspase-8 null fibroblasts [100]. While caspase-8 null hepatocytes show decreased sensitivity to FasL killing [106]. PEA-15 null mice develop normally with no reported defects except increased sensitivity of isolated astrocytes to TNFα [4]. In contrast, DEDD null mice are reported to have normal apoptosis but a shortened mitotic progression [78]. This results in mice with reduced body and organ size. Thus far, no knock-out phenotypes have been published for DEDD2. These observations indicate that DED family proteins function in multiple cell signaling events during development including apoptosis, cell growth and differentiation. However, it is worth emphasizing that the predominant embryonic phenotypes are more easily attributed to defects in proliferation and differentiation.

Death effector domain proteins in disease

DED-containing proteins have been implicated in a diverse array of diseases. Human diseases linked to DED proteins include Huntington's Disease, Type II diabetes, autoimmune lymphoproliferative disease, arthritis, and cancer. Huntington's disease (HD) is a fatal neurodegenerative disorder characterized by hyperkinetic involuntary movements, slowing of voluntary movements, and cognitive impairment. HD is associated with the expansion of a polyglutamine tract in the HD gene product, huntingtin. Overexpression of the polyglutamine repeat can induce apoptosis in primary cerebellar and striatal neurons. Moreover, HD patients have increased activated caspase-8 in an insoluble fraction of the affected brain regions [107]. This is now thought to be due in part to the decreased binding of the mutated huntingtin to the huntingtin interacting protein-1 (Hip-1) [108]. The free Hip-1 in this context interacts with the Hip-1 protein interactor (HIPPI) via its pseudo-DED domain and additionally binds the DED protein caspase-8 in a complex, whereby the apoptotic cascade is initiated [109]. Since there is no causal treatment for Huntington's disease so far, modulation of the caspase-8 mediated apoptotic cascade may be a promising approach.

Defects in death receptor-mediated apoptosis result in autoimmune disease in both humans and mice. The autoimmune lymphoproliferative syndrome (ALPS) is a human childhood condition, characterized clinically by lymphoadenopathy, splenomegaly, autoimmune hemolytic anemia, thrombocytopenia, and hypergammaglobinemia [110–112]. Most ALPS patients have dominant mutations in the genes encoding the CD95 (FAS/APO-1) receptor, the CD95 ligand (CD95L), or caspase-10 [111–113]. These mutations have been associated with the survival and the expansion of an auto reactive population of Thy1+ B220+ CD4− CD8−, which may be responsible for the observed autoimmunity [114, 115]. Mutations in caspase-10 result in the instability of the larger subunits of the protease domain, which results in a decrease in apoptotic activity. Two patients with ALPS-like disorders who have normal *CD95*, *CD95L*, or *caspase-10* genes have also been identified. These patients have a C-to-T mutation in the *caspase-8* gene that reduces protein stability and abrogates the

enzymatic activity of the caspase 8 protein [65]. In contrast to other ALPS patients with mutations in *CD95*, *CD95L*, or *caspase-10* genes, the *caspase-8* mutant patients exhibit clinical immunodeficiency characterized by poor response to immunization and a reduction in T-cell activation and proliferation, natural killer cell activation, and immunoglobulin production. Thus, in humans as in mice, caspase-8 not only plays an important role during the developmental process but also in the proper functioning of the immune system [65].

Deregulation of apoptosis in osteoblasts and T cells in the rheumatoid synovium is a hallmark of bone-destructive diseases such as rheumatoid arthritis (RA) and periodontal diseases [116, 117]. In RA, T cells infiltrate the synovium and interact with synovial fibroblasts where they are activated [118]. Infiltrated T cells in RA are resistant to Fas-induced apoptosis and show high levels of anti-apoptotic molecules like Bcl2 and c-FLIP [119–121]. Indeed, RA macrophages have elevated c-FLIP expression and their resistance to Fas-induced apoptosis persists despite higher levels of Fas expression. In addition, c-FLIP expression in RA is dependent on the stage of the disease. In patients with long-term RA, increased levels of apoptosis were associated with low levels of c-FLIP, whereas patients with short-term RA showed decreased levels of apoptosis accompanied by high expression of c-FLIP [122]. Furthermore, down regulation of c-FLIP in RA synovial fibroblasts sensitizes them to Fas-induced apoptosis, suggesting that c-FLIP has its effect at least in part through blocking apoptosis [123]. Fas triggering may also contribute to RA through caspase-8/p43c-FLIP-dependent activation of NF-κB and AP-1 [124]. Thus c-FLIP may act both by blocking apoptosis and up-regulating NF-κB activation.

DED proteins have been implicated in cancer in many distinct ways. FADD is localized on chromosome 11q13.3 in humans and this locus is frequently associated with malignancies [125]. The role of the FADD adaptor in cancer was initially demonstrated by generating recombination activating gene-1 (RAG-1) deficient transgenic mice that target expression of a FADD dominant negative mutant in lymphocytes. With age these mice developed thymic lymphoblastic lymphoma, whereas FADD+/+ RAG-1-/- mice did not [126]. Moreover, thymic lymphoblastic lymphomas were never observed in FADD-/- RAG-1+/+ or FADD-/- RAG-1+/- mice, demonstrating that the absence of FADD expression was necessary but not sufficient to induce tumor development in this model [126]. These results were the first demonstration that FADD can act as a tumor suppressor *in vivo*. In humans, FADD expression was also either lowered or completely lost in acute myeloid leukemia cells derived from patients [127]. In general it is thought that loss of FADD expression allows tumor cells to avoid cell death mediated by death receptors. Indeed acute myeloid leukemia (AML) cells lacking FADD expression showed resistance to apoptosis [128].

FADD phosphorylation and localization can also contribute to tumorigenesis. Previously, FADD was thought to be exclusively localized to the cytoplasm, but recently it was found that FADD has an NLS (nuclear localization sequence) and can translocate into the nucleus [129]. The role of FADD in the nucleus is still largely unknown. FADD has been reported to interact with the nuclear proteins MBD4 (involved in the repair of GT mismatches in chromatin) and PIDD (a p53 downstream target regulated by DNA damage) [130, 131] . In addition, the phosphorylation of FADD plays an important role in its translocation to the nucleus and in cell-cycle progression, and mutation of serine at position 194 to alanine leads to uniform localization throughout the cytoplasm and aberrant cell-cycle progression [132, 133]. Nuclear FADD might regulate DNA damage during apoptosis by binding to MBD4 and PIDD. Immunohistochemical analysis of tissue arrays revealed that aggressive lung carcinoma has increased nuclear localization and phosphorylation of FADD [134]. Increased phosphorylation and nuclear localization is associated with the poor survival of patients. Moreover, in this study increased phosphorylation of FADD resulted in elevation of NF-κB activation along with an increase in cell proliferation [134]. These results suggest that phosphorylation of FADD plays a major role in aggressive tumor behavior by modulating the NF-κB pathway.

Like FADD, caspases-8 and -10 can act as tumor suppressors [135]. Indeed, both caspase-8 and caspase-10 have been reported to be deregulated in pediatric tumors and neuroendocrine lung tumors as a result of gene silencing through methylation [136]. Mutations of the caspase-10 gene are also found in gastric tumors and similar results have been reported for caspase-8 in small cell lung carcinoma (SCLC) cell lines [137]. In many of these cases, the downregulation of the caspases in the tumor leads to an increased resistance to chemotherapy and may also contribute to metastasis [138]. In particular neuroblastoma metastasis is enhanced by loss of caspase-8 expression and this is necessary for the establishment of the metastases *in vivo* [9]. Moreover, reconstitution of caspase-8 expression in these neuroblastoma cells suppressed their ability to form metastases by promoting apoptosis initiated by unligated integrins

(integrin-mediated death) [9]. In this way caspases are potential suppressors of metastasis [139].

PEA-15 is reported to both activate and suppress tumor growth. For example, PEA-15 enhances 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced skin cancer in mouse models. Mice overexpressing PEA-15 developed four fold more papilloma, and the malignant conversion frequency was 24 % when compared to controls [140]. In these transgenic mice PEA-15 blocks apoptosis by inhibiting caspase-3 activation, and knock-down of PEA-15 by siRNA makes the cells sensitive to apoptosis. Thus PEA-15 was suggested to enhance tumor growth by preventing apoptosis. Similarly, PEA-15 may mediate chemo-resistance in other cancers. Many breast cancer cells have elevated levels of PEA-15 along with increased AKT activity [141]. In several instances, the increase in PEA-15 expression corresponds to increased AKT activity. Furthermore expression of dominant negative AKT in these cells results in down regulation of PEA-15 and increased sensitivity to chemotherapy-induced cell death. In a similar fashion, increased PEA-15 expression is reported to inhibit apoptosis in glioma [142, 143], non-small cell lung cancer [144], B cell chronic lymphocytic leukemia [145], and thyroid cancer [141]. Thus, increases in PEA-15 expression levels may represent a key molecular mechanism for chemoresistance in cancer and therefore provide a novel target to enhance chemotherapy.

More recently, PEA-15 has been reported to enhance glioblastoma cell proliferation by protecting the cells from glucose deprivation-induced apoptosis [146]. PEA-15 had been shown previously to affect glucose metabolism and was cloned as a phosphoprotein up-regulated in Type II diabetes [147]. In this respect overexpression of PEA-15 (also called PED/PEA15) contributes to diabetes by impairing glucose-stimulated insulin secretion and insulin action [148]. In glioblastoma, knockdown of PEA-15 sensitizes cancer cells to glucose withdrawal-mediated death and abolishes tumorigenicity [146]. Furthermore, PEA-15 regulates ERK activity in glioblastoma and this ERK activity is required for up-regulation of glucose transporter 3 and protection from glucose deprivation. This provides an alternative mechanism by which PEA-15 can support survival of tumor cells.

PEA-15 can also suppress tumor cell growth in some instances due to its binding of ERK. For example, the adenoviral gene E1A, which is known to suppress tumorigenicity by down-regulating the human epidermal growth factor receptor 2 (HER2), also up-regulates PEA-15 in ovarian cancers. Here, PEA-15 reduces proliferation by reducing ERK translocation to the nucleus [149]. On the other hand, E1A is also reported to rescue H-Ras mediated senescence by down-regulating PEA-15 expression and hence increasing ERK translocation to the nucleus. These results indicate that PEA-15 promotes H-Ras induced senescence [150]. This appears to be contradictory to the report that E1A up-regulates PEA-15 expression, even though in both cases PEA-15 suppresses tumorigenicity. The observation that E1A induced up-regulation of PEA-15 in ovarian cancer and E1A induced down-regulation of PEA-15 in H-Ras transformed cells might be due to the presence or absence of H-Ras or to differences in cell type. This remains to be determined.

Viral death effector domain-containing proteins

One final group of DED proteins is not encoded by mammalian cells but is instead produced by viruses. DED-containing proteins such as E8 from equine herpesvirus 2, MC159 and MC160 from molluscum contagiosum virus (MCV), K13 from human herpesvirus 8 (HHV-8), ORF71 from herpesvirus saimiri, and E1.1 from bovine herpesvirus have been collectively called viral FLICE inhibitory proteins (v-FLIPs). It is thought that viruses might have evolved these DED proteins to prevent host cell death during infection and replication and thus improve production of the virus by host cells. MC159 binds FADD and thereby prevents FADD self-association and formation of higher order structures in the DISC. This prevents activation of procaspase-8 and thereby blocks apoptosis of infected cells [48]. In contrast, MC160 is unable to protect from Fas-induced apoptosis. Interestingly, MC159 expression prevented MC160 from degradation, indicating cooperation between the two proteins [151].

More recently it has become clear that v-FLIPs have additional effects on host cells. Both MC159 and MC160 inhibit NF-κB activation and may do so in coordination with each other at different points in the pathway [151, 152]. MC160 can inhibit TNF-alpha-mediated NF-κB activation by inhibiting the IKK kinase activity and IKK subunit phosphorylation [152;152]. Whereas MC159 has both a TNF receptor-associated factor 2 (TRAF2) binding domain and TRAF3 binding domain [153, 154]. MC159 association with TRAF3 appears to be a key factor in inhibiting apoptosis because it in turn helps in TRAF2 recruitment into the Fas DISC. TRAF-binding-deficient mutants of MC159 showed impaired inhibition of FasL-induced caspase-8 processing and Fas internalization, and had reduced anti-apoptotic activity [154]. This suggests that formation of a MC159/TRAF2/TRAF3 complex is indispensable for MC159 to inhibit Fas-mediated apoptotic activity

and TNF-alpha induced inflammatory responses. Thus in addition to possible effects on FADD aggregation and activation of caspase-8, MC159 and MC160 may also increase virus replication and survival by preventing the infected cell from responding to TNF-alpha. This prevents the cellular production of pro-inflammatory and immuno-attractant molecules and prevents cell death from CD95-mediated apoptosis.

v-FLIPs act as a class of tumor progression factors that promote tumor progression by inhibiting death receptor-mediated apoptosis. For example, human herpesvirus 8 is a gamma-2-herpesvirus associated with three human malignancies: Kaposi's sarcoma (KS) [155], primary effusion lymphoma (PEL) [156], and multicentric Castleman's disease [156, 157]. HHV-8 encodes the v-FLIP K13 which contains two DEDs. K13 inhibits Fas-mediated apoptosis in B lymphocytes by inhibiting caspase-8 and caspase-3 activity. Furthermore, injection of K13 transduced murine B lymphoma cells into immunocompetent mice results in the development of an aggressive tumor with a high rate of tumor growth and survival [158]. The v-FLIPS also influence tumor growth and progression through the NF-κB pathway. Indeed, it is now thought that the primary mechanism of K13 tumor promotion is through the NF-κB pathway. K13 activation of the NF-κB pathway promotes cell survival, proliferation, transformation and cytokine production [159–162]. K13 interacts with the NF-κB pathway primarily through its binding to IKK. Of the v-FLIPs, only K13 binds IKK and activates the NF-κB pathway specifically through this interaction [153, 163]. Moreover, K13 binds IKK specifically in the nucleus where NF-κB along with K13 bind to various promotors and thus regulate viral and cellular genes [164]. Therefore v-FLIPs can promote tumor growth by mechanisms dependent on NF-κB as well as through effects on caspase-8 activation.

Conclusions

Over the last decade it has become clear that DED-containing proteins have diverse functions beyond the well characterized roles played in both activating and inhibiting apoptosis. These additional roles in proliferation, transcription and migration also contribute to the diseases that arise from perturbation of DED function. As such, drugs developed to target these signaling cascades in apoptosis (e.g. TRAIL therapies in cancer) may have unforeseen and complex side-effects. A full understanding of how DED proteins function in all these roles will not only allow more strategic development of drugs targeting death receptor initiated apoptosis, but may also allow the development of drugs that specifically target the function of these proteins in proliferation or migration. This might prove particularly beneficial in new therapies to prevent cancer growth and metastasis.

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