Review

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The death-fold superfamily of homotypic interaction motifs

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The death-fold superfamily encompasses four structurally homologous subfamilies that engage in homotypic, subfamily-restricted interactions. The Death Domains (DDs), the Death Effector Domains (DEDs), the CAspase Recruitment Domains (CARDs) and the PYrin Domains (PYDs) constitute key building blocks involved in the assembly of multimeric complexes implicated in signaling cascades leading to inflammation and cell death. We review the molecular basis of these homotypic domain–domain interactions in light of their structure, function and evolution. In addition, we elaborate on three distinct types of asymmetric interactions that were recently identified from the crystal structures of three multimeric, death-fold complexes: the MyDDosome, the PIDDosome and the Fas/FADD-DISC. Insights into the mechanisms of interaction of death-fold domains will be useful to design strategies for specific modulation of complex formation and might lead to novel therapeutic applications.

Structure and function of a death-fold domain

Signal transduction pathways controlling immunity, inflammation and apoptotic or necroptotic cell death depend to a large extent on proteins containing homotypic interaction motifs belonging to the death-fold superfamily. The 102 members discovered in humans so far can be categorized in four subfamilies: 39 Death Domains (DDs), 8 Death Effector Domains (DEDs), 33 CAspase Recruitment Domains (CARDs) and 22 PYrin Domains (PYDs) [1]. Despite the considerable overall divergence in sequence of its members (up to 90%), this superfamily is hallmarked by its structure, the so-called death-fold, which consists of a globular structure wherein six amphipathic α-helices are arranged in an antiparallel α-helical bundle with Greek key topology [2–5] (Figure 1). This topology determines the folding process, which starts with the formation of a hydrophobic core containing nearly all the conserved residues of the death-fold superfamily [6,7]. Structural differences between individual domains, which are smaller within a subfamily than between subfamilies, are the result of alterations in the length and orientation of the α-helices, and the distribution of charged and hydrophobic residues along the surface. Consequently, each member obtains a specific array of homotypic interaction partners that, in general, do not cross the boundaries of the subfamily. This homotypic trait is probably the result of some subfamily-specific structural characteristics: a more flexible, exposed third helix in the DDs [8–11]; some conserved surface-exposed hydrophobic patches and the presence of a RxDL-motif in the DEDs [3,12,13]; an interrupted first helix in the CARDs [4,14–17]; and a small third helix combined with a large, hydrophobically stabilized loop connecting helices 2 and 3 in the PYDs [5,18–20] (Figure 1). Moreover, phylogenetic analysis provides an evolutionary basis for the observed subfamily-restricted interaction repertoire because each subfamily constitutes a separate, defined cluster (Box 1). Still, some findings challenge the concept of exclusivity concerning homotypic interactions. For example, the apoptosis repressor with a CARD protein (ARC) interacts directly with the DDs of Fas and the Fas-Associating Death Domain-containing protein (FADD) [21].

Functionally, death-fold domains mediate the assembly of large, oligomeric signaling complexes in which crucial effectors, such as caspases and kinases, gain activity. However, until recently there was little structural information concerning the conformation of protein assemblies that contain death-fold domains. Using X-ray analysis, three-dimensional models have been described for the PIDDosome [22], the death-inducing signaling complex (DISC) formed by the Fas receptor [23,24], the apoptosome and its *Caenorhabditis elegans* counterpart [15,25], and the MyDDosome with its *Drosophila* counterpart, the Pelle/Tube complex [26,27] (summarized in Table 1; for an extensive list of characterized death-fold structures see Table S1 in the supplementary material online). These complexes are, respectively, responsible for the activation of procaspase-2, procaspase-8, procaspase-9 and the Interleukin-1 Receptor-Associated Kinases 2 and 4 (IRAK2 and IRAK4). These novel models provide fundamental knowledge on the formation and constitution of death-fold docking platforms, which is important for the development of new compounds that modulate complex formation. Starting from these newly characterized crystal structures, we provide a detailed overview of the individual death-fold subfamilies, their physiologic role and the interaction mechanisms employed.

Death-fold domains engage in three distinct interaction types

The ability of death-fold domains to mediate the formation of intricate, higher order complexes resides in their ability

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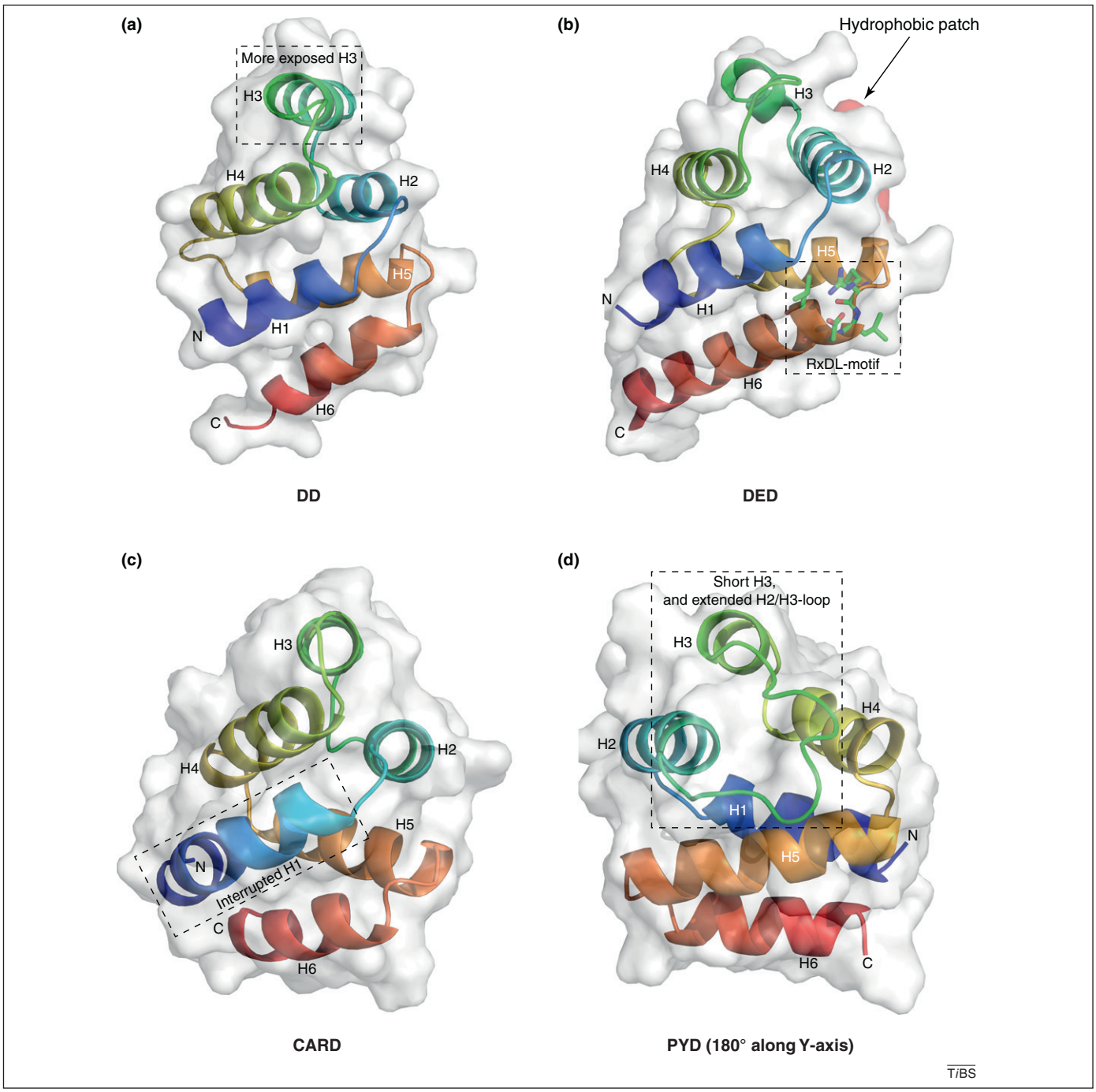

Figure 1. **Tertiary structure of the different death-fold subfamilies**. Cartoon and surface models of death-fold subfamily members with indication of subfamily-specific characteristics. **(a)** The Death Domain (DD) of PIDD (PDB ID: 2OF5). **(b)** The second Death Effector Domain (DED) of MC159 (PDB ID: 2BBR). **(c)** The CAspase Recruitment Domain (CARD) of Apaf-1 (PDB ID: 3YGS). **(d)** The PYrin Domain (PYD) of ASC (PDB ID: 1UCP). The six α-helices are numbered. All structural representations were generated using PyMol (Schrodinger, Portland, United States).

to engage in three distinct types of asymmetric interactions [28]. A type I interaction is formed when residues from helices 1 and 4 (patch Ia) of one death-fold domain interact with residues from helices 2 and 3 (patch Ib) of another (Figure 2a). A type II interaction is formed when residues from helix 4 and the loop between helices 4 and 5 (patch IIa) of one death-fold domain interact with residues of the loop between helices 5 and 6 (patch IIb) of the other death-fold domain (Figure 2b). A type III interaction is formed when residues from helix 3 (patch IIIa) of one death-fold domain interact with residues located on the loops between helices 1 and 2 and between helices 3 and 4 (patch IIIb) of the other death-fold domain (Figure 2c). Because the six interaction patches of a death-fold domain are non-overlapping, each domain can potentially engage up to six binding partners by using each of the three types of pairwise interactions twice (Figure 2d). So far, only members of the DD subfamily have been characterized in this regard [22,24,26]. CARDs and DEDs have been reported only in 1:1 interaction of the first type [12,15,29]. Considering the similar role of the different death-fold subfamilies in the formation of multimeric

Box 1. Evolution of the death-fold superfamily

Given the high homology in structure and the considerable divergence in sequence of the death-fold superfamily members, we examined whether a sequence-based phylogenetic analysis would provide an evolutionary explanation for the emergence of four conserved death-fold subfamilies, the DDs, the DEDs, the CARDs and the PYDs. CARD- and DD-containing proteins have been identified in organisms such as *Caenorhabditis elegans* and *Drosophila melanogaster*, whereas zebrafish and rodents are the most distant organisms wherein PYD- and DED-containing proteins have thus far been found.

This phylogenetic analysis was performed with the death-fold domains of human descent that form an integral part of well-characterized signaling complexes, including the apoptosome [52], the PIDDosome [73], the death receptor complexes [65], the inflammasomes [30], the NOD complexes [74], the RIG-like receptor complexes [67], the TLR/IL-1 receptor complexes [75] and the B cell and T cell receptor proximal complexes [68]. Apart from one exception, the second CARD of RIG-I, each of the subfamilies clusters in a separate branch, indicating that each subfamily had its own ancestor. This analysis suggests that the founding father of the death-fold superfamily gave rise to a CARD and a DD–DED–PYD ancestor (Figure I). The latter diverged in a DD and a DED–PYD ancestor, which in turn resulted in the emergence of the DED and PYD ancestor (Figure I). This clear subfamily separation probably explains why these domains were almost exclusively found to engage in homotypic, subfamily-confined interactions. Considering the structural similarity and the low sequence similarity of the death-fold superfamily, these ancestor death-folds might be the result of a convergent evolution toward the same function [76]. Additional death-fold domains most probably originated through divergent evolution involving gene duplication events within each subfamily [63]. A striking example is given by the caspase-1-related CARD-only proteins, COP, INCA and ICEBERG, which localize at the *caspase-1* locus, share a high degree of sequence identity and acquired premature nonsense mutations resulting in a CARD-only protein [77,78]. In addition, several members of the *Herpesviridae* and *Poxviridae*, two families of dsDNA viruses, acquired death-fold domain-only proteins (DED-containing vFLIPs and PYD-containing vPOPs) through horizontal gene transfer that enable them to evade the host defense system [49,64]. Taken together, the parallel evolution of the four death-fold subfamilies forms the basis for their ability to engage in homotypic interactions within the boundaries of the subfamily.

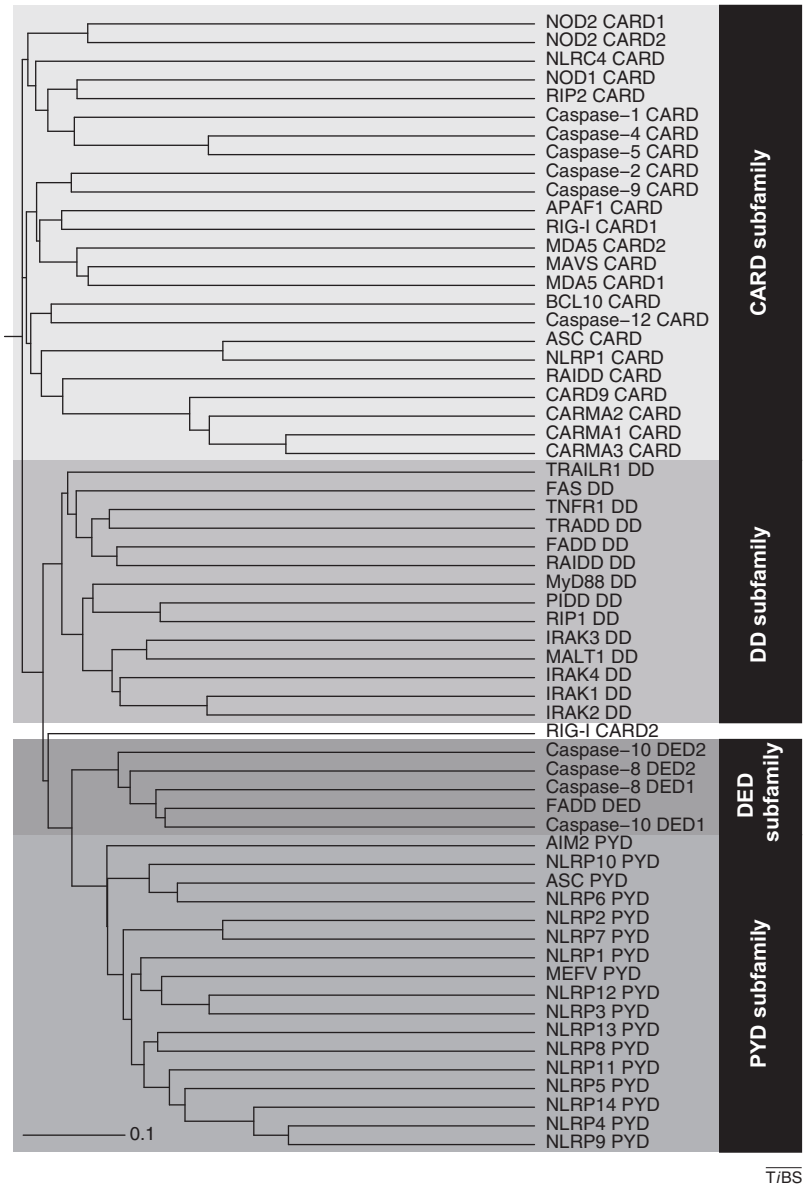

Figure I. Phylogenetic analysis of the death-fold superfamily. The sequences of the death-fold domains were aligned using Clustal X (Gonnet matrix, gap penalty: 10, gap extension penalty: 0.1). The Neighbor Joining bootstrap tree was constructed using Clustal X (http://www.clustal.org) and visualized with iTol (http://itol.embl.de).

Table 1. Overview of all described crystal structures of multimeric death-fold complexes[a]

	Protein/complex	Species	Residues	Purification and crystallization conditions	PDB ID	Resolution	Ref.
DD	PIDDosome	human	PIDD: 778–883 RAIDD: 94–199	Domains individually expressed in *Escherichia coli* and mixed. Crystallized at pH 6.5.	2OF5	3.2 Å	[22]
	Fas/FADD-DISC	human	Fas: 223–335 FADD: 93–208	Domains individually expressed in *E. coli* and mixed. Crystallized at pH 4.0.	3EZQ	2.7 Å	[23]
		mouse, human	Fas: 210–310 FADD: 93–184	Domains coexpressed in *E. coli* and copurified. Crystallized at pH 8.5.	3OQ9	6.8 Å	[24]
	MyDDosome	human	MyD88: 20–117 IRAK4: 4–106 IRAK2: 1–112	Domains coexpressed in *E. coli* and copurified. Crystallized at pH 8.0. IRAK2 contains a mutation (R50W)	3MOP	3.4 Å	[26]
	Pelle–Tube complex	*Drosophila*	Pelle: 26–129 Tube: 1–184	Domains individually expressed in *E. coli* and mixed. Crystallized at pH 7.2–8.4.	1D2Z	2.0 Å	[27]
DED	MC159 (v-FLIP)	*Molluscum contagiosum*	1–187	Expressed in *E. coli*. Crystallized at pH 6.5.	2BBR	1.2 Å	[12]
			1–183	Expressed in *E. coli*. Crystallized at pH 8.5.	2F1S	1.4 Å	[29]
CARD	Apoptosome	human	Apaf-1: 1–97 procaspase-9: 1–112	Domains individually expressed in *E. coli* and mixed. Crystallized at pH 4.6.	3YGS	2.5 Å	[15]
		C. elegans	Full-length CED4 Full-length CED3	Domains individually expressed in *E. coli* and mixed. Crystallization pH was not indicated. No apparent electron density for CED3 in the crystals.	3LQQ 3LQR	3.5 Å 3.9 Å	[25]

[a]The species, the residues included to purify the death-fold domain, some purification and crystallization particulars, the PDB ID and the resolution of the obtained crystals are indicated for each complex.

protein assemblies, one can assume that all death-fold domains might use the three types of interaction. Determining the crystal structure of the CARD- and PYD-containing inflammasomes, which activate procaspase-1 to enable Interleukin (IL)-1β production and pyroptotic cell death [30], could be of particular interest to support this assumption. Moreover, the insight gained on the interaction mechanism of the inflammasomes could provide novel avenues for the development of compounds that interfere with excessive inflammasome formation, an important factor in the pathogenesis of multiple inflammatory disorders (e.g. gout, type II diabetes and cryopyrin-associated periodic syndromes) [30]. Indeed, by targeting upstream complexes, such inflammasome interfering compounds could be valuable, more specific alternatives for the currently used IL-1β-inhibitors anakinra, rilonacept and canakinumab, which target the effector molecules [31].

Although the hitherto reported structures suggest that the three interaction types are conserved throughout the death-fold superfamily, there are marked differences between individual pairwise interactions of the same type. The type I interaction between the CARDs of Apaf-1 and procaspase-9 depends on a network of intermolecular hydrogen bonds and van der Waals interactions [15], whereas the type I interaction between FADD–DED and procaspase-8–DED2 is mediated by a hydrophobic interface [12,13]. The difference in utilization of non-covalent binding modes combined with the complementarity in surface are two important factors that determine the interaction partners within the death-fold superfamily.

Homotypic DD interactions

Most knowledge concerning interactions of death-fold domains in the context of oligomeric protein assemblies was obtained from crystal structures containing DDs. In this section of the review, the functional context and the structural organization of the PIDDosome, the MyDDosome and the Fas/FADD-DISC are highlighted.

The PIDDosome

The crystal structure of the DD assembly containing the p53-Inducible protein with a Death Domain (PIDD) and RIP-Associated protein with a Death Domain (RAIDD) was the first description of an oligomeric complex involving members of the death-fold superfamily [22]. Interactions mediated by these DDs form the core of the PIDDosome, a complex acting as a molecular switch that determines life or death following genotoxic stress [32]. Depending on its proteolytic processing status, PIDD mediates the assembly of two separate PIDDosomes [33]. A 51-kDa C-terminal fragment obtained by single autoprocessing enables formation of the NF-κB-activating, pro-survival PIDDosome. In this complex, the PIDD-DD engages the DD-containing Receptor Interacting serine/threonine-Protein kinase 1 (RIP1) to assemble a platform for the recruitment of the NF-κB Essential MOdulator (NEMO), an essential adaptor protein for the activation of NF-κB [32,33]. The formation

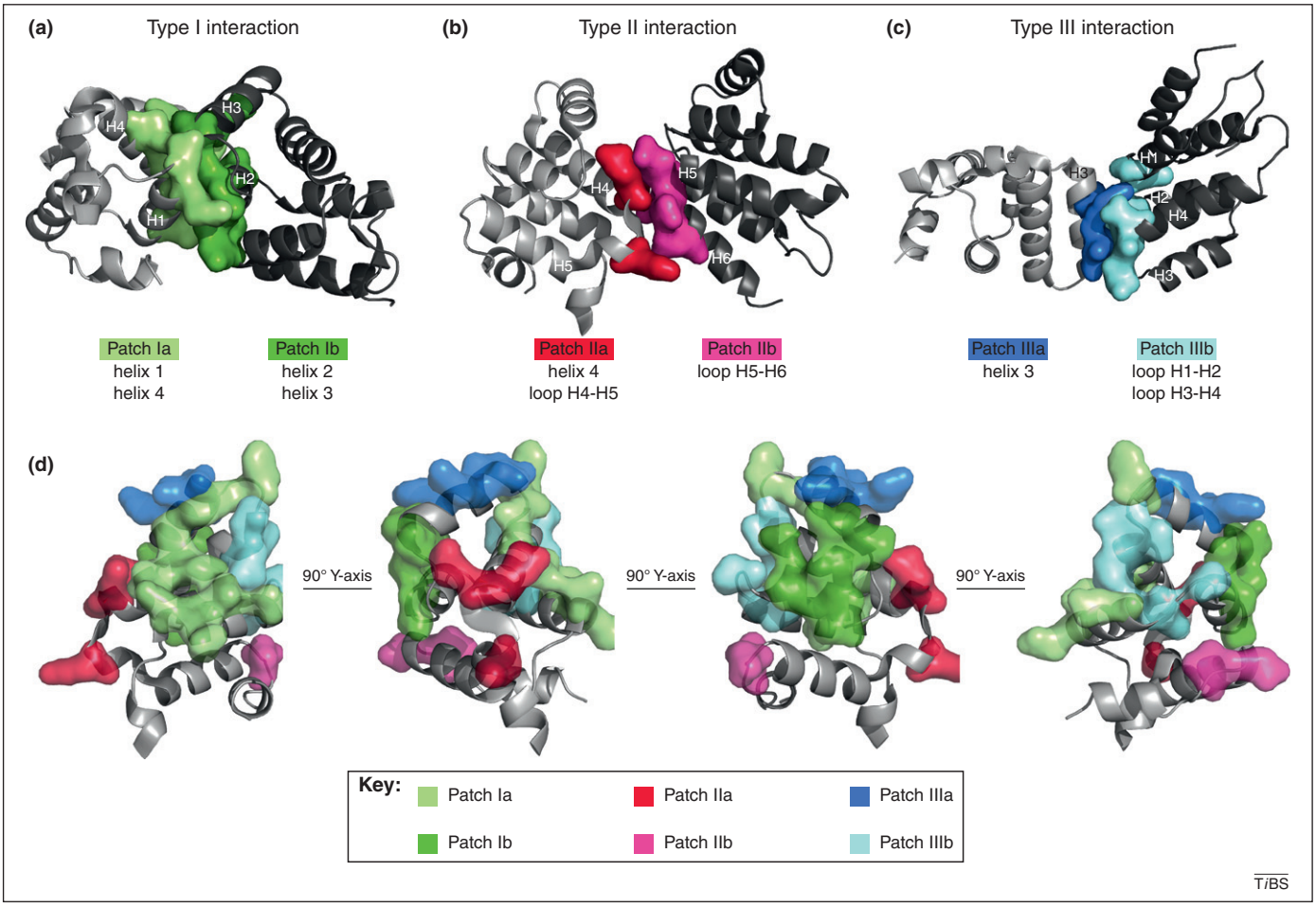

Figure 2. A Death Domain (DD) mediates three interaction types through six interaction patches. The three asymmetric interaction types: type I **(a)**, type II **(b)** and type III **(c)** derived from the PIDDosome (PDB ID: 2OF5). The DDs of RIP-Associated protein with a Death Domain (RAIDD) (on the left, light gray) and p53-Inducible protein with a Death Domain (PIDD) (on the right, dark gray) are shown as cartoon drawings for each interaction, whereas the interaction patches are displayed as surface (type I: green; type II: red; type III: blue). Helices and loops contributing to the interactions are indicated. **(d)** The six possible interaction patches on the DD of RAIDD. The composite model is rotated in four stages of 90° around the *Y*-axis.

of the pro-death PIDDosome relies on a subsequent cleavage that generates a 37-kDa, DD-only PIDD fragment [33]. Under these circumstances, the DD of PIDD interacts with the DD of the CARD- and DD-containing adaptor protein RAIDD, which subsequently recruits and activates the CARD-containing procaspase-2 through a CARD/CARD interaction [33,34]. This pro-death PIDDosome represents the functional context for the crystal structure of the PIDD-DD/RAIDD-DD assembly. An asymmetric, three-layered, globular complex was reported in which the bottom, middle and top layers contain five PIDD-DDs, five RAIDD-DDs and another two RAIDD-DDs, respectively (Figure 3a) [22]. Based on the stoichiometry of this complex, the procaspase-2 activating PIDDosome would have a molecular mass of 696.8 kDa, which is in good agreement with the observed molecular mass of 670 kDa when the molecular composition of the PIDDosome was determined through gel filtration analysis [34,35]. Despite the apparent asymmetry within the complex as a whole, each DD appears to be in a quasi-equivalent environment and employs the three types of interactions (Figure 3b) [22]. More than a decade ago, the interaction between the CARDs of RAIDD and procaspase-2 was predicted based on mutagenic analysis and appeared to be mediated by electrostatic interaction [4]. Still, a crystal structure elucidating this CARD/CARD interaction is needed to unambiguously determine the exact interaction type.

The MyDDosome

Recently, another ternary complex containing DDs of MyD88, IRAK4 and IRAK2 was characterized by X-ray crystallography and was termed the MyDDosome [26]. This complex encompasses an essential component of the signal transduction cascade that links the Toll-like receptors (TLRs) and the receptors for the proinflammatory cytokines IL-1β and IL-18 to the activation of transcription factors important for effective immune responses [36]. Interestingly, the overall structural appearance of the MyDDosome differs rather substantially from the PIDDosome. In contrast to the globular PIDDosome, the MyDDosome seems to be a left-handed, helical tower-shaped structure in which six MyD88-DDs constitute the bottom two layers, four IRAK4-DDs make up the third layer and another four IRAK2-DDs make up the fourth layer (Figure 3d) [26]. Still, similar to the PIDDosome, all the DDs of the MyDDosome were found to be in a quasi-equivalent environment and use the three types of interaction. Type III interactions connect adjacent DDs within a

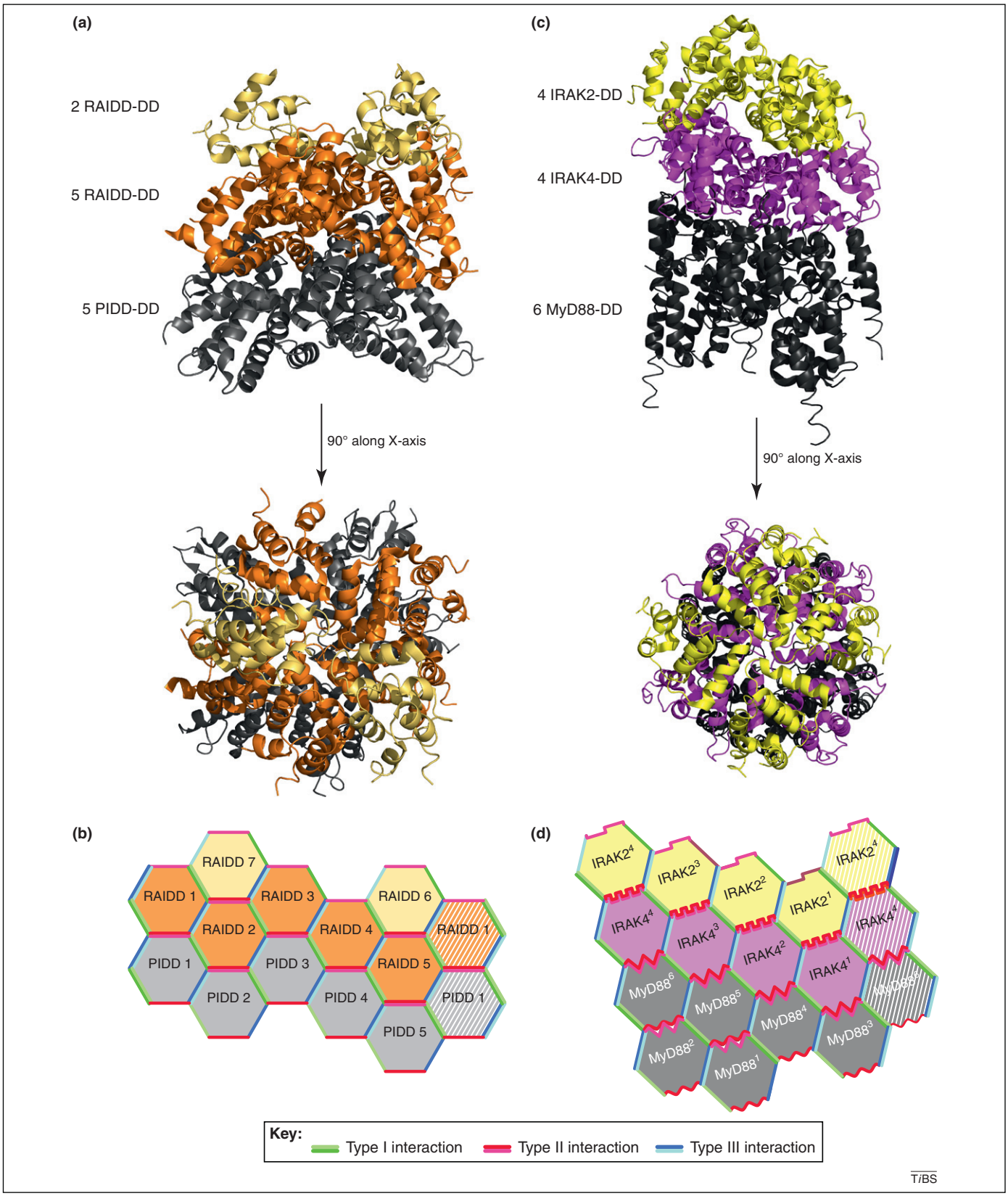

Figure 3. The PIDDosome and MyDDosome complex. (a) Side and top views of the PIDDosome complex (PDB ID: 2OF5) represented as cartoon drawings. The bottom layer contains five PIDD (p53-Inducible protein with a Death Domain) Death Domains (DDs) (gray), the middle layer contains five RAIDD (RIP-Associated protein with a Death Domain) DDs (orange) and the top layer contains two RAIDD DDs (dark yellow). The top view is a 90° rotation around the *X*-axis of the side view. **(b)** Schematic, planar illustration of the three interaction types used to assemble the PIDDosome. **(c)** Side and top views of the MyDDosome complex (PDB ID: 3MOP) represented as cartoon drawings. The bottom layer contains six MyD88-DDs (black), the middle layer contains four IRAK4-DDs (magenta) and the top layer contains four IRAK2-DDs (yellow). The top view is a 90° rotation around the *X*-axis of the side view. **(d)** Schematic, planar illustration of the three interaction types used to assemble the MyDDosome. The different interaction patches are colored as in Figure 2.

layer, whereas type I and II interactions connect the layers (Figure 3d).

Based on the MyDDosome structure a model can be put forward wherein complex assembly encompasses a hierarchical and sequential process that is tightly controlled by multiple mechanisms. Complex assembly can be considered cooperative because the sequential recruitment of each DD increases stability. Complementarity in charge and shape between the bottom and top surfaces of the DDs, involved in the formation of type II interactions, is one of the key determinants of hierarchy. For instance, IRAK2 is recruited to the complex only when IRAK4 is present because the bottom surface of IRAK2 finds its best match in the top surface of IRAK4; the top surfaces of MyD88 and IRAK2 are not sufficiently complementary (Figure 3d) [26]. Furthermore, the observation that the top and bottom surfaces of IRAK2 do not fit properly to each other limits the recruitment of IRAK2-DDs to four.

The functional significance of the MyDDosome lies in the activation of the kinase activity of both IRAKs: IRAK4 undergoes autophosphorylation and cross-phosphorylates IRAK2. Subsequently, active IRAK2 leaves the complex to interact with TRAF6 and to propagate an intricate signaling cascade involving phosphorylation and ubiquitination events that lead to the activation of transcription factors such as NF-κB, AP-1 and c-Jun [36]. Interestingly, the conserved, homologous signaling pathway in *Drosophila* uses similar players, dMyD88, Tube and Pelle, but in this case it does not lead to the assembly of an oligomeric, MyDDosome-like complex [37,38]. Rather, the dMyD88–Tube–Pelle complex was found to be a 1:1:1 kidney-shaped ternary complex in which two type II interactions connect the DDs [26,27,38].

The Fas/FADD-DISC

Two fundamentally different models of the cell death-inducing Fas/FADD-DISC were proposed based on two different crystal structures [23,24]. This membrane-proximal procaspase-8-activating complex forms an integral part of the extrinsic, apoptosis-inducing signaling pathway that is triggered upon Fas-receptor crosslinkage by membrane-bound Fas ligand (FasL), a protein predominantly expressed by T lymphocytes and NK cells [39]. Although the first description of a Fas-DD NMR analysis dates back to 1996, a satisfactory molecular model explaining all particular features of DISC formation could not be proposed despite numerous efforts [2,40,41]. The first crystal structure reported for this complex resulted in a major breakthrough because it indicated that a DD could employ an unprecedented mode of interaction [23]. Apart from the typical globular death-fold, the DD of Fas could be in an 'open' configuration in which its sixth helix translocates and fuses with the fifth helix to form a large 'stem helix', and an additional, small C-terminal helix (C-helix) (Figure 4a) [23]. This conformational rearrangement would not only expose part of the hydrophobic core to enable binding of FADD-DD but would also accommodate the formation of a 'Fas-Fas bridge' between two 'open' Fas-DDs (Figure 4b). Such 'Fas-Fas bridges' would also be formed under resting conditions, though a stimulus, FasL, would be necessary to stabilize these bridges and induce signaling. In brief, binding of FasL to Fas would result in receptor trimerization and a local, intracellular concentration of 'opened' Fas-DDs. This concentration would then allow the formation of multiple 'Fas-Fas bridges', which would eventually lead to the assembly of a honeycomb-like network at the plasma membrane (Figure 4c). FADD incorporation would further stabilize the network and enable recruitment of procaspase-8 through a homotypic DED/DED interaction between the DED of FADD and the second DED of the procaspase-8 DED tandem [12,13]. According to the proposed model the interaction between 'opened' Fas-DD and FADD-DD would induce an overall conformational rearrangement in FADD that results in the exposure of known procaspase-8-binding residues in the DED of FADD [13,23].

Still, this atypical conformation and mode of interaction for a death-fold domain fails to explain some key observations regarding Fas/FADD-DISC biology. NMR analysis indicated that the small C-helix of Fas-DD does not contribute to complex formation [42,43]. Most of the residues reported to be important for mediating the interaction between the DDs of Fas and FADD do not localize to the interaction interface created by Fas-DD opening [8,9,13,44,45]. Moreover, this 'opened' interaction interface does not contain any of the Fas-DD mutations associated with the autoimmune lymphoproliferative syndrome (ALPS) and that were shown to disrupt the Fas/FADD-DISC [8,46,47]. Finally, the crystals demonstrating the 'Fas-Fas bridge' were generated at high salt and pH 4, a condition at which the complex was reported to dissociate and which could increase the chance of observing atypical conformations [23,42].

A second crystal structure of the Fas/FADD-DISC was reported and dealt with many of these considerations [24]. Although this complex was crystallized at low salt and a more neutral pH, caution is still warranted regarding data interpretation because the crystal structure was derived from a mouse/human hybrid complex (mFas/hFADD) and the X-ray data were obtained at a very low resolution of 6.8 Å [24]. This hybrid Fas-DD/FADD-DD crystal structure was proposed to reflect rather well the physiological complex because analysis of this complex in solution through electron microscopy and mass spectrometry indicated that the size, shape and stoichiometry are similar in both conditions and compare well to the PIDDosome [22,24]. Consequently a model of the Fas/FADD-DISC was based on the PIDDosome model and revealed an asymmetric two-layered structure with a bottom layer of five FADD-DDs and a top layer of five Fas-DDs (Figure 4d) [24]. Furthermore, the DED of FADD seems to point outwards, which renders procaspase-8 recruitment easy [24].

Again the three death-fold interaction types were shown to contribute essentially to complex assembly [22,24,26,28]. Type I interactions predominantly connect the two layers, type II interactions exclusively connect the two layers and type III interactions mostly connect adjacent DDs within a layer (Figure 4e) [24]. The observation that most ALPS-associated Fas-DD mutations map to the identified interaction patches underscores further the validity of this Fas/FADD-DISC model [24]. However, how the 5-fold nature of the model fits to the hexameric receptor

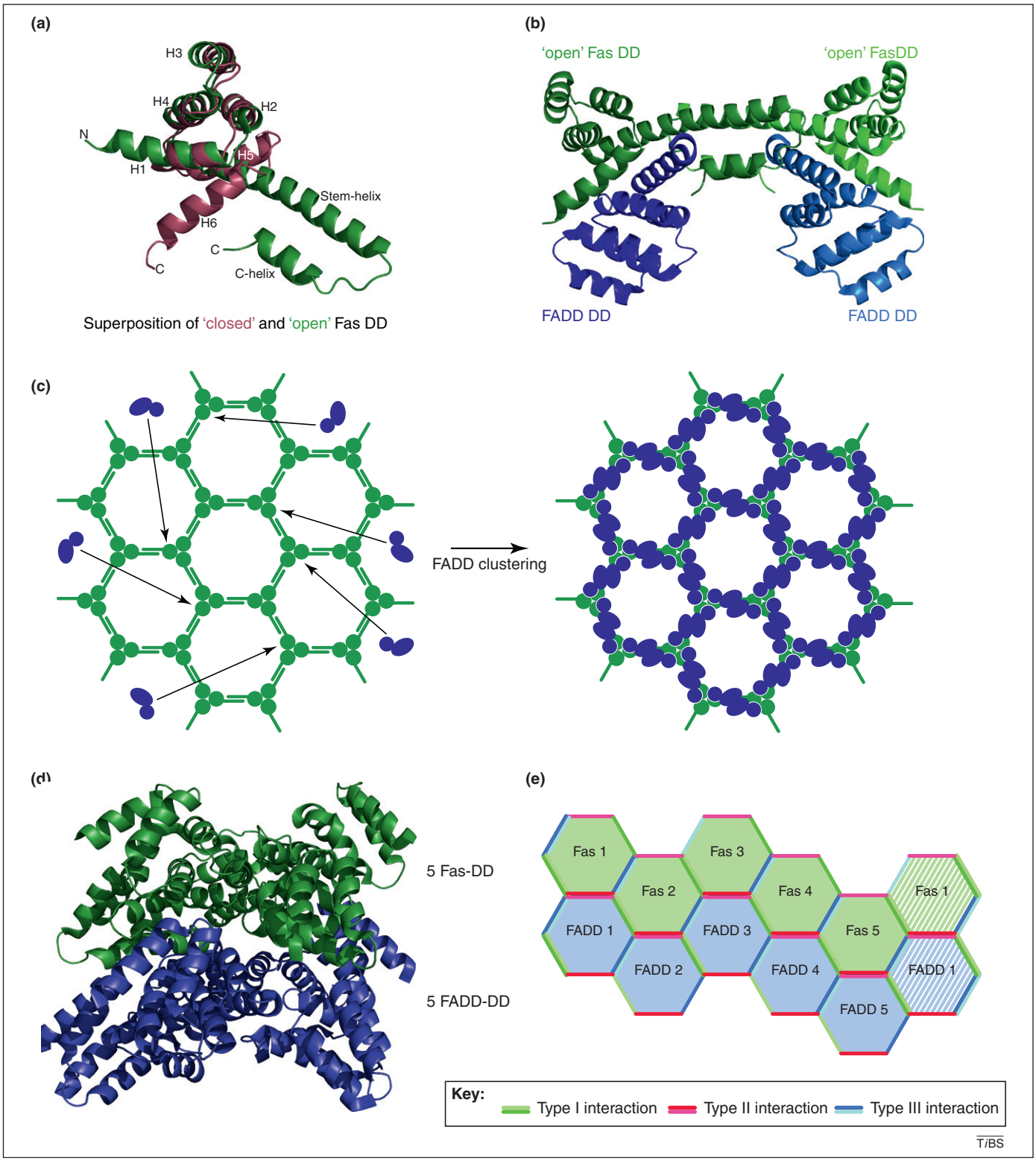

Figure 4. **The Fas/FADD-DISC**. **(a)** Structural alignment of the 'closed' (red) and 'opened' (green) conformation of Fas-DD (PDB ID: 3EZQ). The six α-helices of the 'closed' form are numbered and the newly formed 'stem-helix' and C-helix of the 'opened' form are indicated. **(b)** Diagrammatic representation of a Fas-Fas bridge (green) to which two FADD-DDs (blue) are recruited. **(c)** Model illustrating Fas/FADD-DISC formation. Fas-ligand stimulation triggers trimerization of Fas, which brings together many Fas molecules (green) because they cluster in lipid rafts. The larger number of 'opened' Fas-DDs ensures stabilization of the Fas-Fas bridge, which leads to the emergence of hexagonal Fas clusters. Subsequently, FADD is recruited to the growing complex to stabilize the bridges and the complex as a whole. **(d)** Side view of the second Fas/FADD-DISC crystal structure (PDB ID: 3OQ9). The bottom layer contains five FADD-DDs (blue) and the top layer contains five Fas-DDs (green). **(e)** Schematic, planar illustration of the three interaction types used to assemble the Fas/FADD-DISC. The different interaction patches are colored as in Figure 2.

complexes that get formed by a dimer of FasL trimers, the minimal signaling component of FasL, is not clear [24,48].

Homotypic DED interactions

In contrast to DD subfamily members, which form higher order, multivalent DD complexes, no multivalent complexes involving DEDs have been identified so far. Two studies reported a DED/DED interaction based on the crystal structure of MC159, a viral FLICE/caspase-8 Inhibitory Protein (vFLIP) from the *Molluscum contagiosum* virus, which in analogy to procaspase-8 and procaspase-10 contains a DED tandem [12,29,49]. vFLIPs, mainly found in *Poxviridae* and γ-*herpesviridae*, bind to the DED of FADD and prevent apoptotic cell death of infected cells by interfering with the assembly of the apoptosis-inducing DISC [49]. Thereby, these viral proteins contribute one important mechanism through which the virus might escape the immune surveillance of the host. In analogy, two cellular proteins, $cFLIP_L$ and $cFLIP_S$, regulate death receptor signaling within the cell [50].

The crystal structure of the MC159 DED tandem shows that the DEDs associate tightly through a hydrophobic interface and form a dumbbell-shaped structure [12,29]. Each MC159 DED uses one of its two DED-characterizing hydrophobic patches to form the interaction interface in which helices 2 and 5 of DED1 line up against helices 1 and 4 of DED2 [12,29]. Remarkably, this (helix–helix)/(helix–helix) interaction pattern is consistent with the hallmark type I interaction first described between the CARDs of Apaf-1 and procaspase-9 [15]. In this case, DED1 and DED2 of MC159 contribute patches Ib and Ia, respectively [12,29]. Despite the considerable structural deviation of MC159 DED1 – the third α-helix is reduced to a rigid loop because a few conserved residues in that region are lacking – all DED tandems will probably have a similar compact structure because the residues at the interaction interface are well conserved within the DED subfamily [3,12,29].

Crystal structures describing DED/DED interactions between two proteins have not been reported yet. Nevertheless, combining the information from three studies (the NMR structure of complete FADD, a mutagenesis-based study and a docking study) indicates that the DEDs of FADD and procaspase-8 could be arranged perpendicularly to each other [12,13,51]. In this arrangement, the DED of FADD would use a patch formed by helices 1 and 4 to interact with a patch formed by helices 2 and 5 of the second DED of procaspase-8, a typical type I interaction. The first DED of procaspase-8 is not involved in the interaction with FADD–DED because helices 2 and 5 of procaspase-8–DED1 are necessarily involved in the type I interaction within the procaspase-8 DED tandem.

Homotypic CARD interactions

Knowledge of CARD/CARD interactions is predominantly based on the crystal structure describing the 1:1 interaction between Apaf-1–CARD and procaspase-9–CARD, the first death-fold interaction to be characterized and the prototype of the type I interaction [15]. Functionally, this interaction forms one of the core elements of the apoptosome, a multimeric, procaspase-9-activating complex crucially involved in the intrinsic apoptotic pathway, and triggered by cellular stress or damage-provoking stimuli [52]. In this CARD/CARD interaction, each CARD uses a surface patch complementary in shape and charge to the other one. The convex, acidic patch Ib of Apaf-1–CARD, composed of helices 2 and 3, interacts with the concave, basic patch Ia of the procaspase-9–CARD, composed of the kinked helix 1 and helix 4 [15]. Crystallography, NMR spectroscopy and mutagenic analysis corroborated the lack of tendency for the basic patch of Apaf-1–CARD to interact with the acidic patch of procaspase-9–CARD [15,53,54]. Because conditions of high ionic strength do not disrupt this interaction, electrostatic interactions appear to be involved only in the recognition and correct positioning of the domains [15]. Extensive hydrogen bonding and van der Waals interactions seem to be the driving force for this CARD/CARD interaction [15].

The CARD subfamily as a whole might use this interaction mechanism because most CARDs contain at least one defined charged patch [4,14,16,55]. Indeed, an NMR structure of Nucleotide-binding Oligomerization Domain-containing protein (NOD)1–CARD combined with a homology model of RIP2–CARD suggests that the acidic patch of NOD1–CARD interacts with the basic patch of RIP2–CARD [55]. These results collectively indicate that CARDs would mainly engage in 1:1 interactions in which the correct positioning of the domains relies to a large extent on charge-mediated interactions.

However, crystallographic analysis of the *C. elegans* apoptosome recently indicated that the CARD of CED-4, the *C. elegans* homolog of Apaf-1, can simultaneously interact with at least two other CARDs [25]. Whether these interactions can be categorized as any of the three death-fold interaction types is still unclear. In this complex, the CARDs of CED-4 appear to be organized in two staggered, tetrameric rings wherein the CARDs of one ring are interspersed by the CARDs of the other ring [25]. How the CARD of CED-3, the *C. elegans* homolog of procaspsae-9, integrates in this model is less clear because a 1:1 interaction between CED-4–CARD and CED-3–CARD would cause steric hindrance [25]. Similarly, the 1:1 interaction between Apaf-1–CARD and procaspase-9–CARD within the human apoptosome was questioned [56]. In particular, the disk-like module containing the CARDs located on top of the 7-fold shaped apoptosome cannot fit seven Apaf-1–CARD/procaspase-9–CARD dimers [56]. Taken together these observations question the generalization that CARD/CARD interactions are 1:1. As exemplified by the CED-4 apoptosome more intricate interaction mechanisms can be envisioned for CARDs, although this would require the elucidation of more CARD-containing complexes.

Homotypic PYD interactions

The PYDs are the most recent subfamily to be recognized as part of the death-fold superfamily [57–60]. Many PYD-containing proteins, including ASC, NLRP1, NLRP3 and AIM2, fulfill a crucial role in inflammasome biology. The assembly of inflammasome complexes induced by Pathogen/Danger-Associated Molecular Patterns (PAMP and DAMP) forms an integral part of the innate immune system because it induces an inflammatory condition through procaspase-1 activation, which leads to the proteolytic activation of the

pleiotropic inflammatory cytokines IL-1β and IL-18 [30]. Although these PYD-containing proteins have been extensively studied at the functional level [30], little is known about their tertiary structures or the mechanisms of their molecular interactions. So far, no crystal structures have been reported for this subfamily (Table S1 in the supplementary material online). Structures obtained through NMR analysis confirmed the globular death-fold and gave indications of possible interaction mechanisms [5,18–20,61,62]. However, because the proposed interaction models are still too speculative they are not discussed in this review.

Concluding remarks

Domains of the death-fold superfamily play key roles in the assembly and regulation of complexes important for apoptotic and inflammatory signaling because they enable recruitment of proteins through homotypic interactions. Despite the strong structural homology, an organized bundle of six α-helices being the hallmark of the superfamily, each domain has a well-defined set of interaction partners that are in general confined to members of its own subfamily. An important factor determining this confinement of interaction partners comes from the subfamily-specific structural features. These probably find their origin in divergent evolution by gene duplication, which resulted in the emergence of four death-fold subfamilies that have been well-conserved during evolution (Box 1) [63]. Interestingly, double-stranded DNA (dsDNA) viruses belonging to the *Herpesviridae* and *Poxviridae* acquired through horizontal gene transfer genetic material encoding death-fold domain containing proteins that aid their dissemination within susceptible hosts. To date, two death-fold subfamilies, DEDs and PYDs, have been identified in this context. Tandem DEDs found in the poxvirus *Molluscum contagiosum* virus and several *Herpesviridae* prevent apoptosis by targeting death receptor complexes [49]. Pyrin-Only Proteins (vPOPs) encoded by certain *Poxviridae*, by contrast, were shown to interfere with the inflammatory response of the host by preventing assembly of inflammasome complexes [64]. This adaptive evolution illustrates the importance of death-fold based complexes.

Crystal structures of three higher order, multimeric DD complexes, the PIDDosome, the MyDDosome and the Fas/FADD-DISC, demonstrate that a single DD can engage in up to six interactions through three distinct, well-defined, conserved interaction types involving different helix/loop combinations in the interacting DDs. Whether the utilization of these interaction types is a common feature of the death-fold superfamily as a whole remains speculative because crystal structures of higher order non-DD complexes have not been reported.

Comparing the models of the PIDDosome, the MyDDosome and the second model of the Fas/FADD-DISC indicates that the overall organization of these DD complexes can be regarded as truncated helical oligomers. However, at this point one has to remain cautious in generalizing this model for DD complexes because the first reported crystal structure of the Fas/FADD-DISC illustrated that Fas-DDs might interact with each other through the formation of a 'Fas-Fas bridge' [23], an interaction mechanism not witnessed by others. One possible explanation for this inconsistency could be that the ratio between the different proteins of the complex determines the overall structural organization. Whether this is the case and whether death-folds belonging to other subfamilies can also form helical oligomers remain unanswered questions. Reports on other complexes will shed more light on this issue and are greatly anticipated. Complexes of particular interest include the DD- and DED-containing death receptor and necrosome complexes [65,66], the CARD- and PYD-containing inflammasomes [30], the CARD-containing RIG-I-like receptor complexes [67], and the CARD-containing B cell and T cell receptor proximal complexes [68].

In addition to providing fundamental knowledge, models of death-fold complexes could be helpful for the development of novel therapeutics that could prevent or trigger complex formation. The apoptosome and RIP1 containing complexes are attractive targets for interference because they play key roles in the initiation of apoptosis and necroptosis, respectively. Excessive cell death is associated with multiple pathological conditions, including stroke and neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's disease [66,69]. The inflammasome complexes are valid candidates for therapeutic intervention and also because excessive inflammasome formation is an important etiological factor in the development of multiple (auto-)inflammatory disorders such as gout, type 2 diabetes, silicosis, asbestosis, atherosclerosis and the cryopyrin-associated periodic syndromes [70]. The latter groups three inheritable diseases that are characterized by recurrent episodes of fever and is associated with mutations in the *cias1* gene, which encodes the inflammasome assembling protein cryopyrin/NLRP3 [71]. ALPS is one disease that might be responsive to complex-inducing therapeutics because ALPS-associated mutations in the DD of Fas preclude formation of the Fas/FADD-DISC, which results in the accumulation of autoimmune T cells [72]. Together, these diseases exemplify the enormous potential of therapeutics targeting death-fold containing complexes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tibs.2011.06.006.

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