

The Fas–FADD death domain complex structure unravels signalling by receptor clustering

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The death inducing signalling complex (DISC) formed by Fas receptor, FADD (Fas-associated death domain protein) and caspase 8 is a pivotal trigger of apoptosis^{1–3}. The Fas–FADD DISC represents a receptor platform, which once assembled initiates the induction of programmed cell death. A highly oligomeric network of homotypic protein interactions comprised of the death domains of Fas and FADD is at the centre of DISC formation^{4,5}. Thus, characterizing the mechanistic basis for the Fas–FADD interaction is crucial for understanding DISC signalling but has remained unclear largely because of a lack of structural data. We have successfully formed and isolated the human Fas–FADD death domain complex and report the 2.7 Å crystal structure. The complex shows a tetrameric arrangement of four FADD death domains bound to four Fas death domains. We show that an opening of the Fas death domain exposes the FADD binding site and simultaneously generates a Fas–Fas bridge. The result is a regulatory Fas–FADD complex bridge governed by weak protein–protein interactions revealing a model where the complex itself functions as a mechanistic switch. This switch prevents accidental DISC assembly, yet allows for highly processive DISC formation and clustering upon a sufficient stimulus. In addition to depicting a previously unknown mode of death domain interactions, these results further uncover a mechanism for receptor signalling solely by oligomerization and clustering events.

As with other oligomeric signalling platforms, the DISC acts as a cellular switch, which exists in the ‘off’ position in the absence of a stimulus followed by oligomerization of its constituents to form the active (‘on’ position) oligomeric platform⁶. In the case of the Fas–FADD DISC the apoptotic signal per se is the binding of Fas ligand (FasL), which in a basic view (Supplementary Fig. 1a) leads to recruitment of FADD via death domain interactions. FADD in turn recruits caspase 8 through death effector domain (DED) interactions leading to activation of this apical caspase^{1,2,7}. However, from a cell signalling point of view this string of events remains on a descriptive level, as the binding partners do not (and should not) interact in the absence of a sufficient stimulus. Productive DISC formation is only observed when ligand binding occurs in a permissive environment, such as predisposition of Fas in membrane rafts, and is furthermore characterized by the formation of highly oligomeric DISC clusters^{8–14}. Because the death domain interaction of Fas and FADD is at the heart of the DISC interaction network (Supplementary Fig. 1b), the question arises about how a simple death domain interaction mediates such complexity. Other signalling platforms, for example the apoptosome or inflammasomes^{15,16}, are proposed to elegantly exploit a nucleotide-dependent regulation to properly signal oligomerization as opposed to the seemingly simple death domain interaction of Fas and FADD.

In addition to mutations implicated in disease states, a variety of mutants have been generated in order to define interfaces in the Fas–FADD complex (Supplementary Table 2; reviewed in refs 5, 14). However, despite this information and effort, the nature of the primary Fas–FADD interaction and its implication for the actual mechanism of formation of the Fas–FADD death domain network remains elusive¹⁴. In our study we were able to gain an insight into this mechanism by elucidating the crystal structure of the Fas–FADD death domain complex from which we conclude a model explaining how observed contacts are mediated by a primary Fas–FADD complex, which acts as a sensitive switch governing DISC formation. This extends and completes our view of death domains, which initiated from rigid defined domain complexes such as the constitutive 1:1 complex seen in the APAF-1–caspase 9 CARD–CARD interaction, to current views which include plasticity and asymmetry of complexes in the death domain superfamily⁵ (see also Supplementary Discussion). Thus, the model presented here can furthermore serve as a template for other signalling platforms lacking enzymatic components which are solely mediated by oligomerization and clustering events.

Despite its elusiveness and solubility difficulties¹⁴, we succeeded in producing a soluble Fas–FADD complex when *Escherichia coli* lysates of recombinantly expressed Fas death domain and FADD death domain were combined before purification. After purification we optimized conditions to obtain crystals of the complex and solved the 2.7 Å resolution structure. Crystals indexed in the hexagonal space group $P6_1$ with two tetrameric assemblies each comprising four Fas death domains and four FADD death domains in the asymmetric unit (Fig. 1 and Supplementary Table 1). The tetrameric arrangement represents a dimer of two Fas–FADD death domain complex dimers. In this arrangement Fas provides all contacts (see also Supplementary Discussion). All residues of the Fas death domain are well defined in the electron density. FADD is defined from G93 to G191 with the region around residues 107–149 showing more diffuse density due to a high degree of overall motion caused by a lack of crystal contacts (Supplementary Fig. 1c).

Inspection of the interfaces shows that the actual Fas–FADD complex is formed by helix one and six of the FADD death domain through employment of a hydrophobic patch surrounded by polar residues (Fig. 2a, d). Unlike the FADD death domain, the Fas death domain in the complex deviates from the typical death domain fold^{17–19}. Comparison with the solution structure of the isolated Fas death domain²⁰ reveals that Fas in the complex has undergone an opening in which helix six has shifted and fused with helix five to form a long helix that we term the stem helix (Fig. 2b, c). In addition to Fas opening, a new helix at the carboxy terminus of Fas, dubbed the C-helix, is observed (Fig. 2a). The main interaction interface on

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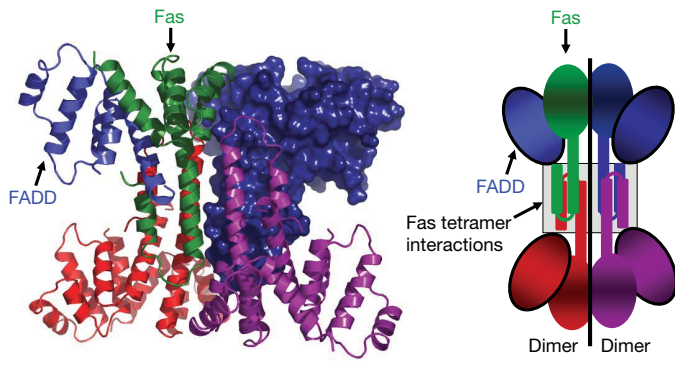


Figure 1 | Overall structure of the Fas–FADD death domain complex. The structure (left panel) shows a tetrameric arrangement of Fas–FADD death domain complexes. Contacts between the complexes are solely mediated by Fas molecules. The right panel shows a cartoon representation of the Fas–FADD complex structure. One Fas–FADD death domain complex is displayed in green (Fas) and blue (FADD) ribbons, whereas the remaining three complexes are coloured red, magenta and blue (surface representation).

Fas is formed by hydrophobic residues that become available only upon Fas opening (Fig. 2d). Thus, the opening of Fas discovered in the crystal structure is a crucial event in its interaction with FADD. Notably, other FADD interacting death receptors show similar residues as those observed in Fas at the complex interface (see Supplementary Fig. 4c and Supplementary Discussion).

Broader investigation of the Fas–FADD interface divulges the disperse nature of the interaction surface, which lacks the presence of defined and focused interaction sites often referred to as hotspots^{21–23}. This phenomena is used in regulatory complexes characterized by weak binding despite sufficiently large interaction surfaces^{22,24,25}. In addition to the lack of hotspots, a loss of compact tertiary structure owing to the opening of Fas further enhances flexibility of the system and thus additionally weakens the complex. Besides providing the primary interface for binding of FADD, a second consequence of the opening of Fas is to allow for interaction with another open Fas molecule forming a Fas–Fas bridge. The predominant Fas–Fas association observed in the structure is formed by stem helices and C-helices of the two Fas molecules building a Fas–Fas dimer (Fig. 2e), which compared to the tetramer, better approximates structural requirements in the context of the natural membrane association (see Supplementary Discussion). Thus, opening of Fas provides both the basis for FADD binding and the formation of a regulatory bridge between Fas molecules. Owing to the difficult nature of the Fas–FADD complex, biochemical analysis is limited (Supplementary Fig. 2a), but indeed confirms a weak primary Fas–FADD complex which dissociates at concentrations as high as 50 μM (Fig. 2f and Supplementary Fig. 2d). Mutational analysis of the Fas–FADD interface reveals predominately mild phenotypes (Supplementary Fig. 3a–c, and data not shown), in line with previous observations for complexes lacking binding hotspots such as IFN α 2/IFNAR1²⁶.

In solution, the Fas–FADD death domain complex indeed shows a tetrameric behaviour (Supplementary Fig. 2b–d), which is further corroborated by previously reported results of a solubility enhanced complex that also pointed to a tetrameric arrangement²⁷. Taken together, these results establish that the observed tetramer in the crystal structure reflects the Fas–FADD death domain arrangement present in solution and is not a result of crystal formation. In addition, the tendency of the tetramer to form higher oligomers can be observed (Supplementary Fig. 2c, d).

To obtain further insight into the binding of FADD, we overlaid the structure of full-length FADD¹⁸ onto the death domain of FADD in the primary complex (Fig. 3a). The marked conformational

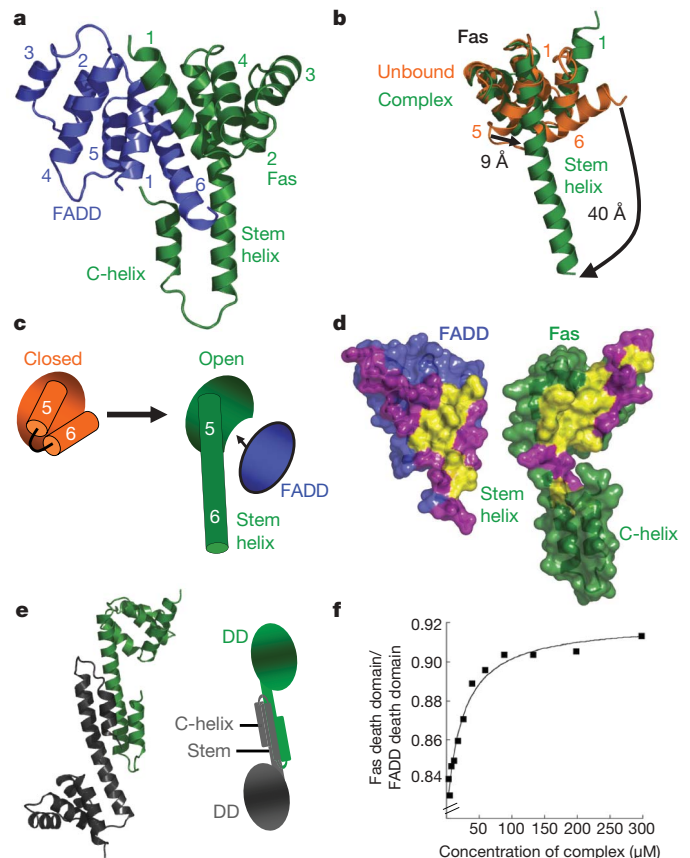


Figure 2 | Fas–FADD death domain complex: Fas–FADD and Fas–Fas interactions are dependent on Fas opening. **a**, Primary Fas–FADD death domain complex. FADD (blue) adopts the characteristic death domain fold with helices one and six participating in the main interaction site. In Fas (green) only helices one to four approximately adopt a death-domain-like fold, whereas a long helix (stem helix) is found in place of helices five and six, which together with helix one provides the main interaction residues for FADD binding. Additionally, a helix at the C terminus of Fas (C-helix) is observed. **b**, Conformational change in Fas. Comparison of the structure of unbound Fas death domain (closed form in orange; Protein Data Bank entry 1DDF) and Fas in the Fas–FADD complex. Owing to formation of the stem helix, residues of helix five and six shift significantly. Furthermore, the rearrangement of helix six exposes part of the hydrophobic core of Fas. **c**, Cartoon illustration of Fas opening. **d**, Primary Fas–FADD interface. View onto interfaces governing primary complex formation. Surface representation shows complementary hydrophobic patches (yellow) on FADD and Fas surrounded by polar residues (magenta). The hydrophobic interface on Fas becomes exposed upon Fas opening. **e**, Fas dimer unit. Another consequence of Fas opening is the formation of Fas dimer units, which interact via the stem and C-helix. The right panel is a cartoon representation of the dimer. DD, death domain. **f**, The Fas–FADD death domain complex is weak. A dilution experiment of the isolated Fas–FADD complex shows cooperative dissociation of the complex below concentrations as high as 50 μM (plot derived from quantitative SDS–PAGE analysis of Fas death domain retained on Ni–NTA resin from various Fas death domain/FADD death domain–His₆ complex dilutions).

change in Fas, which is essential to bind FADD, is also accompanied by a change in the FADD death domain. The C-terminal helix of FADD has to shift to avoid a steric clash with the newly formed C-helix of Fas, substantiating results that attribute a negative role in cell death induction to this region of Fas (Fig. 3a and Supplementary Fig. 3d)²⁸. This observation is even more crucial in the context of full-length FADD, as the FADD C terminus is located at the interface of the DED and death domain. Along this line, an overall conformational rearrangement, namely the relative position of the DED and the death domain of FADD, can also be concluded to expose caspase-8-binding residues determined in earlier studies

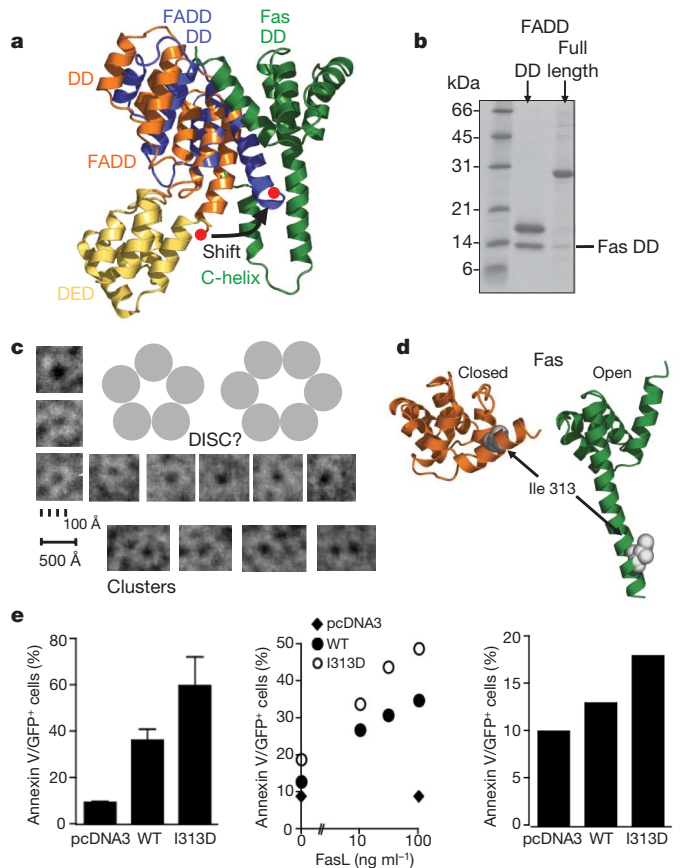


Figure 3 | The Fas-FADD bridge in the DISC: binding of full-length FADD and the key role of Fas opening *in vivo*. **a**, Overlay of the structure of full-length FADD (Protein Data Bank entry 2gf5; orange) onto the Fas-FADD complex structure. (Full-length FADD refers to the well characterized FADD F25Y mutant; see Supplementary Methods.) The last helix of the death domain of unbound FADD (red dot) shifts to avoid clashing with the newly formed Fas C-helix in the Fas-FADD complex. **b**, Conformational change in full-length FADD. Proteins were expressed separately, and His-tagged versions of the death domain of FADD, or full-length FADD, were mixed with untagged Fas. Ni-NTA chromatography demonstrates that full-length FADD shows reduced initial binding to the Fas death domain, when compared to the FADD death domain protein. **c**, Although initial binding of full-length FADD to Fas death domain is reduced, prolonged incubation leads to the formation of DISC-like structures. Incubation of both proteins overnight led to the formation of ring-like structures with a strong tendency to form clusters as determined using electron microscopy. Displayed are single ring-like structures and clusters from several images. Owing to their strong tendency to self-adhere, no consistent monolayer for thorough evaluation could be generated so far. **d**, **e**, Propagating Fas opening results in hyperactive Fas. **d**, Location of Ile 313 in closed (unbound, orange) and open (complex, green) forms of Fas. **e**, Huh7 cells transfected with Fas I313D show elevated cell death, as assessed by annexin V reactivity, compared with Fas wild type on stimulation with Fas antibody (left, error bars indicate standard error of the mean, $n = 3$), FasL (middle), and also in the absence of a stimulus (right). Equal cell surface Fas expression was confirmed by FACS analysis and immunoblot (data not shown).

(summarized in refs 5, 18). This change is readily enabled by the interdomain linker, which is composed exclusively of Ala, Gly and Pro residues. Indeed, our studies found that when full-length FADD is incubated with Fas a reduced initial complex formation can be observed, underlining a conformational change (Fig. 3b), whereas longer incubation of the two proteins leads to the formation of protein clusters (Fig. 3c).

Details revealed in the Fas-FADD death domain structure show that Fas opening is key to both FADD binding and formation of a Fas-Fas bridge. To validate the relevance of Fas opening *in vivo*, we sought to create a mutation that interfered with the closed form of

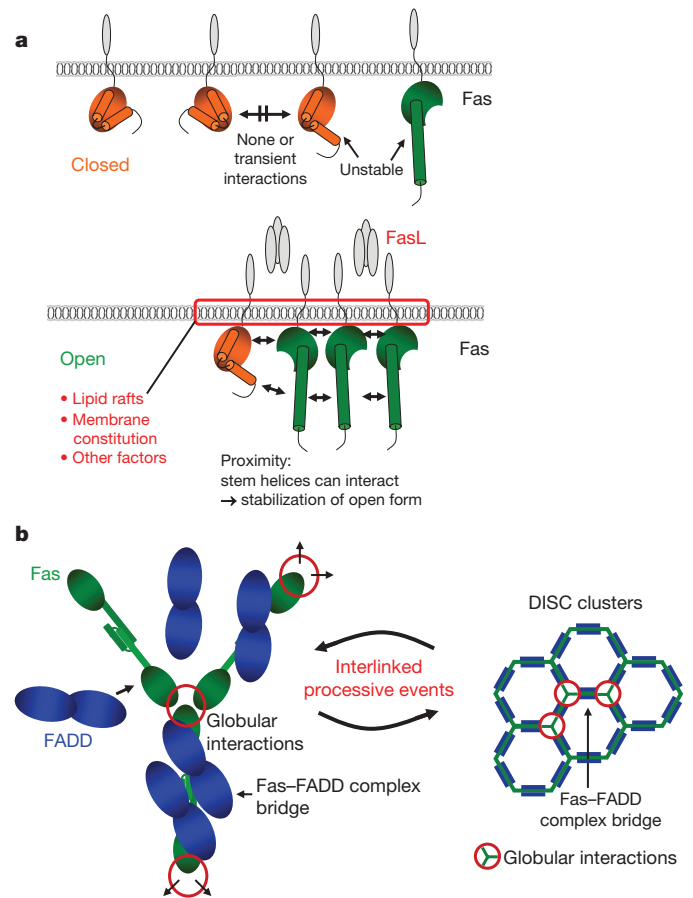


Figure 4 | Model of DISC formation: mechanism of receptor signalling through clustering. Schematic diagram illustrating DISC formation. **a**, Model for Fas opening. Like in any two-state model, it can be assumed that the closed and open forms of Fas exist in equilibrium. In the absence of an apoptotic signal the equilibrium between closed and open forms of Fas strongly favours the closed form whereas open forms are unstable. Upon an apoptotic stimulus the equilibrium shifts to favour the open form of Fas. Fas molecules are brought together by Fas ligand in a permissive environment dependent on several factors, including lipid rafts and membrane constitution. The close proximity of Fas death domains now allows for stem helices to interact, leading to stabilization of the open form. **b**, Opening and formation of the Fas-Fas bridge links trimeric death domain units defined in earlier studies (Supplementary Table 2), which are formed by globular regions of the open Fas. FADD can now bind to the open Fas molecules, further stabilizing the bridge. The consequence is processive interlinked DISC formation and clustering in which open Fas molecules interacting via their globular domains are linked by a multitude of weak Fas-FADD bridges, leading to overall stable DISC clusters. This permits activation of caspase 8, presumably by proximity-enforced dimerization³⁰, and induction of apoptosis.

Fas and propagated Fas opening. If Fas opening is a prerequisite for DISC formation, this forced opening of Fas should produce a hyperactive phenotype. By comparing the structures of closed and open Fas we identified Ile 313 as a candidate. Ile 313 is part of the hydrophobic core and resides in helix 6 of the closed form and is thus at the centre of the opening event. At the same time it does not directly participate in the Fas-FADD interaction or the observed Fas-Fas dimer in the complex structure (Fig. 3d and Supplementary Fig. 4a). Indeed, mutation of Ile 313 to Asp (I313D) resulted in significantly enhanced killing. Enhanced cell death by Fas I313D compared to wild type could be observed when cells were stimulated with Fas antibody or FasL and even in the absence of a stimulus, further establishing the central role of Fas opening (Fig. 3e).

On the basis of the analysis of the Fas-FADD structure, which displays key regulatory features of DISC formation, and taking into

account previous findings, we conclude a model of how receptor signalling in the DISC occurs solely through clustering events. In this model the opening of Fas is central to form the Fas–Fas bridge and to recruit FADD. The Fas–Fas bridge and FADD binding are governed by weak protein–protein interactions only stabilized upon processive clustering, thus representing the actual regulatory switch governing DISC formation (Fig. 4).

Until now, the dominant model for DISC formation proposed that trimeric Fas ligand crosslinked units of preformed trimeric Fas in local membrane rafts^{8–14}, leading to higher order arrangements. This model is substantiated by mutations in the Fas death domain (Supplementary Table 2; reviewed in refs 5, 14) that are not located at the primary Fas–FADD interface, but rather adjacently on the globular units of open Fas observed here. While illustrating the overall process, this initial model did not provide a mechanism for the actual clustering of Fas death domains within the cell and most importantly the relationship between Fas clustering and FADD binding, both of which are explained in our model. In this model only when a sufficient number of Fas molecules are in close proximity, as is expected on Fas ligand binding, can the open forms of Fas become stabilized (Fig. 4a), because the newly formed stem helices can interact with each other. An interlinked consequence of this interaction is that Fas molecules become connected through this sensitive bridge—setting the stage for a chain reaction. Now the formation of Fas bridges linking the Fas death domain trimeric units—which interact via the globular portion of open Fas—directly results in rapid and processive clustering on the inside of the cell membrane (Fig. 4b). Also, FADD is now able to bind the open Fas molecules, key for DISC signalling, and additionally increasing stabilization of the Fas–Fas bridges, thereby further fostering DISC formation and clustering (Fig. 4b).

The proximity-induced stabilization of open Fas, in combination with the formation of the weak bridge, allows timing and strict regulation of DISC formation solely by clustering as opposed to, for example, a nucleotide-exchange-mediated oligomerization as in the apoptosome²⁹.

METHODS SUMMARY

The Fas–FADD death domain complex was obtained by mixing crude *E. coli* lysates from recombinantly expressed proteins at high concentration. Large amounts of pure, soluble and crystallization-grade complex could be obtained after Ni-affinity and ion-exchange chromatography. Crystallization could be achieved under acidic conditions, which after several rounds of optimization resulted in diffracting crystals, one to 2.7 Å resolution. Initial phases were obtained using a Pt-derivative crystal followed by standard structure solution using the native data.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions F.L.S. performed and evaluated *in vivo* studies. J.J.L. and M.K.D. performed cloning, protein expression and crystallization. C.P. performed biochemical analyses. H.R., B.S. and in particular R.S. performed data collection and structure solution. E.M. performed EM-studies. G.S.S. participated in study design and evaluation. All authors discussed the work. S.J.R. participated in and oversaw all aspects of the work and wrote the manuscript.

Author Information Atomic coordinates and experimental structure factors have been deposited within the Protein Data Bank and are accessible under the code 3EZQ. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.J.R. (sriedl@burnham.org) or R.S. (robert.schwarzenbacher@sbg.ac.at).