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Lipid rafts and raft-mediated supramolecular entities in the regulation of CD95 death receptor apoptotic signaling

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Abstract Membrane lipid rafts are highly ordered membrane domains enriched in cholesterol, sphingolipids and gangliosides that have the property to segregate and concentrate proteins. Lipid and protein composition of lipid rafts differs from that of the surrounding membrane, thus providing sorting platforms and hubs for signal transduction molecules, including CD95 death receptor-mediated signaling. CD95 can be recruited to rafts in a reversible way through S-palmitoylation following activation of cells with its physiological cognate ligand as well as with a wide variety of inducers, including several antitumor drugs through ligand-independent intracellular mechanisms. CD95 translocation to rafts can be modulated pharmacologically, thus becoming a target for the treatment of apoptosis-defective diseases, such as cancer. CD95-mediated signaling largely depends on protein–protein interactions, and the recruitment and concentration of CD95 and distinct downstream apoptotic molecules in membrane raft domains, forming raft-based supramolecular entities that act as hubs for apoptotic signaling molecules, favors the generation and amplification of apoptotic signals. Efficient CD95-mediated apoptosis involves CD95 and raft internalization, as well as the involvement of different subcellular organelles. In this review, we briefly summarize and discuss the involvement of lipid rafts in the regulation of CD95-mediated apoptosis that may provide a new avenue for cancer therapy.

Keywords Lipid rafts · CD95 · Fas · Apoptotic signaling · Death receptor

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Introduction

Plasma membrane establishes a boundary that separates the interior of a cell from the environment and is essential for all types of life from the simplest to the most complex. Plasma membrane constitutes a barrier for most substances, thus separating the intracellular milieu and living material within the cell from the non-living environment. Nevertheless, the cell membrane is not entirely an impermeable barrier, and it determines what type of compounds and how can get in and out of the cells, thus allowing some molecules to be concentrated inside the cells. The plasma membrane of eukaryotic cells is selectively permeable for some exogenous molecules, whereas large molecules like DNA, proteins and many drugs are impermeable due to their size or biochemical properties. This compartmentalization enables chemical reactions to take place within the cell that would otherwise be impossible. However, the perception of the plasma membrane as an impermeable barrier has been challenged by the translocation of the so-called cell-permeable peptides or cell-penetrating peptides (CPPs) across the plasma membrane and their subsequent access to the cytosol, even when fused to large hydrophilic proteins by mechanisms that are not fully understood [1–3]. These CPPs are relatively short cationic and/or amphipathic peptides that are able to reach the cytoplasmic and/or nuclear compartments in live cells after internalization,

thus acting as efficient vectors that can internalize hydrophilic cargoes, and so providing valuable biological and potentially therapeutic tools for targeting proteins into cells [2, 4, 5]. CPPs uptake is suggested to involve both endocytosis and direct translocation across the plasma membrane through localized regions of the cell surface [6–8]. In addition, not only plasma membranes separate the inside and outside of a cell, but they receive signals from either outside and inside the cells, interpret and transduce these signals leading to a cell response. The cell membrane, made up of a lipid bilayer with interspersed proteins, is currently viewed as a compartmentalized and dynamic structure showing regions with different fluidity characteristics that modulate diverse cellular functions, including cell signaling. In this regard, strongly packed and rigid cholesterol- and sphingolipid-rich membrane domains, named lipid rafts, function as platforms for the recruitment of signaling proteins to facilitate protein–protein interaction and signal transduction [9, 10]. These lipid rafts have the ability to form specialized and localized domains with distinct composition and physical properties, thus modulating a variety of cellular responses [11]. A large number of survival and proliferation signal transduction pathways, including the insulin-like growth factor-I/phosphatidylinositol 3-kinase (PI3K)/Akt signaling, have been found to locate in lipid rafts for efficient signaling [12], thus supporting a major role of these membrane raft domains in cancer development and progression. However, with the advent of the new millennium new evidence established that efficient apoptotic signaling mediated by the CD95 death receptor also required its translocation and recruitment in lipid rafts [13–15]. Thus, membrane raft domains play a major role in the regulation of cell fate, being involved in the promotion of either survival or cell death. Interestingly, recruitment of CD95 in lipid rafts is not only rendered by the physiological interaction of the death receptor with its cognate ligand, but it can be triggered by a series of different stimuli, including anticancer drugs, thus constituting a new target for apoptosis-directed cancer therapy [16–23].

Lipid rafts and methods used for their analysis

Our current view of membrane organization and structure has profoundly changed since the previous fluid mosaic model introduced by Singer and Nicolson in 1972 [24], who considered the cell membrane as a two-dimensional oriented solution of integral proteins or lipoproteins in the viscous phospholipid bilayer solvent. However, the view of considering the proteins moving freely in a kind of fluid lipid sea was challenged by new evidence that suggested the organization of membrane lipid components into domains [25]. A growing body of evidence indicated the presence of more ordered membrane domains containing sterols, most importantly cholesterol or its analogs in other organisms, as well as the coexistence of liquid-disordered (l_d) and liquid-ordered (l_o) phases in the same lipid bilayer [26–28], thus paving the way for the advent of a particular type of a more ordered membrane domain enriched in sterols and sphingolipids termed as lipid rafts [9–11, 29–33]. The lipid raft hypothesis proposed by Simons and Ikonen in 1997 [29] suggests that cholesterol- and glycosphingolipid-rich domains act as lipid-ordered platforms that float in a sea of disordered phospholipids and where proteins can segregate into or out of these membrane regions.

The size of the lipid raft microdomain depends on the cell type and membrane composition, but it is suggested to be smaller than the optical diffraction limit (250 nm), and thereby direct imaging of these membrane regions is not possible with conventional optical microscopy. However, larger raft platforms are generated following cell activation by clustering of small unstable raft units. A consensus definition of a lipid raft emerged at the 2006 Keystone Symposium of Lipid Rafts and Cell Function, held in Steamboat Springs (CO), as follows: “membrane rafts are small (10–200 nm), heterogenous, highly dynamic, sterol- and sphingolipid-enriched domains that compartmentalize cellular processes. Small rafts can sometimes be stabilized to form larger platforms through protein–protein and protein–lipid interactions” [34]. From an operational point of view, a lipid raft could be defined as a membrane domain that is resistant to extraction in cold 1 % Triton X-100 and that floats in the upper half of a 5–30 % sucrose density gradient. This feature is based on the resistance of lipid rafts to solubilization by nonionic detergents, like Triton X-100, at 4 °C, due to the high enrichment of sphingolipids and cholesterol in rafts. This insoluble fraction or detergent-resistant membrane (DRM) can be isolated by flotation as low-density membranes in density gradients, such as sucrose gradients, and this raft isolation method was the first operational criterion to define a raft-associated protein [29, 35, 36]. A variety of additional detergents have also been employed to isolate DRM domains, but the use of different detergents has rendered domains with distinct composition [37, 38]. The use of Triton X-100 or CHAPS has led to rafts strongly enriched in sphingolipids and cholesterol as compared with total cell membranes, whereas Tween 20, Brij 58 or Lubrol WX yielded DRM domains with little enrichment in the above typical raft lipids [37]. This dependency on the detergent use could indicate heterogeneity within the lipid rafts. In fact, lipid rafts appear to be heterogeneous in their protein and lipid content, as well as they can be localized to different regions of the cell [38–42]. Compositional heterogeneity of lipid

rafts may provide the spatial and temporal regulation of various cellular functions, including cell signaling. Evidence for the association of a protein with cholesterol-rich rafts is strengthened when it becomes detergent-soluble after depletion of cholesterol from the membrane by the use of methyl-β-cyclodextrin or other agents, and when it is supported by fluorescence microscopy approaches.

Nevertheless, the physiological existence of rafts has been challenged by a number of criticisms [43–46], especially those referring to the use of detergents during their isolation and identification that could lead to artifacts and misinterpretations [44, 45]. Thus, the widely used nonionic detergent Triton has been reported to create ordered domains in a homogeneous fluid membrane in model bilayers [47]. The use of synthetic lipid bilayer membranes is of major importance in the studies of lipids in membrane biophysical properties, but due to their drastic differences with physiological biomembranes regarding protein content, membrane–cytoskeleton interactions and natural asymmetry, conclusions should also be taken with caution if not supported by data from physiological biomembranes. Also, a main concern has been raised on the diverse effects that might be expected by depleting cholesterol from the membrane because cholesterol, in addition to be a major component of rafts, has important further functions in the whole plasma membrane. Thus, the presence and functional involvement of rafts in the plasma membrane of living cells as well as the raft localization of proteins, based only on the use of detergents and cholesterol depletion, has been challenged, and therefore caution should be taken before assigning a role of rafts in different biological processes. Trying to deal with the above criticisms and doubts, a number of modifications have been achieved for lipid raft isolation that involve isolation of lipid rafts by detergent-free methods using sodium carbonate buffer and/or shearing followed by density gradient ultracentrifugation along sucrose or Optiprep^{TM} step density gradients [48–51]. Also, fluorescence microscopy has been extensively used in the field in order to visualize raft platforms in the cell, and fluorophores conjugated to cholera toxin B subunit, which binds to ganglioside GM1 [52] that is mainly found in rafts [53], are being broadly used in raft localization studies. A growing number of fluorescent molecular probes are being synthesized to facilitate membrane raft research and the identification of lipid rafts in living cells, including probes for raft lipid components, probes with selective portioning into l_o or l_d phases, and environment-sensitive dyes that partition in both phases but stain them by different color, intensity or lifetime [54].

On these grounds, as mentioned above, a combination of both microscopic (e.g.: fluorescence microscopy) and biochemical (lipid raft isolation) approaches, together with functional studies disrupting rafts by cholesterol synthesis inhibition (using 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors or gene knockdown), sequestration (using filipin, nystatin or amphotericin) or depletion (using methyl-β-cyclodextrin), and the use of well-established raft and non-raft markers further support studies on the role of lipid rafts and raft localization of proteins. The usefulness and reliability of these methods are highly increased to monitor changes in raft composition or raft-association of a determined protein when compared in the absence (control) and presence of physiological or unnatural stimuli.

On the other hand, the evidence for the physiological presence of rafts in the plasma membrane of living cells has, until recently, not been compelling, thus raising some doubts about their physiological existence, mainly because of their difficulty in being visualized due to their small size below the classical diffraction limit of light microscopy. However, the advent of novel microscopy techniques has finally demonstrated the existence of lipid rafts in the cell. Compelling evidence by using high temporal and spatial resolution techniques, including stimulated emission depletion (STED) microscopy, fluorescence resonance energy transfer (FRET), differential polarization laser scanning confocal microscopy (DP-CLSM), fluorescence polarization anisotropy (FPA), total internal reflection fluorescence (TIRF) microscopy, single quantum dot tracking, single particle tracking (SPT), fluorescence correlation spectroscopy (FCS), two-photon microscopy, and near-field scanning optical microscopy (NSOM) in combination with quantum dots, have conclusively demonstrated the transient trapping of certain proteins with reduced mobility in cholesterol-dependent clusters in live cells, the existence of phase separation in vivo, and that the fluidity of lipid rafts are principally governed by cholesterol content [55–66]. The use of non-invasive optical recording of molecular time traces and fluctuation data in tunable nanoscale domains is a powerful approach to study the dynamics of biomolecules and rafts in living cells.

The biophysical mechanisms responsible for the formation and regulation of lipid rafts enriched in saturated lipids and cholesterol remains largely elusive, and the mechanisms by which cells regulate the size, lifetime, and spatial localization of these raft domains are rather poorly understood at the moment. The use of stimulated emission depletion (STED) far-field fluorescence nanoscopy to detect single diffusing (lipid) molecules in nanosized areas in the plasma membrane of living cells, which allows to spot sizes approximately 70-fold below the diffraction barrier, has shown that unlike phosphoglycerolipids, sphingolipids and glycosylphosphatidylinositol-anchored proteins are transiently ($\sim$10–20 ms) trapped in cholesterol-mediated molecular complexes dwelling within <20-nm diameter areas in living cells [63]. A number of scenarios have been

proposed for the formation of lipid rafts including: (a) thermal equilibrium fluctuations near the critical temperature, which results in the formation of small, short lifetime raft domains and non-raft domains in the membrane; (b) immobile surfactant membrane proteins that pin compositional lipid interfaces and induce finite-sized rafts; (c) presence of proteins that bind specifically to raft components leading to raft domain stabilization through protein–raft interactions and an increase in raft lifetime and size; (d) membrane coupling to a lipid reservoir; (e) vesicular and non-vesicular lipid recycling that could result in rafts with a broad size distribution [67–70]. Protein–raft clusters might result from short range attraction, which induces clustering, and long range repulsion, which prevents the formation of large clusters [70]. Biophysical approaches have recently shown that 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and cholesterol mix in the l_o state, but exhibit repulsive interactions in the l_d phase, thus leading to a kind of "push and pull" forces in the interactions between unsaturated phospholipids and cholesterol in eukaryotic cell membranes for lipid raft formation [71].

CD95 death receptor

Mammalian cells express fully functional receptors at the cell surface that trigger suicide when bound to their cognate ligands, thus triggering a cell-extrinsic pathway of apoptosis by ligand engagement of death receptors. Death receptors are members of the tumor necrosis factor (TNF) receptor (TNFR) superfamily characterized by an ~80–88 amino acid long α-helical fold in the cytoplasmic region termed the death domain (DD), a homotypic protein interaction module composed of a bundle of six α-helices that plays a critical role in the induction of apoptosis [72–74]. DD-containing TNFRs have been reported in vertebrates including zebrafish [75], avian [76], and mammalian cells [77, 78]. In addition, Eiger and its receptor, Wengen, comprise the *Drosophila* orthologs of the TNF/TNFR system, but the amino acid sequence of Wengen reveals no DD in the cytoplasmic region [79–82]. Eight members of the death receptor family have been identified in mammalian cells, which can be divided into four structurally homologous clades, namely: the p75(NTR) clade (consisting of ectodysplasin A receptor, death receptor 6 (DR6) and p75 neurotrophin (NTR) receptor); the tumor necrosis factor receptor 1 clade (TNFR1 and DR3); the CD95 clade (CD95, a.k.a. Fas or APO-1); and the TNF-related apoptosis-inducing ligand (TRAIL) receptor (TRAILR) clade (TRAILR1/DR4 and TRAILR2/DR5) [83]. The amino acid sequence and structure of the distinct death receptors has diversified during evolution, suggesting that divergence among death receptors has led to their particular functional specialization [84]. Members of a particular death receptor clade participate in similar processes, further suggesting that structural diversification enables functional specialization. Thus, some receptors appear to be mainly involved in the immune response, whereas others play critical roles in cell-mediated processes during development and tissue differentiation [84]. CD95 and TRAILR clades seem to have specialized in inducing cell death, and CD95 can be considered as a paradigm of this receptor-mediated cell response. Death receptors diversified during early vertebrate evolution indicating that the DD fold has plasticity and specificity that can be easily adjusted to attain additional functions.

CD95 death receptor was originally identified by antibodies that induced apoptosis in human cell lines. Shin Yonehara reported an IgM monoclonal antibody in 1989 that could kill several human cell lines, and termed FS7-associated cell surface antigen (Fas) the cell surface protein recognized by the antibody [85, 86]. Likewise in the same year, Dr. Peter H. Krammer and his associates reported a mouse monoclonal antibody (anti-APO-1 antibody) that promoted apoptosis in human leukemic cells and activated lymphocytes [87]. Soon afterwards, it turned out that both antibodies recognized the same cell surface antigen. Shigekazu Nagata and his associates cloned in 1991 the membrane protein, Fas antigen, recognized by the Fas killing antibody [88], which turned out to be identical to the APO-1 cell surface antigen after being purified and cloned a year later in Krammer's group [89]. Subsequently, the physiological ligand of the Fas death receptor, named Fas ligand (FasL), was cloned in 1993 [90], thus identifying the Fas/FasL system as the major regulator of apoptosis at the cell membrane in mammalian cells through a receptor/ligand interaction [91, 92]. The above antibodies recognizing Fas and APO-1 were included in the CD95 differentiation cluster of the 5th International Workshop on Human Leukocyte Differentiation Antigens, held in Boston in November 1993 [93, 94]. FasL/CD95L belongs to the TNF family [90], can be found as a 40-kDa membrane-bound or a 26-kDa soluble cytokine [95], and was assigned as CD178 in the 7th International Workshop on Human Leukocyte Differentiation Antigens, held in Harrogate, UK in June 2000 [96, 97]. Human mature CD95 (Fig. 1) is a 45–48 kDa type I transmembrane receptor of 319 amino acids (after subtraction of the 16-amino acid signal peptide) with a single transmembrane domain of 17 amino acids (from Leu-158 to Val-174), an N-terminal cysteine-rich extracellular domain (18 cysteine residues in 157 amino acids) and a C-terminal cytoplasmic domain of 145 amino acids that is relatively abundant in charged amino acids (24 basic and 19 acidic amino acids). The cytoplasmic portion of CD95 contains a domain of about 88 amino acids termed

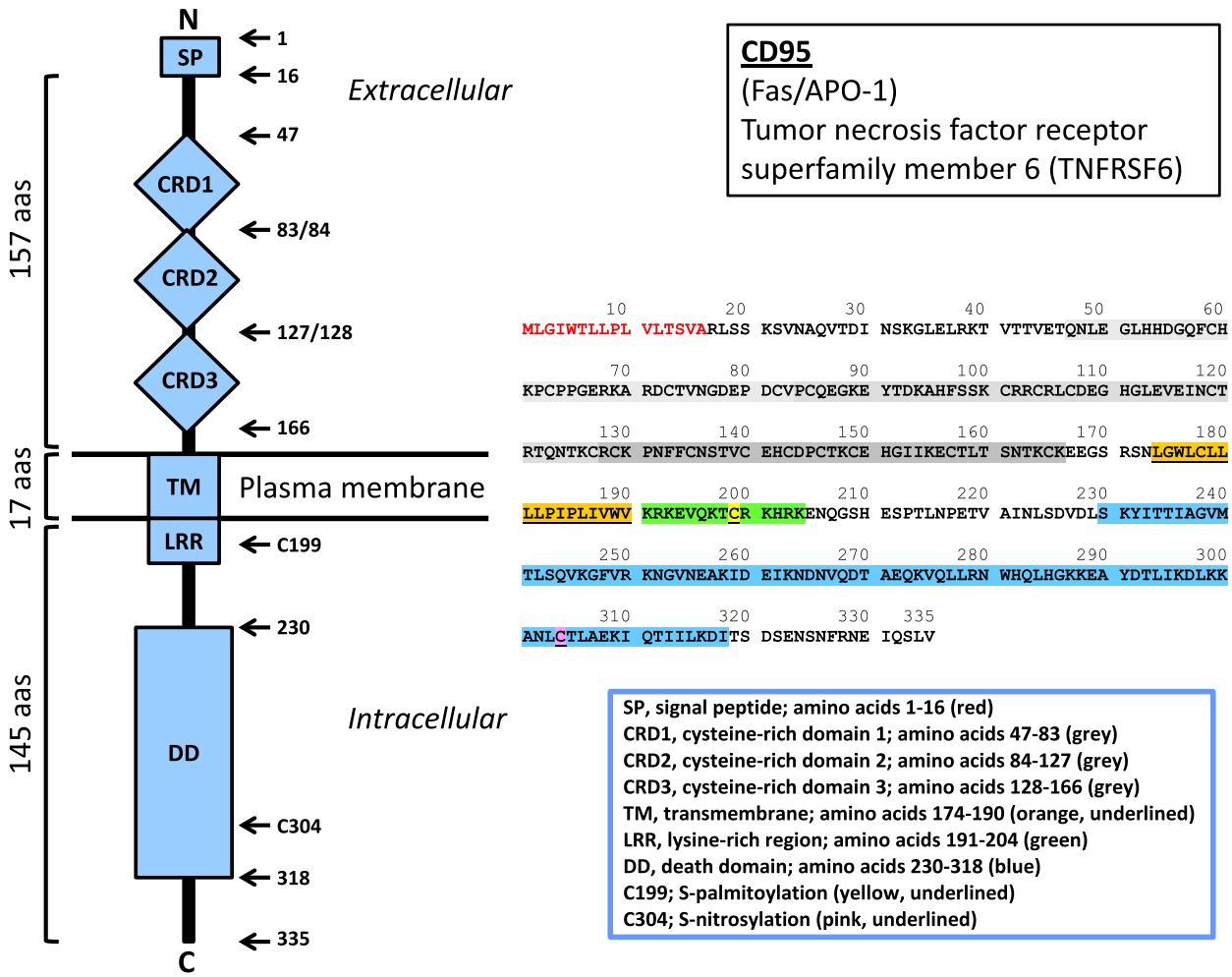

Fig. 1 Primary sequence and schematic diagram of human CD95 death receptor. Human CD95 contains a signal peptide (SP; aminoacid residues 1–16), and thus the mature CD95 protein consists of 319 amino acids (aas) with a N-terminal extracellular domain of 157 aas, a short (17 aas) transmembrane region and a C-terminal cytoplasmic domain of 145 aas. Relevant domains and amino acid residues for CD95 binding to lipid rafts (LRR, C199 and C304) and apoptotic activity (DD), as well as the three cysteine-rich domains (graded *grey* intensity) involved in ligand binding, are indicated. See text for further details

"death domain" (DD, from Ser-230 to Ile-318) (Fig. 1), which is homologous to other death receptors and plays a crucial role in transmitting the death signal from the cell surface to intracellular pathways [16, 74, 88, 92, 98, 99]. Unlike the intracellular regions of other transmembrane receptors involved in signal transduction, the DD does not possess enzymatic activity, but mediates signaling through protein–protein interactions. The DD has the propensity to self-associate, forming large aggregates in solution. The tertiary structure of the CD95-DD consists of six antiparallel amphipathic α helices, and the presence of a high number of charged amino acids in the surface of the death domain is probably responsible for mediating the interactions between DDs [72–74]. CD95 is present at the plasma membrane as a preassociated homo-oligomeric receptor [100–102], and its interaction with FasL/CD95L or agonistic antibodies triggers reorganization of CD95 multimers [103] and recruitment of the adaptor molecule Fas-associated death domain-containing protein (FADD) [104, 105] through interaction between its own DD and the clustered receptor DDs. FADD comprises an N-terminal death effector domain (DED) tethered to the C-terminal FADD-DD, and interaction of FADD with CD95 via their DDs leads to binding of the FADD–DED with an analogous DED domain repeated in tandem within the zymogen form of procaspase-8 [106]. Upon recruitment by FADD, procaspase-8 oligomerization drives its activation through self-cleavage, activating downstream effector caspases and triggering apoptosis [77]. Thus, activation of CD95 by its cognate ligand FasL/CD95L results in receptor aggregation and formation of the so-called "death-inducing signaling complex" (DISC) [107], made up of CD95, FADD and procaspase-8. The interaction of the CD95-DD with the adaptor protein FADD is a critical step in assembling the

DISC leading to an oligomeric structure composed of 5 CD95-DD and 5 FADD-DD [108, 109]. This complexation is independent of the C-terminal 12 residues of CD95, which are dispensable for complex formation between the two DDs [110].

Accumulating evidence gathered by different laboratories [99] in the late 1990s and early noughties following studies on the killing mechanism by distinct inducers, including campothecin [111], CDDP [112], curcumin [113], edelfosine [13, 114, 115], perifosine [116], resveratrol [117], vinblastine [112], etoposide/VP16 [112], ultraviolet light [118–120], c-Jun N-terminal kinase (JNK) activation [121], glutamine-deprivation-mediated cell shrinkage [122], hepatitis C core protein [123], transforming growth factor-β1 [124], and herpes simplex thymidine kinase/ganciclovir system [125], led to the conclusion that CD95 could also be activated in a FasL/CD95L-independent manner. We and others found FasL/CD95L-independent activation of CD95 in the mechanism of action of a number of antitumor drugs, such as the antitumor alkylphospholipid analog edelfosine, cisplatin, etoposide/VP16 and vinblastine [112, 115]. The presence of cell surface CD95 correlated well with the sensitivity of tumor cells to the proapoptotic activity of edelfosine, and cells deficient in CD95 were resistant to the alkylphospholipid analog, but became sensitive to the antitumor drug when transfected with CD95 [114–116]. Edelfosine activated CD95-mediated signaling and apoptosis in hematopoietic tumor cells, and blockade of CD95-FasL/CD95L interactions did not prevent edelfosine-induced apoptosis [115, 116]. Silencing of CD95 by RNA interference, transfection with a FADD dominant-negative mutant that blocks CD95 signaling, and specific inhibition of caspase-8 prevented the apoptotic response triggered by edelfosine in T cell leukemia Jurkat cells, hence demonstrating the functional role of DISC in drug-induced apoptosis [126]. Likewise, a CD95-deficient multiple myeloma subline was resistant to edelfosine, but CD95 retrovirus transduction bestowed edelfosine sensitivity in these cells [116]. Infection of the CD95-deficient multiple myeloma subline with retroviruses containing a truncated version of human CD95 lacking the 57 C-terminal amino acids (amino acids 279–335) that included part of the CD95 death domain [98] led to cells that expressed high levels of cell-surface CD95 but were resistant to edelfosine [116]. Furthermore, drug microinjection experiments in cells unable to incorporate edelfosine that were transfected for ectopic expression of active and inactive versions of CD95 showed that edelfosine should be inside the cell to activate CD95-mediated apoptotic signaling, thus demonstrating the intracellular activation of CD95 without the participation of extracellular FasL/CD95L [114]. These data indicated that CD95 could also receive orders from within the cell. Thus, cells could dictate its own demise through activation of CD95 death receptor without the external influence of its cognate ligand. Down-regulation of FADD by transient transfection of an antisense FADD construct inhibited tumor cell sensitivity to cisplatin, etoposide or vinblastine, whereas overexpression of FADD sensitized tumor cells to drug-induced cell death [112]. Transfection of cells with FADD dominant negative decreased apoptosis induced both by cisplatin [112] or alkylphospholipid analogs [126, 127]. Transient transfection with either MC159 or E8, two viral proteins that inhibit apoptosis at the level of FADD and procaspase-8 respectively [128], protected cells from cisplatin-induced cytotoxicity [112]. Incubation with neutralizing anti-CD95 antibodies (such as ZB4 and SM1/23 antibodies) or with the soluble CD95-IgG chimera fusion protein to block the interaction of CD95 with FasL/CD95L failed to inhibit drug-induced apoptosis [112, 115, 116]. Taken together, these data indicate that some antitumor drugs induce cell death through a CD95–FADD pathway in a FasL/CD95L-independent manner.

CD95 in non-apoptotic signaling

CD95 is not only able to trigger apoptosis but can also promote non-apoptotic functions including cell proliferation [129–134], differentiation [134], inflammatory cytokine expression [135, 136], tumor progression [137], motility, and invasiveness [138–140], involving a number of survival and proinvasive pathways, including transcription nuclear factor-kappa B (NF-κB), extracellular-signal-regulated kinase (ERK), cofilin, PI3K and metalloproteinase-9 (MMP-9) [141–146]. In addition to the above non-apoptotic functions, CD95 plays a major role in inflammation and trigger the recruitment of peripheral myeloid cells to the inflammatory site [147]. In this regard, expression of CD95L on tumor cell xenografts has been shown to induce inflammation by massive neutrophil infiltration [148, 149]. On these grounds, CD95 acts as a double-edge sword promoting either apoptosis or survival leading to tumor regression or protumorigenic responses [150]. A major question that remains to be fully answered is what determines the outcome from the same ligand receptor of the above different and sometimes diametrically opposed signaling pathways, either apoptosis or survival, as well as how these CD95-mediated divergent tumor responses are fully regulated. Recent studies have suggested that at least part of this regulation leading to distinct outcomes might occur at the level of incorporation of specific protein components into lipid rafts [151]. As discussed below CD95-mediated apoptotic signaling involves CD95 translocation and clustering into lipid rafts [13, 114], and expression of sphingomyelin causes CD95-mediated

apoptosis with translocation of CD95 into lipid rafts and subsequent CD95 clustering [152]. Interestingly, redistribution of TRAILR and DISC assembly in lipid rafts promotes caspase-8-initiated apoptosis, whereas TRAILR–DISC assembly in the non-raft phase of the plasma membrane, mediated by receptor-interacting protein (RIP) and cellular Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein (c-FLIP), leads to the inhibition of caspase-8 cleavage and NF-κB and ERK1/2 activation in non-small cell lung carcinoma (NSCLC) [153]. Thus, these studies suggest that RIP and c-FLIP-mediated assembly of the DISC in non-rafts is a critical upstream event in TRAIL resistance and death receptor-mediated survival signaling, and it is tempting to envisage that redistribution of TNFR family members and DISC components from non-raft domains to lipid rafts could control the switch between survival signals and apoptosis [153].

CD95 apoptotic signaling and lipid rafts

In 2000 researchers in Italy [154] and Spain [115] independently found polarization and capping of CD95 in the cell surface of human T cells susceptible of CD95-mediated apoptosis and of human T-cell leukemia Jurkat cells treated with the antitumor drug edelfosine, respectively. The antitumor ether phospholipid edelfosine (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine, ET-18-OCH_3) is the prototype of a family of compounds collectively known as synthetic alkylphospholipid analogs [18, 74, 155–157], and is a stark example of how the study of the mechanism of action of a chemotherapeutic agent can contribute to unveil basic processes in cell biology, leading to significant conceptual and practical advances in the intersection of chemistry and biology. Soon after the finding of the antitumor drug-mediated aggregation of CD95 at one pole of the cell to form caps on the cell surface of hematopietic cancer cells [115], we found in 2001 that edelfosine induced translocation of CD95 into lipid rafts in human T-cell acute leukemia Jurkat and human acute myeloid leukemia HL-60 cells, leading to a co-clustering of CD95 and membrane rafts, which was visualized by confocal microscopy using the raft marker cholera toxin B subunit and was further assessed by raft isolation through sucrose gradient centrifugation [13]. Raft disruption following treatment with the cholesterol-depleting agent methyl-β-cyclodextrin, inhibited both edelfosine-induced CD95 clustering and apoptosis [13]. Also, in the early noughties, subsequent studies showed that the natural ligand FasL/CD95L also induced translocation of the cognate receptor in rafts [14, 158]. Lipid rafts had been previously shown to act as platforms for cell survival and proliferation signaling pathways [12], but taken together the above data provided clear-cut evidence for the role of lipid rafts in harboring cell death signaling molecules, such as death receptor CD95 [16].

Also in the early nougthies the lipid molecule ceramide was involved in the clustering of CD95 into ceramide-rich rafts [15, 159–161]. However, C_{16}-ceramide did not trigger CD95 clustering in the absence of a stimulatory anti-CD95 antibody or FasL/CD95L [15], suggesting that ceramide acted as a mediator of the clustering process, but not as an initiator of the process, amplifying the primary CD95 signaling events. Accumulating evidence [15, 159, 160, 162, 163] suggests that following CD95 engagement CD95–FasL/CD95L complexes enter initially into small membrane rafts and induce a weak formation of the DISC, leading to caspase-8 activation. This rather weak caspase-8 activation could then generate ceramide by sphingomyelinase translocation to the small lipid rafts and subsequent activation. Because sphingomyelin is present in rafts in high amounts (as much as 70 % of all cellular sphingomyelin can be found in rafts [164]), the generated ceramide could induce coalescence of elementary rafts [165], leading to the formation of large patches containing CD95–FasL/CD95L complexes that would further potentiate DISC formation, thereby favoring CD95 signaling. Thus, sphingomyelinase and ceramide serve to amplify the signaling of CD95 at the membrane level after the initial CD95–FasL/CD95L interaction. This notion is supported by biophysical studies showing that, by increasing membrane order, ceramide reduces the lateral diffusion of membrane proteins and lipids, trapping and clustering those molecules [166]. Ceramide does not alter the preferred localization of the CD95 transmembrane domain in l_d non-raft membrane region, and thereby neither the CD95 transmembrane domain membrane organization nor its conformation are affected by ceramide [167], but diffusion of CD95 oligomer would be reduced in lipid rafts containing ceramide, thus minimizing complex dissociation and receptor translocation out of those raft domains [167]. As a result, the presence of ceramide could favor the formation of large and stable apoptotic CD95 clusters that might interact with other proteins of the apoptotic machinery, thus enhancing apoptosis signaling.

The above data indicate that lipid rafts play a major role in the regulation of death receptor-mediated apoptosis, and research conducted in the noughties set up the basis for future raft-mediated cell death studies. It might be envisaged that edelfosine mimicked to some extent the action of the natural ligand FasL/CD95L on fostering death receptor clustering in lipid rafts, but in a FasL/CD95L-independent manner. These data also indicated that CD95 aggregation in rafts and its ensuing activation could be modulated

pharmacologically. In subsequent studies, we found that edelfosine accumulated in lipid rafts, promoting the formation of raft clusters or platforms, and not only induced recruitment of the death receptor CD95 in lipid rafts, but also promoted translocation of downstream signaling molecules into rafts, including the apoptotic complex DISC, required to launch apoptotic signals, in various hematopoietic cancer cells [114, 116, 126]. Formation of the apoptotic complex DISC in lipid rafts upon edelfosine treatment was assessed by electron microscopy (Fig. 2) and co-immunoprecipitation assays [116, 126]. Several reports have shown that additional antitumor drugs promote the recruitment of the three major constituents of DISC, namely CD95, FADD and procaspase-8/10, into lipid rafts, including perifosine [116], cisplatin [168], resveratrol [117, 169], aplidin [170], rituximab [171], and avicin D [172] (Fig. 3). The above DISC apoptotic complex can also be formed with the TRAILR death receptors [173–175], and these TRAILR-mediated DISCs are also suggested to be recruited into rafts following treatment with a number of antitumor drugs [116, 169, 176]. These data indicate that recruitment of death receptor signaling in lipid rafts constitute a novel and prominent mechanism in cancer chemotherapy. Table 1 shows a list of inducers that have been reported to promote the recruitment of death receptors and downstream signaling molecules to lipid rafts. The recruitment of death receptors and downstream signaling molecules in lipid rafts would enhance protein–protein interactions, thus facilitating the generation and delivery of apoptotic signals, and could promote synergy with death receptor ligands to achieve cell death. In this context, a number of antitumor drugs, including resveratrol [169, 176], aplidin [170], edelfosine [20, 116] and perifosine [116] have been reported to recruit death receptors CD95, TNFR1 and the TRAIL receptors death receptor (DR) 4 and DR5 into lipid rafts (Table 1), and this protein redistribution sensitized the cells to death receptor stimulation by their cognate ligands or agonistic cytotoxic antibodies [116, 176].

However, despite CD95 activation takes place mainly within the milieu of lipid rafts and raft disruption blocks CD95-dependent apoptosis [12, 13, 16, 20, 21, 114, 177], in some cell types the opposite has also been reported, where exclusion of CD95 from lipid rafts leads to the spontaneous, ligand-independent activation of this death receptor. Disruption of lipid rafts by cholesterol-depleting compounds (methyl-β-cyclodextrin, filipin III, cholesterol oxidase, and mevastatin) in epidermal keratinocytes leads to a spontaneous clustering of CD95 in the non-raft compartment of the plasma membrane, formation of CD95-FADD complexes, activation of caspase-8, and apoptosis [178]. Likewise, cholesterol depletion in radiosensitive tongue squamous cell carcinoma SCC61 line by the cholesterol-depleting agent methyl-β-cyclodextrin resulted in the triggering of apoptosis, involving clustering of CD95, formation of CD95–FADD complexes and cleavage of procaspase-8 [179]. On the other hand, ginsenoside Rh2, which is structurally similar to cholesterol, exhibits antitumor activity, increases membrane fluidity in HeLa cells, and behaves as novel lipid raft disruptor leading to CD95 oligomerization and apoptosis [180]. Ginsenoside Rh2 treatment leads to internalization of lipid rafts and caveolae and inactivation of Akt [180, 181]. These data suggest that CD95-mediated apoptosis and trafficking is modulated by lipid rafts by mechanisms that remain to be fully elucidated and that appear to be cell type specific, regarding the clustering and activation of CD95 inside or outside the lipid rafts.

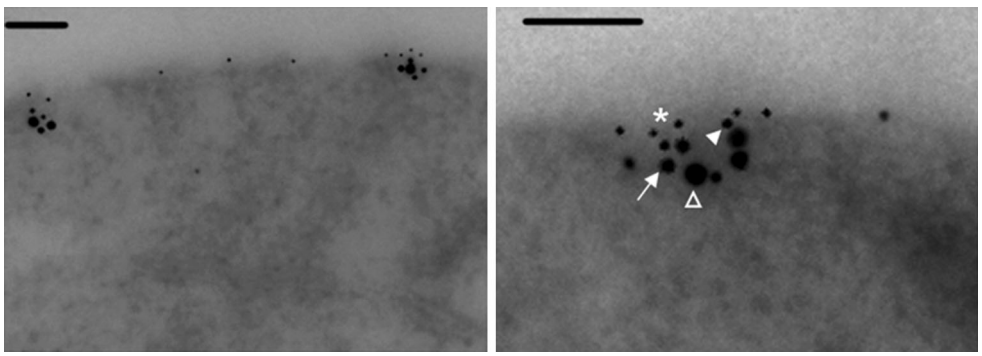

Fig. 2 Electron microscopy evidence for CD95-DISC localization in clustered lipid rafts in edelfosine-treated leukemic cells. Sections of edelfosine-treated human acute T-cell leukemia Jurkat cells were labeled with the raft marker ganglioside GM1 using cholera toxin B subunit (6-nm gold, *asterisk*), anti-CD95 antibody (10-nm gold, *closed arrowhead*), anti-FADD antibody (15-nm gold, *arrow*), and anti-procaspase-8 antibody (20-nm gold, *open arrowhead*). Lipid rafts are labeled on the external face of the membrane, whereas DISC components are located in the internal face of raft-enriched membrane domains. *Bar* 400 nm. Data from Ref. [99]. ©Public Library of Science

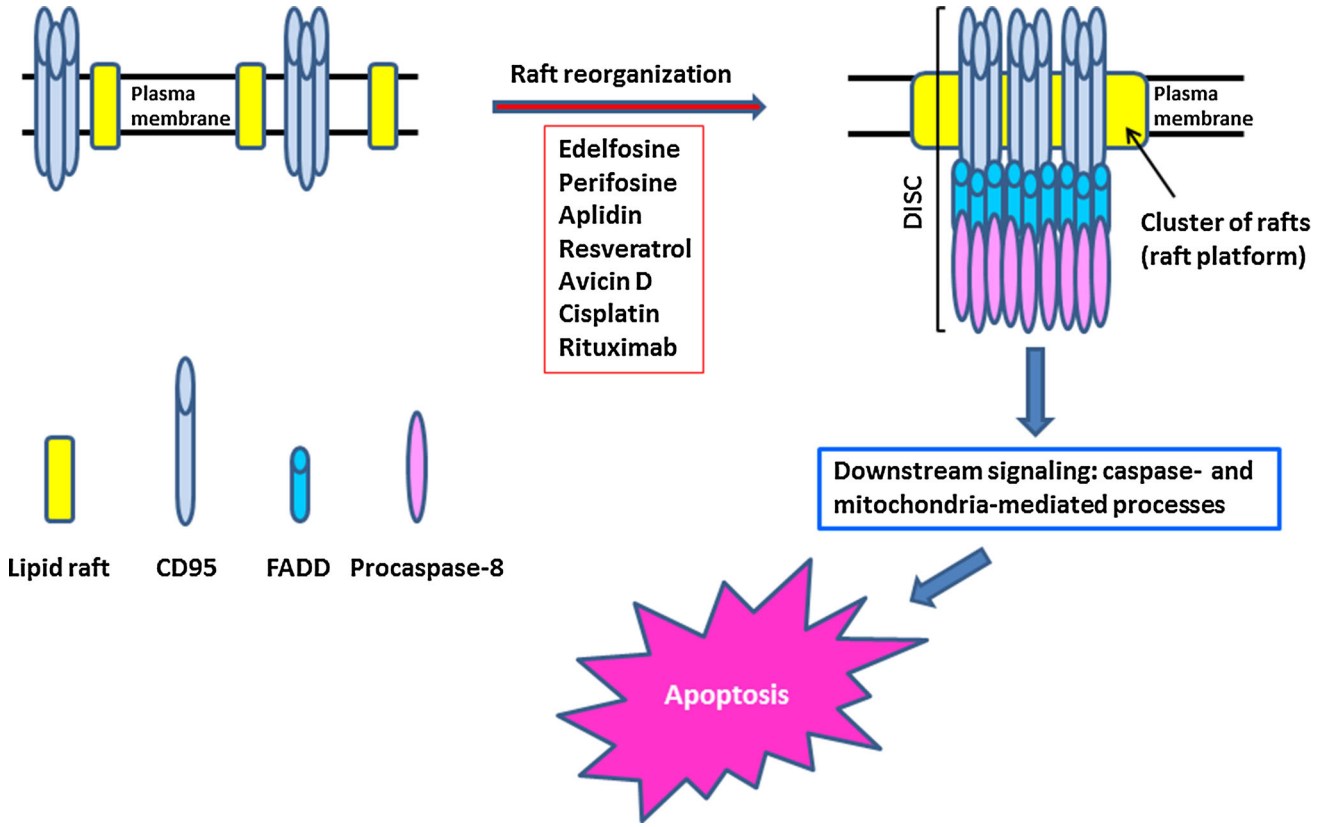

Fig. 3 Recruitment and clustering of CD95-DISC in lipid raft platforms following treatment with antitumor drugs. The indicated anticancer drugs induce apoptosis in distinct cancer cells through recruitment of CD95, FADD and procaspase-8 to clusters of rafts or large raft platforms, thus leading to the triggering of apoptotic signals through caspase activation- and mitochondria-mediated processes. Both protein recruitment and induction of apoptosis is independent of the physiological ligand FasL/CD95L. See text for further details

Phosphatidylinositol 3-kinase as an intracellular signaling route regulating CD95 location in lipid rafts

Activation of PI3K after ligation of CD3 in Th2 cells has been reported to block CD95 aggregation and subsequent caspase-8 cleavage, by altering lateral diffusion of CD95 and blocking the formation of SDS-stable CD95 aggregates and disrupting proper DISC function [182, 183]. On these grounds, it could be envisaged that one of the actions of PI3K signaling in promoting cell survival might be the inhibition of CD95 clustering required to trigger downstream apoptosis signaling. Inhibition of PI3K by using either edelfosine or the specific inhibitors LY294002 and wortmannin leads to the clustering of CD95 in lipid rafts in human leukemic cell lines, thus triggering CD95-mediated apoptosis independently of the CD95-FasL/CD95L interaction [184].

Class I PI3K and its downstream target Akt (a.k.a. protein kinase B) are major survival signaling molecules involved in the control of cell proliferation, apoptosis and oncogenesis, and aberrant activation of the PI3K/Akt pathway contributes to the development and invasiveness of cancer cells [185, 186]. Accumulating evidence has shown that the PI3K/Akt pathway is compartmentalized in plasma membrane rafts [187], and cholesterol-rich rafts mediate Akt signaling in cancer cells [188–191]. We have recently found that edelfosine displaces PI3K/Akt signaling from lipid rafts in mantle cell lymphoma cells, leading to Akt inhibition and CD95 recruitment in membrane rafts [21, 192]. Thus, the above data point out that an intracellular stimulus (PI3K activation) is able to control the raft localization and aggregation of the death receptor CD95. The constitutive activation of PI3K/Akt signaling in numerous types of cancer cells, including mantle lymphoma cells [193, 194], could explain the absence of CD95 in the rafts from untreated mantle lymphoma cells, and the recruitment of CD95 into rafts upon Akt inhibition following edelfosine treatment [192]. These data provide a regulatory connection between survival and apoptotic signaling pathways involving lipid rafts.

Palmitoylation is required for CD95 binding to lipid rafts, subsequent internalization, and efficient CD95 cell death signaling

Raft l_o membranes have a bilayer thickness of 40–48 Å [195, 196], with a hydrophobic region of ~30–38 Å,

Table 1 Recruitment of death receptors and downstream signaling molecules to lipid rafts by distinct inducers in a FasL/CD95L-independent manner

Inducer	Cell line	Death receptor recruited in rafts	Downstream apoptotic signaling molecules recruited in rafts	References
Akt inhibition	Jurkat	CD95		[287]
Anandamide	Mz-ChA-1	CD95		[288]
Aplidin	Jurkat	CD95, DR5, TNF-R1	FADD, procaspase-8, procaspase-10, JNK, Bid	[170]
Avicin D	Jurkat	CD95	FADD, procaspase-8, procaspase-7, Bid	[172]
Cisplatin	HT29	CD95	FADD, procaspase-8	[168]
Edelfosine	Jurkat	CD95	FADD, procaspase-8, procaspase-10, JNK, Bid	[13, 114, 126]
Edelfosine	MM144	CD95, DR4, DR5, TNF-R1	FADD, procaspase-8, procaspase-9, procaspase-10, JNK, Bid, cytochrome *c*, APAF-1	[20, 116, 233]
Perifosine	MM144	CD95, DR4, DR5	FADD, procaspase-8, Bid	[116]
PI3K inhibition (LY294002, wortmannin	Jurkat, CEM	CD95		[184]
Resveratrol	HT29	CD95, DR4, DR5	FADD, procaspase-8	[176]
Resveratrol	Jurkat	CD95, DR5	FADD, procaspase-8, procaspase-10, JNK, Bid	[169]
Resveratrol	MM144	CD95, DR4, DR5	FADD, procaspase-8, procaspase-10, JNK, Bid	[169]
Resveratrol	SW480	CD95	FADD, procaspase-8	[117]
Rituximab	Ramos	CD95	FADD, procaspase-8	[171]
Ultraviolet light	M624	CD95	FADD, procaspase-8	[289]

CEM human acute T-cell leukemia cell line, *HT29* human colon carcinoma cell line, *Jurkat* human acute T-cell leukemia cell line, *M624* human melanoma cell line, *MM144* human multiple myeloma cell line, *Mz-ChA-1 cells* human cholangiocarcinoma cell line, *SW480* human colon carcinoma cell line, *Ramos* human Burkitt's lymphoma cell line

considering the distance from the headgroup peak to the edge of the bilayer for phosphatodylcholine or sphingomyelin is ~5 Å. Non-raft l_d membranes have a thickness of 36–38 Å with a hydrophobic region of 26–38 Å [195]. CD95 has a 17-residue-long hydrophobic transmembrane domain (residues 174–190 in the CD95 amino acid sequence: LGWLCLLLLPIPLIVWV) (Fig. 1) [16] with α-helical structure and a putative length of ~26 Å, considering the spacing between the amino acids in the α-helix is 1.5 Å. Thus, there is a mismatch between the length of the CD95 transmembrane domain and the hydrophobic thickness of l_o membranes. In addition, biophysical approaches with lipid model membranes indicate that the CD95 transmembrane domain is preferentially localized in non-raft l_d membrane regions [167].

Because of the absence of a transmembrane domain compatible with the location of CD95 in lipid rafts, the localization of this protein in these l_o domains should be explained by a protein post-translational modification that could change the CD95 membrane interacting features. In this regard, it has been shown that palmitoylation is required for CD95 localization in lipid rafts and efficient cell death signaling [197, 198]. Using site-directed mutagenesis, CD95 resulted palmitoylated in the membrane proximal intracellular region at the cysteine residue 199 for human (Fig. 1) and 194 for mouse CD95 [197, 198], whereas mutation of the respective C residue for V [197] or S [198] prevented incorporation of [^{3}H] palmitate into CD95 following labeling with [^{3}H] palmitic acid and subsequent immunoprecipitation. Palmitoylation has been found to be required for the redistribution of CD95 to actin cytoskeleton-linked rafts following CD95 stimulation, raft-dependent, ezrin-mediated cytoskeleton association of CD95, and efficient caspase-8-independent receptor internalization [197]. The location of CD95 in membrane rafts is critical as CD95 internalizes from these specialized membrane domains [199]. Internalization of CD95 is essential for the recruitment of DISC components to the death receptor, taking place after CD95 has moved into an endosomal compartment, leading to apoptosis signaling [103, 200]. In contrast, the absence of CD95 internalization results in activation of proliferative and survival ERK and NF-κB signaling pathways [200]. Thus, the subcellular localization and internalization pathways of CD95 seem to play critical roles in triggering distinct signaling routes that could lead to divergent cellular fates and outcome [200, 201].

CD95-mediated apoptosis requires the formation of higher-order receptor aggregates that can be detected as

SDS- and β-mercaptoethanol resistant species of CD95 on SDS-PAGE (a.k.a. SDS-stable CD95 aggregates) that migrate in the range of 90–200 kDa depending on the cell type [107, 202]. These SDS-stable CD95 aggregates accumulate in lipid rafts, due in part to an increase in the amount of FADD and processed caspase-8 [198], but further recruitment of DISC components, and in fact most of DISC formation, occur after internalization of CD95 into endosomal/lysosomal compartments [200]. Interestingly, palmitoylation at the membrane proximal cysteine 199 leads to the formation of SDS-stable CD95 aggregates, which as mentioned above correspond to very high molecular weight DISC complexes and are the sites of caspase-8 activation [198]. Inhibition of palmitoylation with the palmitate analogue, 13-oxypalmitate, known to inhibit protein palmitoylation [203] markedly inhibits [^{3}H] palmitate into CD95 as well as cell death after FasL/CD95L engagement [197]. Also, the palmitoylation inhibitor 2-bromo-palmitic acid severely reduces CD95 internalization and formation of SDS-stable CD95 aggregates [198].

Covalent attachment of lipophilic and hydrophobic moieties is essential to target otherwise soluble proteins to cell membranes, thus influencing cell signaling. These post-translational protein modifications include N-myristoylation to internal cysteine residues by N-myristoyl transferase, as well as protein prenylation by the addition of an isoprenyl lipid (farnesyl- or geranylgeranyl-isoprenoid moieties) via thio-ether linkage to a cysteine residue proximal to the C terminus by farnesyl or geranylgeranyl transferases [204, 205]. These modifications occur in the cytosol and are irreversible. In contrast, thioester linkage of the 16-carbon saturated fatty acid palmitate to cysteine residues (S-palmitoylation) is a reversible modification catalyzed by membrane-bound palmitoyl acyltransferases [206]. Thus, palmitoylation occurs with proteins that are already in the membrane via weak or transient interactions, or in proteins that are already tightly associated with membranes, including transmembrane proteins. Protein S-palmitoylation is the most common acylation process of proteins in eukaryotic cells, and provides an important mechanism for regulating protein subcellular localization, stability, trafficking, aggregation, interaction with effectors, and translocation to lipid rafts [207]. Reversible S-palmitoylation regulates the assembly and compartmentalization of several signaling proteins at specific subcellular sites and is dynamically regulated by two opposing types of enzymes which add (palmitoyl acyltransferases) or remove (acyl protein thioesterases) palmitate from proteins. The unique reversibility of protein S-palmitoylation allows proteins to rapidly shuttle between intracellular membrane compartments in a controlled way, and it might be envisaged that this aspect could be of importance in the internalization of CD95 from lipid rafts to endosomal compartments. Palmitoylation has been shown to be required for raft partitioning of the majority of integral raft proteins. About 35 % of surface raft located proteins are anchored to membrane rafts by palmitoylation (DTT sensitive), ~33 % are glycosylphosphatidylinositol (GPI)-anchored (phosphatidylinositol-phospholipase C sensitive), and the mechanism of raft residence for the remaining ~32 % of raft proteins remains unassigned [208].

In human cells, S-palmitoylation is controlled by 23 members of the DHHC (aspartate–histidine–histidine–cysteine) family through their palmitoyl acyltransferase activity, and DHHC7 has recently found to regulate CD95 palmitoylation and stability [209].

Additional determinants for CD95 localization in lipid rafts

A lysine-rich region (LRR) in the cytoplasmic, membrane-proximal region of CD95 (Fig. 1) constitutes another determinant for CD95 localization in the lipid raft domains and modulates CD95 interaction with ezrin/actin cytoskeleton [210]. Nitric oxide (NO)-induced S-nitrosylation at cysteine 304 (Fig. 1) has also been shown to promote redistribution of CD95 to lipid rafts, formation of the DISC, and induction of cell death [211]. In addition, human FasL/CD95L has also been reported to be located in lipid rafts in order to exert efficiently its apoptotic activity [170, 212], and human FasL/CD95L has been shown to be palmitoylated at the cysteine residue 82, which is located at the N-terminal region of the transmembrane domain [213]. Furthermore, the death receptor DR4, but not DR5 or TNFR1, has been shown to be also palmitoylated to promote its raft localization and oligomerization [214].

DISC and death-effector filaments (DEFs)

From the above it can be suggested that CD95-mediated apoptosis involves the recruitment and clustering of CD95 in membrane rafts, its aggregation, and DISC formation. However, how the apoptotic signals are transmitted into the cell remains to be fully elucidated. This apoptotic signaling generation and transmission seems to largely depend on the formation of protein complexes. DED is a critical protein interaction domain that recruits caspases into complexes with members of the TNFR superfamily. Overexpression of certain DED-containing proteins, especially FADD, has led to the formation of the so called "**d**eath-**e**ffector **f**ilaments" (DEFs) that at a great extent may represent oligomerized FADD that recruit and activate procaspases

and induce apoptosis [215, 216]. Treatment of Jurkat cells with cycloheximide led to apoptosis and to the formation of FADD- and caspase-8-containing perinuclear filaments, similar in appearance to those shown following overexpression of FADD or caspase-8 [217]. These aggregates did not colocalize with tubulin and were detected even in Jurkat cells transfected with a dominant-negative version of FADD lacking the DD required for CD95 binding [217]. However, cycloheximide only induced apoptosis in Jurkat cells, but not in Jurkat expressing the FADD dominant-negative protein, thus suggesting that DEF formation might be necessary, but not sufficient, for apoptosis [217].

Signaling protein oligomerization transduction structures (SPOTS)

CD95 receptor ligation leads to the formation of surface receptor oligomers that have been termed **s**ignaling **p**rotein **o**ligomerization **t**ransduction **s**tructures (SPOTS), which depend on the presence of an intact CD95-DD and FADD [218]. Formation of SPOTS depends on recruitment of FADD to the DD in the cytoplasmic tail of CD95, forming early mediators of CD95-induced apoptosis at the plasma membrane [218]. Then SPOTS aggregate together to form caps followed by receptor internalization [200, 219].

Keratins are the intermediate filament (IF) proteins of epithelial cells, expressed as pairs in a lineage/differentiation manner. Keratin filaments form a complex network that extends from the periphery of the nucleus to the plasma membrane and their role is not only structural, but are also involved in intracellular signaling pathways, including those related to cell death providing resistance to death receptor-mediated apoptosis [220–223]. Hepatocyte IFs are made solely of keratins 8/18 (K8/K18), and the absence of K8/K18 IFs in K8-null hepatocytes leads to more efficient CD95-mediated signaling and subsequent apoptosis. This apoptotic response is associated with accelerated SPOTS formation at the cell surface in a lipid raft-dependent way, followed by the capping and internalization of these structures [224]. In addition, the absence of K8/K18 IFs also perturbs the organization of lipid rafts at the cell surface and alters ezrin compartmentalization [224]. The piwi-like 2 (piwil2) gene is widely expressed in tumors and protects cells from apoptosis by a variety of stimuli, including CD95-mediated apoptosis. This protection is mediated by the interaction of piwil2 with the major IF protein K8, leading to K8 phosphorylation at Ser-73 and inhibition of its ubiquitin-mediated degradation, thus protecting the cell from CD95-mediated apoptosis [225]. Keratins undergo caspase-mediated cleavage during apoptosis [226]. Mutation of caspase-digestion sites in K18 interferes with filament organization and makes hepatocytes more susceptible to cell death following CD95 stimulation or severe hypo-osmotic stress [227]. Likewise, K8-null mice are highly sensitive to CD95-mediated liver cell apoptosis, likely due to the inability to form K8/K18 major intermediate filaments that protect hepatocyte survival by interacting and activating cell survival-related protein kinases and transcription factors, including ERKs and NF-κB [228]. K8/K18 silencing in human endometrial carcinoma KLE cells leads to an increase in CD95 at the membrane through a claudin1-dependent mechanism and sensitizes cells to cisplatin [229].

On the other hand, cytosolic death effector domain containing DNA-binding protein (DEDD), a highly conserved and ubiquitous death effector domain containing protein, and its close homologue DEDD2 form filaments that interact with both the K8/18 intermediate filament network and pro-caspase-3 upon DEDD diubiquitination, suggesting that DEDD acts as a scaffold protein that directs the effector caspase-3 to certain substrates facilitating their ordered degradation during apoptosis [230]. Interestingly, cytokeratin filaments are suggested to provide a scaffold whereby caspases and additional proteins, including DEDD, are recruited forming an amplification loop of caspase activation and apoptosis [231, 232]. Taken together, these data suggest a link between keratin IFs and raft-mediated CD95 signaling that can modulate cell demise in different ways.

Cluster of apoptotic signaling molecule-enriched rafts (CASMER)

The translocation and recruitment of CD95 death receptor together with downstream signaling molecules to lipid rafts (Table 1) facilitate and potentiate protein–protein interactions and cross-talk between different signaling pathways, which ultimately would lead to the triggering of cell death signals. The recruitment and clustering of death receptors, together with downstream apoptotic signaling molecules, in raft platforms, forming cell death-promoting scaffolds, has led us to coin the term CASMER as an acronym for "**c**luster of **a**poptotic **s**ignaling **m**olecule-**e**nriched **r**afts" [22, 126, 170, 233, 234]. CASMER refers to the recruitment of death receptors together with downstream apoptotic signaling molecules in aggregated rafts or raft platforms [22, 234], and represents a novel raft-based supramolecular entity, acting as a rendezvous point for apoptotic signaling molecules (Fig. 4). Thus, formation of death-promoting platforms or CASMERs would facilitate protein–protein interactions and the transmission of apoptotic signals. The efficiency in promoting CASMER formation as well as CASMER protein composition depend on the cell phenotype and the triggering stimulus [22, 234]. CASMER protein composition can vary in complexity, and it could be envisaged that the higher number of apoptotic

proteins recruited in CASMERs the more efficient the apoptosis response to be launched. A basic protein composition of CASMER would include the recruitment of death receptors in aggregated rafts [22, 234], but CASMERs could increase in complexity by recruiting additional downstream signaling molecules in lipid rafts, including DISC components FADD and procaspase-8, and additional proapoptotic molecules [16, 22, 116, 126, 233, 234]. We have found that edelfosine can promote the recruitment of CD95, FADD, procaspase-8, procaspase-10, c-Jun amino-terminal kinase (JNK) and BH3-interacting domain death agonist (Bid) in lipid rafts in several hematopoietic cancer cells [16, 22, 116, 126, 233, 234]. Because Bid acts as a bridge between CD95 signaling and mitochondria [235, 236], its recruitment in membrane rafts highlights a major role of rafts as a putative linker between extrinsic and intrinsic signaling pathways in apoptosis [114, 116, 169, 170, 233], which might amplify the apoptotic response through both caspase activation and mitochondria-related processes (Fig. 4). Following edelfosine treatment, heat shock protein 90 (Hsp90) was also found to bind JNK in lipid rafts and Hsp90–JNK clusters were identified at the plasma membrane by immunoelectron microscopy [237]. These data suggest a chaperoning role of Hsp90 on JNK-mediated edelfosine-induced apoptosis when both Hsp90 and JNK are recruited in lipid rafts [237, 238] (Fig. 4). These data suggest that CASMERs serve as a hub in death receptor-mediated apoptosis signaling and as a connecting platform among different subcellular structures to facilitate cross-talk processes occurring during apoptosis.

CD95 internalization modulates cell fate

Efficient CD95-mediated cell death requires the recruitment of CD95 in lipid rafts followed by its internalization. As stated above, in addition to the proapoptotic function of CD95, this receptor has also been involved in proliferative and survival signaling [150, 239, 240]. In this regard, the spatial and temporal regulation of membrane dynamics and internalization of CD95 constitutes a key regulatory function in determining cell fate. Internalization of CD95 leads

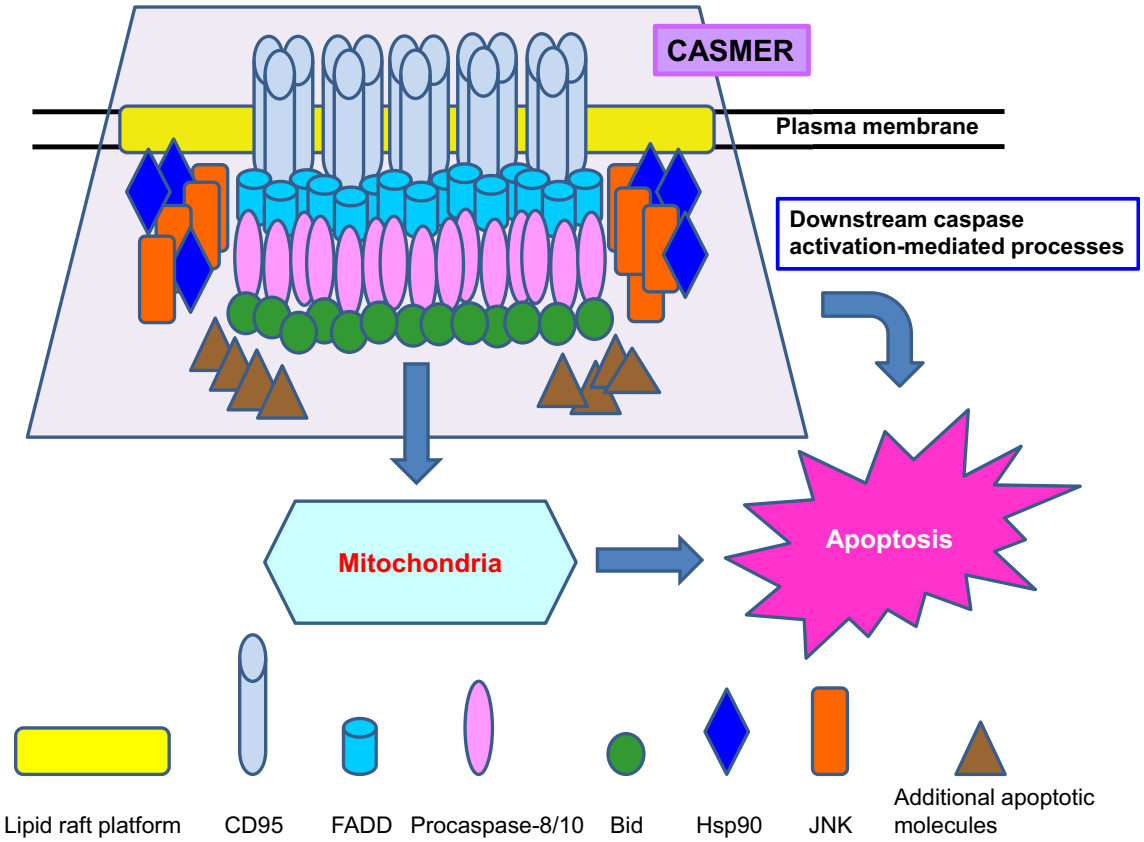

Fig. 4 CASMER formation. A number of apoptotic signaling molecules, including CD95, FADD and procaspase-8/10, forming the DISC, and downstream apoptotic signaling molecules are recruited and brought together in close proximity in large lipid raft platforms or raft clusters to generate the CASMER supramolecular entity. CASMER formation facilitates protein–protein interaction and cross-talk signaling, thus favoring the generation and amplification of apoptotic signals through both caspase activation- and mitochondria-mediated processes. See text for further details

to apoptosis, whereas the lack of CD95 internalization enables the activated receptor to engage signaling pathways, such as activation of ERK1/2 and NF-κB transcription factor [200], that promote cell survival and proliferation. Interaction of CD95 and/or DISC with different proteins seems to be crucial for the triggering of distinct cellular outcomes. Thus, under conditions in which proliferation of CD3-activated human T lymphocytes is increased by recombinant FasL/CD95L, CD95-recruited cellular FADD-like IL-1β-converting enzyme (FLICE)-like inhibitory protein (c-FLIP) can interact with TNF-receptor associated factors 1 and 2 (TRAF1 and TRAF2), as well as with the kinases RIP and Raf-1, resulting in the activation of the NF-kB and ERK signaling pathways, which eventually lead to interleukin-2 production [241]. Thus, c-FLIP acts as a major protein in CD95-mediated cellular fate decision [242, 243]. c-FLIP is a master anti-apoptotic regulator and resistance factor that suppresses TNF-α-, FasL/CD95L- and TRAIL-induced apoptosis, as well as apoptosis triggered by chemotherapy agents in malignant cells. c-FLIP is expressed as long (c-$FLIP_L$) and short (c-$FLIP_S$) splice variants in human cells. c-FLIP binds to FADD and/or procaspase-8 or -10 and forms an apoptosis inhibitory complex. This interaction in turn prevents DISC formation and subsequent activation of the caspase cascade. c-$FLIP_L$ and c-$FLIP_S$ are also known to have multifunctional roles in various signaling pathways, and can activate and/or upregulate several cytoprotective and pro-survival signaling proteins including Akt, ERK, and NF-kB. Upregulation of c-FLIP has been found in various tumor types, and its silencing restores apoptosis triggered by cytokines and various chemotherapeutic agents. Hence, c-FLIP is an important target for cancer therapy [244]. A number of reports underscore the view that as levels of c-FLIP increase CD95 signaling is diverted from induction of apoptosis to pathways leading to cell effector function and cell survival. Dendritic cells are resistant to CD95-induced death due to expression of high levels of the CD95 inhibitor c-FLIP, thus diverting CD95-mediated signals from the death-promoting caspase cascade to the survival ERK and NF-κB pathways [245].

On the other hand, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced activation of ERK and NF-kB is required for maximal inhibition of CD95-induced apoptosis in Jurkat T-cell leukemia cells [246]. Thus, the specific ERK kinase inhibitors PD98059 and U0126 and the repression of TPA-induced NF-κB activation by the irreversible inhibitor of IκBα phosphorylation BAY11-7082 abrogated TPA-mediated suppression of CD95-induced apoptosis [246]. An NF-κB and ERK stimulating, caspase-dependent factor has been suggested to operate downstream of the DISC in a number of apoptosis-resistant pancreatic tumor cells leading to upregulation of proinflammatory genes and proinflammatory signaling [247]. Moreover, stimulation of CD95 has been reported to induce production of pro-inflammatory mediators, including matrix metalloproteinase (MMP)-9 and interleukin-8, through ERK/JNK-dependent activation of NF-κB in the human macrophage-like cell line THP-1 [248].

After stimulation, CD95 is recruited to lipid rafts in human leukemic cells, which, in turn, is followed by DD-dependent translocation of FADD and procaspase-8 to the lipid rafts forming the DISC, and subsequent CD95 internalization. Electron microscopy analysis has shown that CD95 triggering induced the formation and internalization of CD95-containing raft macroaggregates that were internalized in endosomal vesicles, where caspase-8 underwent massive processing, indicating that CD95 was internalized in raft aggregates [199].

An extracellular motif in cysteine-rich domain (CRD)2 and CRD3 regions of CD95 shares some sequence similarity to the V3 loop of HIV-1 gp120, which has been reported as a glycosphingolipid-binding domain [249]. This glycosphingolipid-binding motif is conserved in CD95 across species and among TNFR superfamily members, and has been reported as one of the regulatory elements in the selection of the internalization route of CD95 and consequently the signals transmitted upon ligand binding. This motif is required for clathrin-mediated internalization of CD95, which allows the transduction of its cell death signal. A defect in this motif drives the activated receptor to an alternative clathrin- and raft-independent, but ezrin-dependent internalization route that promotes the non-death functions of CD95 [250]. In this regard, a membrane proximal ezrin-binding domain outside the CD95-DD has been identified [251], and the interaction of CD95 with ezrin correlates well with the stimulation of non-apoptotic signaling routes [251, 252]. Ezrin deficiency did not affect the internalization of CD95 after CD95 ligation. Instead, an enhanced formation of DISC was observed in H9 T lymphoma cells with ezrin knockdown, leading to accelerated caspase-8 activation, and supporting a role for ezrin as a negative regulator of death receptor-induced apoptosis [252].

Following FasL/CD95L stimulation, a complex of CD95, caspase-8 and tubulin has been described, suggesting the involvement of microtubular cytoskeleton in FasL/CD95L-induced apoptosis [253]. Interestingly, the raft component ganglioside GD3 associates with microtubular cytoskeleton at very early time points following CD95 triggering [254], a process that is mediated by CLIPR-59 [255], a CLIP-170-related protein that has been identified as a microtubule binding protein associated with lipid rafts [256, 257].

Inhibition and termination of CD95 receptor internalization has been shown to be mediated in B cells by switching off the CD95–ezrin–actin linkage through the

tyrosine phosphatase SHP-1-regulated Vav dephosphorylation [258]. Ezrin and moesin, two members of the ezrin–radixin–moesin (ERM) protein family, have been shown to link CD95 to the actin cytoskeleton, thus promoting the formation of SDS-stable high molecular mass CD95 aggregates and apoptosis through a Rho-associated protein kinase (ROCK)-mediated phosphorylation of ezrin and moesin on T567 and T558 respectively [154, 259]. Other reports have shown that ezrin, but not moesin or radixin, bind to CD95, thus connecting the death receptor with actin cytoskeleton and allowing CD95 polarization [260]. The ezrin region specifically and directly involved in the binding to CD95 was located in the middle lobe of the ERM domain, between amino acids 149 and 168 [154, 260]. CD95 capping and subsequent polarization, but not apoptosis, has been reported to be ROCK-dependent in the human leukemic T cell lines Jurkat and H9 [261]. On the other hand, merlin (moesin–ezrin–radixin-like protein), a protein that shares significant sequence homology with the ERM family, functions as a tumor suppressor in a wide variety of cells and has been shown to link the actin cytoskeleton with transmembrane proteins [262] and lipid rafts [263]. In addition, Raf-1 has been shown to regulate CD95 expression [264] as well as CD95 internalization and efficient DISC formation [265], and thereby sets the threshold of CD95 sensitivity in distinct cell types.

CD95 stimulation and raft-mediated connection between different subcellular organelles

Interestingly, raft-like microdomains can be detected in mitochondrial membranes [266] as well as a directional movement of endocytic vesicles towards the mitochondrial compartment [267] after CD95 triggering. Gangliosides are major components of lipid rafts, and recent evidence suggest that ganglioside GD3 can traffic to mitochondria, likely through a CLIPR-59-mediated GD3-microtubule interaction and transport [254, 255], and triggers apoptosis by interacting with mitochondrial raft-like microdomains [268, 269]. Hence, microtubules could act as tracks for ganglioside redistribution following apoptotic stimulation [254]. Among the 23 mammalian DHHC palmitoyltransferases, DHHC17 is the major CLIPR-59 palmitoyltransferase. DHHC17 interacts with CLIPR-59 and palmitoylates CLIPR-59 at Cys-534 and Cys-535, thus regulating CLIPR-59 plasma membrane association [270].

The alkylphospholipid antitumor drug edelfosine accumulates in lipid rafts [20, 114, 126, 271–276], and shows a high affinity for cholesterol [277] and cholesterol-enriched membranes [271], increasing the thickness and fluidity of model membranes for lipid rafts [271]. However, edelfosine has also been located in the endoplasmic reticulum [278, 279] and mitochondria [280]. Edelfosine also promotes a redistribution of lipid rafts from the plasma membrane to mitochondria, suggesting a raft-mediated link between plasma membrane and mitochondria [233, 280]. Edelfosine-induced apoptosis is mediated by mitochondria [116, 278, 281, 282], and edelfosine induces swelling in isolated mitochondria, indicating an increase in mitochondrial membrane permeability [280]. Taken together, it is tempting to envisage a putative link between lipid rafts, endoplasmic reticulum and mitochondria in the action of a raft-targeting and proapoptotic drug like edelfosine [18], thus highlighting the role of the poorly understood inter-organelle communication in the transmission of apoptotic signaling. In this regard, endoplasmic reticulum and mitochondria can interact physically [283], and the presence of the endoplasmic reticulum-mitochondria encounter structure (ERMES) protein complex at the interface of the two organelles [284, 285] leads to lipid movement between both subcellular structures [286].

Concluding remarks

Current evidence indicates that the molecular ordering of the initial events in both physiological and non-physiological death receptor CD95-mediated apoptotic signaling includes three major steps that involve the active role of lipid rafts: (a) recruitment of CD95 and downstream signaling molecules into lipid rafts; (b) formation of raft-mediated large clusters and complexes of apoptotic molecules; (c) internalization of apoptotic molecules and rafts.

The advent of lipid rafts has changed our view of the role of cellular membranes in the regulation of signal transduction. Cholesterol-rich raft membrane domains provide platforms where a number of receptors and downstream signaling molecules are brought together, thus acting as scaffolds for signaling pathways in the cell membrane. Despite lipid rafts have been extensively involved in the effective functioning of a wide number of survival and proliferating signaling pathways, recent data collected in the last 15 years have established the critical role of lipid rafts in the efficient activation of CD95 leading to cell demise. The recruitment and concentration of death receptor CD95 as well as of downstream signaling molecules in lipid rafts facilitate a cascade of protein–protein interactions that are critical in apoptosis signaling. This high concentration of apoptotic molecules in a rather reduced area would favor the generation of supramolecular structures that could modulate cell fate, such as the formation of: (a) DEFs, filaments composed of oligomerized FADD and procaspase-8 that result in the efficient activation of caspases [215, 216]; (b) SPOTS, made up by the recruitment of FADD to the CD95-DD, thus forming

oligomers that lead to aggregates or caps, which are modulated by keratin filaments and constitute early mediators of CD95-induced apoptosis at the plasma membrane [218]; (c) CASMERs, which represents raft-based supramolecular entities where death receptors together with downstream apoptotic signaling molecules are recruited in raft clusters or platforms [22, 126, 170, 233, 234], thus largely facilitating protein–protein interactions and the transmission of apoptotic signals. CASMER formation and apoptotic protein composition depend on the cell phenotype and the triggering stimulus, and it could be envisaged that cells prone to elicit CASMER formation would be more susceptible to undergo apoptosis. Likewise, stimuli that behave as potent inducers of CASMER formation could constitute lead compounds in a putative apoptosis-targeted therapy. A profound molecular and functional characterization of these dynamic death-promoting raft platforms will be of major importance to understand how death receptors dictate cell fate. This knowledge could provide novel insights in developing new frameworks and strategies for target identification in cancer therapy. In addition, CD95 apoptotic signaling involves CD95 and raft internalization by mechanisms that remain to be fully understood. Furthermore, raft-like domains are being identified in the membranes of different subcellular organelles where their role remains unclear. Recent evidence suggests interconnection among distinct organelles and that rafts or raft constituents can traffic to distinct subcellular organelles. This opens a fascinating network of communication between plasma membrane and different subcellular organelles that could modulate cell demise.

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