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Seminars in Cancer Biology

CD95/Fas and metastatic disease: What does not kill you makes you stronger

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ABSTRACT

CD95 (also known as Fas) is the prototype of death receptors; however, evidence suggests that this receptor mainly implements non-apoptotic signaling pathways such as NF- κ B, MAPK, and PI3K that are involved in cell migration, differentiation, survival, and cytokine secretion. At least two different forms of CD95L exist. The multi-aggregated transmembrane ligand (m-CD95L) is cleaved by metalloproteases to release a homotrimeric soluble ligand (s-CD95L). Unlike m-CD95L, the interaction between s-CD95L and its receptor CD95 fails to trigger apoptosis, but instead promotes calcium-dependent cell migration, which contributes to the accumulation of inflammatory Th17 cells in damaged organs of lupus patients and favors cancer cell invasiveness. Novel inhibitors targeting the pro-inflammatory roles of CD95/CD95L may provide attractive therapeutic options for patients with chronic inflammatory disorders or cancer. This review discusses the roles of the CD95/CD95L pair in cell migration and metastasis.

Keywords: Apoptosis, calcium, cytoskeleton, migration, TNF.

Introduction

Metastatic disease remains the main cause of mortality among cancer patients, accounting for 90% of cancer-associated deaths [1]. Improving our understanding of metastatic development is essential to improve the outcomes of cancer patients. Decades of cancer research have described metastasis as a multi-step process by which cancer cells disseminate from the primary tumor to surrounding tissues and distant organs. Metastatic spread is mediated by a biological sequence in which metastatic cells invade surrounding tissue, disseminate to blood and lymphatic vessels, survive in the blood circulation, extravasate through the vascular wall, and colonize new organs [2]. This complex process implies that metastatic cells harbor distinct capacities that can be activated sequentially for progression through the metastatic stages.

Primary tumors, which fuel metastatic development, are characterized by marked intratumoral heterogeneity. The origin of this cellular heterogeneity is mainly explained by the hierarchical organization of tumor tissues, where several subpopulations of self-renewing cancer stem cells (CSCs) maintain the long-term oligoclonal nature of the neoplasm [3]. A direct consequence is the pivotal role of CSCs in leading clinical evolution and metastatic progression [4-6]. Growing evidence indicates that CD95 is a key regulator of metastatic spread. Accordingly, this so-called death receptor is involved in the migration of cancer cells in triple-negative breast cancers (TNBCs) [7] and glioblastoma [8] as well as the survival and regulation of CSC fate [9].

Although CD95 is considered the prototype of the death receptors, this paradigm has been challenged. It is becoming clear that, similar to tumor necrosis factor receptor (TNFR)-1, CD95 mediates non-apoptotic signaling pathways involved in many cellular processes including cell proliferation, survival, differentiation, and migration. This review focuses on the role of CD95 and its ligand CD95L in cell migration [10].

Structural features of CD95 and its ligands

Death receptors of the TNFR superfamily include TNFR1, Fas (CD95), death receptor 3 (DR3), TNF-related apoptosis-inducing ligand receptors 1 and 2 (TRAIL-R1 and TRAIL-R2), death receptor 6 (DR6), nerve growth factor receptor (NGFR), and ectodysplasin receptor (EDAR). These receptors share an intracellular death domain (DD) that is required for their apoptosis-inducing activity [11]. Although the non-apoptotic functions of CD95 were reported soon after its cloning [12], they have been neglected over the years, as research efforts focused on deciphering the molecular basis of apoptosis induction by CD95 and the biological functions of CD95 in light of this role [13, 14].

CD95 is a 319 amino acid type I transmembrane glycoprotein [15, 16]. This receptor harbors extracellular amino-terminal cysteine-rich domains (CRDs) [17, 18] that define its ligand specificity [19] and a conserved 80 amino acid sequence located in the cytoplasmic tail called the death domain (DD) that is necessary for the induction of apoptosis [20, 21]. Its corresponding ligand, CD95L (FasL/CD178), is a type II transmembrane protein with a cytoplasmic domain, a transmembrane domain, a stalk region, and a C-terminal TNF homology domain (THD). The THD mediates both homotrimerization of CD95L and binding to CD95 (**Figure 1**). This transmembrane ligand (m-CD95L) can be cleaved at the stalk region by several metalloproteases (MMPs), namely, MMP3, MMP7, MMP9, and a disintegrin and metalloprotease domain-containing protein (ADAM)-10 [22], or at the THD by plasmin [23]. The resulting soluble form of CD95L (s-CD95L) is released into the bloodstream as a homotrimer [24]. s-CD95L can interact with CD95 but does not induce cell death, and was initially thought to compete with its membrane-bound counterpart for CD95 binding [25, 26]. s-CD95L accumulates in the bloodstream of patients with various pathologic conditions, including certain cancers such as NK cell lymphomas [27] and TNBC, in which s-CD95L levels are associated with the risk of relapse [7].

CD95-mediated cell signaling pathways

The interaction of m-CD95L with CD95 leads to the recruitment of the adaptor protein Fas-associated protein with death domain (FADD) via homotypic DD-mediated interactions. FADD recruits the protease caspase-8 and the long form of the regulator of apoptosis cellular FADD-like interleukin-1- β -converting enzyme-inhibitory protein (cFLIP_L) via death effector domain (DED)-mediated interactions. Together, these proteins form the death-inducing signaling complex (DISC) (**Figure 2**) [28]. Tumor necrosis factor receptor-associated factor 2 (TRAF2) can interact with caspase-8 at the DISC [29]. TRAF2 induces K48-linked polyubiquitination of caspase-8 and its rapid proteasomal degradation, protecting cells from death decisions [29]. In addition, TRAF2 orchestrates the implementation of non-apoptotic signaling pathways including NF- κ B by interacting with c-IAP1/2 [30, 31].

CD95 stimulation can induce apoptotic and non-apoptotic signaling pathways. The apoptotic signaling pathway is abrogated before completion in most cancer cells, suggesting that, in these cells, the CD95-mediated non-apoptotic signaling pathways including NF- κ B and the p38, ERK, and JNK MAPKs promote cell proliferation, survival, and migration and cytokine production [32]. The CD95-mediated apoptotic and non-apoptotic signaling pathways share many factors such as the apoptotic factors FADD and caspase-8, which are also required for NF- κ B activation upon m-CD95L stimulation [32, 33]. Unlike m-CD95L, s-CD95L does not efficiently recruit FADD and caspase-8, and thus fails to trigger the DISC and the subsequent apoptotic program. Our group and others showed that binding of the trimeric s-CD95L to CD95 induces the formation of an alternative signaling complex that results in cell migration, which we therefore designated as the motility-inducing signaling complex (MISC) (**Figure 2**) [7, 34]. MISC formation relies on the recruitment of phospholipase C γ 1 (PLC γ 1) to a membrane-proximal intracellular domain of CD95 that has been designated calcium-inducing domain (CID). PLC γ 1 generates inositol triphosphate (IP3) and induces the release of endoplasmic

reticulum (ER) calcium stores by activating IP3 receptors. The intracellular increase in calcium (Ca^{2+}) activates a store-operated current (SOC) through the plasma membrane Ca^{2+} channel ORAI1, resulting in Ca^{2+} entry close to CD95 and limiting the recruitment of FADD and caspase-8 by activating protein kinase C $\beta 2$ [35]. The recruitment and activation of NADPH oxidase 3 in the MISC triggers reactive oxygen species production, resulting in the activation of the src kinase designated c-yes and the subsequent induction of cell migration. This pathway has been observed in T cells, TNBC cells, and glioblastoma cells [8, 36, 37].

CD95/CD95L and immune homeostasis

Although the CD95/CD95L pair exerts pleiotropic effects in physiological and pathological contexts that will be discussed in this review, the main role of this couple involves the regulation of the adaptive immune response [38]. *Lpr* (*lymphoproliferation*) mice harbor a transposable element inserted into intron 2 of CD95 encoded by the *APT1* gene that prematurely terminates CD95 transcription [39]. *Gld* (*generalized lymphoproliferative disease*) mice express a mutated CD95L in which the phenylalanine at position 273 is replaced by leucine (F273L), resulting in reduced affinity for CD95 [40]. *Lpr^{cg}* mice (*lpr gene complementing gld*) bear a mutation within the CD95 DD (N238V) that impinges on FADD recruitment and the induction of apoptosis [41]. Knock-in mice in which full length CD95L was replaced by its soluble counterpart revealed that the non-apoptotic ligand causes a severe lupus-like autoimmune disorder and promotes carcinogenesis [42]. These lupus-prone animal models, which are characterized by lymphadenopathy and splenomegaly, with an accumulation of $\text{CD3}^+\text{CD8}^-\text{CD4}^-$ double-negative T (DNT) cells, revealed the pivotal role of the CD95L/CD95 pair in immune homeostasis. Consistent with these animal models, several germinal mutations of CD95 in humans are causative for autoimmune lymphoproliferative syndrome (ALPS) type Ia (also known as ALPS-FAS) [43]. ALPS patients show chronic lymphoproliferation,

lymphadenopathy, and/or splenomegaly and an aberrant accumulation of DNT cells. Because they share many symptoms with lupus patients, ALPS patients are often initially diagnosed with lupus. ALPS-FAS patients exhibit heterozygous germline mutations in *APT1* that are mainly located in the DD; such mutations prevent the induction of CD95-mediated apoptosis, whereas they allow non-apoptotic signaling [44, 45]. Animal models and ALPS patients confirm the pivotal role of CD95 in immune homeostasis, and suggest that its non-apoptotic signaling function contributes to inflammation and oncogenesis.

CD95 is responsible for immune contraction by mediating the apoptotic deletion of activated T cells in the periphery, a process called activation-induced cell death (AICD) [46-49]. Re-expression of CD95 in the T cells of *lpr* mice prevents lymphoproliferation associated with the accumulation of DNT cells but does not prevent autoimmunity [50, 51]. On the other hand, loss of CD95L in T cells favors the accumulation of CD11c⁺ dendritic cells (DCs) and B cells, thereby contributing to prolonged antigen presentation, and is responsible for the autoimmune disorder [52]. In addition to its pro-apoptotic role, CD95L expression in memory CD4⁺ or CD8⁺ T cells can induce a CD95-mediated PI3K/Akt signaling pathway in naïve T cells, altering their differentiation and activation. This represents an alternative route for CD95 to terminate the immune response in addition to AICD [53]. This CD95-mediated non-apoptotic process is designated “precocious differentiation”.

CD95-mediated cell migration and calcium (Ca²⁺)

Ca²⁺ is a universal and versatile second messenger involved in the activation of pleiotropic signaling cascades [54, 55]. Ca²⁺ pumps, channels, and exchangers and Ca²⁺-binding proteins present at the plasma membrane and in different cellular compartments (ER, lysosome, mitochondria, and Golgi apparatus) tightly regulate the [Ca²⁺]_{cyt}, which is close to 100 nM in the resting state [56-60]. In response to various stimuli, [Ca²⁺]_{cyt} can reach concentrations of

10 μM and higher in microdomains, including the interface between the ER and the mitochondria (mitochondria-associated ER membrane or MAM) [56] or beneath the plasma membrane. This increased $[\text{Ca}^{2+}]_{\text{cyt}}$ can be evoked by mobilizing Ca^{2+} from intracellular stores, such as the ER and Golgi apparatus (**Figure 3**), or through Ca^{2+} entry from the extracellular environment via Ca^{2+} channels, such as Voltage-Gated Calcium (VGC) channels or SOC channels (**Figure 3**). Upon elevation of $[\text{Ca}^{2+}]_{\text{cyt}}$, energy-dependent active transporters, including pumps and exchangers, work to refill stores and reset Ca^{2+} levels to the resting state (**Figure 3**).

Most of the hallmarks of cancer, if not all, involve Ca^{2+} signaling, which mediates critical cellular processes such as therapeutic resistance [61], metastasis, bypass of apoptosis [62], and breast CSC enrichment [63]. CD95 engagement leads to a rapid, localized, and transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ [64-66], which negatively controls CD95 apoptotic signaling [35], and promotes cell migration [34] and metastasis [7] through a c-yes/PLC γ 1/IP3/SOC channels/ Ca^{2+} /PI3K signal (**Figures 2 and 3**). The versatility of Ca^{2+} as an intracellular messenger stems largely from its complex spatiotemporal organization of the Ca^{2+} signals.

Spatiotemporal calcium patterns

Ca^{2+} signaling regulates multiple aspects of adaptive metastatic cancer cell behaviors, including migration, local angiogenesis induction, intravasation, and epithelial-mesenchymal transition (EMT) [67]. EMT is a central process required for normal embryonic development that not only contributes to metastatic dissemination [68], but is also involved in the chemotherapy resistance of cancer cells [69].

$[\text{Ca}^{2+}]_{\text{cyt}}$ plays a key role in directional sensing, as oscillations in $[\text{Ca}^{2+}]_{\text{cyt}}$ activate cytoskeletal remodeling, actin contraction, and focal adhesion (FA) turnover necessary for changes in contractility and directional movement (**Figures 3 and 4**). Cell movement begins with the

formation of protrusions of the cell membrane (lamellipodia, filopodia, and podosomes), followed by the establishment of new FAs at the leading edge to anchor the cytoskeleton to the extracellular matrix (ECM). Traction forces move the cell forward, and the cycle ends with disassembly of the FA at the cell rear [70, 71]. Most of these events are spatiotemporally regulated by Ca^{2+} signaling [72, 73]. Indeed, oscillations of cytosolic Ca^{2+} induce actin remodeling through the activation of the small GTPases RhoA, cdc42, and Rac1, which are activated in cells exposed to s-CD95L [74] (**Figure 3**). During the rear-to-end retraction phase, actomyosin contraction is regulated by phosphorylation of the myosin light chain (MLC) mediated by the calmodulin Ca^{2+} -dependent kinase MLCK (**Figure 3**) [67, 75]. Finally, the disassembly of the FA is controlled by calpains, which are Ca^{2+} -dependent proteases (**Figure 3**) [76]. During migration, polarized cells exhibit a cytosolic Ca^{2+} gradient in which Ca^{2+} concentration is low at the leading edge [77]. This gradient is produced by increased activity of the plasma membrane calcium ATPases (PMCA pumps) at the leading edge to extrude intracellular Ca^{2+} [78] (**Figure 4**). This low intracellular Ca^{2+} concentration allows the different components of the cell migration machinery to respond to local and transient Ca^{2+} spikes called “ Ca^{2+} flickers” at the front of the migrating cells (**Figures 3 and 4**). These “pulses” locally promote the disassembly of FA proteins [79], and steer the migrating cell in the direction of the chemoattractant [80]. In migrating cells, mitochondria actively move along cytoskeletal filaments to different cellular topographies with high energetic demands (**Figure 4**). In addition to ATP production, one of the main functional roles attributed to mitochondria is the ability to spatially remodel intracellular Ca^{2+} signaling. Accordingly, inhibition of the mitochondrial Ca^{2+} uniporter (MCU) by ruthenium red or specific small interfering RNAs induces mitochondrial depolarization in the triple-negative breast adenocarcinoma cell line MDA-MB-231, inhibiting SOC Entry (SOCE) through Orai1/STIM1 channels and abrogating cancer cell migration [81]. In summary, hotspots of Ca^{2+} are crucial for cell migration and cell steering, and rely on many

cellular factors including SOCE [78, 79] and the activity of stretch-activated calcium channels (SAC) [77, 80].

Non-apoptotic effect of CD95 engagement and Ca²⁺ response

Many highly invasive cancer cell types possess specialized matrix-degrading organelles called invadopodia [82, 83]. Invadopodia are dynamic actin-based cell protrusions composed of an actin core surrounded by integrins and actin-associated proteins. The assembly of invadopodia is initiated in response to the focal generation of phosphatidylinositol bi and trisphosphate (PIP₂ and PIP₃) and the activation of the nonreceptor tyrosine kinase Src [84-86]. The assembled invadopodia recruit proteolytic enzymes, such as membrane type 1 (MT1)-matrix metalloproteinase (MMP), MMP2, and MMP9, via localized Ca²⁺ influx through SAC [87-89] and extracellular medium acidification through the Na⁺/H⁺ exchanger NHE1 [90] to facilitate the focal degradation of the ECM and cell invasion [91] (**Figure 4**). The membrane-bound apoptotic CD95L is cleaved by these MMPs [92] to generate the pro-migratory s-CD95L, which in turn induces CD95-mediated NHE1 activation [74].

s-CD95L induces the formation of migrating pseudopods at the leading edge of which the engaged CD95 and SOC channels are capped [34]. Therefore, the Ca²⁺ response that promotes CD95-mediated cell motility does not display a homogeneous cytosolic distribution, but instead results in Ca²⁺ microdomains localized at the leading edge of the cell protrusion emitted by the migrating cells [34]. CD95L-induced cell migration depends, at least in part, on another Ca²⁺ microdomain between the ER and mitochondria called MAM. The anti-apoptotic factors Bcl-2 and BclxL, which are localized in these intracellular compartments, collaborate to induce CD95-mediated mitochondrial Ca²⁺ upload, increasing ATP production and thereby promoting cell migration [93]. Capping of CD95 is associated with F-actin polarization in activated T cells, and this process occurs independently of the apoptotic program because inhibition of actin

polymerization using cytochalasin D prevents CD95 capping without affecting DISC formation [94]. CD95 capping requires activation of the serine/threonine kinase Rho kinase (ROCK) [94], which is also necessary to activate NHE1 in cells exposed to s-CD95L [74]. In the fibroblast cell line PS120, s-CD95L enhances cell migration by activating NHE1 via Akt and RhoA-dependent but Ca^{2+} -independent pathways [74]. This does not rule out the participation of STIM1/Orai1 in CD95L-mediated PS120 cell migration, and Ca^{2+} -independent effects of STIM1/Orai1 on cell migration were recently described in relation to the RhoA/ROCK/MLC transduction pathway [95-97]. Interaction of these proteins, which colocalize in the CD95 cap, would be sufficient to trigger the effects of CD95L on cell migration.

NHE1 catalyzes an electroneutral exchange of extracellular Na^+ for intracellular H^+ , and its activity is necessary for cell migration [98, 99]. The NHE1-driven increase in intracellular pH affects cell migration by changing the structure of cofilin, presumably through the deprotonation of His¹³³, and impairing its recruitment by phosphatidylinositol 4,5-bisphosphate ($\text{PI}_{(4,5)}\text{P}_2$), thus stimulating its actin-severing activity [100] (**Figure 4**). Increased assembly of a branched actin filament network requires filament severing to increase the abundance of actin-free barbed ends, and is a driving force for membrane protrusion through a cofilin and Arp2/3-dependent mechanism [101] (**Figure 4**). Cortactin recruits the adaptor protein Nck1, which in turn binds N-WASP, and cofilin [102]. This molecular complex is disrupted through src kinase-dependent phosphorylation of cortactin and NHE1-dependent pH increase [103] to release cofilin, which promotes actin polymerization and invadopodia formation (**Figure 4**). NHE1 activity is also instrumental for the activation of central determinants of cell polarity such as cdc42 [98]. Activation of $\text{PLC}\gamma$, which hydrolyzes $\text{PI}_{(4,5)}\text{P}_2$, is necessary for cofilin-dependent actin-free barbed end formation [104]. Interaction of s-CD95L with CD95 triggers the rapid accumulation of the receptor at the leading edge of motile cells [34, 105], and activates $\text{PLC}\gamma 1$ [34] and NHE1 [74], engendering a cell migration program that requires cofilin [106].

Activation of cell migration and survival mediated by CD95 relies on the recruitment of receptor tyrosine kinases (RTKs) including epidermal growth factor receptor (EGFR) [7, 107] and platelet-derived growth factor receptor (PDGFR) [106] and the subsequent activation of members of the src kinase family.

Adhesion and regulation of CD95-mediated cell death

We and others observed that CD95L is ectopically expressed by endothelial cells covering blood vessels in inflamed organs of lupus patients [37] and in tumor tissues [7, 108], suggesting that CD95L could serve as a barrier [108], a chemoattractant following its shedding by metalloproteases [37, 109], or an adhesion molecule for the recruitment of neutrophils [110]. Indeed, Coukos and colleagues report that CD95L expression by endothelial cells in tumor tissues represents a physical barrier, killing CD8⁺ T cells while sparing T regulatory (Treg) cells, whose accumulation in the tumor might cause an immunosuppressive environment [108]. Nonetheless, the transmembrane CD95L at the surface of endothelial cells also has non-apoptotic functions, as it promotes the binding and recruitment of myeloid cells by inducing rolling, adhesion, and transmigration processes [110]. Therefore, CD95L expression by endothelial cells is instrumental for mounting an efficient anti-infectious response [110]. After metalloprotease-mediated cleavage, the generation of a s-CD95L gradient promotes the trafficking of pro-inflammatory Th17 cells in inflamed organs of lupus patients, thereby contributing to aggravate their clinical symptoms [37]. This s-CD95L gradient in TNBC patients favors migration of cancer cells and increases the risk of metastasis [7].

The expression of plasminogen activator inhibitor-1 (PAI-1) by endothelial cells is a marker of poor prognosis in colorectal cancer [111]. CD95L expression by endothelial cells controls neo-angiogenesis through a PAI-1-dependent mechanism [23]. PAI-1 is the inhibitor of urokinase-type plasminogen activator (uPA), which converts plasminogen into its active form, plasmin.

Plasmin in turn can cleave CD95L in its trimerization domain at Arg144-Lys145, releasing a cytotoxic ligand that eliminates endothelial cells and abrogates neo-angiogenesis [23]. PAI-1 overexpression inhibits this process and promotes neovessel formation in cancers.

Overall, these studies raise the question of the exact role of CD95L in endothelial cells covering neovessels, and suggest that the co-expression of certain metalloproteases/PAI-1 may account for these pleiotropic roles.

Alteration of adherens junctions in epithelia is associated with loss of cell-cell contacts, an initial step in EMT and the metastatic dissemination of carcinoma cells [112]. Loss of these contacts generates a cell death program that depends on CD95 [113]. Hueber et al. recently showed that the C-terminal domain of CD95 is important for the distribution of the receptor within adherens junctions in epithelial cells [113]. This region of CD95 also interacts with the polarity molecule Dlg1, thereby preventing DISC formation [113]. Depolarization of CD95 and its dissociation from Dlg1 and E-cadherin might represent a control mechanism to eliminate abnormal cells within epithelial tissues, thereby preventing the initial step of metastatic dissemination. The ERM proteins (ezrin, radixin, and moesin) are found in the microvilli, filopodia, membrane ruffles, and cell-to-cell contact sites, where they colocalize with actin and connect various cell-surface proteins to the actin cytoskeleton (**Figure 4**). Similar to Dlg1, ezrin interacts with the C-terminal PDZ-binding site of CD95 [105]; however, Dlg1 binding to CD95 prevents DISC formation, whereas ezrin interaction with the receptor is instrumental for implementing the apoptotic program, at least in activated CD4⁺ T cells [105].

The link between CD95 and the cytoskeleton could reflect different stages of tumor development. Peter et al. showed that cancer cells with mesenchymal markers have a strong link between CD95 and the actin cytoskeleton and are sensitive to actin disruptors (*i.e.*, cytochalasin D), whereas epithelial cells exhibit a stronger association between CD95 and microtubule structures and are sensitive to microtubule-disrupting compounds [114].

Most of the studies on the cytoskeleton and CD95 focus on the apoptotic outcome and neglect to evaluate the obvious effect on cell migration and invasiveness.

Cell migration and the apoptotic machinery

Although caspase-8 is well-known as an apoptotic inducer, this zymogen also acts through its scaffolding function to drive cytokine production in various cancer cell lines upon m-CD95L stimulation [115]. Production of pro-inflammatory chemokines in dying cells results in the recruitment of monocytes and neutrophils that engulf the dying cells expressing the “find me” signal [116]. As recently detailed elsewhere [92], Src kinases phosphorylate multiple targets, and also mediate the recruitment of various RTKs such as EGFR into close proximity to CD95 through mechanisms that remain to be elucidated. MISC formation leads to the activation of the PI3K/Akt pathway, which is instrumental for cancer cell migration [7]. Although caspase-8 is not readily detected in the MISC by western blotting, it can be phosphorylated by Src on Y380, a molecular event that not only limits its activation but also promotes the recruitment and activation of the p85 regulatory subunit, initiating PI3K/Akt activation [117-119]. Although the CID is required for inducing the CD95-mediated Ca²⁺ response, PI3K activation is DD-dependent [34], suggesting that the CD95-mediated non-apoptotic signal stems from both the CID and the DD.

Together with caspase-8, TRAF2 may also contribute to the CD95-mediated migration process. TRAF2 overexpression triggers a positive feedback loop in pancreatic tumor cells, because exposure to CD95L activates the NF-κB signal, which in turn fuels TRAF2 overexpression in tumor cells [120]. In the presence of CD95L, pancreatic tumor cells overexpressing TRAF2 induce the secretion of MMP2 and MMP9, uPA, and IL-8, factors involved in the invasive process. Although partial TRAF2-dependent inhibition of caspase-8 activation at the DISC level is required for the secretion of IL-8 and uPA and cell migration, complete abrogation of

caspace activity using the pharmacologic inhibitor zVAD-fmk blocks invasiveness, strongly supporting that the fine-tuned regulation of caspace activity orchestrates the cellular response [120].

Cancer stem cells and CD95

Increasing evidence supports the existence of functionally distinct subsets of CSCs within primary tumors with tumor-propagating and/or metastatic capacity [121]. This functional heterogeneity within the CSC population was first described in pancreatic cancers, in which CXCR4 expression defines two different CD133⁺ CSC subsets. The CD133⁺CXCR4⁻ population solely maintains the tumorigenic potential of the primary tumor, whereas the CD133⁺CXCR4⁺ cell population is essential for metastatic development [122]. A similar subpopulation of colorectal CSCs expressing CD26 was identified as the population responsible for liver metastasis, and was predictive of distant metastasis in patients [123]. The origin of this functional heterogeneity within the CSC population recently evolved with the notion of CSC state plasticity. In this regard, metastatic CSCs and non-metastatic CSCs may not be two distinct populations, but rather represent a gradient of stem cell programs that are expressed at higher or lower levels in response to intracellular and intercellular signals [124]. EMT plays an essential role in these CSC transition states [125, 126]. This concept was demonstrated by Liu et al., who showed that breast CSCs are highly plastic, transiting between two different cell states: a more proliferative epithelial-like state characterized by expression of ALDH, and a more invasive mesenchymal-like state characterized by the expression of the CD44⁺/CD24⁻ phenotype [127]. Over the last decade, the connection between CSCs and EMT attracted considerable attention [128]. Studies initially suggested that overexpression of EMT transcription factors was sufficient to generate CSCs [129, 130]. However, cumulative clues indicate that cancer stemness and EMT are in fact convergent concepts by which the EMT

process confers CSCs the ability to switch between two CSC states. Although recent findings raise doubts about the critical role of EMT during metastasis in mouse models of breast and pancreatic cancers [69, 131], evidence supports that EMT is alternatively switched on during the first step of metastatic spread (tissue invasion, circulation in blood, and extravasation), and then switched off to allow the metastatic colonization of new organs. This cell plasticity was directly observed by intravital microscopy, confirming the role of epithelial-mesenchymal plasticity in metastatic development [132].

In this context, a thorough understanding of the associated tumor microenvironment factors that control the balance between the different CSC states is required to improve our understanding of metastatic disease. Emerging evidence suggests that tumor-infiltrated immune cells may play this role, bringing back the “seed and soil” hypothesis of S. Paget [133]. Myeloid-derived suppressor cells (MDSCs) are a subset of immature myeloid cells that can be recruited to tumor sites and display immunosuppressive activity [134]. Ouzounova et al. used mammary tumor models to demonstrate that monocytic (M-MDSCs) and granulocytic (G-MDSCs) subsets of MDSCs regulate spatiotemporal tumor cell plasticity. M-MDSCs facilitate tumor cell dissemination from the primary site by inducing EMT, whereas pulmonary G-MDSC infiltrates support metastatic colonization by reverting the EMT process [135]. Consistent with this idea, the IL-1 β inflammatory response facilitates invasion and extravasation of metastatic-initiating cells by promoting EMT, and this signaling must be shut down at the metastatic site to induce EMT reversion and secondary tumor formation [136]. Similarly, neutrophils specifically support metastatic initiation by priming pre-metastatic sites, and neutrophil-derived leukotrienes aid the colonization of distant tissues by selectively expanding the sub-pool of cancer cells that acquire a stemness phenotype and retain high tumorigenic potential [137].

In mice, MDSCs are characterized by the expression of CD11b⁺Gr-1⁺ cell markers; G-MDSCs show CD11b⁺Ly6G⁺Gr-1^{High} staining, whereas M-MDSCs show a CD11b⁺Ly6C⁺Gr-1^{Low}

phenotype. The homeostasis of these myeloid cells relies on CD95L expression because loss of CD95L in mice leads to an increase in these populations [138]. Resistance to immunotherapy including the checkpoint inhibitors anti-CTLA4 and anti-PD1 in a melanoma mouse model is associated with increased infiltration of G-MDSCs expressing CD95L, which eliminates activated anti-tumor CD95^{high}-expressing CD8⁺ T cells [139]. Another study showed that CD95L deficiency skews tumor-infiltrating MDSCs from G-MDSCs to the M-MDSC subset, which displays high immunosuppressive activity mostly through the overexpression of PD-L1 [138]. These results emphasize the pivotal role of the CD95/CD95L pair in modulating the immune landscape by recruiting immunosuppressive cells within the primary tumor and/or participating in the elimination of anti-tumor CD8⁺ T cells. Nonetheless, the cells in tumor tissues that express CD95L and modulate the “soil” and, the CD95-dependent molecular mechanisms involved in the cancer/immune cell unbalance remain to be elucidated; recent data indicate that endothelial cells covering neovessels could represent the major source of CD95L [7, 108], .

Marcus Peter and colleagues proposed that CD95 stimulation can also modulate the phenotype of the “seed” itself. Accordingly, CD95 stimulation in various cancers (*i.e.*, colon, breast, and renal cancer cell lines) induces a conversion from non-CSCs to CSCs through an apoptotic-independent program, and in addition renders these cells resistant to the CD95-mediated apoptotic program [140]. From a molecular standpoint, CD95 stimulation in cancer cells induces the secretion of type I interferons (IFN α or IFN β), which interact with IFN type I receptors (IFNAR1 and IFNAR2) and activate STAT1-promoting cancer stemness [9]. These experiments confirmed that CD95 is not a receptor uniquely able to implement cell death; however, the downstream molecular mechanisms controlling the implementation of cell death at the expense of this non-apoptotic and stemness response and vice versa remain unclear.

Taken together, these studies suggest that the “seed and soil” concept needs to be revised to fit the concept of CSC state plasticity, and the CD95/CD95L pair might affect both seed and soil properties affecting cancer cell fate decisions and function. This assumption, if validated, will strengthen the use of CD95/CD95L inhibitors to improve cancer care.

Conclusion and therapeutic perspectives

Spatiotemporally coordinated calcium gradients generated by SOCs and SACs, calcium efflux systems, and calcium microdomains orchestrate cellular behaviors as complex as directional cell movement. CD95 participates in this phenomenon by activating localized Ca^{2+} influx through SOCs. The effect of CD95 engagement on SAC activity has not been described to date; however, CD95 is linked to structures such as lipid rafts/caveolae or the cytoskeleton, which contribute to the activation of these channels. In muscle fibers, for example, similarities in electrophysiological properties and sensitivity to pharmacological inhibitors suggest that SOC and SAC belong to the same channel population or share common constituents, suggesting that both channels express the TRPC1 protein [141]. This mechanosensitive SOC regulates the cell polarity involved in the migration of human bone osteosarcoma cells [142] and breast cancer cells (for review, see [143]). Future investigation of a potential molecular link between CD95 and the mechanosensitive channels would open new avenues to better understand the relationship between cell migration and the pathophysiological functions of the so-called death receptor.

The capability of proteinases to catalytically cleave substrate proteins post-synthesis is essential for sustaining life in all of its forms, from viruses to humans. Limited, site-specific proteolysis is an important post-translational modification, especially because this post-translational modification, in contrast to others, is irreversible under physiological conditions. The fact that two forms of CD95L exist and are associated with the presence or absence of MMPs raises the

question of what and where metalloproteases cleave CD95L. Similar to TNF, which is processed by TACE (ADAM-17) [144, 145] to release a soluble and inflammatory factor, CD95L-cleaving MMP(s) cause a signaling shift that transforms the apoptotic ligand into a pro-inflammatory factor. Targeting soluble CD95L, its non-apoptotic signaling pathways, or the MMPs responsible for its cleavage may represent a novel therapeutic strategy to alleviate clinical symptoms in chronic inflammatory disorders such as lupus or to prevent metastatic dissemination in cancer patients. In this context, a “niche therapy” based on CD95-oriented targeting of the CSC-niche signals may be a potent alternative to prevent tumor progression [146].

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Figure legends

Figure 1. CD95 and CD95L structures. The death receptor CD95 encompasses three amino-terminal cysteine-rich domains (CRDs) and is expressed at the plasma membrane as a homotrimer through homotypic interactions occurring via its Pre-Ligand Assembly Domain. Binding of its cognate ligand CD95L induces receptor oligomerization and triggers pro-apoptotic signals through its death domain (DD). CD95 also implements an anti-apoptotic Ca^{2+} response through its calcium-inducing domain (CID). CD95L is a type II transmembrane protein harboring a unique proline-rich domain (PRD) for the recruitment of SH3 and WW domain-containing proteins. Membrane-bound CD95L (m-CD95L) binds to CD95 through cell-cell contact and induces cell death. Certain metalloproteases can release a soluble and non-cytotoxic CD95L designated s-CD95L for soluble CD95L. The self-assembly domain (SA) is required for trimerization of the ligand. Plasmin can cleave CD95L, releasing a soluble and cytotoxic ligand. The TNF homology domain (THD) region of CD95L is responsible for CD95 binding.

Figure 2. s-CD95 and m-CD95L trigger different CD95-mediated signaling pathways. A. Interaction of transmembrane CD95L (m-CD95L) with CD95 favors the recruitment of FADD to the death domain (DD) of CD95. FADD binds procaspase-8 and FADD-like IL-1 β -converting enzyme-inhibitory protein (cFLIP) to form the Death-Inducing Signaling Complex (DISC). Oligomerization of procaspase-8 promotes its autocatalytic activation and the release of a mature tetramer to the cytosol, which implements the apoptotic response. **B.** Stimulation of CD95 by soluble CD95L (s-CD95L) induces the recruitment of the PLC γ 1 to the CD95 Calcium-Inducing Domain (CID). The phospholipase generates inositol triphosphate (InsP $_3$) by hydrolyzing phosphoinositol bisphosphate (PIP $_2$). InsP $_3$ activates IP $_3$ -R present at the membrane of the endoplasmic reticulum (ER) releasing Ca^{2+} . This calcium response is

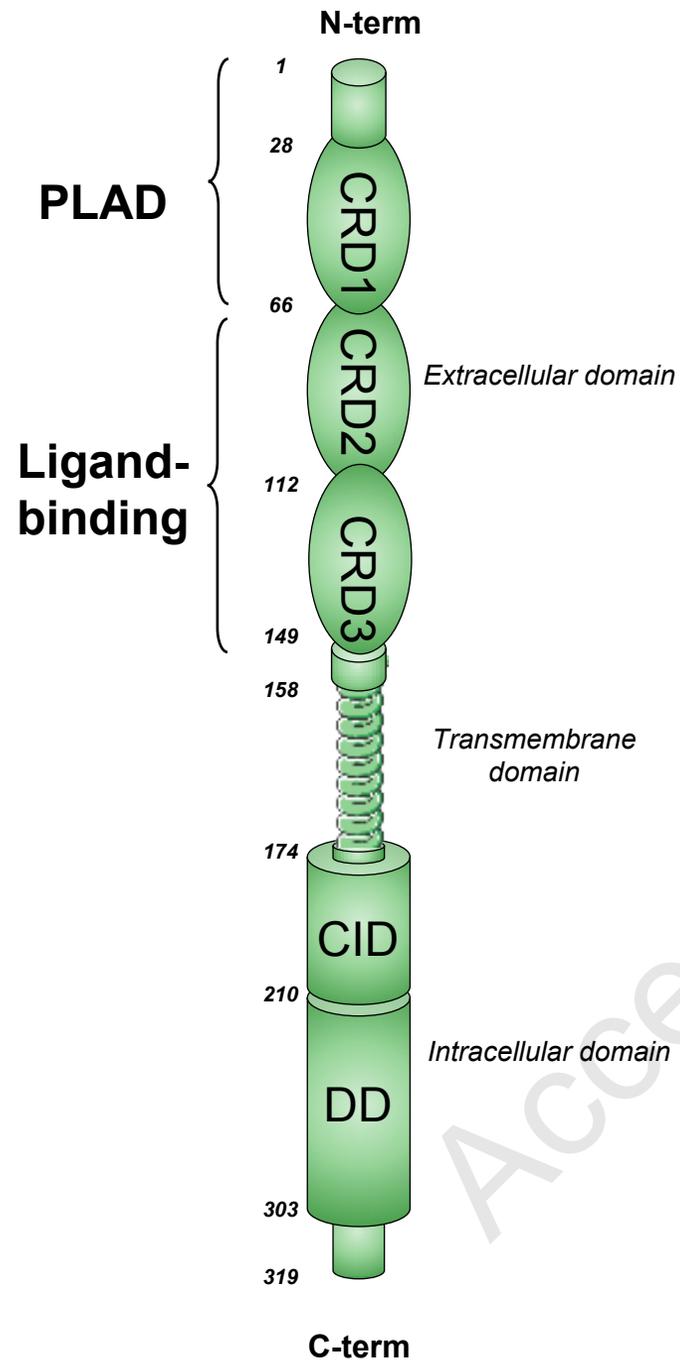
enhanced through the activation of the ORAI1 channel. The increased concentration of Ca^{2+} underneath the plasma membrane activates PKC- β 2, which interferes with the recruitment of FADD to the DISC. In the MISC, NADPH oxidase 3 (NOX3) is also recruited and produces reactive oxygen species (ROS), which activate c-Yes. This Src kinase can recruit receptor tyrosine kinases and phosphorylate different proteins localized in the MISC that are responsible for PI3K/Akt signaling pathway activation and cell migration.

Figure 3. Spatial and temporal regulation of intracellular Ca^{2+} concentration control cell migration. In the front, CD95 engagement induces the activation of phospholipase $\text{C}\gamma$ ($\text{PLC}\gamma$), leading to the activation of inositol triphosphate (IP3) receptors and the depletion of calcium from the endoplasmic reticulum (ER). This in turn stimulates the calcium sensor STIM1, which transactivates the ORAI1 channel and induces store-operated calcium (SOC) entry. This localized SOC entry promotes the autophosphorylation of Focal Adhesion Kinase (FAK³⁹⁷) via activation of calcium-dependent PKC β in the front and the degradation of (FAK) in the rear by the calcium-dependent μ -calpain protease, enhancing focal adhesion (FA) turnover and polarized cell motility. In the rear, local Ca^{2+} pulses activate Rho-associated protein kinase (ROCK) and myosin light chain kinase (MLCK), which phosphorylates myosin II for proper actin treadmilling and recycling. In the front, the Pyk2/rac1 pair regulates cytoskeleton organization via a calcium-dependent mechanism. DAG: diacylglycerol; PMCA: plasma membrane Ca^{2+} -ATPase.

Figure 4. Model for steering a migrating cell. During migration, cells exhibit a typical rear-to-front polarization. A $[\text{Ca}^{2+}]$ gradient is observed in the polarized cell by which high $[\text{Ca}^{2+}]$ at the back and low $[\text{Ca}^{2+}]$ at the leading edge facilitate the formation of functional local Ca^{2+} pulses. This $[\text{Ca}^{2+}]$ gradient is ensured by the accumulation of the plasma membrane Ca^{2+}

ATPase (PMCA) pump and intracellular Ca^{2+} compartments (ER, mitochondria) at the cell leading edge, which mediate Ca^{2+} extrusion and pumping, respectively. Intracellular Ca^{2+} entry controlled by a CD95-induced ORAI1-dependent mechanism establishes local Ca^{2+} flickers/pulses at the leading edge. Stretch-activated channels (TRPM7 and TRPV) are involved in the polarized calcium influx in migrating cells. CD95-stimulated NHE1 activity increases intracellular pH and promotes actin polymerization through a cofilin-dependent mechanism. Cdc42, a small pH-dependent Rho-GTPase, regulates actin polymerization by binding to the neural Wiskott-Aldrich syndrome protein (N-WASP), which activates the Arp2/3 complex. H^+ extruded by NHE1 causes extracellular acidification, which promotes the activity of matrix metalloproteinases (MMPs). Ca^{2+} influx through ORAI channels stimulates calpain to cleave cortactin. ERM: Ezrin Radixin Moesin, FA: Focal Adhesion.

CD95



CD95L

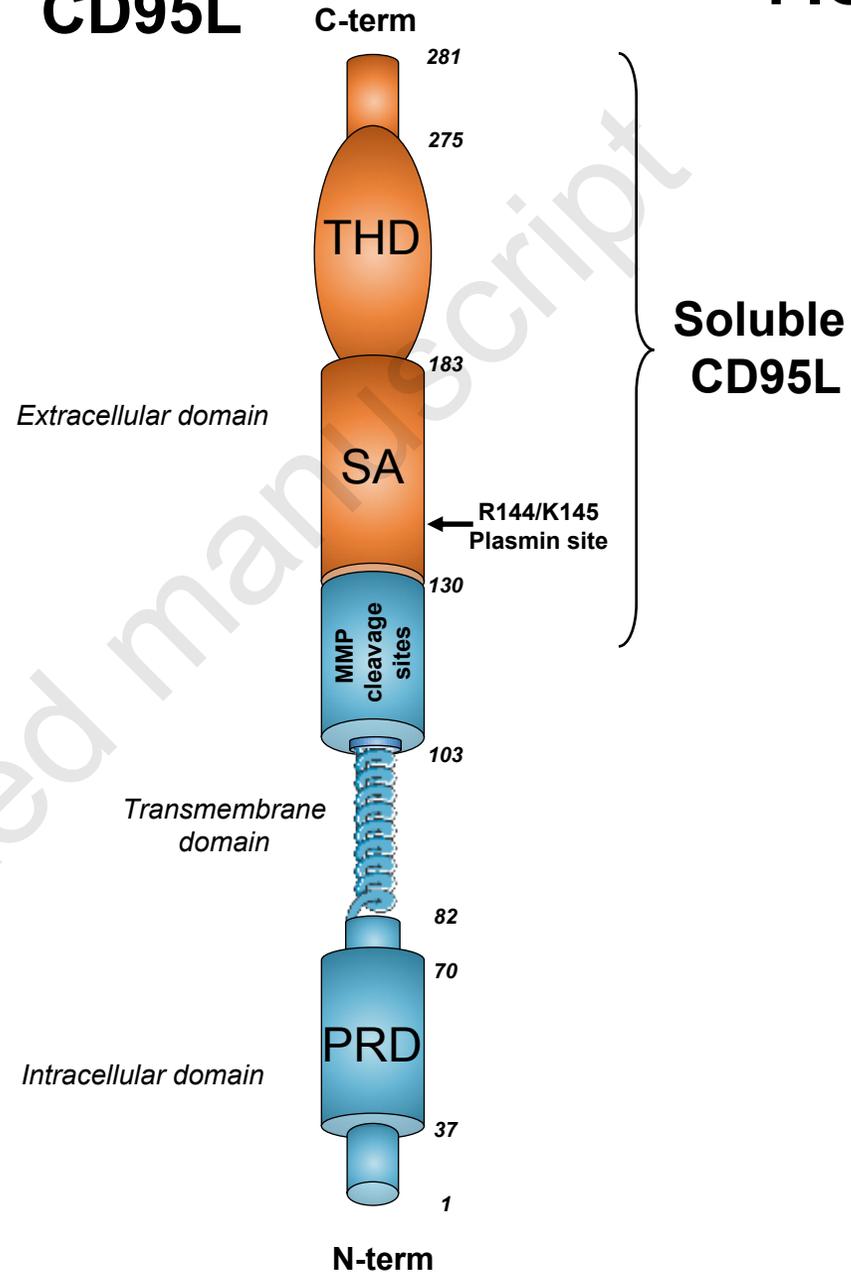


FIGURE 1

FIGURE 2

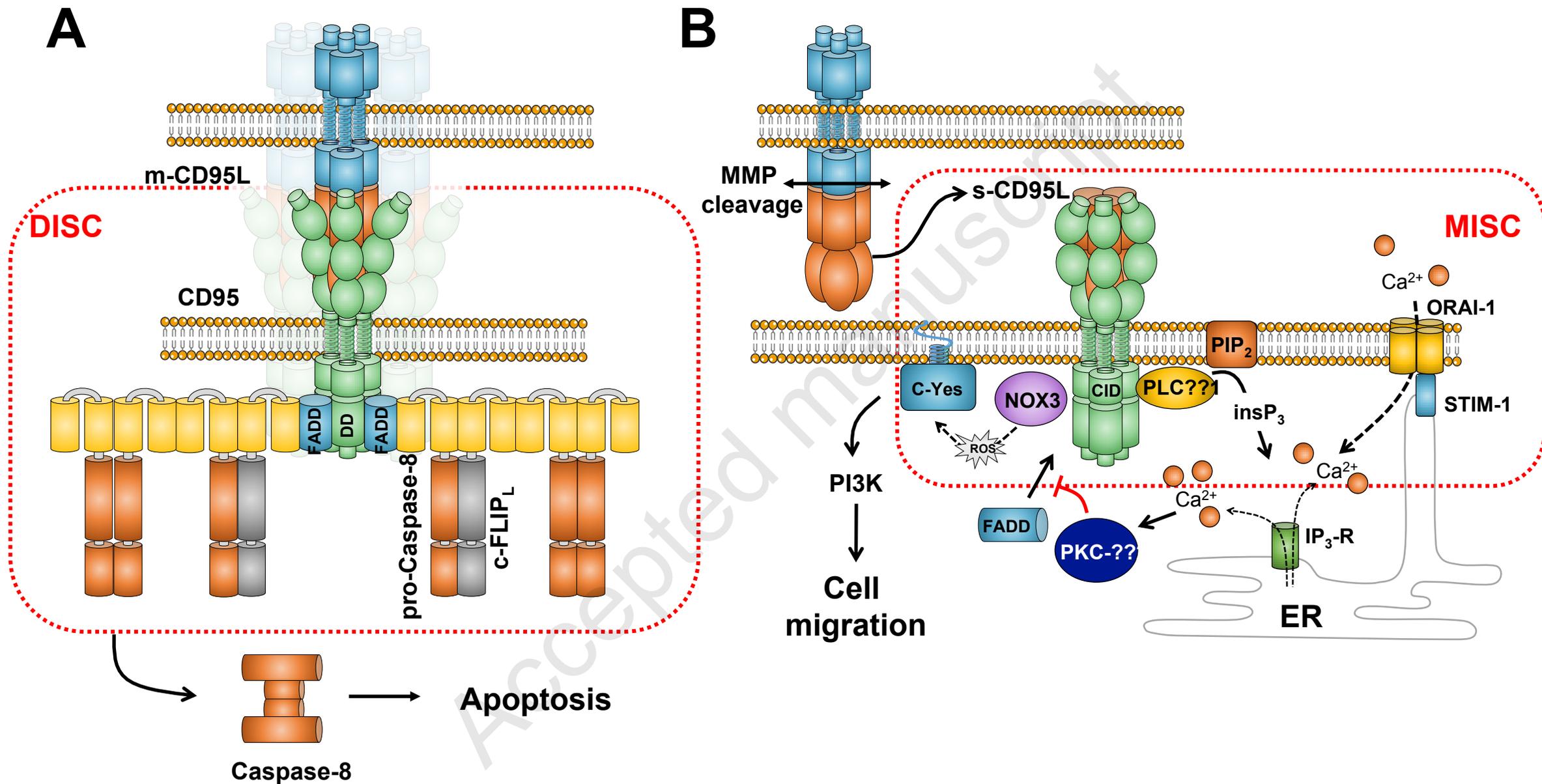


FIGURE 3

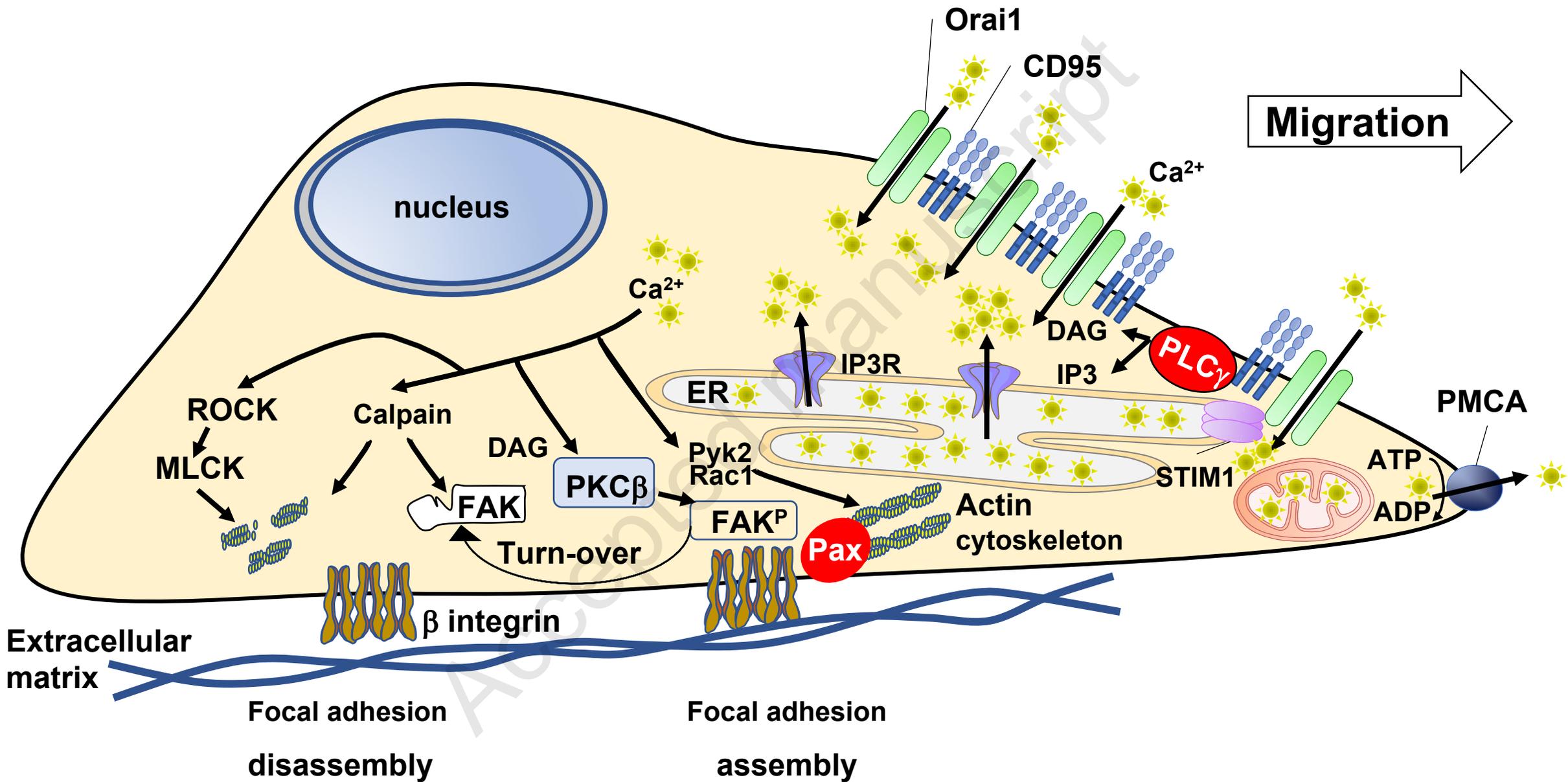


FIGURE 4

Cell migration 