Cellular IAPs inhibit a cryptic CD95-induced cell death by limiting RIP1 kinase recruitment

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A role for cellular inhibitors of apoptosis (IAPs [cIAPs]) in preventing CD95 death has been suspected but not previously explained mechanistically. In this study, we find that the loss of cIAPs leads to a dramatic sensitization to CD95 ligand (CD95L) killing. Surprisingly, this form of cell death can only be blocked by a combination of RIP1 (receptor-interacting protein 1) kinase and caspase inhibitors. Consistently, we detect a large increase in RIP1 levels in the CD95 death-inducing signaling complex (DISC) and in a secondary cytoplasmic complex (complex II) in the presence of IAP antagonists and loss of RIP1-protected cells from CD95L/IAP antagonist–induced death. Cells resistant to CD95L/IAP antagonist treatment could be sensitized by short hairpin RNA–mediated knockdown of cellular FLICE-inhibitory protein (cFLIP). However, only $cFLIP_L$ and not $cFLIP_S$ interfered with RIP1 recruitment to the DISC and complex II and protected cells from death. These results demonstrate a fundamental role for RIP1 in CD95 signaling and provide support for a physiological role of caspase-independent death receptor–mediated cell death.

Introduction

The initiators of the extrinsic cell death pathway are a subclass of TNF superfamily (TNFSF) receptors called death receptors (DRs). A common feature of DR signaling is the formation of a primary plasma membrane–associated death-inducing signaling complex (DISC) and a secondary independent signaling platform in the cytoplasm (complex II). Complex II was first demonstrated for TNF-R1 (Micheau and Tschopp, 2003) but subsequently was also shown for other DR pathways (Varfolomeev et al., 2005; Lavrik et al., 2008). However, the mechanisms leading to the formation of these secondary complexes and their significance to signaling outcome are still unknown. DR signaling pathways are controlled by inhibitors such as cellular FLICE-inhibitory protein (FLIP [cFLIP]) or X-linked inhibitor of apoptosis (IAP [XIAP]; for review see Meier and Vousden, 2007). The *cFLIP* gene can give rise to 11 distinct isoforms, but in most cells, a long ($cFLIP_L$) and a short isoform ($cFLIP_S$) are the only ones readily detected (for reviews see Kataoka, 2005; Budd et al., 2006). $cFLIP_L$ has a caspase-like domain lacking the critical catalytic residues present in caspase-8 in addition to two death effector domains, whereas $cFLIP_S$ contains only two death effector domains and is structurally related to viral FLIP (vFLIP; Thurau et al., 2006). cFLIP isoforms interact with FADD (Fas-associated protein with death domain [DD]) and caspase-8, are recruited to the DISC, and interfere with caspase activation within this signaling platform (Lavrik et al., 2005; Falschlehner et al., 2007).

DRs can also cause nonapoptotic, caspase-independent cell death and elicit nonapoptotic responses (for reviews see Wajant et al., 2003; Kroemer et al., 2009). The significance of these caspase-independent DR pathways is debated, and there is a need to provide additional examples in more physiological scenarios. RIP1

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Abbreviations used in this paper: 4-HT, 4-hydroxy-tamoxifen; CD95L, CD95 ligand; DD, death domain; DISC, death-inducing signaling complex; DKO, double knockout; DL, death ligand; DR, death receptor; FADD, Fas-associated protein with DD; FLIP, FLICE-inhibitory protein; IAP, inhibitor of apoptosis; MEF, mouse embryonic fibroblast; PI, propidium iodide; RIP1K, RIP1 kinase; SCC, squamous cell carcinoma; shRNA, short hairpin RNA; TRAIL, TNF-related apoptosis-inducing ligand; vFLIP, viral FLIP; XIAP, X-linked IAP.

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(receptor-interacting protein 1) belongs to the RIP kinase family but is the only family member with a C-terminal DD (Stanger et al., 1995; for review see Festjens et al., 2007). RIP1 knockout mice are born but die rapidly because of an increased sensitivity to TNF (Kelliher et al., 1998). RIP1, and specifically its DD, was reported to be critical for CD95-mediated necrosis independent of NF-κB–inducing activity or RIP1 kinase (RIP1K) activity (Holler et al., 2000; Degterev et al., 2005). The development of specific RIP1K inhibitors has facilitated experiments examining the functional role of RIP1K in necrosis (Degterev et al., 2008), but the precise role or potential targets of the kinase activity of RIP1 are unknown (Hitomi et al., 2008).

A major goal of tumor therapies such as DR agonists is to overcome transformation-induced apoptosis resistance (Hanahan and Weinberg, 2000; Ashkenazi, 2008). However, unfortunately, resistant tumor cells are frequently selected during treatment, exemplifying the need for novel treatments that can further sensitize tumors to DR-mediated apoptosis. IAP antagonists are synthetic compounds that were modeled on the N-terminal IAP-binding motif of the mitochondrial protein Smac/DIABLO (Wright and Duckett, 2005). The XIAP-interfering function of Smac-mimetic compounds (IAP antagonists) is crucial for therapeutic efficiency of TNF-related apoptosis-inducing ligand (TRAIL) in xenograft tumor models (Vogler et al., 2008). Recently, it has become apparent that compounds principally designed to target XIAP also target cIAPs by rapid autoubiquitylation and proteasomal degradation of cIAP1 and -2 (Gaither et al., 2007; Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007; Bertrand et al., 2008).

Previous studies have shown that cIAPs can inhibit CD95- and TRAIL-R–induced apoptosis (McEleny et al., 2004; Wang et al., 2005). It is unlikely that their role will be as direct caspase inhibitors because cIAPs are rather poor inhibitors of caspase activity (Eckelman and Salvesen, 2006). Because cIAPs regulate RIP1 in TNF-R1 and RIP1 plays a role in CD95 signaling, we have investigated the mechanism of DR cell death in the context of IAP inhibition. We show that cIAPs block DR-mediated cell death and that in their absence, cell death proceeds in a caspase- and RIP1K-dependent manner. Loss of cIAPs results in increased RIP1 recruitment to the DISC and in increased formation of complex II, which contains FADD, caspase-8, RIP1, and cFLIP isoforms. Surprisingly, different cFLIP isoforms have distinct signaling capabilities whenever cIAPs are repressed. This function of cIAPs might be used as a target to overcome apoptosis resistance in tumor therapy and might also be relevant during virus infection or tumor immunity, in which the mode of cell death is important (Lotze et al., 2007).

Results

The IAP antagonist sensitizes to death ligand (DL)–mediated cell death

We characterized the sensitivity of different keratinocyte cell lines and squamous cell carcinoma (SCC) cells with a recently described IAP antagonist, compound A (Vince et al., 2007). A rapid degradation of cIAP1 and, to a lesser extent, cIAP2 was detected after IAP antagonist treatment (Fig. S1 A). HaCaT and MET1 cells but not A5RT3 were sensitized to TRAIL- or CD95 ligand (CD95L)–mediated cell death in a TNF-independent manner in both short-term (Fig. 1 A) and clonogenic assays (Fig. 1 B). DL-induced cell death correlated with phosphatidylserine externalization (Fig. 1 C and Fig. S4 A) and hypodiploidy (Fig. 1 D). Consistent with an apoptotic cell death, we observed processing of caspases and PARP (poly[ADP-ribose] polymerase) cleavage within 4 h of stimulation with DLs in the presence of the IAP antagonist (Fig. 1 E and not depicted). To determine whether IAP antagonist–induced loss of cIAPs was important for this increased sensitization, we tested SV40 large T immortalized mouse embryonic fibroblasts (MEFs) lacking XIAP, cIAP1, cIAP2, or both cIAP1 and -2 (double knockout [DKO]) for their sensitivity to CD95L and TRAIL. DKO MEFs demonstrated an increased sensitivity to DLs, and the IAP antagonist slightly decreased the viability of these cells, suggesting that sensitivity was largely regulated by both cIAP1 and -2 in MEFs (Fig. 2 A). Consistent with this hypothesis, loss of XIAP did not sensitize MEFs to DLs (Fig. 2 A). Inducible reconstitution of cIAP1 or -2 into DKO MEFs (Fig. 2 B) increased the CD95L resistance of the DKOs that was lost when cells were cotreated with the IAP antagonist (Fig. 2 A, right). In contrast, increased expression of XIAP into DKO MEFs did not substantially alter sensitivity of these MEFs to the combination of CD95L and IAP antagonist (Fig. 2 C).

Similarly, knockdown of cIAP1 or both cIAPs sensitized HaCaT cells to CD95L-induced death despite the fact that there was a compensatory rise in the levels of cIAP2 (Fig. 2 D). This rise in cIAP2 levels might be explained by posttranslational regulation of cIAPs (Conze et al., 2005) or by increased NF-κB induced by loss of cIAP1 (Vince, J., personal communication). Specific knockdown of cIAP2 provides further evidence that it plays a less important role than cIAP1 in these cells as reported previously (Diessenbacher et al., 2008), although the knockdown of cIAP2 was weaker when compared with cIAP1 knockdown. We also inducibly expressed cIAP1 or -2 in HaCaT. Overexpression of cIAP2 conferred protection from CD95L-induced cell death in the presence of the IAP antagonist (Fig. 2 E). In contrast, inducible overexpression of cIAP1 did not alter sensitivity to CD95L nor was it able to protect against the IAP antagonist–mediated sensitization to CD95L death, presumably because of the efficient degradation of endogenous and overexpressed cIAP1 (Fig. S4, C and D). These data argue that endogenous levels of cIAPs in HaCaT are sufficient to confer resistance to CD95L-induced cell death but that loss of both cIAPs sensitizes to CD95-induced cell death. Our results demonstrate that cIAPs play an important role in limiting DL toxicity in both human and mouse cell lines.

IAP antagonist/DR-mediated cell death is neither entirely caspase dependent nor independent and requires RIP1K activity

DR-mediated apoptosis is initiated by DISC-activated caspase-8 (Peter and Krammer, 2003; Walczak and Haas, 2008). To investigate whether caspases caused DR-mediated cell death in the presence of the IAP antagonist, we used the caspase inhibitor zVAD-fmk. zVAD-fmk blocked cell death when cells were stimulated with DLs for 24 h. However, DL-mediated cell death

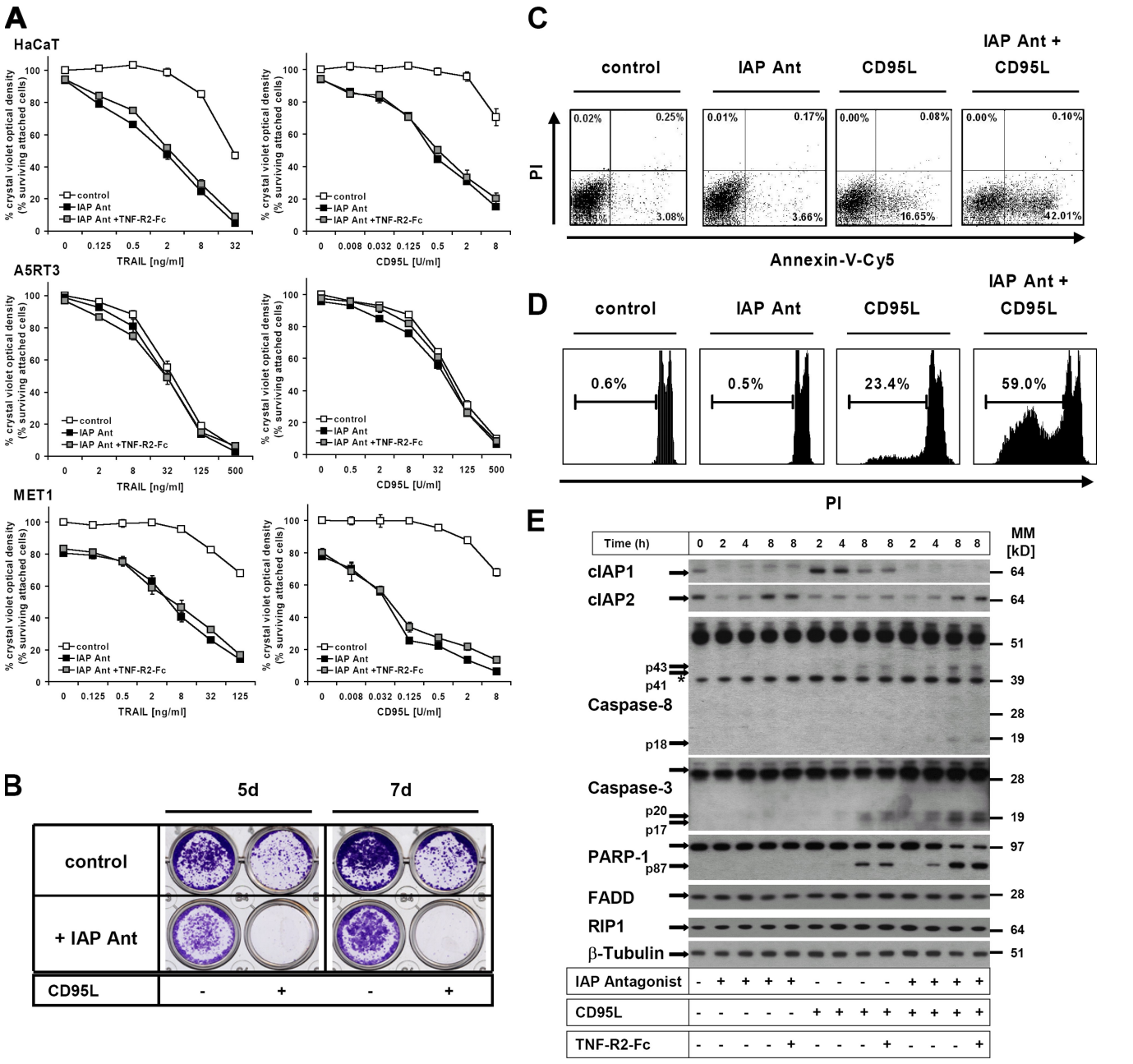

Figure 1. **The IAP antagonist sensitizes SCC and HaCaT to DL-mediated apoptosis independent of autocrine TNF secretion.** (A) HaCaT, MET1, or A5RT3 cells were either pretreated with 100 nM of the IAP antagonist (Ant) alone or in combination with 10 μg/ml TNF-R2-Fc for 30 min and then stimulated with TRAIL or CD95L. The viability of cells was analyzed by crystal violet assay after 18–24 h as indicated in Materials and methods. Mean and SEM of four independent experiments are shown. (B) For clonogenic assays, HaCaT cells were prestimulated with 100 nM of the IAP antagonist for 30 min followed by co-stimulation with 2.5 U/ml CD95L for 24 h. Colony formation was assayed as indicated in Materials and methods. One representative experiment of a total of three independent experiments is shown. (C–E) HaCaT cells were either prestimulated with 100 nM of the IAP antagonist for 30 min alone or stimulated/co-stimulated with 10 U/ml CD95L. (C) Cells were stained with annexin V–Cy5 and PI after 4 h and analyzed by FACS. (D) Cells were incubated for 8 h and subsequently analyzed for hypodiploidy by FACS analysis (see Materials and methods). (E) Cells were treated with 100 nM of the IAP antagonist, 2.5 U/ml CD95L, or the combination of both in the presence or absence of 10 μg/ml TNF-R2-Fc for the indicated time points. Western blot analysis was performed for the expression of cIAP1 and -2, caspase-8 and -3, PARP-1, FADD, and RIP1. β-Tubulin served as an internal loading control. One of two representative experiments is shown. The asterisk marks an unspecific band. MM, molecular mass.

in the presence of the IAP antagonist was only partially blocked by zVAD-fmk (Fig. 3 A). One potential explanation for these data is that DL treatment in the presence of the IAP antagonist induced a caspase-independent form of cell death. To characterize the morphology of cell death in these cells, we performed fluorescence microscopy experiments. Increased numbers of typical apoptotic cells demonstrating membrane blebbing, DNA condensation, and fragmentation were detectable after CD95L treatment and zVAD-fmk fully protected cell death and membrane integrity (Fig. 3 B, left). Caspase inhibition only partly protected from cell death in CD95L + IAP antagonist–treated cells (Fig. 3 B, right). Dying cells under those conditions showed

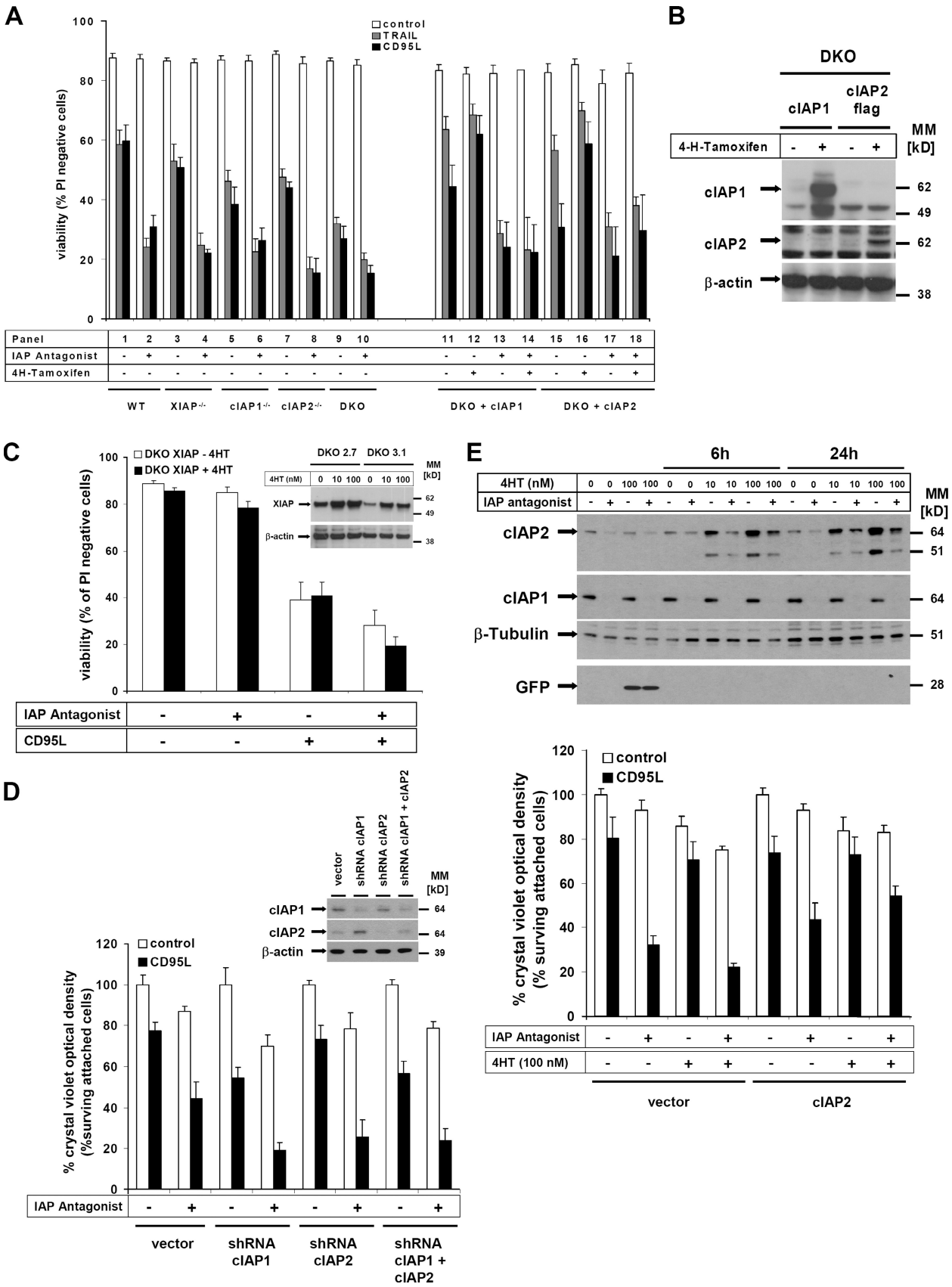

Figure 2. **cIAPs specifically block CD95L-induced cell death of MEFs and human SCCs.** (A) Transformed single knockout or DKO MEFs and respective control wild-type (WT) MEFs were treated with 10 ng/ml CD95L or 500 ng/ml TRAIL for 24 h in the presence or absence of 500 nM of the IAP antagonist (left). Cells were stained with PI and analyzed by flow cytometry. Four independent cIAP1 and -2 DKO MEFs were infected with inducible mouse cIAP1 or Flag-cIAP2 and induced with 10 nM 4-HT for 24 h. Cells were then treated with CD95L and TRAIL for 24 h in the presence or absence of the IAP antagonist,

a rounded shape, a lack of DNA condensation, and a later disruption of cell membranes, which are indicative of caspase-independent cell death (Fig. 3 B). These data suggested that cIAPs inhibit a cryptic caspase-independent death pathway that emanates from DRs. CD95 has the potential to activate a caspase-independent form of cell death via RIP1 (for review see Festjens et al., 2007). Because cIAPs are essential to ubiquitylate RIP1 in the TNF-R1 pathway (Bertrand et al., 2008), we hypothesized that RIP1 was required for this form of cell death. Therefore, we generated cell lines with decreased levels of RIP1 using stable short hairpin RNA (shRNA) expression (Fig. 3 C) and tested for sensitivity to CD95L/IAP antagonist–induced death. Interestingly RIP1 knockdown cells were remarkably resistant to sensitization to DL-mediated cell death by the IAP antagonist in short-term viability (Fig. 3 D) or clonogenic assays (Fig. 3 E).

To rigorously test the requirement of RIP1 in our CD95L/IAP antagonist–induced death, we tested RIP1 knockout MEFs. Consistent with our experiments in human cells, we found that RIP1 knockout cells were not sensitized to DL-mediated cell death in the absence of cIAPs (Fig. 4 A). However, the sensitivity of RIP1 knockout MEFs to CD95L was increased when compared with wild-type cells, which is indicative of a more complex role of RIP1. Our results imply that RIP1 is a required component of a cryptic caspase-independent cell death that is revealed when IAPs are antagonized. However, RIP1 also blocks a cell death pathway in the presence of cIAPs. This dual role is evident in TNF-R1 signaling where in the presence of cycloheximide, RIP1 is protective, but in the presence of the IAP antagonist, RIP1 is required for cell death (Kelliher et al., 1998; Kreuz et al., 2004; Gaither et al., 2007; Petersen et al., 2007). To determine whether the kinase activity of RIP1 was required for this death, we treated cells with the RIP1K inhibitor Necrostatin-1 (Fig. 4 B). When added to DL- and IAP antagonist–treated cells, Necrostatin-1 was unable to protect cells (Fig. 4 B). However, coaddition of Necrostatin-1 and zVAD-fmk resulted in complete protection from cell death (Fig. 4 B), annexin/propidium iodide (PI) positivity (Fig. S4 A), or clonogenic survival of DKO MEFs (Fig. 4 C). It has been suggested that the release of HMGB-1 (high mobility group Box-1 protein) in the cellular supernatant represents a characteristic of necrotic cell death (Scaffidi et al., 2002). When we investigated HMBG-1 release in IAP antagonist–treated cells, zVAD-fmk failed to block HMGB-1 release (Fig. S4 B). These data indicate that DLs activate a cell death pathway that results in activated caspases that are, in most situations, sufficient to kill cells. However, in the absence of cIAPs, a latent RIP1K-dependent pathway is revealed. To fully block cell death, inhibition of both caspases and RIP1K is necessary.

IAPs inhibit recruitment of RIP1 to the DISC and suppress the formation of complex II

To characterize how cIAPs negatively regulated CD95-mediated cell death, we examined the DISC and the receptor-independent complex II (Lavrik et al., 2008) in a cell line responsive to the IAP antagonist (MET1) and compared it with cells resistant to the IAP antagonist (A5RT3). We were readily able to detect recruitment of cFLIP, caspase-8, and FADD after CD95L stimulation (Fig. 5 A, left). Stimulation of CD95 led to SDS- and β-mercaptoethanol–insoluble CD95 complexes of higher molecular mass (Feig et al., 2007), as seen in the CD95 Western blots of our DISC precipitates (compare Fig. 5 with Fig. 7). We also detected small amounts of RIP1 in the CD95 DISC in both cell types (Fig. 5 A, lanes 3 and 7). Given the differential sensitivity, recruitment of FADD, FLIP, and caspase-8 to the DISC was remarkably similar in MET1 and A5RT3s, either in the presence or absence of the IAP antagonist. In contrast, RIP1 recruitment was dramatically increased in the CD95 DISC of MET1 in the absence of cIAPs (Fig. 5 A, lanes 3 and 4), whereas RIP1 recruitment was weaker in A5RT3 cells, although still increased by the IAP antagonist (Fig. 5 A, lanes 7 and 8). When we examined complex II, we observed a similar stimulation-dependent interaction of FADD, cFLIP, and RIP1 with caspase-8. These experiments were performed in the presence of zVAD-fmk during stimulation because caspase inhibitors stabilize complex II (Wang et al., 2008). As in the CD95 DISC, there was a substantial increase in RIP1 recruitment to complex II in the absence of cIAPs compared with CD95L-treated cells alone (Fig. 5 A, lanes 19 and 20 and 23 and 24). Our data suggested that loss of cIAPs increased the DISC recruitment of RIP1 or repressed RIP1 degradation, which translated to an increased level of RIP1 in complex II.

zVAD-fmk stabilizes both the DISC and complex II and allows for the easier detection of DISC and complex II components (Micheau et al., 2002; Wang et al., 2008). Thus, we tested whether zVAD-fmk affected DISC and complex II composition. Reassuringly, the qualitative recruitment of RIP1 was almost identical whether cells were treated with zVAD-fmk or not, except that RIP1 was cleaved in the absence of caspase inhibitor as previously published (Fig. 5 B; Kim et al., 2000; Martinon et al., 2000). Importantly, this experiment showed that both DISC recruitment and complex II formation increase over 1 h and then remain at steady levels for the next hour, finally decreasing in abundance within 4 h. It also demonstrated the effect of zVAD-fmk in increasing the stability of both CD95 DISC and complex II, confirming previous studies (Micheau et al., 2002; Wang et al., 2008).

stained with PI, and analyzed by flow cytometry. The mean + SEM is shown throughout. (B) DKO 1.5 MEFs, infected with inducible mouse cIAP1 and Flag-cIAP2, were induced for 24 h with 4-HT. cIAP1 or Flag-cIAP2 expression was analyzed by Western blotting. β-Actin served as a loading control. (C) DKO 2.7 and DKO 3.1 MEFs were infected with inducible mouse XIAP and induced with the indicated concentrations of 4-HT for 24 h. Induction of XIAP was verified by Western blot analysis. Cells were treated with 20 ng/ml CD95L for 24 h in the presence or absence of 500 nM of the IAP antagonist, stained with PI, and analyzed by FACS. The mean + SEM of a total of six independent experiments is shown. (D) HaCaT cells were transduced with control vector or shRNA against cIAP1 or -2 or both. Western blot analysis shows expression of cIAP1 and -2. β-Actin served as a loading control. Subsequently, cells were analyzed for sensitivity to 2.5 U/ml CD95L in the presence or absence of 100 nM of the IAP antagonist 24 h later by crystal violet assay. (E) HaCaT cells were transduced with lentiviral control vector or inducible cIAP2 as previously described (Diessenbacher et al., 2008). Cells were induced with 4-HT as indicated and then treated with 2.5 U/ml CD95L for 24 h in the presence or absence of 100 nM of the IAP antagonist. Viability was examined by crystal violet assay 24 h later. (D and E) Mean + SEM of three independent experiments is shown. MM, molecular mass.

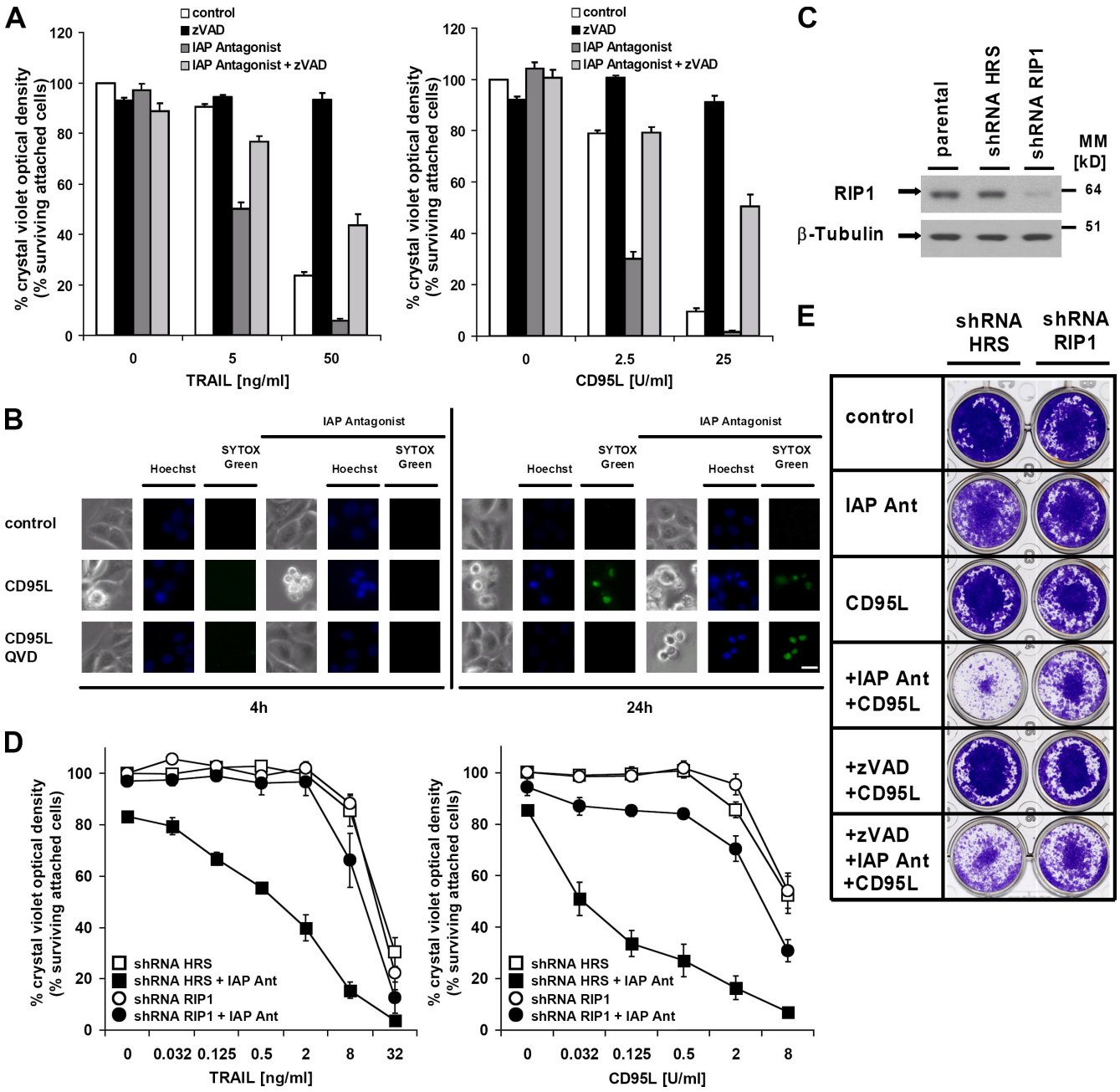

Figure 3. **DR-mediated cell death in the presence of the IAP antagonist uses caspases and caspase-independent signaling pathways.** (A) HaCaT cells were pre- or co-stimulated with 10 µM zVAD-fmk for 1 h and 100 nM of the IAP antagonist for 30 min. Subsequently, cells were stimulated with the indicated concentration of TRAIL or CD95L. The viability of cells was analyzed by crystal violet assay 18–24 h later as indicated in Materials and methods. Mean + SEM for three (TRAIL) or six (CD95L) independent experiments is shown. (B) HaCaT cells were either pretreated with 10 µM zVAD-fmk for 1 h or 100 nM of the IAP antagonist for 30 min. Cells were subsequently stimulated with 5 U/ml CD95L for 4 or 24 h. 5 µg/ml Hoechst 33342 and 5 pM SYTOX green were added for 15 min at 37°C, immediately followed by transmission (left) or fluorescence (right) microscopy. One of two independent experiments is representatively shown. (C) Stable knockdown of RIP1 in HaCaT cells was performed as indicated in Materials and methods and controlled by Western blot analysis for RIP1. Reprobing of the membrane with β-tubulin antibodies served as a control for protein loading. MM, molecular mass. (D) Transduced HaCaT cells as shown in C were prestimulated for 30 min with 100 nM of the IAP antagonist or diluent alone and subsequently stimulated with the indicated concentrations of TRAIL or CD95L for 18–24 h followed by crystal violet assay. Mean ± SEM of three (TRAIL) or four (CD95L) independent experiments is shown. (E) Transduced HaCaT cells, as described in C, were preincubated with 100 nM of the IAP antagonist (Ant) for 30 min and then stimulated with 0.5 U/ml CD95L. After 24 h, colony formation was assayed as indicated in Materials and methods. One of four representative independent experiments is shown. Bar, 10 µm.

Collectively, our results demonstrate that the IAP antagonist leads to a consistent increase in recruitment of RIP1 to the DISC and does not merely delay or block the transition of RIP1 to complex II.

Cells resistant to the sensitizing effect of the IAP antagonist contained cIAP2 within both the DISC and complex II despite treatment with IAP antagonist, opening up the possibility that increased levels of cIAP2 in A5RT3 could account for the

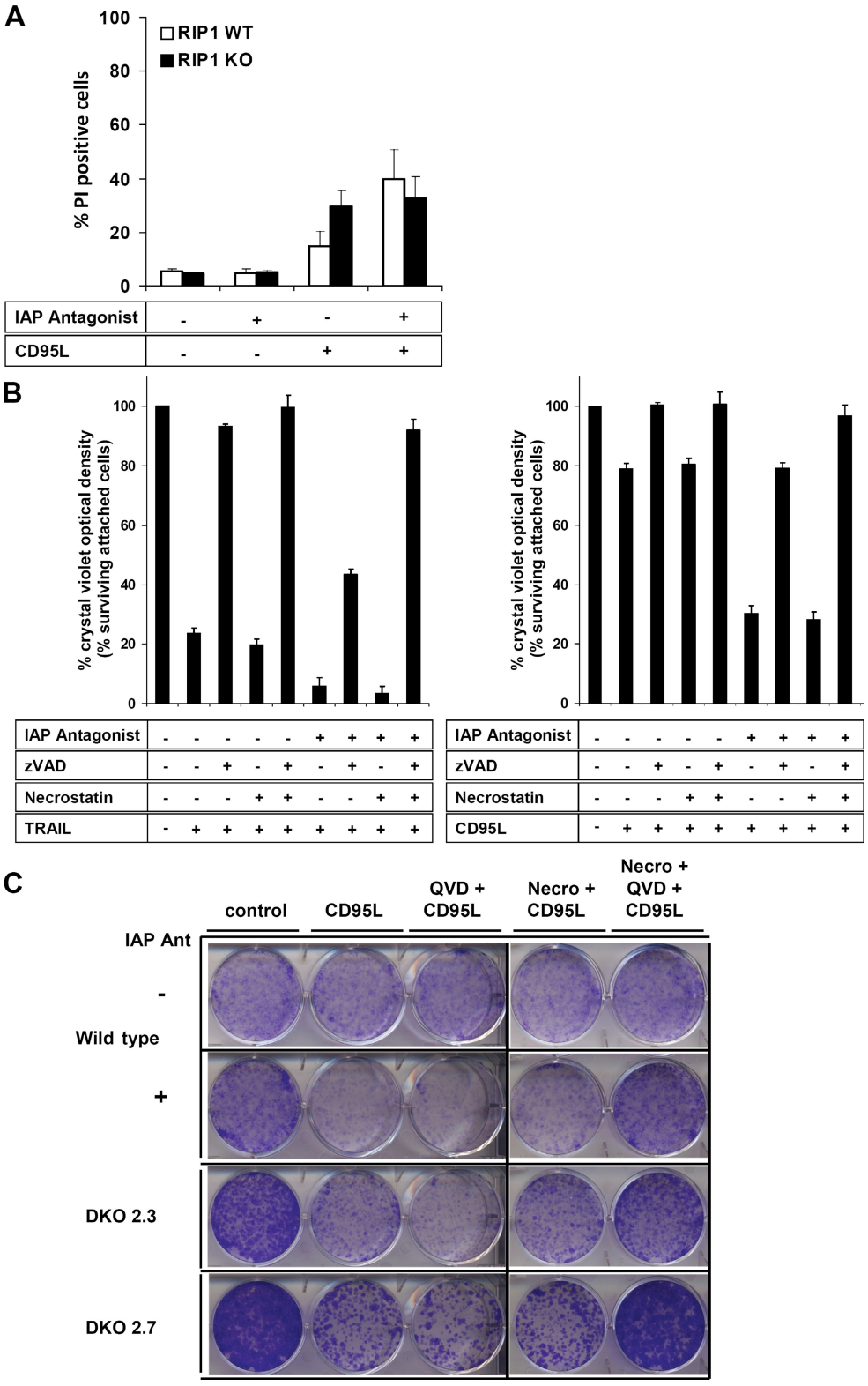

Figure 4. **RIP1 is an important regulator of DL-mediated cell death in the absence of cIAPs.** (A) RIP1 knockout (KO) or wild type (WT) MEFs were stimulated with 10 ng/ml CD95L or 10 ng/ml CD95L and 500 nM of the IAP antagonist for 24 h and then assayed for cell viability using PI and flow cytometry. The mean + SEM of a minimum of three independent experiments is shown. (B) The combination of caspase inhibitor zVAD-fmk and RIP1K inhibitor Necrostatin-1 completely protects HaCaT cells from DL-mediated cell death in the presence of the IAP antagonist. HaCaT cells were separately prestimulated with 10 μM zVAD-fmk for 1 h, 50 μM Necrostatin-1 for 1 h, and 100 nM of the IAP antagonist for 30 min, followed by stimulation with 50 ng/ml TRAIL or 2.5 U/ml CD95L for 18–24 h and subsequent crystal violet assay. Mean + SEM of three (TRAIL) or six (CD95L) independent experiments is shown. (C) Transformed cIAP1 and -2 DKO 2.3 and 2.7 MEFs were treated with 10 ng/ml CD95L for 24 h in the presence or absence of 10 μM QVD and/or 50 μM Necrostatin-1 (Necro). Respective control wild-type MEFs were treated as the DKO MEFs but in the presence or absence of the IAP antagonist (Ant). Subsequently, cells were harvested, and an aliquot was replated in 6-well plates and cultured for another 4 d followed by crystal violet staining of colonies.

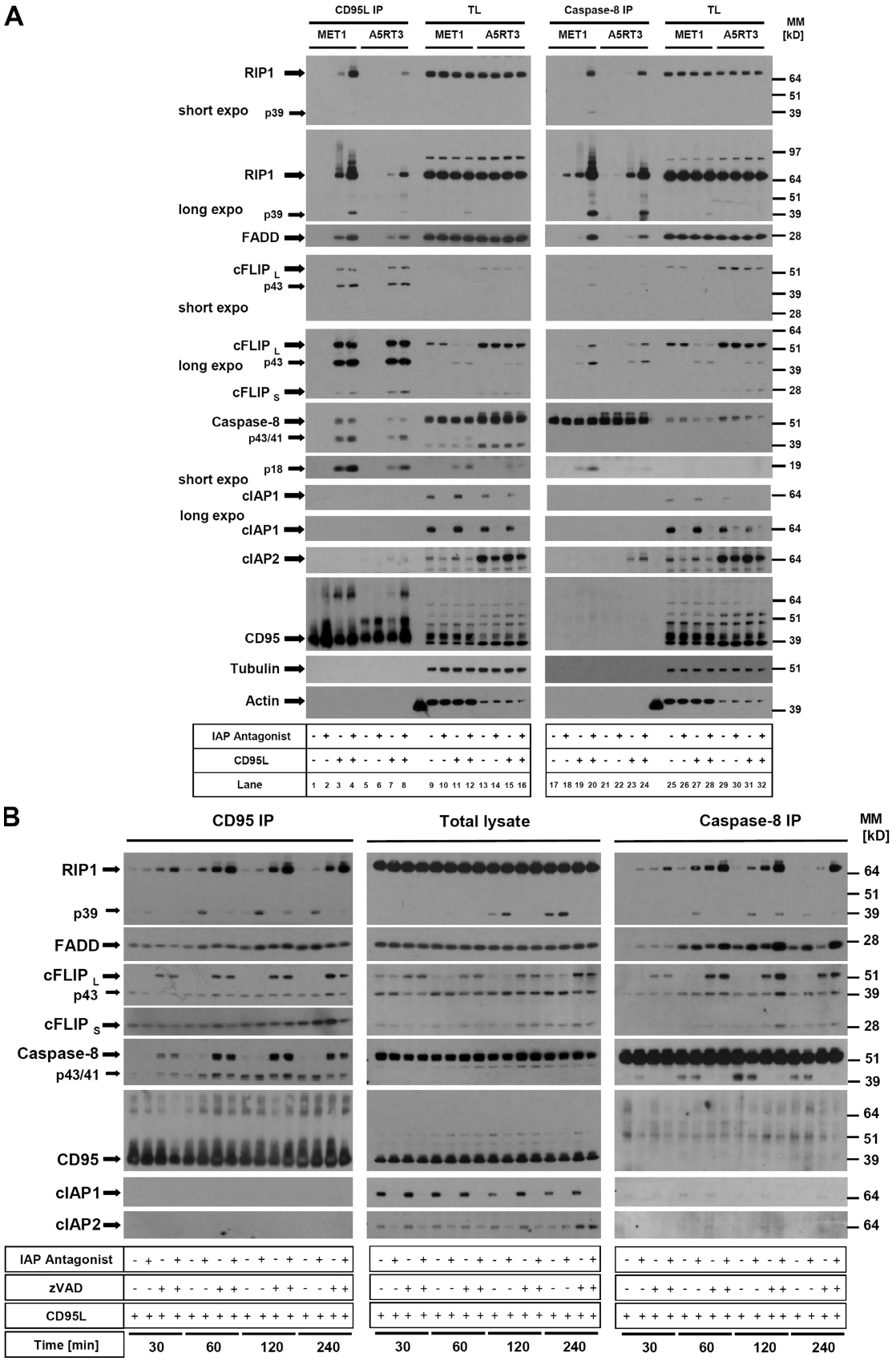

Figure 5. **Induction of ligand-induced receptor-bound CD95 complex (DISC) or intracellular caspase-8–containing complex (complex II) in the presence or absence of the IAP antagonist.** (A) The CD95 DISC was precipitated from MET1 or A5RT3 cells preincubated with 10 µM zVAD-fmk and 100 nM of the IAP antagonist for 1 h and subsequently treated with CD95L-Fc for 2 h. CD95L DISC (left) was precipitated as detailed in Materials and methods. Precipitation of receptor complexes after lysis (−) served as internal specificity control when compared with ligand affinity precipitates (IP; +). Equal amounts of DISC (CD95L IP)

resistance of these cells to CD95L/IAP antagonist–induced death. However, when we performed knockdown of cIAP2, A5RT3 cells were not sensitized to CD95L-induced cell death by the IAP antagonist (Fig. S2 C). Moreover, overexpression of TRAF2 (Fig. S2 B), which is expressed at substantially lower levels in A5RT3 cells than in MET1 cells (Fig. S2 A), did not render A5RT3 cells capable of being sensitized by the IAP antagonist, largely excluding TRAF2 as a critical candidate explaining the difference between IAP antagonist–sensitive and –resistant cells.

cFLIP isoforms differentially contribute to resistance to DL-mediated cell death in the absence of IAPs

There are several conflicting results with respect to signaling capabilities of different cFLIP isoforms (for review see Yu and Shi, 2008). Although cFLIP recruitment appeared very similar in the three human cell lines tested, we wished to test whether cFLIP isoforms could confer resistance to IAP antagonist–mediated sensitization to DLs. Knockdown of both expressed isoforms of cFLIP sensitized A5RT3 to IAP antagonist–DL cell death (Fig. S3). Therefore, we generated HaCaT (Fig. 6 C) and MET1 (Fig. S5 A) cell lines expressing different cFLIP isoforms. We observed that sensitivity to the IAP antagonist alone was increased in cells overexpressing $cFLIP_S$ but not $cFLIP_L$ (Fig. 6, A and B; and Fig. S5, B and C). Furthermore, overexpression of $cFLIP_S$ was unable to protect from DR-mediated cell death in the presence of the IAP antagonist (Fig. 6 B and Fig. S5 B, panels 7 and 8) even if zVAD-fmk was added and despite the fact that it was perfectly competent at protecting cells from CD95L treatment alone (Fig. 6 B and Fig. S5 B, panels 2 and 3). Intriguingly, Necrostatin-1 prevented cell death in short-term assays under those conditions (Fig. 6 B and Fig. S5 B, panel 9). In contrast, $cFLIP_L$ was very effective in blocking CD95L/IAP antagonist–induced cell death (Fig. 6, A and D; and Fig. S5 C). These experiments show that $cFLIP_S$ and $cFLIP_L$ differentially regulate cell death pathways in the absence of cIAPs in a previously unsuspected manner.

cFLIP isoforms differentially influence CD95-induced recruitment of RIP1 to the DISC and complex II

To elucidate the molecular mechanism of this cFLIP isoform phenomenon, we precipitated the CD95 DISC and complex II in cells expressing $cFLIP_L$ or $cFLIP_S$. This experiment was performed in the absence of zVAD-fmk to allow detection of differences in caspase activity. We observed a dramatic increase in RIP1 levels in the DISC of control cells treated with the IAP antagonist despite the fact that the majority of RIP1 was now cleaved within the DISC (Fig. 7 A, lanes 1–4). Consistent with previous studies for the TRAIL DISC (Harper et al., 2001; Wachter et al., 2004), $cFLIP_L$ repressed the recruitment of RIP1 to the CD95 DISC, and caspase-8 and cFLIP were recruited and cleaved as previously published (Krueger et al., 2001; Geserick et al., 2008). In complex II, an increased amount of RIP1, FADD, and $cFLIP_L$ (pro form as well as p43) was detected in control cells (Fig. 7 A, lanes 18–21). In contrast, $cFLIP_L$ and, to a substantially lesser extent, $cFLIP_S$ blocked the formation of complex II (Fig. 7 A, lanes 22–29). Interestingly, complex II formation in $cFLIP_S$-expressing cells was detected at low levels in the absence of DL stimulation (Fig. 7 A, lane 27). Inhibition of RIP1K activity with Necrostatin-1 blocked RIP1–caspase-8 interaction in complex II, suggesting that RIP1K activity may facilitate transition from the DISC to complex II, which is important for caspase-independent cell death (Fig. 7 A, lanes 13–16 and 30–33). To analyze this particular aspect also in parental HaCaT cells, we tested DISC and complex II formation in the presence of zVAD-fmk, the IAP antagonist, Necrostatin-1, and the combination thereof (Fig. 7 B). Because of the known stabilization of the DISC and complex II by zVAD-fmk, comparisons should be made between similarly treated samples. Comparable levels of RIP1 were retained in the DISC in the presence or absence of Necrostatin-1 (Fig. 7 B, compare lane 12 with lane 16 for the CD95 IP, RIP1 vs. FADD). Comparison of lane 8 with lane 14 for the CD95 DISC and lane 25 with lane 31 for complex II indicates that Necrostatin-1 did not detectably change RIP1 association with the CD95 DISC, if the DISC-associated cleavage of RIP1 is taken into account (Fig. 7 B). In contrast, the level of RIP1 in complex II was decreased by Necrostatin-1 (Fig. 7 B, compare lane 29 with 33, low exposure RIP1). Thus, RIP1K activity contributes to the translocation of a complex II in the parental HaCaT cells, which is in line with our overexpression data. Thus, cIAPs normally limit RIP1 recruitment to the DISC and maturation of a RIP1-containing DISC into a RIP1-containing complex II. In the absence of cIAPs, overexpressed $cFLIP_L$ is able to block this increased recruitment and prevent cell death, whereas overexpressed $cFLIP_S$ is not. Thus, $cFLIP_S$ is unable to block increased RIP1 recruitment even though it is able to completely block caspase-8 activation and caspase activity in the DISC. However, $cFLIP_S$ is nevertheless insufficient to block CD95-induced cell death.

TWEAK sensitizes to CD95L-induced cell death comparable with the IAP antagonist in cells lacking XIAP

To further understand the physiological role of cIAPs for CD95-induced cell death, we studied the treatment of cells with TWEAK, which was recently shown to induce cIAP

or complex II (caspase-8 IP) were subsequently analyzed by Western blotting for the indicated molecules. Equal amounts of total cellular lysates (TL) were loaded on the same gels to allow comparison of signal strength between CD95L-IP, complex II, and total cellular lysates. (B) Kinetics of DISC (left) or complex II (right) in the presence or absence of the IAP antagonist. The CD95 DISC was precipitated from parental HaCaT cells either prestimulated with 100 nM of the IAP antagonist and 10 μM zVAD-fmk for 1 h alone or the combination of both and subsequently stimulated with 250 U/ml CD95L for the indicated times. CD95L DISC (left) or complex II (right) was precipitated as detailed in Materials and methods and specified for A. MM, molecular mass.

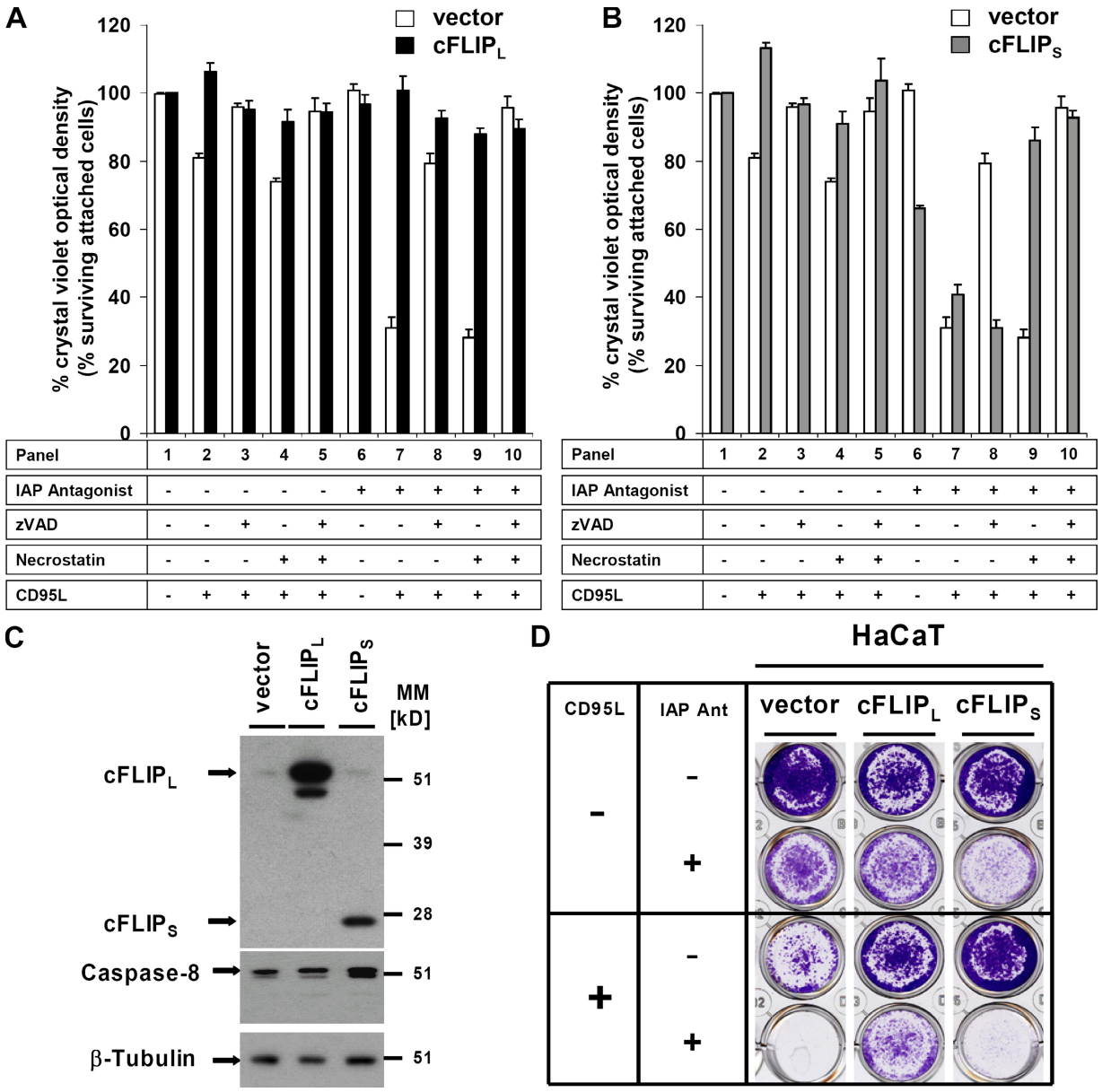

Figure 6. **cFLIP is an important regulator of DL-mediated cell death in the absence of cIAPs.** (A and B) HaCaT cells were transduced with cFLIP$_L$ (A) or cFLIP$_S$ (B) or control vector. Total cellular lysates were analyzed for cFLIP and caspase-8. β-Tubulin served as an internal control for protein loading. The mean + SEM of six independent experiments is shown. (C) Cells were prestimulated with 10 µM zVAD-fmk for 1 h, 50 µM Necrostatin-1 for 1 h, and 100 nM of the IAP antagonist for 30 min or diluent alone. Subsequently, cells were stimulated with 2.5 U/ml CD95L. The viability of cells was analyzed by crystal violet assay after 18–24 h. MM, molecular mass. (D) Transduced HaCaT cells were prestimulated with 100 nM of the IAP antagonist (Ant) for 30 min followed by co-stimulation with 2.5 U/ml CD95L. 24 h later, colony formation assay was performed as described in Materials and methods. One representative experiment of a total of three independent experiments is shown.

degradation and sensitize cells to TNF-mediated cell death (Vince et al., 2008; Wicovsky et al., 2009). TWEAK treatment of HaCaT cells caused down-regulation of cIAP1 and -2 similarly to the IAP antagonist (Fig. 8 B). HaCaT cells were sensitized to CD95 killing by TWEAK to the same extent as HaCaT cells treated with the IAP antagonist and were similarly protected by the combination of zVAD-fmk and Necrostatin-1 (Fig. 8 A). MET1 cells that expressed high levels of XIAP (Fig. S2 A) were substantially less sensitized when comparing TWEAK with the IAP antagonist (Fig. 8 A). These data confirm that XIAP can contribute to DL resistance in keratinocytes (Leverkus et al., 2003b), which is similar to the role of XIAP in type II cells (Jost et al., 2009). TWEAK-induced sensitization similarly uncovered a caspase-independent, RIP1-dependent form of cell death (Fig. 8 C). Most interestingly, TWEAK-induced sensitization was blocked by cFLIP$_L$ but not cFLIP$_S$ (Fig. 8 D), indicating that the findings for the IAP antagonist depicted in Figs. 6 and 7 and Fig. S5 are also relevant in a physiological setting. Of particular interest, cells expressing cFLIP$_S$ were fully protected by Necrostatin-1 alone, indicating that TWEAK-induced degradation of cIAPs may be sufficient to allow a CD95-induced

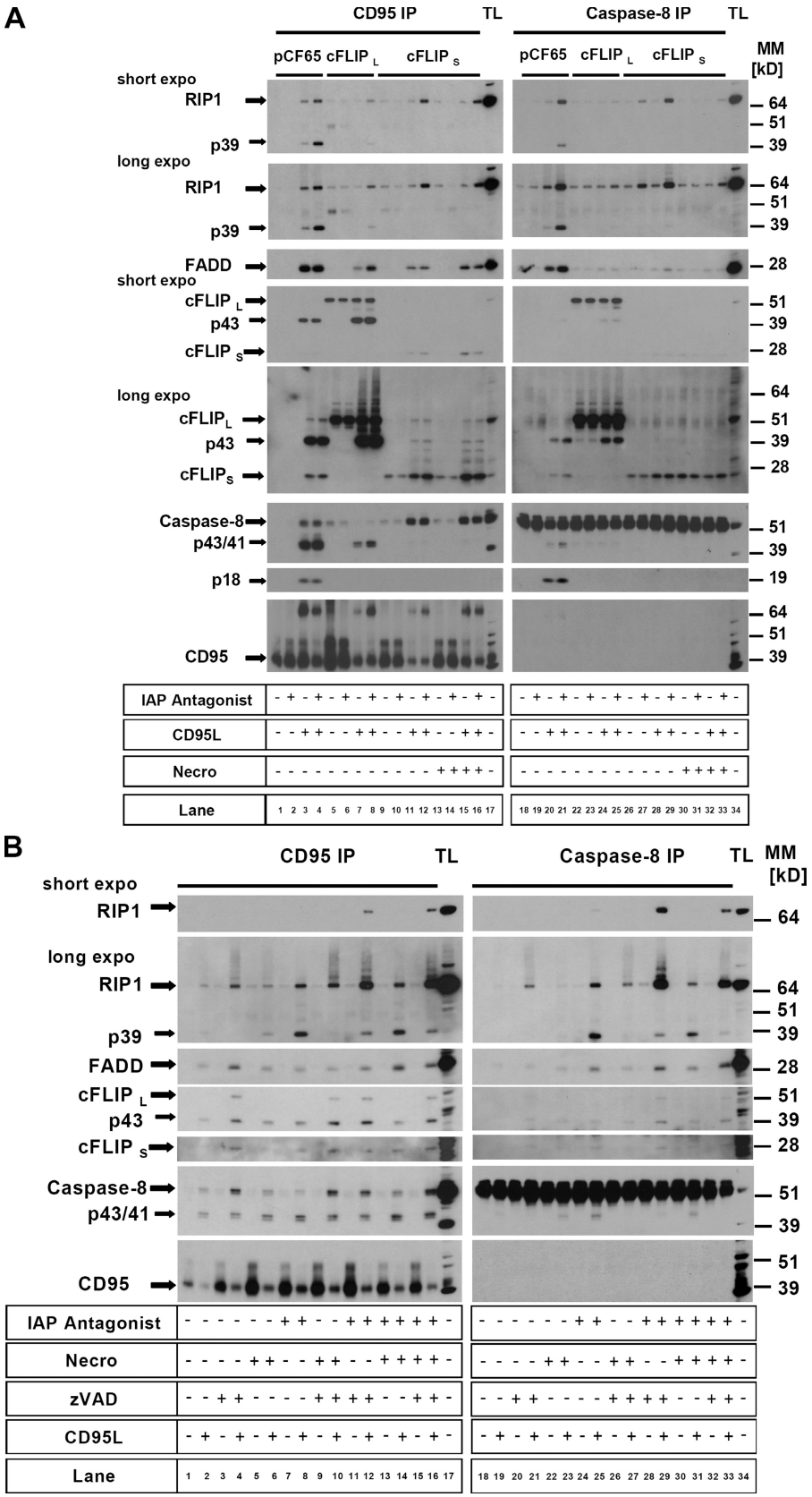

Figure 7. **cFLIP$_L$ but not cFLIP$_S$ blocks the formation of complex II.** (A) DISC or complex II formation in the presence or absence of the IAP antagonist. To allow for caspase activity, these experiments were performed without zVAD-fmk. HaCaT cells were stimulated with CD95L-Fc for 2 h. Subsequently, the CD95L DISC (left) was precipitated using ligand affinity precipitation as detailed in Materials and methods. Precipitation of receptor complexes after lysis (−) served as an internal specificity control when compared with ligand affinity precipitates (IP; +). Equal amounts of DISC (CD95L IP) or complex II (caspase-8 IP) were subsequently analyzed by Western blotting for the indicated molecules. Equal amounts of total cellular lysates (TL) were loaded on the same gels to allow comparison of signal strength between IP and total cellular lysates. (B) DISC or complex II formation in parental HaCaT. Cells were either pre- or co-stimulated with 10 μM zVAD-fmk, 50 μM Necrostatin-1, and 100 nM of the IAP antagonist for 1 h and subsequently stimulated with 250 U/ml CD95L for 2 h. The CD95L DISC (left) or complex II (right) was precipitated as detailed in Materials and methods and specified for A. Equal amounts of DISC (CD95L IP) or caspase-8–interacting proteins (complex II) were subsequently analyzed by Western blotting for the indicated molecules. MM, molecular mass.

caspase-independent signaling pathway to be unblocked. Moreover, these data suggest that the stoichiometry of cFLIP$_L$ and cFLIP$_S$ might be important to determine the sensitivity to caspase-independent cell death once cIAPs are inactivated in a given cell irrespective of the mode of their degradation.

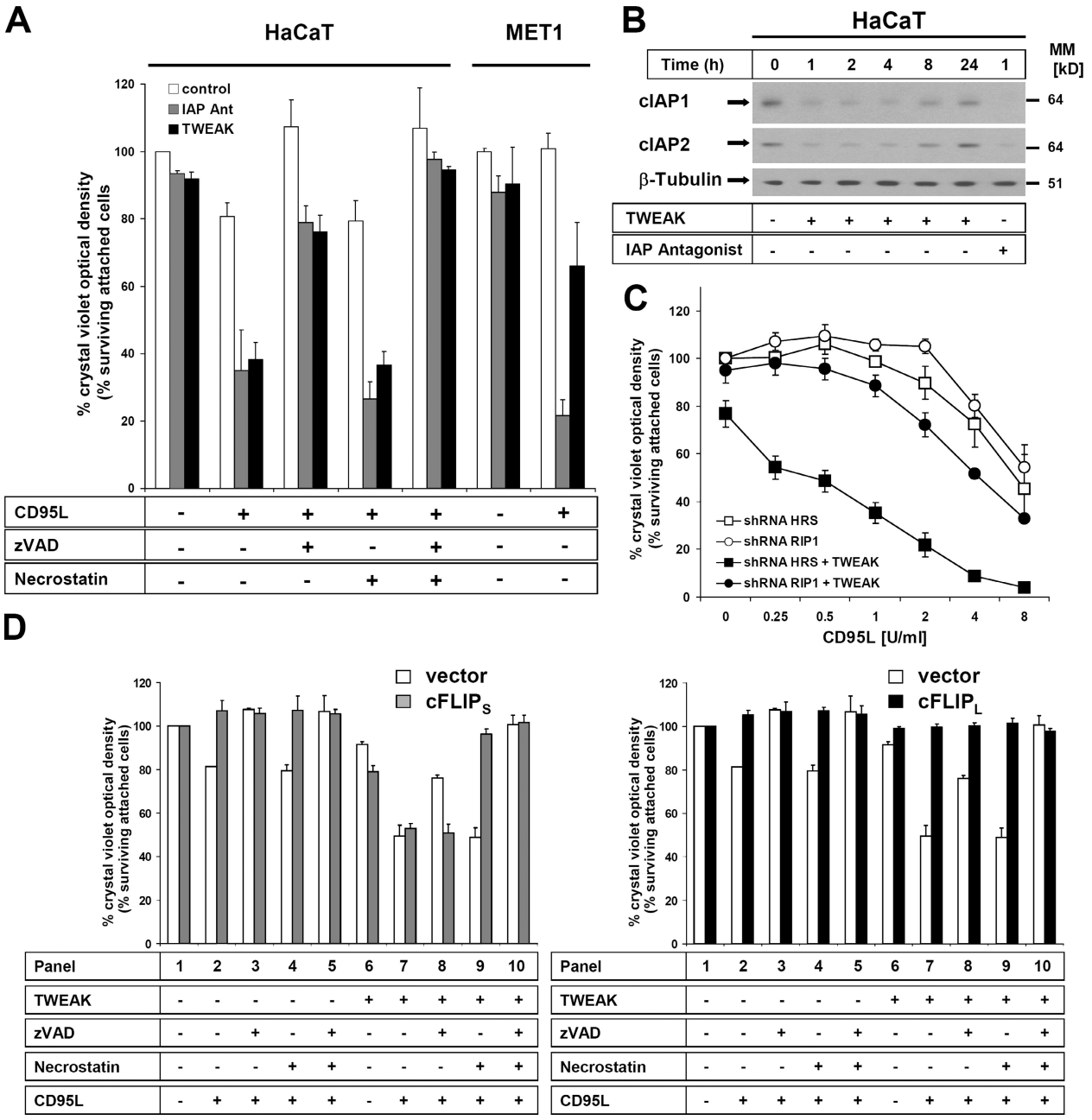

Figure 8. **TWEAK sensitizes to CD95L-induced cell death in a RIP1-dependent manner and is negatively regulated by cFLIP$_L$ but not cFLIP$_S$.** (A) HaCaT and MET1 cells were either prestimulated with 0.5 ng/ml TWEAK for 2 h or 100 nM of the IAP antagonist (Ant) for 30 min and then treated with 2.5 U/ml CD95L. In the same experiments, HaCaT cells were treated with 10 µM zVAD-fmk for 1 h, 50 µM Necrostatin-1 for 1 h, and 0.5 ng/ml TWEAK for 2 h. Subsequently, cells were stimulated with 2.5 U/ml CD95L. Viability was analyzed by crystal violet assay after 18–24 h. (B) TWEAK leads to rapid down-regulation of cIAP1 and -2 expression in HaCaT. Cells were stimulated with 0.5 ng/ml TWEAK for the indicated time. Subsequently, total cellular lysates were analyzed for expression of cIAP1 or -2 by Western blotting. β-Tubulin served as a loading control. MM, molecular mass. (C) Stable knockdown of RIP1 protects HaCaT cells from CD95L-induced cell death in the presence of TWEAK. Transduced HaCaT cells as shown in Fig. 3 C were prestimulated for 2 h with 0.5 ng/ml TWEAK or diluent alone, subsequently stimulated with CD95L for 18–24 h, and assayed by crystal violet assay. The mean ± SEM of three independent experiments is shown. (D) cFLIP$_L$ but not cFLIP$_S$ protects HaCaT cells from CD95L-induced cell death in the presence of TWEAK. cFLIP$_L$, cFLIP$_S$, and vector-transduced control HaCaT as specified in Fig. 6 C were prestimulated with 10 µM zVAD-fmk for 1 h, 50 µM Necrostatin-1 for 1 h, and 0.5 ng/ml TWEAK for 2 h or diluent alone. Cells were then stimulated with 2.5 U/ml CD95L, and viability of cells was analyzed by crystal violet assay after 18–24 h. (A and D) The mean + SEM of three independent experiments is shown.

Discussion

Our study contributes several important findings for the understanding of signaling pathways activated by DRs. We found that IAP antagonists, which sensitize cells to TRAIL-induced cell death as shown previously (Fulda et al., 2002), also dramatically alter sensitivity of cells to CD95L. We studied these aspects in human SCC cells treated with a pharmacological inhibitor that induces degradation of IAPs within minutes and cIAP-deficient MEFs. Our data demonstrate that DL-induced signaling

pathways are profoundly regulated by cIAPs. XIAP appeared to play only a minor role because even overexpression of XIAP in cIAP1- and cIAP2-deficient MEFs failed to increase resistance to CD95L. Furthermore, TWEAK-FN14 signaling, which leaves XIAP levels unaffected (Vince et al., 2008; Wicovsky et al., 2009), duplicates the effects of the IAP antagonist. Although XIAP undoubtedly plays a role in regulating CD95 signaling in type II cells (Jost et al., 2009), CD95 signaling in type II cells requires amplification via the mitochondrial pathway to effectively kill cells, and therefore, XIAP inhibits caspases at a late point in the signaling pathway. In our cells, we show that cIAPs regulate death signaling at the level of the DISC and complex II and suggest that this alters the cell death signal strength or character such that it can no longer be effectively inhibited by XIAP.

CD95L induces apoptosis in many cell types. Our data show that the presence of cIAPs favors the apoptotic pathway and therefore, in the presence of cIAPs, CD95 killing can be blocked by caspase inhibitors such as zVAD-fmk, $cFLIP_S$, and $cFLIP_L$. However, in the absence of cIAPs, a cryptic alternative pathway is revealed. This type of death shows hallmarks of apoptosis, including phosphatidylserine exposure and cleavage of substrates such as PARP by caspases. However, it also shows features of a necrotic type of cell death, including HMGB-1 release and other morphological characteristics. This CD95L/IAP antagonist–induced death cannot be blocked by either zVAD-fmk or Necrostatin-1 alone. Therefore, the fact that the specific RIP1 inhibitor Necrostatin-1 (Degterev et al., 2008) in combination with zVAD-fmk inhibits CD95L/IAP antagonist killing indicates that RIP1 plays a pivotal role in this death. Support for this concept comes from a previous study by Holler et al. (2000) that showed DISC recruitment of RIP1 in the complete absence of FADD. Several other studies have shown that blocking the CD95 or TNF apoptotic pathway, often with chemical caspase inhibitors, induces an alternative cell death pathway variously called programmed necrosis or necroptosis (Vercammen et al., 1998; Matsumura et al., 2000), and in the absence of FADD or caspase-8, a caspase-independent cell death is operative (Bell et al., 2008; Ch'en et al., 2008). In line with our findings, a recent study has identified extracellular pH as a possibility to switch DR-induced apoptotic cell death to RIP1-dependent necrotic cell death in the absence of pharmacological caspase inhibitors such as zVAD-fmk (Meurette et al., 2007). However, despite these insights, the molecular mechanisms that regulate each response under physiological conditions are unresolved (for review see Festjens et al., 2007).

Using a combined genetic and biochemical approach, we demonstrate that cIAPs inhibit a caspase-independent cell death and thereby facilitate an apoptotic death. In the absence of cIAPs, a cryptic alternative death pathway is revealed. Using $RIP1^{-/-}$ MEFs and a specific RIP1K inhibitor, Necrostatin-1, we show that this alternative death pathway is dependent on RIP1K activity. The recruitment of RIP1 to the CD95 membrane-bound complex (CD95 DISC) is decreased by cIAPs, whereas caspase-8 recruitment and processing is unaltered, thus suggesting a molecular mechanism for how cIAPs inhibit this RIP1K-dependent cell death pathway. Not only is the total amount of RIP1 increased in the CD95 DISC and complex II in the absence of cIAPs, the degree of RIP1 modification is also less. Because several studies have shown that RIP1 is a direct target of the E3 ligase activity of cIAPs, this decrease in RIP1 modification is likely to be caused by a reduction in RIP1 ubiquitylation (Park et al., 2004; Bertrand et al., 2008; Varfolomeev et al., 2008). However, our experiments cannot distinguish whether cIAPs limit RIP1 recruitment into the DISC or whether they limit the accumulation of RIP1 within the DISC by K48 ubiquitylating RIP1, leading to its proteasomal degradation. Future studies using ubiquitin-specific antibodies will be able to address this issue (Newton et al., 2008). It is tempting to speculate that the increased amount of RIP1 in the DISC and complex II subsequently leads to autoactivation of the kinase within the complex (Fig. 9), which is consistent with the activation mechanism of other kinases (for review see Eswarakumar et al., 2005). Identifying targets of RIP1K will undoubtedly promote greater insight into this caspase-independent cell death program (Hitomi et al., 2008). While this manuscript was under revision, RIP3, a kinase involved in the apoptosis/necrosis shift in TNF-mediated cell death, was reported to interact with RIP1 (Cho et al., 2009; He et al., 2009) and autophosphorylate in a RIP1K-dependent manner. In turn, activated RIP3 may regulate metabolic enzymes that could promote the necrotic phenotype (Zhang et al., 2009).

The precise physiological relevance of complex II formation after DL stimulation remains unresolved to date (Varfolomeev et al., 2005; Lavrik et al., 2008). We have now studied this complex under conditions in which cIAPs are absent and find that complex II formation is increased whenever cIAPs are downregulated and contain high amounts of RIP1 in association with caspase-8. Remarkably, less RIP1 is detected in complex II in cells insensitive to IAP antagonists, which suggests that it is a shift of the stoichiometric balance of complex II–associated proteins that is relevant for the outcome of apoptotic/necrotic signaling. RIP1 is not only ubiquitylated in the DISC but is also cleaved by caspases. This fact raises a question concerning the impact of caspase activation on RIP1-dependent cell death. Our kinetic analysis of DISC and complex II formation showed a marked increase of full-length RIP1 in the CD95 DISC and complex II in the presence of zVAD-fmk when compared with control cells or in cleaved form in the absence of zVAD-fmk. A large body of evidence about alternative DR-induced cell death pathways for CD95 (Vercammen et al., 1998; Holler et al., 2000) or TNF (Chan et al., 2003) has been generated by studies using chemical caspase inhibitors (e.g., zVAD-fmk). Based on our data, increased RIP1 recruitment to the DISC and/or complex II in the absence of cIAPs may explain the increase in DR-induced cell death in the presence of zVAD-fmk. However, zVAD-fmk is unable to fully block DISC-associated activity of caspase-8 in the DISC (Wachter et al., 2004) and does not block the enzymatic activity of the pro form of caspase-8 (Boatright et al., 2004), which makes it difficult to draw further mechanistic conclusions from such experiments. Thus, we attempted to clarify the role of caspases by investigating the impact of cFLIP isoforms as potent caspase-8 inhibitors that can block DL-mediated cell death. However, surprisingly, we showed that $cFLIP_S$ is insufficient to prevent the alternative RIP1 cell death pathway that proceeds independent of inhibition of caspases by zVAD-fmk. In contrast, $cFLIP_L$, with its equivocal caspase inhibitory

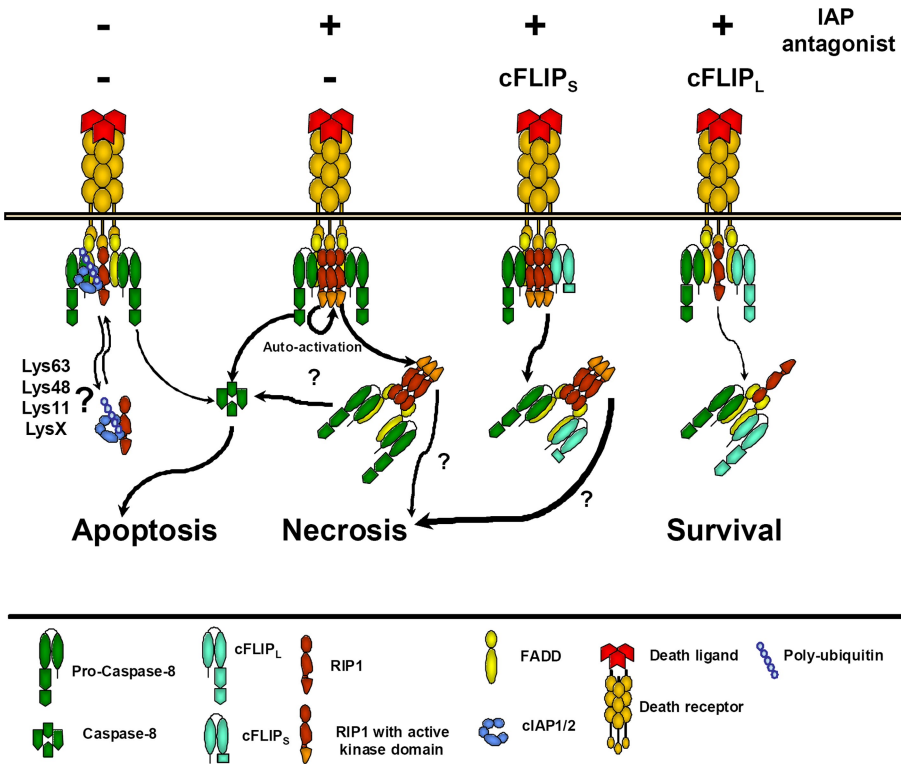

Figure 9. **The role of cIAPs during DR-mediated cell death.** cIAPs block recruitment to or degradation of RIP1 in the DISC. This signaling platform induces cell death in a caspase-dependent as well as -independent manner. A secondary complex II, which is critical for necrotic cell death, also contains the initiator caspase-8 and FADD. In the presence of high levels of RIP1, RIP1 might be autoactivated and induce necrotic cell death or may require additional binding partners such as RIP3 (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). cFLIP$_L$ but not cFLIP$_S$ is able to block complex II formation and the pronecrotic activity of RIP1 in complex II. In contrast, both isoforms block apoptotic cell death initiated by caspase-8 at the DISC. Caspase-8–mediated cleavage of RIP1 is one hypothetical mechanism of down-regulation of RIP1 within the complexes. Alternatively, RIP1 is only recruited to the DISC when ubiquitylated. cIAPs transfer ubiquitin chains of currently debated specificity (e.g., Lys63, -48, or -11) to its substrate RIP1 (Park et al., 2004; Bertrand et al., 2008; Varfolomeev et al., 2008; Blankenship et al., 2009).

capability, effectively blocks RIP1K-dependent death, and this inhibitory potential is associated with its ability to repress the efficient formation (or maintenance) of RIP1 in association with caspase-8 within the DISC and complex II. In particular, the interaction of RIP1 with caspase-8 in complex II is repressed by cFLIP$_L$ but not cFLIP$_S$. These data point to a critical role of the caspase-like domain of cFLIP$_L$ that may allow cleavage of RIP1 within the DISC, thereby limiting its ability to form complex II. Alternatively, cFLIP$_L$ might simply be better than cFLIP$_S$ at inhibiting RIP1 recruitment because of steric hindrance. Finally, it is also possible that cFLIP isoforms act independently of the DR complex, as previously suggested in lymphoid cell (Golks et al., 2006). For example, it is possible that nonubiquitylated forms of RIP1 bind to FADD independently of DISC formation, subsequently leading to DR-independent complex II formation and thereby facilitating necrotic cell death.

Our study using isoforms of cFLIP has the obvious limitation of ectopic overexpression of both isoforms. HaCaT cells express low levels of endogenous cFLIP as compared with primary keratinocytes (Leverkus et al., 2000) and are therefore ideally suited for these experiments (Wachter et al., 2004). However, overexpression experiments can result in nonphysiological responses, and in the case of cFLIP, a lack of any clear consensus about the physiological relevance of different isoforms makes it challenging to interpret such experiments. Even in the case of vFLIP isoforms, which are highly similar to cFLIP isoforms, it is unknown what the intracellular protein levels of vFLIP are during a virus infection or how endogenous cFLIP could affect vFLIP effects. Of note, vFLIP of HHV8 dramatically increases its expression during late stages of HHV8-induced Kaposi sarcomas (Stürzl et al., 1999). Thus, a major shift of the ratio of cFLIP$_L$ to cFLIP$_S$, such as that which occurs in our overexpression experiments, may be of pathophysiological relevance. To test whether the phenotype we described is unique to HaCaT cells, we generated cFLIP-expressing MET1 cells that are p53 wild type and express XIAP. Reassuringly, overexpression of cFLIP$_L$ or cFLIP$_S$ resulted in very similar results in respect to the isoform-specific effects of cFLIP, indicating a more general phenomenon. One intriguing observation is that cFLIP$_L$ p43 recovered in the DISC in HaCaT cells was only found at very low levels in cells expressing cFLIP$_S$. Thus, one added hypothesis explaining the functional difference of both cFLIP isoforms in the absence of cIAPs may be that high levels of cFLIP$_S$ inhibit incorporation of cFLIP$_L$ into the DISC, and this may result in increased RIP1 in the DISC. Further experiments are required to test this hypothesis. With these considerations in mind, we believe our experiments suggest a remarkable and previously unsuspected specificity concerning the mechanism of death inhibition by cFLIP isoforms and open up interesting questions for future studies.

cFLIP$_L$ was reported to mediate binding to proteins such as TRAFs, RIP1, or others (for review see Kataoka, 2005). TRAF2 is a binding partner of cIAPs and RIP1, and in some cells, cFLIP$_L$ overexpression results in increased TRAF2 recruitment to the CD95 DISC (Siegmund et al., 2007). We were consistently unable to specifically detect TRAF2 in the DISC or complex II using either CD95L-Fc– or antibody-mediated precipitation, and because TRAF2 overexpression did not alter the sensitivity of A5RT3 cells to IAP antagonist CD95L death in our cells, TRAF2 is unlikely to be the critical molecule mediating the different effects of cFLIP$_L$ and cFLIP$_S$. In summary, our data point to novel and differential functions of cFLIP isoforms in the absence of cIAPs. Intriguingly, we detect an increased spontaneous complex II in IAP antagonist–treated cells as well

as increased DL-induced formation of complex II in $cFLIP_S$-expressing cells. Because $cFLIP_L$ can block these events, it suggests that the caspase-like domain of $cFLIP_L$ is involved in negatively regulating complex II formation, but whether and which $cFLIP_L$ interacting proteins are required for this effect are unclear.

Our discoveries concerning the role of RIP1 in an alternative RIP1-dependent death emanating from the CD95 receptor were made initially with IAP antagonist drugs that rapidly deplete cIAP levels and antagonize XIAP. Although IAP antagonists have attracted great interest as cancer therapeutics, it could be questioned whether these findings have any physiological relevance. TWEAK is able to promote the rapid degradation of cIAPs in an analogous manner to IAP antagonists (Vince et al., 2008; Wicovsky et al., 2009). TWEAK and other ligands such as TRAIL, CD95L, or TNF are likely to be present in the same physiological scenario (Vince and Silke, 2006). Thus, stimulation of FN14, as shown in this manuscript, or possibly other receptors such as CD30 or -40 able to recruit cIAPs based on their protein structure (for example DR6, TRAIL-R2 or -R4, CD27, or EDAR) could deviate DR-mediated apoptotic to necrotic cell death with major physiological and pathophysiological consequences during tumorigenesis or the inflammatory response in multicellular organisms (Leverkus et al., 2008; Kerstan et al., 2009).

Materials and methods

Materials

The following antibodies were used for Western blot analysis: antibodies to caspase-8 (C-15 [provided by P.H. Krammer, German Cancer Research Center, Heidelberg, Germany] and C-20 [Santa Cruz Biotechnology, Inc.]); cFLIP (NF-6; Enzo Life Sciences, Inc.); XIAP, FADD, and RIP1 (BD); caspase-3 (CPP32; provided by H. Mehmet, Merck Frosst, Kirkland, Quebec, Canada); PARP-1 (clone C-2-10; Enzo Life Sciences, Inc.); rat antibodies to cIAP1 (Silke et al., 2005) and cIAP2 (Vince et al., 2009); and β-tubulin (clone 2.1; Sigma-Aldrich). Polyclonal antibodies to HMGB-1 were purchased from Abcam, and TRAF2 (C-20) and CD95 antibodies (C-20) were purchased from Santa Cruz Biotechnology, Inc. β-Actin antibodies were purchased from Sigma-Aldrich, and HRP-coupled monoclonal antibody to GFP was purchased from Clontech. His-Flag-TRAIL (HF-TRAIL) was produced as previously described (expression construct provided by H. Walczak, Imperial College London, London, England, UK; Diessenbacher et al., 2008). For expression of Fc-CD95L or Fc-TWEAK, we used constructs previously published (Bossen et al., 2006), which were provided by P. Schneider (University of Lausanne, Epalinges, Switzerland). 1 U of Fc-CD95L was determined as a 1:500 dilution of the stock Fc-CD95L supernatant, and 1 U/ml of Fc-CD95L supernatant was sufficient to kill 50% (LD50) of A375 melanoma cells, as previously described (Geserick et al., 2008). Ligand-mediated cell death was completely blocked by the addition of either soluble TRAIL-R2-Fc protein or CD95-Fc protein. HRP-conjugated goat anti–rabbit, goat anti–rat IgG, and goat anti–mouse IgG antibodies and HRP-conjugated goat anti–mouse IgG1, IgG2a, IgG2b, and IgG1-κ were obtained from SouthernBiotech. TRAIL-R1 (HS 101) and TRAIL-R2 (HS 201) monoclonal antibodies for FACScan analysis of surface receptor expression were used as previously described (Leverkus et al., 2003b) and are available from Enzo Life Sciences, Inc. CD95 antibodies (Apo-1 IgG1 and IgG3a) were provided by P.H. Krammer. Cy5-conjugated annexin V was purchased from BD. The IAP antagonist (compound A) was provided by TetraLogic Corp.

Cell culture

The generation of MEFs has previously been described (Vince et al., 2007, 2008). RIP knockout mice were provided by M. Kelliher (University of Massachusetts Medical School, Worcester, MA). The spontaneously transformed keratinocyte line HaCaT and the derived metastatic clone A5RT3 (Mueller et al., 2001) were provided by P. Boukamp (German Cancer Research Center). MET1 cells (Popp et al., 2000) were provided by I. Leigh (Cancer Research UK, London, England, UK). Cell lines were cultured exactly as previously described (Boukamp et al., 1988; Proby et al., 2000; Mueller et al., 2001).

Inducible lentiviral reconstitution of murine or human IAPs

The generation of MEFs and lentiviral particles have previously been described (Vince et al., 2007, 2008). In brief, MEFs were generated from embryos in accordance with standard procedures and were infected with SV40 large T antigen–expressing lentivirus. In vivo DKO cIAP1 and -2 MEFs were obtained from LoxP/LoxP cIAP1 and FRT/FRT cIAP2 mouse crossed first with Cre transgenic mouse followed by Flp transgenic mouse. DKO MEFs were generated at embryonic day (E) 10 instead of E15. To generate DKO MEFs expressing mouse cIAP1 or Flag-cIAP2 or mouse XIAP, 293T cells were transfected with packaging construct pCMV δR8.2, VSVg, and the relevant lentiviral plasmid. DKO MEFs were infected with packaged lentivirus, and polyclonal MEFs were obtained after puromycin (2–5 μg/ml: pF 5xUAS selection) and hygromycin selection (100–500 μg/ml: GEV16 selection). HaCaT cells were transduced to express GFP or Flag-tagged cIAP1 or -2 in an inducible manner (Diessenbacher et al., 2008). Cells were subsequently tested for expression of the respective proteins after 24 h of induction with 10 or 100 nM 4-hydroxy-tamoxifen (4-HT).

Retroviral infection

For infection of HaCaT cells, the pCFG5-IEGZ retroviral vector containing cDNA inserts of $cFLIP_L$, $cFLIP_S$, or TRAF2 (provided by H. Wajant, University of Würzburg, Würzburg, Germany) was used as previously described (Leverkus et al., 2003a; Geserick et al., 2008). In brief, the amphotropic producer cell line ΦNX was transfected with 10 μg of the retroviral vectors by Ca phosphate precipitation. Cell culture supernatants containing viral particles were generated by incubation of producer cells with HaCaT medium (DME containing 10% FCS) overnight. After filtration (45-μm filter; Schleicher & Schuell), culture supernatant was added to HaCaT cells seeded in 6-well plates 24 h earlier in the presence of 1 μg/ml polybrene. After centrifugation for 3 h at 30°C, viral particle–containing supernatants were replaced by fresh medium. After 10–14-d Zeocin selection of bulk-infected cultures, FACS analysis for GFP expression (always >90%) and Western blot analysis were performed on polyclonal cells to confirm ectopic expression of the respective molecules. The empty retroviral vector served as control. Aliquots of cells were used for the experiments between passages 2 and 6 after initial characterization for all subsequent experiments.

Stable siRNA expression

We used stable expression of siRNA against cFLIP or cIAP1 or -2 as recently published (Diessenbacher et al., 2008; Schmidt et al., 2009). RIP1 siRNA as well as a hyper random sequence not matched by any gene in the National Center for Biotechnology Information database (HRS; Vogler et al., 2007) were used. The HRS construct was provided by S. Fulda (Ulm University, Ulm, Germany). For generation of the constructs, cDNA 64-mer oligomers containing RIP1-targeting sequence (nt start position +193) were cloned into the pSuper.retro retroviral vector (pRS) using HindIII and BglII restriction sites. The resulting vectors or control vector containing a sequence not found in the human genome were transfected into the amphotropic producer cell line exactly as outlined in the previous section. The retrovirus-containing supernatant was then used to infect A5RT3 and MET1 cells with HRS or cFLIP shRNA, respectively. HaCaT cells were infected either with HRS or RIP1 or cIAP1 or -2 shRNA, and infected cells were selected with 1 μg/ml puromycin (Sigma-Aldrich) for 3 d to obtain puromycin-resistant bulk-infected cultures for further analysis. The respective control constructs served as internal control. FACS analysis of GFP expression (always >90%) and Western blot analysis were performed on polyclonal cells to confirm ectopic expression of the respective molecules. Aliquots of cells were used for cytotoxicity assays and biochemical characterization between passage 2 and 6 after the antibiotic selection.

FACScan analysis

For surface staining of TRAIL receptors (TRAIL-R1 and -R2) and CD95, cells were trypsinized, and 4×10^5 cells were incubated with monoclonal antibodies against TRAIL-R1 or -R2, CD95, or isotype-matched control IgG for 60 min followed by incubation with biotinylated goat anti–mouse secondary antibodies (BD) and Cy5-phycoerythrin–labeled streptavidin (Invitrogen) as described previously (Wachter et al., 2004). For all experiments, 2×10^4 cells were analyzed by FACScan (BD).

Western blot analysis

After stimulation as indicated, cells were washed twice with ice-cold PBS and lysed for 30 min on ice by the addition of lysis buffer (30 mM Tris-HCl,

pH 7.5, at 21°C, 120 mM NaCl, 10% glycerol, 1% Triton X-100, and Complete protease inhibitor cocktail [Roche]). Cellular debris was removed by centrifugation at 20,000 *g* for 10 min. 5 µg of total cellular proteins was supplemented with fourfold concentrated Laemmli buffer and boiled at 95°C. Proteins were separated by SDS-PAGE on 4–12% gradient gels (Invitrogen) and followed by transfer to nitrocellulose or polyvinylidene fluoride membranes (GE Healthcare). Membranes were blocked with 5% nonfat dry milk and 3% BSA in PBS/Tween (1× PBS containing 0.05% Tween 20) for 1 h, washed with PBS/Tween, and incubated in PBS/Tween containing 3% nonfat dry milk and primary antibodies as indicated overnight. After washing in PBS/Tween, blots were incubated with HRP-conjugated isotype-specific secondary antibody in PBS/Tween. After washing of the blots with PBS/Tween, bands were visualized with ECL detection kits (GE Healthcare).

Cytotoxicity assay

Crystal violet staining of attached, living cells was performed 18–24 h after stimulation with the indicated concentrations of DL in 96-well plates. Plates were washed two times with PBS. Subsequently, 50 µl of staining solution (0.5% crystal violet and 20% methanol) were added per well. After incubation for 20 min at room temperature, plates were washed four times with water. Plates were air dried, and 200 µl methanol was added per well and incubated for 30 min. The OD of the wells was subsequently measured by a plate reader (Victor3; PerkinElmer). The OD of control cultures was normalized to 100% and compared with stimulated cells. For statistical analysis, the SEM was determined for three to seven independent experiments of each cell line and stimulatory condition.

Hypodiploidy analysis

Subdiploid DNA content was analyzed as previously described (Wachter et al., 2004). In brief, cells were stimulated with the indicated reagents for 8 h. Cells were then detached, washed with cold PBS, and resuspended in buffer N (0.1% [wt/vol] Na citrate, 0.1% [vol/vol] Triton X-100, and 50 µg/ml PI). Cells were kept in the dark at 4°C for 36–48 h, and then diploidy was measured by FACScan analysis.

Immunofluorescence microscopy

For detection of nuclear morphology and integrity of the cell membrane, 5×10^4 cells of the respective cells were seeded per well in a 12-well plate. After 24 h of incubation for adherence, cells were stimulated as indicated in the figure legend for 4 or 24 h. Subsequently, cells were incubated with 5 µg/ml Hoechst 33342 (Polysciences Europe GmbH) and 5 pM SYTOX green (Invitrogen) for 15 min at 37°C, immediately followed by phase-contrast or fluorescence microscopy in DME + 10% FCS at room temperature. Images were taken with an epifluorescence microscope (Axiovert 40 CFL; Carl Zeiss, Inc.) equipped with a camera (18.0 monochrome without IR; Nikon) using a 20× NA 0.30 Ph 1 objective (Carl Zeiss, Inc.). All digital images were identically processed using the advanced SPOTSOFTWARE version 4.6 (Diagnostic Instruments, Inc.).

Annexin V externalization

For the detection of phosphatidylserine externalization, cells were stimulated as indicated in the figure legends. 4 or 24 h after incubation of cells, trypsinized cells were resuspended in 1× annexin V–binding buffer (10 mM Hepes, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$), and $2–4 \times 10^5$ cells were subsequently stained with Cy5-conjugated annexin V exactly according to the manufacturer (BD), followed by counterstaining (10 µg/ml PI) for 15 min in the dark at room temperature. For all experiments, 2×10^4 cells were analyzed by FACScan.

Colony formation assays

For colony formation assay, 10^4 cells of parental as well as of retrovirally transduced HaCaT cells (HRS, shRNA RIP1, $cFLIP_L$ or $cFLIP_S$, and the respective control vectors) were seeded per well in a 24-well plate. After 24 h of incubation, adhering cells were either separately prestimulated with 100 nM of the IAP antagonist for 30 min, 10 µM zVAD-fmk for 1 h, or 50 µM Necrostatin-1 for 1 h or in combination of all compounds followed by co-stimulation with CD95L for 24 h. At that time, medium was removed, cells were washed two times with sterile PBS, and complete medium was added. Cells were cultured for 3, 5, or 7 d, and subsequently, colonies of viable cells were stained by crystal violet as indicated in Cytotoxicity assay.

Ligand affinity precipitation of receptor complexes

For precipitation of the CD95L DISC, 5×10^6 cells were used for each condition. Cells were washed once with medium at 37°C and subsequently preincubated for 1 h with 10 µM zVAD-fmk and, as indicated, with 100 nM of the IAP antagonist at 37°C. Subsequently, cells were treated with 250 U/ml CD95L-Fc for 2 h. Receptor complex formation was stopped by washing the monolayer four times with ice-cold PBS. Cells were lysed on ice by the addition of 2 ml of lysis buffer (30 mM Tris-HCl, pH 7.5, at 21°C, 120 mM NaCl, 10% glycerol, 1% Triton X-100, and Complete protease inhibitor cocktail). After 30-min lysis on ice, the lysates were centrifuged two times at 20,000 *g* for 5 min and 30 min, respectively, to remove cellular debris. A minor fraction of these clear lysates was used to control for the input of the respective proteins. For the precipitation of the CD95 receptor and stimulation-dependent recruited proteins, Apo-1 IgG3 antibodies (provided by P.H. Krammer) were added to the lysates prepared from nonstimulated as well as stimulated cells to precipitate the CD95-interacting proteins. The levels of receptor precipitated by either ligand affinity precipitation or caspase-8 immunoprecipitation was compared in all experiments by Western blotting for CD95, although direct comparison was obscured by the induction of SDS-stable high molecular mass complexes upon stimulation of CD95 (compare Fig. 5 with Fig. 7; Feig et al., 2007). Receptor complexes were precipitated from the lysates using 40 µl protein G beads (Roche) for 16–24 h on an end over end shaker (SB2 rotator; Stuart) at 4°C. Ligand affinity precipitates were washed four times with lysis buffer before the protein complexes were eluted from dried beads by the addition of standard reducing sample buffer and boiling at 95°C. Subsequently, proteins were separated by SDS-PAGE on 4–12% NuPAGE gradient gels (Invitrogen) before the detection of DISC components by Western blot analysis.

Caspase-8 immunoprecipitation of complex II

After precipitation of the CD95 DISC, remaining lysates were centrifuged two times at 20,000 *g* for 5 min. Subsequently, 1 µg caspase-8 antibody (C-20; Santa Cruz Biotechnology, Inc.) was added to all lysates. The caspase-8–containing complexes were precipitated from the lysates by coincubation with 40 µl of protein G beads for 16–24 h on an end over end shaker at 4°C. Ligand affinity precipitates were washed four times with lysis buffer before the protein complexes were eluted from dried beads by the addition of standard reducing sample buffer and boiling at 95°C. Subsequently, proteins were separated by SDS-PAGE on 4–12% NuPAGE gradient gels before the detection of caspase-8–interacting proteins by Western blot analysis. To exclude remaining receptor-bound DISC complexes, all caspase-8–interacting complexes were analyzed for the presence of CD95 (compare Fig. 5 with Fig. 7).

Online supplemental material

Fig. S1 shows that the IAP antagonist leads to down-regulation of cIAP1 and -2 in HaCaT and SCC cell lines without changes in DR expression. Fig. S2 shows that the resistance of A5RT3 cells to CD95L/IAP antagonist treatment is independent of endogenous TRAF2 or cIAP2 expression. Fig. S3 shows that cFLIP regulates sensitivity to the combination of DL and IAP antagonist in A5RT3 cells. Fig. S4 shows that the IAP antagonist sensitizes HaCaT cells to apoptotic and nonapoptotic cell death. Fig. S5 shows that $cFLIP_L$ but not $cFLIP_S$ blocks IAP antagonist DL-induced cell death in MET1 cells. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200904158/DC1.

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