

ORIGINAL ARTICLE

DISC-mediated activation of caspase-2 in DNA damage-induced apoptosis

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The tumor suppressor p53 protein supports growth arrest and is able to induce apoptosis, a signaling cascade regulated by sequential activation of caspases. Mechanisms that lead from p53 to activation of individual initiator caspases are still unclear. The present model for caspase-2 activation includes PIDDosome complex formation. However, in certain experimental models, elimination of complex constituents PIDD or RAIDD did not significantly influence caspase-2 activation, suggesting the existence of an alternative activation platform for caspase-2. Here we have investigated the link between p53 and caspase-2 in further detail and report that the latter is able to utilize the CD95 DISC as an activation platform. The recruitment of caspase-8 to this complex is required for activation of caspase-2. In the experimental system used, the DISC is formed through a distinct, p53-dependent upregulation of CD95. Moreover, we show that caspase-2 and -8 cleave Bid, and that both act simultaneously upstream of mitochondrial cytochrome *c* release. Finally, a direct interaction between the two caspases and the ability of caspase-8 to cleave caspase-2 are demonstrated. Thus, the observed functional link between caspase-8 and -2 within the DISC represents an alternative mechanism to the PIDDosome for caspase-2 activation in response to DNA damage.

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Introduction

Apoptosis is an evolutionarily conserved process that can lead to cell disassembly by activation of the caspase family of cysteine proteases. On the basis of their primary structure and function in the apoptotic signaling cascade, caspases are defined as regulatory, apical

caspases (caspase-2, -8, -9, -10) or effector caspases (caspase-3, -6, -7). Significant for apical caspases is the presence in their N-terminal pro-domain of protein–protein interaction motifs, either a death domain (DD) or a caspase recruitment domain (CARD) (Park *et al.*, 2007). There are two main routes for caspase activation: the extrinsic, death receptor pathway and the intrinsic, mitochondrial pathway. In the former, extracellular ligands interact with members of the tumor necrosis factor (TNF) receptor family to recruit the adapter molecule, Fas (CD95)-associated protein with death domain (FADD). The resulting death-inducing signaling complex (DISC) is required for caspase-8 activation. Genome damage and cytotoxic stress lead to the activation of the second pathway, which is characterized by mitochondrial outer membrane (OMM) permeabilization and release of cytochrome *c* into the cytosol. In the presence of dATP, cytochrome *c* interacts with the adapter molecule APAF-1 to promote the formation of the apoptosome complex, which is essential for caspase-9 processing and activation. Both routes converge into downstream activation of effector caspases that execute apoptotic cell death by their proteolytic activity. Caspases in their active form are dimers of identical catalytic units, and all three-dimensional structures identified support the view that each catalytic unit is composed of one large (~20 kDa) and one small (~10 kDa) subunit, which are derived from the same precursor molecule by internal cleavage (Lavrik *et al.*, 2005).

Activation of apical caspases is compulsory for the initiation of the apoptotic process, and a model for caspase-8 and -9 activation has been proposed and verified in various experimental systems (Boatright *et al.*, 2003; Pop *et al.*, 2006, 2007). These reports indicate that activation of the apical caspases occurs on dimerization of monomeric zymogens, and that internal proteolysis is a secondary event resulting in partial stabilization of the activated dimers. The function of adapter-mediated DISC and apoptosome formation is to facilitate progression of the process.

Accumulating data indicate that caspase-2 can function as an initiator regulatory enzyme upstream of the mitochondria during apoptosis induced by DNA damage (Guo *et al.*, 2002; Robertson *et al.*, 2002; Vakifahmetoglu *et al.*, 2006). The mechanisms of caspase-2 activation were found to be similar to those described for other apical caspases, although both dimerization and cleavage of the overexpressed zymogen

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seem to be required for its ability to promote apoptotic cell death *in vivo* (Baliga *et al.*, 2004). A polymeric activation platform, known as the PIDDosome, was found to be essential for caspase-2 activation in response to genotoxic stress (Tinel and Tschopp, 2004). It was shown that the PIDDosome forms when the adapter protein RAIDD (receptor-interacting protein (RIP)-associated ICH1/CED-3-homologous protein with a death domain) interacts with the CARD domain in caspase-2 and the DD domain in PIDD (p53-induced protein with a death domain) by homotypic domain interaction. PIDD can operate as a bifurcated molecular switch with the ability to induce either apoptosis by means of caspase-2 activation, or cell survival by promoting the NF- κ B pathway (Janssens *et al.*, 2005; Tinel *et al.*, 2007). PIDD-mediated caspase-2 activation is strongly associated with the tumor suppressor p53 (Baptiste-Okoh *et al.*, 2008). However, the PIDDosome model suffers from the fact that RAIDD-deficient mice show no apparent phenotype, and RAIDD^{-/-} thymocytes remain sensitive to various cytotoxic agents. This was contrasted by inhibition of caspase-2 activation and cell death in RAIDD^{-/-} cells but not in the parental cell line when PIDD overexpression was used to induce apoptosis (Berube *et al.*, 2005).

Clearly, the function and sites of caspase-2 activation differ between experimental systems, and examples of its processing downstream of caspase-3 have also been reported (Paroni *et al.*, 2001; O'Reilly *et al.*, 2002). This is in accordance with data showing that procaspase-2 is efficiently cleaved by recombinant caspase-3 *in vitro* (Van de Craen *et al.*, 1999). Because very few cellular proteins have been identified as substrates for caspase-2 (Zhivotovsky and Orrenius, 2005), these data suggest an amplifying, rather than an effector function for this protease in the apoptotic cascades discussed above.

Our previous findings demonstrate a functional link between p53 and caspase-2 activation and subsequent downstream apoptotic signaling in HCT116 colon carcinoma cells (Vakifahmetoglu *et al.*, 2006). However, neither PIDD nor RAIDD was needed for caspase-2 activation in this particular cell death pathway, as determined by siRNA methodology. Here, we present evidence that caspase-2 is able to use the DISC as a platform for its activation. The recruitment of caspase-8 to this complex is crucial for caspase-2 activation. As a consequence, two apical caspases operate simultaneously to regulate apoptotic signaling. We propose that this represents an alternative mechanism of apical caspase-2 activation in response to DNA lesions.

Results

Kinetics of apoptosis in HCT116 colon carcinoma cells

The HCT116 colon carcinoma cell line is a widely used tumor cell line that is sensitive to a number of apoptosis-inducing agents (Yamaguchi *et al.*, 2003; Galligan *et al.*, 2005). Here, we used the chemotherapeutic drug 5-fluorouracil (5-FU) to trigger apoptosis, and to

examine potential mechanisms for caspase-2 activation in this cell line. In response to 5-FU, processing of initiator caspases, including caspase-2, -8 and -9, as well as effector caspase-3 and -7, could be observed by western blotting approximately 13 h after treatment (Figure 1a). Moreover, apoptotic markers, such as mitochondrial cytochrome *c* release and poly-(ADP-ribose)polymerase (PARP) cleavage became evident at about the same time (Figure 1a and b). The lag period between stabilization of the tumor suppressor p53 at 3 h and subsequent activation of caspases were associated with a halt in the cell cycle and transactivation of p21waf1 (Figure 1a; data not shown). In contrast, two other DNA-damaging agents, cisplatin and etoposide, are unable to induce complete processing of apical caspase-2 and caspase-8 24 h after treatment (Figure 2b). Together, these data demonstrate that progression of apoptosis, from the activation of apical caspases and mitochondrial cytochrome *c* release, to the activation of effector caspases and cleavage of cellular proteins, occurs in a rapid manner. In the following experiments we used various approaches to dissect the order of this sequence of events.

The succession of activation of apical caspases

Our previous findings placed caspase-2 activation upstream of the mitochondria as a regulatory enzyme that was able to trigger cytochrome *c* release (Vakifahmetoglu *et al.*, 2006). Apart from caspase-2, active forms of caspase-8 and -9 were also identified in the experimental system used (Figure 1a). Because caspase-9 has been reported to induce processing of both caspase-2 and -8 in some experimental systems (Samraj *et al.*, 2007), we investigated this possibility in apoptotic HCT116 cells by means of siRNA methodology (Figure 2a). Despite efficient silencing of caspase-9, processing of both caspase-2 and -8 appeared unchanged in response to 5-FU treatment (Figure 2a and b). This was contrasted by an almost complete inhibition of caspase-2 processing when caspase-8 was targeted by siRNA under similar conditions (Figure 2c). This effect was unidirectional because downregulation of caspase-2 failed to provoke any inhibition of caspase-8 cleavage (Figure 2d). Because downregulation of caspase-3 also failed to block caspase-8 processing, a potential involvement for this effector caspase in feedback amplification was ruled out (Figure 2d). These results suggest that caspase-8, but not caspase-9, is able to act upstream of and regulate the processing of caspase-2 in this experimental system.

Next, we investigated how individual apical caspases might relate to the sequence of events given by cleavage of the proapoptotic BH3-only Bcl-2 family member Bid, mitochondrial cytochrome *c* release and activation of effector caspase-3 and -7. Bid has a critical function upstream of mitochondrial permeabilization in selected apoptotic routes, and Bid activation by either caspase-8- or -2-mediated cleavage leading to formation of truncated Bid (tBid) has been reported (Li *et al.*, 1998; Wagner *et al.*, 2004). Here, siRNA-mediated silencing of caspase-2 or -8, but not of caspase-9 or -3, resulted in

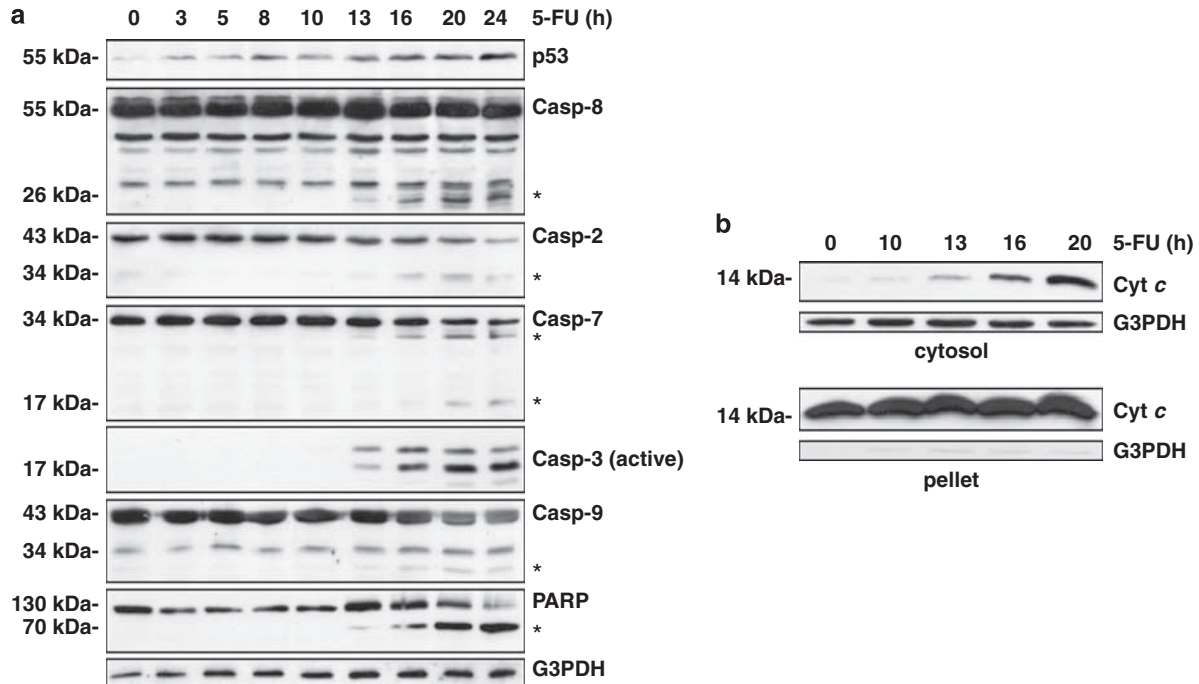


Figure 1 Analysis of apoptotic markers in 5-fluorouracil (5-FU)-treated HCT116 cells. Untreated or treated with 5-FU HCT116 cells were harvested at the time points indicated, separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by western blotting. **(a)** Immunoblots showing a time-dependent processing of caspase-2, -3, -7, -8 and -9; cleavage of poly-(ADP-ribose)polymerase (PARP) as well as accumulation of the tumor suppressor p53 in response to 5-FU. Cleaved fragments are indicated with asterisks. **(b)** Immunoblots show cytochrome *c* distribution between the mitochondrial and the cytosolic fractions in 5-FU-treated cells at different time points. Blots were re-probed for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) to confirm equal loading of the samples.

diminished tBid accumulation in cells treated with 5-FU (Figure 3a). Similarly, release of cytochrome *c* was not affected when caspase-9 was targeted with specific siRNA (Figure 3b). In contrast, downstream activities of effector caspase-3 and -7 were reduced by down-regulation of all apical caspases, including caspase-9 (Figure 3c). Taken together, these data support the notion that both caspase-2 and -8, but not caspase-9, contribute to the generation of tBid with subsequent cytochrome *c* release, events that are followed by activation of effector caspases.

p53-dependent transactivation of CD95 in 5-FU-treated HCT116 cells

Apical caspase-8 activation relies on formation of a DISC through stimulation of one of several death receptors (TNF, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) or CD95). Previous reports indicate that 5-FU also exerts its cytotoxic effect in colorectal carcinoma cells through a CD95-dependent apoptotic pathway that involves Bax translocation to mitochondria and permeabilization of the OMM (Borrhalho *et al.*, 2007). Therefore, an investigation of the CD95 expression at the mRNA level by semiquantitative RT-PCR and protein localization by immunohistochemistry were performed. Prominent transactivation of CD95 mRNA expression was detected at the initiation of the apoptotic cascade (Figure 4a and b). Because the CD95 mRNA in 5-FU-treated HCT116

p53^{-/-} cells remains at basal level, we concluded that elevation of the mRNA is strictly dependent on p53 activity (Figure 4a and b). Increased content of CD95 protein in plasma membranes of 5-FU-treated cells supports and extends our reverse transcriptase (RT)-PCR-generated data (Figure 4c).

Because activation of caspase-2 and -8 also is dependent on p53 function (Vakifahmetoglu *et al.*, 2006; Figure 4d) this indicates that a p53-CD95-mediated pathway regulates activity of both caspases. In a comparative analysis of the response of HCT116 cells to two other DNA damaging agents, cisplatin and etoposide, it was shown that etoposide, similar to 5-FU, induced p53 activation and CD95 upregulation (Figure 4e). However, these events did not lead to caspase-8 processing. In contrast to etoposide and 5-FU, cisplatin-induced p53 activity did not lead to transactivation of CD95. Partial processing of caspase-8 was observed in response to cisplatin although this might be mediated through DISC-independent mechanism(s) (Figure 4e).

In conclusion, our data indicate that p53-mediated CD95 transactivation is required but not sufficient for DISC-mediated caspase-8 activation.

Cumulative caspase-8 and -2 recruitment to the CD95 DISC in response to 5-FU treatment of HCT116 cells

To verify and further support our finding that 5-FU-induced apoptosis in HCT116 cells is mediated through

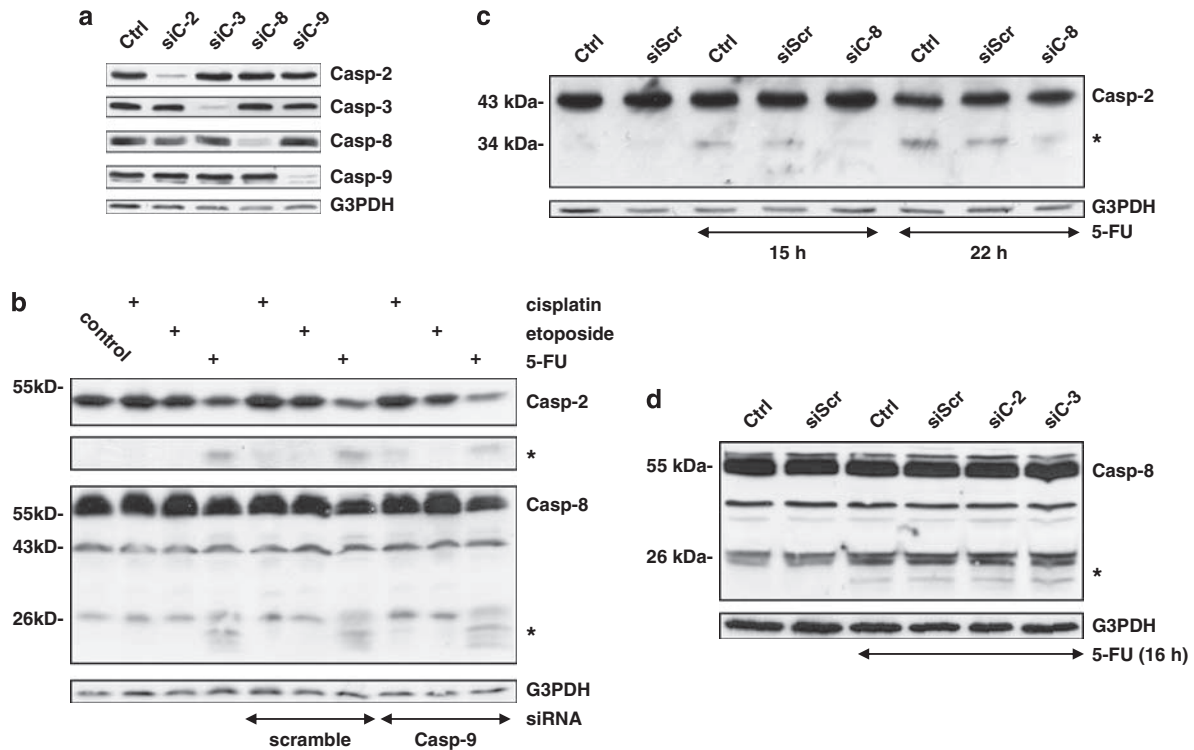


Figure 2 siRNA silencing of procaspase-8 inhibits 5-FU-induced caspase-2 processing. HCT116 cells were cultured and transfected with caspase-2, -3, -8 or -9 siRNA for 48 h before 5-FU treatment. Lysates were harvested from post-treated cells at time points indicated and analysed by western blotting. (a) Representative western blot image of efficiency of caspase-2, -3, -8 and -9 siRNAs. (b) Processing of caspase-8 and -2 in cisplatin-, etoposide- and 5-FU-treated HCT116 cells (24 h) transfected with caspase-9 siRNA. (c) Processing of caspase-2 in 5-FU-treated HCT116 cells transfected with caspase-2 or caspase-3 siRNA. (d) Processing of caspase-8 in 5-FU-treated HCT116 cells transfected with caspase-2 or caspase-3 siRNA. Cleaved fragments are indicated with asterisks. In all experiments, scrambled siRNA was used as a negative control and G3PDH was used as control for equal loading of the samples.

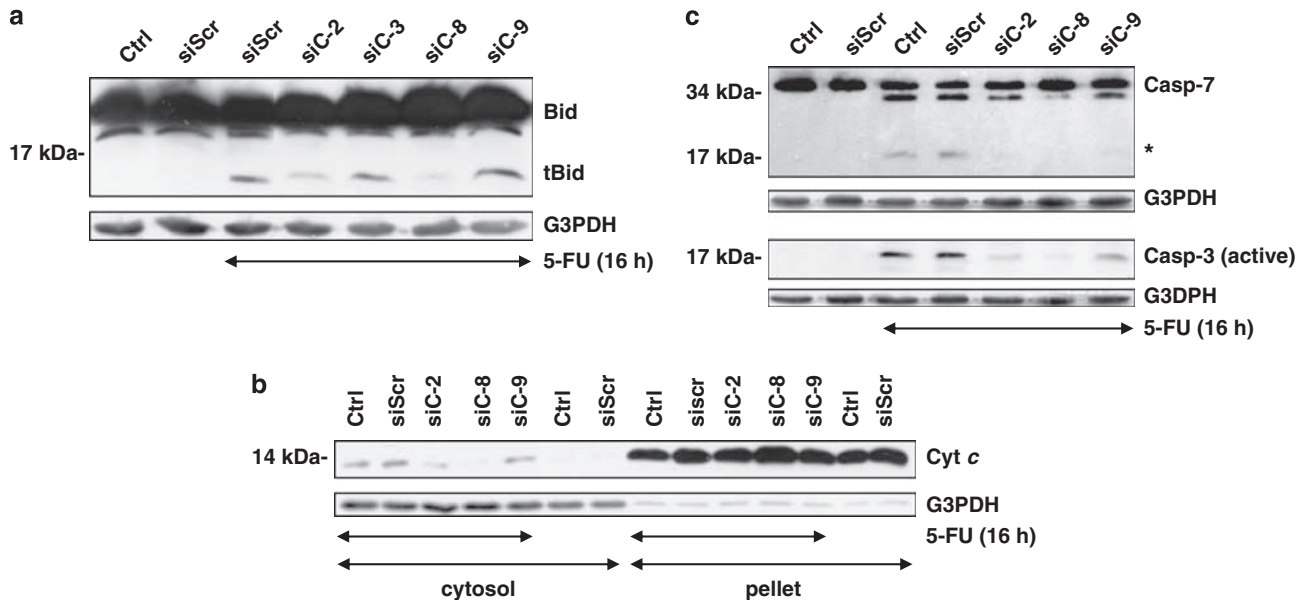


Figure 3 siRNA silencing of apical procaspase-2 and -8 inhibits Bid cleavage, cytochrome *c* release and processing of effector caspase-3 and -7. Caspase-2, -3, -8 or -9 siRNA transfected HCT116 cells were incubated for 48 h and subsequently treated with 5-FU. Protein lysates from cells, treated as indicated in figures, were analysed by western blotting. To analyse cytochrome *c* release cells were incubated with digitonin and cytosolic and mitochondrial fractions were separated by centrifugation. (a) Effect of siRNA's targeting caspase-2, -3, -8 or -9 on accumulation of proapoptotic truncated Bid (tBid) in 5-FU-treated cells. (b) Analysis of mitochondrial cytochrome *c* release as a result of 5-FU treatment combined with siRNA silencing of caspase-2, -3, -8 or -9. Both mitochondrial and cytosolic fractions are shown. (c) Effect of siRNA's targeting caspase-2, -8 or -9 on caspase-3 and -7 processing in cells treated with 5-FU. Cleaved fragments are indicated with asterisks. In all experiments scrambled siRNA was used as a negative control and G3PDH was used as loading control.

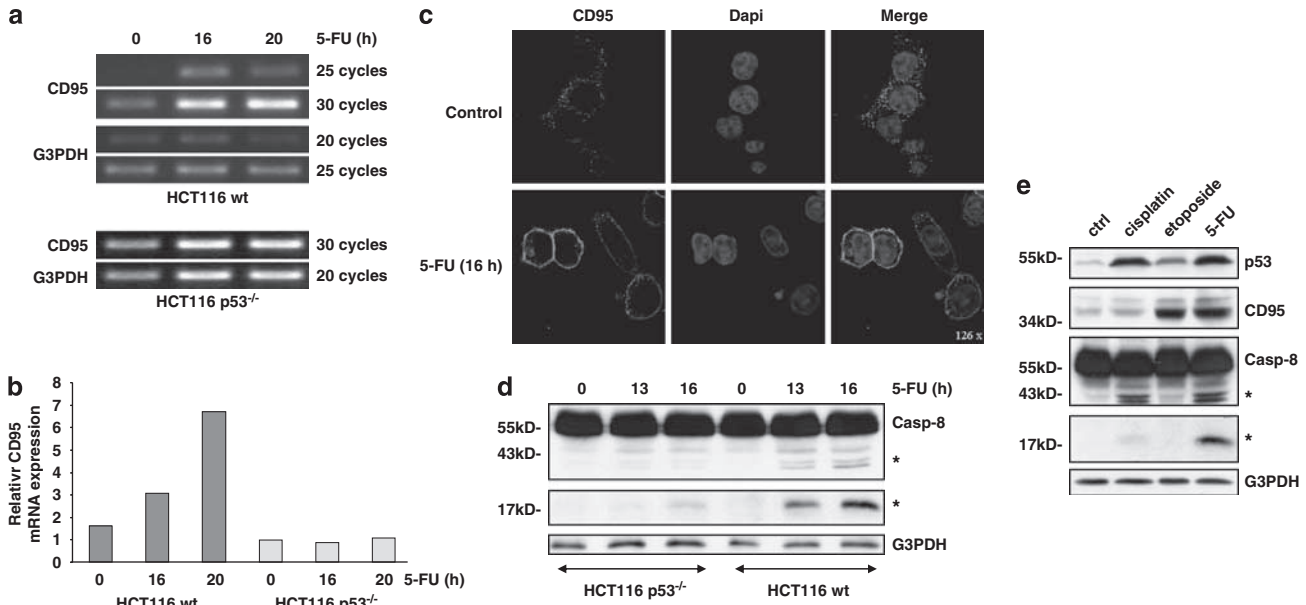


Figure 4 Upregulation of the CD95 expression at protein and mRNA levels as a result of 5-FU treatment in wt HCT116 but not in HCT116 p53^{-/-} cells. Total RNA was isolated from cells as described in Materials and methods. CD95 mRNA expression was then analysed in HCT116 wt and HCT116 p53^{-/-} cells upon treatment with 5-FU using reverse transcriptase (RT)-PCR (a). G3PDH RT-PCR samples were served as an internal expression control. (b) Quantification of the CD95 mRNA level in wt HCT116 and HCT116 p53^{-/-} cells treated with 5-FU for various time periods. (c) Expression and localization of CD95 protein in wt HCT116 cells analysed by immunohistochemistry. Co-staining of nuclei was performed using 4,6-diamidino-2-phenylindole (DAPI). Representative results for all samples are shown by staining of untreated and 5-FU-treated cells. (d) SDS-PAGE analysis of caspase-8 activity in HCT116 wt and HCT116 p53^{-/-} cells upon treatment with 5-FU. (e) Response of HCT116 cells to DNA-damaging agents cisplatin, etoposide and 5-FU with respect to p53 activation, CD95 upregulation and caspase-8 processing. Cleaved caspase-8 fragments are indicated with asterisks. G3PDH was used as control for equal loading of the samples.

a p53-CD95-caspase-8-caspase-2 axis, co-immunoprecipitation analysis was performed. We first found that specific antibodies to either caspase-2 or -8 failed to co-precipitate DISC-related proteins, including CD95 and FADD, in apoptotic cells (data not shown), possibly because the antibodies used bound equally well to the soluble form of analysed caspases, which represents the main pool of these enzymes. On the other hand, when we used specific antibodies to CD95 in a methodologically similar approach, co-precipitation of CD95, caspase-2 and the DISC proteins, FADD and caspase-8, was detected in lysates originating from 5-FU-treated HCT116 cells, but not in lysates from untreated cells (Figure 5). However, in contrast to caspase-8, mainly processed caspase-2 is accumulated in the immunoprecipitated material, indicating different binding properties between these proteins.

Overexpression of c-FLIP_L inhibits the processing of caspase-8 and -2

The long-splice isoform of c-FLIP (c-FLIP_L) is enzymatically inactive but identical to caspase-8 with respect to its protein-protein interaction potential, as both proteins contain N-terminal tandem death effector domains. The structural features of c-FLIP_L fulfill the criteria for a dominant-negative inhibitor, a view that is supported by most data (Yeh *et al.*, 2000). Given that c-FLIP_L also localizes to the CD95 DISC, we decided to

perform retroviral transduction of this protein and thereby generate a stable HCT116 cell line containing elevated c-FLIP_L protein level (Figure 6a). Indeed, overexpression of c-FLIP_L efficiently blocked apical caspase-8 and -2 activities as well as downstream activation of effector caspases (Figure 6b). This experiment verified our hypothesis, suggesting that in response to 5-FU the CD95 induces DISC formation, which in turn mediates sequential caspase-8 and -2 activation. It also indicates that the action of these two proteolytic enzymes significantly contributes to the apoptotic cascade examined. The antiapoptotic Bcl-2-like protein Bcl-X_L antagonizes proapoptotic members of the Bcl-2 family, inhibiting the apoptotic signaling at the mitochondrial level (Gross *et al.*, 1999). Activation of effector caspases, which occurs downstream of the mitochondria, was inhibited in response to ectopic expression of Bcl-X_L (Figure 6a and b). Surprisingly, also caspase-2 and -8, which are activated upstream of mitochondria and stimulate cytochrome *c* release through Bid cleavage (Figure 3a), remained partially unprocessed in Bcl-X_L overexpressing cells (Figure 6b). One possible explanation for these findings could be that Bcl-X_L obstructs an amplifying loop leading from effector caspases back to apical caspases. Reports that caspase-2 and -8 have been identified as caspase-3 substrates support this explanation (Harvey *et al.*, 1996; Van de Craen *et al.*, 1999). Alternatively, recent findings suggest that endogenous Smac released from the

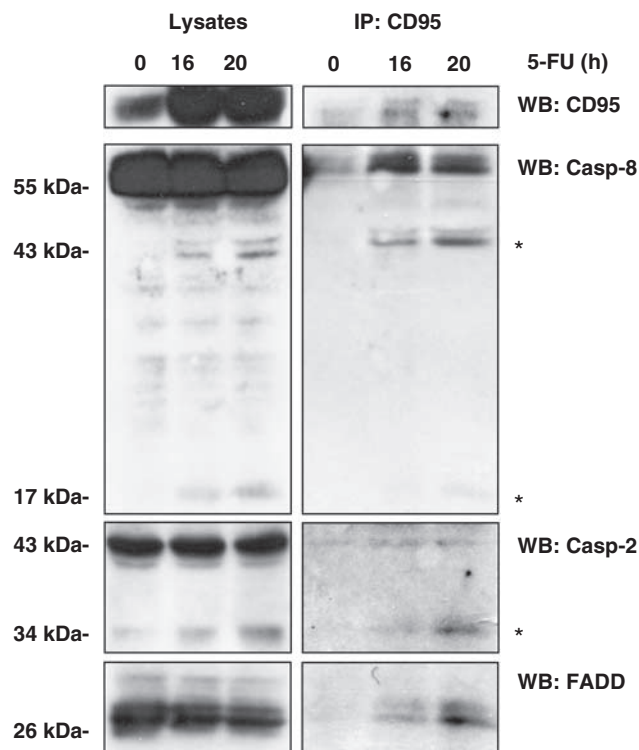


Figure 5 Analysis of CD95 co-immunoprecipitation. Total protein isolates from untreated or 5-FU-treated HCT116 cells were immunoprecipitated with CD95-specific antibodies (Apo-1). Precipitates were separated by SDS-PAGE and analysed by immunoblotting. Presence of DISC-related proteins, including caspase-8 and FADD, as well as DISC nonrelated caspase-2, was examined by specific antibodies. Untreated samples were served as an internal negative control. Cleaved caspase fragments are indicated with asterisks.

mitochondria as well as Smac mimetic can trigger autodegradation of cIAP1 and cIAP2, leading to the release of receptor-interacting protein kinase (RIPK1) from an activated TNF receptor complex to form a caspase-8-activating complex consisting of RIPK1, FADD and caspase-8 (Wang *et al.*, 2008). In this scenario, caspase-8 activation downstream of the mitochondria may therefore be challenged by Bcl-X_L-mediated decrease of mitochondrial Smac release. Indeed, it has been shown that overexpression of Bcl-X_L delays Smac translocation into the cytosol during apoptosis (Dirsch *et al.*, 2003). The latter explanation is in fact in line with our results.

Proteolytic cleavage of caspase-2 by caspase-8 in vitro
Our data demonstrate that once activated, two apical caspases can act simultaneously to amplify the apoptotic cascade in response to DNA damage. They also establish caspase-8 activity as a prerequisite for downstream processing of caspase-2. To determine if intermediate proteolytic enzymes or cofactors are needed for this process, an *in vitro* cleavage assay was performed. Inactive, [³⁵S]-methionine-labeled caspase-2 and -8 were generated and incubated with recombinant, active

enzymes. Although active caspase-2 was able to cleave full-length caspase-2, it did not cleave procaspase-8 (Figure 7a and b). Like caspase-2, active caspase-8 was to some extent also able to cleave its own precursor (Figure 7a). Importantly, the ability of caspase-8 to directly interact and process the pro-form of caspase-2 was demonstrated by this experiment (Figure 7b).

The biochemical mechanism of caspase-2 activation has been analysed in detail and is based on the presence of three proteolytic cleavage sites localized in the primary structure of caspase-2 at D169, D333 and D347 (Figure 7d; Baliga *et al.*, 2004). Interestingly, the pattern of caspase-2 processing differs between its autocatalytic and caspase-8-mediated cleavage. Fully processed caspase-2 forms a (p19-p12)₂ dimer. In autocatalytic samples, the caspase-2 p18 subunit was detected, but the signal from the p12 subunit was below detection level, probably due to low numbers of methionine residues (Figure 7b). In samples treated with active caspase-8, caspase-2 was not completely processed and only fragments originating from cleavage events at D333 and D347, but not D169, were identified (Figure 7b and c). The partial processing of caspase-2 by caspase-8 indicates that other mechanisms, such as autocatalytic activation, are needed to generate separate p12 and p18 subunits. Notably, fragments corresponding to both D333 and D347 cleavage were produced when procaspase-2 was incubated with recombinant active caspase-8, whereas only the D347 fragment was detected when the former was incubated with recombinant active caspase-2 (Figure 7c). A possible explanation for these findings is that the differences seen reflect variation in proteolytic efficiency, rather than specificity, meaning that more caspase-8 activity is required to reach the same level of caspase-2 processing as seen in autocatalytic samples.

Discussion

At present, the best-characterized apoptotic pathways are the receptor-mediated (extrinsic) and cellular stress-induced (intrinsic) pathways, in which caspase-8 and -9 serve as the most apical activating caspases, respectively. In some models of stress-induced apoptosis caspase-2 was also shown to have an apical function, although both caspase-2 and -8 have been placed downstream of caspase-3 in these experimental systems (Guerrero *et al.*, 2008).

However, their primary structures with long pro-domains containing specific protein-protein interaction motifs suggest regulatory functions for these enzymes upstream in most apoptotic pathways. Indeed, activation of caspase-8 as a result of DISC formation triggered by CD95 stimulation is well characterized and represents the initiation of cell-specific apoptotic cascades. In type I cells, sufficient amount of active caspase-8 is generated at the DISC to directly process effector caspase-3, ultimately leading to apoptosis. In type II cells, however, the amount of caspase-8 processed in the

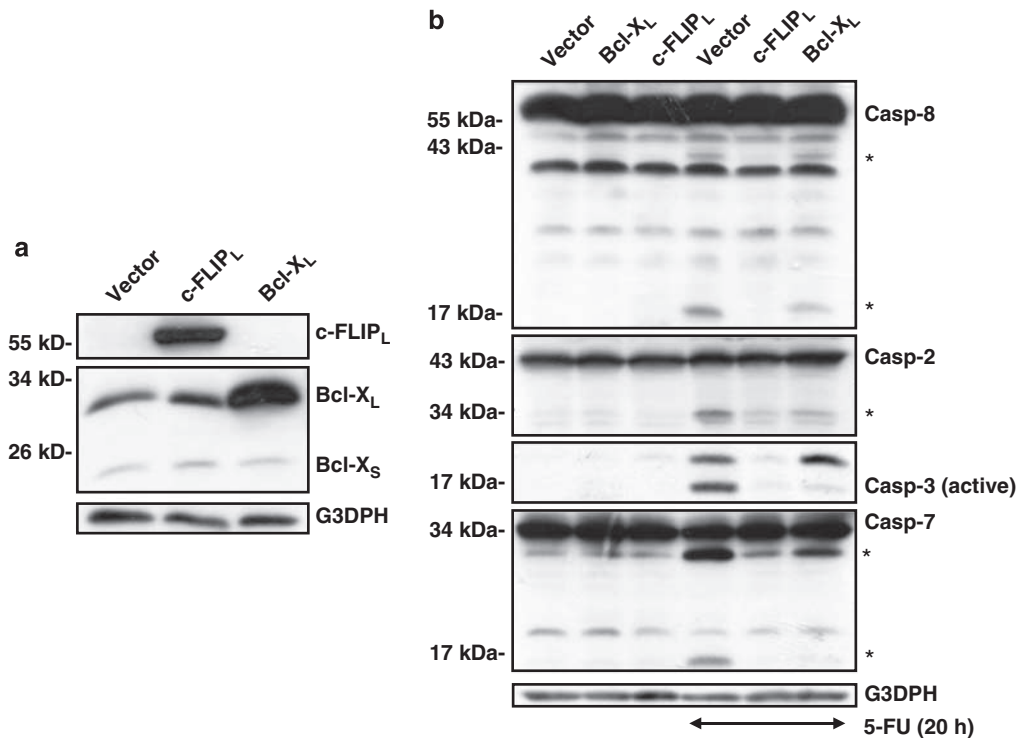


Figure 6 Decreased processing of apical and effector caspases during 5-FU-induced apoptosis in HCT116 cells with elevated c-FLIP_L or Bcl-X_L expression levels. c-FLIP_L, Bcl-X_L or empty expression vectors were introduced into HCT116 cells by retroviral transduction. Genetically heterogeneous cell populations were selected by antibiotic pressure. **(a)** Immunoblot analysis of lysates harvested from HCT116 cells, transduced with c-FLIP_L, Bcl-X_L or empty vector. **(b)** Immunoblot analysis of protein lysates from genetically modified cell cultures harvested at the indicated time after 5-FU treatment. Proteolytic processing of apical caspase-2 and -8, as well as effector caspase-3 and -7 is shown. Blots were re-probed for G3DPH to confirm equal loading of the samples. Cleaved fragments are indicated with asterisks.

DISC is not sufficient to directly activate downstream effector caspases. Apoptosis in these cells is dependent on the cleavage of the proapoptotic Bcl-2 family protein Bid. tBid leads to oligomerization and activation of Bax or Bak, followed by the opening of specific pores in the OMM, permitting the release of cytochrome *c* and other proteins from the mitochondrial intermembrane space to the cytoplasm (Peter and Krammer, 2003).

In several reports an association between caspase-2 and DISC-mediated apoptosis has been suggested. For example, caspase-2 was shown to be required for Bid cleavage and optimal development of TRAIL-induced apoptosis (Wagner *et al.*, 2004), and leukemic caspase-2 knockdown cell lines were characterized by a reduced sensitivity to CD95-mediated apoptosis (Droin *et al.*, 2001). However, a direct interaction was not described until caspase-2 was found to be recruited to the DISC in T and B cells on CD95 stimulation (Lavrik *et al.*, 2006). Yet, despite its presence at the DISC, caspase-2 activation was not detected in response to CD95 stimulation of a caspase-8-deficient Jurkat T cell line. This is in line with our finding that DISC- and caspase-8-mediated activation of caspase-2 might occur in response to p53-dependent DNA damage-induced signaling. Induction of the CD95 occurs in a wide selection of cell types by various DNA-damaging agents (Muller *et al.*, 1998). The CD95 death receptor gene

contains a p53-responsive element, but the transactivation mechanism does not discriminate between wild-type (wt) and p53 mutants, which are unable to induce apoptosis (Muller *et al.*, 1998; Munsch *et al.*, 2000). It has, therefore, been questioned whether the p53-dependent upregulation of CD95 *per se* induces apoptosis, or if it sensitizes the cell to other proapoptotic signals. Etoposide, like 5-FU, induces p53-dependent transactivation of CD95, but caspase-8 remains unprocessed in response to this DNA-damaging agent (Figure 4e). Therefore, results using HCT116 cells indicate that although p53-mediated CD95 transactivation is required, it is not sufficient for DISC-mediated caspase-8 activation.

Our data clearly establish that DISC formation and caspase-8 activation are essential for cleavage of procaspase-2 in 5-FU-treated HCT116 cells. Both caspases then promote apoptosis by Bid cleavage, mitochondrial cytochrome *c* release and activation of effector caspases in a parallel manner. FADD serves as an adapter protein by linking caspase-8 to the CD95 receptor through homotypic domain interaction. The existence of an analogous protein that might connect caspase-2 to the DISC remains elusive. The CARD and the DD required for this purpose are found in RAIDD (Duan and Dixit, 1997). However, we and others were unable to detect RAIDD in DISC immunoprecipitates (data not shown; Lavrik *et al.*, 2006). In addition to the

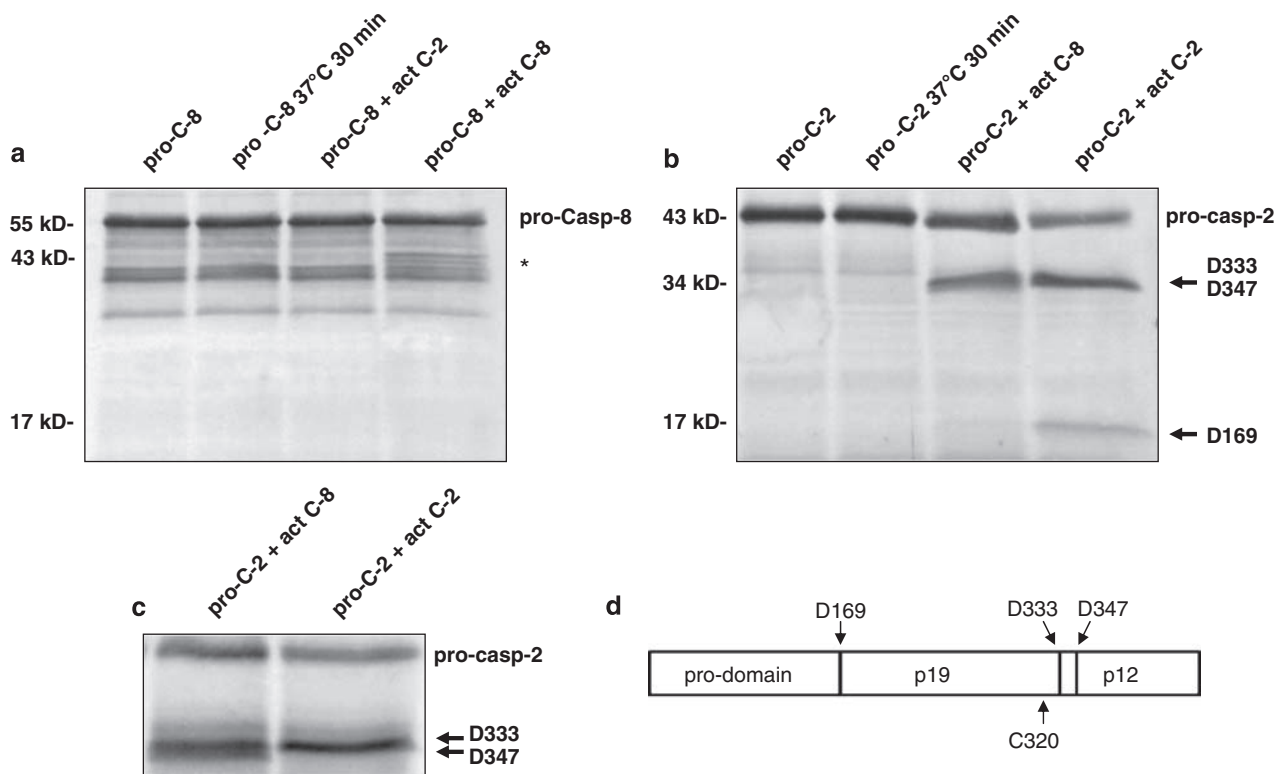


Figure 7 Caspase-8 is able to mediate a direct proteolytic cleavage of caspase-2 *in vitro*. [³⁵S]-methionine-labeled procaspases were incubated for 30 min at 37 °C with recombinant active caspases in cleavage buffer, and then analysed by 15% SDS-PAGE. (a) Cleavage of procaspase-8. (b) Cleavage of procaspase-2. (c) Magnification of autoradiography showing differences in cleavage pattern of procaspase-2 by recombinant caspase-2 and -8. (d) Schematic illustration of the procaspase-2 primary structure. The D169, D333 and D347 cleavage sites, as well as the C320 active site are depicted.

PIDDosome, caspase-2 might also be recruited to a large and inducible protein complex along with RIP1 and TRAF2, an oligomerization that enables activation of the NF- κ B pathway (Lamkanfi *et al.*, 2005). Thus, due to the presence of a N-terminal DD, RIP1 would theoretically function to unite caspase-2 to the DD in the CD95 receptor. This possibility, however, suffers from the observation that RIP1 mainly is involved in inflammatory or cell survival signaling pathways (Festjens *et al.*, 2007). Although the pro-forms of caspase-2 were found in CD95 immunoprecipitates, pull-down of the p36 processed fragment was more prominent. This caspase-2 immunoprecipitate pattern was distinct from caspase-8, which led us to hypothesize that caspase-2 may accumulate at the DISC by transient binding where caspase-8-mediated cleavage occurs, and that this accumulation does not require any particular adapter protein(s).

Caspase-2 knockout mice lack an apparent phenotype, and cells that originate from these animals have normal susceptibility to apoptosis in response to multiple stimuli, including DNA damage (Bergeron *et al.*, 1998). In contrast, crucial proapoptotic function for caspase-2 in diverse experimental settings *in vitro* is supported by several reports (Troy and Shelanski, 2003; Zhivotovsky and Orrenius, 2005). The lack of a global apoptotic defect in caspase-2-deficient mice has, for that reason, been explained by the existence of compensatory

mechanisms. Experimental evidence for the existence of such mechanisms has been provided by data showing that the caspase-9 pathway adopt caspase-2 functions in caspase-2^{-/-} sympathetic brain cells (Troy *et al.*, 2001). Our findings are consistent with the presence of compensatory mechanisms *in vivo*, but place caspase-8, rather than caspase-9, as the main compensatory caspase, at least in DNA damage-induced apoptosis mediated through DISC formation.

Overexpression of PIDD has been used to support the requirement of the PIDDosome platform for caspase-2 activation (Tinel and Tschopp, 2004; Berube *et al.*, 2005; Cuenin *et al.*, 2008), and the presence of PIDD, RAIDD and caspase-2 in the PIDDosome complex even in untreated cells was documented (Tinel and Tschopp, 2004; Vakifahmetoglu *et al.*, 2006). However, elimination of either PIDD or RAIDD did not significantly influence caspase-2 activation in our experimental model (Vakifahmetoglu *et al.*, 2006). This suggests that in some situations, caspase-2 might use another activation platform. Indeed, present findings demonstrate such an alternative mechanism, where DISC formation and caspase-8 activity promote processing and activation of caspase-2 (Figure 8). It is important to note that both platforms exist in HCT116 cells. However, it is still unclear whether both of them contribute to caspase-2 activation simultaneously, or if they have cell and/or trigger specificity. The relative importance of these

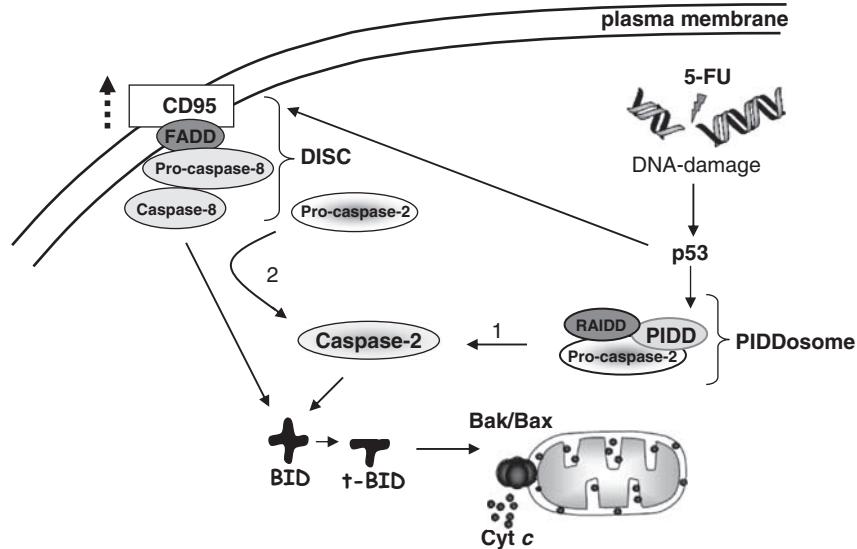


Figure 8 Schematic illustration of the apoptotic signaling cascade in HCT116 cells upon treatment with 5-FU. PIDDosome-mediated (1) and DISC-mediated (2) caspase-2 activation is indicated.

pathways for cell elimination in response to genotoxic stress also requires further clarification.

Materials and methods

Cell culture

The HCT116 cell line and its derivative, deficient in p53 protein expression, were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM). Cells were grown in a humidified 5% CO₂ atmosphere at 37 °C and maintained in a logarithmic growth phase. DNA-damaging agents 5-FU (375 µM; Hospira, Lake Forest, IL, USA), cisplatin (40 µM; Meda, Solna, Sweden) and etoposide (20 µM; Bristol Myers Squibb, New York, NY, USA) were used as apoptotic inducers. Cell culture reagents were purchased from Invitrogen (San Diego, CA, USA) unless otherwise stated.

siRNA methodology

Post-transcriptional silencing of protein expression levels in HCT116 cells was achieved by transfection of 21-nucleotide RNA duplexes. Design and synthesis of siRNAs were performed by Dharmacon (Lausanne, Switzerland). Transfection of caspase-2 (L-003465-00), caspase-3 (L-004307-00), caspase-8 (L-003466-00), caspase-9 (L-003309-00) and control (D-001810-10) ON-TARGET-*plus* SMARTpool siRNAs was carried out using the INTERFERin transfection reagent (Polyplus transfection, Illkirch, France) according to the manufacturer's instructions. In brief, 3 × 10⁵ cells were seeded and on the following day transfected with a final concentration of 10 µM siRNA and 3.65 µl INTERFERin per ml. Repression of protein translation was monitored by SDS-polyacrylamide gel electrophoresis (PAGE). Protein downregulation was confirmed as early as 24 h after transfection and lasted for up to 5 days.

Gel electrophoresis and immunoblotting

Cells were harvested, washed once in phosphate-buffered saline (PBS) and lysed for 5 min in complete Lysis-M and 1 × Complete Protease Inhibitor Cocktail (Roche Diagnostics,

Mannheim, Germany). Protein concentration was determined by the BCA Protein assay (Thermo Fisher Scientific, Waltham, MA, USA) and mixed with Laemmli's loading buffer. Equal quantities of protein from each sample were boiled for 5 min and subjected to SDS-PAGE at 40 A followed by electroblotting to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA) for 2 h at 100 V.

Membranes were blocked for 1 h with 5% non-fat milk, 2% bovine serum albumin (BSA) and 0.05% NaN₃ in PBS and subsequently probed with the primary antibody of interest at 4 °C overnight. Membranes were then washed in PBS and incubated with horseradish-peroxidase-conjugated secondary antibodies for 1 h at room temperature. Blots were revealed by ECL (GE Healthcare Biosciences, Uppsala, Sweden).

Antibodies

The following primary antibodies were used: anti-Bcl-X_L (S18) pAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA), c-FLIP_L mAb, clone Dave-2 (Alexis Biochemicals, San Diego, CA, USA), anti-p53 mAb, clone 184721 (R&D Systems, Minneapolis, MN, USA); anti-cytochrome *c* mAb, clone 7H8.2C12, anti-caspase-2 mAb, clone 35, anti-caspase-7 mAb, clone B94-1, anti-caspase-9 pAb, anti-PARP mAb, clone 4C10-5, anti caspase-8 pAb (used in Figures 1 and 2; all from BD Biosciences, Franklin Lakes, NJ, USA); anti-cleaved-caspase-3 pAb, anti-Bid pAb (both from Cell Signaling, Danvers, MA, USA); anti-FADD pAb (Upstate Biotechnologies, Lake Placid, NY, USA); polyclonal anti-G3PDH (glyceraldehyde-3-phosphate dehydrogenase) serum (Nordic Biosite, Täby, Sweden); anti-caspase-8 mAb, clone C15 (used in Figures 5 and 6; kindly provided by Professor PH Krammer, German Cancer Research Center, Heidelberg, Germany). All primary antibodies were diluted in PBS containing 1% BSA and 0.05% NaN₃. Secondary antibodies were diluted in blocking buffer without NaN₃. Horseradish-peroxidase-conjugated secondary antibodies were purchased from Thermo Fisher Scientific. For immunoprecipitation (IP) anti-CD95 mAbs, clone APO-1, were used (kindly provided by Professor PH Krammer). For immunofluorescence and western blot analysis as a primary anti-CD95 (C-20) pAbs (Santa Cruz Biotechnology) were used. Secondary fluorescein isothiocyanate-conjugated Alexa Fluor

594 goat anti-rabbit IgG (Invitrogen) were used for immunofluorescence.

Immunofluorescence

HCT116 cells were seeded on coverslips and fixed for 20 min in 4% formaldehyde at 4 °C. Incubations with primary antibodies, diluted (1:400) in PBS containing 0.3% Triton X-100 and 0.5% BSA, and secondary antibodies (1:200) were performed at 4 °C overnight in a humid chamber and at room temperature for 60 min, respectively. Nuclei were counterstained with Hoechst 33342 (10 µg/ml in PBS solution) by 10 min incubation at room temperature. Between all steps, cells were washed for 3 × 10 min in PBS. Stained slides were mounted using Vectashield H-1000 (Vector Laboratories Inc., Peterborough, UK) and examined under a Zeiss LSM 510 META confocal laser scanner microscope (Carl Zeiss MicroImaging, Göttingen, Germany).

Mitochondrial cytochrome c release

HCT116 cells (1×10^6), treated as indicated in figures, were washed once in PBS, resuspended in 100 µM of permeabilization buffer (5 mM Tris-HCl (pH 7.4), 50 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 5 mM succinate, 1 mM KH₂PO₄ and 140 mM manitol) and incubated for 5 min at room temperature in the presence of digitonin (100 µg/ml; Merck, Darmstadt, Germany). Mitochondria and nuclei were then separated from the cytosolic fraction by centrifugation at 16 000 g for 5 min. Cytochrome c, remaining in mitochondria and released into supernatants, was analysed by immunoblotting.

RT-PCR

Total RNA was isolated from HCT116 wt and HCT116 p53^{-/-} cells, treated as indicated in figures, by taking advantage of the TRIzol reagent (Invitrogen). The protocol used followed the recommendations from the manufacturer. RNA quality was controlled by separation of 18S and 28S ribosomal RNA in denaturing agarose gel electrophoresis and by visualization using ethidium bromide staining. For first-strand cDNA synthesis, the RevertAid M-MuLV RT enzyme (Fermentas, Vilnius, Lithuania) was used in combination with an oligo(dT)18 primer according to the instructions of the manufacturer. The PCR was performed using the Platinum Pfx DNA polymerase system (Invitrogen) and the following specific primers (Thermo Fisher Scientific):

CD95 receptor, 5', 5'-CAAGGGATTGGAATTGAGGA-3'
CD95 receptor, 3', 5'-GACAAAGCCACCCCAAGTTA-3'
G3PDH, 5', 5'-CCTGGCCAAGGTCATCCATG-3'
G3PDH, 3', 5'-TGAGGTCCACCCTGTTG-3'

The conditions for all PCR reactions were as follows: 94 °C for 1 min followed by 95 °C for 15 s, 60 °C for 30 s and 68 °C for 50 s. Samples were removed every five cycle to ensure exponential product growth. PCR yield was analysed on a 2% agarose gel in the presence of an appropriate DNA ladder. CD95 mRNA expression was quantified with respect to the amount of G3PDH cDNA in each specific PCR sample.

Immunoprecipitation

The method to determine the composition of the CD95 DISC by IP has been described (Lavrik *et al.*, 2006). In brief, 5×10^6 cells untreated and treated with 5-FU for the indicated time points were washed twice in PBS and subsequently lysed in buffer A (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100 and 10% glycerol, 1 mM phenylmethylsulfonyl fluoride and 1 × Complete Mini Protease Inhibitor Cocktail; Roche Diagnostics) for 15 min on ice. After sonication, 500 µg of total protein extract (1 µg/µl) was

incubated with 2 µg of the anti-CD95 antibody on ice overnight. The CD95 DISC was then immunoprecipitated for 3 h with 100 µl (10%, w/v) of Protein G Sepharose 4 Fast Flow (GE Healthcare Biosciences) at 4 °C. Finally, beads were washed 3 × 5 min with 20 volumes of lysis buffer. Immunoprecipitates were analysed by SDS-PAGE.

Expression vectors and retroviral transduction

The retroviral expression vectors pLXIN-h Bcl-X_L and pXIN-hFLIP_L have been described previously (Djerbi *et al.*, 2001). Retroviral particles were produced by transient transfection of the Phoenix-Ampho packaging cell line (kindly provided by Dr GP Nolan, Stanford University, Stanford, CA, USA). Production of viral particles and retroviral transductions was performed as described previously (Djerbi *et al.*, 2001). Transduced cells were selected with 0.8 mg/ml of Geneticin (Invitrogen).

In vitro analysis of the proteolytic interaction between caspase-8 and -2 cDNAs for human caspase-2 and -8 was introduced into the pET28b(+) vector (Merck) and the pCDNA3.1(+) vector (Invitrogen), respectively. L-[³⁵S]-methionine-labeled, proteolytically silent enzymes were then generated by taking advantage of the TNT T7 Coupled Reticulocyte Lysate System (Promega, Madison, WI, USA) and by following the instructions from the manufacturer. Translation products were verified with specific antibodies targeting the enzymes of interest. *In vitro* translated caspase-2 and -8 proteins were incubated at 37 °C for 30 min in cleavage buffer (50 mM HEPES-KOH (pH 7.5), 2 mM EDTA (pH 8.0), 10 mM DTT and 1 mM PSMF) with or without 100 ng recombinant active caspase-8 (BD Biosciences) or 100 ng recombinant active caspase-2. Proteolytic activities were analysed by SDS-PAGE and autoradiography using Hyperfilm MP (GE Healthcare Biosciences). Recombinant caspase-2 was expressed in *Escherichia coli* (strain BL21[DE3]) as a C-terminal His6-tagged protein using the pET-28a vector (Novagen, Gibbstown, NJ, USA) and purified by standard Ni²⁺-affinity chromatography. Recombinant caspase-2 and -8 activities were confirmed by measurement of cleavage of VDVAD-AMC, or IETD-AMC (Peptide Institute, Osaka, Japan), respectively using a fluorometric assay reported previously (Robertson *et al.*, 2002).

Abbreviations

5-FU, 5-fluorouracil; DISC, death-inducing signaling complex.

Conflict of interest

The authors declare no conflict of interest.

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