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FLASH links the CD95 signaling pathway to the cell nucleus and nuclear bodies

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Caspase-8-binding protein FLICE-associated huge protein (FLASH) has been proposed to regulate death receptor CD95-induced apoptosis through facilitating caspase-8 activation at the death-inducing signaling complex. Here, we found that FLASH interacts with the PML nuclear body component Sp100 and predominantly resides in the nucleus and nuclear bodies (NBs). In response to CD95 activation, FLASH leaves the NBs and translocates into the cytoplasm where it accumulates at mitochondria. The nucleo-cytoplasmic translocation of FLASH requires CD95-induced caspase activation and is facilitated by the Crm1-dependent nuclear export pathway. Downregulation of FLASH by RNA interference or inhibition of its nucleo-cytoplasmic shuttling reduced CD95-induced apoptosis. Furthermore, we show that the adenoviral anti-apoptotic Bcl-2 family member E1B19K traps FLASH and procaspase-8 in a ternary complex at mitochondria, thereby blocking CD95-induced caspase-8 activation. Knock-down of Sp100 potentiated CD95-activated apoptosis through enhancing nucleo-cytoplasmic FLASH translocation. In summary, our findings suggest that CD95 signals via a previously unrecognized nuclear pathway mediated by nucleo-cytoplasmic translocation of FLASH.

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Introduction

Death receptor CD95 (Fas/APO-1) belongs to the TNF-receptor superfamily and plays a crucial role in controlling immune response and tumor suppression (Nagata, 1999; Krammer, 2000). After binding its ligand (CD95L/CD178) or agonistic antibodies (e.g., anti-APO-1), a multimeric protein complex termed death-inducing signaling complex (DISC) composed of FADD, procaspase-8/FLICE, procaspase-10 and c-FLIP is recruited to the CD95 receptor (Kischkel *et al*, 1995; Boldin *et al*, 1996; Muzio *et al*, 1996; Irmler *et al*, 1997; Lavrik *et al*, 2005). Death receptor-induced apoptosis requires caspase-8 activation (Varfolomeev *et al*, 1998), which culminates in the activation of downstream caspases and facilitates cellular apoptotic destruction (Fischer *et al*, 2003).

CD95 signals apoptosis via two pathways (Scaffidi *et al*, 1998). Type I cells activate high amounts of caspase-8 at the DISC, facilitating direct activation of caspase-3. In contrast, type II cells activate low amounts of caspase-8 and rely on a mitochondrial amplification pathway triggered by caspase-8-dependent Bid cleavage, loss of the mitochondrial transmembrane potential and release of mitochondrial proapoptotic factors, leading to caspase-3 activation through the apoptosome, a caspase activation platform formed by multimerized APAF-1, cytochrome *c* and procaspase-9 (Wang, 2001). Type II cells are susceptible to apoptosis inhibition by antiapoptotic Bcl-2 protein family members (Scaffidi *et al*, 1998), including adenoviral E1B19K protein (Cuconati and White, 2002). Interestingly, independent studies reported an association of endogenous caspase-8 with mitochondria, where it is apparently accessible for activation through a currently incompletely defined mechanism (Qin *et al*, 2001; Stegh *et al*, 2000, 2002; Chandra *et al*, 2004).

A previous report showed that E1B19K inhibits CD95-mediated apoptosis downstream of FADD but upstream of caspase-8 (Perez and White, 1998), suggesting that E1B19K targets an unknown factor functionally positioned between FADD and caspase-8. Later, E1B19K has been reported to target FLICE-associated huge protein (FLASH), a caspase-8-binding protein that interacts with the death-effector domains (DEDs) of FADD and caspase-8 (Imai *et al*, 1999). FLASH binds the tandem DEDs of procaspase-8 via an interaction module termed death-effector domain recruiting domain (DRD), thereby regulating caspase-8 activation in response to CD95 stimulation (Imai *et al*, 1999). The interaction between procaspase-8 and FLASH was proposed to take place at the DISC (Imai *et al*, 1999).

However, the role of FLASH in CD95 signaling was challenged by the observation that FLASH harbors several nuclear localization signals, and as the presence of a postulated CED4 homology signature could not be confirmed by others (Imai *et al*, 1999; Koonin *et al*, 1999). Hence, the function of FLASH in CD95 signaling remained controversial (Peter and Krammer, 2003). This issue was further substantiated by recent findings demonstrating interaction of FLASH with nuclear proteins, including the nuclear hormone receptor coactivator GRIP and various nuclear hormone receptors (Kino and Chrousos, 2003; Obradovic *et al*, 2004).

In the present study, we identified FLASH as an interaction partner of Sp100 (speckeled 100-kDa protein), a well-established constitutive constituent of PML nuclear bodies (PML-NBs), macromolecular structures playing a role in DNA

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damage response and in apoptosis signaling (Bernardi and Pandolfi, 2003; Hofmann and Will, 2003; Takahashi *et al*, 2004; Everett, 2006). Concordantly, we observed that endogenously expressed FLASH predominantly localizes to the cell nucleus and NBs. These findings motivated us to analyze whether FLASH may provide a link between CD95 signaling and the cell nucleus and NBs.

Results

Identification of FLASH an ak Sp100 interacting protein

To gain insight into the cellular function of Sp100, we performed a yeast two-hybrid screen to identify novel interaction partners. Using the amino-terminal part of Sp100 as bait, we isolated a cDNA clone coding for the carboxy-terminal 277 amino acids (aa) of human FLASH, a polypeptide that comprises 1982 aa in total (Figure 1A). To determine whether Sp100 and FLASH interact *in vivo*, we expressed Sp100 and Flag epitope-tagged FLASH in 293T cells and performed immunoprecipitations. Immunoblot analysis demonstrated specific co-immunoprecipitation of Sp100 with FLASH (Figure 1B). Furthermore, reciprocal immunoprecipitation also revealed specific co-immunoprecipitation of FLASH with Sp100 (Figure 1C). In contrast, ectopically expressed PML did not co-immunoprecipitate with FLASH (Figure 1D), implying that FLASH interacts specifically with Sp100, and that the interaction is not mediated by PML. Using different FLASH deletion mutants (Figure 1E), we mapped the *in vivo* interaction domains of FLASH and Sp100 to the C-terminal domain of FLASH (Figure 1F), which is consistent with our yeast two-hybrid data (Figure 1A). Moreover, endogenous FLASH was co-immunoprecipitated with endogenous Sp100 protein from HT1080 cell lysates, further confirming the physical interaction of both proteins (Figure 1G). Taken together, these results establish FLASH as an Sp100 interacting protein.

FLASH localizes to the cell nucleus and nuclear bodies

To analyze the subcellular localization of endogenous FLASH, we performed confocal immunofluorescence analyses in HT1080 cells using two different antibodies, one raised against the amino terminus and one against the carboxy terminus of FLASH. Both antibodies revealed a similar distribution of endogenous FLASH protein, with the main fraction localizing in the nucleus and NBs and a minor fraction to the cytoplasmic compartment (Figure 2A and B). A similar distribution of endogenous FLASH protein was observed in 293T and HeLa cells (Figure 2C and not shown).

Sp100 and PML are the only known constitutive PML-NB components and are well-established markers for these nuclear domains (Negorev and Maul, 2001; Hofmann and Will, 2003). Confocal microscopy revealed that Sp100-NBs

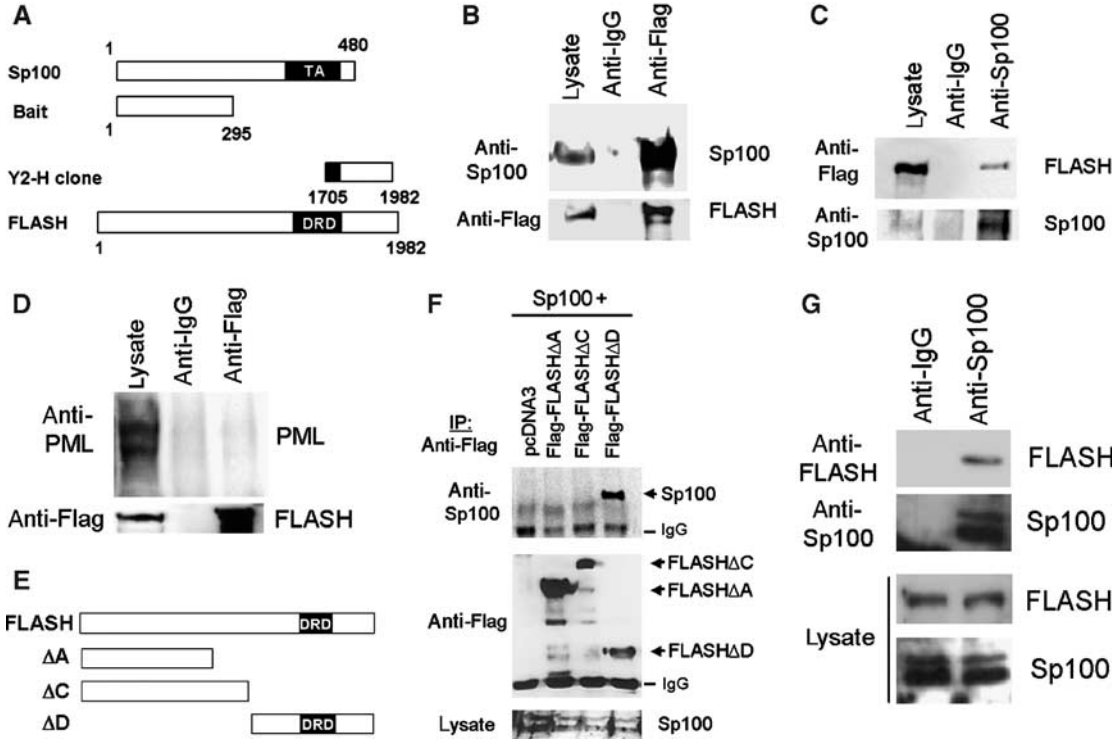

Figure 1 Identification of FLASH as an Sp100 interacting protein (**A**) Schematic representation of the Sp100 bait used for yeast two-hybrid screening and the isolated human FLASH sequence. DRD, death-effector domain recruiting domain (B, C) 293T cells were transfected with Sp100 and Flag-FLASH constructs. (**B**) After immunoprecipitation of FLASH (lower panel) with Flag antibodies, co-precipitated Sp100 was detected by immunoblotting (upper panel). (**C**) Sp100 was precipitated with Sp100 antibodies and co-precipitated FLASH was detected by immunoblotting with Flag antibodies. (**D**) 293T cells were transfected with PML-IV and Flag-FLASH constructs. After immunoprecipitation of FLASH (lower panel), no co-precipitation of PML was detected by immunoblotting (upper panel). (**E**) Schematic diagram showing FLASH and mutants thereof. (**F**) Lysates from 293T cells expressing Sp100 and the indicated Flag-FLASH proteins were immunoprecipitated (IP) with Flag antibodies (middle panel) and co-immunoprecipitated Sp100 was detected by immunoblotting with Sp100 antibodies (upper panel). (B–D, F, G) Lysate inputs (20%) were analyzed by immunoblotting. (**G**) HT1080 cell lysates were subjected to immunoprecipitation using either control antibodies or Sp100 (GH3) antibodies. Precipitates were analyzed by immunoblotting.

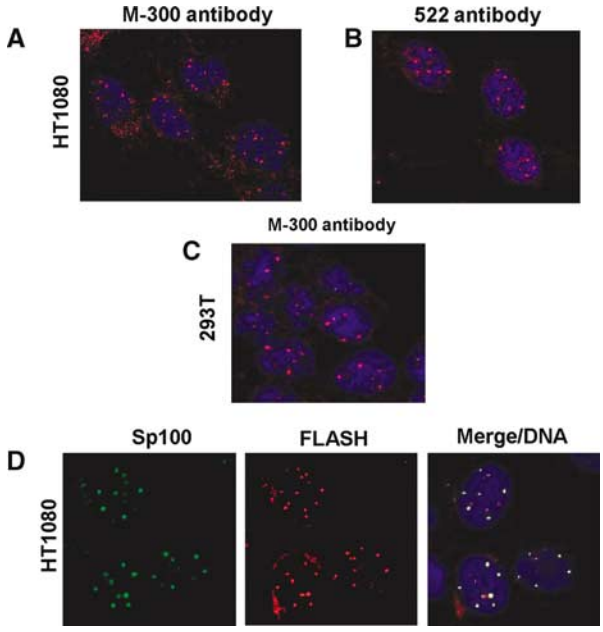

Figure 2 FLASH localizes to the cell nucleus and nuclear bodies. (**A, B**) HT1080 cells stained for endogenous FLASH (red) using different FLASH antibodies (M-300 and 522). (**C**) Endogenous FLASH protein (red) in 293T cells stained with FLASH antibodies. (**D**) HT1080 cells were analyzed for localization of endogenous Sp100 (green) detected by rat Sp26 antibodies and of endogenous FLASH (red) stained with FLASH antibodies (M-300). (A–D) Nuclear DNA was stained with Draq5 and visualized in blue. Images were generated using a confocal laser scanning microscope.

colocalized with FLASH-NBs, indicating an overlap of FLASH-NBs and PML-NBs (Figure 2D). Of note, only a fraction of FLASH-NBs overlapped with PML-NBs (Figure 2D), indicating that FLASH does not exclusively associate with PML-NBs but also with other, not further determined, NBs. Consistent with previous reports demonstrating that Sp100 and PML are constitutive PML-NB components, Sp100 localized completely to PML-NBs in HT1080 cells (data not shown), further confirming FLASH as not exclusively a PML-NB component. PML expression was not required for FLASH-NB formation, as FLASH readily localized in NBs in *PML*-deficient cells (data not shown). These data identify FLASH as a component of PML-NBs and other nuclear domains.

CD95 triggers nucleo-cytoplasmic translocation of FLASH

Next, we analyzed the subcellular distribution of endogenous FLASH after CD95 activation using treatment with agonistic APO-1 antibodies. Remarkably, endogenous FLASH was released from NBs and the nucleus and accumulated in the cytoplasm upon CD95 activation (Figure 3A–C). Similar results were obtained using recombinant CD95 ligand (data not shown). Also, a fraction of Sp100 accumulated in the cytoplasm after CD95 activation (Figure 3D). In contrast, PML, the organizer of PML-NBs (Ishov *et al*, 1999; Zhong *et al*, 2000), remained in PML-NBs following CD95 activation (Figure 3E and F), indicating that redistribution of FLASH and

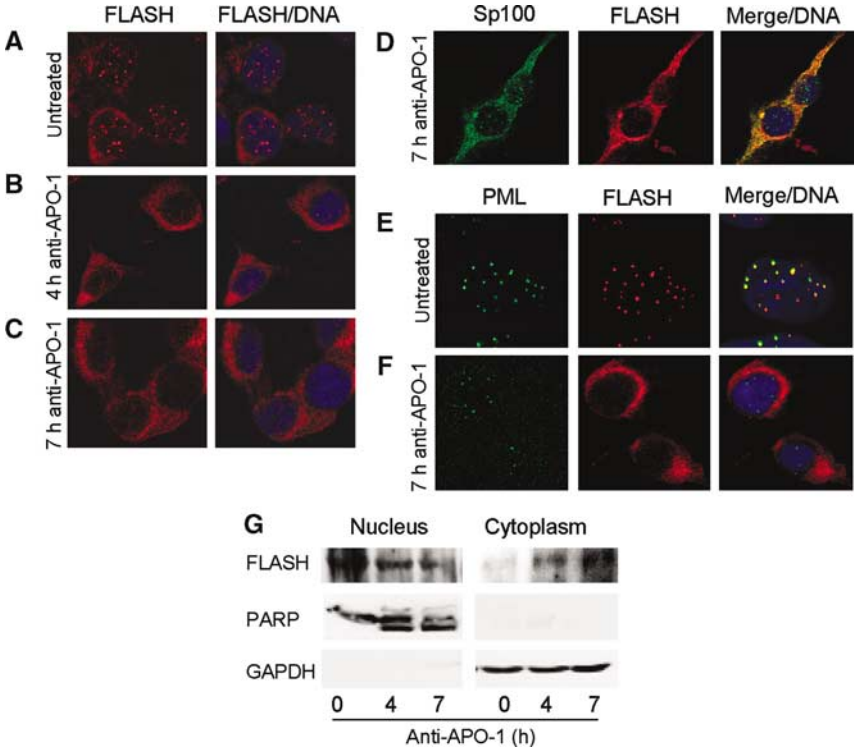

Figure 3 Nucleo-cytoplasmic translocation of FLASH following CD95 activation. (**A–C**) HT1080 cells were treated with APO-1 antibodies for the time indicated and localization of endogenous FLASH (red) was analyzed by indirect immunofluorescence stainings. Nuclear DNA was stained with Draq5 (blue). (**D**) Subcellular localization of endogenous Sp100 after CD95 activation. (**E, F**) Localization of endogenous FLASH (red) and endogenous PML (green) in HT1080 cells analyzed by confocal microscopy before and after CD95 activation. (**G**) Equal amounts of nuclear and cytoplasmic fractions of HT1080 cells harvested at the indicated time points after CD95 activation were analyzed by immunoblotting. PARP (a nuclear caspase substrate; caspase-cleaved form appears after CD95 activation) and GAPDH (cytoplasmic protein) were used as markers for the purity of the fractions.

Sp100 to the cytoplasm is specific and not caused by apoptosis-associated NB disruption and nuclear protein leakage.

Immunoblot analyses of nuclear and cytoplasmic fractions confirmed increased cytoplasmic FLASH levels paralleled by decreased nuclear FLASH levels after CD95 activation (Figure 3G). The purity of the nuclear and cytoplasmic fractions, as well as activation of CD95-mediated apoptosis, was verified by immunoblotting with antibodies against the nuclear caspase substrate poly-(ADP-ribose) polymerase (PARP) and the cytoplasmic enzyme GAPDH, respectively (Figure 3G). Collectively, these results demonstrate that FLASH leaves NBs and the nucleus and accumulates in the cytoplasm after CD95 activation.

FLASH translocation is mediated by Crm1 and could be important for CD95-mediated apoptosis

Next, we investigated whether FLASH is actively exported from the nucleus to the cytoplasm after CD95 activation. Therefore, we blocked the Crm1-dependent nuclear export pathway with leptomycin B (LMB) (Fukuda *et al*, 1997). The LMB concentration used showed no cytotoxic effects in our experimental system and efficiently blocked nuclear export of an HIV Rev-GFP fusion protein transiently expressed in HT1080 as control (data not shown). Pretreatment with LMB before CD95 activation readily inhibited cytoplasmic accumulation of endogenous FLASH (Figure 4A and B). Notably, LMB did not interfere with recruitment and activation of caspase-8 at the DISC (Supplementary Figure 1), excluding a general blockade of the CD95 pathway.

To determine the relevance of FLASH translocation for CD95-induced apoptosis we inhibited FLASH nuclear export by LMB and measured CD95-induced apoptosis. Blocking nuclear export of FLASH resulted in a strong inhibition of CD95-induced mitochondrial damage (Figure 4C). Similar results were obtained when apoptosis was determined by subdiploid DNA content measurement using FACS analysis (data not shown). These findings indicate that a factor/s exported from the nucleus is/are required for efficient CD95-mediated apoptosis.

FLASH depletion impairs CD95-induced apoptosis and caspase-8 activation

To investigate a direct role of FLASH in CD95-mediated apoptosis, we depleted endogenous FLASH by RNA interference (Figure 4D). Depletion of FLASH resulted in a marked inhibition of CD95-induced apoptosis (Figure 4E). Furthermore, FLASH knock-down clearly impaired CD95-induced caspase-8 processing, as evident by reduced formation of the active p18 subunit (Figure 4F). However, levels of the caspase-8 substrate protein Bid were only slightly reduced

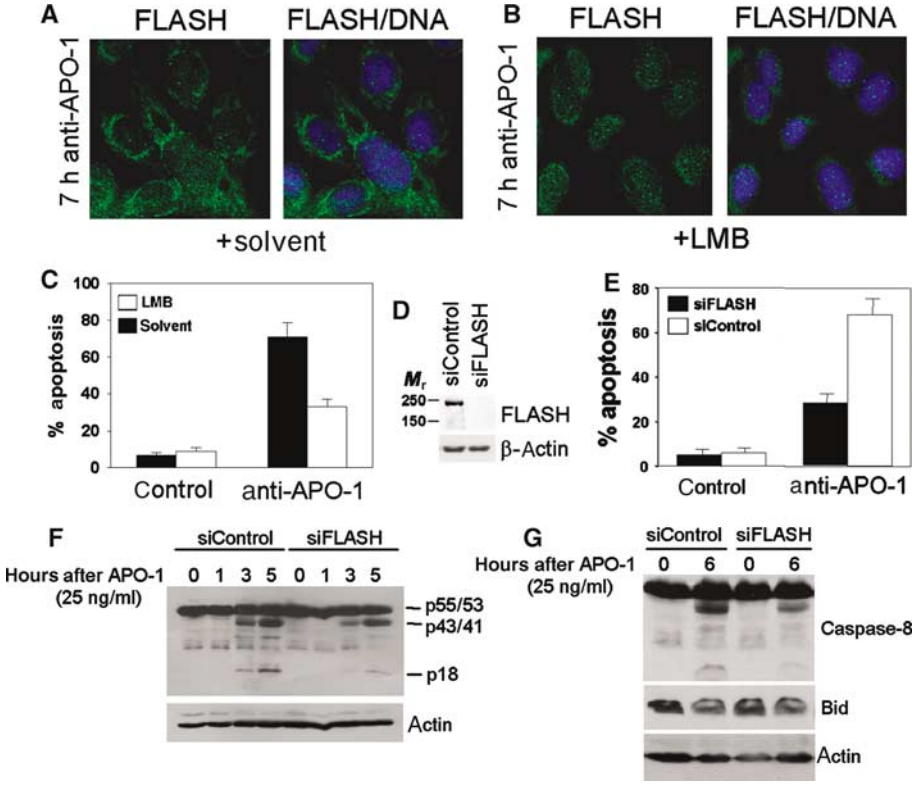

Figure 4 Nucleo-cytoplasmic translocation of FLASH is mediated by Crm1 and is important for CD95-mediated apoptosis. (**A, B**) HT1080 cells pretreated with solvent or leptomycin B (LMB; 20 ng/ml) for 1.5 h were stimulated with APO-1 antibodies and endogenous FLASH (green) and DNA (blue) were stained. (**C**) HT1080 cells pretreated with solvent or LMB were stimulated with APO-1 antibodies as indicated. Apoptosis was determined by Mitotracker Red CM-H_2XRos staining and apoptosis-associated nuclear DNA condensation by analyzing 200 cells under a flourescence microscope. Standard deviations from three independent experiments are indicated. (**D**) HT1080 cells were transfected with pSUPER-Luci (siControl) or pSUPER-FLASH (siFLASH). FLASH was detected by immunoblotting with FLASH antibodies. β-Actin is shown as loading control. (**E**) HT1080 cells were cotransfected with GFP and pSUPER-Luci (siControl) or pSUPER-FLASH (siFLASH) expression vectors. Cells were treated 30 h later with APO-1 antibodies for 7 h. Apoptosis was determined by analyzing 250 GFP-positive cells under a fluorescence microscope as specified in (C). Standard deviations from three independent experiments are indicated. (**F**, **G**) HT1080 cells were transfected with siRNAs specific for FLASH (siFLASH) or firefly luciferase (siControl). After 48 h, cells were treated with APO-1 antibodies and total cell lysates were analyzed by immunoblotting.

after CD95 activation under those conditions (not shown). Even at later time points, when a clear reduction of full-size Bid was detectable, no significant difference between control and FLASH knock-down cells was observed (Figure 4G), implying that other caspase-8 substrates may be regulated by the FLASH–caspase-8 axis. Of note, immunoblot analysis of FADD and CD95 revealed no changes in their expression levels after FLASH knock-down (Supplementary Figure 2).

Interestingly, FLASH depletion more efficiently impaired caspase-8 activation at low APO-1 concentrations than at high concentrations (not shown). These findings suggest that FLASH is required to amplify weak CD95-induced death signals, which presumably reflect the physiologically relevant situation. In sum, these data suggest that FLASH and its nucleo-cytoplasmic translocation are important for CD95-mediated apoptosis.

Nucleo-cytoplasmic translocation of FLASH requires caspase activation

Our finding that endogenous FLASH shuttles from the nucleus to the cytoplasm upon CD95 activation indicates that this event is regulated by a CD95-initiated signaling pathway. To define this pathway, we inhibited CD95-induced pathways using pharmacological inhibitors and analyzed the kinetics of FLASH translocation by immunofluorescence analyses. Pretreatments with small molecule inhibitors specific for the mitogen-activated protein kinases (MAPKs) Erk (PD98059), p38 (SB203580) or JNK (SP600125) were not capable of inhibiting the redistribution of endogenous FLASH in response to CD95 ligation (data not shown), indicating that MAPK signaling is dispensable for this process. By contrast, inhibition of caspase activity by the caspase-8-specific peptide inhibitor zIETD-fmk prevented cytoplasmic translocation of FLASH (Figure 5A).

To provide independent evidence for a role of CD95-mediated caspase activation in the translocation process, we generated HT1080 cell pools stably expressing a transdominant negative FADD version (HT1080$^{\text{FADD-DN}}$; Figure 5B), which blocks CD95-induced apoptosis by interfering with procaspase-8 activation directly at the DISC (Chinnaiyan *et al*, 1996). HT1080$^{\text{FADD-DN}}$ cell pools, in contrast to HT1080$^{\text{vector}}$ control pools, were fully resistant toward CD95-induced apoptosis (data not shown). Consistent with a role of CD95-induced caspase-8 activation in the translocation process, FLASH resided in PML-NBs in HT1080$^{\text{FADD-DN}}$ cells following CD95 ligation (Figure 5C). In an attempt to further elucidate the role of caspases in FLASH translocation, we analyzed caspase-dependent cleavage of FLASH, Sp100 and PML in CD95-treated HT1080 by immunoblotting. However, neither FLASH nor Sp100 and PML were found to be cleaved during CD95-mediated apoptosis (data not shown). In sum, these data imply that caspase-8 activation at the DISC is required to trigger cytoplasmic translocation of FLASH.

FLASH is targeted to mitochondria where it colocalizes with caspase-8

FLASH has been previously identified by virtue of its interaction with the DEDs of procaspase-8 (Imai *et al*, 1999). Taking

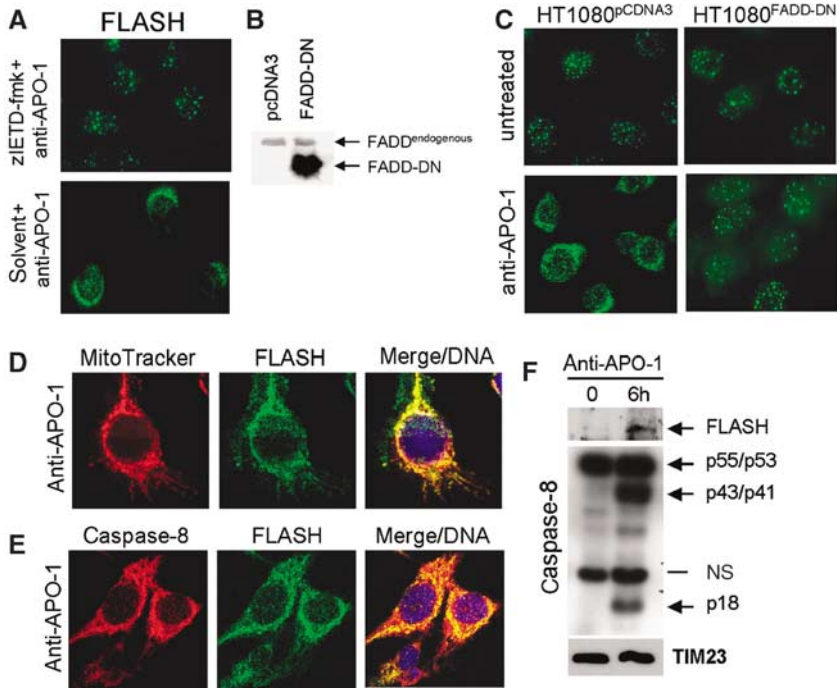

Figure 5 Requirement of CD95-induced caspase activation for translocation and mitochondrial accumulation of FLASH. (**A**) HT1080 cells were pretreated with caspase-8 inhibitor zIETD-fmk or solvent (DMSO) 1 h before CD95 activation. Localization of endogenous FLASH (green) was analyzed by confocal laser scanning microscopy. (**B**) Total lysates of HT1080$^{\text{pCDNA3}}$ and HT1080$^{\text{FADD-DN}}$ cells was analyzed by immunoblotting with FADD antibodies. (**C**) HT1080$^{\text{pCDNA3}}$ and HT1080$^{\text{FADD-DN}}$ cells untreated (upper panel) or treated for 7 h with APO-1 antibodies (lower panel) were stained for endogenous FLASH (green). (**D**) HT1080 cells treated with APO-1 antibodies for 7 h were stained with Mitotracker Red CMXRos. Localization of endogenous FLASH (green) and mitochondria (red) was analyses by confocal laser scanning microscopy. Colocalization is shown in yellow and nuclear DNA in blue. (**E**) Localization of endogenous FLASH (green) and endogenous caspase-8 (red) in HT1080 cells 7 h after CD95 activation. Colocalization is shown in yellow and nuclear DNA in blue. (**F**) Immunoblot analyses of mitochondrial fractions prepared from untreated and APO-1-treated HT1080 cells. The mitochondria-specific TIM23 protein was used as a loading control. NS; nonspecific.

this and the previously determined localization of caspase-8 at mitochondria (Stegh *et al*, 2000, 2002; Qin *et al*, 2001; Chandra *et al*, 2004) into consideration, we tested whether FLASH and caspase-8 may colocalize at these organelles after CD95 activation. In fact, staining with Mitotracker Red, a stain specific for mitochondria, confirmed that endogenous FLASH translocates to mitochondria (Figure 5D), where it partially colocalizes with endogenous caspase-8 (Figure 5E). Similar results were obtained when cells were treated with recombinant CD95 ligand (data not shown). Endogenous caspase-8 showed a similar staining pattern in untreated cells (Supplementary Figure 3) and was clearly detected in the mitochondrial fraction by immunoblotting (Figure 5F).

Immunoblotting of mitochondrial fractions also revealed that after CD95 activation the mitochondrial translocation of FLASH correlated with the proteolytic activation of mitochondria-associated caspase-8 (Figure 5F). Thus, after CD95 ligation, FLASH is translocated from the nucleus to caspase-8-rich regions at the mitochondria.

Ternary complex formation of E1B19K, FLASH and caspase-8 at the mitochondria

The adenoviral antiapoptotic Bcl-2 family member E1B19K blocks CD95-mediated apoptosis downstream of FADD and upstream of caspase-8 (Perez and White, 1998) through

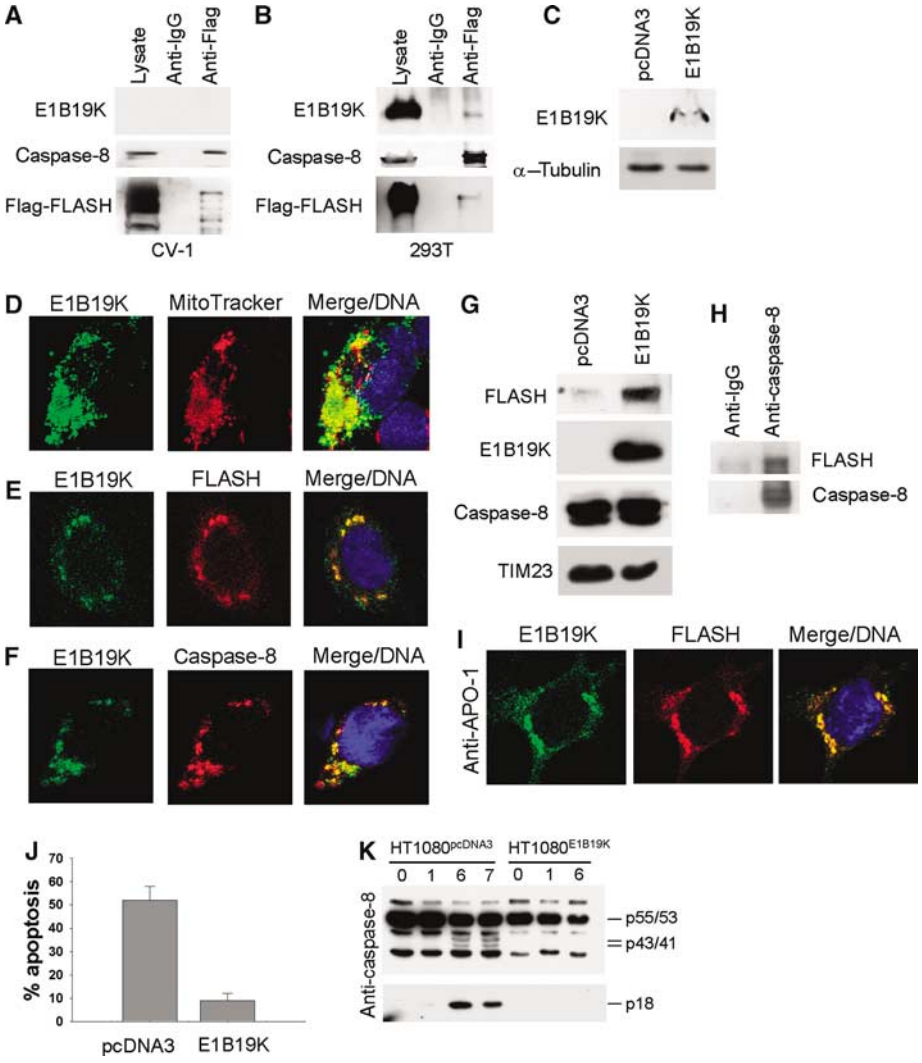

Figure 6 Ternary complex formation of E1B19K, FLASH and procaspase-8 at mitochondria. (**A**) FLASH was immunoprecipitated from lysates of CV-1 cells expressing GFP-caspase-8 C360S and Flag-FLASH using Flag antibodies and the immunecomplex was analyzed by immunoblotting. (**B**) FLASH was immunoprecipitated from lysates of 293T cells expressing E1B19K, GFP-caspase-8 C360S and Flag-FLASH using Flag antibodies and the immunecomplex was analyzed by immunoblotting. (**C**) Immunoblot analysis of stably transfected HT1080^{pcDNA3} and HT1080^{E1B19K} cells. (D–F) Localization of E1B19K, endogenous FLASH and procaspase-8 in HT1080^{E1B19K}-expressing cells. Colocalization is shown in yellow and nuclear DNA is stained in blue. (**D**) E1B19K (green) localizes predominantly to mitochondria (red) in HT1080^{E1B19K} cells. (**E**) Localization of E1B19K (green) and endogenous FLASH (red) in HT1080^{E1B19K} cells. (**F**) Localization of E1B19K (green) and endogenous caspase-8 (red) in HT1080^{E1B19K} cells. (**G**) Immunoblot analyses of mitochondrial fractions prepared from HT1080^{pcDNA3} and HT1080^{E1B19K} cells. (**H**) Caspase-8 was immunoprecipitated from the mitochondrial fraction of HT1080^{E1B19K} cells and the immunecomplex was analyzed by immunoblotting as indicated. (**I**) Localization of E1B19K and endogenous FLASH in HT1080^{E1B19K} cells 7 h after CD95 activation. (**J**) HT1080^{pcDNA3} and HT1080^{E1B19K} cells ($n = 200$) treated for 10 h with 100 ng/ml anti-APO-1 antibodies were analyzed with Mitotracker Red CM-H_2XRos staining and DAPI staining for apoptosis-induced mitochondrial transmembrane potential breakdown and DNA condensation. Standard deviations from three independent experiments are shown. (**K**) Caspase-8 immunoblot of total cell lysates from HT1080^{pcDNA3} and HT1080^{E1B19K} cells treated with 100 ng/ml APO-1 antibodies as indicated.

association with FLASH (Imai *et al*, 1999) by a not yet determined mechanism.

Owing to use of adenoviral immortalized 293 cells, it remained unclear whether the interaction of caspase-8 and FLASH can occur in the absence of E1B19K (Imai *et al*, 1999). To investigate whether complex formation of FLASH and caspase-8 may require E1B19K, we performed a similar experiment in CV-1 cells. CV-1 cells, in contrast to 293T cells, are not adenovirally transformed and therefore lack any E1B19K expression. Importantly, caspase-8 was co-immunoprecipitated with FLASH from CV-1 cell lysates (Figure 6A), demonstrating that FLASH–caspase-8 interaction is independent of E1B19K.

In order to test whether E1B19K may interfere with the interaction of FLASH and procaspase-8, we performed co-immunoprecipitation experiments. To avoid apoptosis initiation by coexpression of FLASH and caspase-8 (Imai *et al*, 1999), we used proteolytically inactive caspase-8, which contains the C360S active site point mutation. Interestingly, caspase-8 co-immunoprecipitated with FLASH in the presence of E1B19K (Figure 6B). Moreover, the presence of caspase-8 and E1B19K in the FLASH immunecomplexes indicated ternary complex formation of E1B19K, FLASH and caspase-8.

To gain more insight into the molecular mechanism of how E1B19K prevents CD95-mediated apoptosis, we established HT1080 cells stably expressing E1B19K ($HT1080^{E1B19K}$; Figure 6C). It has been previously reported that a large fraction of E1B19K localizes to mitochondria during adenovirus infection (Lomonosova *et al*, 2005). Concordantly, confocal microscopy detected stably expressed E1B19K predominantly at the mitochondria (Figure 6D), indicating a physiological distribution of E1B19K. Remarkably, E1B19K redistributed endogenous FLASH from the nucleus to mitochondria (Figure 6E), where it colocalized with endogenous caspase-8 (Figure 6F). By contrast, in $HT1080^{pcDNA3}$ control cells, FLASH showed a comparable predominantly nuclear distribution to that found in parental HT1080 cells (data not shown). Consistent with our immunofluorescence data, FLASH, E1B19K and caspase-8 were also detected in the mitochondrial fractions of $HT1080^{E1B19K}$ cells by immunoblotting (Figure 6G). Co-immunoprecipitation analysis of mitochondrial fractions further confirmed complex formation of FLASH and caspase-8 at the mitochondria in $HT1080^{E1B19K}$ cells (Figure 6H).

Colocalization of E1B19K and FLASH was still detectable after CD95 activation (Figure 6I), suggesting that the E1B19K–FLASH–caspase-8 protein complex remains stable after CD95 ligation. Moreover, $HT1080^{E1B19K}$ cells were protected against CD95-mediated apoptosis (Figure 6J) and failed to activate caspase-8, as evident by the absence of the p18 proteolytic cleavage fragment (Figure 6K). Taken together, these findings imply that E1B19K interferes with caspase-8 activation at the mitochondria by trapping FLASH and procaspase-8 in a ternary complex.

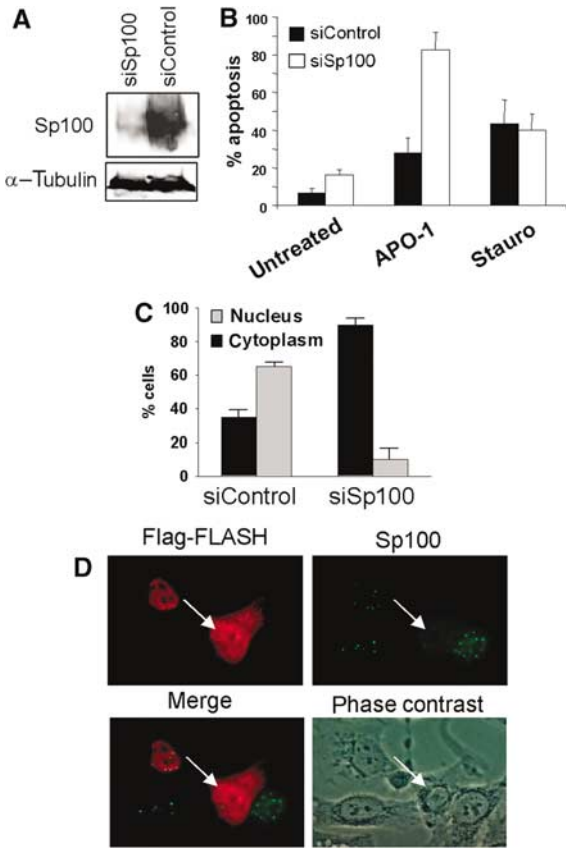

Figure 7 Sp100 depletion potentiates CD95-mediated apoptosis. (**A**) HT1080 cells transfected with pSUPER-Luci (siControl) or pSUPER-Sp100 (siSp100) plasmids were analyzed by immunoblotting 36 h post transfection with the antibodies indicated. (**B**) HT1080 cells were transfected with pSUPER-Luci or pSUPER-Sp100 along with a pEGFP vector. Thirty-six hours later, cells were treated with anti-APO-1 (20 ng/ml) or staurosporine (1 μg/ml) for 19 h as indicated or left untreated. Apoptosis in GFP expressing cells was assayed. Standard deviations of three independent experiments are shown. (**C**) HT1080 cells were transfected with Flag-FLASH and pSUPER-Luci or pSUPER-Sp100 constructs as indicated. The percentage of cells showing nuclear and NB-associated (nucleus) versus cytoplasmic FLASH (cytoplasm) is given with standard deviations from three independent experiments. (**D**) Endogenous Sp100 (green) and Flag-FLASH (red) were stained 36 h after transfection with Flag-FLASH and pSUPER-Sp100. The arrow indicates an Sp100-depleted cell with increased cytoplasmic distribution of FLASH.

Sp100 knock-down sensitizes for CD95-mediated apoptosis

Our findings that FLASH interacts and partially colocalizes with Sp100 in nuclear bodies prompted us to analyze whether Sp100 was capable of regulating CD95-mediated apoptosis. To this end, we knocked down endogenous Sp100 expression through RNA interference (Figure 7A) and measured apoptosis induction in response to CD95 activation. Potentiation of CD95-mediated apoptosis was observed specifically after Sp100 knock-down (Figure 7B). In contrast, reduced Sp100 levels did not sensitize the cells for staurosporine-induced apoptosis (Figure 7B), which utilizes an intrinsic apoptosis pathway via the proapoptotic Bcl-2 family members Bax and Bak (Wei *et al*, 2001).

To learn more about the mechanism of how Sp100 depletion affects CD95-mediated apoptosis, we analyzed the subcellular localization of FLASH upon Sp100 downregulation using fluorescence microscopy. Knock-down of Sp100 led to a significant increase in the proportion of cytoplasmically localized FLASH (Figure 7C and D), which correlated with the activation of its apoptotic function (data not shown). These results suggest that Sp100 depletion specifically sensi-

tizes cells for CD95-mediated apoptosis through enhancing nucleo-cytoplasmic translocation of FLASH.

Discussion

Our data presented here elucidate a novel CD95 signaling pathway and imply that CD95 signaling is coupled to a nuclear pathway via nucleo-cytoplasmic translocation of FLASH. This signaling pathway finally impinges on mitochondria where FLASH meets caspase-8.

FLASH is a nuclear body constituent

Visualization of FLASH at endogenous expression levels by confocal microscopy revealed a predominant distribution to the cell nucleus and NBs. Consistent with our data identifying FLASH as an interaction partner for Sp100, a well-established constitutive PML-NB component, we observed colocalization of FLASH and Sp100 in the PML-NBs. Interestingly, FLASH-NBs only partially overlapped with PML-NBs and endogenous FLASH was also capable of forming NBs independent of PML expression in PML−/− MEFs (data not shown). These results argue that FLASH, like most proteins, is a dynamic PML-NB component, rather than a constitutive one. Very recently, FLASH has been identified as a Cajal body component (Barcaroli *et al*, 2006b) and was shown to regulate progression through S-phase of the cell cycle in normal dividing cells (Barcaroli *et al*, 2006a). These findings suggest that Sp100-negative FLASH-NBs are Cajal bodies, implying that FLASH provides a link between both nuclear domains. The mechanisms regulating PML-NB and Cajal body association of FLASH remain to be clarified.

FLASH exits nuclear bodies and the nucleus after CD95 activation

In response to CD95 activation, endogenous FLASH was mobilized from NBs and the nucleus and translocated into the cytoplasm. This process was facilitated through the Crm1-dependent nuclear export pathway. Such a shuttling mechanism is consistent with previous reports describing both nuclear localization signals and a nuclear export signal in the amino-acid sequence of FLASH (Imai *et al*, 1999; Koonin *et al*, 1999). The cytoplasmic translocation of FLASH required CD95-mediated caspase activation. Concordantly, inhibition of caspase-8 activation by a synthetic peptide as well as by stable expression of a transdominant negative version of FADD (FADD-DN), which prevents caspase-8 activation at the DISC (Chinnaiyan *et al*, 1996), blocked FLASH translocation. As neither FLASH nor Sp100 and PML were found to be proteolytically processed in a caspase-dependent manner in response to CD95 activation (data not shown), the detailed mechanism of how caspase activity impacts on FLASH translocation remains to be elucidated.

FLASH and caspase-8 at the mitochondria

FLASH was originally identified as a caspase-8 interacting protein capable of enhancing CD95-mediated caspase-8 activation (Imai *et al*, 1999). Later reports demonstrated association of a significant fraction of endogenous caspase-8 with mitochondria, where it is accessible for activation after CD95 ligation (Stegh *et al*, 2000, 2002; Qin *et al*, 2001). The mechanism governing activation of mitochondria-associated caspase-8 remained unclear. Our data here propose a role of FLASH, which is targeted from NBs and the nucleus to caspase-8-rich regions at the mitochondria, in this process. In line with this hypothesis, blocking of Crm1-mediated nucleo-cytoplasmic export with LMB, which inhibits FLASH translocation, strongly inhibited CD95-mediated apoptosis. Our concept was further substantiated by our finding that downregulation of FLASH impaired CD95-induced caspase-8 activation and apoptosis induction. Remarkably, LMB blocked CD95-mediated apoptosis to a similar extent as depletion of endogenous FLASH by RNA interference, suggesting a role of nucleo-cytoplasmic FLASH translocation in CD95-mediated apoptosis. However, we cannot rule out the possibility that also other factors released from the nucleus play a role in CD95 signaling.

E1B19K traps FLASH and caspase-8 at the mitochondria

Previously, complex formation of FLASH with the adenoviral antiapoptotic Bcl-2 family member E1B19K has been shown, thus providing a possible explanation for its inhibitory effect on CD95-induced caspase-8 activation (Perez and White, 1998; Imai *et al*, 1999). However, the molecular mechanism of how E1B19K inhibits CD95-induced apoptosis remained unknown. In addition, owing to use of adenoviral immortalized 293 cells, it remained unclear whether the interaction of caspase-8 and FLASH can occur in the absence of adenoviral E1B19K (Imai *et al*, 1999). Our results here clearly demonstrate that complex formation of FLASH and caspase-8 is independent of E1B19K. Moreover, our data indicate that E1B19K does not interfere with FLASH–caspase-8 interaction, but instead forms a ternary complex with these factors. Analysis of the subcellular distribution of this protein complex revealed that E1B19K traps endogenous FLASH and procaspase-8 at the mitochondria. Importantly, a large fraction of E1B19K localizes to mitochondria during adenovirus infection (Lomonosova *et al*, 2005), which is indicative of a physiological subcellular distribution of E1B19K in our cell system. As CD95-induced caspase-8 proteolysis and apoptosis were blocked in E1B19K-expressing cells, and the E1B19K-FLASH complex remained stable after CD95 activation, we conclude that E1B19K interferes with caspase-8 activation at the mitochondria.

Although a recent report suggested an association of FLASH with the CD95 DISC (Imai *et al,* 1999), we failed to detect FLASH, in contrast to FADD and caspase-8 (data not shown), in the DISC of HT1080 cells by using the classical DISC precipitation protocol (Kischkel *et al*, 1995). This discrepancy may be explained by a different DISC precipitation protocol used by Imai *et al* (1999). Although our data cannot exclude DISC recruitment of small amounts of FLASH (below the detection limit of the antibodies used here), our results imply that the major fraction of FLASH promotes caspase-8 activation at the mitochondria.

Model for the role of FLASH in CD95 signaling

In conclusion, our data support a model that DISC-activated caspase-8 facilitates a feedforward loop leading to Crm1-mediated nucleo-cytoplasmic translocation of FLASH in response to CD95 ligation (Figure 8). Cytoplasmic FLASH is targeted to mitochondria, where it can form a molecular complex with caspase-8, thereby presumably activating the mitochondrial apoptosis pathway by regulating caspase-8 activity. The adenoviral Bcl-2 homolog E1B19K can interfere

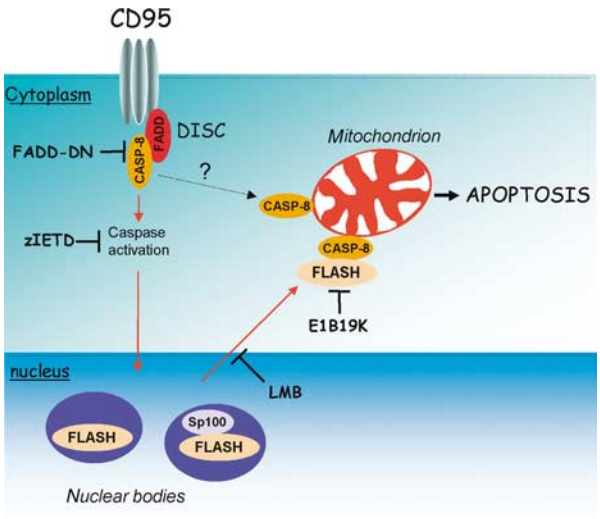

Figure 8 Model for the role of FLASH in the CD95 signaling pathway. In response to CD95 activation, DISC-activated caspase-8 triggers a feedforward loop triggering FLASH mobilization from nuclear bodies and its Crm1-dependent nucleo-cytoplasmic translocation. Cytoplasmic FLASH accumulates at the mitochondria, where it interacts with mitochondria-associated caspase-8, thereby regulating its proteolytic activation. CD95-induced FLASH translocation is blocked by FADD-DN expression, by caspase-8 inhibition through zIETD-fmk and by treatment with the nuclear export inhibitor leptomycin B (LMB). E1B19K inhibits CD95-induced caspase-8 activation through trapping FLASH and caspase-8 in a ternary complex at the mitochondria.

with CD95-induced apoptosis through complex formation with FLASH and caspase-8 at the mitochondria. Collectively, our data provide evidence that CD95 signals apoptosis via a nuclear amplification pathway. As apoptosis induction by other death receptors also depends on caspase-8 activation (Varfolomeev *et al*, 1998), the signaling pathway discovered here may also be relevant for other death receptor systems.

Materials and methods

Cell culture and transfections

HT1080, 293T cells (both from ATCC) and CV-1 cells were maintained in DMEM/10% FCS/1% (w/v) penicillin/streptomycin/20 mM HEPES. Transient transfections were carried out using FuGene 6 (Roche Molecular Biochemicals), standard calcium phosphate precipitation or HiPerFect (Qiagen). $HT1080^{E1B19K}$, $HT1080^{FADD\text{-}DN}$ and $HT1080^{pcDNA3}$ stable cell lines were generated by selecting transfected cells with 850 μg/ml G418 (Gibco) and establishing cell pools.

Yeast two-hybrid screening

The cDNA encoding the N-terminal 295 amino acids of human Sp100 was cloned into vector pFBL32, transformed into L40 yeast and tested for reporter auto-transactivation and cytotoxicity. A human Matchmaker B-cell cDNA library (Clontech) was screened. Positive clones were identified using selective media and lacZ reporter gene activation. Preys were isolated from yeast and subsequently retransformed with bait or empty vector to confirm the specificity of these interactions. Prey cDNAs were sequenced and BLAST (NCBI) searches were performed with the nucleotide sequence obtained.

Expression plasmids and antibodies

Sp100 expression vectors were previously described (Sternsdorf *et al*, 1999). FLASH expression vectors were kind gifts from Y Imai and YH Choi. GFP-caspase-8 (C360S) vector was kindly provided by MJ Lenardo, AU1-FADD and FADD-DN by C Vincenz and GFP-Rev by J Hauber. E1B19K plasmid and antibodies were a gift from E White. Flag (M2) and the α-tubulin (clone B-5-1-2) antibodies were purchased from Sigma, HA (12CA5) from Roche, caspase-8 (12F5) from Apotech, PARP and FADD antibodies from BD Transduction Laboratories, BID from Cell Signaling and PML (PG-M3), FLASH (M-300), GAPDH (V-18), CD95 and caspase-8 (C-20) antibodies were from Santa Cruz Biotechnology. Rabbit and rat Sp100 antibodies were previously described (Sternsdorf *et al*, 1997). APO-1 and caspase-8 (C1 and C15) antibodies (Scaffidi *et al*, 1997) were generous gifts from PH Krammer. The rabbit FLASH antibody (FLASH-522) was generated by immunizing rabbits with the indicated peptide coupled to KLH: NH_2-CLEKEGKPHSDKRSTS-CO_2H (aa 198–212 of the human FLASH). The sera were affinity purified against the peptide before use.

Immunofluorescence microscopy

Immunofluorescence stainings were performed as described (Hofmann *et al*, 2002). The following primary antibodies were used: mouse Flag (M2, Sigma), rabbit AU1 (Covance Research Products), mouse PML (PG-M3), rabbit and rat Sp100 (Sternsdorf *et al*, 1997), rabbit FLASH (M-300 and own rabbit antibody), mouse caspase-8 C1 and mouse and rabbit E1B19K. The secondary antibodies used were Alexa-488-coupled goat anti-mouse and Alexa-594-coupled goat anti-rabbit (Molecular Probes). Mitochondria were stained with Mitotracker Red CMXRos or with Mitotracker Red CM-H_2XRos (both from Molecular Probes) as indicated 30 min before cell fixation. DNA was visualized by Draq5 (Apotech), which was false colored in blue. LMB was a kind gift from M Yoshida. Cells were examined using a confocal laser scanning microscope (LSM 510 META, Zeiss) with a $\times 63$ oil objective. All images were collected and processed using the AxioVision software (Zeiss) and sized in Adobe Photoshop 7.0.

Subcellular fractionations

For nuclear and cytoplasmic fractionation, 3×10^7 HT1080 cells were resuspended in hypotonic buffer (250 mM sucrose, 10 mM KCl, 20 mM HEPES, pH 7.5, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA and 1 mM DTT) supplemented with complete protease inhibitor mix (Roche), incubated for 15 min on ice and lysed using a dounce homogenizer (lysis was about 85–95% as judged by Trypan blue staining of aliquots). Subsequently, nuclei and cell debris were pelleted for 10 min at 800 *g* and supernatants containing the cytoplasmic fraction (including mitochondria and other cellular organelles) were collected. Equal amounts of nuclear and cytoplasmic fractions of cells harvested at the indicated time points were analyzed by immunoblotting with the antibodies indicated. PARP (a nuclear-localized caspase substrate) and GAPDH (cytoplasmic protein) were used as markers for the purity of the fractions generated by differential centrifugations. Mitochondrial fractions were isolated at the time points indicated using dounce homogenization as published previously (Vander Heiden *et al*, 1997).

RNA interference

For RNA interference, the following targeting sequences were inserted into the pSUPER vector (Brummelkamp *et al*, 2002) to knock down human Sp100 or FLASH, or dsRNA against the same regions synthesized by Dharmacon was used. The following target sequences were used: Sp100:

5′-TGCGACTGGTGGATATAAA-3′; FLASH: 5′-GATTGTCTGAGTTT CCACA-3′. For the control experiments, an siRNA targeting GL2 firefly luciferase

(5′-CGTACGCGGAATACTTCGA-3′) was used (Elbashir *et al*, 2001). All siRNA sequences were verified to confirm their specificity to the respective target mRNA.

Immunoprecipitation and immunoblotting

293T cells were lysed in buffer containing 300 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM EDTA, 0.5% NP-40 and protease inhibitors (Roche). For immunoprecipitation, mouse Flag (M2) or rabbit Sp100 (SpGH) antibodies were used with protein-A/G-coupled Sepharose beads (Santa Cruz Biotechnology). After incubation at 4°C on a rotating wheel, the beads were washed three times in NP-40 buffer (150 mM NaCl, 50 mM HEPES, pH 7.5, 5 mM EDTA and 0.5% NP-40). Immunoblotting was performed as described (Hofmann *et al*, 2002). For co-immunoprecipitation of endogenous FLASH and Sp100, cells were lysed in buffer containing 0.5% SDS, 1% NP-40, 300 mM NaCl, 50 mM HEPES pH 7.5 and 5 mM EDTA

supplemented with protease inhibitors (Roche), sonified, centrifuged and supernatants were diluted with SDS-free buffer to a final concentration of 0.1% SDS. Immunoprecipitation was performed using either 5 μg rabbit IgG control antibodies or Sp100 GH3 rabbit antibodies and protein-A/G-coupled Sepharose beads. Beads were washed three times in cold PBS and analyzed by immunoblotting. For co-immunoprecipitation of endogenous FLASH and caspase-8 from mitochondrial fractions, 450 μg of cleared lysates of isolated mitochondria lysed in 1% CHAPS, 150 mM NaCl, 50 mM HEPES pH 7.5 and 5 mM EDTA supplemented with protease inhibitors was used. Immunoprecipitations were performed using 10 μg mouse IgG antibodies or caspase-8 antibodies (12F5) and protein-A/G-coupled Sepharose beads.

APO-1 treatment and apoptosis measurement

APO-1 treatment was performed as described (Kischkel *et al*, 1995). If not directly indicated in the figure legend, cells were treated with 25 ng/ml APO-1 antibody and 1 ng/ml protein A for crosslinking. CD95L treatment was performed using Flag-tagged CD95L (Apotech). Apoptosis was determined by analyzing the loss of mitochondrial membrane potential, which is indicated by strongly diminished staining of cells with Mitotracker Red CM-H_2XRos (Molecular Probes) under a fluorescence microscope, and in addition by analyzing apoptosis-associated chromatin condensation and changes in nuclear morphology using DNA staining and fluorescence microscopy, as described (Hofmann *et al*, 2002). FACS-based apoptosis determination by calculating the percentage of hypoploid cells (sub-G_1 DNA content) was carried out as published (Hofmann *et al*, 2000). For caspase inhibition, cells were preincubated for 1 h using 50 μM zIETD-fmk (Calbiochem) before CD95 ligation.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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References

Barcaroli D, Bongiorno-Borbone L, Terrinoni A, Hofmann TG, Rossi M, Knight RA, Matera AG, Melino G, De Laurenzi V (2006a) FLASH is required for histone transcription and S-phase progression. *Proc Natl Acad Sci USA* **103:** 14808–14812

Barcaroli D, Dinsdale D, Neale MH, Bongiorno-Borbone L, Ranalli M, Munarriz E, Sayan AE, McWilliam JM, Smith TM, Fava E, Knight RA, Melino G, De Laurenzi V (2006b) FLASH is an essential component of Cajal bodies. *Proc Natl Acad Sci USA* **103:** 14802–14807

Bernardi R, Pandolfi PP (2003) Role of PML and the PML-nuclear body in the control of programmed cell death. *Oncogene* **22:** 9048–9057

Boldin MP, Goncharov TM, Goltsev YV, Wallach D (1996) Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* **85:** 803–815

Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296:** 550–553

Chandra D, Choy G, Deng X, Bhatia B, Daniel P, Tang DG (2004) Association of active Caspase 8 with the mitochondrial membrane during apoptosis: potential roles in cleaving BAP31 and Caspase 3 and mediating mitochondrion–endoplasmic reticulum cross talk in etoposide-induced cell death. *Mol Cell Biol* **24:** 6592–6607

Chinnaiyan AM, Tepper CG, Seldin MF, O'Rourke K, Kischkel FC, Hellbardt S, Krammer PH, Peter ME, Dixit VM (1996) FADD/MORT1 is a common mediator of CD95 (Fas/APO-1) and tumor necrosis factor receptor-induced apoptosis. *J Biol Chem* **271:** 4961–4965

Cuconati A, White E (2002) Viral homologs of BCL-2: role of apoptosis in the regulation of virus infection. *Genes Dev* **16:** 2465–2478

Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411:** 494–498

Everett RD (2006) Interactions between DNA viruses, ND10 and the DNA damage response. *Cell Microbiol* **8:** 365–374

Fischer U, Janicke RU, Schulze-Osthoff K (2003) Many cuts to ruin: a comprehensive update of caspase substrates. *Cell Death Differ* **10:** 76–100

Fukuda M, Asano S, Nakamura T, Adachi M, Yoshida M, Yanagida M, Nishida E (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **390:** 308–311

Hofmann TG, Hehner SP, Droge W, Schmitz ML (2000) Caspase-dependent cleavage and inactivation of the Vav1 proto-oncogene product during apoptosis prevents IL-2 transcription. *Oncogene* **19:** 1153–1163

Hofmann TG, Moller A, Sirma H, Zentgraf H, Taya Y, Droge W, Will H, Schmitz ML (2002) Regulation of p53 activity by its interaction with homeodomain-interacting protein kinase-2. *Nat Cell Biol* **4:** 1–10

Hofmann TG, Will H (2003) Body language: the function of PML nuclear bodies in apoptosis regulation. *Cell Death Differ* **10:** 1290–1299

Imai Y, Kimura T, Murakami A, Yajima N, Sakamaki K, Yonehara S (1999) The CED-4-homologous protein FLASH is involved in Fas-mediated activation of caspase-8 during apoptosis. *Nature* **398:** 777–785

Irmler M, Thome M, Hahne M, Schneider P, Hofmann K, Steiner V, Bodmer JL, Schroter M, Burns K, Mattmann C, Rimoldi D, French LE, Tschopp J (1997) Inhibition of death receptor signals by cellular FLIP. *Nature* **388:** 190–195

Ishov AM, Sotnikov AG, Negorev D, Vladimirova OV, Neff N, Kamitani T, Yeh ET, Strauss III JF, Maul GG (1999) PML is critical for ND10 formation and recruits the PML-interacting protein Daxx to this nuclear structure when modified by SUMO-1. *J Cell Biol* **147:** 221–234

Kino T, Chrousos GP (2003) Tumor necrosis factor alpha receptor- and Fas-associated FLASH inhibit transcriptional activity of the glucocorticoid receptor by binding to and interfering with its interaction with p160 type nuclear receptor coactivators. *J Biol Chem* **278:** 3023–3029

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J* **14:** 5579–5588

Koonin EV, Aravind L, Hofmann K, Tschopp J, Dixit VM (1999) Apoptosis. Searching for FLASH domains. *Nature* **401:** 662; discussion 662–663

Krammer PH (2000) CD95's deadly mission in the immune system. *Nature* **407:** 789–795

Lavrik I, Golks A, Krammer PH (2005) Death receptor signaling. *J Cell Sci* **118:** 265–267

Lomonosova E, Subramanian T, Chinnadurai G (2005) Mitochondrial localization of p53 during adenovirus infection and regulation of its activity by E1B-19K. *Oncogene* **24:** 6796–6808

Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* **85:** 817–827

Nagata S (1999) Fas ligand-induced apoptosis. *Annu Rev Genet* **33:** 29–55

Negorev D, Maul GG (2001) Cellular proteins localized at and interacting within ND10/PML nuclear bodies/PODs suggest functions of a nuclear depot. *Oncogene* **20:** 7234–7242

Obradovic D, Tirard M, Nemethy Z, Hirsch O, Gronemeyer H, Almeida OF (2004) DAXX, FLASH, and FAF-1 modulate mineralocorticoid and glucocorticoid receptor-mediated transcription in hippocampal cells—toward a basis for the opposite actions elicited by two nuclear receptors? *Mol Pharmacol* **65:** 761–769

Perez D, White E (1998) E1B 19K inhibits Fas-mediated apoptosis through FADD-dependent sequestration of FLICE. *J Cell Biol* **141:** 1255–1266

Peter ME, Krammer PH (2003) The CD95(APO-1/Fas) DISC and beyond. *Cell Death Differ* **10:** 26–35

Qin ZH, Wang Y, Kikly KK, Sapp E, Kegel KB, Aronin N, DiFiglia M (2001) Pro-caspase-8 is predominantly localized in mitochondria and released into cytoplasm upon apoptotic stimulation. *J Biol Chem* **276:** 8079–8086

Scaffidi C, Fulda S, Srinivasan A, Friesen C, Li F, Tomaselli KJ, Debatin KM, Krammer PH, Peter ME (1998) Two CD95 (APO-1/Fas) signaling pathways. *EMBO J* **17:** 1675–1687

Scaffidi C, Medema JP, Krammer PH, Peter ME (1997) FLICE is predominantly expressed as two functionally active isoforms, caspase-8/a and caspase-8/b. *J Biol Chem* **272:** 26953–26958

Stegh AH, Barnhart BC, Volkland J, Algeciras-Schimnich A, Ke N, Reed JC, Peter ME (2002) Inactivation of caspase-8 on mitochondria of Bcl-xL-expressing MCF7-Fas cells: role for the bifunctional apoptosis regulator protein. *J Biol Chem* **277:** 4351–4360

Stegh AH, Herrmann H, Lampel S, Weisenberger D, Andra K, Seper M, Wiche G, Krammer PH, Peter ME (2000) Identification of the cytolinker plectin as a major early *in vivo* substrate for caspase 8 during CD95- and tumor necrosis factor receptor-mediated apoptosis. *Mol Cell Biol* **20:** 5665–5679

Sternsdorf T, Jensen K, Reich B, Will H (1999) The nuclear dot protein Sp100: characterization of domains necessary for dimerization, subcellular localization and modification by small ubiquitin-like modifiers. *J Biol Chem* **274:** 12555–12566

Sternsdorf T, Jensen K, Will H (1997) Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *J Cell Biol* **139:** 1621–1634

Takahashi Y, Lallemand-Breitenbach V, Zhu J, de The H (2004) PML nuclear bodies and apoptosis. *Oncogene* **23:** 2819–2824

Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB (1997) Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* **91:** 627–637

Varfolomeev EE, Schuchmann M, Luria V, Chiannilkulchai N, Beckmann JS, Mett IL, Rebrikov D, Brodianski VM, Kemper OC, Kollet O, Lapidot T, Soffer D, Sobe T, Avraham KB, Goncharov T, Holtmann H, Lonai P, Wallach D (1998) Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* **9:** 267–276

Wang X (2001) The expanding role of mitochondria in apoptosis. *Genes Dev* **15:** 2922–2933

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292:** 727–730

Zhong S, Müller S, Ronchetti S, Freemont PS, Dejean A, Pandolfi PP (2000) Role of SUMO-1-modified PML in nuclear body formation. *Blood* **95:** 2748–2752