

# PKC-mediated phosphorylation regulates c-FLIP ubiquitylation and stability

A Kaunisto<sup>1,2</sup>, V Kochin<sup>1,3,5</sup>, T Asaoka<sup>1,3,5</sup>, A Mikhailov<sup>4,6</sup>, M Poukkula<sup>1,2,7</sup>, A Meinander<sup>1,3,8</sup> and JE Eriksson<sup>\*,1,3</sup>

Cellular FLICE-inhibitory protein (c-FLIP) proteins are crucial regulators of the death-inducing signaling complex (DISC) and caspase-8 activation. To date, three c-FLIP isoforms with distinct functions and regulation have been identified. Our previous studies have shown that the stability of c-FLIP proteins is subject to isoform-specific regulation, but the underlying molecular mechanisms have not been known. Here, we identify serine 193 as a novel *in vivo* phosphorylation site of all c-FLIP proteins and demonstrate that S193 phosphorylation selectively influences the stability of the short c-FLIP isoforms, as S193D mutation inhibits the ubiquitylation and selectively prolongs the half-lives of c-FLIP short (c-FLIP<sub>S</sub>) and c-FLIP Raji (c-FLIP<sub>R</sub>). S193 phosphorylation also decreases the ubiquitylation of c-FLIP long (c-FLIP<sub>L</sub>) but, surprisingly, does not affect its stability, indicating that S193 phosphorylation has a different function in c-FLIP<sub>L</sub>. The phosphorylation of this residue is operated by the protein kinase C (PKC), as S193 phosphorylation is markedly increased by treatment with 12-O-tetradecanoylphorbol-13-acetate and decreased by inhibition of PKC $\alpha$  and PKC $\beta$ . S193 mutations do not affect the ability of c-FLIP to bind to the DISC, although S193 phosphorylation is increased by death receptor stimulation. Instead, S193 phosphorylation affects the intracellular level of c-FLIP<sub>S</sub>, which then determines the sensitivity to death-receptor-mediated apoptosis. These results reveal that the differential stability of c-FLIP proteins is regulated in an isoform-specific manner by PKC-mediated phosphorylation.

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Apoptosis is a crucial instrument of cellular homeostasis that enables organisms to remove damaged and potentially dangerous cells (reviewed by Degterev and Yuan<sup>1</sup>). Apoptosis is induced by a plethora of physiological and pathological stimuli that activate the apoptotic machinery in a stimulus-specific manner. Stresses such as DNA damage and cytokine deprivation activate the intrinsic apoptotic pathway, leading to the disruption of the mitochondrial membrane potential and the formation of a caspase-activating complex, apoptosome.<sup>2</sup> In contrast, the extrinsic pathway is induced by the binding of death ligands to their cognate death receptors at the cell surface. Activated death receptors oligomerize, thereby inducing the formation of the death-inducing signaling complex (DISC) on their intracellular parts.<sup>3</sup> The DISC recruits caspase-8 molecules that dimerize and activate each other forming an active tetramer,<sup>4–7</sup> the release of which initiates the caspase cascade causing the proteolysis of hundreds of target proteins and eventually cell death (reviewed by Danial and Korsmeyer<sup>8</sup>).

Cellular FLICE-inhibitory protein (c-FLIP) is a caspase-8 homolog that binds to the DISC and regulates the recruitment and activation of procaspase-8.<sup>9</sup> Three c-FLIP splice variants

have been detected at the protein level, namely c-FLIP long (c-FLIP<sub>L</sub>), c-FLIP short (c-FLIP<sub>S</sub>), and c-FLIP Raji (c-FLIP<sub>R</sub>).<sup>9,10</sup> All c-FLIP proteins share their N-terminal 202 amino acids. The C-terminal part of c-FLIP<sub>L</sub> comprises an inactive caspase-like domain, whereas c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub> only contain short C-terminal splicing tails. In the DISC, the c-FLIP<sub>L</sub>–caspase-8 heterodimer is cleaved into p43/41 and p12/10 fragments, but as the second cleavage cannot occur, no active caspase-8 is released.<sup>11,12</sup> In contrast, the short c-FLIP isoforms remain uncleaved in the DISC.

Protein kinase C (PKC) is a family of serine/threonine protein kinases well known for their tumor-promoting abilities (reviewed by Griner and Kazanietz<sup>13</sup>). The PKC isozymes are classified into classical, novel, and atypical subgroups, according to activating stimuli. PKCs have versatile biological functions including differentiation and cell-cycle progression, as well as apoptosis.<sup>14,15</sup> For example, PKC $\alpha$  and PKC $\beta$  have been found to interfere with the formation of the Fas DISC in Jurkat T cells by blocking FADD recruitment and subsequent caspase-8 activation.<sup>16,17</sup> Inhibition of DISC formation by PKC activation has also been observed in HeLa cells during tumor necrosis factor (TNF)-related apoptosis-inducing ligand

<sup>1</sup>Turku Centre for Biotechnology, University of Turku and Åbo Akademi University, FIN-20521, Turku, Finland; <sup>2</sup>Department of Biology, University of Turku, FIN-20014, Turku, Finland; <sup>3</sup>Department of Biology, Åbo Akademi University, FIN-20520, Turku, Finland and <sup>4</sup>Department of Biochemistry and Pharmacy, Åbo Akademi University, FIN-20520, Turku, Finland

\*Corresponding author: JE Eriksson, Department of Biology, Åbo Akademi University, Tykistökatu 6 A, FIN-20520, Turku, Finland. Tel: +358 2 215 3313;

Fax: +358 2 215 4748; E-mail: jeriksso@abo.fi

<sup>5</sup>These authors contributed equally to this work.

<sup>6</sup>Current address: School of Medicine, University of Tampere, FIN-33520, Tampere, Finland

<sup>7</sup>Current address: Temasek Life Sciences Laboratory, Singapore

<sup>8</sup>Current address: The Breakthrough Toby Robins Breast Cancer Research Centre, Institute of Cancer Research, London SW3 6JB, UK

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**Abbreviations:** c-FLIP, cellular FLICE-inhibitory protein; CHX, cycloheximide; DISC, death-inducing signaling complex; PKC, protein kinase C; TNF $\alpha$ , tumor necrosis factor  $\alpha$ ; TPA, 12-O-tetradecanoylphorbol-13-acetate; TRAIL, TNF-related apoptosis-inducing ligand

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(TRAIL)-induced apoptosis.<sup>18</sup> However, because these effects were reported not to be mediated by FADD phosphorylation, the specific mechanism how PKC operates in this context remains unclear.

We have previously shown that the ubiquitylation and proteasomal degradation of c-FLIP is isoform specific.<sup>19</sup> Here, we show that all c-FLIP proteins are phosphorylated on S193 in a PKC-regulated manner. Phosphorylation of S193 was markedly enhanced by 12-O-tetradecanoylphorbol-13-acetate (TPA), an inducer of the classical PKC. Conversely, expression of kinase-dead PKC $\alpha$  and PKC $\beta$  mutants, as well as treatment with pharmacological inhibitors of these kinases, leads to decreased S193 phosphorylation. Importantly, phosphorylation regulates the ubiquitylation of all c-FLIP proteins and selectively affects the stability of the short isoforms. Furthermore, these changes in stability are reflected in sensitivity to death-receptor-mediated apoptosis. Our results demonstrate that although S193 phosphorylation *per se* does not dictate the anti-apoptotic potential of c-FLIP, it specifically affects the half-lives of the short isoforms, thereby connecting PKC to DISC signaling in a previously undescribed manner.

## Results

**c-FLIP proteins are phosphorylated on S193.** Because c-FLIP is a critical regulator of death-receptor-mediated apoptosis, we aimed at understanding how it is modified posttranslationally. Although we showed earlier that the levels of c-FLIP are determined by ubiquitylation, others have reported that c-FLIP is modulated by phosphorylation.<sup>19–22</sup> Hence, we studied how these posttranslational modifications regulate c-FLIP in concert.

To investigate c-FLIP phosphorylation, we performed *in vivo* <sup>32</sup>P labeling of K562 cells stably overexpressing Flag-c-FLIP<sub>S</sub> and Flag-c-FLIP<sub>L</sub> treated with type 1 and type 2A phosphatase inhibitor calyculin A to boost the signal of individual phosphorylation sites. After immunoprecipitating the exogenous c-FLIP with anti-Flag agarose, the immunoprecipitates were separated by SDS-polyacrylamide gel (SDS-PAGE). The autoradiographs showed clear <sup>32</sup>P-labeled bands in c-FLIP-overexpressing cells, but not in the cell line overexpressing an empty vector (Figure 1a), demonstrating that c-FLIP is phosphorylated *in vivo*.

To compare the phosphorylation patterns of the c-FLIP isoforms, we performed tryptic phosphopeptide mapping of c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub>. When tryptic digests of *in vivo* <sup>32</sup>P-labeled c-FLIP were analyzed, both isoforms displayed a similar phosphopeptide pattern, indicating the same peptides are phosphorylated on both isoforms. The c-FLIP<sub>S</sub> phosphopeptide maps displayed one prominent <sup>32</sup>P spot (Figure 1b), representing a phosphorylated peptide from c-FLIP<sub>S</sub>. MALDI mass spectrometry (MS) analysis of this spot revealed phosphopeptide 193-pSLKDPSNNFR-202 (Figure 1d). Correspondingly, Edman sequencing of the <sup>32</sup>P-labeled peptide extracted from this spot showed release of the <sup>32</sup>P label at the first cycle, confirming the phosphorylation of S193 (Figure 1c). A <sup>32</sup>P phosphopeptide spot similar to the one in the c-FLIP<sub>S</sub> map was also observed in the c-FLIP<sub>L</sub> phosphopeptide map

(Figure 1e; spot 1), which also contained another prominent phosphopeptide (Figure 1e; spot 2). Further analysis by manual Edman sequencing and MALDI MS revealed that these two <sup>32</sup>P spots contained phosphopeptides 193-pSLKDPSNNFR-202 (Figure 1e, spot 1; Figure 1f–g) and 184-NVLQAAIQKpSLK-195 (Figure 1e, spot 2; Figure 1f; MS data on the peak of *m/z* 1392.78 corresponding to the peptide mass not shown), representing trypsin-cleavage variants of the same phosphorylation motif.

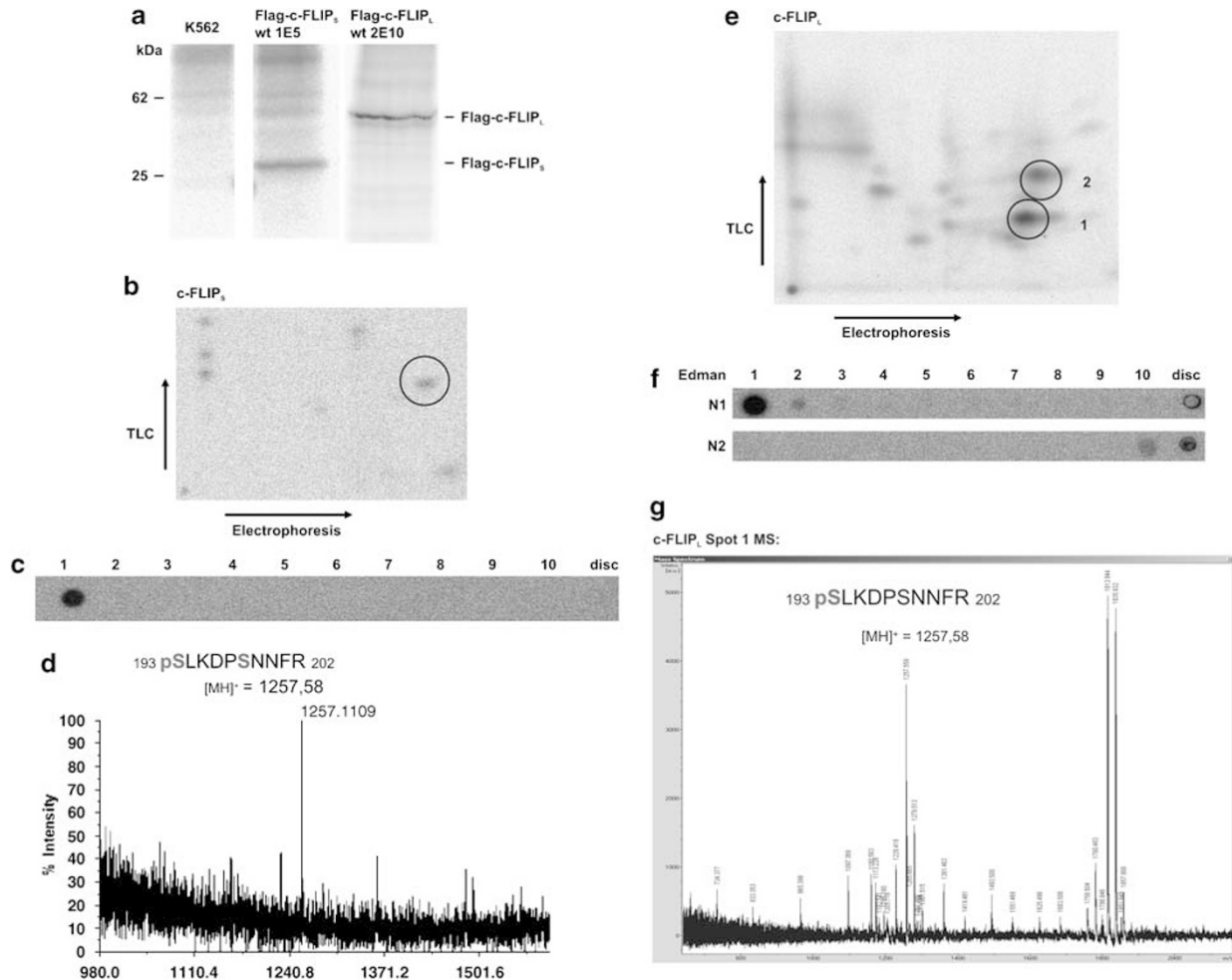
To verify c-FLIP phosphorylation on S193 *in vivo*, we performed <sup>32</sup>P labeling in K562 cell lines stably overexpressing the phosphorylation-deficient c-FLIP S193A mutants. As expected, the <sup>32</sup>P spot corresponding to the c-FLIP<sub>S</sub> WT phosphopeptide 193-pSLKDPSNNFR-202 (Supplementary Figure 1A and B) was diminished in the c-FLIP<sub>S</sub> S193A phosphopeptide map, faint remaining signal most likely originating from endogenous phosphorylated c-FLIP complexed with immunoprecipitated Flag-c-FLIP. Similarly, the spots corresponding to the c-FLIP<sub>L</sub> WT phosphopeptides 193-pSLKDPSNNFR-202 (Supplementary Figure 1C, spot 1) and 184-NVLQAAIQKpSLK-195 (Supplementary Figure 1C, spot 2) were absent from the c-FLIP<sub>L</sub> S193A phosphopeptide map (Supplementary Figure 1D). Together, these data demonstrate that S193 is a novel *in vivo* phosphorylation site of c-FLIP.

In addition, we generated a polyclonal antibody against phosphorylated c-FLIP S193. As seen in Figure 2a, our antibody recognized immunoprecipitated Flag-c-FLIP from calyculin A-treated K562 cells, but did not detect the phosphorylation-deficient Flag-c-FLIP S193A mutants. Although the phosphopeptide antibody recognized immunoprecipitated c-FLIP well, the antibody titer was not high enough to identify the low levels of endogenous phosphorylated c-FLIP.

**c-FLIP S193 phosphorylation is mediated by PKC $\alpha$  and PKC $\beta$ .** As the amino acids surrounding S193 correspond to a PKC consensus sequence, we determined the phosphorylation status of S193 after inhibiting or activating PKC. We saw a clear reduction in S193 phosphorylation in response to GÖ6976, a specific inhibitor of the classical PKC isozymes  $\alpha$  and  $\beta$  (Figure 2b). We also studied the effect of PKC $\alpha$  and PKC $\beta$  pseudosubstrate on c-FLIP S193 phosphorylation, and found that pseudosubstrate treatment decreased S193 phosphorylation (data not shown). These effects were more pronounced in c-FLIP<sub>S</sub>, indicating that c-FLIP<sub>L</sub> may be subjected to phosphorylation by a wider array of kinases than c-FLIP<sub>S</sub>.

As PKC $\alpha$  and PKC $\beta$  can be activated with TPA, we were interested in whether TPA treatment would upregulate c-FLIP S193 phosphorylation. Indeed, significant increase in S193 phosphorylation occurred already after 1 h of TPA addition also without phosphatase inhibition (Figure 2c), confirming that this mechanism is physiologically relevant. TPA also induced S193 phosphorylation in HeLa cells (data not shown), indicating that induction of c-FLIP S193 phosphorylation by the PKC is not cell-type specific.

To verify that the TPA-induced phosphorylation of c-FLIP S193 was dependent on PKC $\alpha$  and PKC $\beta$ , we pretreated K562 cells with GÖ6976 before adding TPA, performed Flag-immunoprecipitation, and detected S193 phosphorylation by



**Figure 1** c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub> are phosphorylated on S193. To identify c-FLIP phosphorylation sites, *in vivo* <sup>32</sup>P labeling of Flag-tagged c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub> (a) was conducted in stably overexpressing cell lines treated with 50 nM calyculin A to boost the phosphorylation. Phosphopeptide mapping of the labeled c-FLIP<sub>S</sub> (b) and c-FLIP<sub>L</sub> (e) shows that both proteins are phosphorylated. Edman degradation (c, f) and MALDI mass spectrometry (d, g) of the most prominent <sup>32</sup>P-spots from the phosphopeptide maps confirmed S193 to be phosphorylated in c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub>. Numbers in Edman degradation data correspond to the amino-acid number in a typically generated peptide starting from its N terminus. When the <sup>32</sup>P label is released at a certain cycle, the cycle number corresponds to the position of serine in the peptide being sequenced (i.e., amino acid 1 in phosphopeptide 193-pSLKDPSNNFR-202 (c, f), and amino acid 10 in 184-NVLQAAIQKpSLK-195 (f)). ‘Disc’ designates the amount of remaining <sup>32</sup>P label on the Sequelon-AA disc after 10 Edman cycles (the reaction was continued for 10 cycles)

western blotting. As expected, the TPA-induced phosphorylation was decreased by GÖ6976 pretreatment, supporting the notion of TPA-induced S193 phosphorylation occurring mainly through PKC $\alpha$  and PKC $\beta$  (Figure 2d). However, as this effect was more pronounced in c-FLIP<sub>S</sub> than in c-FLIP<sub>L</sub>, we cannot rule out the possibility that c-FLIP<sub>L</sub> S193 phosphorylation is regulated by other kinases as well.

The newly identified c-FLIP splice variant c-FLIP<sub>R</sub> has been reported to behave similarly to c-FLIP<sub>S</sub>.<sup>10</sup> Because many cell lines primarily express either c-FLIP<sub>S</sub> or c-FLIP<sub>R</sub> (Supplementary Figure 2), we wanted to see if c-FLIP<sub>R</sub> was also inducibly phosphorylated on S193. When K562 cell lines stably overexpressing Flag-c-FLIP<sub>R</sub> were treated with TPA, GÖ6976, and calyculin A, we found that c-FLIP<sub>R</sub> was phosphorylated on S193 in a manner similar to c-FLIP<sub>S</sub> (Figure 2e).

To specify whether PKC $\alpha$ , PKC $\beta$ , or both were involved in the phosphorylation of S193, we overexpressed kinase-dead,

dominant-negative (dn) mutants of PKC $\alpha$  and PKC $\beta$  and investigated the phosphorylation of S193. Consistent with our previous results, we saw that expression of either dnPKC $\alpha$  or dnPKC $\beta$  interfered with TPA-induced S193 phosphorylation (Figure 2f). Again, the effect on S193 phosphorylation was somewhat stronger on c-FLIP<sub>S</sub> than on c-FLIP<sub>L</sub>, agreeing with our data obtained by pharmacological inhibitors. Neither dnPKC was able to completely inhibit S193 phosphorylation, likely indicating functional redundancy between PKC $\alpha$  and PKC $\beta$ . Taken together, our results demonstrate that S193 phosphorylation is common to all c-FLIP isoforms and that it is mediated by PKC $\alpha$  and PKC $\beta$ .

### S193 phosphorylation regulates c-FLIP ubiquitylation.

The C-terminal part of c-FLIP<sub>S</sub> is required for ubiquitylation and proteasomal degradation during K562 erythroid differentiation.<sup>19</sup> As S193 is located near this critical region, we investigated the potential effect of S193 phosphorylation



were substantially more ubiquitylated, whereas all S193D mutants were ubiquitylated similarly to or somewhat less than the corresponding WT c-FLIP (Figure 3a–c). However, loss of S193 phosphorylation did not enhance the ubiquitylation of a truncated c-FLIP 1-202 mutant lacking the C-terminal tail (Figure 3d), suggesting that S193 phosphorylation regulates c-FLIP ubiquitylation in concert with the splicing tail. In addition, we detected a moderate increase in the ubiquitylation of WT, but not S193D mutant c-FLIP<sub>R</sub>, and a stronger effect on the ubiquitylation of c-FLIP<sub>L</sub> in response to GÖ6976 (Figure 3e), supporting our hypothesis of c-FLIP ubiquitylation being regulated through PKC-mediated S193 phosphorylation.

**S193 phosphorylation increases the stability of the short c-FLIP proteins.** As polyubiquitylation is often coupled to proteasomal degradation, we determined how effects on ubiquitylation reflected on c-FLIP stability. We analyzed the half-lives of c-FLIP proteins using the translation inhibitor cycloheximide (CHX). K562 cells were transiently transfected with low amounts of WT and mutant c-FLIP constructs simulating the half-lives of endogenous c-FLIP, and their stability was detected after a time-course treatment with CHX. Compared to WT c-FLIP<sub>S</sub>, and c-FLIP<sub>R</sub>, abrogation of phosphorylation by S193A mutation led to destabilization of the proteins. In contrast, when constitutive S193 phosphorylation was mimicked by S193D mutation, c-FLIP<sub>S</sub> (Figure 4a) and c-FLIP<sub>R</sub> (Figure 4b) were stabilized. Instead, mutations of S193 did not affect the stability of c-FLIP<sub>L</sub> (Figure 4c), indicating that S193 phosphorylation regulates c-FLIP isoforms differently.

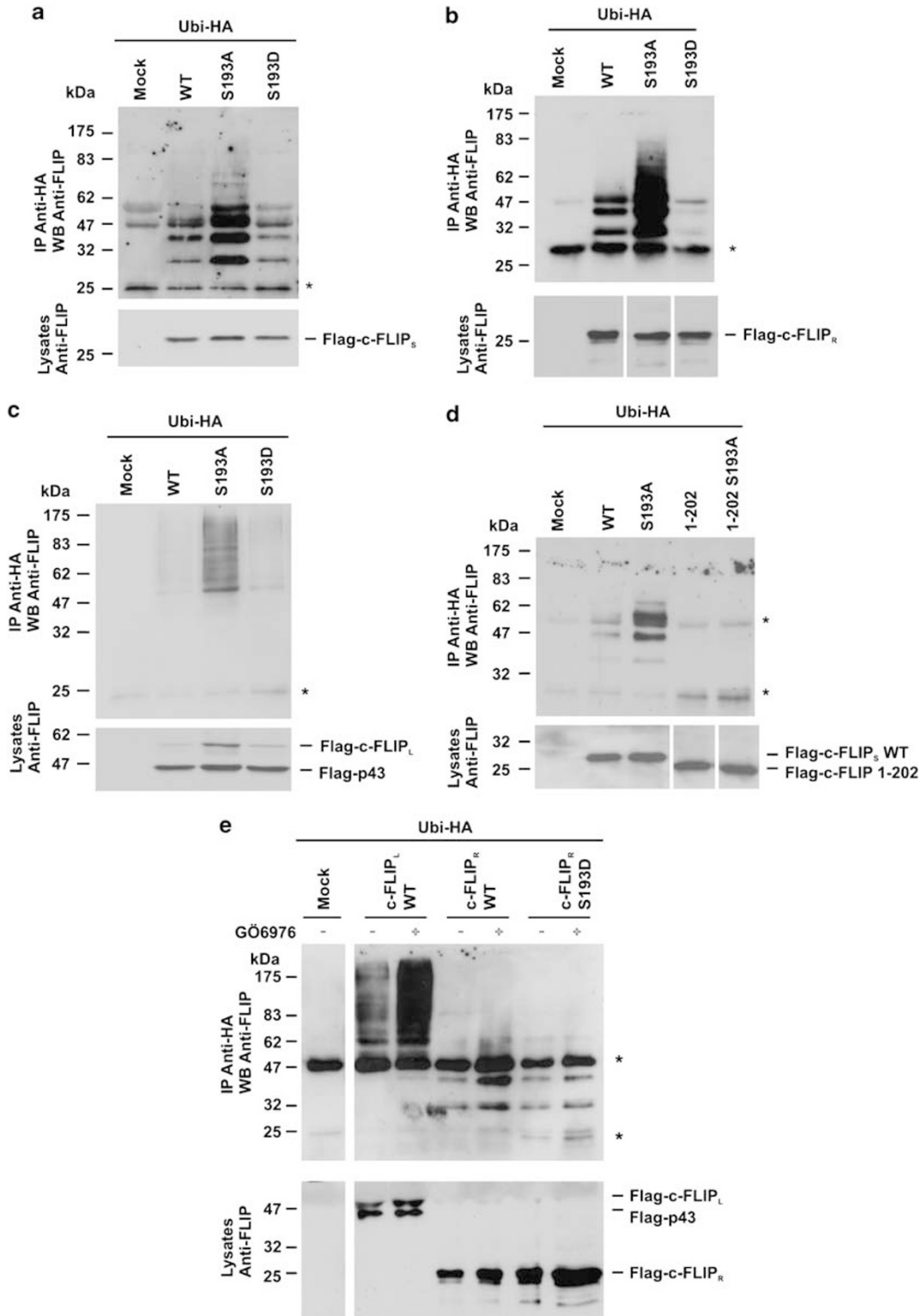
To verify the effect of S193 phosphorylation on the stability of c-FLIP, we performed <sup>35</sup>S metabolic labeling of K562 cells stably overexpressing WT or mutant c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub>. As in our CHX chase experiments, c-FLIP<sub>S</sub> S193A was less stable, whereas the S193D mutant was more stable than WT c-FLIP<sub>S</sub> (Figure 4d). Again, we could neither detect differences in the half-lives of WT and phosphomutant c-FLIP<sub>L</sub> (Figure 4e), nor did S193 mutations affect the stability of c-FLIP<sub>L</sub> at shorter time points (data not shown), indicating that the ubiquitylation of non-S193-phosphorylated c-FLIP<sub>L</sub> does not lead to degradation. Taken together, our data show that the phosphorylation status of S193 differentially affects the stability of c-FLIP isoforms.

### S193 phosphorylation does not affect the TRAIL DISC binding of c-FLIP or its effects on caspase-8.

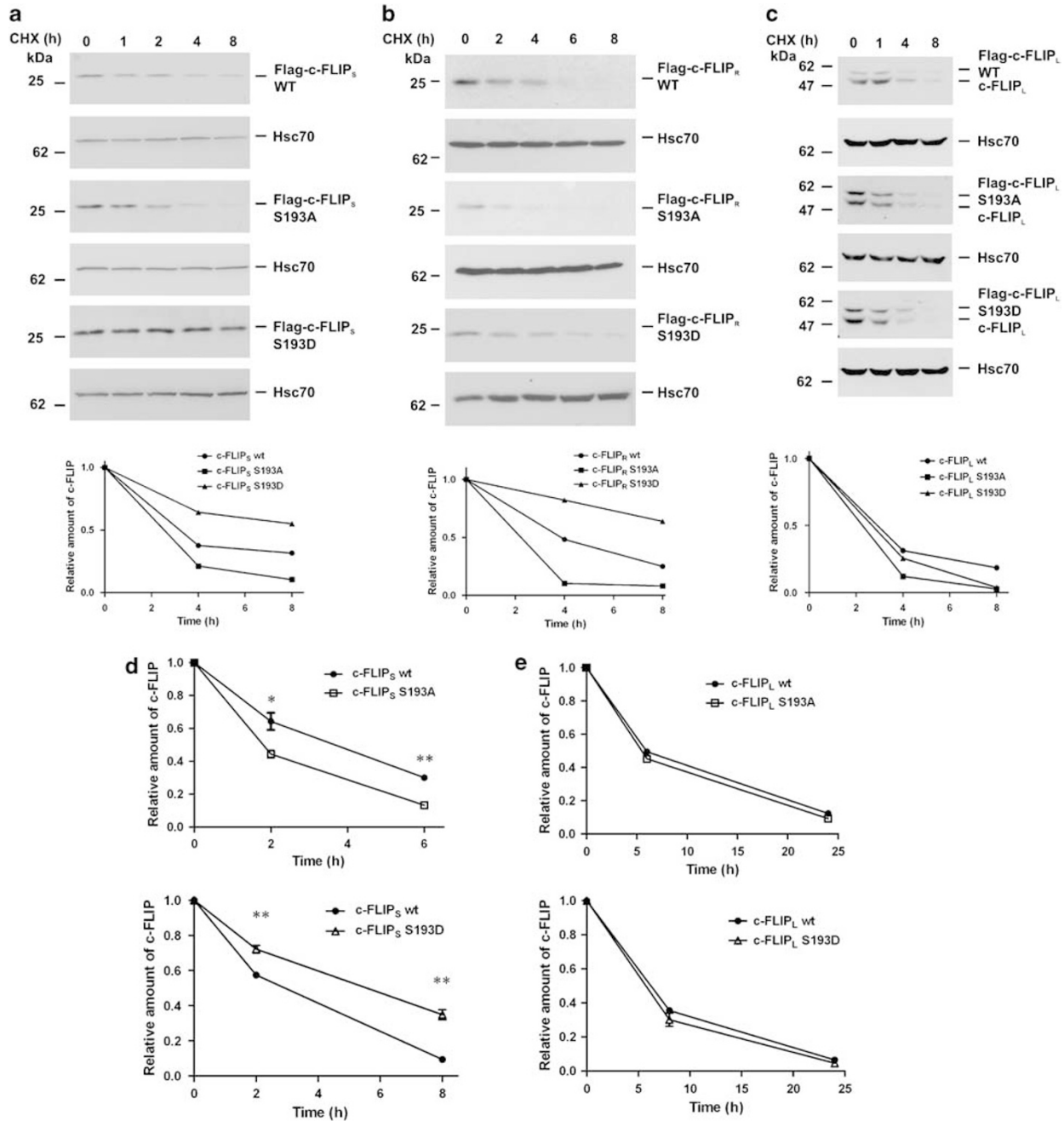
Because the best-known functions of c-FLIP occur in protein complexes induced by death receptor activation, we investigated how death receptor stimulation affects the phosphorylation of S193. K562 cells were treated with either TRAIL (Figure 5a) or TNF $\alpha$  (Figure 5b) for 1 h followed by calyculin A, after which c-FLIP S193 phosphorylation was analyzed. Following either TNF $\alpha$  or TRAIL treatment, S193 phosphorylation was clearly increased. The effect was more pronounced after TNF $\alpha$  treatment, demonstrating that S193 phosphorylation is adjusted in a stimulus-dependent manner. To explore whether S193 phosphorylation affects the recruitment of c-FLIP to the DISC, we performed TRAIL receptor immunoprecipitation with monoclonal antibodies against DR4 (TRAIL-R1) and DR5 (TRAIL-R2) in K562 cell lines stably overexpressing WT, S193A, or S193D c-FLIP. After TRAIL treatment, both WT and mutant c-FLIP associated with the DISC in proportion to the exogenous c-FLIP expression levels of the individual cell lines (Figure 5c). This is consistent with S193 residing outside the DEDs that mediate DISC binding. Both WT and mutant c-FLIP<sub>L</sub> allowed the cleavage of procaspase-8, whereas in the presence of WT or mutant c-FLIP<sub>S</sub>, little caspase-8 cleavage occurred (Figure 5c).

In addition to regulating c-FLIP turnover, S193 phosphorylation might also qualitatively affect procaspase-8 regulation by c-FLIP. To study this, we induced the TRAIL DISC in the K562 cell lines stably overexpressing WT or mutant c-FLIP proteins, and immunoprecipitated the DISC with antibodies against DR4 and DR5. The immunoprecipitated DISC was incubated in Caspase 8 Glo reagent, and cleavage of an artificial caspase-8 substrate was measured as release of luminescence. The measurements demonstrated that WT and mutant c-FLIP did not differ in their ability to regulate caspase-8 activation (Figure 5d). As previously described,<sup>11,12</sup> c-FLIP<sub>S</sub> inhibited the activation of caspase-8, whereas c-FLIP<sub>L</sub> overexpression did not. The mutations of S193 did not alter these properties (Figure 5d). Small, statistically insignificant differences between WT and mutant cell lines occurred, most likely due to their differing expression levels. These results indicate that S193 phosphorylation does not directly affect the intrinsic ability of c-FLIP to regulate caspase-8 activation.

**Figure 2** c-FLIP S193 phosphorylation is decreased upon inhibition of PKC $\alpha$  and PKC $\beta$  and induced by TPA. (a) To study phosphorylation, we generated a phosphospecific antibody that recognizes phosphorylated c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub> after calyculin A treatment (20 nM, 20 min), but does not recognize S193A mutant c-FLIP. (b) To observe S193 phosphorylation upon PKC inhibition, K562 stably overexpressing Flag-c-FLIP<sub>S</sub> WT (clone 1E5), Flag-c-FLIP<sub>S</sub> S193A (clone 2G7), Flag-c-FLIP<sub>L</sub> WT (clone 2E10), or Flag-c-FLIP<sub>L</sub> S193A (clone 1G7) were treated with 40  $\mu$ M GÖ6976 for 4 h, followed by 20 nM calyculin A (CalA) for 30 min. Flag immunoprecipitation was conducted and the immunoprecipitated proteins were subsequently eluted from the beads with Flag peptide. c-FLIP phosphorylation was detected by western blotting with the phosphopeptide antibody against phosphorylated S193 (upper panels). The presence of c-FLIP in the lysate is shown in the lower panels. (c) c-FLIP S193 phosphorylation was induced by TPA treatment (20 nM, 2 h) and can be also detected without phosphatase inhibition (CalA 20 nM, 30 min). (d) The induction of S193 phosphorylation by TPA is dependent on PKC $\alpha$  and PKC $\beta$ . The pretreatment of cells with GÖ6976 (40  $\mu$ M, 2 h) attenuates the promoting effect TPA (20 nM, 1 h) has on S193 phosphorylation. Calyculin A was used at 20 nM for 30 min. (e) Similar to c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub>, the S193 phosphorylation of c-FLIP<sub>R</sub> can be induced by TPA in a PKC $\alpha$ - and  $\beta$ -dependent manner. (f) TPA-induced S193 phosphorylation is decreased by the overexpression of kinase-dead PKC $\alpha$  and PKC $\beta$ . Kinase-dead, GFP-tagged PKC $\alpha$  and PKC $\beta$  were transiently transfected into cell lines overexpressing c-FLIP<sub>S</sub> (clone 1E5), c-FLIP<sub>L</sub> (clone 2E10), or empty plasmid (Mock). PKC-mediated phosphorylation was induced by TPA (20 nM, 1 h) and boosted by calyculin A (20 nM, 20 min), after which S193 phosphorylation was analyzed. Overexpression of kinase-dead, dominant-negative PKC $\alpha$  and PKC $\beta$  inhibited the TPA-mediated induction of S193 phosphorylation, indicating that PKC $\alpha$  and PKC $\beta$  mediate S193 phosphorylation. Relative phosphorylation was determined by performing densitometry on the phospho-S193 signal and normalizing it against the densitometry values of the total c-FLIP in the lysate



**Figure 3** S193 phosphorylation regulates c-FLIP ubiquitylation. The ubiquitylation of c-FLIP mutants was detected from K562 cells transfected transiently and thereafter left untreated (**a–d**) or treated with  $10\ \mu\text{M}$  GÖ6976 for 14 h (**e**). The cells were lysed according to the denaturing SDS lysis protocol, and ubiquitylated proteins were immunoprecipitated using anti-HA antibody and protein G-Sepharose. c-FLIP was detected from the immunoprecipitates by western blotting. WT and mutant ubiquitylated c-FLIP<sub>S</sub> (**a**), c-FLIP<sub>R</sub> (**b**), and c-FLIP<sub>L</sub> (**c**) are shown in the upper panels, the presence of c-FLIP in the lysates before immunoprecipitation is shown in the lower panels. (**d**) The ubiquitylation of truncated c-FLIP 1-202 is not affected by S193A mutation. (**e**) The inhibition of PKC $\alpha$  and PKC $\beta$  by GÖ6976 moderately increases the ubiquitylation of WT, but not S193D mutant c-FLIP<sub>R</sub>, and has a more pronounced effect on the ubiquitylation of c-FLIP<sub>L</sub>. Asterisks denote unspecific bands



**Figure 4** S193 phosphorylation regulates the stability of the short c-FLIP isoforms. K562 cells were transiently transfected with WT and mutant c-FLIP<sub>S</sub> (a), c-FLIP<sub>R</sub> (b), or c-FLIP<sub>L</sub> (c) constructs and treated with 50  $\mu$ M cycloheximide (CHX) for indicated times. Samples from 1% Triton X-100 lysates are shown and equal loading is presented by Hsc70 blotting. The densitometry was performed from experiments in question. The stability of c-FLIP was also studied by <sup>35</sup>S metabolic labeling. K562 cells stably overexpressing WT and mutant c-FLIP<sub>S</sub> (d) or c-FLIP<sub>L</sub> (e) proteins were labeled with radioactive <sup>35</sup>S-methionine and cysteine, and the loss of radioactivity, as an indicator of protein degradation, was followed at indicated time points. The relative half-lives were measured by phosphorimager analyses and illustrated using GraphPad Prism. Statistical significance was analyzed by Student's unpaired *t*-test (*n* = 3), \**P* = 0.05, \*\**P* = 0.005. Graphs show standard errors of mean

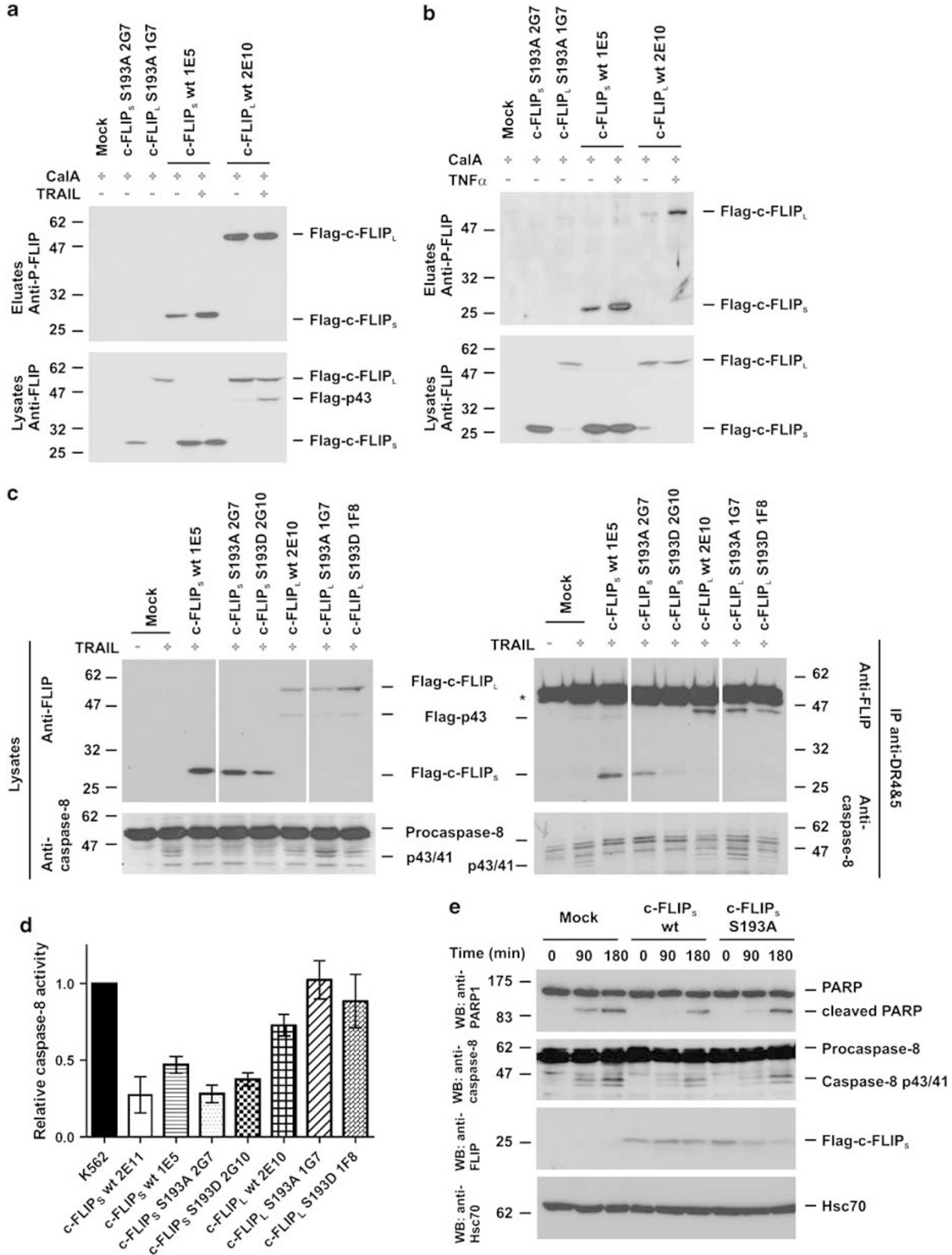
**S193-mediated differences in c-FLIP stability are reflected in death receptor sensitivity.** As S193 phosphorylation did not contribute to the intrinsic ability of c-FLIP to regulate procaspase-8, we speculated that the function of S193 phosphorylation in the turnover of c-FLIP<sub>S</sub> would, however, affect the outcome of death receptor stimulation. We transfected small amounts of WT and

S193A c-FLIP<sub>S</sub> into K562 cells and pretreated them with CHX to inhibit protein synthesis. Thereafter, we induced apoptosis with recombinant human TRAIL and detected the levels of c-FLIP, as well as the cleavage of caspase-8 and the caspase substrate PARP1, by western blotting (Figure 5e). Indeed, in the presence of CHX, the rapid degradation of S193A mutant led to a more pronounced

cleavage of caspase-8 and PARP1. Therefore, although S193 phosphorylation does not govern the anti-apoptotic properties of c-FLIP *per se*, it affects cell survival indirectly by regulating the stability of the short c-FLIP proteins.

## Discussion

The critical function of c-FLIP in determining death receptor responses requires careful control of c-FLIP levels according





to the particular needs of a cell. c-FLIP is regulated by a variety of posttranslational modifications, such as nitrosylation<sup>23</sup> and ubiquitylation.<sup>19,24,25</sup> Our previous studies have shown that ubiquitylation-mediated isoform-specific regulation of c-FLIP stability is vital for death receptor responses upon erythroid differentiation and lymphocyte persistence during hyperthermia.<sup>26,27</sup> In this study, we uncovered how c-FLIP is regulated by phosphorylation, as we identified S193 as a novel *in vivo* phosphorylation site of all c-FLIP proteins and demonstrated that S193 phosphorylation opposes c-FLIP ubiquitylation. Furthermore, although S193A mutation rendered all c-FLIP proteins susceptible for ubiquitylation, S193 phosphorylation selectively increased the stability of c-FLIP<sub>S</sub> and c-FLIP<sub>R</sub>.

S193 is found within a PKC consensus sequence, suggesting that its phosphorylation is mediated by the PKC. Indeed, the phosphorylation of S193 was markedly enhanced by TPA-induced PKC activation and inhibited by overexpression of dnPKC $\alpha$  and dnPKC $\beta$  as well as by PKC $\alpha$ - and PKC $\beta$ -specific inhibitors. In contrast, S193 phosphorylation was not affected by Rottlerin, an inhibitor of the novel PKCs  $\theta$  and  $\delta$  (data not shown). These data delineate PKC isozymes  $\alpha$  and  $\beta$  as being involved in the modification of S193. Interestingly, c-FLIP phosphorylation was elevated by death receptor stimulation. As the activation of the classical PKC involves translocation of the kinase to the membrane (reviewed by Gomez-Fernandez *et al.*<sup>28</sup>), the increased S193 phosphorylation upon death receptor stimulation may result from the recruitment of c-FLIP to a protein complex at the cell membrane, augmenting the physical interaction with the kinase. Interestingly, TNF $\alpha$  induced S193 phosphorylation more strongly than TRAIL, possibly due to dissimilar compositions of the TRAIL DISC and the TNFR complexes, differences in the signaling pathways activated by the respective receptor systems, and the altering kinetics of c-FLIP recruitment to these complexes. Moreover, these complexes differ in their subcellular localization, as c-FLIP binds to TRAIL DISC at the membrane, whereas TNFR complex II recruits c-FLIP in the cytosol.<sup>29</sup> In addition, although TRAIL and TNF $\alpha$  induce diverse signaling pathways, the PKC activities probably respond to them with varying kinetics.

Apart from regulating ubiquitylation and stability, phosphorylation of c-FLIP S193 may have additional effects related to

DISC assembly. Although the phosphorylation of S193 was increased by the stimulation of TRAIL and TNF receptors, it did not affect the ability of c-FLIP proteins to bind to the DISC, consistent with S193 residing outside the death effector domains.<sup>30</sup> Nonetheless, when in the DISC, S193 phosphorylation of c-FLIP may influence the recruitment of other proteins. Furthermore, it is possible that S193 phosphorylation regulates interactions with binding partners yet to be identified. These speculations are especially interesting in terms of c-FLIP<sub>L</sub> whose stability was not influenced by S193 phosphorylation.

Posttranslational modifications create complex regulatory networks. Interestingly, S193 is found in a region we have previously shown to regulate c-FLIP<sub>S</sub> ubiquitylation,<sup>19</sup> prompting us to explore its potential interplay with phosphorylation. Indeed, the disruption of S193 phosphorylation led to increased ubiquitylation in all isoforms, whereas the phosphomimetic S193D mutants were more resistant to ubiquitylation. In addition, pharmacological inhibition of PKC $\alpha$  and PKC $\beta$  especially increased the ubiquitylation of c-FLIP<sub>L</sub>. Furthermore, we demonstrated that the C-terminal parts, unique to individual isoforms, are needed for their degradation, regardless of S193 phosphorylation. However, because c-FLIP1-202 mutant is phosphorylated *in vivo* as efficiently as the WT c-FLIP (data not shown), the C-terminal parts are not essential for kinase recruitment or action.

Ubiquitylation is an extremely versatile mode of posttranslational modification, and an increasing body of evidence shows that proteasomal degradation is only one among many variable outcomes of ubiquitin conjugation. In addition to proteolysis, ubiquitylation can mediate endocytosis, kinase activation, and DNA repair (reviewed by Ikeda and Dikic *et al.*<sup>31</sup>). Importantly, our data do not reveal the kind of polyubiquitylation the c-FLIP S193A mutants are subjected to, and it can therefore be speculated that particularly c-FLIP<sub>L</sub> may be modified by ubiquitin chains that, instead of mediating proteasomal degradation, affect its signaling properties.<sup>31</sup> Hence, S193 phosphorylation regulates ubiquitylation in both isoforms whereas affecting different processes in the c-FLIP<sub>S</sub> and c-FLIP<sub>L</sub> proteins. This outcome may be related to the differential physiological functions of c-FLIP isoforms. The linkage type within a ubiquitin chain is determined by the E3 ligase conjugating ubiquitin moieties. Because the isoform-specific splicing tails of c-FLIP proteins are required for ubiquitylation, it

**Figure 5** S193 phosphorylation *per se* does not affect the TRAIL DISC binding of c-FLIP or its effects on caspase-8, but determines death receptor sensitivity by regulating c-FLIP stability. The K562 cell clones stably overexpressing WT and mutant c-FLIP were treated with SuperKiller TRAIL (a) or murine recombinant TNF $\alpha$  (b) for 1 h, followed by calyculin A treatment (20 nM, 20 min). Phosphorylated c-FLIP (upper panels) was immunoprecipitated and detected as described in Figure 2. The presence of c-FLIP in the lysate is shown in the lower panels. (c) The ability of WT and mutant c-FLIP proteins to bind to the TRAIL DISC was analyzed in K562 cells stably overexpressing Flag-c-FLIP<sub>S</sub> WT (clone 1E5), Flag-c-FLIP<sub>S</sub> S193A (clone 2G7), Flag-c-FLIP<sub>S</sub> S193D (clone 2G10), Flag-c-FLIP<sub>L</sub> WT (clone 2E10), Flag-c-FLIP<sub>L</sub> S193A (clone 1G7), or Flag-c-FLIP<sub>L</sub> S193D (clone 1F8). The formation of the TRAIL DISC was induced by adding human recombinant TRAIL, followed by immunoprecipitation of the DISC complex with monoclonal antibodies against DR4 and DR5. Cellular lysates and immunoprecipitates were immunoblotted with antibodies against c-FLIP and caspase-8 (panels on the right). The presence of the respective proteins in the lysate is shown in the panels on the left. Asterisks denote unspecific bands. (d) S193 mutation does not affect the effects of c-FLIP on caspase-8 in the TRAIL DISC. Caspase-8 activity was measured by a luminometric assay after TRAIL immunoprecipitation, and the activity in cell lines stably overexpressing WT or S193 mutant c-FLIP<sub>S</sub> or c-FLIP<sub>L</sub> was compared to caspase-8 activity in the parental K562 cells. Both WT and S193 mutant c-FLIP<sub>S</sub> overexpressing cell lines were able to inhibit caspase-8 activation, whereas WT and S193 mutant c-FLIP<sub>L</sub> allowed caspase-8 activation. The cell lines presented small, statistically insignificant differences compared to each other. (e) The stability of c-FLIP<sub>S</sub> affects the progression of apoptotic signaling in K562 cells. K562 cells transiently overexpressing small amounts of WT, S193A, or S193D c-FLIP<sub>S</sub> were pretreated with 40  $\mu$ M cycloheximide for 15 min before apoptosis was induced by human recombinant TRAIL (90 ng/ml). The progression of apoptosis was followed at 90 and 180 min time points by immunoblotting against PARP1 and caspase-8. Equal loading was ensured by blotting against Hsc70

is tempting to speculate that these C termini may be able to recruit different ligases, possibly explaining the alternative physiological outcomes of ubiquitylation. Our ongoing investigations focus on resolving the type of ubiquitylation the c-FLIP isoforms are subjected to, as well as on describing the biological functions of these modifications.

This study complements earlier observations regarding c-FLIP phosphorylation. Although others did not identify individual phosphorylation sites, they suggested that CaMKII phosphorylates c-FLIP<sub>L</sub> on threonine,<sup>20–22</sup> and that phosphorylation is involved in the detachment of c-FLIP from the TRAIL DISC.<sup>32</sup> However, because CaMKII inhibition did not affect S193 phosphorylation (data not shown), CaMKII is unlikely to be involved in S193 phosphorylation. As our study is the first observing the effects of an individual site rather than overall c-FLIP phosphorylation, the differences between our results and previous reports probably stem from the dissimilarity of the model systems. Furthermore, the effects of S193 phosphorylation are presumably context dependent, leading to different responses depending on the costimulatory signals and binding partners present.

In summary, this report describes for the first time how c-FLIP is modified by site-specific phosphorylation. Our data show that S193 phosphorylation is mediated by PKC $\alpha$  and PKC $\beta$  and reveal a connection between c-FLIP phosphorylation and ubiquitylation, as S193 mutations differently affect c-FLIP ubiquitylation. Surprisingly, although all c-FLIP proteins are prone to ubiquitylation by S193A mutation, disruption of this phosphorylation site selectively affects the half-lives of the c-FLIP<sub>S</sub> proteins. We propose that S193 phosphorylation determines the level of death receptor sensitivity by regulating the isoform-specific turnover of c-FLIP.

## Materials and Methods

**Cell culture and treatments.** Human K562 erythroleukemia cells were cultured in a humidified 5% CO<sub>2</sub> atmosphere at 37°C in RPMI-1640 medium (Sigma-Aldrich, St Louis, MI, USA) supplemented with 10% fetal calf serum (FCS), antibiotics (penicillin and streptomycin), and 2 mM L-glutamine. K562 cells stably overexpressing c-FLIP<sub>S</sub>, c-FLIP<sub>R</sub>, and c-FLIP<sub>L</sub> WT isoforms (1E5, 3A6, and 2E10, respectively) or c-FLIP mutants (c-FLIP<sub>S</sub> S193A 2G7, S193D 2G10; c-FLIP<sub>L</sub> S193A 1G7, S193D 1F8) were maintained in RPMI-1640 containing G418 (500  $\mu$ g/ml; Calbiochem, Darmstadt, Germany). The protein synthesis inhibitor CHX (Sigma-Aldrich) was used at either 5, 10, or 50  $\mu$ M concentration for the indicated time periods. Formation of the DISC was induced by adding 1  $\mu$ g of human soluble recombinant Flag-tagged TRAIL (Alexis, San Diego, CA, USA) together with 2  $\mu$ g/ml cross-linking M2 anti-FLAG antibody (Sigma-Aldrich) (DISC immunoprecipitation), or either human recombinant soluble SuperKiller TRAIL (Alexis) or isoleucine zipper human recombinant TRAIL (kind gift from Dr. Henning Walczak, Imperial College, London, UK), neither of which requires a cross-linking enhancer, in the final concentration of 250 ng/ml for 1 h (detection of c-FLIP phosphorylation upon death receptor activation) or 90 ng/ml for indicated time points (detection of apoptosis markers after CHX pretreatment).

K562 cells were treated with 20 nM TPA (Sigma-Aldrich) to activate the PKCs for indicated times. To inhibit type 1 and type 2A phosphatases, we treated K562 cells with 20 nM calyculin A (Sigma-Aldrich) for 30 min before harvesting. To specifically inhibit PKC $\alpha$  and PKC $\beta$ , we treated the cells with 40  $\mu$ M GÖ6976 (Sigma-Aldrich) for 4 h or 10  $\mu$ M pseudosubstrate (PKC inhibitor 20–28; Calbiochem) for 2 h. To stimulate TNF receptors, we added murine recombinant TNF $\alpha$  (R&D Systems, Minneapolis, MN, USA) to the cells (10 ng/ml) for 1 h before calyculin A treatment.

**In vivo <sup>32</sup>P labeling, Edman sequencing, and MS.** K562 cells stably overexpressing Flag-c-FLIP<sub>S</sub> or Flag-c-FLIP<sub>L</sub> were grown in RPMI-1640 medium supplemented with 10% serum, L-glutamine, and antibiotics. Cells (2.5  $\times$  10<sup>6</sup>

ml) were preincubated in 6 ml for 2.5 h with 0.3 mCi/ml <sup>32</sup>P-orthophosphate (ICN Pharmaceuticals, Washington, DC, USA) in phosphate-free minimum essential medium Eagle (Sigma-Aldrich) supplemented with 10% fetal bovine serum. After treatment for 30 min with 50 nM of the phosphatase inhibitor calyculin A (Sigma-Aldrich), the cells were collected by centrifugation, washed with ice-cold PBS, and subjected to the Flag immunoprecipitation. The resulting immunoprecipitates were run on 12.5% SDS-PAGE followed by autoradiography with an imaging plate using Fujifilm BAS-1800 Bioimaging analyzer (Figure 1a).

In-gel tryptic digestion of the <sup>32</sup>P-labeled Flag-c-FLIP<sub>S</sub> (26.5 kDa band) and Flag-c-FLIP<sub>L</sub> (55 kDa band), followed by two-dimensional phosphopeptide mapping on a thin layer chromatography (TLC) sheet, was carried out as previously described.<sup>33</sup> Labeled Flag-c-FLIP<sub>S</sub> or Flag-c-FLIP<sub>L</sub> bands were excised from the dried gel and in-gel digested overnight at 37°C with 2  $\mu$ g/ml sequencing grade trypsin (Promega, Madison, WI, USA) in 50 mM ammonium bicarbonate solution (pH 8). The tryptic digest was applied on a cellulose TLC sheet (20  $\times$  20 cm; Merck KgaA, Darmstadt, Germany) and separated in two dimensions by electrophoresis and TLC. The first dimension, electrophoresis, was performed in a pH-1.9 buffer (formic acid 2.3%, acetic acid 2.9% v/v) at 750 V for 1.5 h using the Hunter Thin Layer Peptide Mapping System, model HTLE-7000 (C.B.S. Scientific Co., Solana Beach, CA, USA). The sheet was dried and ascending TLC in the second dimension performed for 14 h in a chromatography tank saturated with a mobile phase containing 30% water, 37.5% n-butanol, 7.5% acetic acid, and 25% pyridine. The sheet was air-dried and the <sup>32</sup>P phosphopeptides visualized by autoradiography on a Fuji phosphorimager plate. For MALDI MS and Edman sequencing, corresponding <sup>32</sup>P peptides were extracted from cellulose sheets by scraping off the powder into an Eppendorf tube and then eluted twice with 100  $\mu$ l of 30% ACN, 0.1% TFA solution. The resulting extract was vacuum-evaporated and MALDI MS and Edman sequencing were performed exactly as described by Kochin *et al.*<sup>33</sup> For Edman degradation, phosphopeptides were immobilized on arylamine membrane discs (Sequelon-AA membrane; Applied Biosystems, Foster City, CA, USA) using water-soluble carbodiimide. The Sequelon-AA membranes consist of a PVDF matrix that has been derivatized with arylamine groups. Individual c-FLIP phosphopeptides are immobilized on the Sequelon-AA discs through their C-terminal carboxyl groups. Therefore, the N termini of such peptides are free, and amino acids are clipped off during 10 Edman degradation cycles. The collected fractions were spotted on a Whatman filter paper, which was then visualized by autoradiography on a Fuji phosphorimager plate to reveal the cycle at which the radiolabel is released, which corresponds to the position of the phosphorylated amino acid, as counted from the N terminus (Figure 1c–f; Kochin *et al.*<sup>33</sup>).

**Plasmid constructs.** The Flag-tagged c-FLIP<sub>L</sub> and c-FLIP<sub>S</sub> were a kind gift from Dr. Jürg Tschopp (Institute of Biochemistry, University of Lausanne, Switzerland). Flag-tagged c-FLIP<sub>R</sub> was constructed by PCR using Flag-tagged c-FLIP<sub>L</sub> as a template (forward primer, 5'-ACAGTTGAATTCATGCTGCTGAAGT C-3'; reverse primer, 5'-TCTAGACTCGAGTCATGCTGGGATTCATATGTTTTCT CCAGACTCACCTGAAGTTATTTGAAGG-3') and was subcloned into the EcoRI and XhoI sites in frame with the N-terminal Flag tag in pCR3-Met-Flag. c-FLIP<sub>S</sub>, c-FLIP<sub>R</sub>, and c-FLIP<sub>L</sub> point mutations were made using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and confirmed by sequencing. The Flag-tagged c-FLIP<sub>S</sub> deletion mutant was constructed as described before.<sup>19</sup> The HA-tagged ubiquitin was a kind gift from Dr. Dirk Bohmann (University of Rochester, Rochester, NY, USA). The GFP-tagged kinase-dead PKC $\alpha$  and PKC $\beta$  constructs were kindly provided by Dr. Christer Larsson (University of Lund, Lund, Sweden).

**Transient transfections and stable cell lines.** For transfections, 5  $\times$  10<sup>6</sup> K562 cells were centrifuged and resuspended in 0.4 ml OptiMEM (Gibco BRL, Gaithersburg, MD, USA), and up to 40  $\mu$ g of plasmid DNA was added. Cells were subjected to a single electric pulse (220 V, 975  $\mu$ F) in 0.4 cm electroporation cuvettes (BTX, Holliston, MA, USA) using a Bio-Rad Gene Pulser electroporator, followed by dilution to 5  $\times$  10<sup>5</sup> cells per ml in RPMI-1640 with 10% FCS and antibiotics. Cells were incubated at 37°C for 24–48 h before the experimental treatments. The stable neomycin resistant c-FLIP cell lines were selected by G418 (500  $\mu$ g/ml; Calbiochem) for 2 weeks, the resistant pool was serially diluted on a 96-well plate in the presence of G418, and the single cell clones were upscaled and screened for Flag-c-FLIP expression by western blotting.

**SDS-PAGE and western blotting.** For western blot analysis, cells were harvested by centrifugation and washed once with PBS. Cells were lysed either in

the Laemmli SDS sample buffer or in lysis buffer (30 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 1 × Complete mini protease inhibitor cocktail (Roche, Basel, Switzerland)). Resulting Triton X-100 lysis buffer lysates were centrifuged to remove insoluble material and the protein concentrations were determined by Bradford assay. Each lysate containing 30–50 μg protein was loaded and resolved by SDS-PAGE and transferred to nitrocellulose membrane (Protran nitrocellulose; Schleicher & Schuell, Dassel, Germany) by using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA, USA). Western blotting was performed using antibodies against c-FLIP (NF6; Alexis), Hsc70 (SPA-815; Stressgen, Ann Arbor, MI, USA), GFP (JL-8; Clontech, San Francisco, CA, USA), caspase-8 (C15; Alexis), PARP1 (C-2-10; Sigma-Aldrich), and c-FLIP phosphorylated on S193. HRP-conjugated secondary antibodies were purchased from Amersham and Southern Biotechnology. The bands were visualized using the enhanced chemiluminescence method (Amersham, Buckinghamshire, UK).

**Preparation and use of the anti-pS193 phosphopeptide antibody.** To generate an antibody that specifically recognizes human c-FLIP phosphorylated on S193, we conjugated the phosphopeptide (Ac-)CLQAAIQK(pS)LKDPSNN(-CONH<sub>2</sub>) to keyhole limpet hemocyanin and 150 μg of the conjugate was repeatedly injected into rabbits (NZ white, 5 kg). The antiserum against pS193-c-FLIP was collected on day 7 after injections and positively and consequently negatively affinity purified using (Ac-)CLQAAIQK(pS)LKDPSNN(-CONH<sub>2</sub>) and (Ac-)CLQAAIQKSLKDPSNN(-CONH<sub>2</sub>) conjugated to NHS-activated matrix columns (GE Bioscience, Chalfont St Giles, UK), respectively. Whole-cell extracts, as well as lysis buffer lysates, were subjected to 10% SDS-PAGE and tested for their specificity.

Immunoblotting with the anti-pS193-c-FLIP antibody was performed after SDS-PAGE in reducing conditions and subsequent electric transfer to nitrocellulose membranes. Membranes were briefly washed in MOPS buffer saline (MOPS 25 mM, NaCl 125 mM (pH 7.4), Tween 20 0.5%) and blocked in 5% BSA in the same buffer overnight. After three washes, the membranes were exposed to the primary antibodies (1:500) overnight. After antibody incubation, the membranes were washed three times, incubated with HRP-conjugated secondary antibody (Pierce, Rockford, IL, USA), triple washed three times again, after which the membranes were developed with ACL chemiluminescent substrate (Amersham) and registered on X-ray films (Fuji, Tokyo, Japan).

**Metabolic labeling by <sup>35</sup>S.** K562 cells (1–5 × 10<sup>6</sup>) stably overexpressing c-FLIP were washed once with methionine and cysteine-free RPMI-1640 supplemented with 10% dialyzed FCS, antibiotics, and L-glutamine, and pulse labeled at 37°C for 3 h with 200 μCi (0.1 mCi/ml) [<sup>35</sup>S]methionine and cysteine (MP Biomedicals, Irvine, CA, USA). The labeled cells were washed with supplemented RPMI-1640 containing unlabeled methionine and cysteine 100 times in excess (Sigma-Aldrich). The cells were chased for indicated times, washed with PBS, and treated according to the Flag-immunoprecipitation protocol. The relative amounts of <sup>35</sup>S-labeled c-FLIP were quantified by phosphorimager analyses, and statistical significance of the differences between WT and mutant c-FLIP<sub>S</sub> from three independent experiments was statistically analyzed.

**Quantitation, densitometry, analysis, and illustration of c-FLIP half-lives and caspase-8 activation.** The relative amounts of c-FLIP in the CHX chases were analyzed by densitometry (Adobe Photoshop) and normalized against the Hsc70 loading control. The amounts of <sup>35</sup>S-labeled c-FLIP were quantified by phosphorimager analysis. The relative half-lives and the relative caspase-8 activation were illustrated using GraphPad Prism (version 5) and statistical significance analyzed by Student's unpaired *t*-test (*n* = 3, \**P* = 0.05, \*\**P* = 0.005). The graphs show mean values and the standard errors of mean.

**Immunoprecipitation.** To immunoprecipitate Flag-tagged c-FLIP, we lysed the cell pellets on ice in coimmunoprecipitation buffer (25 mM HEPES (pH 7.5), 150 mM sodium chloride, 5 mM EDTA, 0.5% Triton X-100, 20 mM sodium pyrophosphate, 0.5 mM PMSF, 0.1 mM sodium orthovanadate, 1 mM DTT, Complete Protease Inhibitor Cocktail Tablets (Roche Diagnostics GmbH, Germany) and 10% of the lysates were used as input samples. The cleared cell lysate was immunoprecipitated with 15 μl of M2-agarose Flag-beads (Sigma-Aldrich), at 4°C on a rotamix for 1–2 h. The beads were collected by mild centrifugation and washed twice with TEG buffer (20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10% glycerol, 150 mM sodium chloride, 0.5% Triton X-100) and three times

with Flag buffer (10 mM Tris-HCl (pH 7.5), 50 mM sodium chloride, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 5 μM zinc chloride, 10% glycerol, 0.5% Triton X-100). Flag-tagged c-FLIP was eluted from the beads by adding 250 μg/ml of Flag-peptide (Sigma-Aldrich) in Flag buffer and incubating in shaking overnight at 4°C. The supernatant was collected and mixed with 3 × Laemmli sample buffer, boiled, and separated by SDS-PAGE, followed either by autoradiography with an imaging plate using Fujifilm BAS-1800 Bioimaging analyzer or western blotting.

For immunoprecipitation of ubiquitylated Flag-c-FLIP, the cell pellet from transiently transfected cells was resuspended in 75 μl of boiling 1% SDS in PBS, and the resulting lysate was heated at 100°C for 5 min. The lysates were suspended 1:10 in 1% Triton X-100 in PBS. DNA was sheared by sonication, and the particulate material was centrifuged for 15 min at 15 000 × *g*. Samples were taken from the cleared lysates for input control. The lysates were further diluted 1:1 with 1% Triton X-100, 0.5% BSA in PBS, and incubated with anti-HA (HA probe; Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody and 15 μl of a 50% slurry of protein G-Sepharose under rotation for 2 h. After incubation, the Sepharose beads were washed four times with 1% Triton X-100 in PBS, and the immunoprecipitated proteins were run on an 8 or 10% SDS-PAGE, transferred to nitrocellulose membrane (Protran nitrocellulose; Schleicher & Schuell), and immunoblotted with anti-FLIP antibody.

**TRAIL-R immunoprecipitation and DISC analysis.** To stimulate TRAIL receptors, we pelleted 4 × 10<sup>7</sup> K562 cells per sample (500 × *g*, 7 min) and resuspended in 1 ml of RPMI-1640 medium. Thereafter, 1 μg Flag-tagged TRAIL (Alexis) together with 2 μg anti-Flag monoclonal M2 antibody (Sigma-Aldrich) was added to the cell suspension. The cells were incubated at 37°C for 20 min, and the reaction was stopped by adding 10 ml of ice-cold PBS. After washing, the cells were lysed in 1 ml of lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10% glycerol, 0.2% Nonidet P40, and Complete protease inhibitor cocktail (Roche)) for 30 min on ice. The cell debris was removed by centrifugation at 15 000 × *g* for 15 min at 4°C. The amount of protein was determined by the Bradford assay, and an equal amount of protein from each sample was precleared with 50 μl of Sepharose-CL-4B for 2 h at 4°C. A total of 2.5 μg of monoclonal anti-DR5 and 2.5 μg monoclonal anti-DR4 (Alexis) was added to samples and immunoprecipitated with 15 μl protein G beads (Amersham) for 2.5 h at 4°C. The beads were washed six times with 1 ml of lysis buffer, resuspended in 3 × Laemmli sample buffer, and finally boiled for 3 min. The IP samples and corresponding cell lysates were analyzed by 10% SDS-PAGE.

**Luminometric caspase-8 activity assay.** The activity of caspase-8 in the TRAIL DISC overpopulated with WT or phosphomutant c-FLIP was measured after downscaled TRAIL DISC immunoprecipitation (described above) by Caspase-8 Glo kit (Promega) according to manufacturer's protocol. Luminescence was measured by Tecan Ultra luminometer at MediCity, Turku. Caspase-8 activity induced in the parental K562 cells was given the value 1, and the activity readings from c-FLIP overexpressing cell lines were related against this value. Relative caspase-8 activation in different cell lines was illustrated using GraphPad Prism (version 5). The graphs show mean values and the standard errors of mean (*n* ≥ 2).

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (<http://www.nature.com/cdd>)