LY303511 amplifies TRAIL-induced apoptosis in tumor cells by enhancing DR5 oligomerization, DISC assembly, and mitochondrial permeabilization

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Certain classes of tumor cells respond favorably to TRAIL due to the presence of cell surface death receptors DR4 and DR5. Despite this preferential sensitivity, resistance to TRAIL remains a clinical problem and therefore the heightened interest in identifying compounds to revert tumor sensitivity to TRAIL. We recently demonstrated that the phosphatidylinositide-3-kinase (PI3K) inhibitor, LY294002, and its inactive analog LY303511, sensitized tumor cells to vincristine-induced apoptosis, independent of PI3K/Akt pathway. Intrigued by these findings, we investigated the effect of LY303511 on TRAIL-induced apoptosis in HeLa cells. Preincubation of cells with LY30 significantly amplified TRAIL signaling as evidenced by enhanced DNA fragmentation, caspases 2, 3, 8, and 9 activation, and reduction in the tumor colony formation. This increase in TRAIL sensitivity involved mitochondrial membrane permeabilization resulting in the egress of cytochrome c and second mitochondrial activator of caspase/direct IAP-binding protein with low PI, cleavage of X-linked inhibitor of apoptosis protein, and activation of caspase 9. We link this execution signal to the ability of LY30 to downregulate cFLIP$_S$ and oligomerize DR5, thus facilitating the signaling of the death initiating signaling complex. The subsequent exposure to TRAIL resulted in processing/activation of caspase 8 and cleavage of its substrate, the BH3 protein Bid. These data provide a novel mechanism of action of this small molecule with the potential for use in TRAIL-resistant tumors.
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Tumor necrosis factor-related apoptosis inducing ligand (TRAIL) has been shown to induce apoptosis preferentially in tumor cells.[1,2] The selective responsiveness of TRAIL is attributed to the increased surface expression of TRAIL receptors (DR4 and DR5) on tumor cells.[3–6] In contrast, non-transformed cells express decoy receptors that provide a mechanism for evading apoptotic signaling initiated by TRAIL, and hence its tumor selectivity.[7,8] Unfortunately, as with most chemotherapeutic compounds, TRAIL-responsive tumors acquire a resistant phenotype which renders TRAIL therapy ineffective.[9,10] This has stimulated an enormous interest in identifying small molecule compounds that, when used in combination with TRAIL could sensitize tumor cells to TRAIL-induced apoptosis. The desired consequence would be the need for a much lower therapeutic dose of TRAIL and at the same time an increase in the efficacy of the sensitizing drug. To that end, various groups have demonstrated that a variety of compounds and proteins (either upon silencing or upregulation) sensitize several classes of tumor cells to TRAIL-induced apoptosis.[11,12]

LY303511 (LY30) is an inactive analog of LY294002 (LY29), a widely used inhibitor of the phosphatidylinositide-3-kinase (PI3K)/Akt survival pathway.[13] Previously, LY30 has been purported to have no effect on cells in contrast to its active counterpart, LY29. However, recent studies from our laboratory and other groups have demonstrated that LY30 does have activity of its own.[14–16] In this regard, we recently demonstrated that LY30 significantly enhanced sensitivity of tumor cells to vincristine-induced apoptosis via the generation of intracellular hydrogen peroxide (H_2O_2).[16] As LY30 is a cell-permeable small molecule compound, here we investigated if it could behave similarly in the settings of TRAIL-induced apoptosis, thus providing a novel combinatorial regimen for targeting TRAIL-responsive tumor cells.

We report that preincubation of TRAIL-responsive human cervical cancer cells (HeLa) with LY30 significantly increased their sensitivity to low non-apoptotic concentrations of TRAIL. In addition, results indicate that LY30 facilitates TRAIL-induced death signaling by inducing DR5 oligomerization and downregulation of FLIP$_S$ in HeLa cells, which significantly

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amplifies TRAIL-evoked assembly of the death-inducing signaling complex (DISC). DISC formation in these cells is then followed by robust activation of caspases 8, 2, 9, and 3, as well as mitochondrial apoptotic events such as release of cytochrome *c* (cyt *c*) and second mitochondrial activator of caspase/direct IAP-binding protein with low pI (Smac/Diablo). These data reinforce our earlier report that the supposedly inactive analog of LY29, LY30, possesses strong antitumor activity of its own and suggest the therapeutic potential of LY30-like small molecule compounds in enhancing tumor cell responsiveness to TRAIL-induced apoptosis.

Results

Preincubation with LY30 increases TRAIL sensitivity and inhibits tumor colony formation. The dose of TRAIL used throughout this study was established by analyzing the effect of increasing TRAIL concentrations (20–100 ng/ml) for 18 h on the viability of HeLa cells, which responded in a dose-dependent manner ($LD_{50} \sim 50$ ng/ml), with relatively modest effect on cell viability ($<20\%$) at concentrations of 20 ng/ml (Figure 1a). TRAIL (20 ng/ml) was therefore used in subsequent experiments to assess the sensitizing activity of LY30. Preincubation of HeLa cells with LY30 (2.5–25 μM) for 1 h followed by treatment with 20 ng/ml of TRAIL for 18 h significantly decreased cell viability (Figure 1b). For comparison, HeLa cells were separately preincubated with LY29 before TRAIL treatment; however, there was no significant reduction in cell viability (Figure 1b) despite the ability of LY29 to inhibit PI3K (Figure 1c). These data prompted us to analyze further the mechanism underlying LY30-induced sensitization to TRAIL. Results show that pretreatment of HeLa cells with 25 μM LY30 (optimal concentration eliciting synergistic effect, used in subsequent experiments) for 1 h followed by exposure to 20 ng/ml of TRAIL for 12 h strongly amplified the enzymatic activities of caspases 2 and 3 over the single agent-treated cells. This was confirmed by kinetic analyses of the processed forms of both caspases by Western blotting; LY30 + TRAIL-treated cells displayed significantly enhanced processing of both caspases to their active fragments, 18 and 17 kDa for caspases 2 and 3, respectively (Figure 1d lower panel and Figure 1e).

The augmented caspase activation in LY30 + TRAIL-treated cells was also accompanied by a significant increase in subdiploid (sub-G1) DNA content indicative of apoptosis-associated DNA fragmentation. Of note, whereas single agent treatment with LY30 or TRAIL for 24 h induced a minimal increase in sub-G1 fraction, there was a marked synergistic increase in this fraction upon pretreatment with LY30 and subsequent exposure to TRAIL (39.1%), which became more pronounced after 48 h (76.7%, Figure 1f). Moreover, to assess the potential of LY30-induced sensitization to TRAIL on long-term survival/progression of tumor cells, we evaluated the effect of this combinatorial treatment on tumor colony forming units as reported previously.[16] There was a strong reduction in colony formation (20% of untreated cells) upon exposure to LY30 + TRAIL, compared to single agent-treated cells (Figure 1g).

LY30 enhances TRAIL-mediated signaling by engaging mitochondrial death pathway. Having shown that LY30-induced sensitization to TRAIL involved caspase activation, we next investigated if the amplification of death signaling by LY30 was a function of mitochondrial outer membrane permeablization (MOMP) by studying the effect of LY30 and TRAIL on mitochondrial transmembrane potential ($\Delta\Psi$m) and the release of intermembranous proteins, cyt *c* and Smac/Diablo. Despite the minimal effect on $\Delta\Psi$m (Figure 2a), Western blot analysis of the kinetics of the release of mitochondrial amplification factors revealed that LY30 + TRAIL treatment resulted in a significant increase of cytosolic cyt *c* levels followed by the egress of Smac/Diablo, whereas TRAIL alone had no effect on the cytosolic translocation of these proteins (Figure 2b). The release of cyt *c* could be partially inhibited by the mitochondrial permeability transition (MPT) pore inhibitor cyclosporine A (CsA; 10 nM) (Figure 2c), suggesting that the MPT pore may have a role in MOMP associated with LY30-induced sensitization to TRAIL. Interestingly, despite the increase in cytosolic Smac/Diablo in LY30 + TRAIL-treated cells, there was no significant change in the level of the X-liked apoptosis inhibitory protein, XIAP (Figure 2d). Instead, a 30 kDa cleaved form of XIAP was detected, which was accompanied by a significant increase in the enzymatic activity of caspase 9 (Figure 2e).

LY30 enhances DR5 oligomerization. Our data has shown that LY30-mediated sensitization to TRAIL-induced apoptosis triggered the mitochondrial death pathway, but the upstream signal involving receptor-mediated extrinsic pathway remained elusive. Therefore, we evaluated the effect of LY30 on the surface expression of DR5 and DR4 by flow cytometry. Results showed that 1 h incubation with 25 μM LY30 resulted in a slight increase in DR5, but not DR4, immunostaining at the cell surface (Figure 3a); however, Western blot analysis of total DR5 levels remained unchanged (Figure 3b). Of note, as the increase in DR5 surface expression was not significant enough to be attributed to a classical upregulation of membrane receptor, we hypothesized that the increase in DR5 immunostaining could be a result of DR5 surface oligomerization in LY30-treated cells, thus increasing their affinity for the anti-DR5-PE compared to the single non-oligomerized surface DR5. In contrast, preincubation with LY29 did not significantly affect the status of DR4 or DR5 in our system, reiterating the inability of LY29 to amplify TRAIL signaling (data not shown).

Involvement of DR5 oligomerization in LY30-induced TRAIL sensitization was corroborated by studies demonstrating that LY30 treatment induced DR5 oligomerization using limiting (0.4 μg) concentration of anti-DR5 (Figure 3d, upper panel) in the presence of the non-cell-permeable cross-linker, 3,3′-dithiobis sulfosuccinimidylpropionate (DTSSP), demonstrated previously in DR5 oligomerization studies.[17] Cross-linked oligomerized DR5 complexes are immunoprecipitated more readily in the presence of limiting anti-DR5 (0.4 μg) as compared to non-oligomerized DR5 or cross-linked complexes made up of DR5 and other receptors. This was observed in LY30-treated whole-cell lysates, compared to untreated cells with (Figure 3d, upper panel) or without
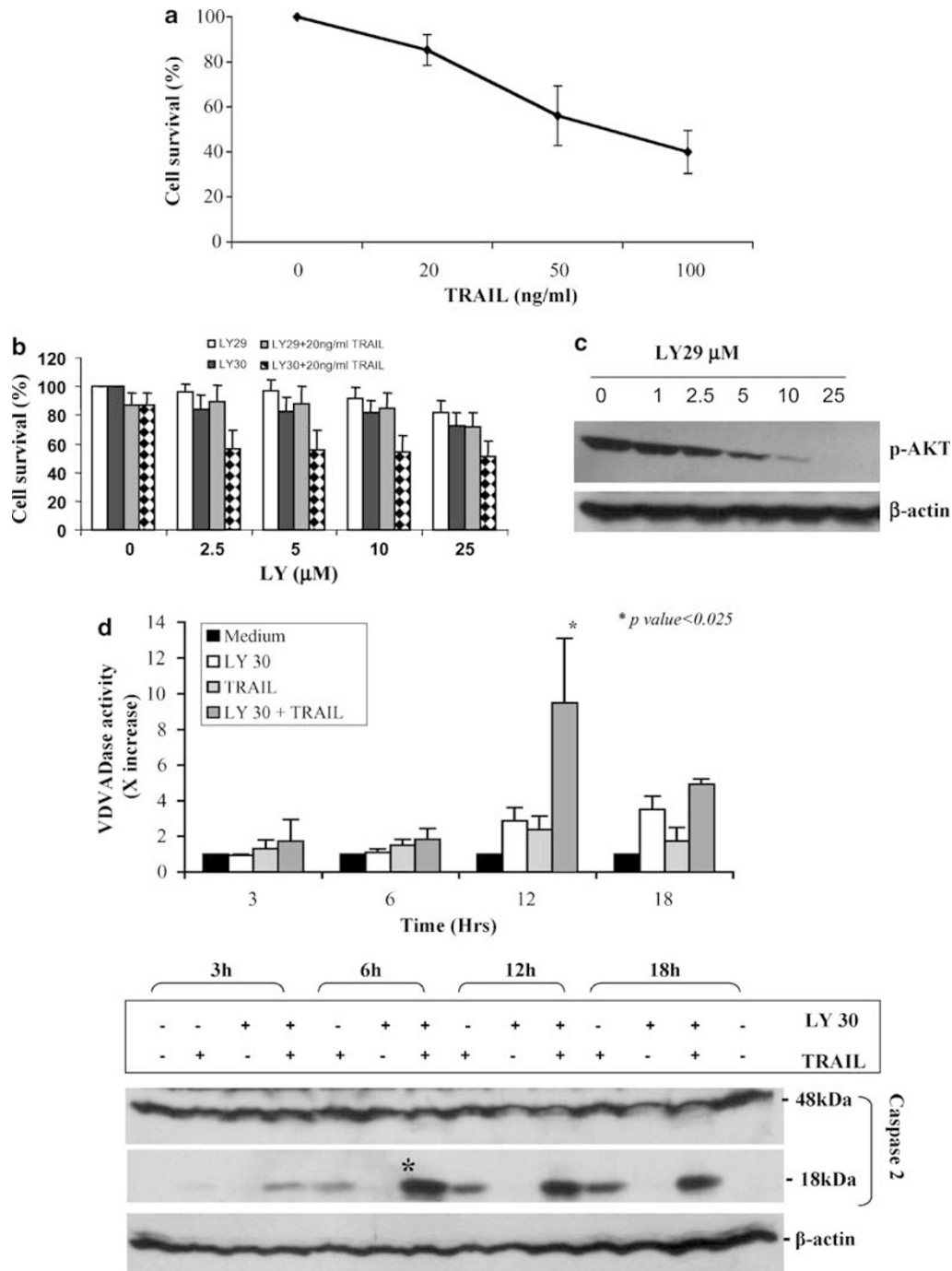
Figure 1 LY30 sensitizes HeLa cells to TRAIL-induced apoptosis with activation of caspases 2 and 3, and reduction of tumor colony forming ability. (**a**) HeLa cells (1×10^5) were exposed to TRAIL (20–100 ng/ml) for 18 h or (**b**) treated with 20 ng/ml of TRAIL for 18 h $\pm$ an hour pretreatment with various doses of LY29 or LY30. Cell viability was determined by the crystal violet assay. (**c**) HeLa cells (1×10^6) were exposed to various doses of LY29 for 1 h and whole-cell lysates were then used to assay for phospho-Akt by Western blotting. (**d** and **e**) HeLa cells (1×10^5) were exposed to 20 ng/ml TRAIL (3–18 h) following 1 h pretreatment with 25 μM LY30. Whole-cell lysates were then used to determine the activity kinetics of caspases 2 and 3 and processing of their proforms by Western blotting (NS: non-specific band). (**f**) HeLa cells (1×10^5) were exposed to TRAIL for 24 and 48 h $\pm$ an hour pretreatment with LY30. PI staining was performed for cell-cycle analysis as described in Materials and Methods. Cell-cycle profiles shown are representative of three independent experiments. (**g**) HeLa cells were exposed to TRAIL for 8 h $\pm$ an hour pretreatment with LY30 before being re-seeded for colony formation assay. The error bars in **a**, **b**, **d**, **g** and **e** represent the mean $\pm$ S.D. ($n = 3$)

DTSSP (Figure 3c, lower panel), suggesting increased surface oligomerization of DR5. Additionally, gel filtration followed by Western blot analysis was performed and higher molecular weight fractions (200–400 kDa) of DR5 oligomers in lysates of LY30-treated cells were detected (Figure 3d, lower panel). Furthermore, confocal imaging of LY30 + TRAIL-treated cells for 5 min showed oligomerizaton of DR5, evidenced by the punctuate pattern of DR5 staining
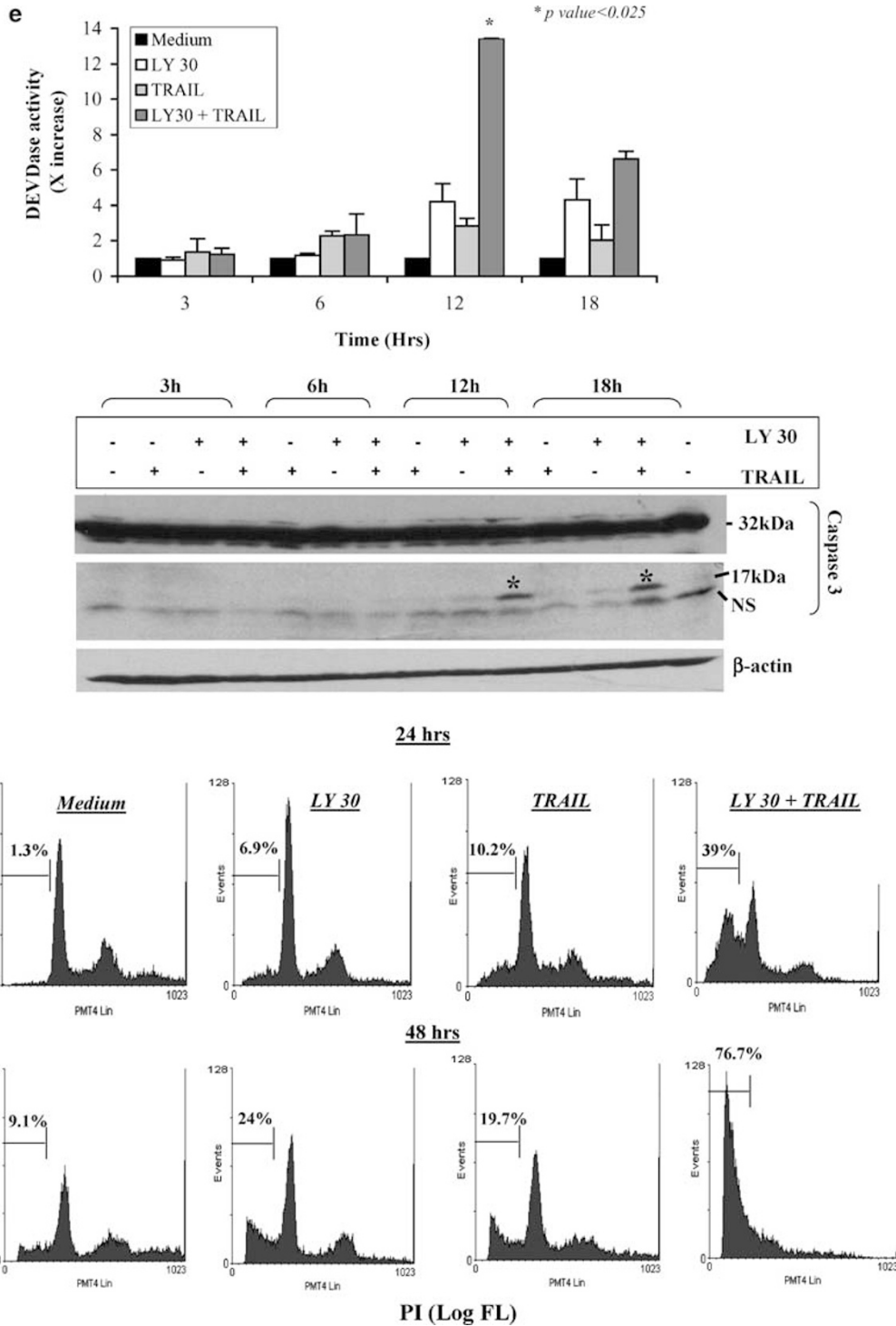
Figure 1 Continued

(Supplementary Figure S1). Intriguingly, this was only observed at 1 min post-TRAIL treatment of cells preincubated with LY30, which is in concordance with recent reports that TRAIL ligation could be a signal for rapid DR5 internalization.[18,19] Taken together, these data suggest a probable mechanism whereby LY30 enhances TRAIL responsiveness by increasing the oligomerization of DR5.

LY30 enhances DISC assembly and downstream caspase 8 processing. To provide functional significance to the data on surface DR5 oligomerization, we questioned whether the effect of LY30 was mediated through an increase in DISC assembly. To assess DISC formation, HeLa cells were first incubated for 1 h in the presence or absence of LY30, and then treated with TRAIL for 5–30 min.
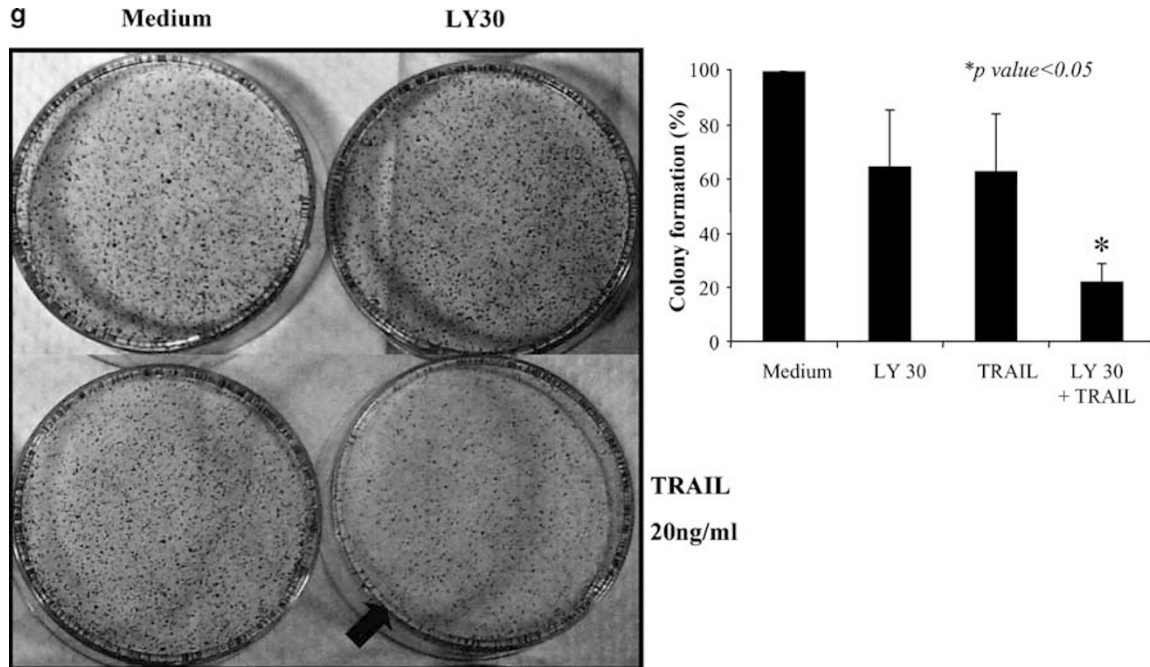
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Lysates were immunoprecipitated with anti-DR5 and Western blot analysis was performed using anti-FADD or anti-caspase 8 (Figure 4a). LY30 + TRAIL-treated cells displayed a strong interaction between DR5, the adaptor molecule FADD, and caspase 8 (Figure 4a). Fas-associated death domain-containing protein (FADD), like DR5, has also been shown to appear as a doublet (24/25 kDa) in Western blots and this has been suggested by various groups to be a result of either different FADD phosphorylation states or the presence of two FADD isoforms.[20,21] In our DISC analysis of LY30 + TRAIL-treated cells, we observed that both forms of FADD (24/25 kDa) and processed caspase 8 (43/41 kDa) were associated with immunoprecipitated DR5, compared to cells treated singly with either LY30 or TRAIL, where only the 43 and 25 kDa forms of caspase 8 and FADD respectively, were associated with DR5. The more efficient DISC formation seen in LY30 + TRAIL-treated cells is probably a dual result of LY30-mediated DR5 oligomerization and TRAIL-mediated ligation of DR5 itself. To confirm further this and understand the kinetics of this signaling, DISC assembly was analyzed after 0, 5, 15, and 30 min of TRAIL ligation. Results indicate that the recruitment of the DISC components (FADD and caspase 8) occurred within 5–15 min after TRAIL exposure (Supplementary Figure S4).

Analysis of caspase 8 processing, an event immediately downstream of DISC assembly, showed that pretreatment of cells with LY30 amplified TRAIL-mediated activation of caspase 8 (41/43 and 18 kDa) (Figure 4b). Furthermore, activation of caspase 8 was confirmed by measuring the kinetics of its enzymatic activity, as well as, the processing of its substrate, the Bcl-2 BH3 only protein Bid. Indeed, caspase 8 activity was significantly induced (200% of untreated cells) as early as 4 h and peaked by 12 h (300% of untreated cells) after treatment with LY30 + TRAIL

(Figure 4c). Correspondingly, LY30 + TRAIL-treated cells showed significant Bid cleavage (Figure 4d). Single agent treatment with LY30 appeared to stimulate weak recruitment of the DISC components, 43 and 25 kDa forms of caspase 8 and FADD, respectively (Figure 4a). This could be a result of DR5 oligomerization induced by LY30, which would be conducive for association of these DISC components. However, cell death is induced via processing of caspase 8 to its active 18 kDa form to induce Bid cleavage and MOMP. Without TRAIL stimulation, there was no processing of caspase 8 to its 18 kDa fragment (Figure 4b), no significant enzymatic activity of caspase 8 (Figure 4c), and absence of Bid (caspase 8 substrate) cleavage (Figure 4d) to affect mitochondria-dependent events, which would explain the limited cytotoxicity observed in LY30-treated cells without TRAIL stimulation (Figure 1).

Many compounds have been shown to enhance TRAIL-induced apoptosis by reducing c-FLIP expression and facilitating caspase 8 activation.[22] Indeed, we show that the short isoform of c-FLIP, FLIP$_S$, is downregulated as early as 3 h post-LY30 + TRAIL treatment (Figure 4e). Although the activation of the caspase cascade appeared to be necessary for apoptosis, as pan-caspase inhibitor ZVAD-fmk blocked LY30 + TRAIL-induced decrease in cell survival (Figure 5a) and DNA fragmentation (Figure 5c), incubation with caspase 8 inhibitor, Z-IETD-fmk, was only able to partially salvage cells from LY30 + TRAIL-induced apoptosis (∼20% protection, Figure 5b). A closer analysis of these data show that presence of the caspase 8 inhibitor rescued cells treated with TRAIL (*); however, cell viability/proliferation affected by LY30 treatment alone (∼20%) could not be reverted by caspase 8 inhibition. Individual caspase activity assays in the presence and absence of the specific caspase inhibitors were performed to demonstrate their efficacy (Supplementary Figure S3).
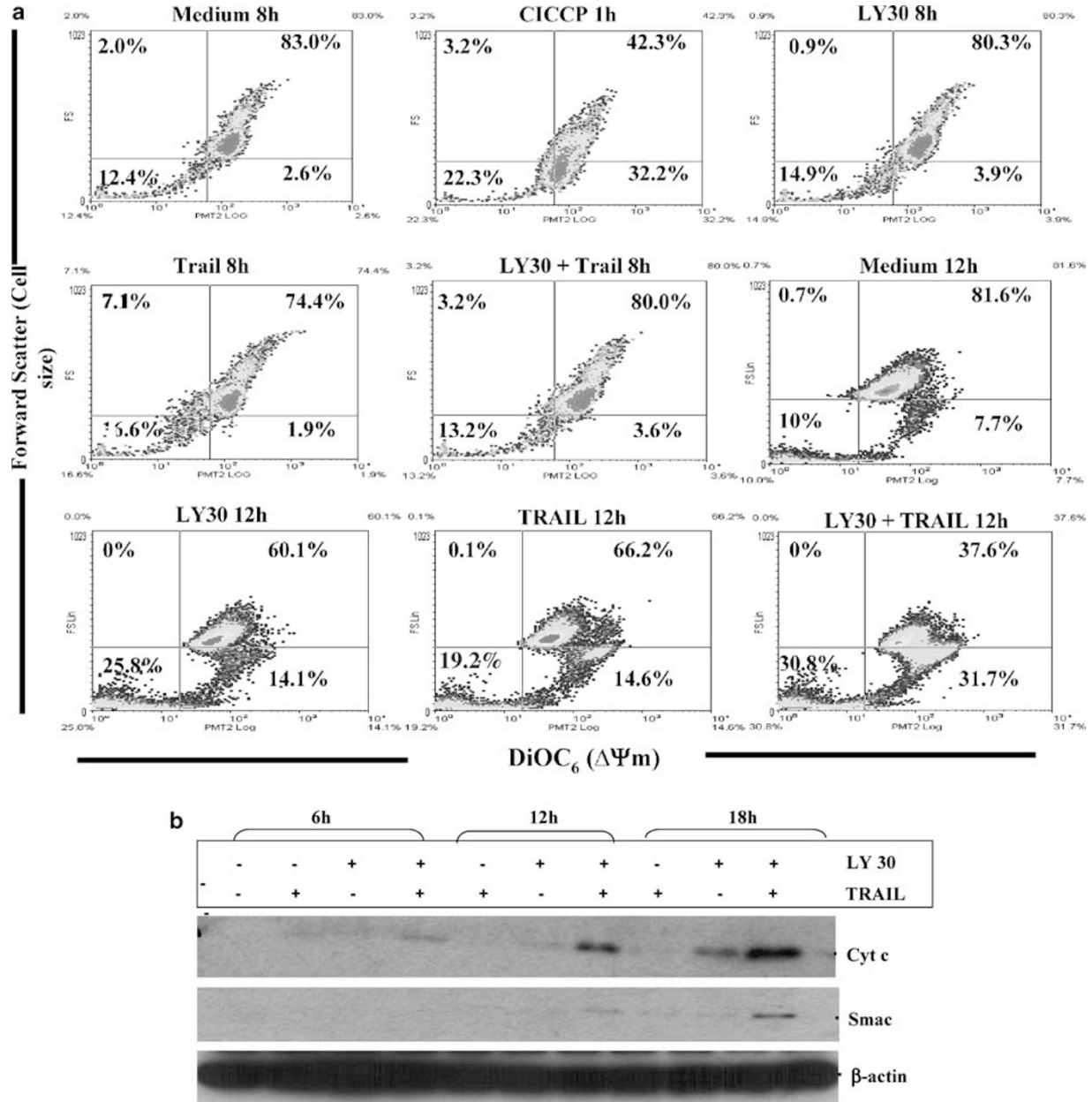
Figure 2 LY30 + TRAIL-induced apoptosis involves MOMP and caspase 9 activation. (**a**) HeLa cells (1×10^5) were exposed to TRAIL (8 or 12 h) $\pm$ an hour pretreatment with LY30. $\Delta\Psi$m of cells was assessed by DIOC6 using flow cytometry with 100 μM CICCP as a positive control. At least 10 000 events were acquired. (**b**) HeLa cells (3×10^6) were exposed to TRAIL (6, 12, or 18 h) $\pm$ an hour pretreatment with LY30. Levels of cyt c and Smac in the cytosolic fractions were then assayed by Western blotting. (**c**) HeLa cells (3×10^6) were first exposed to 10 nM CsA for 1 h followed by LY30 + TRAIL treatment for 18 h before detection of cytosolic cyt c by Western blotting. (**d**) HeLa cells (1×10^6) were treated as in **b** and whole-cell lysates were used to assay for XIAP expression by Western blotting. (**e**) Whole-cell lysates of HeLa cells (1×10^5) after exposure to TRAIL (3–18 h) $\pm$ an hour pretreatment with LY30 were assayed for caspase 9 activity. The error bars represent the mean $\pm$ S.D. ($n = 3$)

LY30 sensitizes HT29 and Jurkat cells to TRAIL-induced apoptosis. To show that the sensitizing effect of LY30 was not restricted to a single tumor cell line (HeLa), similar experiments were performed with another TRAIL-sensitive leukemia cell line, Jurkat. Initial data from these experiments demonstrated that the same dose of LY30 (25 μM) could sensitize these cells to 20 ng/ml of TRAIL with a reduction of cell viability and synergistic activation of caspases 2, 3, 8,

and 9 (Figure 6a and b). TRAIL-resistant colon carcinoma cell line HT29 was also tested in preliminary studies. Cells were preincubated for 1 h with increasing doses of LY30 (25–100 μM) followed by treatment with increasing doses of TRAIL (50–(50–100 ng/ml)) for 18 h. There appeared to be a marked synergistic reduction in cell viability for HT29 cells treated with 100 μM LY30 and 100 ng/ml TRAIL (Figure 6c). These data provide strong evidence that LY30-mediated
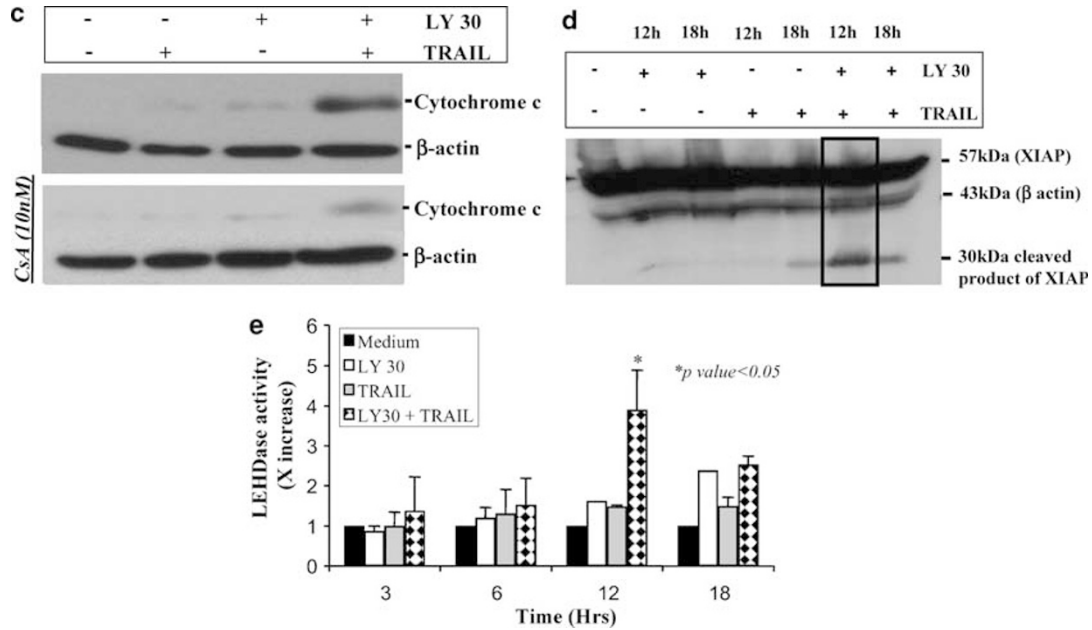
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sensitization to TRAIL-induced apoptosis is not restricted to one cell type and independent of cells' susceptibility to TRAIL.

Discussion

TRAIL-mediated apoptosis is amplified upon pretreatment with LY30. We present evidence that preincubation of HeLa cells with LY30 significantly amplified TRAIL-mediated death signaling with a marked reduction in the tumor colony forming ability. Results also support the involvement of the mitochondrial death pathway in LY30-induced TRAIL sensitization as evidenced by cytosolic translocation of cyt *c* and Smac/Diablo at a time preceding maximal activation of caspase 9 with no significant decrease in $\Delta\Psi m$, an observation in line with recent literature demonstrating that minimal opening of MPT pore can induce cyt *c* release without significantly affecting $\Delta\Psi m$.[23] With regards to the mitochondria in TRAIL sensitization, there are reports demonstrating that drug-induced sensitization to TRAIL might be linked to silencing of the IAP family of proteins.[24,25] Our results show that despite the cytosolic translocation of IAP regulating protein Smac/Diablo, exposure of cells to LY30 + TRAIL did not significantly affect the cellular levels of XIAP. However, cleavage of XIAP to a 30 kDa fragment was detected via Western blot in LY30 + TRAIL-treated cells and this occurred with approximately the same kinetics as activation of caspases 2, 3, and 9, an observation that was paralleled in other studies of XIAP cleavage in apoptosis induced by members of the TNF family.[26,27] These studies have shown that caspases can exert positive feedback by cleaving (and inactivating) members of the IAP family such as XIAP, thus accelerating the apoptotic process. In fact, one of these

studies showed that TNFα induced similar cleavage of XIAP to a 30 kDa fragment enhanced by LY29,[27] again alluding perhaps to the non-specificity of the LY compounds. Collectively, these data indicate involvement of caspases and the mitochondrial pathway in the TRAIL-sensitizing activity of LY30. Furthermore, XIAP cleavage (inactivation) also argues in favor of a post-mitochondria amplification of death signaling subsequent to the initial trigger.

LY30 induces DISC assembly and amplifies DR5 signaling upon TRAIL ligation. TRAIL-mediated apoptosis follows a pathway similar to FasL and TNFα, whereby ligation of the death receptors is necessary to trigger DISC formation and processing/activation of procaspase 8, which can then drive the caspase cascade with (Type II) or without (Type I) the need for amplification from the mitochondria. Selective upregulation of genes for DR4 and/or DR5,[28,29] caspase 8,[30] BH3 protein Bid,[31] or Smac/Diablo[32,33] have been documented as mechanism(s) underlying increased sensitization to TRAIL. In our model, LY30 pretreatment resulted in an increase in sensitivity to TRAIL with a readout involving MOMP and cytosolic translocation of amplification factors. To decipher the upstream signal necessary for the recruitment of these pathways, we analyzed the effect of LY30 on death receptor signaling. Indeed, we show here that the amplification effect could be a function of induced assembly of the DISC components, FADD and caspase 8. Notably, unlike other models of TRAIL-induced apoptosis,[11,29,34,35] the total protein expression of DR5 did not change significantly upon LY30 treatment; however, a selective augmentation of DR5 immunostaining intensity at the cell surface was observed by flow cytometry within 1 h of incubation with LY30. The subsequent addition of TRAIL for, as short as 5 min, resulted in a reduction in the fluorescent anti-DR5-PE signal. This is suggestive of rapid internalization of DR5, a mechanism

consistent with death receptor–ligand interaction. Similar experiments were also performed with anti-DR4-PE and there was no regression in the population of cells staining positive for surface DR4 in the presence of TRAIL, an indication that the

reduction of DR5 signal at the cell surface upon addition of TRAIL was not due to non-specific competition between anti-DR5 and TRAIL. Furthermore, we provide evidence that the increased cell surface immunostaining intensity of DR5 upon
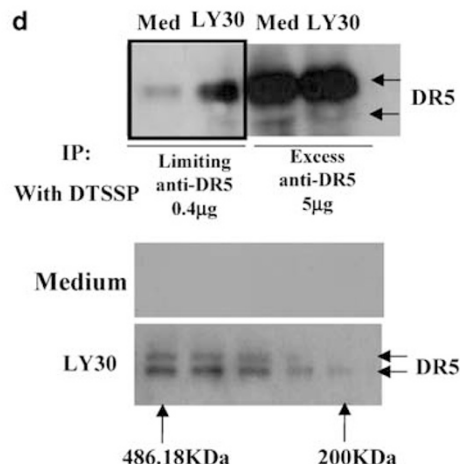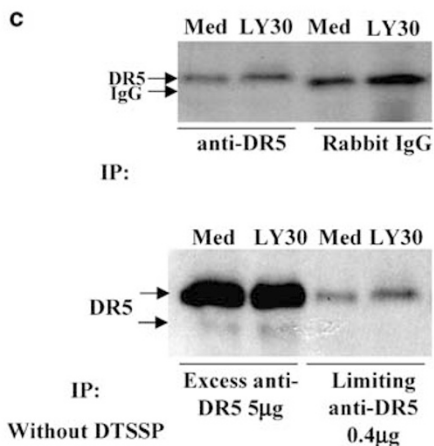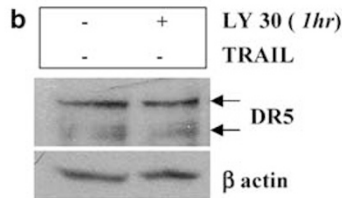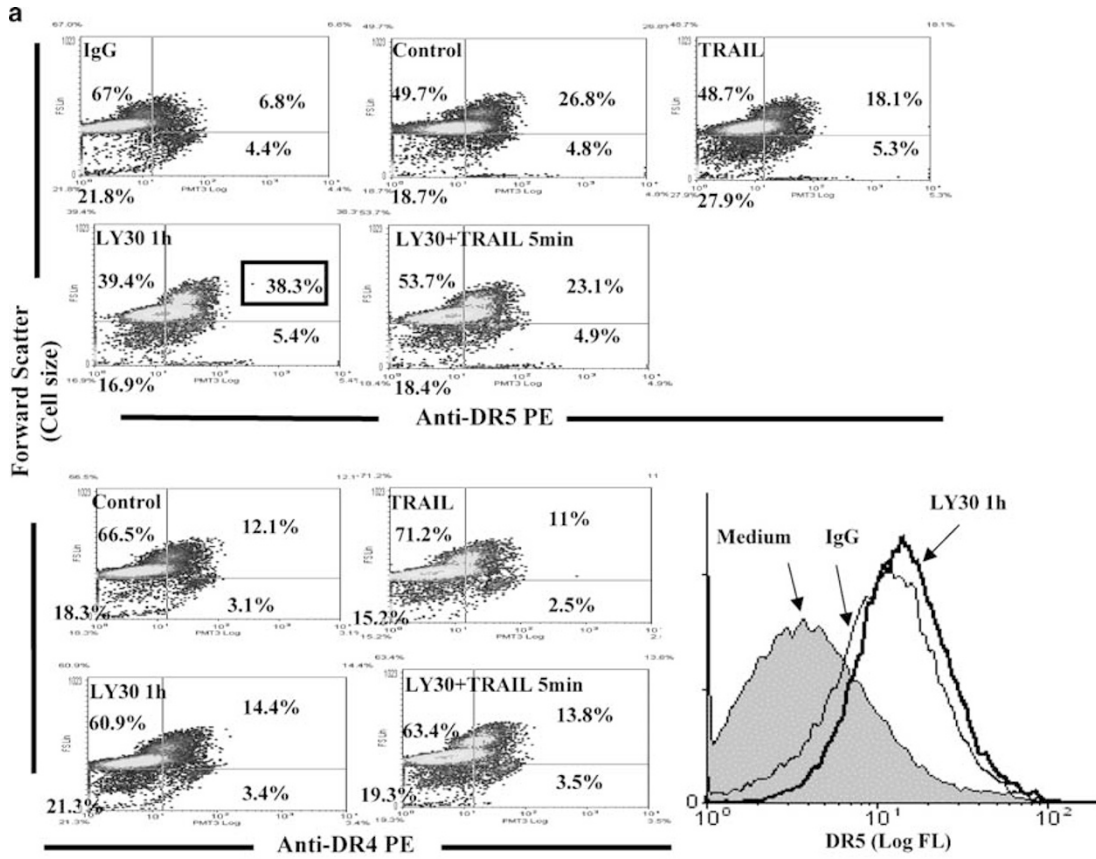
LY30 treatment could be a function of TRAIL-independent death receptor oligomerization through DR5-affinity immunoprecipitation, gel filtration, and confocal studies, which could account for the robust amplification of receptor signaling upon TRAIL ligation.

The precise mechanisms by which LY30 induces these changes in the cell surface receptor and DISC formation are not clearly understood, but a possible target could be cellular FLICE-like inhibitory protein (cFLIP); cFLIP has been shown to bind to either FADD or caspase 8 at the DISC, thus inhibiting death receptor signaling; and conversely, silencing cFLIP has been shown to promote TRAIL-mediated apoptosis.[24,36] Although we were unable to detect cFLIP at the DISC level, even in untreated cells (data not shown), an early downregulation of the short cFLIP isoform, $cFLIP_S$, was observed with LY30 treatment that was more pronounced after subsequent TRAIL treatment. Reduction of $cFLIP_S$ levels by LY30 would facilitate enhanced processing of the apical caspase 8.[37] In fact, our time kinetic analysis of these proteins corroborates the sequence of events where early downregulation of $FLIP_S$ is followed by processing of procaspase 8 processing to its 18 kDa form, resulting in Bid cleavage. This serves to amplify LY30-mediated death sensitization loop by further affecting the mitochondrial death pathway characteristic of Type II (HeLa) cells. The processing of caspase 8 after DISC formation is the main initiating event that drives downstream death execution; however, selective inhibition of caspase 8 in our system did not completely rescue cells from LY30 + TRAIL-induced apoptosis, although it was able to protect TRAIL-treated cells. This suggests that LY30 by itself may also affect cell viability, albeit minimally (<20%), in a manner independent of caspase 8 activity.

A second possible candidate could be intracellular H_2O_2 produced upon LY30 + TRAIL treatment as various studies have implicated intracellular reactive oxygen species (ROS) in receptor-mediated apoptosis,[35,38] and offer ROS-dependent mechanistic explanations such as release of Smac/Diablo from the mitochondria and ROS-induced death receptor upregulation.[35,39] However, in our model, although LY30 and TRAIL induced an increase in intracellular H_2O_2 (Supplementary Figure S2A) neither the scavenging of H_2O_2 by transfection with human catalase (Supplementary Figure S2B and C) nor the addition of exogenous H_2O_2 (data not shown), had any significant effect on LY30-induced sensitization to TRAIL. These data argue against the direct involvement of LY30-mediated intracellular production of H_2O_2 in DR5 oligomerization and DISC assembly and suggest the involvement of death amplification pathways that may operate in parallel or independent of H_2O_2 production.

It should be pointed out that the ability of LY30 to sensitize other TRAIL-responsive (Jurkat) and TRAIL-resistant (HT29) cells demonstrates that the sensitizing ability of LY30 is not restricted to HeLa cells. TRAIL sensitization studies on HT29 cells by others have demonstrated amplification of the mitochondrial death pathway with release of Smac and XIAP downregulation,[12] an observation consistent with our data on HeLa cells.

LY30 and related compounds as novel sensitizers or amplifiers of TRAIL signaling. The myriad ways by which TRAIL-mediated death could be enhanced suggest that there are many factors mediating the effector mechanisms underlying death receptor-dependent death signaling in tumor cells. Our findings suggest an interesting possibility in designing chemotherapeutic strategies aimed at enhancing the response of tumor cells to TRAIL. Interestingly, LY29, a sister compound of LY30, was unable to enhance TRAIL signaling, thus ruling out the involvement of the PI3K pathway in sensitizing HeLa cells to TRAIL-induced apoptosis. These data indicate a novel biological activity of LY30, which could have tremendous implications for enhancing the therapeutic efficacy of TRAIL.

In conclusion, we describe a novel mechanism where LY30 sensitizes cervical carcinoma HeLa cells to TRAIL via DR5 oligomerization and $FLIP_S$ downregulation. This facilitates transduction of the death signal upon TRAIL ligation from the DISC to the mitochondria, triggering MOMP and resulting in amplification of caspase-dependent apoptotic signaling. This is in line with another recent study demonstrating the significance of ligand-independent death receptor clustering in the sensitization to Fas-mediated apoptosis and overcoming of Fas resistance.[40] Also, the observation that LY30 can sensitize Jurkat and HT29 cells to TRAIL indicates that our findings might be relevant across different tumor cell types. Taken together, this work highlights the tremendous potential of LY30 in priming tumor cells for TRAIL-mediated killing, and could have implications for the design of novel strategies to overcome the problem of TRAIL resistance in tumor cells.

Materials and Methods

Determination of cell viability. The cervical cancer HeLa, human leukemia Jurkat and colon carcinoma HT29 cell lines were purchased from ATCC (Rockville, MD, USA) and maintained in DME supplemented with 10% FBS, 1% L-glutamine, and 1% S-penicillin. In a typical survival assay, HeLa cells (1×10^5 cells/well) plated in 24-well plates were preincubated for 1 h with LY30 (2.5–25 μM) (Calbiochem, Darmstadt, Germany and Alexis Phramaceuticals) and then treated with 20 ng/ml of TRAIL (Biomol, Plymouth Meeting, PA, USA) for 18 h. To determine the optimum concentration of TRAIL, a dose–response curve for TRAIL (20–100 ng/ml for 18 h) was obtained. Cell viability was determined by the crystal violet assay

Figure 3 LY30 enhances oligomerization of DR5. (**a**) HeLa cells (1×10^5) were exposed to TRAIL for 5 min after 1 h pretreatment with LY30, stained with anti-DR5-PE or anti-DR4-PE and analyzed by flow cytometry. The density plots of forward scatter and DR5-PE/DR4-PE fluorescence shown are representative of at least three independent experiments. A corresponding histogram of DR5-PE fluorescence intensity *versus* cell number for cells singly treated with LY30 is shown. (**b**) HeLa cells (1×10^6) were incubated with LY30 for 1 h and whole-cell lysates were used to assay for total DR5 levels by Western blotting. (**c**) Immunoprecipitation with rabbit IgG was also used as a control in the immunoprecipitation experiments (upper panel). Control immunoprecipitation experiments with limiting and excess amounts of anti-DR5 in the absence of DTSSP were also similarly performed (lower panel). (**d**) HeLa cells (3×10^6) were exposed to LY30 for 1 h before treatment with DTSSP to cross-link oligomerized DR5 and immunoprecipitated with limiting (0.4 μg) and excess (5 μg) amounts of anti-DR5 as described in Materials and Methods and analyzed by Western blotting (upper panel). High molecular weight fractions of oligomerized DR5 were also obtained via gel filtration as described in Materials and Methods. Data shown is a mean representative blot of three independent experiments depicting the relevant high molecular weight fractions of DR5 oligomers (lower panel)
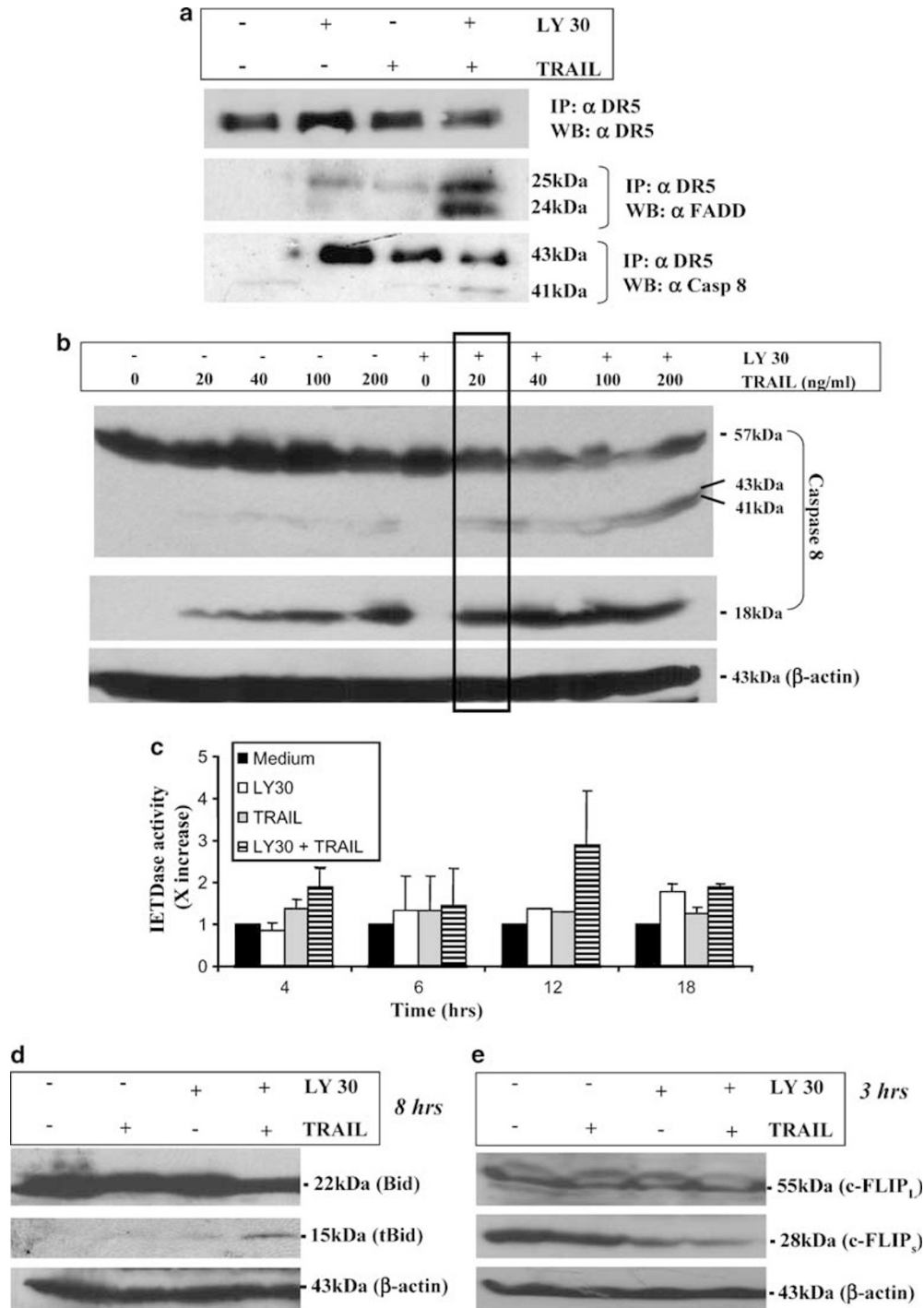
Figure 4 LY30 pretreatment enhances DISC assembly with downstream caspase 8 activation in TRAIL-treated cells. (**a**) HeLa cells (3×10^6) were exposed to TRAIL for 5 min $\pm$ an hour pretreatment with LY30. Cells were harvested and immunoprecipitated with anti-DR5 as described in Materials and Methods. Analyses of DISC components, FADD and caspase 8, were performed by Western blotting using anti-FADD and anti-caspase 8. (**b**) Whole-cell lysates of HeLa cells (1×10^6) were obtained after exposure for 8 h to TRAIL (20–100 ng/ml) with $\pm$ an hour pretreatment with LY30 and used to assay for caspase 8 processing by Western blotting. (**c**) HeLa cells (1×10^5) were also exposed to of TRAIL (4–18 h) $\pm$ an hour pretreatment with LY30. Whole-cell lysates were used to assay for activity kinetics of caspase 8. The error bars represent the mean $\pm$ S.D. ($n = 3$). (**d**) Cleavage of Bid was assessed by Western blotting after 8 h of LY30 + TRAIL treatment. (**e**) c-FLIP$_{S/L}$ levels in the cell were assessed by Western blotting after 3 h of LY30 + TRAIL treatment

as described previously.[16] Cell viability experiments were similarly performed with preincubation for 1 h with 20 μM pan-caspase inhibitor ZVAD-fmk (Biomol), caspase 8 inhibitor Z-IETD-fmk, caspase 2 inhibitor Z-VDVAD-fmk, or caspase 9 inhibitor Z-LEHD-fmk (all from R&D systems, Minneapolis, MN, USA), before

the addition of LY30 and TRAIL. For cell lines where the crystal violet assay could not be used to assess viability (Jurkat), the 3-(4,5-dimethylthiazol- 2-yl)-2,5 diphenyltetrazolium bromide (MTT) assay as described previously,[41] was used instead.
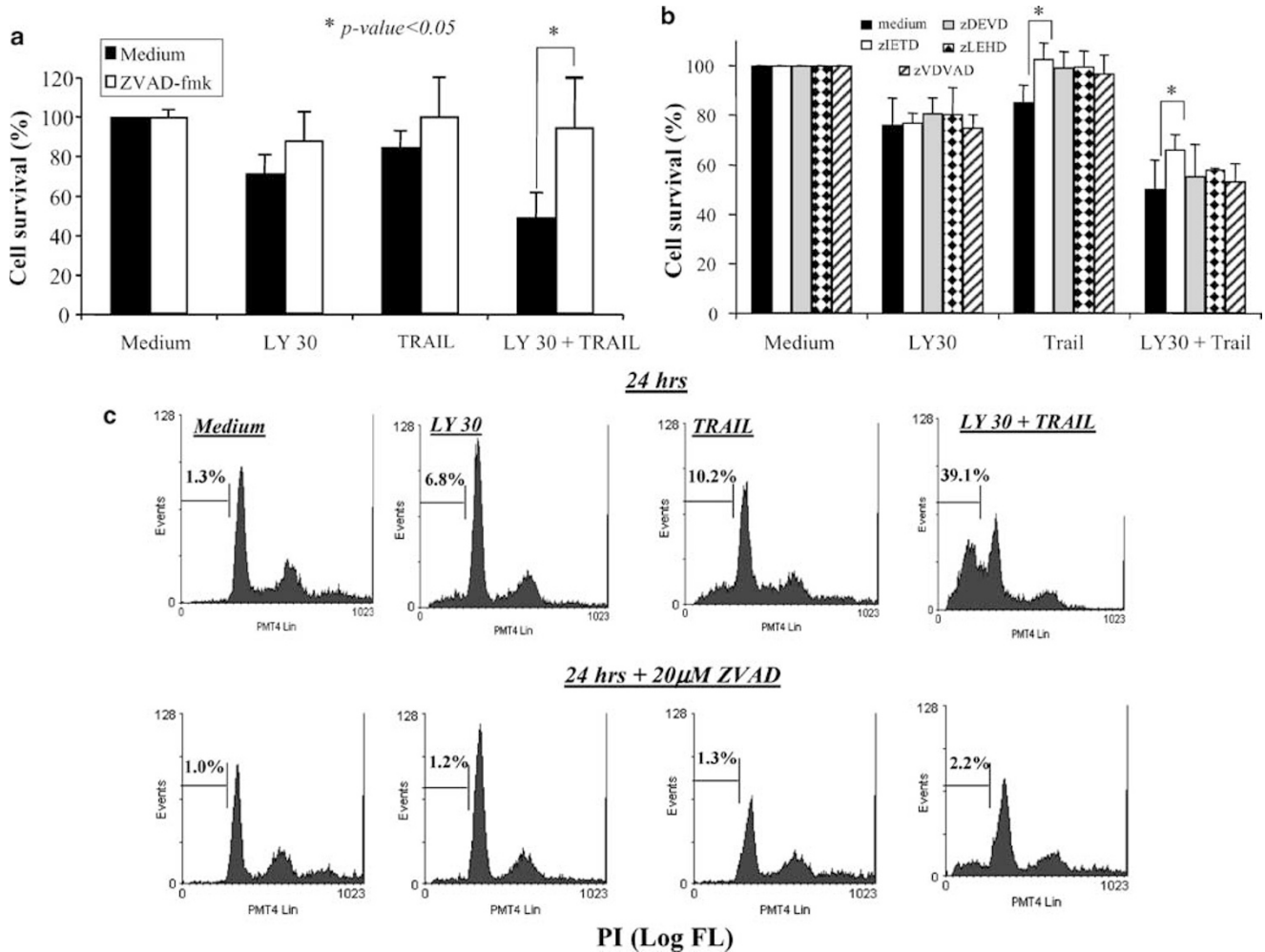
Figure 5 Caspase 8 inhibition partially rescues cells from LY30 + TRAIL-induced caspase-dependent apoptosis (**a** and **b**). HeLa cells (1×10^5) were treated for 1 h with the respective caspase inhibitors, (ZVAD-fmk 20 μM, Z-DEVD-fmk 50 μM, Z-IETD-fmk 50 μM, Z-VDVAD-fmk 50 μM, Z-LEHD-fmk 50 μM) before treatment with LY30 + TRAIL for 18 h. Cell viability was then assessed via crystal violet staining. The error bars represent the mean ± S.D. ($n = 3$). (**c**) HeLa cells (1×10^5) were preincubated with 20 μM ZVAD-fmk before the usual LY30 + TRAIL treatment and stained with PI for analysis of DNA fragmentation by flow cytometry. Cell-cycle profiles shown are representative of three independent experiments

Determination of caspases 2, 3, 8, and 9 activities. Caspases 2, 3, 8, and 9 activities were assayed using 7-amino trifluoromethylcoumarin and 7-amino-4-methycoumarin-conjugated substrates (BioMol). HeLa cells (1×10^5/ml) were preincubated with 25 μM LY30 for 1 h, followed by treatment with 20 ng/ml of TRAIL for 3, 6, 12, and 18 h. Cells were then harvested and washed with $1 \times$ PBS, resuspended in chilled cell lysis buffer (BD Pharmingen, San Diego, CA, USA), and incubated on ice for 10 min before the addition of the respective substrates for caspase activity analysis as reported previously.[16]

Propidium iodide staining for DNA fragmentation. Cells were fixed with 70% ethanol and stained with propidium iodide (PI) for DNA content analysis as described elsewhere.[41] A total of 10 000 events were analyzed by flow cytometry using an excitation wavelength set at 488 nm and emission at 610 nm.

SDS-PAGE and Western blotting. HeLa cells were plated (3×10^6) in a 60 mm Petri dish and treated with various agents for the indicated durations. Cells were harvested and washed once with PBS, then lysed with cell lysis buffer (150 mM NaCl, Tris–HCL 7.4, and 1% Nonidet P40). Cell lysate (100 μg) was then electrophoresed on a 12% polyacrylamide gel before immunoblotting for specific proteins.

For isolation of cytosolic fractions, cells were incubated for 30 min in extraction buffer A (50 mM PIPES-KOH (pH 7.4), 200 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM MgCl$_2$, and 1mM DTT), supplemented with a cocktail of protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin, 20 μg/ml pepstatin A, and

10 μg/ml leupeptin), before homogenization in a dounce homogenizer. The homogenate was spun at 12 000 g for 45 min, and cytosolic supernatants were removed. Rabbit polyclonal anti-caspase 3, anti-Smac (clone 7), anti-XIAP (clone 48), and anti-cyt c (clone 7H8.2C12) were obtained from BD Pharmingen. Anti-caspase 2 (clone C2), anti-caspase 8 (clone 1C13), and anti-Bid (clone 7A3) were purchased from Cell Signaling (Beverly, MA, USA). Protein blots were probed with anti β-actin (Sigma-Aldrich, St Louis, MO, USA) as controls for protein loading and cytosolic fractions.

Determination of mitochondrial transmembrane potential. Potential-sensitive probe 3,3′-dihexyloxacarbocyanine iodide (DiOC$_6$) was used to measure $\Delta\psi$m as described previously.[42] Briefly, cells were washed once with PBS, then incubated with DiOC$_6$ (40 nM) for 15 min at 37°C and immediately analyzed by flow cytometry (Coulter EPICS Elite ESP) with excitation set at 488 nm. At least 10 000 events were analyzed.

Flow cytometric analysis of intracellular H$_2$O$_2$ concentration. Intracellular concentration of H$_2$O$_2$ was determined by staining with the redox-sensitive dye 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescin diacetate (CM-H$_2$DCFDA; Molecular Probes, Eugene, OR, USA), which becomes fluorescent when oxidized by H$_2$O$_2$ and its free radical products. Briefly, cells were washed once with PBS, loaded with 5 μM CM-H$_2$DCFDA at 37°C for 15 min, and analyzed by flow cytometry (Coulter EPICS Elite ESP) using an excitation wavelength of 488 nm. At least 10 000 events were analyzed.
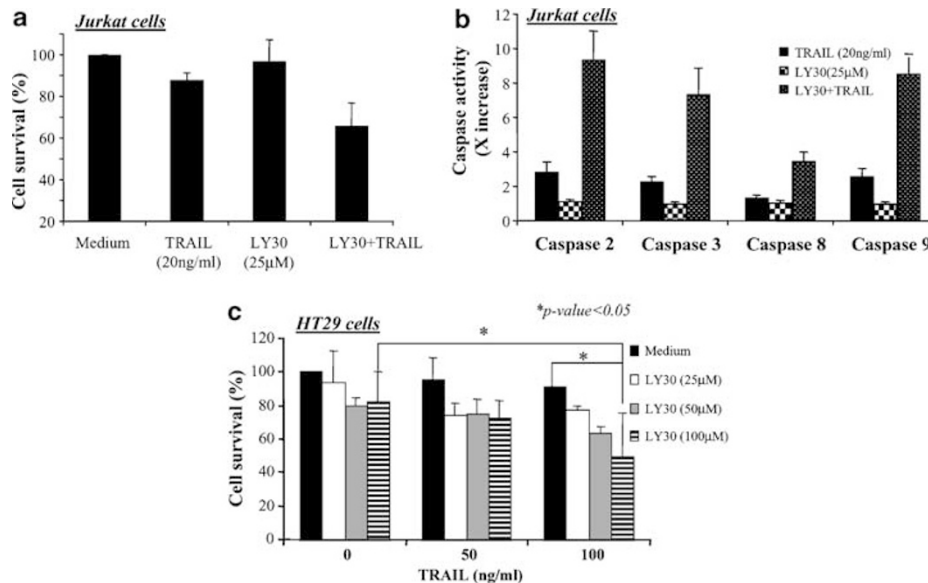
Figure 6 LY30 can also sensitize Jurkat and HT29 cells to TRAIL-induced apoptosis. (**a**) Jurkat cells (1×10^6) were exposed to 20 ng/ml TRAIL for 18 h $\pm$ an hour pretreatment with 25 μM LY30. Cell viability was determined by the MTT assay and (**b**) caspase activation was determined by using the respective caspase substrates. (**c**) HT29 cells (1×10^5) were exposed to TRAIL (50 or 100 ng/ml) for 18 h $\pm$ an hour pretreatment with 25–100 μM LY30. Cell viability was determined by the crystal violet assay. The error bars represent the mean $\pm$ S.D. ($n = 3$)

Determination of tumor colony formation. HeLa cells (1×10^5) were pretreated for 1 h with 25 μM LY30 followed by 20 ng/ml of TRAIL in a 24-well plate for 8 h. As this treatment period did not induce any visible cell death, cells were then washed and 4000 cells from each well were re-plated into 60 mm culture dishes and left to form colonies over a period of 8–12 days. Culture dishes were stained with crystal violet to determine the number of colonies as described in our recent communication.[16]

Analyses of DISC formation and DR5 oligomerization. HeLa cells (5×10^6) were seeded in 100 mm Petri dishes and treated with LY30 and TRAIL accordingly. For DR5 oligomerization studies, cells were treated for 30 min at 25°C with 2 mM DTSSP (Pierce Chemical Co., Rockford, IL, USA), a cleavable cross-linker.[17] The reaction was quenched with 50 mM Tris–HCL for 10 min, and cells were washed twice with ice-cold PBS and incubated for 30 min at 4°C in lysis buffer (30 mM Tris–Hcl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Triton-X supplemented with 1 mM PMSF, 10 μg/ml aprotinin, 20 μg/ml pepstatin A, and 10 μg/ml leupeptin) on a rocker platform. For analysis of DISC formation, cells were washed with ice-cold PBS after LY30 + TRAIL treatment and lysed in a similar fashion. The lysates were then pre-cleared with 20 μl Protein A (Sigma-Aldrich) before incubating overnight on a rocker with 1 μg rabbit polyclonal anti-DR5 (BioVision, Mountain View, CA, USA) and 40 μl Protein A. For assessment of DR5 oligomerization, the lysates were incubated overnight on a rocker with limiting and excess amounts of the same anti-DR5. The limiting (0.4 μg) and excess amounts (5 μg) of anti-DR5 were determined via a titration process where the limiting amount was determined by the highest amount of anti-DR5 that was just not enough to immunoprecipitate DR5. The complexes were washed four times with the cell lysis buffer and centrifuged at 12 000 r.p.m. for 5 min at 4°C before boiling for 10 min in 1 × SDS loading buffer. Lysates were then subjected to SDS-PAGE and immunoblotted with mouse anti-caspase 8 (clone 1C12; Cell Signaling) and mouse anti-FADD (clone A662; BD Transduction Labs, San Diego, CA) for analysis of DISC assembly. Protein blots were probed for equal loading and for confirmation of DR5 in the pulldown with goat anti-DR5 (R&D Systems, Minneapolis, MN, USA). For analysis of DR5 oligomerization, protein blots were also probed with the goat anti-DR5. Rabbit IgG was used as a control in the DR5 immunoprecipitation experiments.

Analysis of cell surface levels of DR5 and DR4. HeLa cells (1×10^5) were treated with LY30 and TRAIL as described above and then washed three times in PBS supplemented with 0.5% BSA. Cells were then stained with phycoerythrin (PE)-conjugated mouse monoclonal anti-human DR5 or DR4 (clone

71908 and 69036, respectively; R&D systems, Minneapolis, MN, USA) for 45 min at 4°C according to manufacturer's instructions before washing and resuspension in PBS for flow analysis using an excitation wavelength of 488. PE-conjugated mouse IgG2B was used as an isotype control.

Gel filtration. Gel filtration chromatography was done on a Superdex 200 10/300 GL column connected to a fast protein liquid chromatography system. Equilibration with buffer containing PBS and calibration was done with β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), albumin (66 kDa), carbonic anhydrase (29 kDa), and cyt *c* (12 kDa). Void volume corresponded to the elution of Blue Dextran 2000. Cellular extracts were prepared and lysed in a 300 μl volume. Soluble extracts (250 μl) were injected onto the column for elution at a flow rate of 200 μl/min. Fractions (200 μl) from untreated/LY30-treated samples were subjected to SDS-PAGE and Western blotting simultaneously. Position of DR5 proteins in the elution profile was determined by immunoblotting using goat anti-DR5.

Confocal analysis of surface DR5. Hela cells (1×10^5) were plated in a 24-well plate on polylysine-coated coverslips. After a 1 h preincubation with 25 μM LY30 followed by 1–5 min incubation with 20 ng/ml TRAIL, cells were washed twice with 1 × PBS and fixed with 4% paraformaldehyde for 20 min at room temperature. After three washes with 1 × PBS, cells were incubated with goat anti-DR5 (Santa Cruz, Santa Cruz, CA) in 1 × PBS + 0.5% BSA at 1 : 100 dilution for 2 h, washed three times with 1 × PBS in 0.5% BSA, and incubated for 1 h with 1 : 200 FITC-conjugated secondary antibody (Santa Cruz) in 1 × PBS + 0.5% BSA. Cells were again washed three times with 1 × PBS + 0.5% BSA before the coverslips were mounted on glass slides and imaged with a Carl Zeiss laser scanning confocal microscope.

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