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TITLE:

Measuring Composition of CD95 Death-inducing Signaling Complex and Processing of Procaspase-8 in this Complex

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CD95 DISC formation, immunoprecipitation, caspase-8

SUMMARY:

Here, an experimental workflow is presented that enables the detection of caspase-8 processing directly at the death-inducing signaling complex (DISC) and determines the composition of this complex. This methodology has broad applications, from unraveling the molecular mechanisms of cell death pathways to the dynamic modeling of apoptosis networks.

ABSTRACT:

Extrinsic apoptosis is mediated by the activation of death receptors (DRs) such as CD95/Fas/APO-1 or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-receptor 1/receptor 2 (TRAIL-R1/R2). Stimulation of these receptors with their cognate ligands leads to the assembly of the death-inducing signaling complex (DISC). DISC comprises DR, the adaptor protein Fas-associated protein with death domain (FADD), procaspases-8/-10, and cellular FADD-like interleukin (IL)-1β-converting enzyme-inhibitory proteins (c-FLIPs). The DISC serves as a platform for procaspase-8 processing and activation. The latter occurs *via* its dimerization/oligomerization in the death effector domain (DED) filaments assembled at the DISC.

Activation of procaspase-8 is followed by its processing, which occurs in several steps. In this work, an established experimental workflow is described that allows the measurement of DISC formation and the processing of procaspase-8 in this complex. The workflow is based on immunoprecipitation techniques supported by western blot analysis. This workflow allows careful monitoring of different steps of procaspase-8 recruitment to the DISC and its processing and is highly relevant for investigating molecular mechanisms of extrinsic apoptosis.

INTRODUCTION:

One of the best-studied death receptors (DRs) is CD95 (Fas, APO-1). The extrinsic apoptotic

pathway starts with the interaction of the DR with its cognate ligand, *i.e.,* CD95L interacts with CD95 or TRAIL binds to TRAIL-Rs. This results in the formation of the DISC at the corresponding DR. DISC consists of CD95, FADD, procaspase-8/-10, and c-FLIP proteins[1,2]. Furthermore, the DISC is assembled by interactions between death domain (DD)-containing proteins, such as CD95 and FADD, and DED-containing proteins such as FADD, procaspase-8/-10, and c-FLIP (**Figure 1**). Procaspase-8 undergoes oligomerization *via* association of its DEDs, resulting in the formation of DED filaments, followed by procaspase-8 activation and processing. This triggers a caspase cascade, which leads to cell death (**Figure 1**)[3,4]. Thus, procaspase-8 is a central initiator caspase of the extrinsic apoptosis pathway mediated by CD95 or the TRAIL-Rs, activated at the corresponding macromolecular platform, DISC.

Two isoforms of procaspase-8, namely procaspase-8a (p55) and -8b (p53), are known to be recruited to the DISC[5]. Both isoforms comprise two DEDs. DED1 and DED2 are located at the N-terminal part of procaspase-8 a/b followed by the catalytic p18 and p10 domains. Detailed cryo-electron microscopy (cryo-EM) analysis of procaspase-8 DEDs revealed the assembly of procaspase-8 proteins into filamentous structures called DED filaments[4,6]. Remarkably, the linear procaspase-8 chains were initially suggested to be engaged in the dimerization followed by procaspase-8 activation at the DISC. Now, it is known that those chains are only a substructure of the procaspase-8 DED filament, the latter comprising three chains assembled into a triple helix[3,4,6,7].

Upon dimerization at the DED filament, conformational changes in procaspase-8a/b lead to the formation of the active center of procaspase-8 and its activation[3,8]. This is followed by procaspase-8 processing, which is mediated *via* two pathways: the first one goes *via* the generation of a p43/p41 cleavage product and the second one *via* the initial generation of a p30 cleavage product. The p43/p41 pathway is initiated by the cleavage of procaspase-8a/b at Asp374, resulting in p43/p41 and p12 cleavage products (**Figure 2**). Further, these fragments are auto-catalytically cleaved at Asp384 and Asp210/216, giving rise to the formation of the active caspase-8 heterotetramer, $p10_2/p18_2$[9–11]. In addition, it was shown that in parallel to the p43/p41 pathway of processing, procaspase-8a/b is also cleaved at Asp216, which leads to the formation of the C-terminal cleavage product p30, followed by its proteolysis to p10 and p18[10] (**Figure 2**).

Procaspase-8a/b activation at the DED filament is strictly regulated by proteins named c-FLIPs[12]. The c-FLIP proteins occur in three isoforms: $c\text{-FLIP}_{Long}$ ($c\text{-FLIP}_L$), $c\text{-FLIP}_{Short}$ ($c\text{-FLIP}_S$), and $c\text{-FLIP}_{Raji}$ ($c\text{-FLIP}_R$). All three isoforms contain two DEDs in their N-terminal region. $c\text{-FLIP}_L$ also has a C-terminal catalytically inactive caspase-like domain[12,13]. Both short isoforms of c-FLIP—$c\text{-FLIP}_S$ and $c\text{-FLIP}_R$—act in an anti-apoptotic manner by disrupting DED filament formation at the DISC[6,14,15]. In addition, $c\text{-FLIP}_L$ can regulate caspase-8 activation in a concentration-dependent manner. This can result in both pro- and anti-apoptotic effects[16–18]. By forming the catalytically active procaspase-8/$c\text{-FLIP}_L$ heterodimer, $c\text{-FLIP}_L$ leads to the stabilization of the active center of procaspase-8 and its activation. The pro- or anti-apoptotic function of $c\text{-FLIP}_L$ is directly dependent on its amount at the DED filaments and the subsequent amount of assembled procaspase-8/$c\text{-FLIP}_L$ heterodimers[19]. Low or intermediate concentrations of $c\text{-FLIP}_L$ at the DISC result in sufficient amounts of procaspase-8/$c\text{-FLIP}_L$ heterodimers at the DED filament, which

supports the activation of caspase-8. In contrast, increased amounts of c-FLIP$_L$ directly lead to its anti-apoptotic effects at the DISC[20].

Taken together, the activation and processing of procaspase-8a/b at the DISC is a highly regulated process involving several steps. This paper discusses the measurement of procaspase-8 processing directly at the DISC as well as the analysis of the composition of this complex. This will be presented using CD95 DISC as the exemplary DR complex.

PROTOCOL:

T cell experiments were performed according to the ethical agreement 42502-2-1273 Uni MD.

1. Preparing cells for the experiment

NOTE: The average number of cells for this immunoprecipitation is 1×10^7. Adherent cells have to be seeded one day before the experiment so that there are 1×10^7 cells on the day of the experiment.

1.1. Preparing adherent cells for the experiment

1.1.1. Seed 5–8 × 10^6 adherent cells in 10 mL of medium (see the **Table of Materials** for the composition) for each condition in 14.5 cm dishes one day before the experiment starts.

1.1.2. On the day of the experiment, ensure that the cells are 80–90% confluent and adherent to the dish. Discard the medium and add fresh medium to the adherent cells.

1.2. Preparing suspension cells for the experiment

1.2.1. Carefully place 1×10^7 suspension cells in 10 mL of culture medium (see the **Table of Materials** for the composition) per condition in 14.5 cm dishes immediately before the experiment starts.

1.2.2. If using primary cells, isolate primary T cells according to the previously described procedure[21]. Treat primary T cells with 1 µg/mL phytohemagglutinin for 24 h, followed by 25 U/mL IL2 treatment for 6 days.

1.2.3. Carefully place 1×10^8 primary T cells in 10 mL of culture medium (see the **Table of Materials** for the composition) per condition in 14.5 cm dishes immediately before the experiment starts.

NOTE: This higher number of primary T cells is recommended, as these cells are smaller.

2. CD95L stimulation

2.1. Stimulate the cells with CD95L (produced as described previously[20] or commercially available

(see the **Table of Materials**)).

NOTE: The concentration of the CD95L and the time of stimulation are cell-type dependent[13,15,22–25]. Prepare one stimulation condition twice to generate a 'bead control' sample in parallel.

2.1.1. Stimulate adherent cells with the selected concentration of CD95L. Hold the plate at an angle and pipet the ligand into the medium without touching the adherent cells.

2.1.2. Stimulate suspension cells with CD95L by pipetting the ligand solution into the cell suspension.

3. Cell harvest and lysis

3.1. Place the cell dishes on ice.

NOTE: Do not discard the medium. Dying cells float in the medium and are important for the analysis.

3.2. Add 10 mL of cold phosphate-buffered saline (PBS) to the cell suspension and scrape the attached cells off the plate. Collect the cell suspension in a 50 mL tube.

3.3. Wash the cell dish with 10 mL of cold PBS twice and place the wash solution into the same 50 mL tube. Centrifuge the cell suspension at 500 × *g* for 5 min, 4 °C.

3.4. Discard the supernatant and resuspend the cell pellet with 1 mL of cold PBS. Transfer the cell suspension into a 1.5 mL tube.

3.5. Centrifuge the cell suspension at 500 × *g* for 5 min, 4 °C. Discard the supernatant and resuspend the cell pellet with 1 mL of cold PBS.

3.6. Centrifuge the cell suspension at 500 × *g* for 5 min, 4 °C. Discard the supernatant and resuspend the cell pellet with 1 mL of lysis buffer (containing 4% protease inhibitor cocktail). Incubate it for 30 min on ice.

3.7. Centrifuge the lysate at maximal speed (~15,000 × *g*) for 15 min, 4 °C.

3.8. Transfer the supernatant (lysate) to a clean tube. Discard the pellet. Take 50 µL of the lysate in another tube. Analyze the protein concentration by Bradford assay and take the amount of lysate corresponding to 25 µg of protein in a vial. Add loading buffer (see the **Table of Materials** for the composition) to the vial. Store it at -20 °C as lysate control.

4. Immunoprecipitation (IP)

4.1. Add 2 µL of anti-APO-1 antibodies and 10 µL of protein A sepharose beads (prepared as

recommended by the manufacturer) to the lysate. Add only 10 µL of the beads to a separate tube containing lysate (stimulated sample) to generate a 'bead control.'

NOTE: Use pipet tips with wide orifices either by cutting the tips or buying special tips for IP while handling the protein A sepharose beads.

4.2. Incubate the mixture of lysate with antibodies/protein A sepharose beads with gentle mixing overnight at 4 °C. Centrifuge the lysates with antibodies/protein A sepharose beads at 500 × *g* for 4 min, 4 °C. Discard the supernatant, add 1 mL of cold PBS to the beads, and repeat this step at least three times.

4.3. Discard the supernatant. Aspirate the beads preferably with a 50 µL Hamilton syringe.

5. Western blot

5.1. Add 20 µL of 4x loading buffer (see the **Table of Materials** for the composition) to the beads and heat at 95 °C for 10 min. Heat the lysate controls at 95 °C for 5 min.

5.2. Load the lysates, IPs, and a protein standard onto a 12.5% sodium dodecyl sulfate (SDS) gel (see the **Table of Materials** for the gel preparation) and run with a constant voltage of 80 V.

5.3. Transfer the proteins from the SDS gel to a nitrocellulose membrane.

NOTE: Here, the semi-dry technique, optimized for the proteins of interest, was used for the transfer over 12 min (25 V; 2.5 A= constant). Soak the nitrocellulose membrane in electrophoresis buffer (see the **Table of Materials** for the composition, prepare according to the manufacturer's instructions) for a few minutes before western blotting.

5.4. Place the blotted membrane in a box and block it for 1 h in blocking solution (0.1% Tween-20 in PBS (PBST) + 5% milk). Incubate the membrane with the blocking solution under gentle agitation.

5.5. Wash the membrane three times with PBST for 5 min each wash.

6. Western blot detection

6.1. Add the first primary antibody at the indicated dilution (see the **Table of Materials**) to the membrane and incubate it overnight at 4 °C with gentle agitation.

6.2. Wash the membrane three times with PBST for 5 min each wash.

6.3. Incubate the membrane with 20 mL of secondary antibody (diluted 1:10,000 in PBST + 5% milk) with gentle shaking for 1 h at room temperature.

6.4. Wash the membrane three times with PBST for 5 min each wash.

6.5. Discard PBST and add approximately 1 mL of horseradish peroxidase substrate to the membrane.

6.6. Detect the chemoluminescent signal (see the **Table of Materials**).

NOTE: The exposure time and the number of captured images depend on the amount of protein in the cell and the specificity of the antibodies used. It must be established empirically for each antibody used for the detection.

REPRESENTATIVE RESULTS:

To analyze caspase-8 recruitment to the DISC and its processing at the CD95 DISC, this paper describes a classical workflow, which combines IP of the CD95 DISC with western blot analysis. This allows the detection of several key features of caspase-8 activation at the DISC: the assembly of the caspase-8-activating macromolecular platform, recruitment of procaspase-8 to the DISC, and the processing of this initiator caspase (**Figure 1** and **Figure 2**). This workflow involves the treatment of sensitive cells with CD95L in a time-dependent manner, followed by their lysis, immunoprecipitation using anti-CD95 (anti-APO-1) antibodies, and subsequent western blot analysis (**Figure 3**).

Cervical cancer HeLa-CD95 cells were used as an example to analyze the DISC formation[26]. Stimulation of these cells with CD95L resulted in a high level of CD95 DISC formation, monitored *via* CD95-immunoprecipitation (**Figure 4**). CD95, FADD, procaspase-8, procaspase-10, and c-FLIPs were observed in these CD95 immunoprecipitations, indicating efficient DISC formation. Importantly, the cleavage products of procaspase-8a/b: p43/p41, p30, and p18 were detected at the DISC, which indicates activation of procaspase-8 and its subsequent processing. In particular, the cleavage products of procaspase-8 p43/p41 and p18 were detected, indicating the two aforementioned steps of the p43 processing pathway. In addition, the p30 product was detected, indicating the alternative pathway of caspase-8 processing. Furthermore, activation of procaspase-8 at the DISC is followed by the cleavage of its substrates such as c-FLIP proteins.

Indeed, the cleavage products of c-FLIP—p43-FLIP and p22-FLIP—were detected in the immunoprecipitations, indicating caspase-8 activation (**Figure 4**). Importantly, neither FADD, procaspase-8, procaspase-10, and c-FLIPs, nor their cleavage products were detected in the immunoprecipitation samples from untreated cells, which underlines the specificity of DISC immunoprecipitation (**Figure 4**). Important information can be obtained from these experiments by quantifying the bands corresponding to the different cleavage products of procaspase-8 (**Figure 4**). This information can be used in the mathematical modeling of apoptosis networks and provides quantitative insights into pathway regulation.

The level of caspase-8 activation at the DISC is modulated by c-FLIPs. Hence, HeLa-CD95 cells that overexpress c-FLIP$_L$ (HeLa-CD95-FL)[15] were selected as the second example (**Figure 5**). The effects of the c-FLIP$_L$ isoform in these experiments could be observed, resulting in a different rate of

procaspase-8a/b processing to p43/p41 at the DISC compared to the corresponding proteolysis of procaspase-8 at the DISC in parental HeLa-CD95 cells, as described by Hillert *et al.*[15]. Similar to the observations in **Figure 4**, no recruitment of FADD, procaspase-8, procaspase-10, c-FLIP proteins, and their cleavage products was detected in the immunoprecipitations from HeLa-CD95-FL cells without CD95L treatment, which supports the specificity of these immunoprecipitations. More evidence for the specificity of immunoprecipitations is the absence of the recruitment of procaspase-3 and poly(ADP-ribose)polymerase 1 (PARP1) to the DISC, which was observed in the CD95L-treated immunoprecipitations (**Figure 5**). These proteins are not part of the complex, and their absence in the immunoprecipitation signals can serve as proof for the absence of nonspecific binding of abundant cellular proteins.

Finally, immunoprecipitations of CD95 DISC from suspension cells were performed as a third example, *e.g.,* activated primary T cells (**Figure 6**). These cells are also characterized by high levels of CD95, FADD, procaspase-8, procaspase-10, and c-FLIPs that were observed in anti-CD95 immunoprecipitations along with their cleavage products (**Figure 6**). The detection of procaspase-8 cleavage products in the immunoprecipitations indicates the activation and processing of this initiator caspase in the DISC immunoprecipitation from primary T cells. These experiments indicate that the DISC can be immunoprecipitated from many adherent and suspension cells and that caspase-8 processing and activation can be validated by western blot analysis.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic presentation of the CD95 signaling pathway. CD95L triggers the DISC assembly. The DISC comprises CD95, FADD, procaspase-8/-10, and c-FLIP. FADD binds to CD95 *via* its DD, whereas procaspase-8, procaspase-10, and c-FLIPs interact *via* their DEDs, forming DED filaments. Formation of the DED filaments serves as a platform for procaspase-8 dimerization, processing, and subsequent activation. The active caspase-8 heterotetramer, $p18_2/p10_2$, activates caspase-3 by cleavage, which leads to apoptosis. Abbreviations: CD = cluster of differentiation; CD95L = CD95 ligand; DISC = death-inducing signaling complex; DD = death domain; FADD= Fas-associated death domain; c-FLIP = cellular FADD-like interleukin (IL)-1β-converting enzyme-inhibitory protein; DED = death effector domain; $c\text{-}FLIP_L$ = $c\text{-}FLIP_{Long}$.

Figure 2: Procaspase-8 processing at the DISC. Two ways of procaspase-8a/b processing at the DISC are shown. The first way involves p43/p41 generation followed by p18 formation. The second way involves p30 generation followed by its processing to p18 and p10. The residues are numbered according to the sequence of procaspase-8a. Abbreviations: DED = death effector domain.

Figure 3: Schematic presentation of the experimental setup of the DISC-IP. Cells are stimulated with CD95L. After stimulation, the cells are harvested and collected, followed by different washing steps. The cells are then lysed, and the lysates are collected. Subsequently, protein A-sepharose beads and anti-APO-1 (anti-CD95) antibodies were added to the lysate and incubated overnight. After several washing steps, the immunoprecipitations were analyzed by western

blotting. Abbreviations: DISC = death-inducing signaling complex; IP = immunoprecipitation; CD95L = CD95 ligand.

Figure 4: CD95L DISC formation in HeLa-CD95 cells. HeLa-CD95 cells were stimulated with 125 ng/mL CD95L for 30 min or 1 h. CD95 DISC-IPs were carried out using anti-APO-1 (anti-CD95) antibodies. The composition of the IPs was examined by western blot analysis using the antibodies for the indicated proteins. Actin was used as a loading control. Inputs are shown. Quantification of procaspase-8 cleavage products at the DISC is shown and normalized to CD95 signal. Abbreviations: l.e. = long exposure; s.e. = short exposure; BC = control IP with 'beads-only', without the addition of antibodies; CD95L = CD95 ligand; DISC = death-inducing signaling complex; IP = immunoprecipitation; FADD = Fas-associated death domain; c-FLIP = cellular FADD-like interleukin (IL)-1β-converting enzyme-inhibitory protein; c-$FLIP_L$ = c-$FLIP_{Long}$; c-$FLIP_S$ = c-$FLIP_{Short}$; M = molecular weight in kiloDalton (kDa).

Figure 5: CD95 DISC formation in c-$FLIP_L$-overexpressing HeLa-CD95 cells. HeLa-CD95-FL cells were stimulated with 250 ng/mL CD95L for the indicated time points (1–3 h). CD95 DISC-IPs were carried out using anti-APO-1 (anti-CD95) antibodies. The composition of the IPs was examined by western blot analysis and analyzed for the indicated proteins. Actin was used as a loading control. Inputs are shown. Quantification of procaspase-8 cleavage products at the DISC is shown and normalized to the CD95 signal. One representative experiment out of two is shown. Abbreviations: l.e. = long exposure; s.e. = short exposure; BC = control IP with 'beads-only', without the addition of antibodies; CD95L = CD95 ligand; DISC = death-inducing signaling complex; IP = immunoprecipitation; FADD = Fas-associated death domain; c-FLIP = cellular FADD-like interleukin (IL)-1β-converting enzyme-inhibitory protein; c-$FLIP_L$ = c-$FLIP_{Long}$; PARP1 = poly(ADP-ribose)polymerase 1; M = molecular weight in kiloDalton (kDa).

Figure 6: CD95 DISC formation in primary T cells. Primary activated T cells were stimulated with 500 ng/mL CD95L for 15 min and 30 min. CD95 DISC-IPs were carried out using anti-APO-1 (anti-CD95) antibodies. The composition of the IPs was examined by western blot analysis and analyzed for the indicated proteins. Actin was used as a loading control. Quantification of procaspase-8 cleavage products at the DISC is shown and normalized to the CD95 signal. Inputs are shown. Abbreviations: l.e. = long exposure; s.e. = short exposure; ; CD95L = CD95 ligand; DISC = death-inducing signaling complex; IP = immunoprecipitation; FADD = Fas-associated death domain; c-FLIP = cellular FADD-like interleukin (IL)-1β-converting enzyme-inhibitory protein; c-$FLIP_L$ = c-$FLIP_{Long}$; M = molecular weight in kiloDalton (kDa).

DISCUSSION:

This approach was first described by Kischkel et al.[27] and has successfully been developed since then by several groups. Several important issues have to be considered for efficient DISC immunoprecipitation and monitoring caspase-8 processing in this complex.

First, it is essential to follow all washing steps during immunoprecipitation. Especially important are the final washing steps of the sepharose beads and the drying of the sepharose beads. This must be done correctly to increase the signal/noise ratio of immunoprecipitation, allowing the

detection of caspase-8 recruitment and processing at the DISC. Importantly, for very sensitive analytical techniques, such as mass spectrometry, a "preclearing step," which includes the incubation of the lysates with only the sepharose beads or isotype control antibodies, can also be important in reducing the noise. However, several studies have shown that this preclearing step is not essential for the detection of caspase-8 recruitment to the DISC by western blotting[7,23]. However, as mentioned, the washing of the beads at the end of immunoprecipitation is essential for obtaining reliable results. Nonspecific binding of abundant cellular proteins to the sepharose beads can essentially decrease the specific signals of the core DISC components. As an important control for the absence of the nonspecific binding, western blot analysis of the proteins, reported not to be present at the DISC, might be performed. An example of this analysis is given in **Figure 5**, in which the recruitment of PARP1 and caspase-3 to the DISC-immunoprecipitation was not observed. This indicates the specificity of the particular immunoprecipitation and sufficient washing of the sepharose beads during the experiment.

Second, it is crucial to perform negative controls such as a beads-only control or an immunoprecipitation control with an antibody with the same isotype as the antibody used for the immunoprecipitation. For anti-APO-1 antibodies, anti-mouse IgG3 antibodies can be used as an isotype control. Third, it is important to monitor the results of the immunoprecipitation from untreated samples, in which only CD95 should be observed. The detection of FADD, c-FLIP, or procaspase-8 in these samples typically indicates the presence of some shortcomings in the immunoprecipitation protocol or washing steps. This could give rise to assumptions on the stimulation-independent association of FADD or c-FLIP with CD95, which might be not entirely correct, and instead indicate flaws in immunoprecipitation. Fourth, for each immunoprecipitation, the inputs or lysates must be carefully analyzed in parallel to measure of the expression and posttranslational modifications of the core proteins analyzed by immunoprecipitation.

Fifth, time-dependent analysis allows changes in the complex to be followed over time and provides yet another important confirmation on the specificity of the proteins recruited to the complex. In this regard, an important issue is that each cell line has a different level of CD95 expression and of intracellular components of this complex. Accordingly, the exact timing of the CD95 DISC formation has to be carefully established for each particular cell type. Finally, the crucial issue for the analysis of the DISC dynamics is the comparison of the amount of protein in each immunoprecipitation. For CD95 DISC immunoprecipitations performed using anti-APO-1 antibodies, the amount of CD95 is a key measure of the equal amount of the complexes being compared. This might be difficult because the intensity of the CD95 signal in the immunoprecipitations is relatively high. However, one must find the optimal time interval for measuring the corresponding western blot signal. Another obstacle is that CD95 is a highly glycosylated protein, which also contributes to the difficulties in its detection in immunoprecipitation due to the presence of a particular pattern of several 'blurry' bands[28].

DISC immunoprecipitation analysis provides an optimal basis for detecting caspase activation and processing. Indeed, immunoprecipitation combined with western blotting allows for quantitative detection of cleavage products of procaspase-8a/b: p43/p41, p30, and p18, as shown in this study

(**Figure 4**, **Figure 5**, and **Figure 6**). This, in turn, enables experimenters to follow changes in procaspase-8a/b processing over time and distinguish different cleavage steps of procaspase-8. This approach has been successfully used to describe caspase-8 activation in mathematical models and distinguish between inter- and intramolecular procaspase-8 processing at the DISC[7,20,29]. Moreover, measuring caspase-8 cleavage products by western blotting has clear advantages compared to the conventional caspase-8 activity assays based on IETD substrate. In the latter case, it is well established that IETD also serves as a substrate for the other caspases. Hence, there is increasing evidence that the detection of IETD activity indicates a general increase of caspase activity in the cell. In contrast, using western blot analysis can help specifically assign the corresponding bands in the western blot to caspase-8, allowing the researcher to be confident that caspase-8 is activated in this complex. Furthermore, as mentioned above, measuring caspase-8 processing at the DISC presents an excellent tool for mathematical modeling and systems biology studies. Taken together, a classical workflow is presented to allow the monitoring of different steps of procaspase-8 activation and processing, which is essential for unraveling the molecular mechanisms of cell death.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

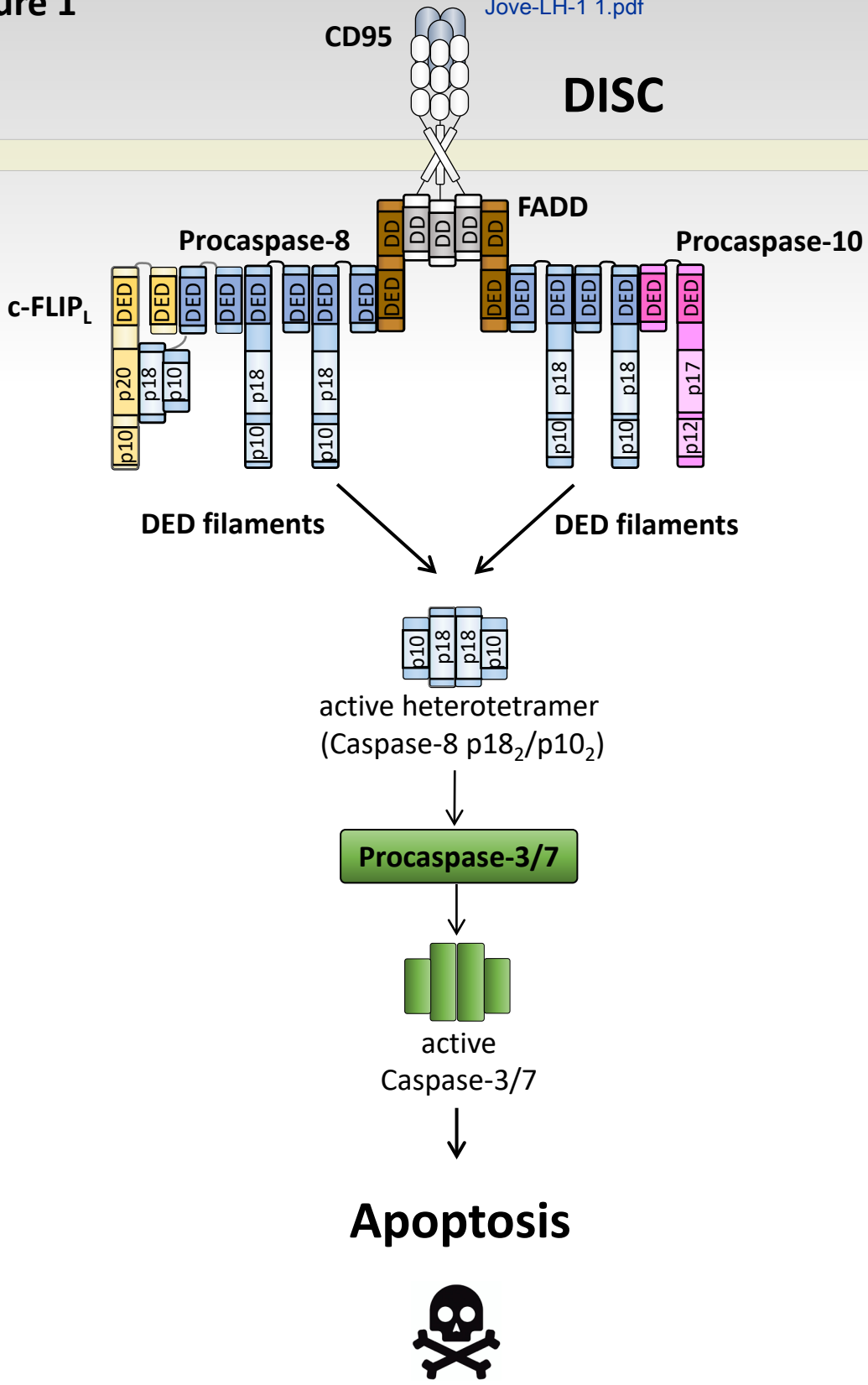


Figure 2

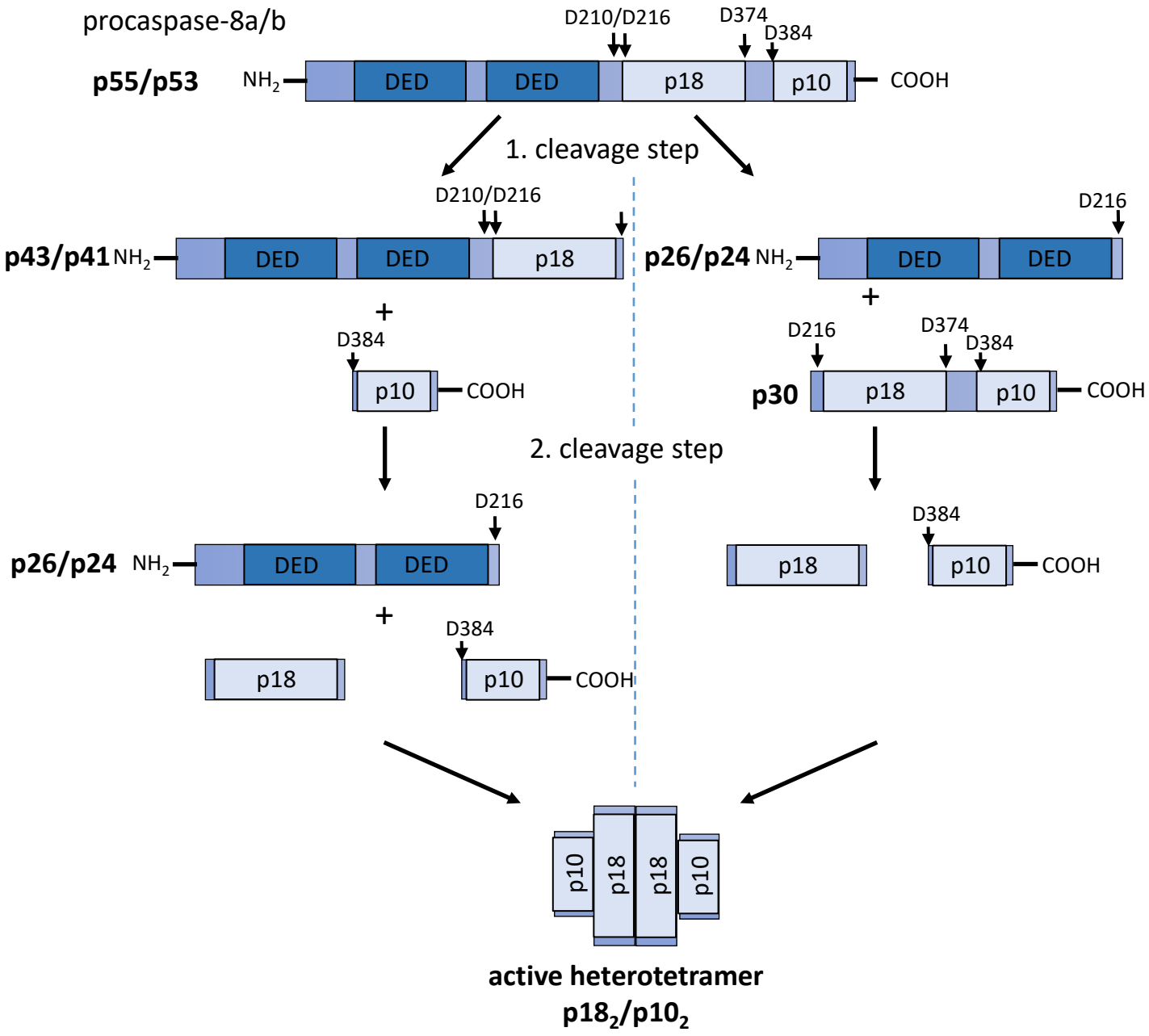


Figure 3

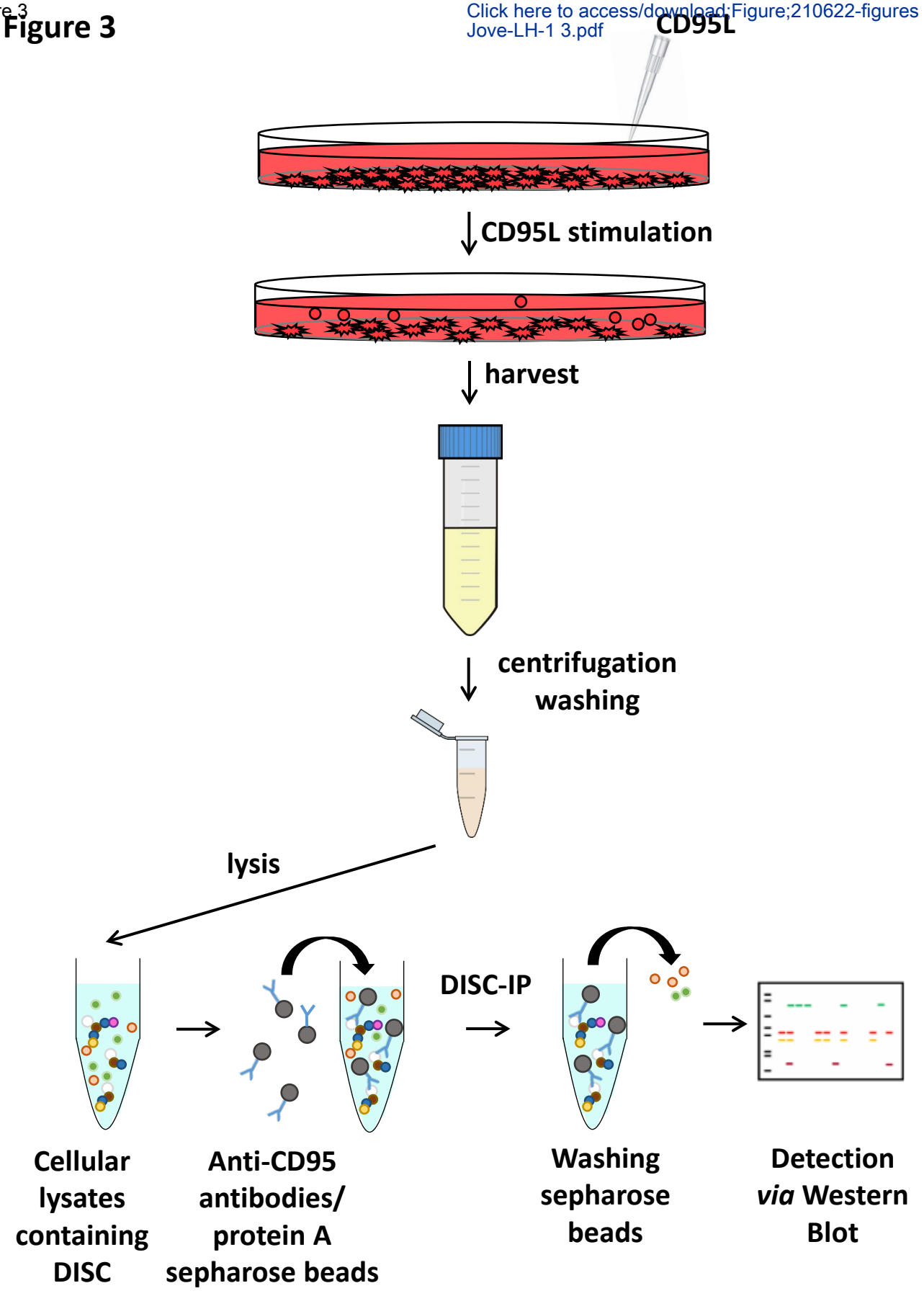


Figure 4

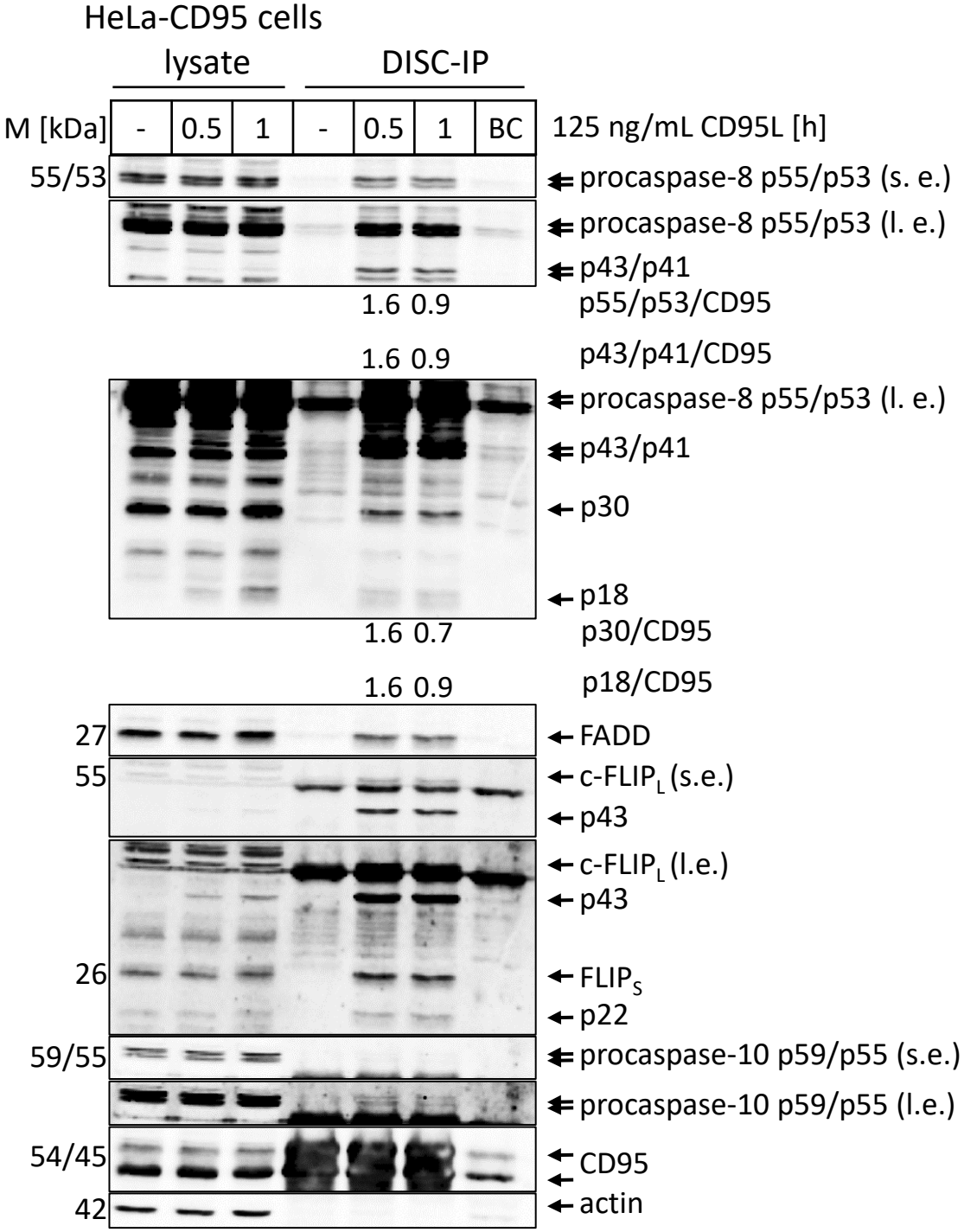


Figure 5

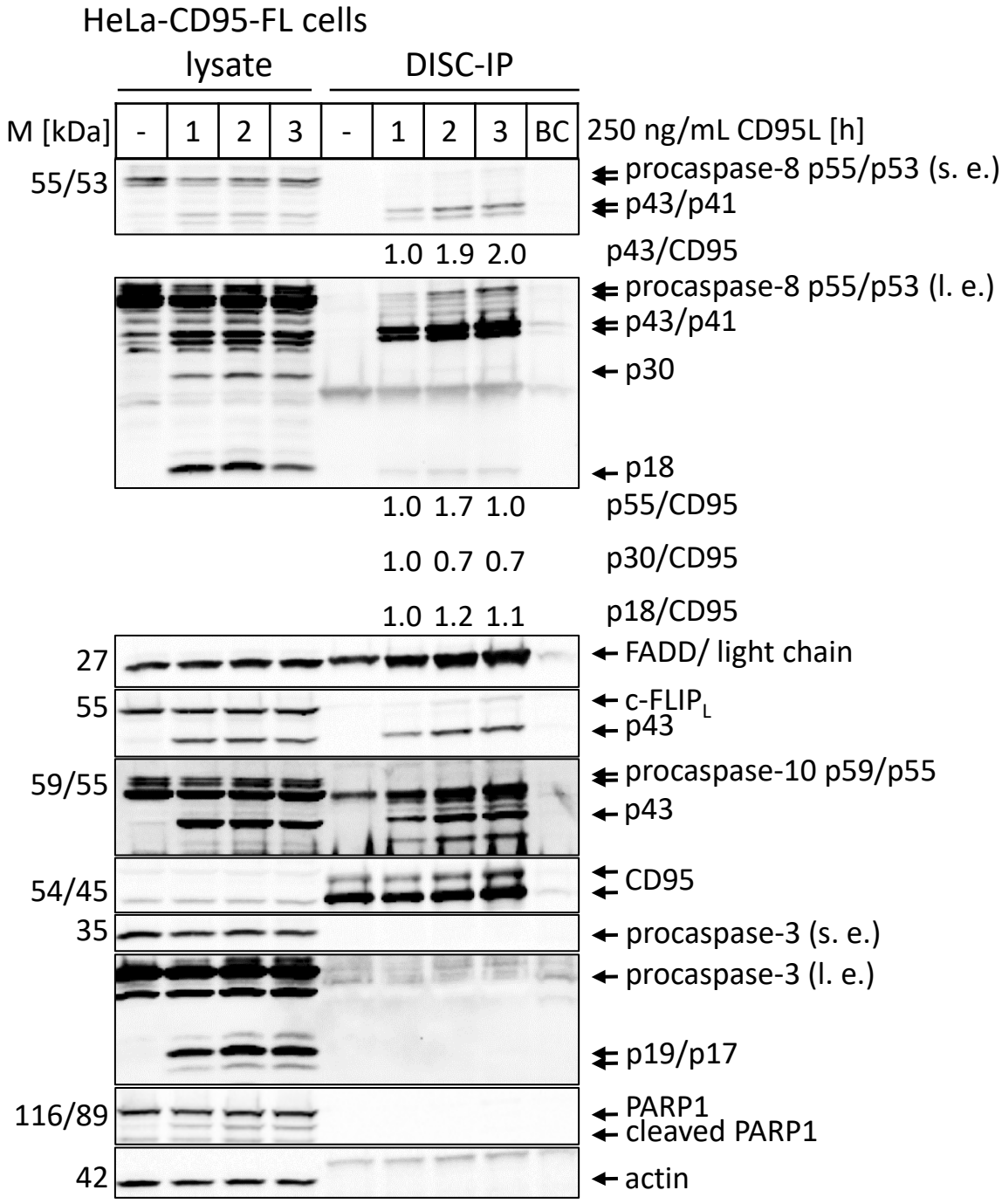
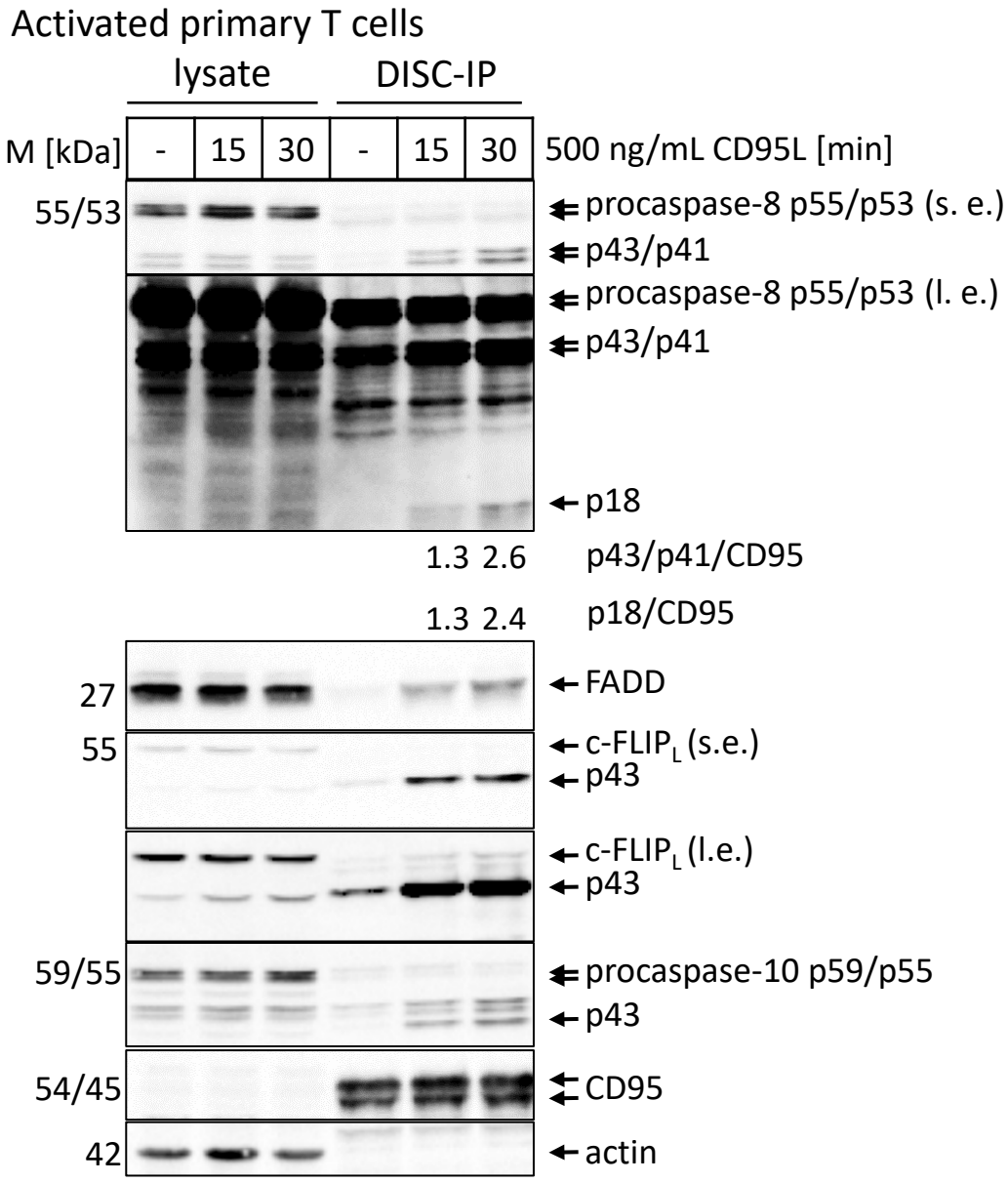


Figure 6



Click here to access/download

Table of Materials

Kopie von 210615-JoVE_Materials-1.xls

Point to point response

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer: We thoroughly proofread the manuscript

2. Please revise the following lines to avoid previously published work: 274-277, 283-285, 291-293

Answer: This part was modified to have a different phrasing than previously published work

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Answer: We thoroughly proofread the manuscript to avoid the use of any personal pronouns

4. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Answer: We added the number of the ethical agreement to the protocol text

5. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Please add more specific details (e.g., button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

Answer: We added more details to our protocol steps to have it contain everything that we would like shown in the video. Please, see the revised version of the protocol with changes highlighted in yellow.

6. Line 106-107: Please specify the constituents of the medium.

Answer: We added the link to the table of materials, where detailed composition of the medium is presented

7. Line 109-110: Please specify how the cell confluence and adhesion are controlled.

Answer: we added the following points:

1.1.2. On the day of experiment: Control cell confluence and cell adhesion microscopically before the experiment starts. Comparing the amount of space covered by cells with unoccupied space gives an estimation of their confluence. The cells with confluence of 80-90 % were typically used for the experiments. Nearly all cells should be attached to the bottom of the dish.

8. Line 116: Please specify from where the primary T cells were isolated.
Answer: we give a citation which describes the preparation of these cells and acknowledge our colleagues that have provided us with primary T cells.

9. Line 126: Please add more details to the CD95L stimulation step

Answer: we added the following details:

2.1. Stimulate the cells with CD95L (produced as previously described[20] or commercially available at ENZO: ALX-522-020-C005).

Note: The concentration of the CD95L and the time of stimulation are cell-type dependent.[13,15,22] Prepare one stimulation condition twice to generate in parallel a 'bead control' sample.

2.1.1. Stimulate adherent cells for example with selected concentration of CD95L (see the note above). Hold the plate at an angle and pipet the ligand into the medium without touching the adherent cells.

2.1.2. Stimulate suspension cells with CD95L by pipetting the ligand solution into the cell suspension.

10. Line 131: Are the flasks/dishes with media put on ice? Is the media discarded before placing it on ice?

Answer: we have expanded this part with the following text:

3.1. Put the cell dishes on ice.

Note: Do not discard the medium. Dying cells float in the medium and are important for the analysis.

3.2. Add 10 mL cold PBS to the cell suspension and scrape the attached cells off the plate.

11. Line 205: Is the detection performed immediately after the addition of substrate. How is the chemiluminescent signal detected?

Answer: we have expanded this part with the following text:

6.6. Detect directly the chemoluminescent signal using ChemiDoc XRS+ Gel Imaging System (Biorad).

Note: The exposure time and the amount of captured images depends on the amount of protein in the cell and the specificity of antibodies used. It has to be established empirically for each antibody that is used for the detection

12. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Answer: This part was highlighted in yellow.

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”

Answer: The figures were generated solely for this manuscript and not published elsewhere.

14. As we are a methods journal, please ensure the Discussion explicitly covers the critical steps within the protocol, modifications, and troubleshooting of the technique, any limitations of the technique, significance with respect to existing methods, and future applications of the technique.

Answer: We expanded the discussion with several technical issues and limitations of the technique. Please, see updated text.

15. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Answer: This was done accordingly.

16. Figure 4: Please revise the X-axis label to “0.5 h” instead of “0,5 h”.

Answer: This was done, accordingly.

17. Figure 4/5/6: Does the Y-axis represent the fold increase? Is there any specific unit?

Please include the details of sample size and statistics performed?

Answer: In the previous version of the manuscript we have shown only quantification of this particular Western Blot and, hence, we do not apply any statistical methods here. This was done only to show that one can quantify the cleavage products at the DISC. However, following this comment, we have decided to take away this graph to avoid the confusion, which apparently it causes, and leave only the numbers that correspond to the quantification of the corresponding bands.

18. Please sort the Table of materials in alphabetical order.

Answer: This was done accordingly.

WE THANK THE EDITOR FOR THE EXCELLENT COMMENTS

_____

Reviewers' comments:

Reviewer #1:

Manuscript summary:

Comment 1. The above-mentioned manuscript aims at providing a detailed method to measure caspase-8 activity. Better said, the procotol described here merely allows visualization of caspase-8 activation. I expected from the title a protocol, based on the immunoprecipitation followed by an enzymatic assay to quantitate caspase-8 activation. This has been described in the literature and is probably not routinely performed in Inna Lavrik's laboratory. The title thus needs to be changed to avoid confusion.

Answer 1: We thank for this excellent comment. The title was changed accordingly. It has be noted, that though we discussed in the previous version of the text that processing of procaspase-8 at the DISC is a direct indication of its activation, however we fully agree that this was certainly not the most straightforward way to claim as a protocol for the activation of caspase-8 at the DISC and the previous title was indeed misleading. We also took care that we more carefully discuss caspase-8 activation at the DISC in the current version of the manuscript and rather use terms, recruitment and processing‘ as suggested by the reviewer, which is really a splendid suggestion.

Comment 2. Second, there are a number of references to antibodies provided by Dr P Krammer. While the latter may be available to selected scientists, I guess that most readers won't get them easily. I would therefore recommend the Author's to make their best to provide either referenced commercially available antibodies that may easily replace Dr P Krammer's antibodies, or to mention whether the latter are available upon request, and if so, who should be contacted.

This also strongly applies to the immunoprecipitating antibody APO1 (Also from Dr Krammer). To get a broader audience, one may also be happy to get a side-by-side comparison with commercially available Fas/CD95 Ligands, and probably some comments, as the aim of the protocol is to follow and sustain cell death induced by Fas/CD95, through, but not exclusively, visualization of capase-8 recruitment and processing within the Fas/CD95 DISC.

Answer 2: We thank for this comment. We have provided referenced commercially available antibodies that may easily replace Dr P Krammer's antibodies. These antibodies are from ENZO. They are all, except for anti-FADD antibodies, are produced from the same hybrodomas as the ones from Krammer’s lab. These hybrydomas to our knowledge were given at some time point to the company (Alexis was the name of company at that time that was later taken over by Enzo). This also corresponds to anti-APO-1 antibody. We also provided the information on commercially available Fas/CD95 ligands. However, for the suggestion to perform more experiments with commercially available FasL/CD95L, and compare it with our results, the time of the revision is too short for these experiments. Hence, we hope that giving the corresponding number for the commercially available ligand would address the comment of the reviewer to the larger extent.

Finally, we decided to mention Dr Krammer’s name in the protocol as it was not mentioned so far.

Comment 3. The Authors also mention that stimulation of the cells with Fas/CD95L is highly

dependent on the cell type/ cell line. It would be nice to share your expertise in a table for cell lines that you have worked with in the past, and for which the DISC analysis is mastered in your laboratory. This could easily be proposed in a table.

Answer 3: We have given a number of references that provide an important information what are the times of stimulation/ stimulation strength for the different cell lines.

Comment 4. In the protocol you provide a number of consideration to increase the Signal/noise ratio of your immunoprecipitation, but you omit to mention that a preclearing step can also help in reducing the noise and favor post WB analysis. Could you comment in this ? Don't you ever had this step ? Do you think it is not mandatory ?

Along the line, in all your figures, "non-DISC components" are cropped see for example Actin (but you also show PARP, or caspase-3); Are these results added as an illustration but not blotted along the caspase-8 or CD95 ? Could be nice to show, if you have done so some of these controls to illustrate the importance of the washings during the IP steps? If these blots are not matching the (Lysate + DISC IP) showing C8 / FLIP FAS/CD95, then you should present them aside from the main DISC analysis and not below, to avoid confusion.

Answer 4: For the prewashing steps, we perform them only in the case of special analysis like Mass spectrometry, however for the conventional protocol, e.g. detecting recruitment of procaspase-8 to the DISC by Western Blot, we never saw the differences with prewashing and without it. Therefore, we use this protocol without pre-clearing and pre-washing. We fully agree that this has to be mentioned and added to the manuscript, which is done in the current version.

Within these lines, we think it is much more important to wash the beads after immunoprecipitation and especially remove the rests of the washing buffer with the syringe and in this and only this way one can reach reliable results. This was also further highlighted in the current version.

We thank for the valuable point with Caspase-3 and PARP1. Indeed, we ‚uncropped' these images and use the absence of the signals in the immunoprecipitation as an additional control for the absence of the unspecific binding to the beads. We added the additional text on this to the results and discussion.

Comment 5. Could you please check whether the amount of cells seeded for adherent cells before DISC analysis (line 106). Is 5 million enough for the IP, whilst for suspension cells you mention that the analysis is perfomed with nearly 20 times more !

Answer 5: This a very valuable point and we are very grateful for pointing it out. The average number of cells for IP is 10^7, which is the same for adherent and suspensions cells. However, we seed adherent cells one day before so that they can reach this number. This was commented in the current version of the protocol.

T cells are exception from the rule as they are rather small and one needs the higher initial number of the cells.

Minor Concerns:
Last some typos remain in the text, please check out:
Ie : adhaerent instead of adherent.

Answer: We have carefully checked the text for the typos. However, as for correction 'adhaerent' instead of 'adherent', we prefer to use the latter version as it is often used as well.

Reviewer #2:
Manuscript Summary:
In this manuscript Laura Hillert_Richter and Inna Lavrik describe in detail a classical immunoprecipitation and Western blotting methodology for assessment of the CD95-activated assembly of the death-inducing signaling complex (DISC) in mediating death receptor (DR)-associated extrinsic apoptosis. The authors summarized the literature in the field, depicted a complete signaling pathway and its analysis workflow for the DR signal initiation, adaptor-associated component recruitment in DISC formation and subsequent caspase 8 activation. The experimental procedures are written in great detail with step-by-step explanations on the enzymatic activation cascade. This protocol deserves to be published as a standard, reliable method for analysis of DR-induced programmed cell death signals.

Minor Concerns:
A few minor comments as follows:
1. It would be appropriate to describe the procedure as a classical, conventional workflow, rather than it is stated in line 41 "a state-of-art…"

2. In line 163, please consider adding in "a separate tube of " front of "the lysate"
3. It appears any pipette rather than Hamilton pipette (line 172) will be able to do the job
4. In line 176, a reducing agent, such as DTT or 2-ME, appears to be overlooked.
5. In line 179, an SDS protein or PAGE gel needs to be specified, and electrophoresis runs at constant 80 volts
6. In line 186, please specify PBST as 0.1% Tween-20 in PBS pH7.5
7. In line 307, please consider define "mouse" IgG3 as the isotype control
8. For Figure 2, it would depict clearer by adding a dashed line in separating the left and right panels in the middle of the figure
9. It would be good to provide the details for electrophoresis buffer and the self-made 12% SDS gel

Answer: We have performed all corrections accordingly. We thank the reviewer for this valuable input

In addition we would like to comment to the point 3. Here it was commented that any pipette would do this, we really strongly recommend to use a syringe rather than a pipette for this step (it was a typo in the previous version as we used pipette instead of syringe). This was corrected in the current version.

Figure 1

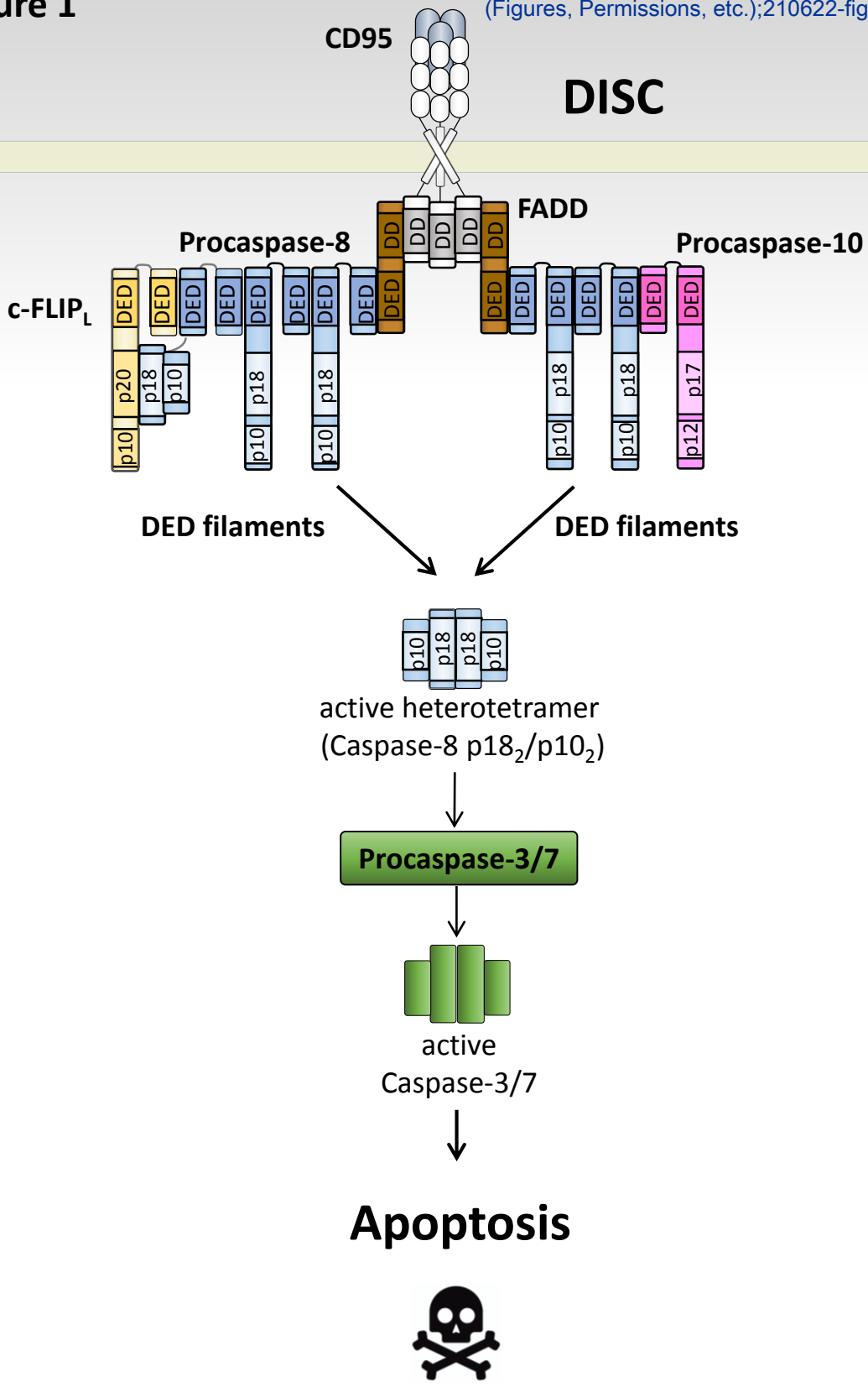


Figure 2

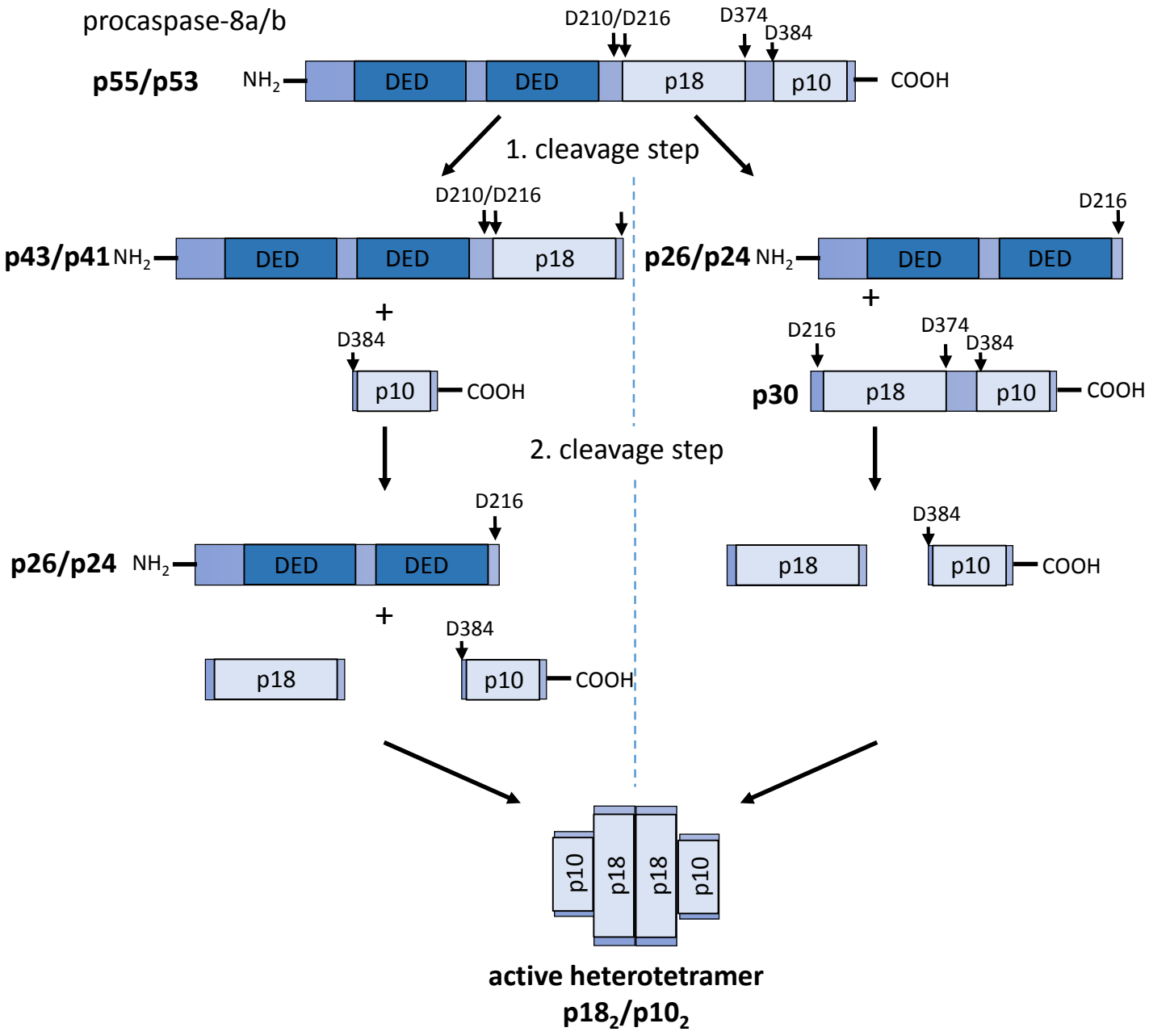

procaspase-8a/b
p55/p53
NH2
DED
DED
p18
p10
COOH
D210/D216
D374
D384
1. cleavage step
p43/p41
p26/p24
D216
+
p30
2. cleavage step
active heterotetramer
p18₂/p10₂

Figure 3

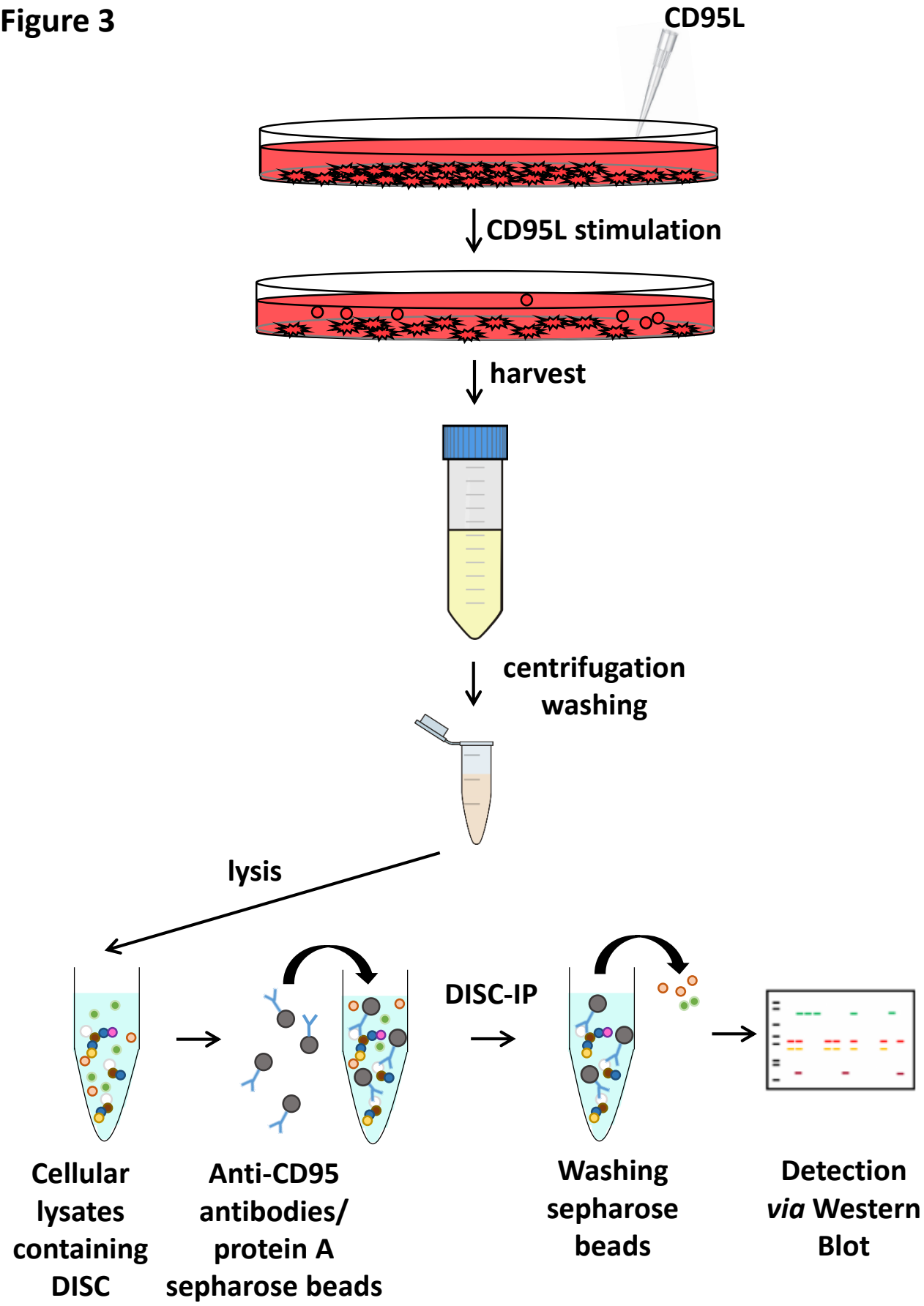

CD95L
CD95L stimulation
harvest
centrifugation
washing
lysis
DISC-IP
Cellular lysates containing DISC
Anti-CD95 antibodies/ protein A sepharose beads
Washing sepharose beads
Detection *via* Western Blot

Figure 4

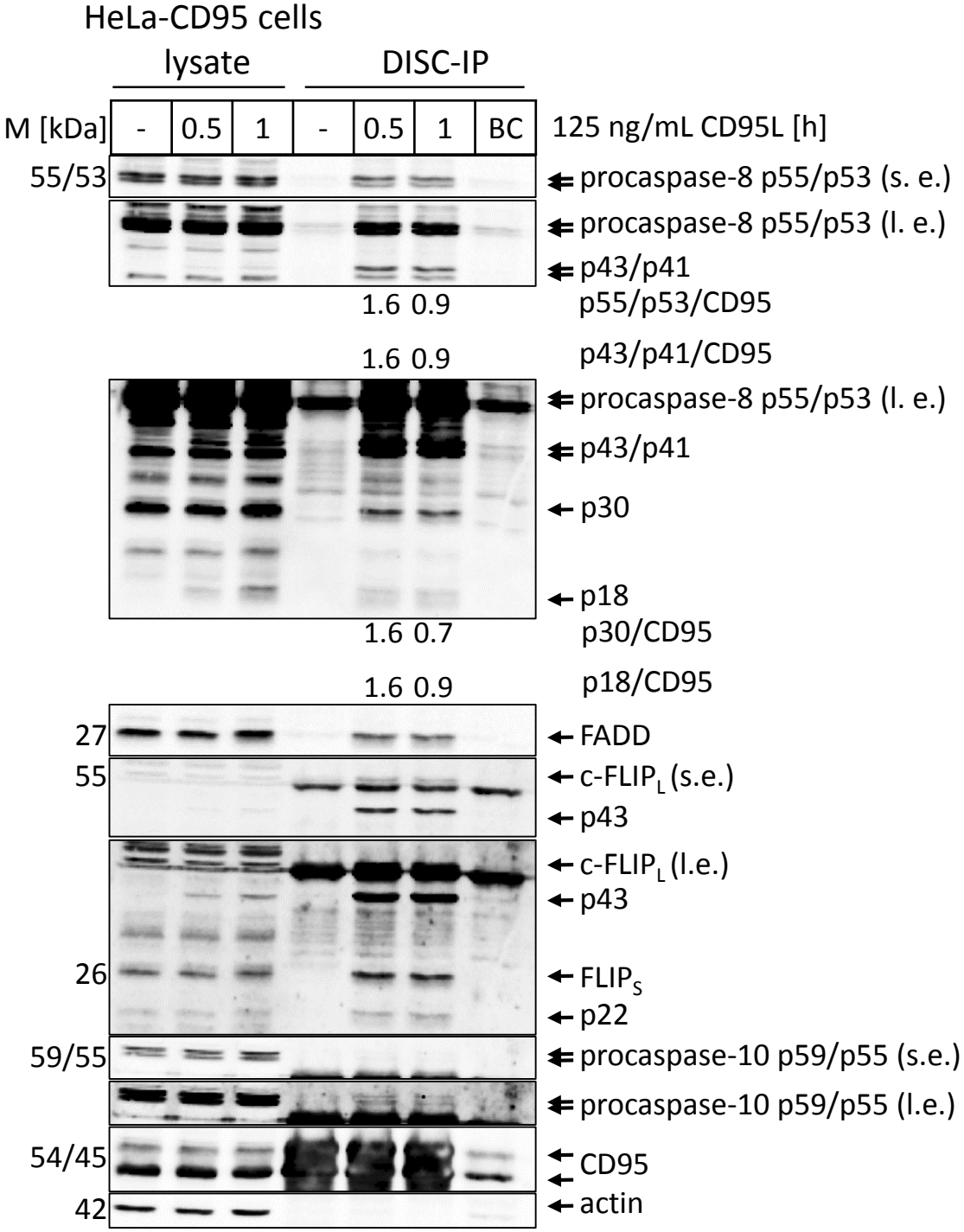
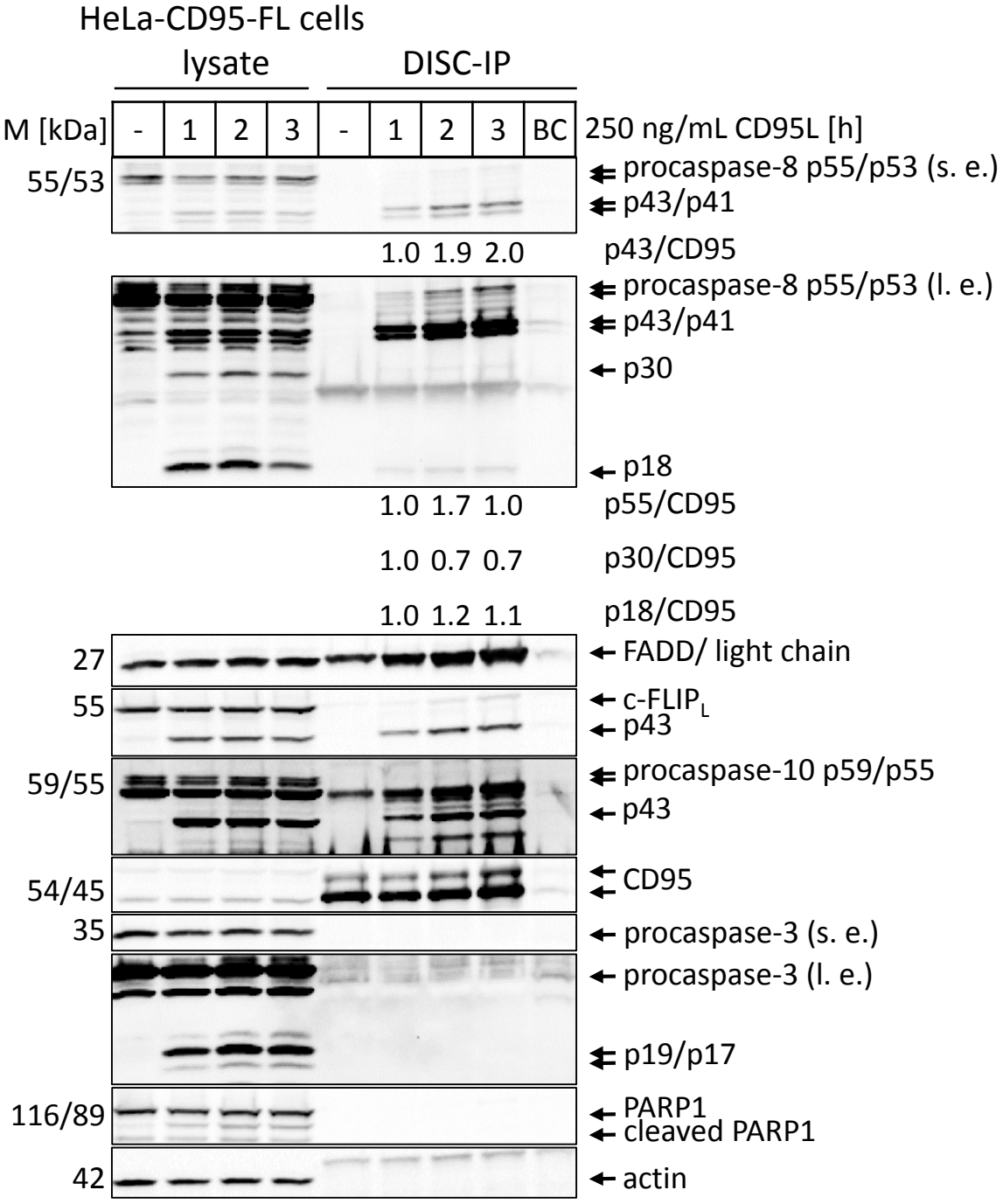

HeLa-CD95 cells
lysate
DISC-IP
M [kDa]
-
0.5
1
-
0.5
1
BC
125 ng/mL CD95L [h]
55/53
procaspase-8 p55/p53 (s. e.)
procaspase-8 p55/p53 (l. e.)
p43/p41
1.6 0.9
p55/p53/CD95
1.6 0.9
p43/p41/CD95
procaspase-8 p55/p53 (l. e.)
p43/p41
p30
p18
1.6 0.7
p30/CD95
1.6 0.9
p18/CD95
27
FADD
55
c-FLIP$_L$ (s.e.)
p43
c-FLIP$_L$ (l.e.)
p43
26
FLIP$_S$
p22
59/55
procaspase-10 p59/p55 (s.e.)
procaspase-10 p59/p55 (l.e.)
54/45
CD95
42
actin

Figure 5


HeLa-CD95-FL cells
lysate
DISC-IP
M [kDa]
- 1 2 3 - 1 2 3 BC
250 ng/mL CD95L [h]
55/53
procaspase-8 p55/p53 (s. e.)
p43/p41
1.0 1.9 2.0
p43/CD95
procaspase-8 p55/p53 (l. e.)
p43/p41
p30
p18
1.0 1.7 1.0
p55/CD95
1.0 0.7 0.7
p30/CD95
1.0 1.2 1.1
p18/CD95
27
FADD/ light chain
55
c-FLIP$_L$
p43
59/55
procaspase-10 p59/p55
p43
54/45
CD95
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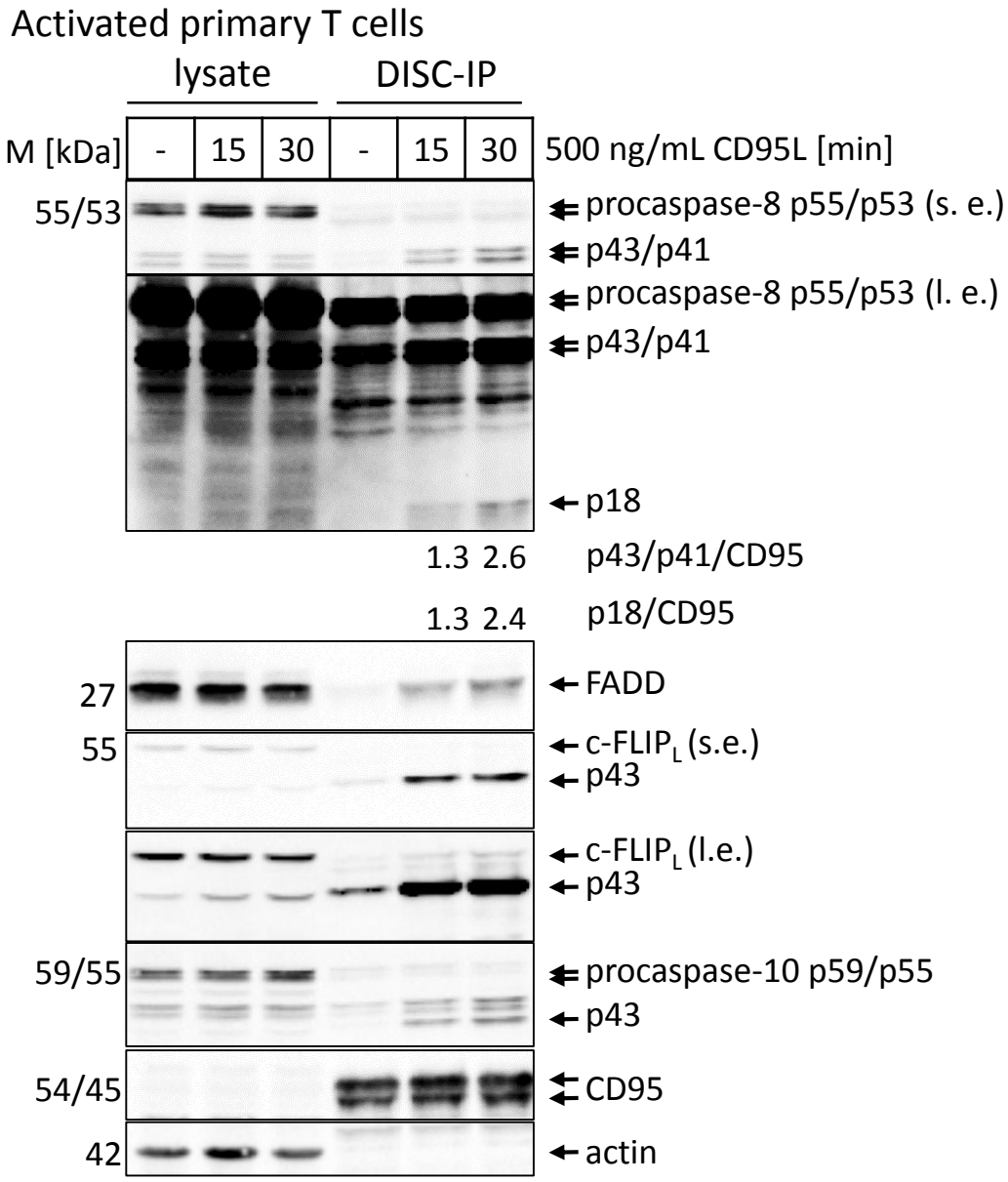
procaspase-3 (l. e.)
p19/p17
116/89
PARP1
cleaved PARP1
42
actin

Figure 6


Activated primary T cells
lysate
DISC-IP
M [kDa]
-
15
30
-
15
30
500 ng/mL CD95L [min]
55/53
procaspase-8 p55/p53 (s. e.)
p43/p41
procaspase-8 p55/p53 (l. e.)
p43/p41
p18
1.3 2.6
p43/p41/CD95
1.3 2.4
p18/CD95
27
FADD
55
c-FLIP$_L$ (s.e.)
p43
c-FLIP$_L$ (l.e.)
p43
59/55
procaspase-10 p59/p55
p43
54/45
CD95
42
actin