Original paper

Rapid and significant induction of TRAIL-mediated type II cells in apoptosis of primary salivary epithelial cells in primary Sjögren's syndrome

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Running Title: Apoptosis and caspases in pSS

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Abstract

Expressions of the effector molecules of Fas-mediated apoptosis in primary cultured salivary gland epithelial cells (SGEC) of primary Sjögren's syndrome (pSS) remain to be clarified. We focused on Fas-mediated caspase cleavage compared to tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-mediated apoptosis. Induction of apoptosis was performed by anti-Fas antibody coupled with PI3K inhibitor, or TRAIL. Activation of caspases, cytochrome C, and apoptotic protease activating factor-1 (Apaf-1) was determined by western blotting or immunofluorescence observed by confocal microscopy.

Fas-mediated apoptosis and activation of caspase 3/8 were induced in the presence of LY294002. TRAIL induced apoptosis in SGEC, which was stronger than that induced by anti-Fas antibody. TRAIL-induced caspase 9 cleavage accompanied by activation of cytochrome C and Apaf-1 were not mediated by anti-Fas antibody. Our results suggest that death receptor-dependent apoptosis in primary cultured SGEC is regulated by the engagement of type II cells in pSS.

Key words: Sjögren's syndrome, caspases, type II cells, Fas, TRAIL

Abbreviations: apoptotic protease activating factor-1 (Apaf-1); epidermal growth factor (EGF); fluorescein isothiocyanate (FITC); labial salivary glands (LSG); phosphate-buffered saline (PBS); phosphatidylinositol 3-kinase (PI3K); primary Sjögren's syndrome (pSS) ; salivary gland epithelial cells (SGEC); tumor necrosis

factor-related apoptosis inducing ligand (TRAIL); tetramethyl rhodamine isothiocyanate (TRITC); Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL).

Introduction

Both Fas and tumor necrosis factor-related apoptosis inducing ligand (TRAIL) are crucial receptors of cell death **(1-3)**. Recently, we demonstrated that Fas-mediated apoptosis was found under the co-existence of phosphatidylinositol 3-kinase (PI3K) inhibitor or IkappaB kinase (IKK) inhibitor in salivary gland epithelial cells (SGEC) of primary Sjögren's syndrome (pSS) **(4, 5)**. As we previously described, single stimulation with anti-Fas antibody induced no apoptosis in SGEC. Since the salivary gland cell line, HSG is sensitive to anti-Fas antibody, the primary cultured SGEC might be relatively resistant to anti-Fas antibody from a perspective of reactivity toward stimulation to Fas. However, the role of the effector molecules in observed apoptosis remains to be clarified. Originally, Scaffidi et al. **(6)** demonstrated that type I cells and type II cells are involved in cell death receptor-mediated apoptosis. The former is initiated by the cleavage of a large amount of caspase 8 at the death-inducing signaling complex (DISC) **(7)** followed by rapid activation of caspase 3, while the latter is characterized by cleavage of caspase 9 accompanied by cytochrome C release from mitochondria **(8)**. Although non-destructive mechanisms including a neurological dysfunction caused by anti-muscarinic type 3 receptor antibodies **(9)** or abnormal distribution of aquaporin 5 **(10)** are considered in pSS, apoptosis of SGEC remains of importance. In this study, we focused on the manner of the caspase cleavage in Fas and TRAIL-mediated apoptosis of the SGEC of pSS patients.

Materials and Methods

Patients

Our study involved 5 female patients with pSS (all female, age; 46.2 ± 13.7). These patients fulfilled the revised criteria for the diagnosis of pSS, as proposed by the American-European Consensus group **(9)**. A 70 year-old female as a normal subject who showed pleural effusion and positive schirmer test without diagnosis of pSS was also entried. Labial salivary glands (LSG) biopsies were obtained with informed consent from all participants. The study was conducted in accordance with the human experimental guidelines of our institution.

Antibodies and reagents

Anti-cleaved caspase 3, 8, and 9 rabbit monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Anti-cytochrome C mouse monoclonal antibody and anti-Apaf-1 goat polyclonal antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-Fas IgM monoclonal antibody (CH-11) which recognizes the human cell surface antigen Fas was purchased from MBL (Nagoya, Japan). Secondary antibodies including donkey anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC), donkey anti-rabbit IgG conjugated with tetramethyl rhodamine isothiocyanate (TRITC), and donkey anti-goat IgG conjugated with TRITC were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA). Hoechst dye 33258 was purchased from Sigma (St. Louis, MO, USA). The selective PI3K inhibitor LY294002 was purchased

from Calbiochem (La Jolla, CA, USA). Recombinant TRAIL was purchased from R&D Systems (Minneapolis, MN, USA).

Culturing of primary salivary epithelial cells

The culturing of SGEC was described in our previous studies **(3, 4)**. Briefly, obtained minor salivary gland tissue was cut with a scalpel several times without collagenase treatment and cultured in a defined keratinocyte-SFM culture medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with hydrocortisone (Sigma) and bovine pituitary extract (Kurabo, Osaka, Japan). For immunofluorescence, the SGEC were cultured on 12-mm^2 cover slips precoated with Type I collagen, Cellmatrix (Nitta Gelatin, Inc., Osaka, Japan).

Immunofluorescence

SGEC on 12-mm^2 cover slips were incubated according to the experiment design. The cells were incubated for 10 min in PBS containing 4% paraformaldehyde at 4°C and immersed in methanol at -20°C for 10 min. After blocking in 5% normal horse serum in PBS, the cells were incubated in the diluted primary antibodies for 1 hour at room temperature, followed by incubation in FITC-labeled and TRITC-labeled secondary antibodies, supplemented with Hoechst dye 33258. After washing in PBS, the cells were mounted in Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA, USA), and scanned by confocal microscopy (LSM5, PASCAL; Carl Zeiss, Jena, Germany). Control experiments were performed to ensure the isotype specificity of each secondary antibody used.

Induction of apoptosis

After 12 hours of starvation of growth supplement, the primary cultured SGEC were treated with 50 μM of LY294002 with 1 μg/ml of anti-Fas antibody (CH-11) for 12 hours **(5)**. On the other hand, the cultured SGEC were treated with 50 ng/ml of TRAIL for up to 3 hours, following the initial 12 hours of starvation of growth supplement.

Terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) staining

For the confirmation of apoptosis by immunofluorescence or flowcytometric analysis, TUNEL staining was employed to detect double-stranded DNA breaks. After the SGEC were fixed in 4% PFA 4°C for 15 minutes followed by immersion in PBS with 0.5 % Tween 20 and 0.2 % bovine serum albumin, we used Mebstain Apoptosis kit direct (MBL, Nagoya, Japan). After the SGEC were rinsed with distilled water, they were incubated with a 50ul terminal deoxynucleotidyl transeferase (TdT) solution at 37°C for one hour. The stained SGEC were analyzed by confocal microscopy.

Agarose gel electrophoresis

DNA ladder was detected by Apoptotic DNA Ladder Kit (Roche Diagnostic Corporation, Indianapolis, IN, USA). Briefly, the cultured SEGC in 60 mm^2 dishes were lysed and centrifuged after addition of isopropanol. After washing, the prewarmed elution buffer was applied to obtain DNA. For DNA ladder detection, the

samples were applied on the 1 % agarose gel with ethidium bromide in 1x Tris, Boric acid and EDTA-buffer. As a positive control, apoptotic U937 cells in the kit were used.

Western blot analysis

Western blot analysis was also described in our previous studies **(4)**.

After the cells were lysed and the protein concentration was determined identical amounts of protein were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to a polyvinylidene fluoride filter, which was blocked for 1 hour using 5% nonfat dried milk in Tris-buffered saline containing 0.1% Tween 20, and incubated at 4°C overnight in a 1:1000 dilution of each antibody. The filter was washed with TBS and incubated with a 1:1000 dilution of donkey anti-mouse IgG or rabbit IgG, coupled with horseradish peroxidase, followed by detection with an enhanced chemiluminescence (ECL) system (Amersham, Arlington Heights, IL, USA). The density of each band against β-actin was measured by Scion analyzing system (Scion Corporation, Frederick, Maryland, USA). Student *t*-test was employed to show the statistical significance in the densitometric analysis ($p<0.05$; significant).

Results

Cleavage of caspase 3 and caspase 8 of primary SGEC stimulated with anti-Fas antibody and PI3K inhibitor

We examined whether stimulation of both anti-Fas antibody and PI3K

inhibitor could induce cleavage of caspases in primary cultured SGEC. After growth factor starvation for 12 hours, the primary cultured SGEC were treated with anti-Fas antibody with LY294002 for another 12 hours. Hoechst staining clearly showed apoptosis of cultured SGEC (**Fig. 1).** When cell death was induced, activation of caspase 3 and caspase 8 was detected as a cytoplasmic staining in immunofluorescence. However, no expression of a cleaved form of caspase 9 was detected, despite the presence of apoptosis in cultured SGEC **(Fig. 1)**.

Then, the primary cultured SGEC were treated with either 50 mM of LY294002 only, 1 mg/ml of anti-Fas antibody only, or anti-Fas antibody with LY294002 for 12 hours. The cleavage of caspases under these types of stimulation was also determined by western blotting **(Fig. 2A)** and immunofluorescence **(Fig. 2BC)**. We found that neither anti-Fas antibody only nor LY294002 only induced cleavage of caspases. The combination of anti-Fas antibody and LY294002 induced cleavage of caspase 3 and caspase 8 along with apoptosis, but no cleavage of caspase 9 was observed. We also analyzed the density of largest fragment against β-actin **(Fig. 2B)**. The density of cleaved caspase 3 and 8 fragments which were treated with both LY294002 and anti-Fas antibody was significantly increased compared to that of control lane ($p<0.05$). However, no significant increase was observed for the bands of cleaved caspase 9.

Determination of TRAIL-induced apoptosis

As a positive control for cleavage of caspase 9, we needed to show positive staining of cleaved-form caspase 9 coupled with apoptosis, because anti-Fas antibody

along with LY294002 showed no staining for the cleaved form of caspase 9. Therefore, we tested the cell death receptor-mediated apoptosis, without inclusion of Fas. We observed that tumor necrosis factor-α induced no apoptosis in the SGEC (unpublished observation). However, we found that TRAIL was able to induce apoptosis in more than 50% of cultured SGEC in 3 hours **(Fig. 3A-a and Fig. 3A-b)**. To determine whether or not the fragments observed by Hoechst staining induced by TRAIL actually indicated apoptosis, TUNEL staining was employed, resulting in the complete coincidence of Hoechst staining and TUNEL staining **(Fig. 3A-c)** by the merged view **(Fig. 3A-d)**. Furthermore, TRAIL-induced apoptosis was observed in the bright field **(Fig. 3A-e and Fig. 3A-f)**. To confirm the TRAIL-mediated apoptosis, we also employed agarose gel electrophoresis to detect DNA ladder **(Fig. 3B)**. Stimulation with TRAIL showed no DNA ladder at 0, 30 and 60 minutes stimulation. However, light DNA ladder appeared at 120 minutes stimulation with TRAIL.

TRAIL-induced cleavage of caspase 9 and release of cytochrome C

Since the frequency of apoptosis induced by TRAIL was greater than that induced by stimulation of Fas, we examined the difference of the effector molecules. Unlike the case with Fas-mediated apoptosis, TRAIL induced cleavage of caspase 9 as well as caspase 3 and 8 on the apoptotic cells **(Fig. 4A)**. Correlated with the degree of apoptosis, cleavage of caspase 3 and 8 in TRAIL-mediated cell death was frequent in immunofluorescence. TRAIL-induced cleavage of caspase 3, 8, and 9 was also detected by western blot analysis **(Fig. 4B)**. The density of largest fragment against β-actin was analyzed by a densitometry **(Fig. 4C)**. The density of cleaved caspase 9

fragments which were treated with TRAIL was significantly increased compared to that with no stimulation. According to the time course, obvious cleavage of caspase 9 was detected by western blotting, which was not detected in Fas-mediated apoptosis **(Fig. 2A)**. Furthermore, cleavage of caspase 9 induced by TRAIL was co-expressed by cytochrome C that was detected as cytoplasmic staining **(Fig. 5A)**. We also observed Apaf-1 expression that is co-expressed by cytochrome C in TRAIL-induced apoptosis **(Fig. 5B)**. Co-expression of cytochrome C and Apaf-1 that was synchronized with the cleavage of caspase 9 was detected in TRAIL-mediated apoptosis; however, such a co-expression was not observed in Fas-mediated apoptosis **(Fig. 5C)**.

Apoptosis and cleavage of caspases in the primary SGEC of a normal subject

Finally, we examined reactivity of the primary SGEC toward anti-Fas antibody or TRAIL in a normal subject. In the bright field image, apoptosis was observed when the SGEC were treated with anti-Fas antibody along with LY294002 or TRAIL in the same way found in the SGEC of pSS patients **(Fig. 6A)**. Especially, massive apoptosis was found when the SGEC were treated with TRAIL. Although cleavage of caspase 3 and 8 was observed in the both stimuli, cleavage of caspase 9 was not detected by the stimulation of anti-Fas antibody and LY294002 **(Fig. 6B)**. As well as cleavage of caspase 9, activation of cytochrome C and Apaf-1 was observed in TRAIL-induced apoptosis **(Fig. 6C)**.

Discussion

As we recently showed, Fas-mediated apoptosis occurred under the presence of PI3K inhibitor **(5)**. Although TNF-α induced no apoptosis in the cultured SGEC, TRAIL induced remarkable cell death. Therefore, we tested the difference between type I cells and type II cells. However, it remains to be clarified that stimulation with anti-Fas antibody along with inhibition of survival factors induces caspases. In our study, we employed TRAIL-induced apoptosis for the positive control toward cleavage of caspase 9. Although TRAIL-induced caspase 3/8 was more remarkable than that in Fas-mediated apoptosis, activation of caspase 9 was detected in TRAIL-mediated cell death only. Accompanied by massive apoptosis of SGEC following 3-hour stimulation with TRAIL, cleavage of caspase 9 was observed in apoptotic cell cytoplasm. Furthermore, the cleavage of caspase 9 entailed Apaf-1 activation and cytochrome C release from mitochondria **(10, 11)**. Krammer **(12)** stated that the apoptosis via mitochondria involved type II cells, in comparison to type I cells that require activation of large amounts of caspase 8 at DISC without a mitochondria pathway. In this study, we did not examine the activation of caspase 8 at DISC. Our observation showing cleavage of caspase 9 coupled with Apaf-1 and cytochrome C release with stimulation of TRAIL was confirmed to involve type II cells.

On the other hand, the Fas-mediated apoptosis we observed in a recent study was considered to involve type I cells, since the apoptotic cells lacked the cleavage of caspase 9. Cultured SGEC are type II cells; however, anti-Fas antibody-induced activation of caspase 9 was not obvious, resulting in low frequency of apoptosis compared to TRAIL-mediated apoptosis. Our observation accorded with the previous

observations showing that the cleavage of caspase 9 was not dominantly found in the labial salivary gland of pSS *in vivo* **(13)**. The results of a previous study showed that apoptosis in the LSG of SS was rarely confirmed **(14)**, while Fas and FasL were observed in inflamed SS LSG in several studies **(15-17)**. Therefore, Fas-mediated caspase activation might be tightly controlled by cell survival molecules for maintenance of the salivary glands in SS.

Jimenez S et al. **(13)** previously reported that activated caspase 3 and a classical substrate poly (ADP-ribose) polymerase (PARP) were strongly expressed in ductal and acinar cells in the labial salivary gland of patients with SS, but no positive staining of caspase 9 was observed. Since the level of activation of caspase 8 in TRAIL-mediated apoptosis is more remarkable than that in Fas-mediated apoptosis, type II cells might be induced in the former condition. To determine the type II cells in TRAIL-mediated apoptosis, it is necessary to demonstrate whether the introduction of caspase 8 into the cultured SGEC could raise the cleavage of caspase 9 *in vitro*.

In summary, we confirmed that our recent study showing Fas-mediated apoptosis as affecting type I cells was achieved by cleavage of caspase 8 and caspase 3, in comparison to the type II cells induced by TRAIL. Because the SGEC in a normal subject demonstrated the same fashion found in pSS patients regarding apoptosis or cleavage of caspases, the present data might not be specific for pSS. Since the number of apoptotic cells differs between Fas-mediated and TRAIL-mediated apoptosis in this study, the balance of type I or type II cells might be regulated by the upsteam stimulus. So far, there was no detailed histopathological study to demonstrate the relationship between apoptosis and caspases *in vivo*. It

should be elucidated how many salivary epithelial cells show apoptosis or whether or not the TUNEL-positive cells have caspase 9 activity in the next phase. Further studies are required to elucidate the complex interaction between caspase activation and anti-apoptotic molecules.

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Figure Legends

Figure 1. Fas-mediated cleavage of caspase 3 and caspase 8 in the primary cultured salivary gland epithelial cells (SGEC)

After 12 hours of starvation of growth supplement, the primary cultured SGEC were treated with 50 μM of LY294002 with 1 μg/ml of anti-Fas antibody (CH-11) for 12 hours. The SGEC were labeled with rabbit anti-cleaved caspase 3, 8, and 9 with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). Apoptosis in the nucleus was determined by Hoechst staining (blue). Shown are representative results of five three independent experiments (Bar 20 μM).

Figure 2. Neither anti-Fas antibody only nor LY294002 only induced any cleavage of caspases in the primary cultured salivary gland epithelial cells (SGEC)

The primary SGEC were treated with either 50 μM of LY294002 only, 1 μg/ml of anti-Fas antibody (CH-11) only, or CH-11 with LY294002 for 12 hours, following growth supplement starvation for 12 hours. **A.** Cleaved caspases 3, 8, and 9 were expressed by western blotting. As a control, β-actin was used. **B.** The density of bands on western blotting film was analyzed by the densitometric analyzer. The density of largest fragment against β-actin was measured and the ratio was demonstrated as black bars with standard deviation. The average of the density of bands against control was calculated by student *t*-test ($^{*}p<0.05$). **BC.** The cells were labeled with rabbit anti-cleaved caspase 3, 8, and 9 with tetramethyl rhodamine isothiocyanate

(TRITC)-conjugated secondary antibody (red) along with Hoechst staining (blue). Shown are representative results of five three independent experiments (Bar 20 μM).

Figure 3. TRAIL-induced apoptosis in the primary cultured salivary gland epithelial cells (SGEC)

The primary cultured SGEC were treated with 50 ng/ml of TRAIL for 3 hours, following 12 hours of starvation of growth supplement. **A.** Apoptosis in the nucleus after 0 **(A-a)** and 3 hours **(A-b)** stimulation with TRAIL was determined by Hoechst staining (Bar 20 μM). To determine whether or not the fragments observed by Hoechst staining induced by TRAIL actually indicated apoptosis, TUNEL staining was employed and the TUNEL image **(A-c)** was merged with Hoechst image by the merged view (Bar 20 μM) **(A-d)**. Compared to the unstimulated condition **(A-e)**, TRAIL-induced apoptosis was observed in the bright field **(A-f)**. **B.** Agarose gel electrophoresis of Fas-mediated and TRAIL-mediated apoptosis. The primary SGEC were treated with 50 ng/ml of TRAIL for up to 3 hours and then DNA was obtained from the cell lysate. The DNA of the SGEC and positive control (apoptotic U937 cells) were were applied on the 1% agarose gel for the detection of DNA ladder. (M; 72-1353 base pair molecular weight marker, PC; positive control)

Figure 4. TRAIL-mediated cleavage of caspases in the primary cultured salivary gland epithelial cells (SGEC)

The primary cultured SGEC were treated with 50 ng/ml of TRAIL for 3 hours, following 12 hours of starvation of growth supplement. **A.** The SGEC were labeled

with rabbit anti-cleaved caspase 3, 8, and 9 with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). Apoptosis in the nucleus was determined by Hoechst staining (blue) (Bar 20 μM). **B.** Cleaved caspase 9 as well as caspases 3 and 8 were expressed by western blotting. As a control, β-actin was used. Shown are representative results of three independent experiments. **C.** The density of bands on western blotting film was analyzed by the densitometric analyzer. The density of largest fragment against β-actin was measured and the average of the density of bands against control was calculated by student *t*-test ($^{*}p<0.05$).

Figure 5. TRAIL-induced cleavage of caspases accompanied by the release of cytochrome C along with Apaf-1 in the primary cultured salivary gland epithelial cells (SGEC)

A. Epithelial cells were treated with 50 ng/ml of TRAIL for up to 3 hours, following the initial 12 hours of starvation of growth supplement. Then, the SGEC were double-labeled using mouse anti-cytochrome C antibody with FITC-conjugated secondary antibody (green) and rabbit anti-cleaved caspase 9 antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). Apoptosis in the nucleus was determined by Hoechst staining (Bar 10 μM). **B.** Likewise, the SGEC were double-labeled using mouse anti-cytochrome C antibody with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (green) and goat anti-Apaf-1 antibody with TRITC-conjugated secondary antibody (red). Apoptosis in the nucleus was determined by Hoechst staining (Bar 10 μM).

C. Then, the primary SGEC were treated with either 50 μM of LY294002 only, 1 μg/ml of anti-Fas antibody (CH-11) only, or CH-11 with LY294002 for 12 hours, following growth supplement starvation for 12 hours. The SGEC were double-labeled using mouse anti-cytochrome C antibody with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (green) and goat anti-Apaf-1 antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). Apoptosis in the nucleus was determined by Hoechst staining (Bar 10 μM). Shown are representative results of four two independent experiments.

Figure 6. Cleavage of caspases in the primary cultured salivary gland epithelial cells (SGEC) of a normal subject

A. Bright field image of the primary SGEC treated with either 50 μM of LY294002 only, 1 μg/ml of anti-Fas antibody (CH-11) only, CH-11 with LY294002 for 12 hours, or 50 ng/ml of TRAIL for 3 hours following growth supplement starvation for 12 hours of a normal subject. **B.** After the primary SGEC were treated with CH-11 with LY294002 for 12 hours, or TRAIL, the SGEC were labeled with rabbit anti-cleaved caspase 3, 8, and 9 with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). Apoptosis in the nucleus was determined by Hoechst staining (blue).**C.** Then, the SGEC were treated with CH-11 with LY294002 or TRAIL, the SGEC were double-labeled using mouse anti-cytochrome C antibody with FITC-conjugated secondary antibody (green) and rabbit anti-cleaved caspase 9 antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). Continuously, the SGEC were double-labeled using mouse

anti-cytochrome C antibody with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (green) and goat anti-Apaf-1 antibody with tetramethyl rhodamine isothiocyanate (TRITC)-conjugated secondary antibody (red). Apoptosis in the nucleus was determined by Hoechst staining (Bar 20 μM).

Fig1

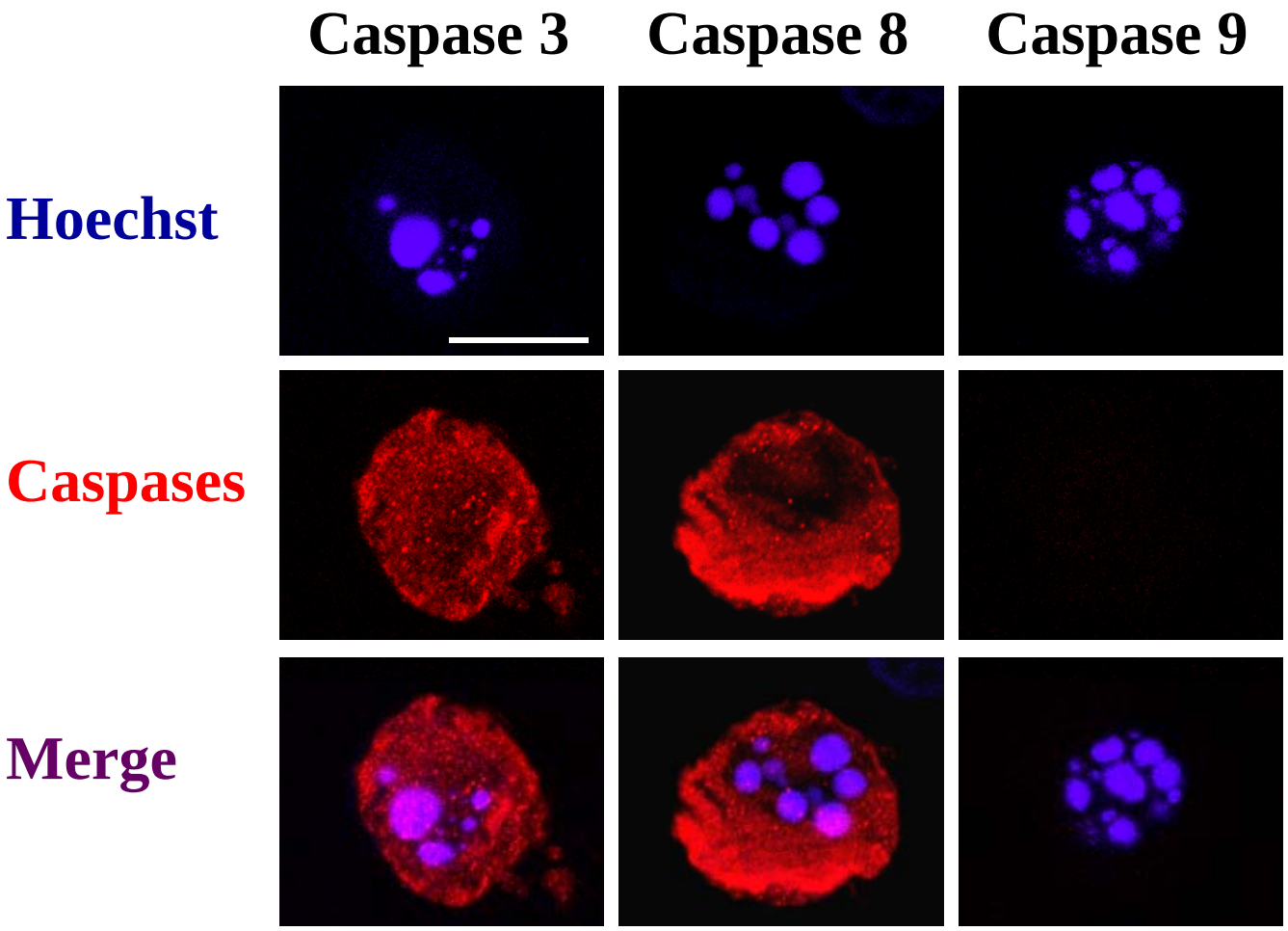

Fig2A

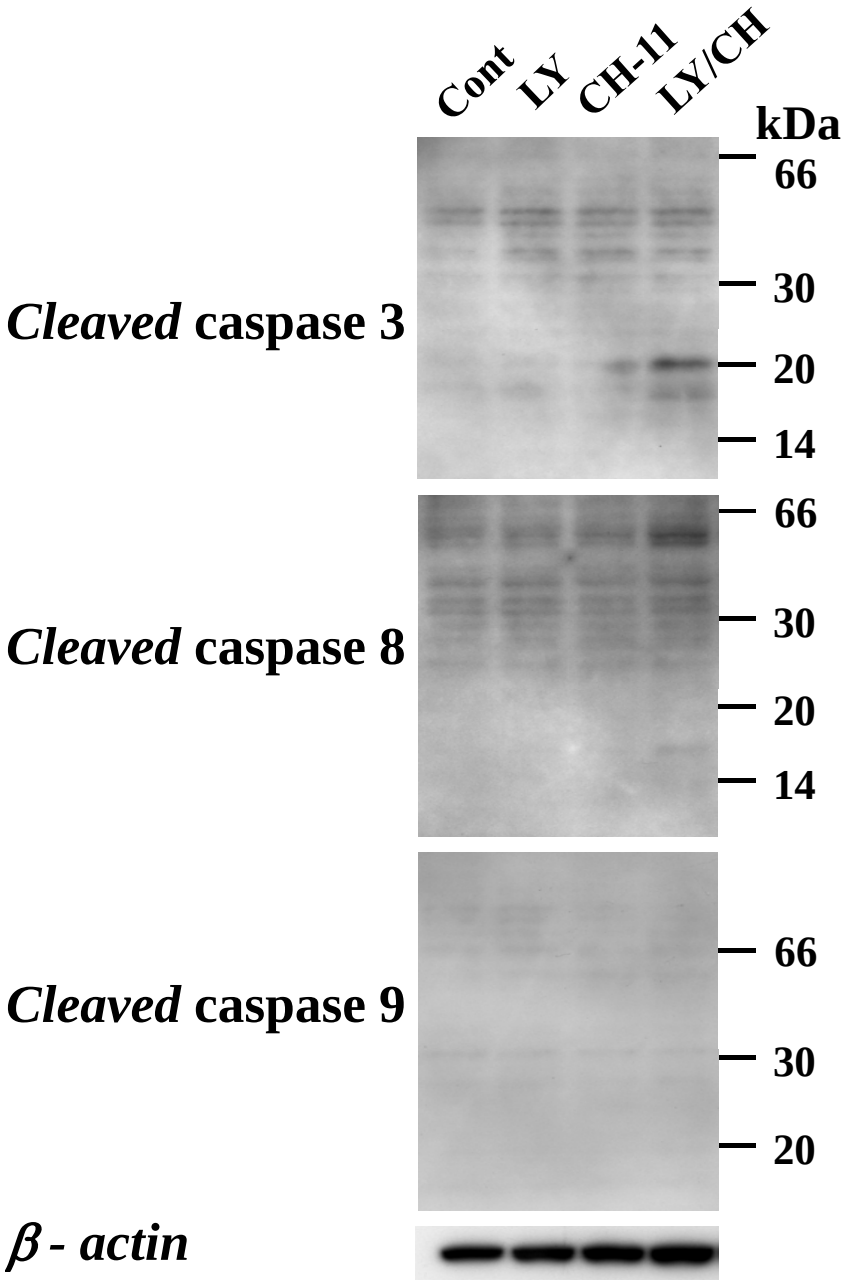
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Fig2B

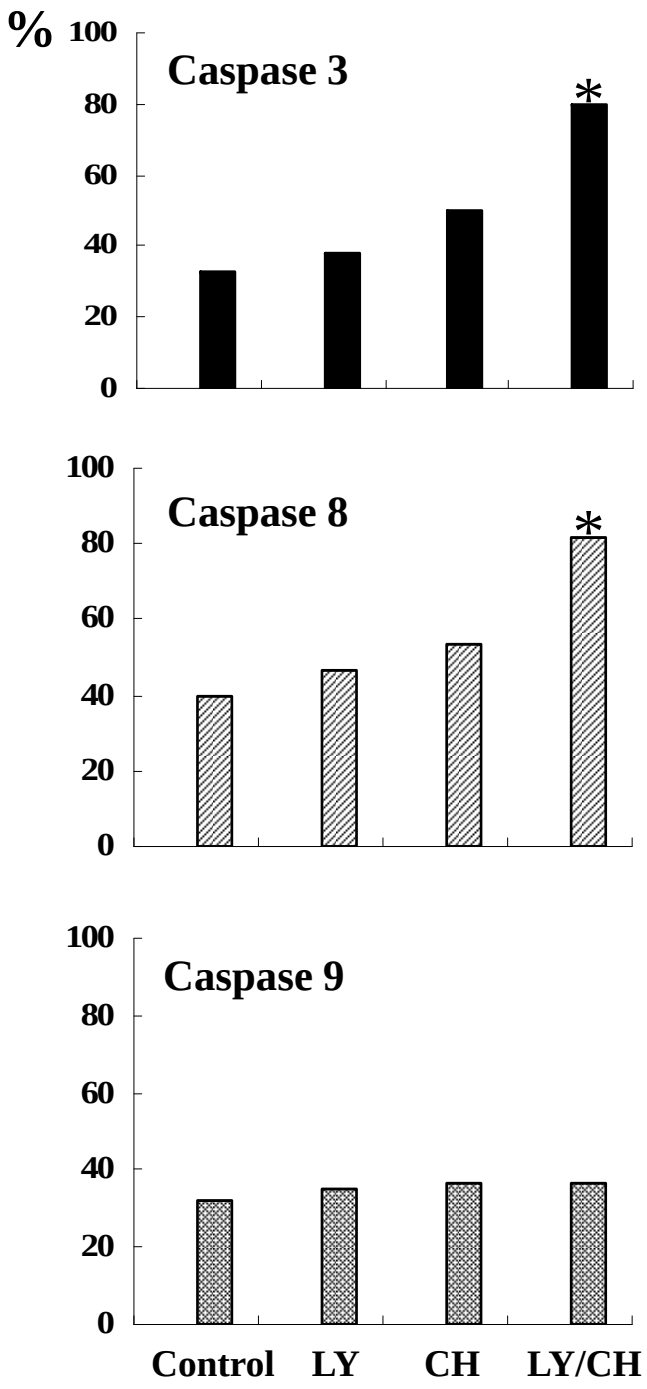
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Fig2C

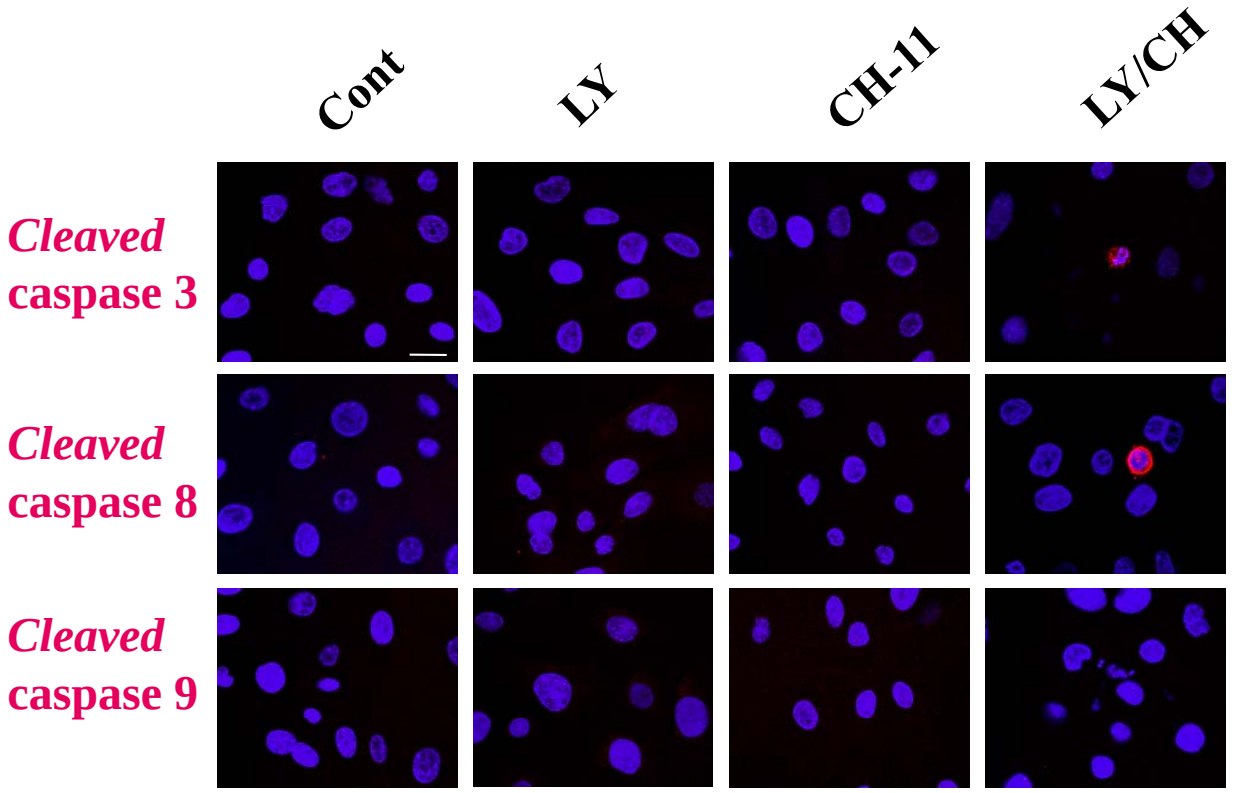
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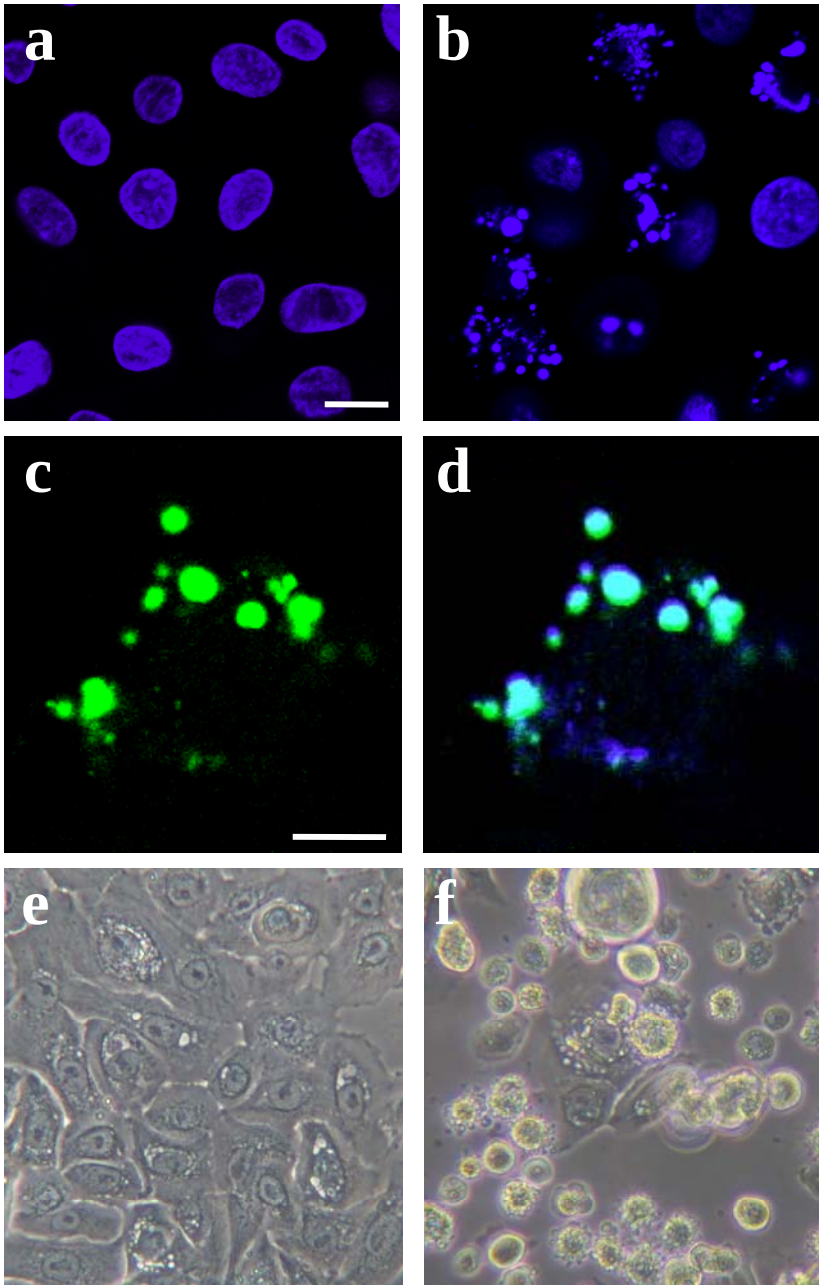
Fig3A



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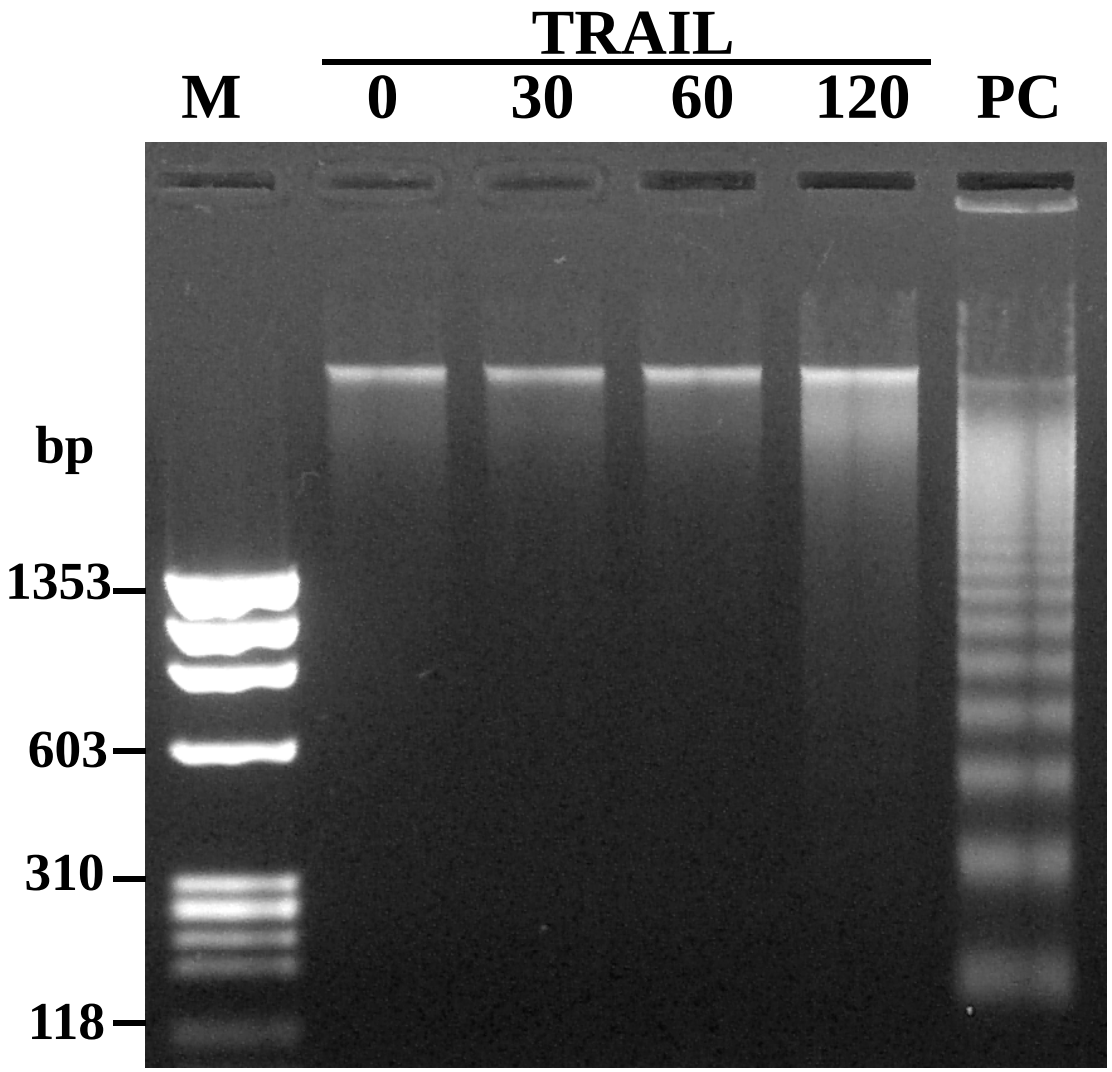

Fig4A

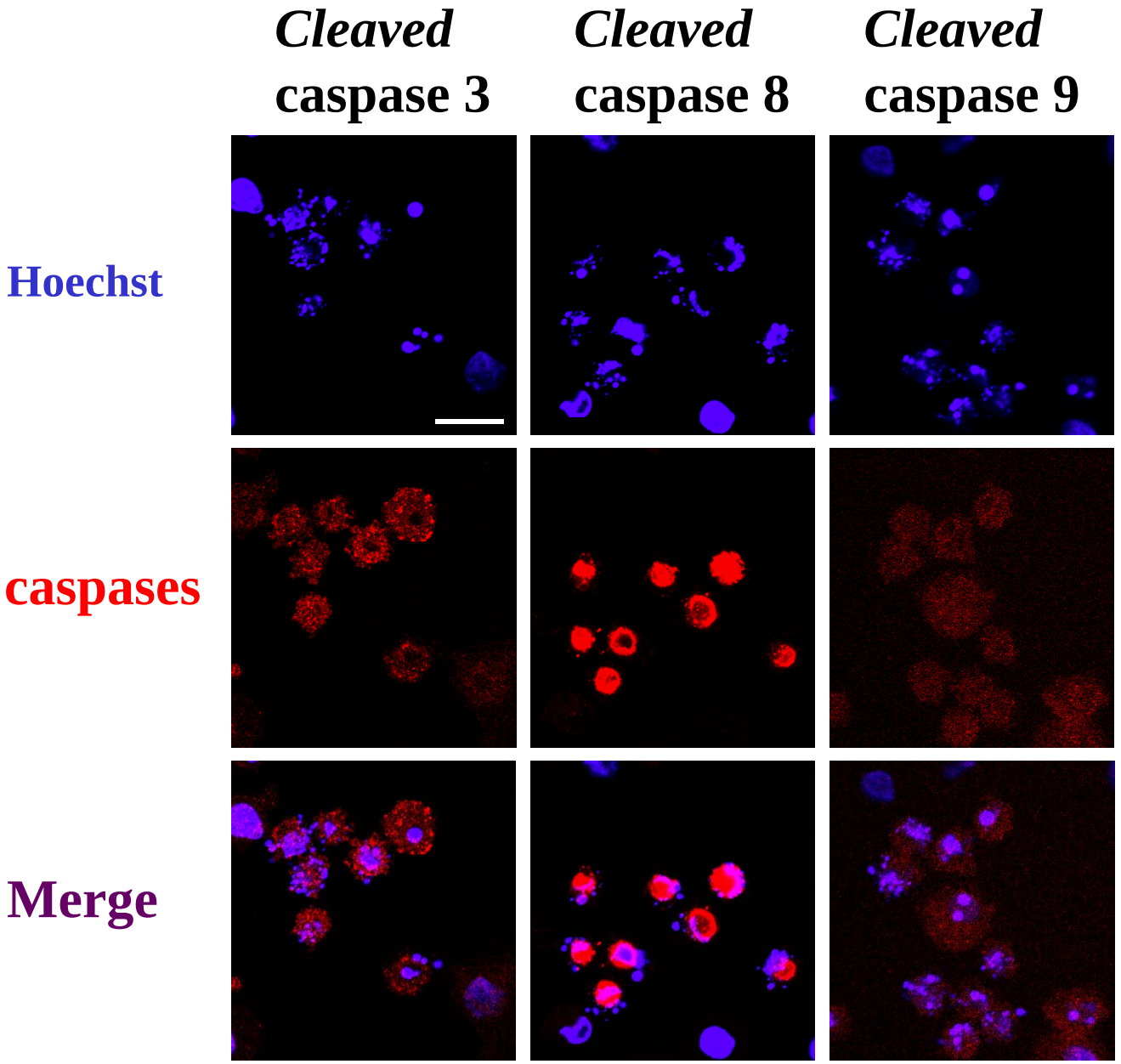
Cleaved caspase 3
Cleaved caspase 8
Cleaved caspase 9
Hoechst
caspases
Merge

Fig4B

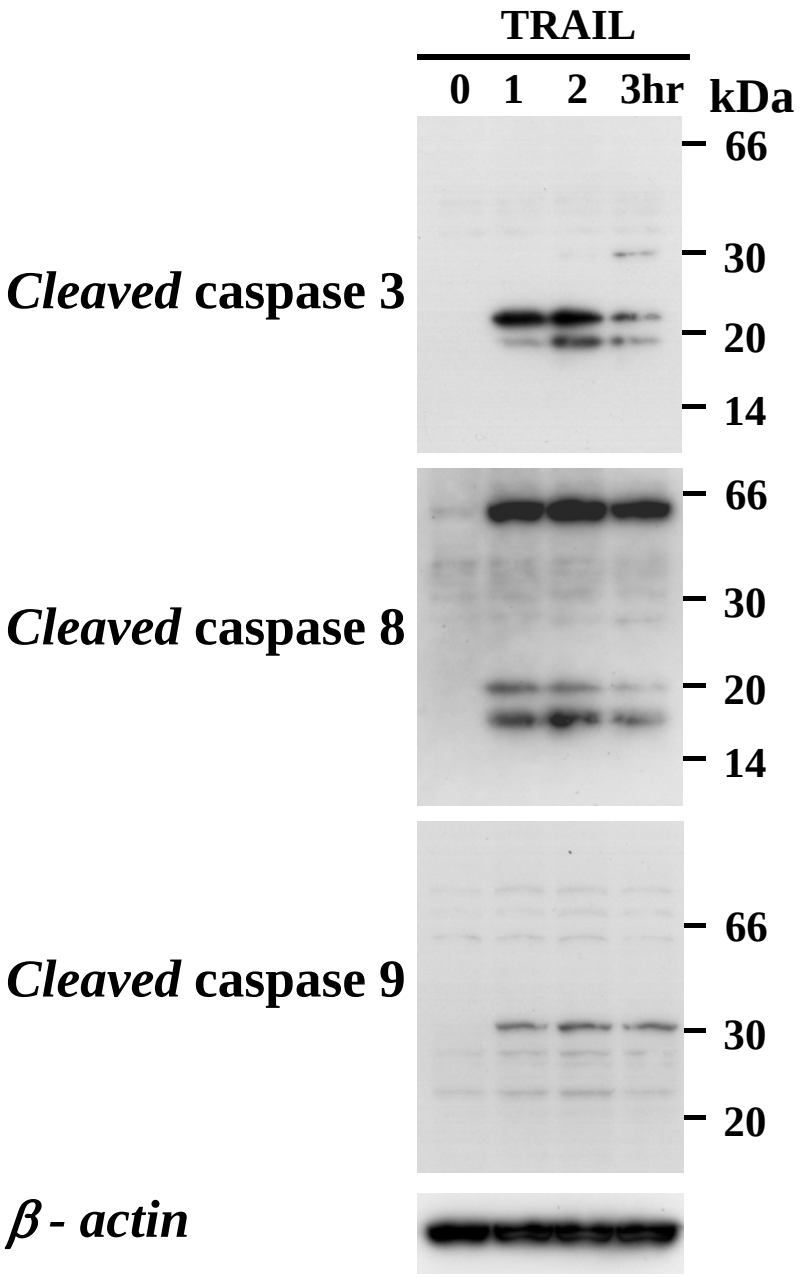
TRAIL
0 1 2 3hr
kDa
Cleaved caspase 3
66
30
20
14
Cleaved caspase 8
66
30
20
14
Cleaved caspase 9
66
30
20
β - actin

Fig4C

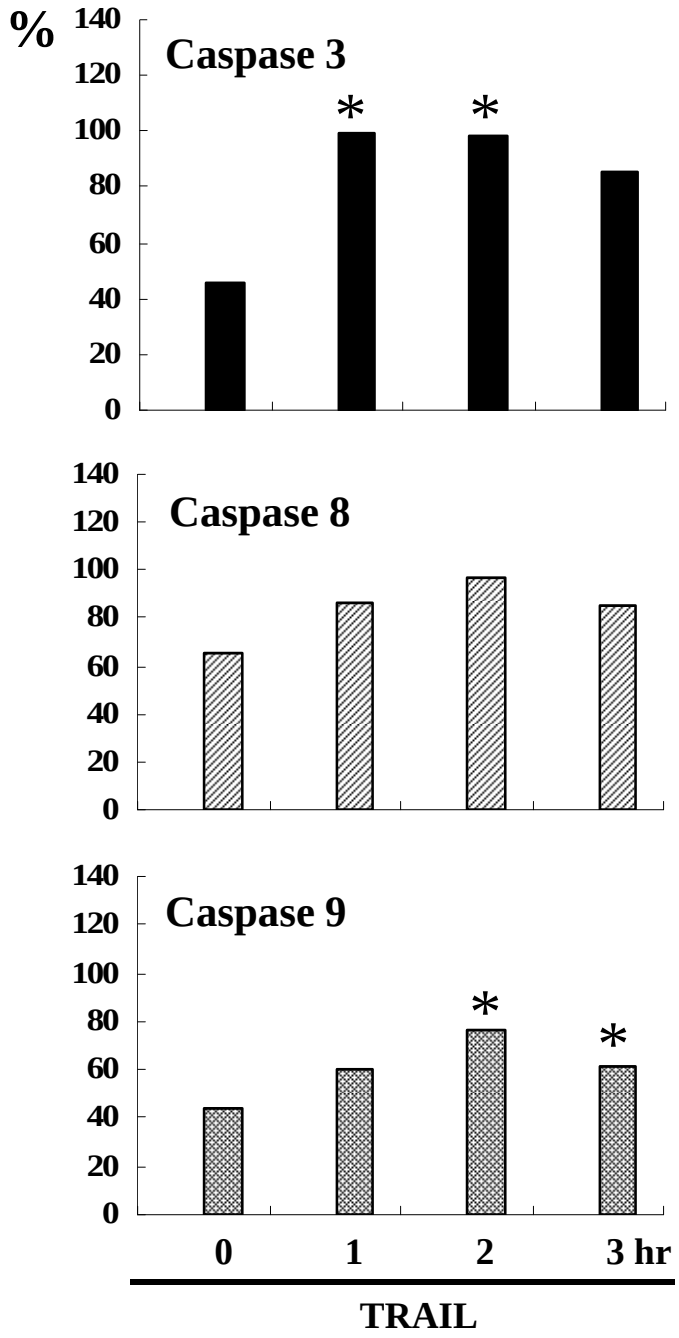
%
Caspase 3
Caspase 8
Caspase 9
140
120
100
80
60
40
20
0
*
0
1
2
3 hr
TRAIL

Fig5A

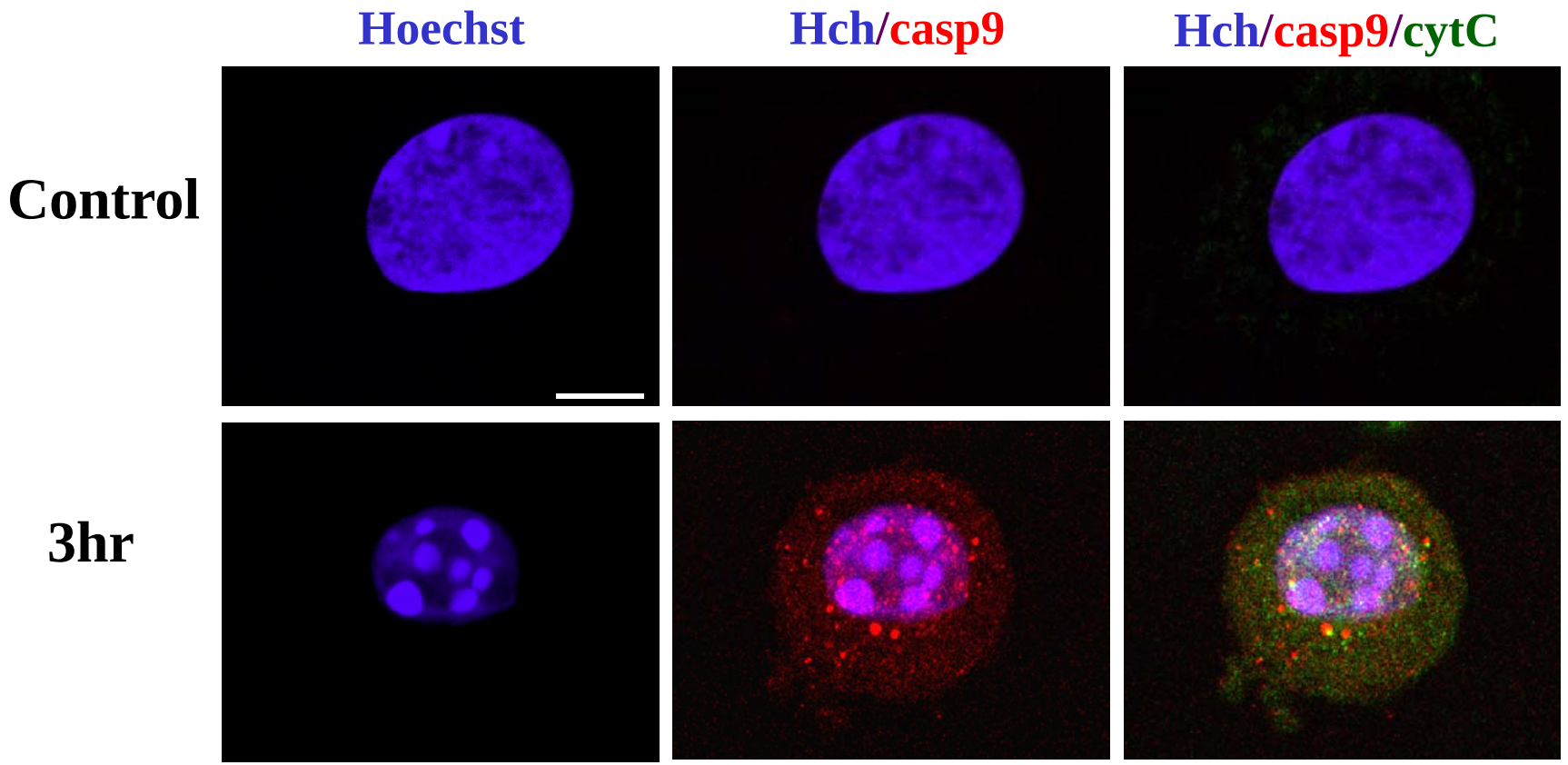

Fig5B

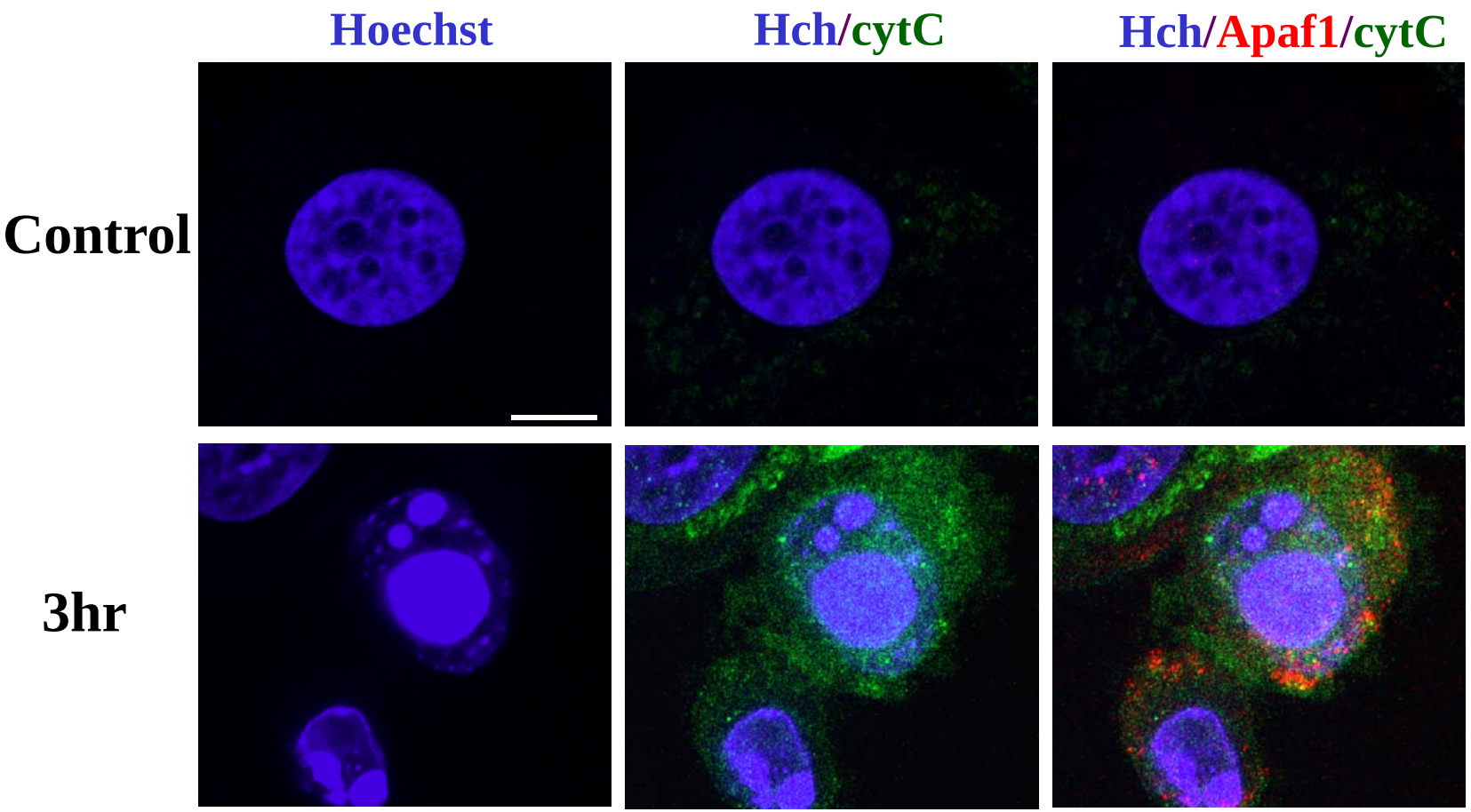

Fig5C

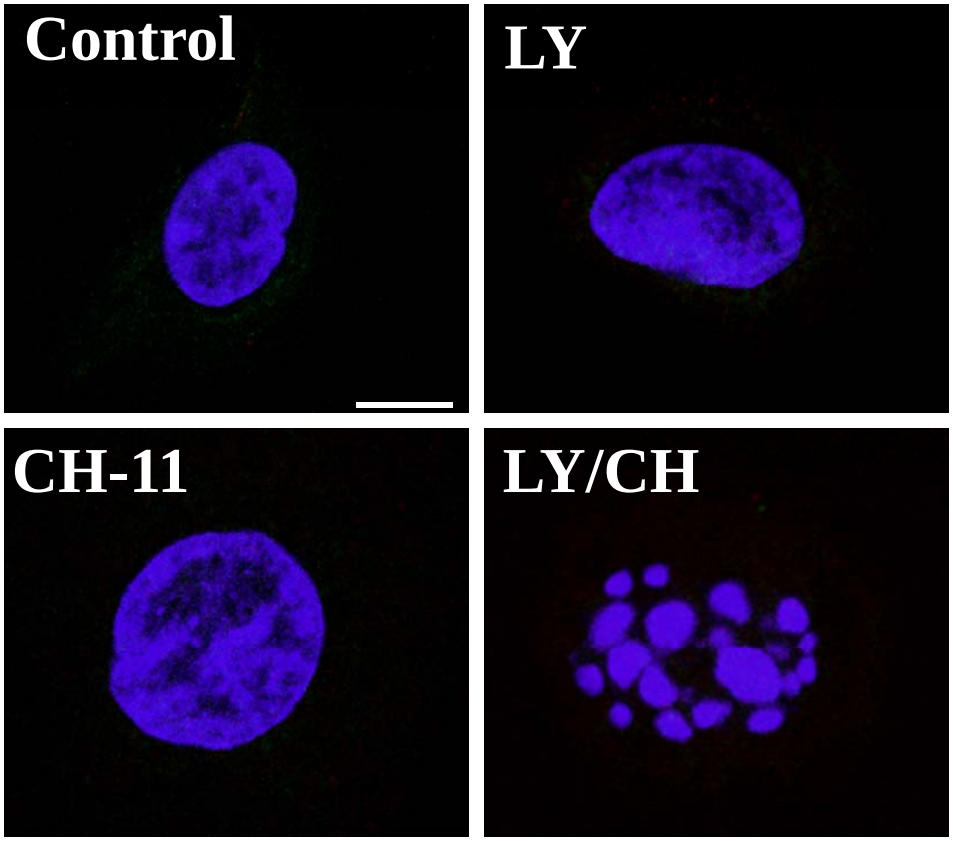

Fig6A

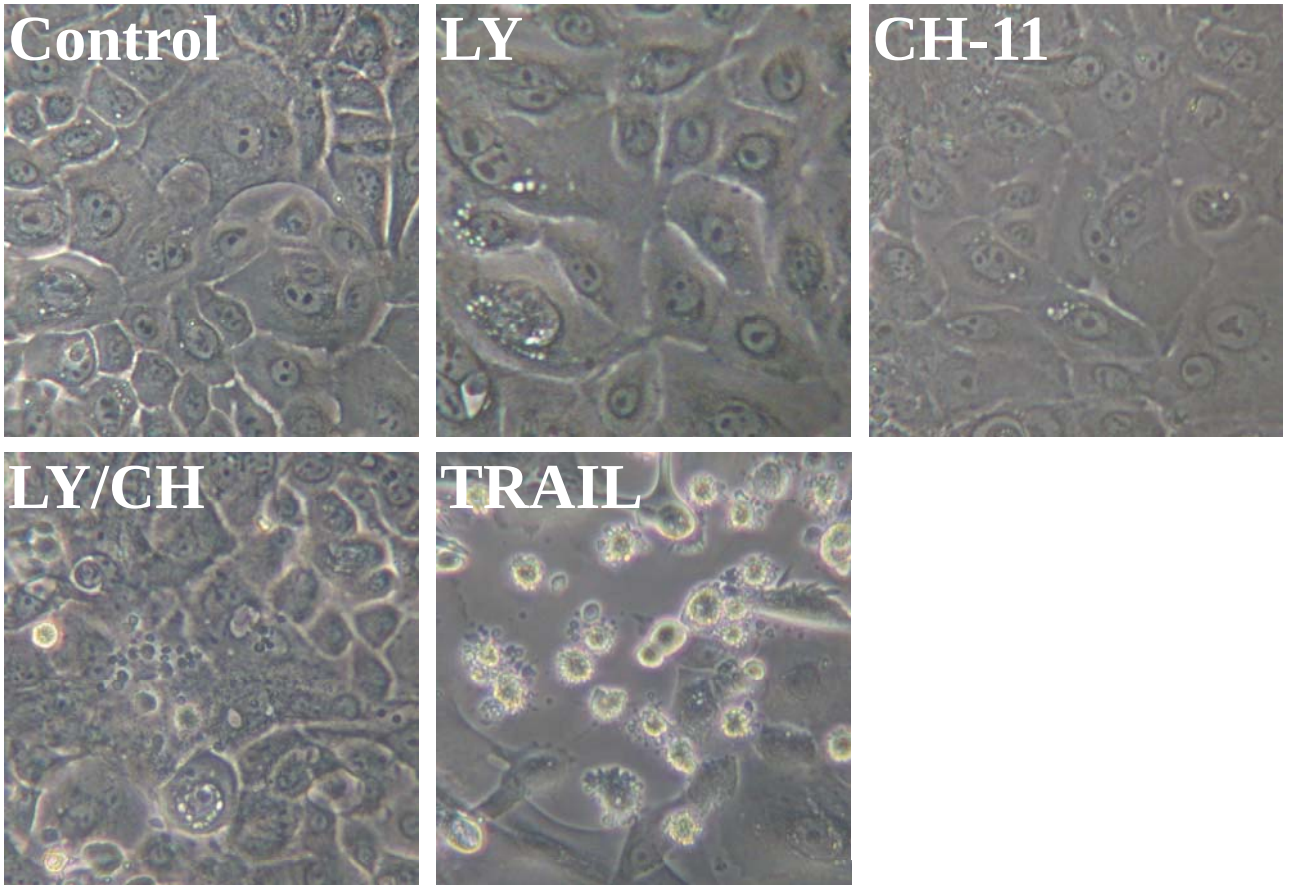

Fig6B

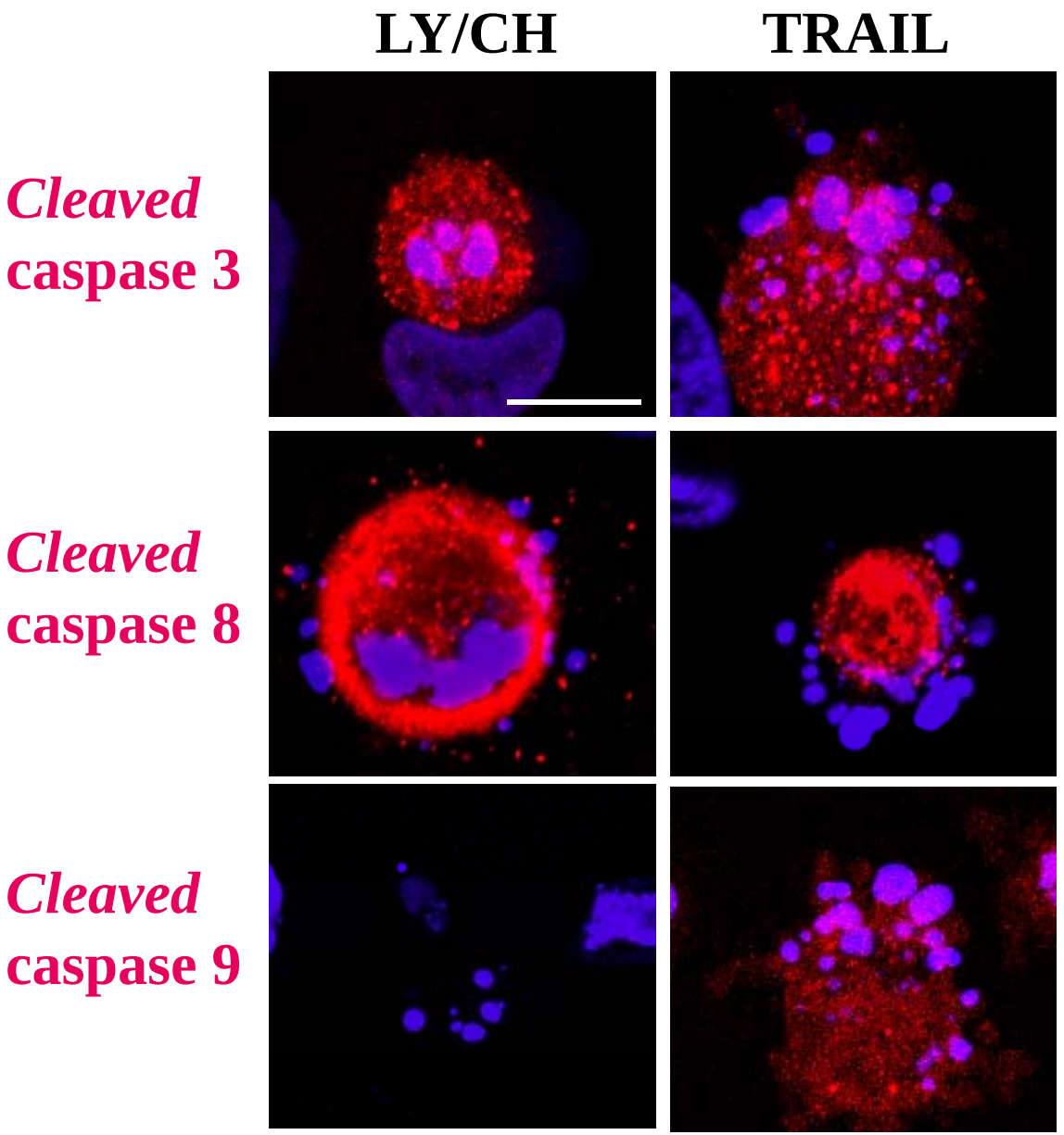
LY/CH
TRAIL
Cleaved caspase 3
Cleaved caspase 8
Cleaved caspase 9

Fig6C