Supporting Information

Exogenous Introduction of Initiator and Executioner Caspases Result in Different Apoptotic Outcomes

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1. Materials and Instruments

**Materials and Instruments.** All chemicals were purchased from commercial sources, such as Thermo Fisher Scientific and Sigma Aldrich, and used without additional purification or modification. Azobisisobutyronitrile (AIBN) was purchased but was used after purification by recrystallization. 1H NMR spectra were recorded on a 400 MHz Bruker NMR spectrometer using the residual proton resonance of the solvent as the internal standards, chemical shifts are reported in parts per million (ppm). Molecular weight of the polymers were estimated by gel permeation chromatography (GPC) in THF using poly(methyl methacrylate) (PMMA) standards with a refractive index detector. UV−Vis absorption spectra and fluorescence spectra were recorded on a Spectravax M5 spectrophotometer with clear or black 96-well plates, accordingly. Dynamic light scattering (DLS) measurements were performed using a Malvern Nanopetasizer. Thermal denaturation was measured using a CFX Connect Real-Time PCR detection system (BioRad Laboratories Inc.). Microscopy images provided by ZOE Fluorescent Cell Imager (BioRad) and Yokogawa Spinning Disc Confocal Microscope (Nikon) with image workup through ImageJ (FIJI). Flow cytometry experiments executed using BD DUAL LSR Fortessa Laser FACS and data analysis through FlowJo™ 10.

2. Additional methods/experimental

2.I Synthesis

2.I.i Random Copolymer synthesis – PEG-PDS random copolymer was synthesized as previously documented using commercially available polyethylene glycol monomethyl ether methacrylate (PEGMA) and synthesized pyridyl disulfide methacrylate (PDSMA) monomers in the presence of AIBN and a commercial RAFT reagent 4-cyano-4-[(dodecylsulfanylthiocarbonyl) sulfanyl]pentanoic acid.1 Pyridyl disulfide methacrylate (PDSMA) was prepared using a previously reported procedure.2 PDSMA (400 mg, 1.56 mmol), PEGMA (784 mg, 1.56 mmol) and RAFT (24 mg, 0.059 mmol) reagent were dissolved in 2000 µL dry THF in a 10 mL vial and a catalytic amount of AIBN (1.9 mg, 0.0038 mmol) in 200 µL dry THF was added to that. The solution was degassed by performing three freeze–pump–thaw cycles with an argon inflow into the reaction. The reaction flask was tightly sealed and placed in a preheated oil bath at 65 °C with overnight stirring. The next day, the polymerization was quenched by lowering the reaction temperature, and the polymer was purified by dialysis against a mixture of acetone and dichloromethane. Finally, the product was evaporated and dried under high vacuum to get a highly viscous yellow polymer. GPC (THF) Mn: 17.746 kDa, PDI: 1.2. The relative ratios between PEGMA and PDSMA were calculated from 1H NMR considering the integration of peaks corresponding end group methyl of PEGMA at 3.3 ppm and aromatic peak of PDSMA at 8.5 ppm which was found to be PEGMA:PDSMA = 55:45. 1H NMR (400 MHz, CDCl3) δ 8.45, 7.69, 7.28, 7.13, 4.23, 4.08, 3.63, 3.56, 3.05, 1.88, 1.04, 0.90.

2.II NMR Characterization

2.II.i PDSOH 1H NMR after synthesis and purification
2.II.ii PDSMA 1H NMR after synthesis and purification

![PDSMA 1H NMR](image)

2.II.iii PEG-PDS polymer 1H NMR after synthesis and purification

![PEG-PDS polymer 1H NMR](image)
2.III Protein expression and purification

2.III.i Casp-3 expression and purification – pET 23b plasmid encoding human WT casp-3 was transformed into BL21(DE3) E.coli cells via electroporation and plated on agar plates containing ampicillin. Single colony cultures were grown in 50 mL LB media with the corresponding antibiotic at 37 °C overnight. The following day 8L of LB was inoculated with ~5 mL per L of the small seed culture and grown at 37 °C until an OD$_{600}$ of ~0.6 was achieved. The incubation temperature was then reduced to 25 °C and cells were induced with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and left to express for ~3 hours. Cells were then harvested via centrifugation for 10 minutes at 5k RPM and stored at -80 °C. Cell pellets were thawed and lysed using a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM Na$_3$PO$_4$, 300 mM NaCl, 2 mM imidazole, pH 8. Lysed cells were centrifuged at ~27k RCF for 55 minutes to remove cellular debris. The lysate supernatant was then loaded onto a pre-charged 5 mL HiTrap Ni-affinity column (GE Healthcare) and the column was subsequently washed with lysis buffer. Following the lysis wash, the column was further washed with an increased imidazole buffer, 50 mM, and the protein was finally eluted using a linear gradient to 300 mM imidazole. The eluted protein (~20 mL) was diluted 7-fold in a buffer containing 20 mM Tris and 2 mM DTT, pH 8. This ~175 mL solution was then loaded onto a 5 mL HiTrap Q column (GE Healthcare) and finally eluted using a steep linear gradient to 1M NaCl in 20 mM Tris, 2 mM DTT, pH 8. The Q-fractions were analyzed for purity via SDS-PAGE and concentration concluded via A$_{280}$ absorbance, using molar extinction coefficients ~25,900 M$^{-1}$cm$^{-1}$ and aliquots were subsequently stored at -80 °C.

2.III.ii Casp-6 expression and purification – A similar procedure to casp-3 expression and purification was adapted here. pET 11a plasmid encoding constitutively two-chain (CT) human WT casp-6 was transformed into BL21(DE3) E.coli cells via electroporation. Single colony cultures were grown and the following day large LB cultures were inoculated with ~3 mL per L of the small seed culture and grown at 37 °C until an OD$_{600}$ of ~0.6 was achieved. The incubation temperature was then reduced to 20 °C and cells were induced with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and left to express for ~3 hours. Cells were then harvested and frozen, cell pellets were then thawed and lysed using a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM Tris, 300 mM NaCl, 5% glycerol, 2 mM imidazole, pH 8.5. Lysed cells were centrifuged to remove cellular debris then loaded onto a pre-charged 5 mL HiTrap Ni-affinity column (GE Healthcare) and the column was subsequently washed with lysis buffer. Following the lysis wash, the column was further washed with an increased imidazole buffer, 50 mM, and the protein was finally eluted using a linear gradient to 300 mM imidazole. The eluted protein (~20 mL) was diluted 7-fold in a buffer containing 20 mM Tris and 2 mM DTT, pH 8. This ~175 mL solution was then loaded onto a 5 mL HiTrap Q column (GE Healthcare) and finally eluted using a steep linear gradient to 1M NaCl in 20 mM Tris, 5% glycerol, 2 mM DTT, pH 8.5. The Q-fractions were analyzed for purity via SDS-PAGE and concentration concluded via A$_{280}$ absorbance and aliquots were subsequently stored at -80 °C.

2.III.iii Casp-7 expression and purification – A similar procedure to casp-3 expression and purification was adapted here. pET 23b plasmid encoding WT human casp-7 was transformed into BL21(DE3) E.coli cells via electroporation. Single colony cultures were grown and the following day large 2xYT cultures were inoculated with ~5 mL per L of the small seed culture and grown at 37 °C until an OD$_{600}$ of ~0.6 was achieved. The incubation temperature was then reduced to 18 °C and cells were induced with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and left to express for ~18 hours. Cells were then harvested and frozen, cell pellets were then thawed and lysed using a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM Na$_3$PO$_4$, 300 mM NaCl, 2 mM imidazole, pH 8. Lysed cells were centrifuged to remove cellular debris then loaded onto a pre-charged 5 mL HiTrap Ni-affinity column (GE Healthcare) and the column was subsequently washed with lysis buffer. Following the lysis wash, the column was further washed with an increased imidazole buffer, 50 mM, and the protein was finally eluted using a linear gradient to 300 mM imidazole. The eluted protein (~20 mL) was diluted 7-fold in a buffer containing 20 mM Na$_3$PO$_4$, 2 mM DTT, pH 8. This ~175 mL solution was then loaded onto a 5 mL HiTrap Q column (GE Healthcare) and finally eluted using a steep linear gradient to 1M NaCl in 20 mM Tris, 2 mM DTT, pH 8. The Q-fractions were analyzed for purity via SDS-PAGE and concentration concluded via A$_{280}$ absorbance and aliquots were subsequently stored at -80 °C. Handcuffed casp-7 was purified similarly to WT casp-7.

2.III.iv Casp-8 expression and purification – A similar procedure to casp-3 expression and purification was adapted here. pET 15b plasmid encoding WT human casp-8 without the death-effector-domain domain (casp-8 ΔDED) was transformed into BL21(DE3) E.coli cells via electroporation. For significant casp-8 expression, it is essential that fresh transformation is executed. After colonies have developed, single colony cultures were immediately grown and the following day large 2xYT cultures were inoculated with ~5 mL per L of the small seed culture and grown at 37 °C until an OD$_{600}$ of ~0.6 was achieved. The incubation temperature was then reduced to 25 °C and cells were induced with a final
concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and left to express for ~3 hours. Cells were then harvested and frozen, cell pellets were then thawed and lysed using a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM Tris, 500 mM NaCl, 0.50% tween20, 2 mM imidazole, pH 8. Lysed cells were centrifuged to remove cellular debris then loaded onto a pre-charged 5 mL HiTrap Ni-affinity column (GE Healthcare) and the column was subsequently washed with lysis buffer. Following the lysis wash, the column was further washed with an increased imidazole buffer, 20 mM, and the protein was finally eluted using a linear gradient to 300 mM imidazole. The eluted protein (~20 mL) was diluted 7-fold in a buffer containing 20 mM Tris, 2 mM DTT, pH 8. This ~175 mL solution was then loaded onto a 5 mL HiTrap Q column (GE Healthcare) and finally eluted using a steep linear gradient to 1M NaCl in 20 mM Tris, 2 mM DTT, pH 8. The Q-fractions were analyzed for purity via SDS-PAGE and concentration concluded via A_{280} absorbance and aliquots were subsequently stored at -80 °C.

2.III.v Casp-9 expression and purification – A similar procedure to casp-3 expression and purification was adapted here but casp-9 protein was eluted in fractions from the Ni column and analyzed via SDS-PAGE prior to the Q column. pET 23b plasmid encoding human WT casp-9 (or Casp-9(C)) was transformed into BL21(DE3) E.coli cells via electroporation. Single colony cultures were grown and the following day large 2xYT cultures were inoculated with ~4 mL per L of the small seed culture and grown at 37 °C until an OD_{600} of ~0.9 was achieved. The incubation temperature was then reduced to 15 °C and cells were induced with a final concentration of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and left to express for ~3 hours. Cells were then harvested and frozen, cell pellets were then thawed and lysed using a microfluidizer (Microfluidics, Inc.) in a buffer containing 50 mM Tris, 300 mM NaCl, 2 mM imidazole, pH 8. Lysed cells were centrifuged to remove cellular debris then loaded onto a pre-charged 5 mL HiTrap Ni-affinity column (GE Healthcare) and the column was subsequently washed with lysis buffer. Following the lysis wash (significant longer wash than the other caspases), the protein was eluted in fractions over a linear gradient to 100 mM imidazole over 270 mL (casp-9 elutes over a significantly broad peak compared to other caspases). Fractions were analyzed via SDS-PAGE for purity and protein size. Fractions containing casp-9 protein were combined and diluted 7-fold in a buffer containing 20 mM Tris, and 5 mM DTT, pH 8.5. This casp-9 protein solution was then loaded onto a 5 mL HiTrap Q column (GE Healthcare) and finally eluted using a steep linear gradient to 1M NaCl in 20 mM Tris, 2 mM DTT, pH 8.5. The Q-fractions were analyzed for purity via SDS-PAGE and concentration concluded via A_{280} absorbance and aliquots were subsequently stored at -80 °C.

2.IV Evaluation of caspase solvent exposed cysteines – As previously accomplished for casp-3, the number of solvent exposed cysteine residues can be measured using quantitative, colorimetric Ellman’s reagent 5,5’-dithiobis-(2-nitrobenzoic acid (DTNB). Since caspases are purified with DTT, the proteins were first buffer exchanged to remove the reducing agent and concentrations quantified using A_{280} absorbance. Caspases were buffer exchanged to 20 mM sodium phosphate, 100 mM NaCl pH 7.5 using 3K MWCO Amicon Filters or NAP5 (GE) column. A saturated, 50 mg/mL, DTNB solution was made in the same buffer and prior to use, the insoluble DTNB was allowed to settle. A DTT standard curve was made by adding 10 μL of the saturated DTNB solution to 50 μL of DTT with concentrations ranging from 0-30 μM. Then, the various caspases were diluted and assayed similarly. Absorbance of each sample is monitored at 420 nm and caspase thios were quantified from the DTT standard curve.

2.V Casp-NG Characterization

2.V.i Casp-NG protein release and encapsulation comparisons – Purified nanogels were removed from dialysis, volumes normalized and concentrations reevaluated. To visualize protein release, 30 μL of the nanogel solution was incubated with either 10 μL of 1M DTT or 1X PBS and left for 15 minutes at RT. 10 μL of SDS-PAGE 3X dye (with reductant) was added to the DTT-NG sample and reductant free SDS-PAGE 3X dye was added to the water-NG sample. The samples were immediately boiled at 95 °C for ~5 minutes and then added to a 16% SDS-PAGE and electrophoresis was executed at 175V for 60 minutes. To compare encapsulation efficiencies, only when ran on the same gel, large subunit band intensities of all casp-NG released proteins were compared to casp-3 using Image Lab™ Software.

2.V.ii Casp-NG size evaluation – Purified nanogels were diluted to ~0.5-1 mg/mL in water and subsequently filtered to remove dust using a 0.45 μm syringe filter. After filtration, samples were subjected to DLS characterization using a Malvern Nanozetasizer operating at 633 nm. For each sample, three readings with 10 runs each were run.

2.V.ii Cy5-Casp labeling, nanogel formation and uptake study – Full length, catalytically inactive versions of all caspase variants (Casp-3 C163A, Casp-6 C163A, Casp-7 C186A, Casp-8 C360A and Casp-9 C287A) were diluted to 32 μM in 0.1M NaHCO3, pH 8.5 to protonate all lysine residues. To these solutions, 1.5 equivalents of Cy5-NHS ester (approximately 0.4 mg) was added in 10% DMSO of the final reaction volume. These solutions were left to react for 3 hours at room temperature. Free dye was removed via purification using NAP5 columns (GE Healthcare) before NG formation on the same day. NG prepared as previously described. As all caspases were fluorescently labeled to different extents, the final uptake data was normalized to the highest NG dose (see supplemental figure). The day of protein labeling and NG formation, HEK293T cells wereseeded at a density of ~5 x 10^3 in a 24-well plate and left to adhere for
~24 hours. The next day, media was removed and purified NG samples added in a media and 1X PBS, pH 7.4 mixture where the 1X PBS comprises 10% of the final solution volume. Samples were left to incubate for 6 hours before flow cytometry analysis. Notably, all caspases demonstrated different extents of fluorescent labeling. Accordingly, after selecting the live, single cell population, the mean fluorescence intensity for the highest dose was normalized to 100% for each casp-NG. The subsequent doses (0.5 mg/mL, 0.25 mg/mL) were quantified based on the maximum 1 mg/mL dose.
3. Supplemental Figures (S#)

**Figure S1.** Approximate apoptotic caspase NG encapsulation efficiencies. Quantification of released caspase large subunit band intensity reveals similar encapsulation across all caspase cargos. Error bars correspond to SEM of at least two independent NG batches, made and tested on separate days.

**Figure S2.** Casp-9<sub>cd</sub> encapsulation and release. Casp-9<sub>cd</sub> was prepared using site directed mutagenesis, replacing five residues in casp-9 (Gly402-Cys-Phe-Asn-Phe406) with those in casp-3 (Cys264-Ile-Val-Ser-Met268). Casp-9<sub>cd</sub> was effectively encapsulated and released from PEG-PDS NG. The amount of casp-9<sub>cd</sub> encapsulated is similar to WT casp-9 (88-94% after normalizing to WT casp-9 large subunit), error bars correspond to SEM of two independent NG batches, made and tested on separate days.
Figure S3. NGReleased caspase activity in lysates workflow. Proteins were kept encapsulated or released (+DTT) before addition of cell lysate and subsequent analysis of substrate proteolysis by western blot.

Figure S4. Delivery of Cy5-labelled apoptotic caspase monitored by flow cytometry. (A) Experimental schematic. (B) NG crosslinking (343 nm) and visualization of different extents of Cy5-labeling (633 nm). (C) Dose dependent example of Cy5-casp-7 NG delivery. (D) Normalized uptake of all casp-NG demonstrate dose dependent delivery.

Figure S5. Casp-8 delivery to different cell lines. The amount of casp-8 protein delivered was quantified by normalizing casp-8 small to alpha tubulin.
Figure S6. Workflow for apoptosis evaluation using 7-AAD. Workflow adapted from literature. 6,7

Figure S7. Casp-9\textsubscript{CD} NG induced cell death. Left: Casp-9\textsubscript{CD} NG induced 1.76X more apoptosis compared to casp-9 cargo (five independent NG batches normalized to casp-9 induced apoptosis, in HEK293T cells). Right: AnnexinV assay also demonstrated more apoptosis by the constitutive dimeric (\textsubscript{CD}) cargo.

Figure S8. Handcuffed casp-7 PEG-PDS NG. (A) NG encapsulation and release of handcuffed casp-7 (casp-7\textsubscript{HC}) in PEG-PDS NG. (B) Casp-7\textsubscript{HC} is successfully delivered intracellularly, (C) induces significant PARP cleavage and (D) apoptotic cell death (error bars represent SEM of two biological replicates, independent NG batches made and tested on separate days).
Figure S9. Workflows for evaluating executioner caspase activity in whole or lysed cells. Top: Workflow for detection of caspase activity toward multiple fluorogenic peptide substrates in whole cells after treatment with various casp-NG. Bottom: Workflow for detection of caspase activity toward multiple fluorogenic peptide substrates in lysed cells, with DTT added, after treatment with various casp-NG indicating casp-NG have been significantly delivered intracellularly but are trapped in endosomes.

Figure S10. Executioner caspase activity in whole cells, with or without DTT. Similar experiment to Figure 8A, but with DTT added (i.e. activity of intracellular exogenous executioner caspases towards different fluorogenic substrates in cells, with DTT present). Bars plotted are one biological replicate in duplicate.
Figure S11. Executioner caspase activity in cell lysates. Data from Figure 8 E-H, plotted as a function of time. Error bars pertain to technical replicates, experiment was executed in biological triplicate.
4. Supplemental Scheme

Scheme S1. Known caspase-procaspase activation and substrate cleavage.
Top panel: Casp-8 and casp-9 are activated in response to extrinsic or intrinsic stimuli, respectively. These initiator caspases then activate the executioner casp-3, -6 and -7, facilitating substrate cleavage and apoptosis propagation.
Bottom panel: Reported possibilities of executioner casp-3, -6, -7 procaspase cleavage. Caspase enzymes are color coded consistently with Figure 1 and their corresponding substrates for clarity: casp-3 (purple), casp-6 (red), casp-7 (green), casp-8 (orange), casp-9 (blue).
5. Supplemental Tables:

Supplemental Table S1. Kinetic Parameters for caspase variants.

| caspase       | description     | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (μM/s$^{-1}$) | reference |
|---------------|-----------------|------------|----------------------|-----------------------------|-----------|
| casp-3        | executioner     | 10         | 14                   | 1.4                         | a         |
| casp-6        | executioner     | 38         | 0.7                  | 0.02                        | b         |
| casp-7        | executioner     | 41         | 2.4                  | 0.06                        | b, c      |
| casp-7$_{HC}$ | handcuffed casp-7| 39         | 2.4                  | 0.06                        | c         |
| casp-8        | extrinsic initiator | 7          | 0.4                  | 0.05                        | a         |
| casp-9        | intrinsic initiator | 430       | 1.4                  | 0.003                       | d         |

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$^{c}$Wilkowski, W. A. and J.A. Hardy. Protein Science. 2011, 20, 1421-1431.
$^{d}$B.P. Serrano et al. and J.A. Hardy. J Biol Chem. 2017, 292(52), 21352-21365.

Supplemental Table S2. Casp-3, -7 methods of inhibition.

| intracellular inhibition | casp-3 | casp-7 |
|--------------------------|--------|--------|
| prosurvival antagonists   |        |        |
| phosphorylation          | IAPs$^{bc}$: XIAP, cIAPs | IAPs$^{bc}$: XIAP, cIAPs |
| metal binding (Zn)       | p38-MAPK (S150)$^a$ | PAK2 (S239)$^d$ |
| S-nitrosylation          | 7 nM (three Zn)$^f$ | 76 nM (one Zn)$^f$ |
| glutathionylation        | 85% of mitochondrial casp-3 (C163S)$^b$ | NO$^b$ |
|                         | C163, C220$^d$ | | |
| phosphorylation          | CK2 (ISL T174,S176)$^d$ | PAK2 (S30)$^h$ |

$^a$Salveson, G. S. and Duckett, C. S. IAP Proteins: Blocking The Road To Death’s Door. Nature Reviews Molecular and Cellular Biology 2002, 3, 401-405.
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$^h$Eron, S. J., MacPherson, D. J., Hardy, J. A. Multiple Mechanisms of Zinc-Mediated Inhibition for the Apoptotic Caspases-3, -6, -7 and -8. ACS Chem. Biol. 2018, 13, 1279-1290.

Supplemental Table S3. Summary of apoptotic casp-NG results

| NG formation | caspase activity | delivery | PARP cleav. | apoptosis | DEVD activity | VEID activity | LEHD activity |
|--------------|------------------|----------|-------------|-----------|---------------|---------------|---------------|
| NG peptide sub. | casp-3 | casp-6 | casp-7 | casp-8 | casp-9 | casp-9$_{cd}$ | PARP | PARP | PARP | PARP | PARP | PARP |
|              | ✓     | ✓      | ✓      | ✓       | ✓      | ✓      | ✓    | ✓      | ✓      | ✓   | ✓    | ✓    |
| NG protein sub. | PARP | DVL3 | procasp-6 | laminA | p23 | PARP | PARP | PARP | PARP | PARP | PARP | PARP |
|               | ✓     | ✓      | ✓      | ✓       | ✓      | ✓      | ✓    | ✓      | ✓      | ✓   | ✓    | ✓    |
| NG-released casp activity | P38   | R36   | R36   | R36   | R36   | R36   | R36   | R36   | R36   | R36   | R36   | R36   |
|               | ✓     | ✓      | ✓      | ✓       | ✓      | ✓      | ✓    | ✓      | ✓      | ✓   | ✓    | ✓    |
| DEVD activity | 1.0x  | 1.0x   | 1.7x   | <0.2x   | 1.2x   | 1.2x   | 1.2x  | 1.2x   | 1.2x   | 1.2x  | 1.2x  | 1.2x  |
| VEID activity | 2.0x  | 2.2x   | 2.2x   | 1.2x    | 1.2x   | 1.2x   | 1.2x  | 1.2x   | 1.2x   | 1.2x  | 1.2x  | 1.2x  |
| LEHD activity | 2.3x  | 5.0x   | 2.1x   | 1.2x    | 1.4x   | 1.4x   | 1.4x  | 1.4x   | 1.4x   | 1.4x  | 1.4x  | 1.4x  |

Figure 2, S1, S2
Figure 3
Figure 4
Figure 5, S4, S5
Figure 6
Figure 7
Figure 8$_{casp-3,-6,-7}$
Figure 8$_{casp-3,-6,-7}$
Figure 8$_{casp-3,-6,-7}$

DEVD recognition sequence of casp-3,-7; VEID of casp-6; LEHD of casp-8,-9
6. Supplemental References:

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