Supporting Information

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SI Materials and Methods

Screening Human Cancer Cell Lines for Apo2L/TRAIL Sensitivity. For cell-line screening studies, two independent screens were performed. For both screens, adherent lines were trypsinized and suspension lines were resuspended. Cells were assessed with a ViCELL Cell Viability Analyzer (Beckman Coulter); viability of at least 90% for adherent cell lines and 85% for suspensions was required for screening. Adherent cells were plated at a seeding density intended to achieve 75% confluency at 96 h, as assessed by Incucyte (Essen Bioscience). Optimal seeding for suspension and mixed suspension/adherent cell lines was 75% $\text{signal}_{\text{max}}$ at 96 h, as assessed by CellTiter-Glo. A Multidrop Combi Reagent Dispenser (Thermo Fisher Scientific, Inc.) was used for plating cells into barcoded Falcon 384-well black clear-bottom plates. Cell plates were placed in a 37 °C humidified incubator maintained at 5% CO_2 overnight. For the Apo2L.0 screen, a nine-point, 1:3 dose dilution, with a maximum dose of 3 μg/mL, was performed using a Bravo Automated Liquid Handling Platform (Agilent Technologies). For the Apo2L.XL screen, the maximum dose was 10 μg/mL and the nine-point 1:3 dose dilution was performed using an Echo 550 (Labcyte). The following day, compound addition was done using an Oasis integrated Liquid Handling Robot Platform (Dynamic Devices) with Liconic incubators and automated barcode tracking. After 72 h, 25 μL of CellTiter-Glo reagent was added using a Biotek Multiflo Microplate Dispenser. Cell lysis was induced by mixing for 2 min on an orbital shaker; plates were then incubated at room temperature for 10 min to stabilize luminescent signal. Luminescence readout was done via a 2104 EnVision Multilabel Plate Reader (PerkinElmer).

Three to four independent biological replicates were produced. Data were processed using the R statistical computing environment and a Genentech-developed analysis package (singleAgentPlots). To reduce noise and the impact of spatial effects, per-dose viability was computed relative to median viability from undrugged wells physically near drugged wells on the plate. To minimize the impact of isolated aberrant wells, a four-parameter log-logistic model relating viability to log dose was fit by minimizing the mean of residual absolute value (as an alternative to standard least-squares fitting). Reported IC_{50} is dose at which cross-run estimated inhibition is 50% relative to undrugged wells (i.e., absolute IC_{50}).

To assess the statistical significance of variations in cell viability at 2×10^2 nM, we performed a one-tailed test, examining whether Apo2L.0 was more potent than Apo2L.XL in all cell lines in the panel. We also performed a similar one-tailed test assessing the opposite hypothesis, that Apo2L.XL is more potent than Apo2L.0. Fig. 1*B* reflects these analyses—red triangles for Apo2L.XL viability scores show statistically significant ($P < 0.05$) differences whereas gray triangles show statistically insignificant differences.

To query a correlation between DR4 or DR5 expression and sensitivity to Apo2L.XL, we used available data for 260 cell lines and calculated Pearson's correlation coefficients, as well as *P* values derived from a two-tailed Student's *t* test for the following pairs: DR4:Apo2L.XL viability, DR5:Apo2L.XL viability, DR4:Ratio of Apo2L.0/Apo2L.XL viabilities, DR5:ratio of Apo2L.0/Apo2L.XL viabilities, $\log_2$ transformations of these variables, DR4:difference of Apo2L.0-Apo2L.XL viabilities, and DR5:difference of Apo2L.0-Apo2L.XL viabilities. Among these, the most strongly correlated combinations were $\log_2$(DR4): $\log_2$(Apo2L.0 viability) and $\log_2$(DR4):$\log_2$(Apo2L.XL viability) (−0.397 and −0.311, respectively).

Formulating Liposomes for Membrane Display of Apo2L/TRAIL. For supported membrane experiments, liposomes were composed of 99.9 mol % DOPC (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) and 0.1 mol % biotin-DOPE [1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine-N-(cap biotinyl)], and hydrated in PBS. Supported membranes were deposited as described previously (1–3).

For all experiments using liposome-displayed Apo2L, liposomes were composed of 90 mol % DOPC, 4.95 mol % PEG (2000)-DSPE (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000]), 4.95 mol % PEG(2000)-DSPE maleimide (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000]), and 0.1 mol % TR-DHPE (Texas Red 1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine), and hydrated in 25 mM Tris and 0.4 M Na_2SO_4, at pH 7.5 (Apo2L EB). TR-DHPE was purchased from Molecular Probes (Life Technologies); all other phospholipids were purchased from Avanti Polar Lipids, Inc.

Disulfide bonds between $\text{Apo2L}^{\text{K179C}}$ molecules were reduced through a 1-h incubation with 10 mM DTT at room temperature. DTT was then removed by running the protein through 7K MW cutoff Zeba spin columns (Thermo Fisher Scientific, Inc.). The protein was then added to freshly prepared liposomes at a 1:1 molar ratio with maleimide moieties on the outer leaflet of the liposomes and allowed to react for 1 h at room temperature. The coupling reaction was quenched by the addition of N-ethylmaleimide (NEM, fivefold molar excess, 1 h, room temperature). NEM was removed by dialysis using Slide-A-Lyzer or Slide-A-Lyzer G2 cassettes (Thermo Fisher Scientific, Inc.) dialyzed in 1 L of Apo2L EB overnight at room temperature, protected from light.

The concentration of the final Apo2L.L mixture was calculated by comparing the fluorescence of bare liposome standards to that of the Apo2L.L solution and then calculating the number of maleimide groups in the solution and assuming a 100% reaction yield. This assumption was in good agreement with the integrated area under the curve for the SDS-HPLC chromatogram of the Apo2L.L solution.

Fluorescence Imaging of Apo2L.M. Supported membranes were deposited into wells of a 96-well plate, to which live cells were added. Live cell imaging was performed using a Nikon Ti-Eclipse fitted with a 100× oil immersion objective and a Cy5 fluorescence cube to excite Alexa Fluor 647, a FITC fluorescence cube to excite Alexa Fluor 488, and a GFP fluorescence cube to excite GFP–caspase-8. Cells were imaged every 20 min for up to 16 h after addition to the wells. Critically, repeated excitation of the same areas of the surface during video acquisition caused photobleaching that decreased the intensity of Apo2L.M as a function of exposure number. Therefore, to accurately quantify receptor transport over time (Fig. S2*A*), samples were fixed and subjected to only a single exposure per site. Before Apo2L.M engagement, cells were incubated with 1 μg/mL of the cell-permeable nuclear dye Hoechst 33342 for 30 min at 37 °C. Cells were then added to each well (2×10^4 cells per well) and fixed at 2-h intervals through the direct addition of 16% (wt/vol) paraformaldehyde [final concentration ∼4% (wt/vol)] for 15 min; 10% (vol/vol) Triton-X was then added (final concentration ∼0.1%) to remove unbound protein on the supported membrane and decrease background fluorescent signal. This helped to remove streptavidin microclusters that form independently of death receptor ligation by Apo2L/TRAIL (Fig. S2*B*).

Fixed cell images used for quantification of Apo2L.M transport were acquired using an ImageXpress Micro System fitted with a

40× air immersion objective. Analysis of receptor transport was performed as described previously (2, 3). Briefly, the MetaXpress image analysis software package was used to choose regions of interest corresponding to cells based on nuclear signal. The radial distribution of Apo2L.M was then calculated for these regions of interest, and the slope of these radial distributions was used as the transport score for each well. For each cell line at each time point, six independent wells were analyzed, with at least 120 cells per well.

Measuring Proapoptotic Signaling in Cell Samples. For in vitro studies, cells were detached using trypsin-EDTA and added to 96-well plates at a concentration of 2×10^4 cells per well. The wells contained varying concentrations of Apo2L.M, Apo2L.L, Apo2L.0, Apo2L cross-linked with streptavidin (Apo2L.STP), or Apo2L cross-linked with a Flag antibody (Apo2L.XL). Cells were treated at 37 °C, 5% CO_2.

Caspase-8 activity was measured using caspase-8 Glo, added at a 1:1 volume ratio, after 4 h of treatment. Caspase-3/7 activity was measured using caspase-3/7 Glo, added at a 1:1 volume ratio, after 6 h of treatment. Cell viability was measured using Cell Titer Glo, added at a 1:1 volume ratio, after 24 or 72 h of treatment. All measurements were normalized to treatment with bare media.

Immunoblotting of Protein, Cell, and Tissue Samples. Samples were lysed for 30 min at 4 °C in RIPA buffer and centrifuged at 14,000 × *g* for 15 min at 4 °C and the supernatants were collected and frozen for later use. Western blots were performed using primary antibodies directed against Apo2L (2E11; Genentech, Inc.), DR5 (3H1; Genentech, Inc.), caspase-8 (C15; Enzo Life Sciences), FADD (BD Biosciences), cleaved caspase-8 (Cell Signaling), and cleaved caspase-3 (Cell Signaling). Samples were labeled with isotype-matched secondary antibodies conjugated to horseradish peroxidase or Alexa Fluor 780.

Fluorescence Imaging of Apo2L.0-Stimulated Cells. Biotinylated Apo2L/TRAIL, labeled with Alexa Fluor 594-streptavidin, was added to Jurkat cells on ice. Cells were then heated to 37 °C for up to 45 min, at which point they were fixed with 4% (wt/vol) paraformaldehyde and injected into slides containing mounting media with the nuclear stain DAPI. Images were acquired using a Nikon TE300 inverted epifluorescence microscope fitted with a 60× oil immersion objective.

Size-Exclusion Chromatography of Apo2L.0-Stimulated Cells. As described earlier (4), Apo2L/TRAIL-treated or untreated cells were lysed in Tris-buffered saline containing 1% Triton X-100 and protease inhibitor mixture (Roche). Lysates were spun at maximum speed then loaded onto a Superdex 200 10/300 GL column (GE Healthcare). Fractions were collected at the indicated volumes.

SDS-HPLC of Apo2L.L. To quantify the amount of lipid-modified $Apo2L^{K179C}$ in each formulation, samples were combined 1:1 (final concentration 1 mg/mL) with 100 mM Tris, 1% SDS, and 0.4 M NaCl, pH 7.0 containing 10 mM iodoacetic acid. The resulting solution was heated for 10 min at 50 °C and injected onto a 7.8- × 300-mm Toso Haas G2000SWXL column run held at ambient temperature and eluted isocratically using a 0.1% SDS and 0.2 M NaCl, pH 7.0 mobile phase at a flow rate of 0.6 mL/min. Injection volume was 10 μL (10 μg) with UV detection at 280 nm. The resulting chromatograms were collected and integrated using Chromeleon (Dionex) and Prism (Graphpad, Inc.) software.

Samples were run both under reduced (with 10 mM DTT instead of iodoacetic acid) and nonreduced (lacking DTT) conditions to determine which peaks were due to the formation of DTT-reducible disulfide bonds. Using these data, peaks were assigned as follows: (*i*) a small peak eluting between 9.5–10 min corresponding to a disulfide-linked Apo2L/TRAIL homodimer, (*ii*) a large peak eluting at 10.5–11.5 min (shifted to 10–11 min upon lipidation) corresponding to Apo2L/TRAIL monomer, and (*iii*) a small peak centered around 11.8 min corresponding to free lipids.

Animal Studies Using Apo2L.L. Nude mice 6–8 wk old were s.c. implanted with human tumor cells. When tumors grew to 150–250 mm^3 in volume, the animals were treated, via i.p. or tail vein i.v. injection, with soluble Apo2L.0, Apo2L.L, or bare liposomes. A control group of animals was left untreated, or treated with Apo2L.0 vehicle.

For pharmacokinetics/pharmacodynamics studies, animals were treated with a single dose of either 25 mg/kg (COLO205 xenograft) or 50 mg/kg (HT-29 xenograft) of either Apo2L.0 or Apo2L.L delivered via i.p. injection. Tumors were collected either 24 or 48 h after treatment and assessed for Apo2L/TRAIL levels as well as caspase activity.

For efficacy studies, animals were treated every 2 d and their tumor volumes and body weights measured at least two times a week. Animals that lost greater than 20% body weight over the course of these studies were killed, and the condition of their internal organs was observed to assess potential toxicity, although no gross changes to any examined tissues (including tumors, livers, and spleens) were observed. A nonlinear mixed-effect modeling approach was used to analyze the repeated measurement of tumor volumes (and of body weights) from the same animals over time. This analytical method uses all of the collected data and reduces the impact of unequal variance, outlier bias, and censoring owing to the removal of animals before the end of the study for reasons not related to the test article. Briefly, cubic regression splines were used to fit a nonlinear profile to the time courses of $\log_2$(tumor volume) at each dose level. The nonlinear profiles were then related to dose within the mixed model. Tumor growth inhibition (TGI) was calculated as percent area under the fitted tumor volume−time curve (AUC) per day of each treatment group in relation to the vehicle, using the following formula:

$$\%TGI = 100 \times \left[1 - (AUC_{treatment} \text{ per day} \div AUC_{vehicle} \text{ per day})\right].$$

Data were analyzed using the R statistical computing environment, version 2.15.2, and the mixed effect models were fit within R using the nlme package, version 3.1−108 (5).

Fluorescence Imaging of Apo2L.L. Nude mice were injected via the tail vein with a bolus of up to 20 mg/kg Apo2L.L. Immediately after injection, animals were anesthetized using isofluorane and imaged using a Leica M165 FC stereo microscope. Texas-Red fluorescence was detected using a Texas Red filter cube. Animals were imaged within 5 min of the Apo2L.L injection, and then at time points up to 6 h later. At this point, animals were killed and dissected for ex vivo imaging. After image acquisition, dashed yellow lines were overlaid onto fluorescence images to denote areas corresponding to tumors based on corresponding bright field images. Fluorescent tissues were collected and flash-frozen in liquid nitrogen for later biochemical characterization.

Measuring Apo2L/TRAIL Quantities and Activities in Tumor Samples. Whole organs and tumors were removed from treated animals and immediately flash-frozen in liquid nitrogen. The samples were then weighed and homogenized at 4 °C in RIPA buffer and centrifuged at 14,000 × *g* for 15 min at 4 °C. The supernatants were collected and their protein concentrations were measured using a BCA protein quantification kit (Thermo Fisher Scientific, Inc.).

For quantification of Apo2L/TRAIL in each sample, an Apo2L/TRAIL ELISA (R&D Systems, Inc.) was used. Fifty micrograms of protein from each sample was diluted to 50 μL with RIPA buffer

and added to each well, and Apo2L/TRAIL standards were prepared according to manufacturer protocols.

For quantification of caspase-8 and caspase-3/7 activity in each sample, 30 μg of protein from each sample was diluted to 50 μL with RIPA buffer. Fifty microliters of either caspase-8 Glo or caspase-3/7 Glo reagent was then added to each sample and measured as above. All samples were compared with corresponding tissues from untreated animals at 24 h.

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2. Nair PM, Salaita K, Petit RS, Groves JT (2011) Using patterned supported lipid membranes to investigate the role of receptor organization in intercellular signaling. *Nat Protoc* 6(4):523–539.
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5. Gong IY, et al. (2013) Determination of clinically therapeutic endoxifen concentrations based on efficacy from human MCF7 breast cancer xenografts. *Breast Cancer Res Treat* 139(1):61–69.

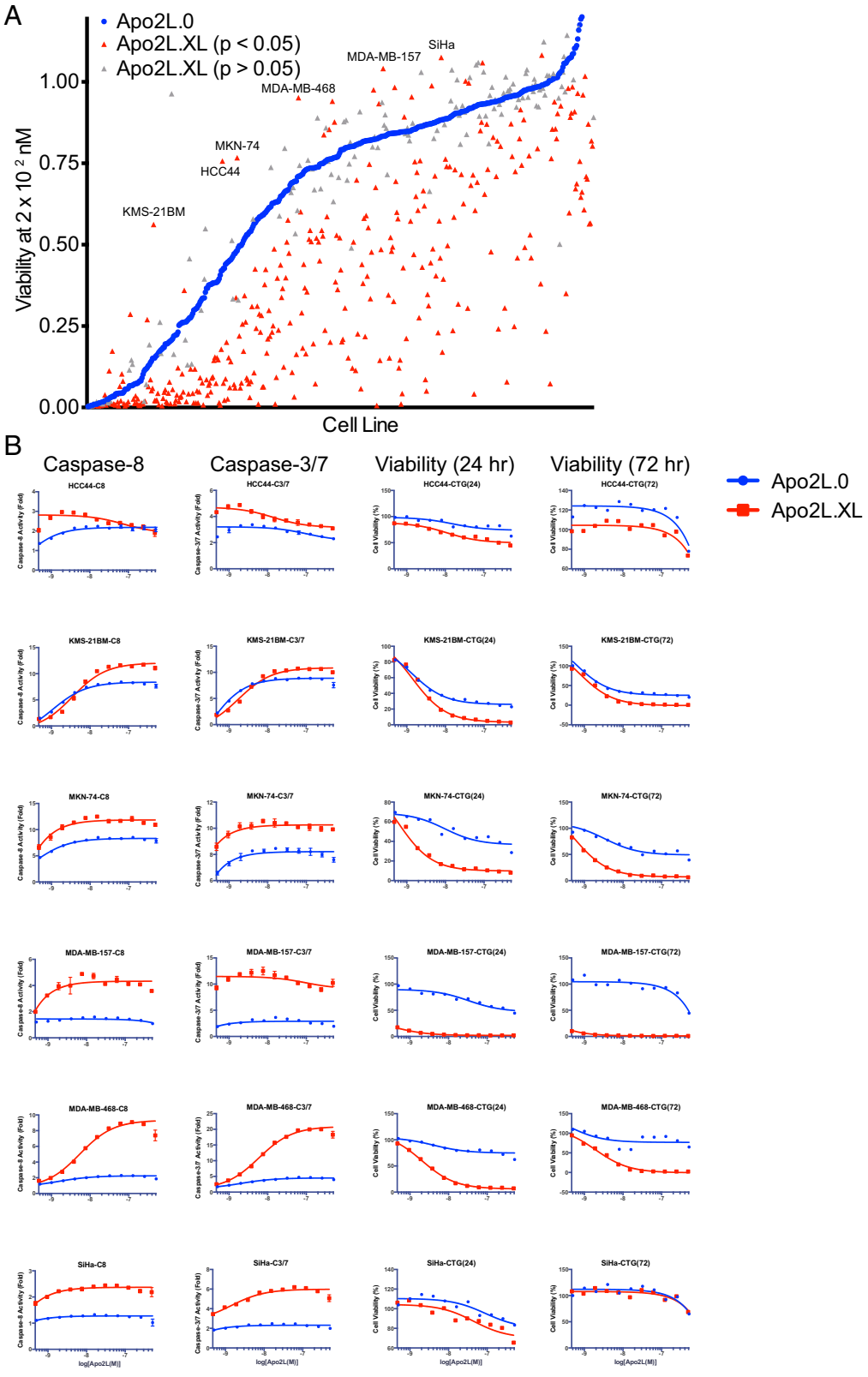


Fig. S1. Multiple cell lines display greater sensitivity to Apo2L.XL than to Apo2L.0. (*A*) Of the cell lines that appeared unexpectedly to be more sensitive to Apo2L.0 than Apo2L.XL by a statistically significant degree in the screen, the six with the largest differential in viability scores between these two treatments were examined further: HCC44, KMS-21BM, MDA-MB-157, MDA-MB-468, MKN-74, and SiHa. (*B*) Cells were treated with increasing concentrations up to 5×10^2 nM Apo2L.0 or Apo2L.XL. Caspase-8 activity was measured after 4 h, caspase-3/7 activity after 6 h, and cell viability after 24 and 72 h. Error bars denote SEM, $n = 3$ or 4 per treatment.

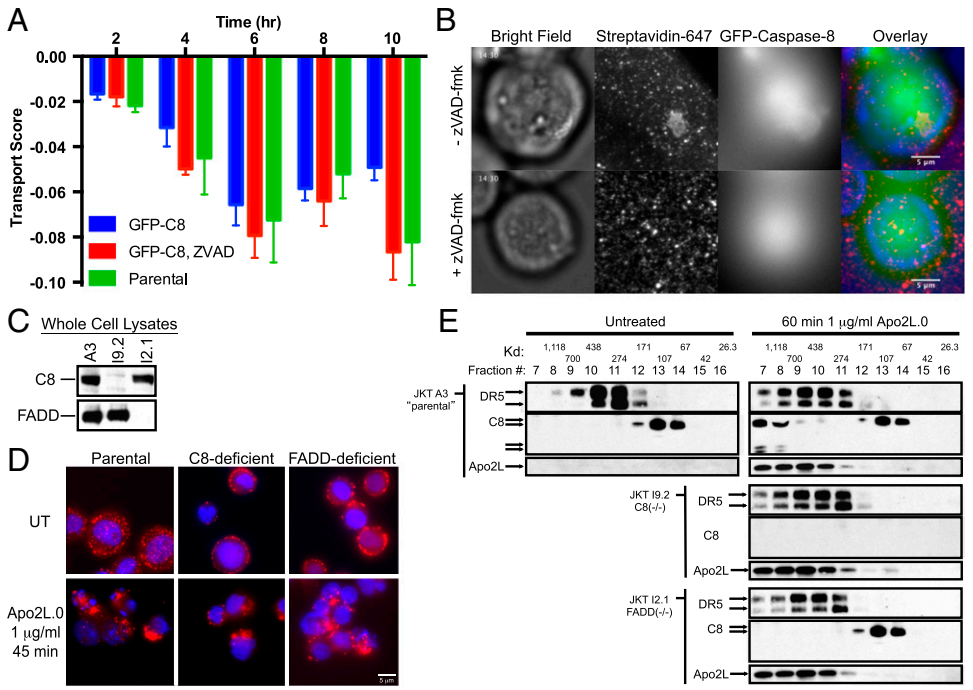


Fig. S2. Apo2L/TRAIL stimulation triggers death receptor transport and clustering. (*A*) The radial distribution of Apo2L clusters engaged by NB-7 cells expressing DR5 was measured at 2-h intervals up to 10 h. (*B*) NB-7^{GFP-C8} cells were deposited on supported membranes lacking Apo2L.M. Corresponding bright field, streptavidin (Alexa Fluor 647), and caspase-8 (GFP) channels were imaged. (*C*) The parental Jurkat line A3, as well as its caspase-8–deficient (I9.2) and FADD-deficient (I2.1) sublines. (*D*) The Jurkat cell lines in *C* were treated for 45 min with 1 μg/mL biotinylated Apo2L.0 labeled with streptavidin-Alexa Fluor 594 then fixed in mounting media containing the nuclear stain DAPI. (*E*) The Jurkat cell lines in *C* were treated with1 μg/mL Apo2L.0 then collected and lysed. Lysates were subjected to size-exclusion chromatography and immunoblotted for Apo2L, DR5, and caspase-8.

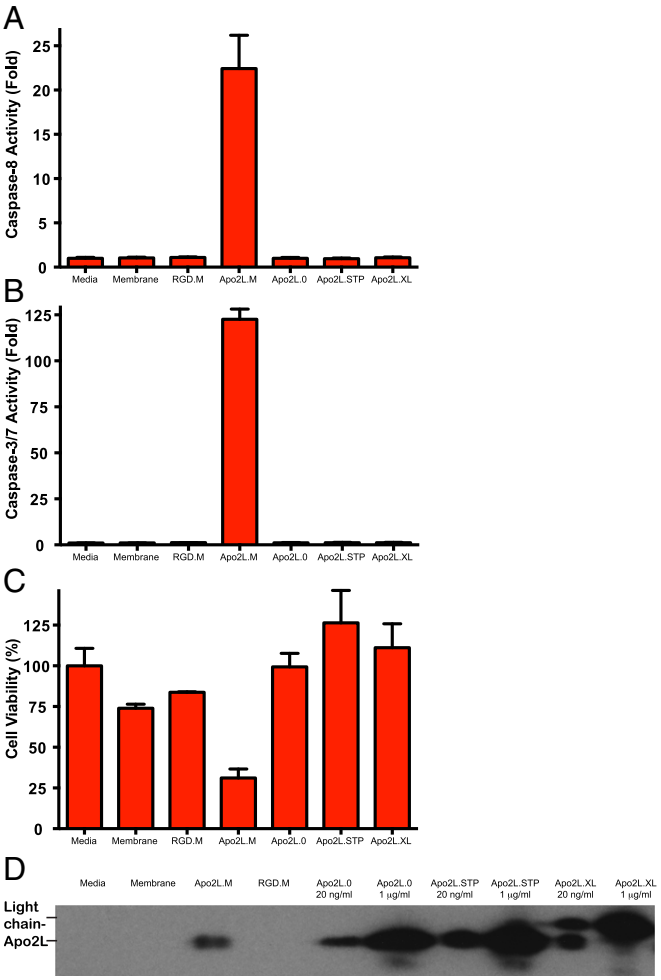


Fig. S3. KPP pancreatic cancer cells are sensitive to membrane-displayed Apo2L but resistant to soluble Apo2L variants. (*A*–*C*) KPP pancreatic cancer cells were treated with equimolar amounts (~20 ng/mL) bare membranes, membrane-displayed RGD (RGD.M), membrane-displayed Apo2L (Apo2L.M), Apo2L.0, streptavidin-crosslinked Apo2L (Apo2L.STP), or antibody-cross-linked Apo2L (Apo2L.XL) and analyzed for caspase-8 activity after 4 h (*A*), caspase-3/7 activity after 6 h (*B*), or cell viability after 24 h (*C*). Error bars denote SEM, $n = 3$ per treatment. (*D*) Glass-bottomed wells were prepared with the noted reagents and then solubilized in an SDS-containing protein loading buffer. The resulting solutions were immunoblotted for Apo2L/TRAIL.

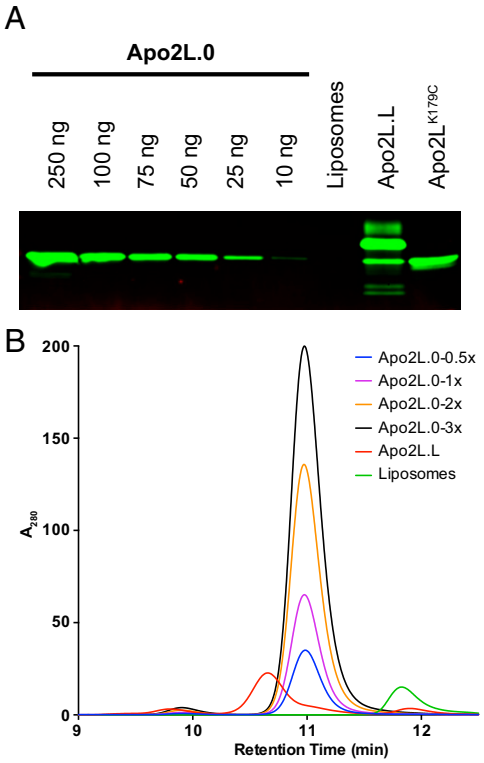


Fig. S4. Apo2L/TRAIL display on liposome surfaces. (*A*) Immunoblot (under reducing conditions) showing nonreducible shift in mass of Apo2L/TRAIL corresponding to the addition of a 3-kDa lipid moiety. (*B*) SDS-HPLC chromatograms used to quantify yield of Apo2L.L by comparing integrated area under the curve to those of different concentrations of Apo2L.0.

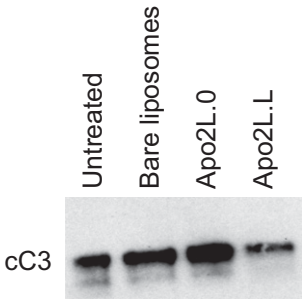


Fig. S5. Caspase-3 cleavage in untreated and treated mouse spleens. HT-29 xenografts were treated with the indicated reagents at 20 mg/kg (or an equivalent concentration of liposomes) for 6 h. Animals were then killed and their spleens were homogenized and immunoblotted for cleaved caspase-3. Equal amounts of protein extracts were loaded per lane.

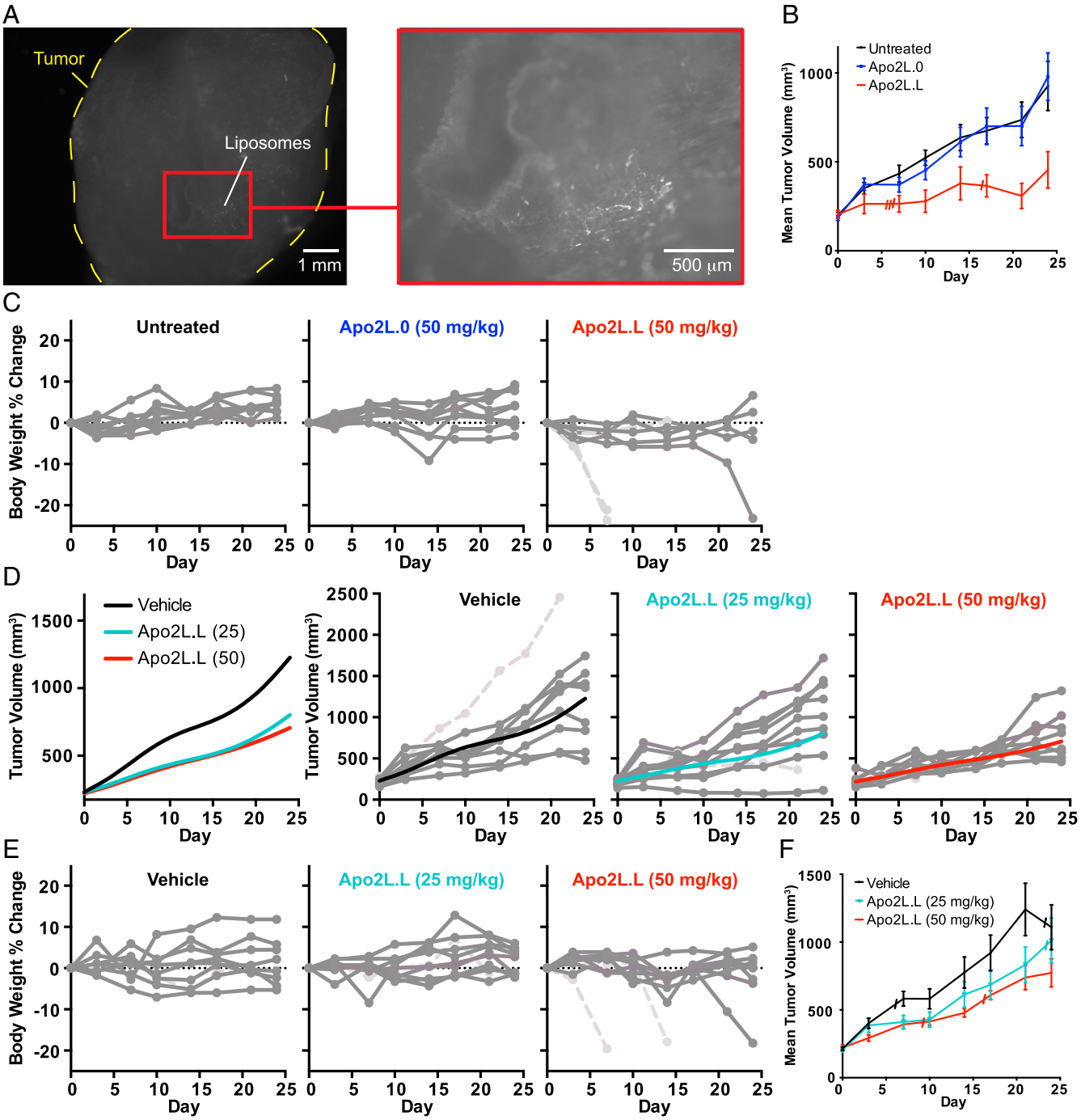
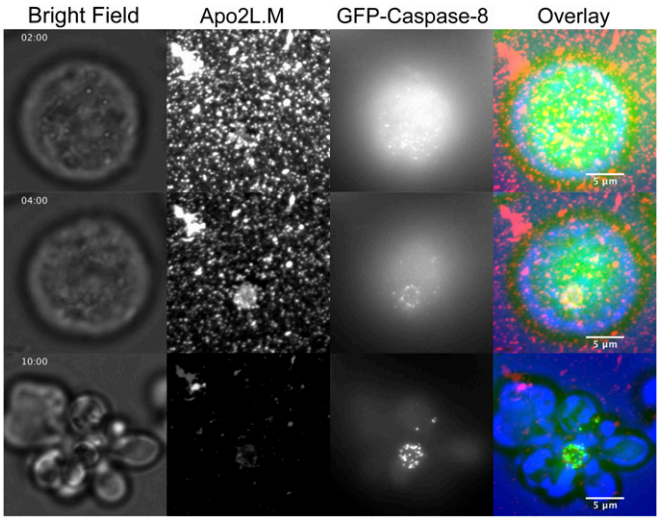


Fig. S6. Detection and efficacy of Apo2L.L in HT-29 xenografts. (*A*) Ex vivo fluorescence imaging of HT-29 xenograft tumor 6 h after a single Apo2L.L injection. (*Inset*) Zoomed-in view of area highlighted in red. (*B*) Mean tumor volumes over the course of treatment with Apo2L.0, Apo2L.L, or no treatment. Hash marks denote animals that were killed before the end of study. Error bars denote SEM. (*C*) Body weights of mice bearing HT-29 xenografted tumors in response to no treatment, 50 mg/kg Apo2L.0, or 50 mg/kg Apo2L.L. (*D*) Tumor volumes of mice bearing HT-29 xenografted tumors in response to vehicle, 25 mg/kg Apo2L.L, or 50 mg/kg Apo2L.L. Dark solid lines denote curves that were fit to a nonlinear mixed-effect model of tumor growth based on the tumor volume data (*SI Materials and Methods*). (*E*) Body weights of mice bearing HT-29 xenografted tumors in response to vehicle, 25 mg/kg Apo2L.L, or 50 mg/kg Apo2L.L. For efficacy studies, all animals were dosed every 2 d via i.p. Dashed lines denote animals that were killed before the end of study. (*F*) Mean tumor volumes over the course of treatment with either 25 or 50 mg/kg Apo2L.L or Apo2L.0 vehicle. Hash marks denote animals that were killed before the end of study. Error bars denote SEM.



Movie S1. Death receptor microclustering, caspase-8 recruitment, and apoptotic blebbing in response to membrane-displayed Apo2L/TRAIL. NB-7$^{GFP\text{-}C8}$ cells were deposited on supported membranes displaying Apo2L.M. Cells were allowed to engage Apo2L.M on the surface for 2 h, at which point bright-field, Alexa Fluor 647, and GFP channels were imaged at 100× magnification every 20 min for 14 h. Samples were maintained in a live-cell imaging enclosure at 37 °C, 5% CO_2 for the duration of the imaging.

Movie S1

Dataset S1. Effect of Apo2L.0 and Apo2L.XL on cell viability across 479 human cancer cell lines

Dataset S1

Summarized data from multiple screens across a panel of 479 human cancer cell lines. Cells were treated with up to 3 μg/mL Apo2L.0 or up to 10 μg/mL Apo2L.XL for 72 h. Cell viability was then measured and normalized to untreated controls.