Supplementary methods

Cell culture and transfections. HeLa cells were maintained in DMEM (Mediatech, Inc.) supplemented with L-glutamine (Gibco), penicillin/streptomycin (Gibco) and 10% foetal bovine serum (Mediatech, Inc.). MCF10A cells were cultured as described[28]. H1299 cells containing FLIP tagged with YFP at the endogenous locus were obtained from the Kahn Dynamic Proteomics Project and maintained at 8% CO_2 in RPMI (Mediatech, Inc.) with 10% foetal bovine serum and penicillin/streptomycin. HeLa cells expressing IMS-RP [11] were transfected using FuGENE 6 (Roche) with pd4-Bid-EGFP (Clontech) to sample expression levels across a wide range. H1666 cells were maintained (and imaged) in RPMI supplemented with 10% FBS, L-Glutamine, penicillin/streptomycin, 1x ITES (Lonza Biosciences #17-839Z), 50 nM hydrocortisone, 10 uM phosphorylethanolamine, 0.1 nM Tri-iodothyronine, 10 mM HEPES, 0.5 mM sodium pyruvate, 2 g/L BSA, and 1 ng/mL EGF. Fresh primary liver cells were obtained from CellzDirect and plated on collagen type I. Cells were maintained (and imaged) in Eagle's Minimum Essential Medium supplemented with 10% FBS, L-Glutamine, 100 nM dexamethasone, 5 ug/mL human insulin, 5 ug/mL transferrin from human serum, 5 ug/mL sodium selenite, and 15 mM HEPES. SKBR3 cells were maintained (and imaged) in RPMI supplemented with 10% FBS, L-Glutamine, and penicillin/streptomycin.

Live-cell microscopy. HeLa cells expressing IMS-RP and FRET reporters EC-RP or IC-RP were imaged in a 37ºC humidified chamber as described[11]. For sister cell experiments, HeLa cells expressing IMS-RP were imaged[11] at 10-minute intervals for 20-30 hours in phenol red-free CO_2 independent medium (Invitrogen) with L-glutamine, penicillin/streptomycin, and 1% serum (Fig. 1 b-e) or at ~5% CO_2 in phenol red-free DMEM with L-glutamine, penicillin/streptomycin, and 10% serum (Fig. 1 f-i, see also Supplementary Fig. 5). The growth media was then replaced with the same media

containing TRAIL (Alexis Biochemicals ALX-201-115) with or without 2.5 μg/ml cycloheximide (Sigma-Aldrich) and images were acquired at 3-minute intervals for an additional 8 hr. Cells still alive at the end of the 8 hr were considered to have survived the treatment. MFC10A were imaged as described[11] but in ~5% CO_2 in phenol red-free assay media[28] without EGF or insulin to reduce cell migration. HeLa cells co-expressing IMS-RP and Bid-GFP and H1299 YFP-FLIP cells were imaged[11] in the same media as described above for Fig. 1b-e but at 20× magnification with frames every 3 min or every 10 minutes, respectively. H1666, SKBR3, and primary liver cells were imaged in 96-well glass bottom plates (Matrical) on a Nikon TE2000E at 10× magnification in a 37ºC chamber with 5% CO2.

Image analysis. Sister-cell tracking was performed manually. To assess whether there is a cell cycle effect on death time, we plotted death time as a function of time since division, as a proxy for cell cycle phase. Because the distribution of time since division is not uniform, one might infer a cell cycle effect but, in fact, a cell cycle effect would appear as a slope in the data, which we do not observe. For sister cell experiments where CO_2 independent medium was used, there was more proliferation early in the divisions movie (and thus more points near "G2") because CO_2 independent medium does not support very long term proliferation (division slows down, but the cells are not dying). Importantly, the lack of cell cycle effect is also apparent for sister cell experiments performed in DMEM, the standard medium for HeLa cells (Supplementary Fig. 5). In these experiments, the cells proliferated continuously during the divisions movie. Cells that divided early in the divisions movie often divided again if they survived stimulation with TRAIL, producing "cousins" which were excluded from the analysis. This resulted in fewer cells in the “G2” region, but this does not indicate a cell cycle effect, as the probability of surviving or dying is constant.

Analysis of IC-RP and EC-RP cleavage and IMS-RP translocation was performed as described [11]. To derive estimates for k_{IC} and θ, individual cell trajectories were fit with an equation derived from mathematical reduction of the differential equation model for the pathway (J.M.B., J.G.A, S.L.S., D. Lauffenburger, P.K.S., manuscript in preparation; see also Supplementary Fig. 5). The equations for the fitted relationship between T_d and k_{IC} and between T_d and θ were as follows: $T_d = 6.9e^{(-53.7\ kIC)}$ and $T_d = 6.8\ \theta + 1.1$. FLIP-YFP fluorescence was quantified at $t = 0$ hr (time of TRAIL addition) by manually outlining the cell and measuring the average fluorescence intensity within the outline. For FLIP-YFP cells, time of death was scored as the first frame where a cell exhibited apoptotic morphology. Bid-GFP fluorescence was quantified at $t = 0$ hr by measuring the average fluorescence intensity from a representative area within the cell. To determine the absolute number of proteins/cell, the average Bid-GFP fluorescence intensity from these movies was set equal to the average number of GFP-tagged proteins per cell as measured by quantitative immunoblot Supplementary Fig. 9).

Flow cytometry. The distributions of initial protein levels were measured in HeLa cells (fixed with paraformaldehyde and permeabilised with methanol) on a FACSCalibur (BD Biosciences). The antibodies were carefully validated by knockout or knockdown and/or by over-expression of GFP-tagged fusion proteins. The following antibodies were used: α-Bid (HPA000722, Atlas Antibodies), α-Bax (MAB4601, Chemicon International), α-Bcl-2 (SC7382, Santa Cruz Biotechnology), α-XIAP (610717, BD Biosciences), α-C3 (SC7272, Santa Cruz Biotechnology). The coefficient of variation (CV) of cells of similar size (as estimated by forward scatter) ranged from 0.21 to 0.28.

Modelling. The details of methods used will be described elsewhere. Briefly, a series of 10^4 simulations of the EARMv1.1 ordinary differential equation model [12] (modified to include general protein degradation) were run in Jacobian (Numerica Technology).

In previous work, only single values for each protein concentration were used[12]. Here, for each run of the model (representing one cell), initial protein levels were independently sampled from log-normal distributions having mean values identical to those previously reported[12] (except for Bcl-2, whose mean was set to 30,000 molecules/cell), and coefficient of variation as measured by flow cytometry (Fig. 2a) or set to 0.25 for proteins that were not measured. Initial protein concentrations and parameter values are listed in Supplementary Tables 1 and 2.

Supplementary Methods References

[28] J. Debnath, S. K. Muthuswamy, and J. S. Brugge, *Methods (San Diego, Calif* **30** (3), 256 (2003).