Fricker et al., http://www.jcb.org/cgi/content/full/jcb.201002060/DC1

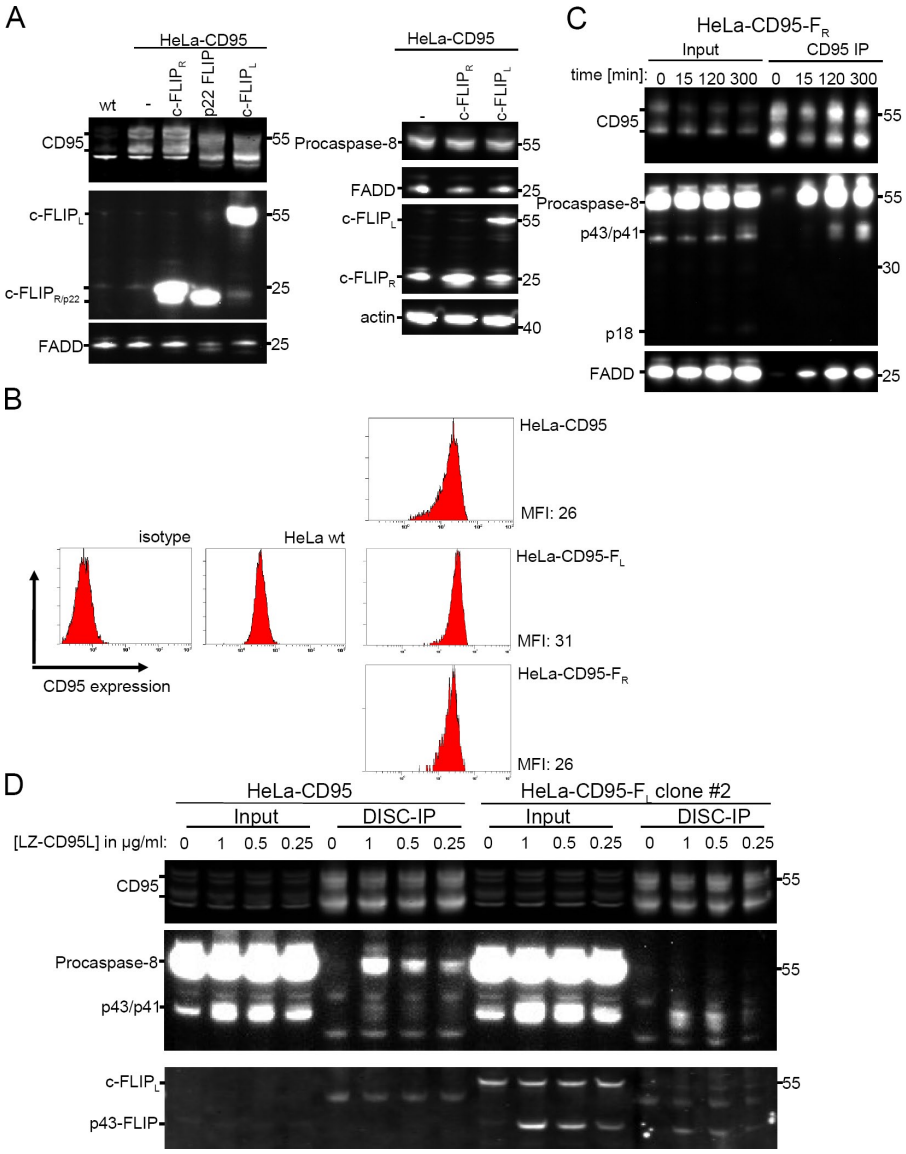

Figure S1. **Characterization of stable cell lines overexpressing different c-FLIP isoforms.** (A) Total cellular lysates of HeLa wt, HeLa-CD95, HeLa-CD95–F_L, HeLa-CD95–p22, and HeLa-CD95–F_R cells were analyzed by Western blotting with antibodies against CD95 (C20), caspase-8 (C15), c-FLIP (NF6), FADD (1C4), and actin. (B) HeLa wt, HeLa-CD95, HeLa-CD95–F_L, and HeLa-CD95–F_R cells were labeled with anti–APO-1 antibody or isotype control antibody. CD95 expression was determined with flow cytometry. (C) Processing of procaspase-8 at the DISC of HeLa-CD95–F_R cells is impaired. HeLa-CD95–F_R cells were stimulated with 1 µg/ml LZ-CD95L for the indicated time points. CD95 DISCs were immunoprecipitated (IP) using anti–APO-1 antibodies and analyzed along with total cellular lysates using Western blotting with antibodies against caspase-8 (C15), c-FLIP (NF6), and CD95 (C20). (D) c-$FLIP_L$ exhibits the same procaspase-8–activating effect in an independent HeLa-CD95–F_L clone. HeLa-CD95 and HeLa-CD95–F_L cells were stimulated with the indicated amounts of LZ-CD95L for 20 min. CD95 DISCs were immunoprecipitated using anti–APO-1 antibodies and analyzed along with total cellular lysates using Western blotting with antibodies against caspase-8 (C15), c-FLIP (NF6), and CD95 (C20).

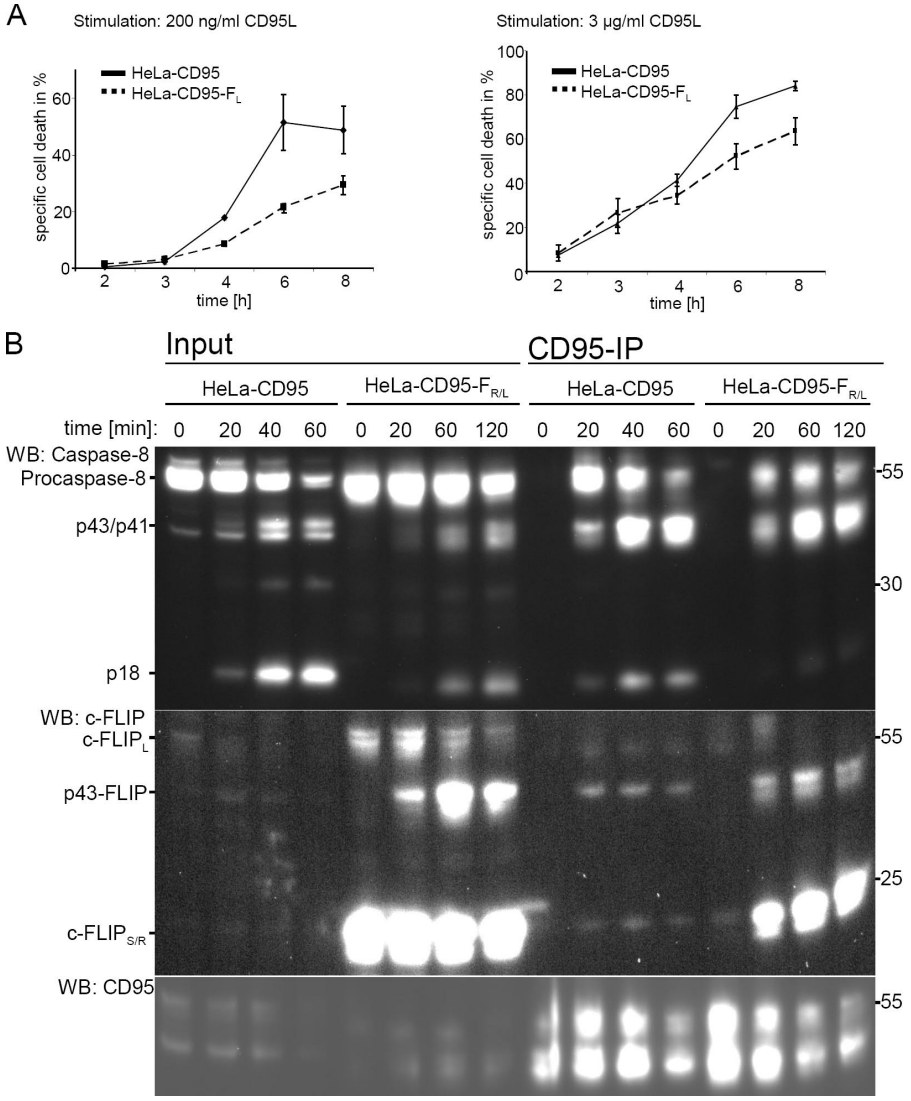

Figure S2. **Effect of c-FLIP$_L$ on procaspase-8 processing and cell death.** (A) HeLa-CD95 and HeLa-CD95–F$_L$ cells were stimulated with 200 ng/ml (left) or 3 µg/ml (right) CD95L. Cell death was measured with PI stain at various time points after stimulation. Mean and SEM of three independent experiments are shown. (B) HeLa-CD95 and HeLa-CD95–F$_{R/L}$ cells were stimulated with 3 µg/ml LZ-CD95L for the indicated time points. CD95 DISCs were immunoprecipitated (IP) using anti–APO-1 antibodies and analyzed along with total cellular lysates using Western blotting (WB) with antibodies against caspase-8 (C15), c-FLIP (NF6), and CD95 (C20). One representative experiment out of three is shown. White lines indicate that intervening lanes have been spliced out.

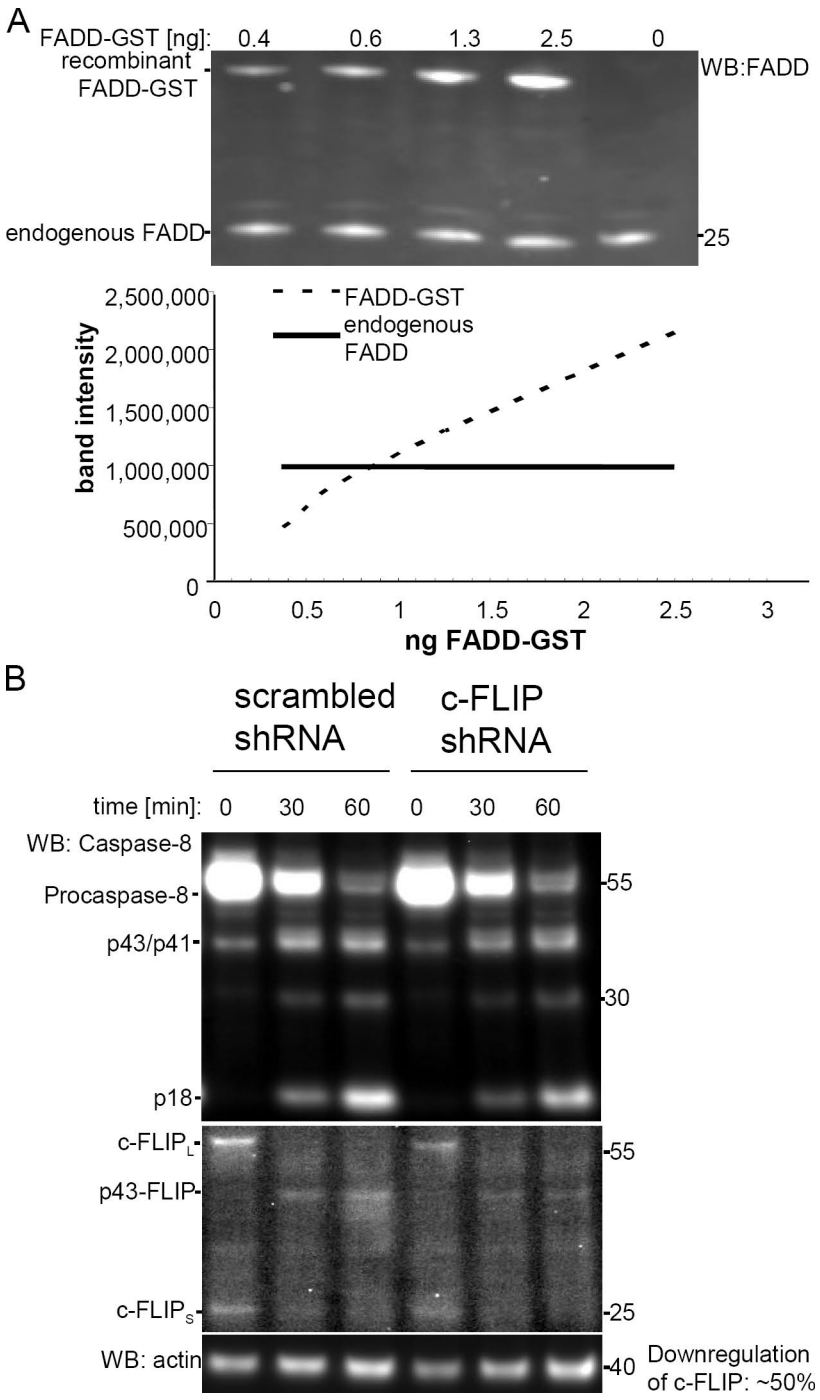

Figure S3. **Protein quantification with Western blots and the effect of c-FLIP down-regulation in HeLa-CD95 cells.** (A) Lysates from HeLa cells were loaded on a 4–12% Bis-Tris gel together with increasing amounts of recombinant FADD-GST (top). A Western blot (WB) against FADD (1C4 antibody) was performed, and intensities of the bands were measured. The amount of endogenous protein in comparison with recombinant protein was determined (bottom). (B) HeLa-CD95 cells were transfected with 1.5 µg pSilencer 3.1-H1 plasmid encoding c-FLIP shRNA or scrambled shRNA. 48 h after transfection, cells were stimulated with 3 µg/ml LZ-CD95L, and total cellular lysates were analyzed using Western blot with antibodies against caspase-8 (C15), c-FLIP (NF6), and actin. White lines indicate that intervening lanes have been spliced out.

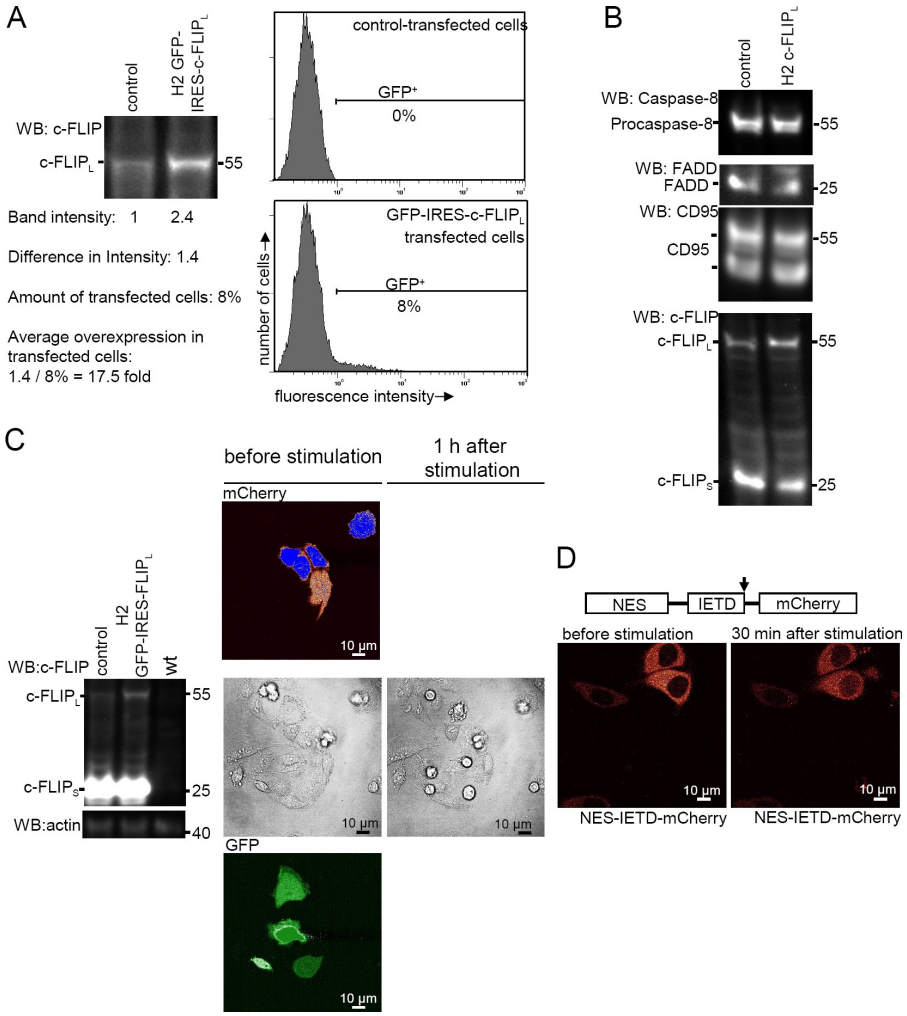

Figure S4. **Live cell imaging of HeLa-CD95 cells.** (A) Transfection with an H2 GFP-IRES–c-FLIP$_L$ plasmid leads to an ~20-fold c-FLIP$_L$ overexpression. HeLa-CD95 cells were transfected with 1.5 µg of an H2 GFP-IRES–c-FLIP$_L$ or empty control plasmid. The amount of c-FLIP$_L$ overexpression was estimated with quantitative Western blotting (WB). The percentage of transfected cells was determined by flow cytometry. (B) Transfection with an H2 GFP-IRES–c-FLIP$_L$ plasmid did not influence CD95, caspase-8, or FADD expression. HeLa-CD95 cells were transfected with 1.5 µg of an H2 GFP-IRES–c-FLIP$_L$ or empty control plasmid. Total cellular lysates were analyzed by Western blotting against CD95 (C20), caspase-8 (C15), c-FLIP (NF6), and FADD (1C4) 48 h after transfection. White lines indicate that intervening lanes have been spliced out. (C) Analysis of cell death with live cell imaging. HeLa cells were transfected with an H2 GFP-IRES–c-FLIP$_L$, CMV c-FLIP$_L$–IRES-GFP, or mCherry-encoding control plasmid. At the beginning of an experiment, fluorescence images were taken to determine transfected cells. GFP$^+$ and mCherry$^+$ cells were followed in time-lapse microscopy. Cell death of GFP$^+$ and mCherry$^+$ cells was measured with live cell imaging. (D) The NES-IETD-mCherry caspase-8 activity probe is shown. (top) Scheme of the caspase-8 activity probe. The probe is a fusion protein consisting of an NES followed by the caspase-8 cleavage sequence IETD fused to mCherry. The arrow indicates the caspase-8 cleavage site. (bottom) HeLa cells were transfected with an NES-IETD-mCherry caspase-8 activity probe. This probe resides in the cytoplasm of unstimulated cells. When cleaved by caspase-8, mCherry is free to translocate to the nucleus. mCherry translocation to the nucleus was measured with confocal time-lapse microscopy.

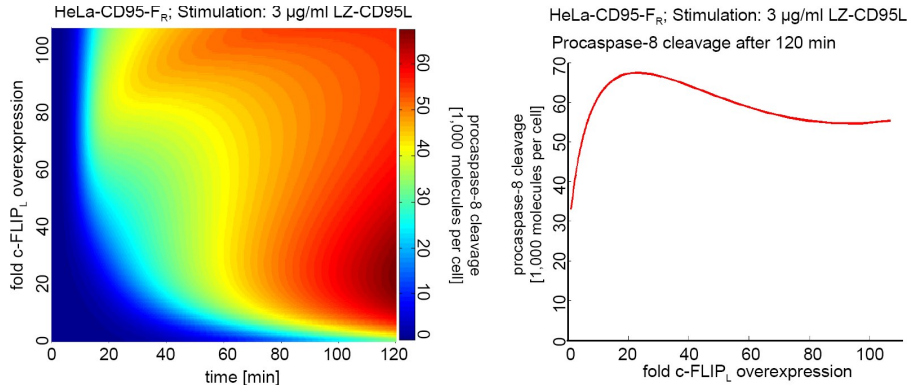

Figure S5. **The model predicts an increase in procaspase-8 processing upon c-FLIP$_L$ overexpression in HeLa-CD95–F$_R$ cells.** (left) Prediction of the amount of procaspase-8 processing depending on time and the amount of c-FLIP$_L$ at a stimulation with 3 µg/ml CD95L in HeLa-CD95–F$_R$ cells. The number of cleaved procaspase-8 molecules is given in thousands of molecules per cell. (right) Prediction of the amount of procaspase-8 processing dependent on the amount of c-FLIP$_L$ after 120 min of stimulation with 3 µg/ml CD95L in HeLa-CD95–F$_R$ cells.

Table S1. **Mean protein numbers per cell in HeLa-CD95 and c-FLIP–overexpressing HeLa-CD95 cells**

Cell type	FADD	c-FLIP$_{R/S}$	c-FLIP$_L$	Procaspase-8
HeLa-CD95	130,000 ± 14,000	530 ± 100	320 ± 80	250,000 ± 25,000
HeLa-CD95–F$_L$	130,000 ± 14,000	530 ± 100	32,000 ± 4,000	250,000 ± 25,000
HeLa-CD95–F$_R$	130,000 ± 14,000	90,000 ± 10,000	320 ± 80	250,000 ± 25,000

********** MODEL NAME
CD95 signaling model
********** MODEL NOTES
Author: Nicolai Fricker
********** MODEL STATES
d/dt(CD95L) = −RCD95LBindCD95R
d/dt(CD95R) = −RCD95LBindCD95R
d/dt(FADD) = −RFADDBindCD95RL
d/dt(C8) = −RC8BindCD95FADD + RFADDC8Dissociate
d/dt(FL) = −RFLBindCD95FADD
d/dt(FS) = −RFSBindCD95FADD + RFADDFSDissociate
d/dt(CD95RL) = RCD95LBindCD95R − RFADDBindCD95RL
d/dt(CD95FADD) = RFADDBindCD95RL − RC8BindCD95FADD − RFLBindCD95FADD − RFSBindCD95FADD + 2×(Rp43homodimerCleavep43homodimer + Rp43heterodimerCleavep43homodimer) + RFADDFSDissociate + RFADDC8Dissociate
d/dt(FADDC8) = RC8BindCD95FADD − 2×RFADDC8BindFADDC8 − RFADDFLBindFADDC8 − RFADDFSBindFADDC8 + 2×RC8homodimerDissociate + RC8FSdimerDissociate − RFADDC8Dissociate
d/dt(FADDFL) = RFLBindCD95FADD − RFADDFLBindFADDC8
d/dt(FADDFS) = RFSBindCD95FADD − RFADDFSBindFADDC8 + RC8FSdimerDissociate − RFADDFSDissociate
d/dt(C8heterodimer) = RFADDFLBindFADDC8 − RC8heterodimerCleaveC8heterodimer − RC8homodimerCleaveC8heterodimer − Rp43homodimerCleaveC8heterodimer − Rp43heterodimerCleaveC8heterodimer
d/dt(C8homodimer) = RFADDC8BindFADDC8 − RC8homodimerCleaveC8homodimer − RC8heterodimerCleaveC8homodimer − Rp43homodimerCleaveC8homodimer − Rp43heterodimerCleaveC8homodimer − RC8homodimerDissociate
d/dt(C8FSdimer) = RFADDFSBindFADDC8 − RC8FSdimerDissociate
d/dt(p43heterodimer) = RC8heterodimerCleaveC8heterodimer + RC8homodimerCleaveC8heterodimer + Rp43homodimer-CleaveC8heterodimer + Rp43heterodimerCleaveC8heterodimer
d/dt(p43homodimer) = RC8heterodimerCleaveC8homodimer + RC8homodimerCleaveC8homodimer + Rp43homodimer-CleaveC8homodimer + Rp43heterodimerCleaveC8homodimer − Rp43homodimerCleavep43homodimer − Rp43heterodimerCleavep43homodimer
d/dt(p18) = Rp43homodimerCleavep43homodimer + Rp43heterodimerCleavep43homodimer
d/dt(apoptosissubstrate) = −Rp43homodimerCleaveApoptosisSubstrate − Rp43heterodimerCleaveApoptosisSubstrate − Rp18CleaveApoptosisSubstrate
d/dt(cleavedsubstrate) = Rp43homodimerCleaveApoptosisSubstrate + Rp43heterodimerCleaveApoptosisSubstrate + Rp18CleaveApoptosisSubstrate
%% Protein amounts are given in thousand molecules per cell.
CD95L(0) = 1,500%% amount ligand
CD95R(0) = 170.999%% amount CD95
FADD(0) = 133.165%% amount FADD
C8(0) = 200.168%% amount Procaspase-8
FL(0) = 0.49995%% amount FLIP-Long
FS(0) = 0.422%% amount FLIP-Short
CD95RL(0) = 0%% amount of CD95–CD95L complexes
CD95FADD(0) = 0%% amount of CD95–FADD complexes
FADDC8(0) = 0%% amount Procaspase-8 bound to FADD
FADDFL(0) = 0%% amount c-FLIPL bound to FADD
FADDFS(0) = 0%% amount c-FLIPS bound to FADD
C8heterodimer(0) = 0%% amount Procaspase-8/c-FLIPL heterodimers
C8homodimer(0) = 0%% amount Procaspase-8 homodimers
C8FSdimer(0) =0%% amount Procaspase-8/c-FLIPS heterodimers
p43heterodimer(0) = 0%% amount p43/p41-Procaspase-8/p43-FLIP heterodimers
p43homodimer(0) = 0%% amount p43/p41-Procaspase-8 homodimers
p18(0)=0%% amount p18 formed
apoptosissubstrate(0)=100
cleavedsubstrate(0) = 0%% amount cleaved apoptosis substrate
********** MODEL PARAMETERS
********** MODEL VARIABLES
p18total = 2×p18

p43Casp8total = 2×p43homodimer + p43heterodimer
procaspase8total = C8 + FADDC8 + C8heterodimer + 2×C8homodimer + C8FSdimer
c8total = p43Casp8total + procaspase8total + 2×p18
cleavedC8 = c8total − procaspase8total
celldeath = cleavedsubstrate/0.10875%% Model readout: percentage of dead cells
********** MODEL REACTIONS
RCD95LBindCD95R = 7.0980e-002 × CD95L × CD95R
RFADDBindCD95RL = 0.0844211 × CD95RL × FADD
RC8BindCD95FADD = 0.00319838 × CD95FADD × C8
RFLBindCD95FADD = 0.0693329 × CD95FADD × FL
RFSBindCD95FADD = 0.0694022 × CD95FADD × FS
RFADDC8Dissociate = 0.1 × FADDC8
RFADDFSDissociate = 0.08 × FADDFS
RFADDC8BindFADDC8 = 1.18581 × FADDC8 × FADDC8
RFADDFLBindFADDC8 = 4.83692 × FADDC8 × FADDFL
RFADDFSBindFADDC8 = 2.88545 × FADDC8 × FADDFS
RC8FSdimerDissociate = 1 × C8FSdimer
RC8homodimerDissociate = 0.1 × C8homodimer
RC8homodimerCleaveC8homodimer = 0.000223046 × C8homodimer × C8homodimer
RC8homodimerCleaveC8heterodimer = 0.000223046 × C8homodimer × C8heterodimer
RC8heterodimerCleaveC8heterodimer = 0.000805817 × C8heterodimer × C8heterodimer
RC8heterodimerCleaveC8homodimer = 0.000805817 × C8heterodimer × C8homodimer
Rp43homodimerCleaveC8homodimer = 0.0014888 × p43homodimer × C8homodimer
Rp43homodimerCleaveC8heterodimer = 0.0014888 × p43homodimer × C8heterodimer
Rp43heterodimerCleaveC8homodimer = 0.013098 × p43heterodimer × C8homodimer
Rp43heterodimerCleaveC8heterodimer = 0.013098 × p43heterodimer × C8heterodimer
Rp43homodimerCleavep43homodimer = 0.000999273 × p43homodimer × p43homodimer
Rp43heterodimerCleavep43homodimer = 0.000982109 × p43heterodimer × p43homodimer
Rp43heterodimerCleaveApoptosisSubstrate = 1.66747e-005 × p43heterodimer × apoptosissubstrate
Rp43homodimerCleaveApoptosisSubstrate = 6.97394e-005 × p43homodimer × apoptosissubstrate
Rp18CleaveApoptosisSubstrate = 4.79214e-08 × p18 × apoptosissubstrate
********** MODEL FUNCTIONS