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## Supplemental information

# Characteristic ERK1/2 signaling dynamics distinguishes necroptosis from apoptosis

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## Supplemental material

## Figure S1

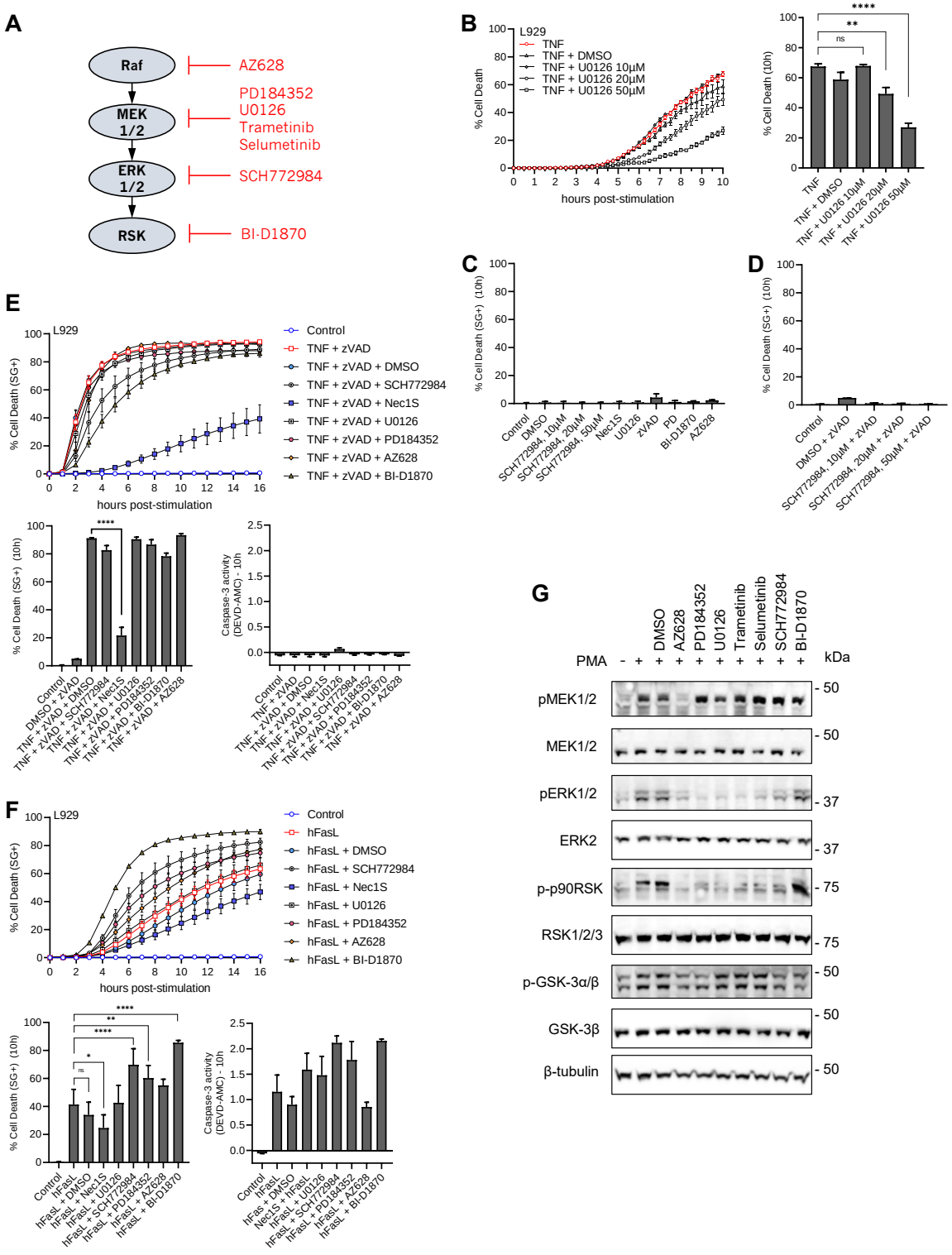


**Figure S1. Effect of MAPK/ERK1&2 signaling pathway inhibition in TNF-induced necroptosis and hFasL-induced apoptosis, related to Figure 1.**

(**A**) Schematic overview of MAPK/ERK1&2 signaling pathway and corresponding inhibitors used in this study. (**B**) L929 wildtype cells were pretreated for 1 hour with U0126 in a dose-dependent manner or DMSO as a control before TNF (red) treatment. Cell death was quantified by the number of propidium positive over the total number of cells, measured as a function of time by video-microscopy (B, left panel), and plotted at 10 hours after TNF stimulation (B, right panel). (**C** and **D**) Cytotoxicity measurements ($SG^+$ positivity) in L929 wildtype cells 10 hours in the control condition. (**E** and **F**) L929 wildtype cells were pretreated for 1 hour with the indicated compounds targeting the MAPK/ERK1&2 pathway or RIPK1 activity before TNF+zVAD (E) or hFasL (F) treatment. Cell death was measured as a function of time by SytoxGreen ($SG^+$) positivity (E-F, left graphs) and plotted at 10 hours after stimulation (E-F, middle graphs). Caspase-3 activity was assessed by DEVD-AMC cleavage efficiency (E-F, right graphs) 10 hours after stimulation. (**G**) L929 cells were stimulated or not with 500 nM PMA for 10 min and then treated for 10 min with the indicated MAPK/ERK pathway inhibitor or DMSO. Cells were then lysed and immunoblotted as indicated on the left of each blot. Data are presented as mean ± SEM of at least two independent experiments. Statistical significance was determined using two-way ANOVA followed by a Tukey *post hoc* test. Significance between samples is indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

**Figure S2**

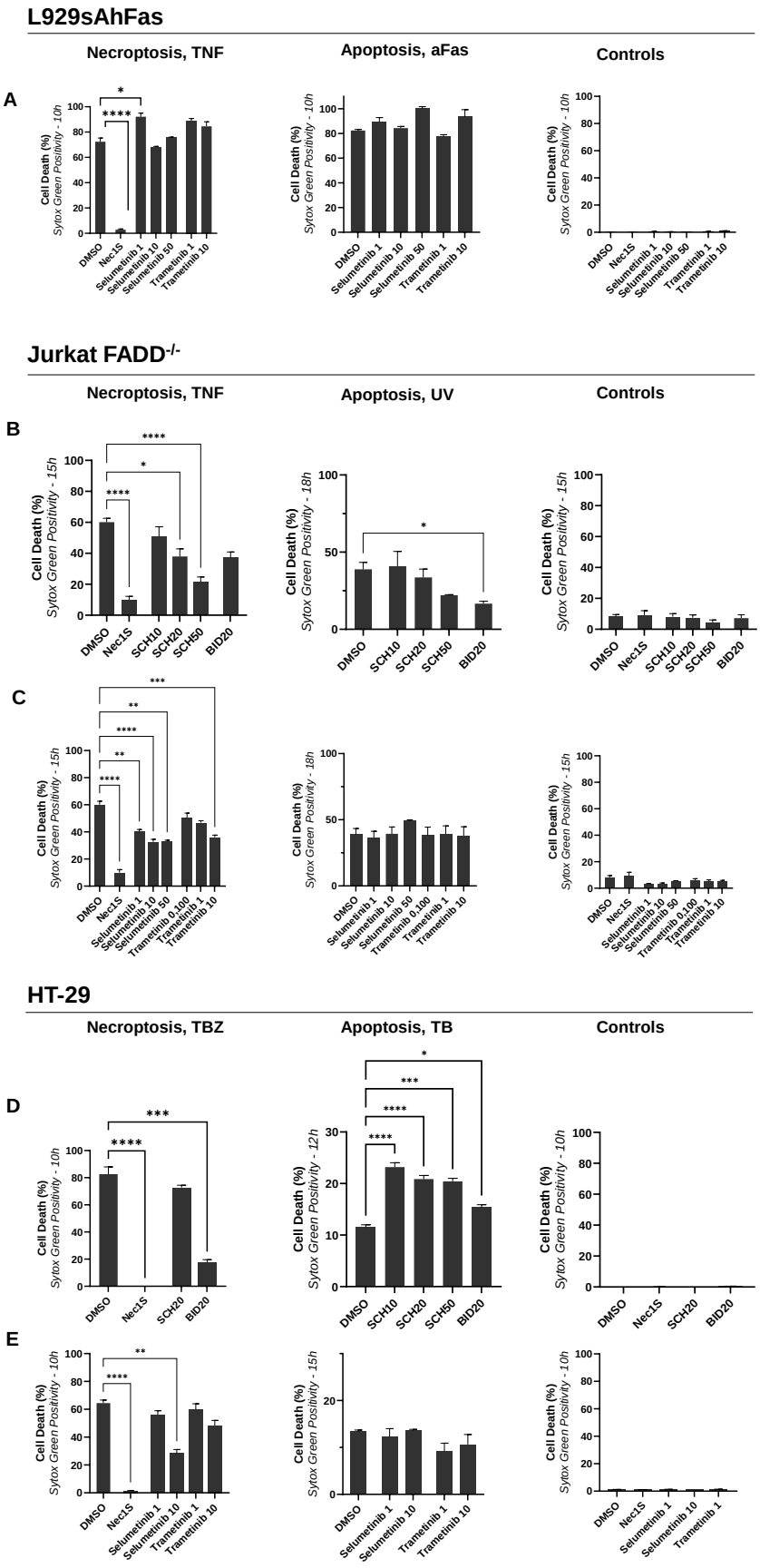

L929sAhFas
Necroptosis, TNF
Apoptosis, aFas
Controls
A
Cell Death (%)
Sytox Green Positivity - 10h
DMSO
Nec1S
Selumetinib 1
Selumetinib 10
Selumetinib 50
Trametinib 1
Trametinib 10
Jurkat FADD-/-
Necroptosis, TNF
Apoptosis, UV
Controls
B
Cell Death (%)
Sytox Green Positivity - 15h
Sytox Green Positivity - 18h
DMSO
Nec1S
SCH10
SCH20
SCH50
BID20
C
Cell Death (%)
Sytox Green Positivity - 15h
Sytox Green Positivity - 18h
DMSO
Nec1S
Selumetinib 1
Selumetinib 10
Selumetinib 50
Trametinib 0.100
Trametinib 1
Trametinib 10
HT-29
Necroptosis, TBZ
Apoptosis, TB
Controls
D
Cell Death (%)
Sytox Green Positivity - 10h
Sytox Green Positivity - 12h
DMSO
Nec1S
SCH20
BID20
SCH10
SCH50
E
Cell Death (%)
Sytox Green Positivity - 10h
Sytox Green Positivity - 15h
DMSO
Nec1S
Selumetinib 1
Selumetinib 10
Trametinib 1
Trametinib 10

**Figure S2. Effect of advanced MEK1/2 inhibitors, ERK1/2, and RSK inhibitors on necroptosis and apoptosis in different cellular models, related to Figure 1.**

(**A**) Dose-dependent response of L929 wildtype cells pretreated for 1 hour with advanced MEK1/2 inhibitors (Selumetinib and Trametinib) or Nec1S before stimulation. (**B-E**) Jurkat FADD-/- (B-C) and HT29 (D-E) were pretreated 1 hour with different concentrations of SCH772984 or with BI-D1870 at 20 µM (B and D) or with advanced MEK1/2 inhibitors (C and E) before stimulation. For each panel, cell death (necroptosis (left), apoptosis (center), or control (right)) was measured by SytoxGreen. TBZ: TNF+BV6+zVAD, TB: TNF+BV6. Data are presented as mean ± SEM of at least two independent experiments. Statistical significance was determined using two-way ANOVA followed by a Tukey *post hoc* test. Significance between samples is indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

**Figure S3**

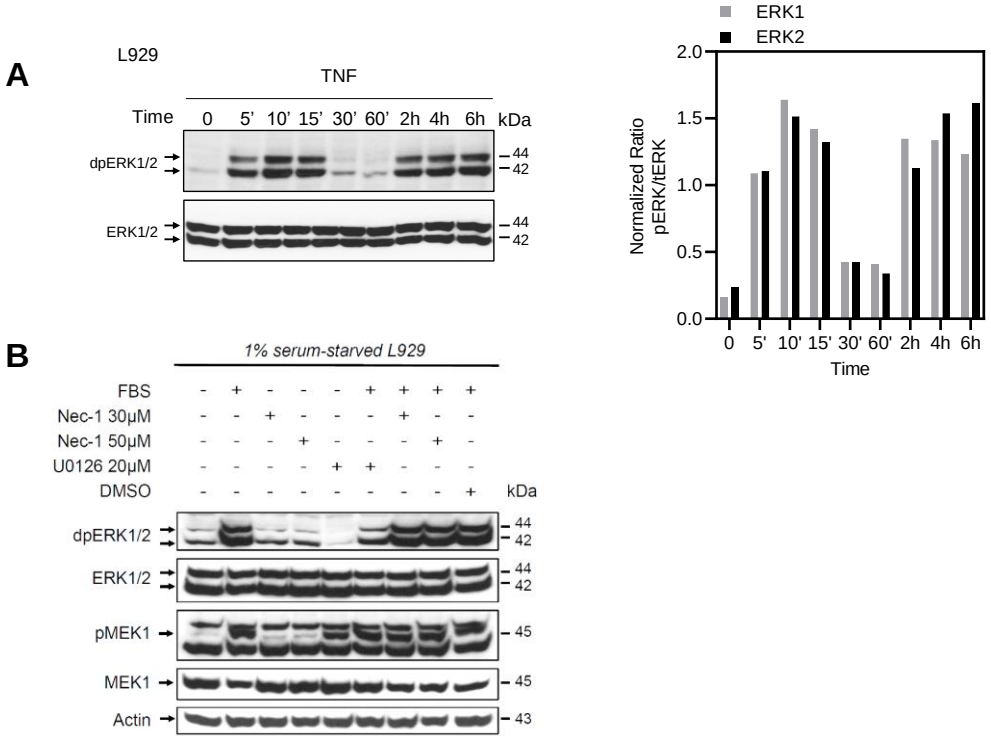

A
L929
TNF
Time
0 5' 10' 15' 30' 60' 2h 4h 6h
kDa
dpERK1/2
ERK1/2
44
42
B
1% serum-starved L929
FBS
Nec-1 30μM
Nec-1 50μM
U0126 20μM
DMSO
pMEK1
MEK1
Actin
45
43
ERK1
ERK2
Normalized Ratio
pERK/tERK
2.0
1.5
1.0
0.5
0.0
0 5' 10' 15' 30' 60' 2h 4h 6h
Time

**Figure S3. RIPK1 activity inhibition does not prevent serum-stimulated MEK1/2 and ERK1/2 activation, related to Figure 2.**

(**A**) L929 cells were serum-deprived (1% fetal calf serum: FCS) for 12 h and then stimulated with TNF for the indicated time points. Cells were then lysed and immunoblotted as indicated on the left of each blot. Histogram of densitometry analysis of phospho-ERK/total ERK1/2 level at each time point is presented both for ERK1 and ERK2 signal. (**B**) L929 cells were serum-deprived (1% FCS) for 12h and then pretreated for 30 min with either Nec1S at 30 μM and 50 μM or U0126 at 20 μM, or DMSO (volume equivalent to 50 μM treatment condition) before being growth factor-stimulated (10% FCS). Cells were then lysed and immunoblotted as indicated on the left of each blot.

**Figure S4**

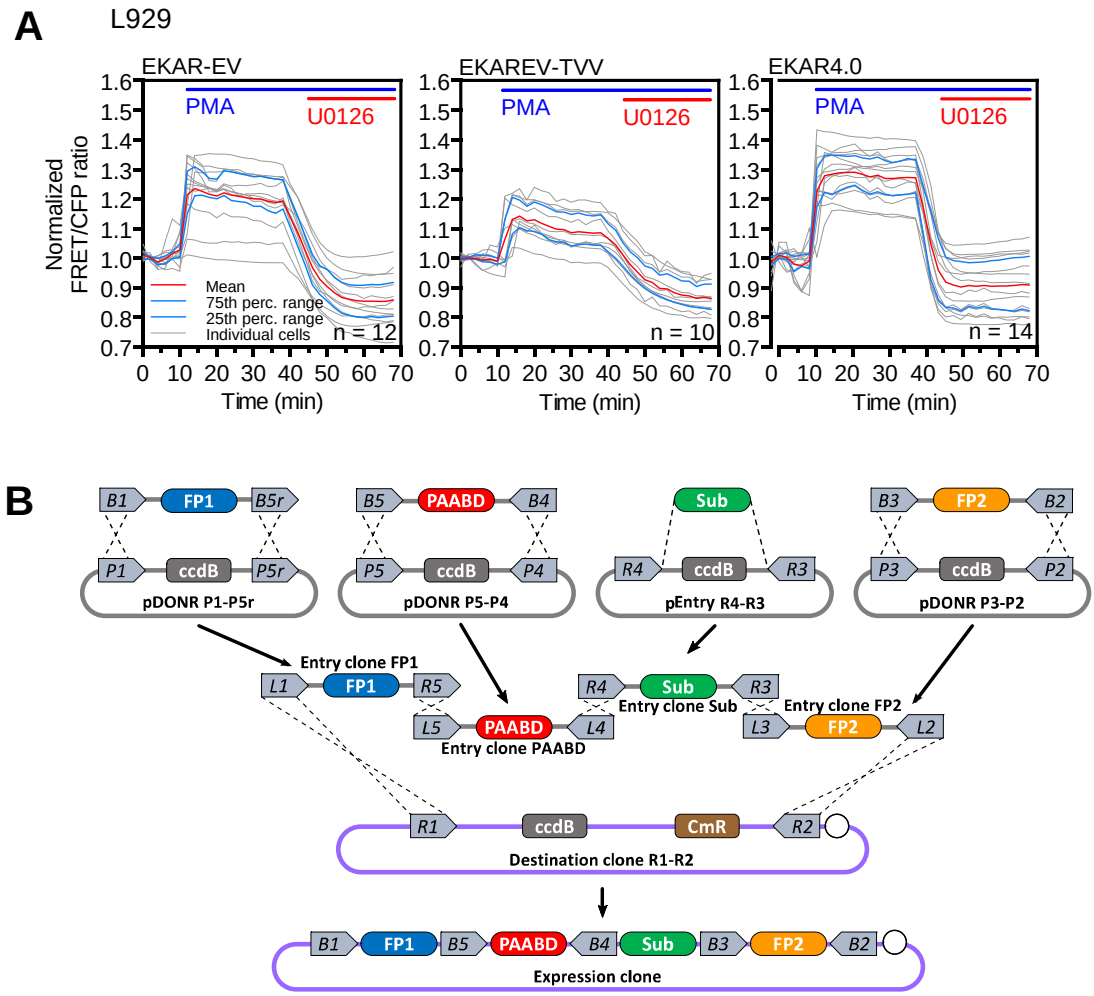

A
L929
EKAR-EV
EKAREV-TVV
EKAR4.0
PMA
U0126
Normalized FRET/CFP ratio
Mean
75th perc. range
25th perc. range
Individual cells
n = 12
n = 10
n = 14
Time (min)
B
B1
FP1
B5r
P1
ccdB
P5r
pDONR P1-P5r
B5
PAABD
B4
P5
P4
pDONR P5-P4
Sub
R4
R3
pEntry R4-R3
B3
FP2
B2
P3
P2
pDONR P3-P2
Entry clone FP1
L1
R5
Entry clone PAABD
L5
L4
Entry clone Sub
Entry clone FP2
L3
L2
R1
CmR
R2
Destination clone R1-R2
Expression clone

**Figure S4. Strategy for versatile generation of genetically encoded FRET biosensors using multisite Gateway cloning, related to Figure 3.**

(**A**) Biosensor reference experiments: L929 cells expressing EKAR-EV (left), EKAR-EV-TVV (middle), and EKAR4.0 (right) were time lapse-imaged by FRET microscopy and treated with PMA (0,5 µM) at t= 10 min and then with U0126 (20 µM) at t= 40 min. Graphs present FRET/CFP ratio values of every single cell normalized to the averaged baseline FRET/CFP ratio value throughout reference experiments. FRET biosensor data are displayed in two ways: all individual cell responses (grey curves), the population mean in red, and the $25^{th}$ and $75^{th}$ percentile values in blue. Cells were imaged at the rate of 1 acquisition every 2 minutes for a total duration of 70 minutes. Cell number (n) is reported on each graph. (**B**) Schematic representation of the cloning strategy based on multisite gateway for the generation of entry vectors of the library containing one functional element of a FRET biosensor (Nt or Ct terminally tagging fluorescent protein (FP1/FP2), phospho-amino acid-binding domain (PAABD), and a specific substrate for the kinase of interest (Sub), and the multisite Gateway recombination att sites. The insertion of *att*B1, *att*B5, *att*B4, *att*B3, and *att*B2 recombination sites connects each functional domain of the resulting FRET biosensor acting as linkers. The detailed cloning strategy can be found in the *Materials & Methods* section.

**Figure S5**

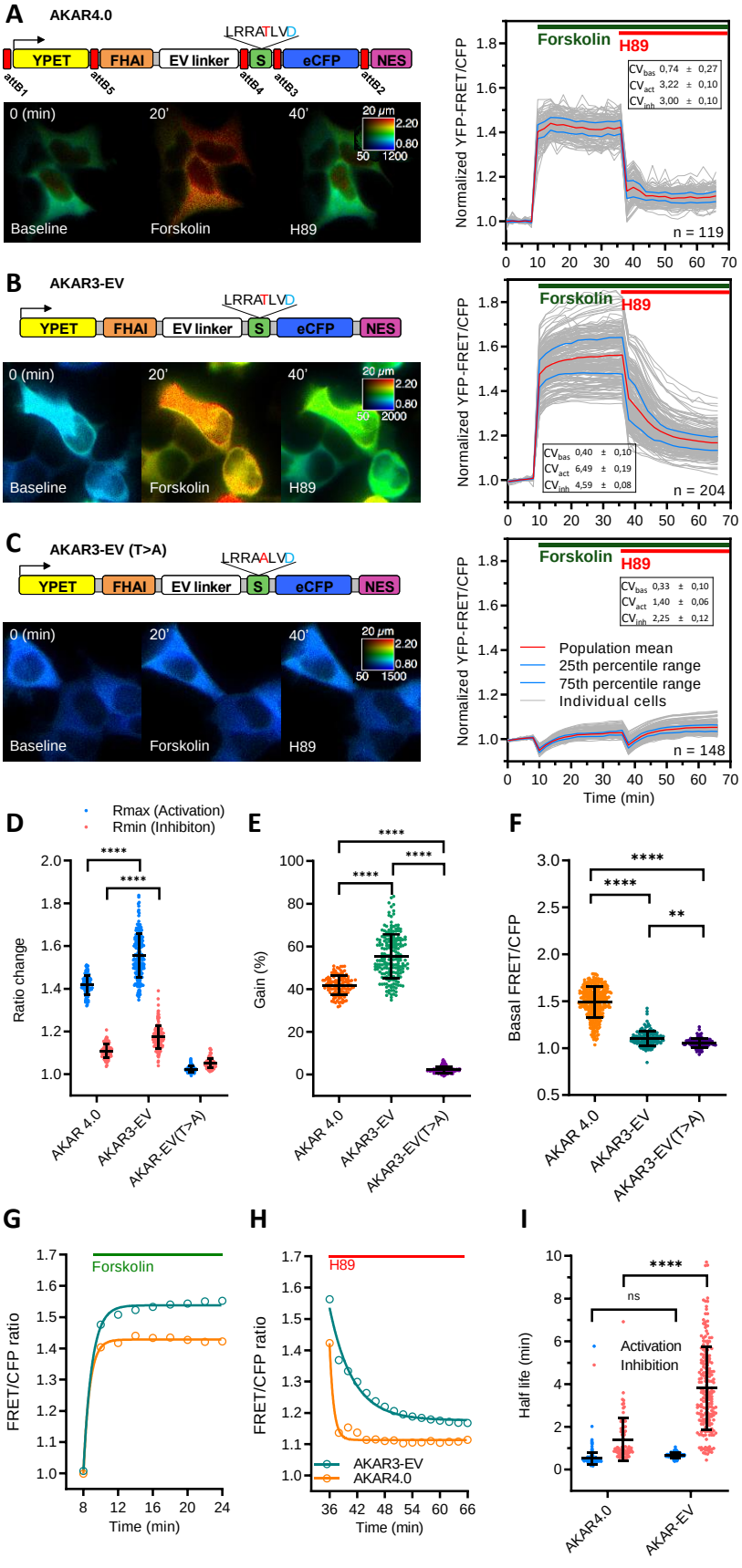
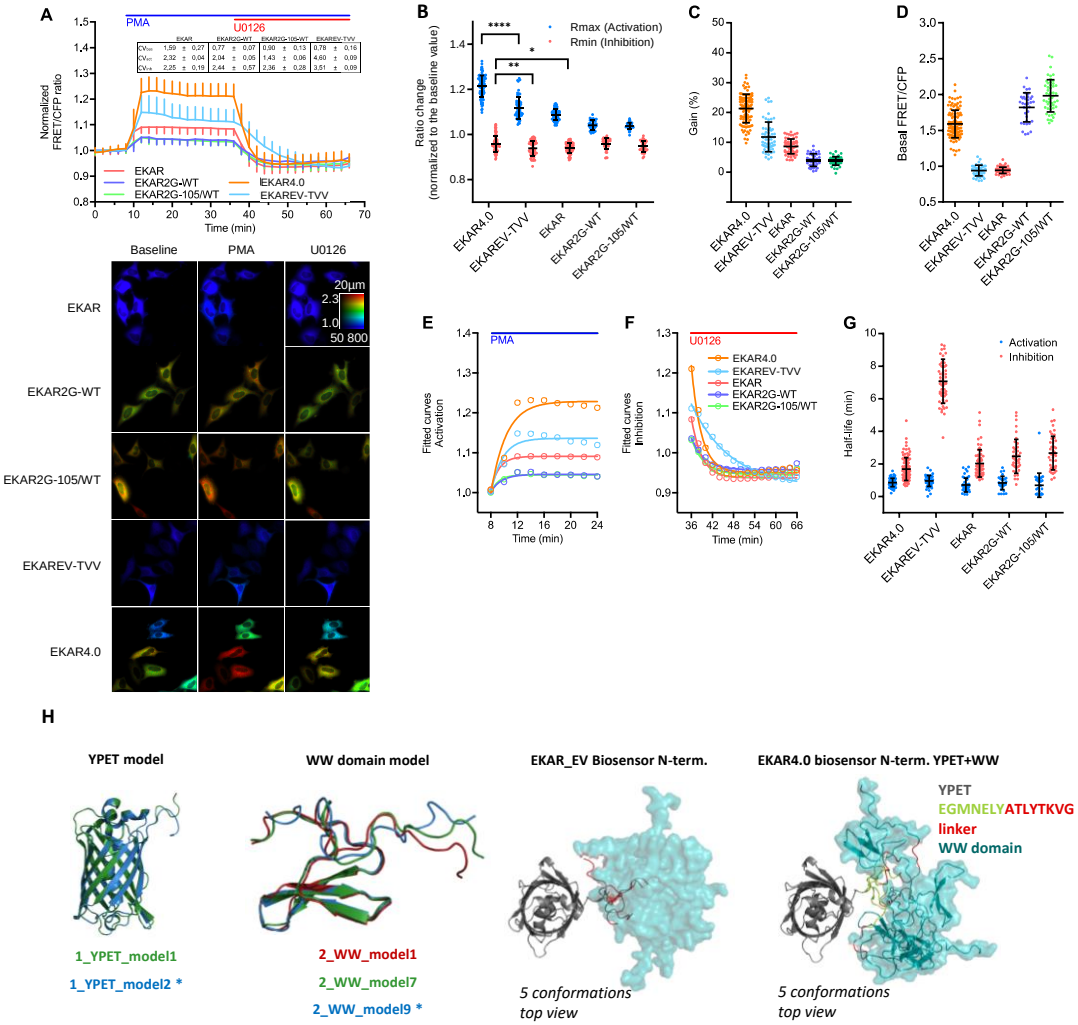

A
AKAR4.0
LRRATLVD
YPET
FHAI
EV linker
S
eCFP
NES
attB1
attB5
attB4
attB3
attB2
0 (min)
20'
40'
20 µm
2.20
0.80
50 1200
Baseline
Forskolin
H89
Forskolin
H89
Normalized YFP-FRET/CFP
n = 119
B
AKAR3-EV
50 2000
n = 204
C
AKAR3-EV (T>A)
LRRAALVD
50 1500
n = 148
Population mean
25th percentile range
75th percentile range
Individual cells
Time (min)
D
Rmax (Activation)
Rmin (Inhibiton)
Ratio change
AKAR 4.0
AKAR3-EV
AKAR-EV(T>A)
E
Gain (%)
F
Basal FRET/CFP
G
FRET/CFP ratio
H
AKAR3-EV
AKAR4.0
I
Half life (min)
Activation
Inhibition
ns
AKAR-EV

**Figure S5. Multisite Gateway cloning improves the reversibility of AKAR3-EV, related to Figure 3.**

(**A**-**C**) Biosensor reference experiments: HEK cells expressing AKAR3-EV GW4.0 (A), AKAR3-EV (B) or its mutant version, AKAR3-EV (T>A) (C) were time lapse-imaged by FRET microscopy and treated with Forskolin (12.5 µM) at t= 10min and then with H89 (10 µM) at t= 34 min. Top of each panel: Schematic representation of PKA Kinase Activity Reporter (AKAR) construct used. The small red rectangles in (A) map the positions of obligated *attB* linkers between each FRET biosensor domain. For the substrate peptide sequence, red letters indicate the phosphorylation site, and blue letters indicate amino-acid substitutions to increase the affinity for the WW domain, Bottom left of each panel: Pseudo-color FRET/CFP ratio images of PKA activity at the steady-state (t= 0 min), upon Forskolin (t= 20 min) and H89 treatment (t= 40 min). Right of each panel: Graph presenting the FRET/CFP ratio values of every single cell normalized to the averaged baseline FRET/CFP ratio value throughout reference experiments. All individual cells (grey curves), as well as the mean (red curve) and the 25th and 75th percentile range (blue curves) from at least 100 cells, are plotted as a function of time. $CV_{bas}$, $CV_{act}$, and $CV_{inh}$ indicate the coefficient of variation for cells at the baseline, cells in PKA activated state, and cells in the PKA inhibited state, respectively. Imaging parameters were kept identical for the different biosensors tested. Cells were imaged for a total duration of 70 minutes at the rate of 1 acquisition every 2 minutes. (**D-I**) Characterization and comparison of different AKAR FRET biosensors responses were performed based on the following parameters: ratio changes (Rmin, Rmax) (D), gain (E), basal FRET /CFP value without any stimulation (F), and fitted curves to determine the speed of activation upon PKA activation (G, I) and the reversibility of the biosensor upon PKA inhibition (H, I). Fitted curves were plotted based on Tau ½, which is the time value representing half of the measured duration of the inhibition or activation phase. Tau ½ was calculated by applying a non-linear regression curve on ration values over the complete set of individual cells during the activation or inhibition phase. Nonlinear regression was calculated between t= 8 min and t= 24 min for the activation, and between t= 36 min and t= 66 min for the inhibition, and plotted as a fitted curved. All calculations were performed using GraphPad PRISM 8. Data are mean ± SD from at least two independent experiments. Scale bar: 10 µm. The number of cells analyzed is indicated by n (A-C). Statistical significance was determined using two-way ANOVA followed by a Tukey *post hoc* test. Significance between samples is indicated as follows: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

## Figure S6


A
PMA
U0126
Normalized FRET/CFP ratio
Time (min)
EKAR
EKAR2G-WT
EKAR2G-105/WT
EKAR4.0
EKAREV-TVV
Baseline
PMA
U0126
20µm
2.3
1.0
50 800
B
Ratio change (normalized to the baseline value)
Rmax (Activation)
Rmin (Inhibition)
****
*
**
C
Gain (%)
D
Basal FRET/CFP
E
Fitted curves Activation
F
Fitted curves Inhibition
G
Half-life (min)
Activation
Inhibition


H
YPET model
WW domain model
EKAR_EV Biosensor N-term.
EKAR4.0 biosensor N-term. YPET+WW
1_YPET_model1
1_YPET_model2 *
2_WW_model1
2_WW_model7
2_WW_model9 *
5 conformations
top view
5 conformations
top view
YPET
EGMNELYATLYTKVG
linker
WW domain

**Figure S6. Comparison of EKAR4.0 to previous EKAR biosensors, related to Figure 3.**

(**A**) Biosensor reference experiments: HeLa cells expressing EKAR, EKAR-EV, EKAR-EV-TVV, EKAR2G-WT, and EKAR2G-105/WT were time lapse-imaged by FRET microscopy and treated with PMA (0.5 µM) at t= 10 min and then with U0126 (20µM) at t= 34 min. Upper panel: graph presenting the FRET/CFP ratio values of every single cell normalized to the averaged baseline FRET/CFP ratio value throughout reference experiments. Data are presented as mean ± SD from at least two independent experiments, and at least 40 cells are plotted as a function of time. $CV_{bas}$, $CV_{act}$, and $CV_{inh}$ indicate the coefficient of variation for cells at the baseline, cells in ERK1/2 activated state, and cells in the ERK1/2 inhibited state, respectively. Imaging parameters were kept identical for the different biosensors tested. Cells were imaged for a total duration of 70 minutes at the rate of 1 acquisition every 2 minutes. Pseudo-color FRET/CFP ratio images of ERK1/2 activity at the steady-state (t= 0 min), upon PMA addition (t= 20 min) and U0126 addition (t= 40 min) are shown. Scale bar: 20 µm. (**B-G**) Characterization and comparison of all ERK1/2 biosensors responses were performed based on the following parameters: ratio changes (Rmin, Rmax) (B), gain (C), basal FRET /CFP value without any stimulation (D), and fitted curves to determine the speed of activation upon ERK1/2 activation (E and G) and the reversibility of the biosensor upon MEK1/2 inhibition (F and G). Fitted curves were plotted based on Tau ½, which is the time value representing half of the measured duration of the inhibition or activation phase. Tau ½ was calculated by applying a non-linear regression curve on ration values over the complete set of individual cells during the activation or inhibition phase. The nonlinear regression was calculated between t= 8 min and t= 24 min for the activation and between t= 36 min and t= 66 min for the inhibition and plotted as fitted curves. All calculations were performed using GraphPad PRISM 8. (**H**) Predicted 3D models of YPET, WW domain, and N-terminal of ERK1/2 biosensors with (EKAR4.0) and without (EKAR_EV) *att* linkers. Green letters correspond to residues from *att* linker within the Eevee linker in red letters. The sampling of their conformational spaces is indicated below each 3D model.

## Figure S7

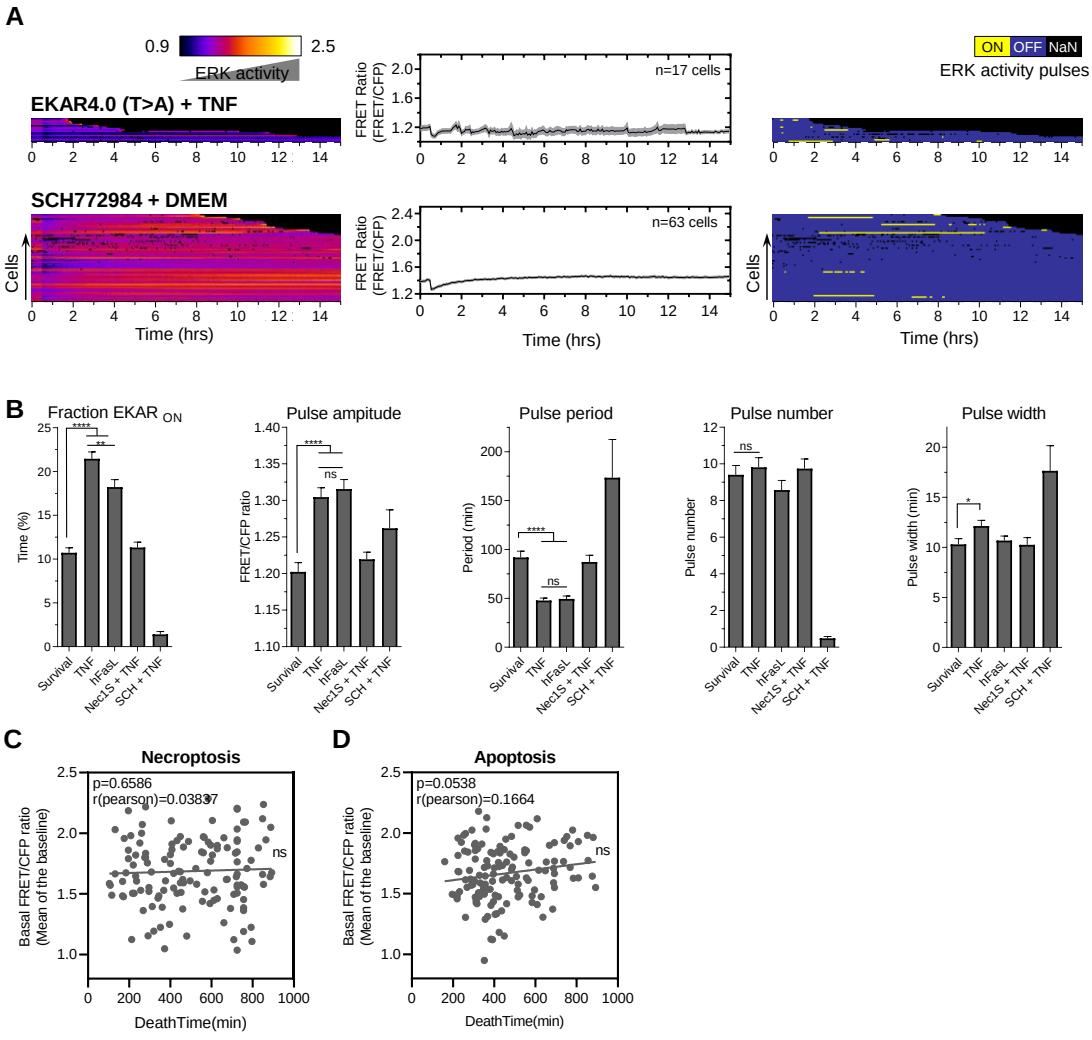

A
0.9
2.5
ERK activity
EKAR4.0 (T>A) + TNF
SCH772984 + DMEM
FRET Ratio (FRET/CFP)
n=17 cells
n=63 cells
ON
OFF
NaN
ERK activity pulses
Cells
Time (hrs)
B
Fraction EKAR ON
Time (%)
Pulse ampitude
FRET/CFP ratio
Pulse period
Period (min)
Pulse number
Pulse width
Pulse width (min)
Survival
TNF
hFasL
Nec1S + TNF
SCH + TNF
C
Necroptosis
p=0.6586
r(pearson)=0.03837
Basal FRET/CFP ratio (Mean of the baseline)
DeathTime(min)
D
Apoptosis
p=0.0538
r(pearson)=0.1664

**Figure S7. Basal ERK1/2 activity does not correlate with cellular death time, related to Figure 4 & 5.**

(**A**) Biosensing experiment: L929 expressing the negative control FRET biosensor EKAR-EV-TA GW4.0 (upper panel) or EKAR4.0 (bottom panel) were treated with TNF or DMEM as internal controls, respectively. From left to right, results are displayed as follows: FRET/CFP ratio of every single cell displayed by a FRET/CFP ratio kymographic representation (left), averaged FRET/CFP ratio (mean±SEM) as a function of time (middle), the corresponding pattern of ERK1/2 pulse dynamics displayed by a binary kymographic representation of $ERK_{ON}$ and $ERK_{OFF}$ states (right). Experimental and analysis protocols, as well as graphical representations, are the same as in Fig 4 and 5. (**B**) Global quantitative analysis of EKAR4.0 pulse dynamics from data presented in Fig 5 were calculated from 1h after the start of imaging until the end of the experiment, for each cell and each condition described in Fig 4. This global data analysis is presented as histograms. It corresponds to the mean±SEM of at least two independent experiments for the following parameters, from left to right: $EKAR_{ON}$ state (calculated for each cell, normalized to the cell life duration of each cell, and expressed in percentage of time of the cell lifespan), pulse amplitude, pulse period (min), pulse number measured during the entire experiment, pulse width (min). Statistical significance was determined using two-way ANOVA followed by a Tukey *post hoc* test. Significance between samples is indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; *ns*, not significant. (**C-D**) From experimental data presented in Fig 4, a scatterplot of single-cell measurements of basal ERK1/2 activity plotted against the time of cell death induced by TNF (C) or hFasL (D) stimulation. The dark line indicates a linear fit, and the corresponding p values are indicated. A Pearson's correlation was performed to assess the linear relationship between variables.

**Figure S8**

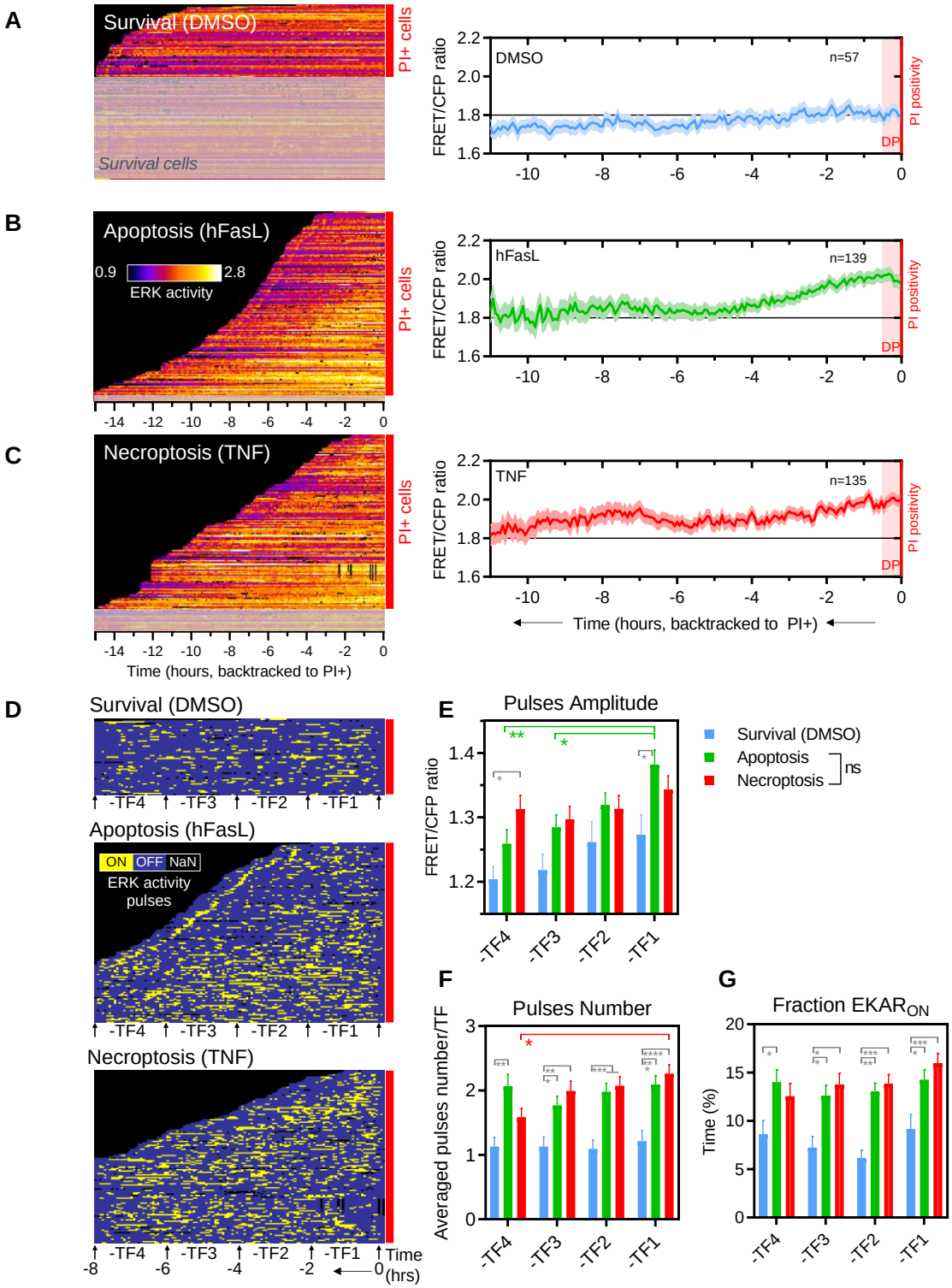

A
Survival (DMSO)
PI+ cells
Survival cells
DMSO
n=57
FRET/CFP ratio
PI positivity
DP
B
Apoptosis (hFasL)
0.9
2.8
ERK activity
hFasL
n=139
C
Necroptosis (TNF)
TNF
n=135
Time (hours, backtracked to PI+)
D
Survival (DMSO)
-TF4
-TF3
-TF2
-TF1
Apoptosis (hFasL)
ON
OFF
NaN
ERK activity pulses
Necroptosis (TNF)
Time (hrs)
E
Pulses Amplitude
Survival (DMSO)
Apoptosis
Necroptosis
ns
F
Pulses Number
Averaged pulses number/TF
G
Fraction EKAR$_{ON}$
Time (%)

**Figure S8. Time-resolved analysis of ERK1/2 activity dynamics preceding death events distinguishes necroptosis from apoptosis, related to Figure 4 & 5.**

(**A-C**) From experimental data presented in Fig 4, dead cells identified using propidium iodide staining were systematically backtracked in three cellular contexts (survival (A), apoptosis (B), necroptosis (C)). The FRET/CFP ratio is displayed using a kymographic representation (left panels) and is then averaged (mean ± SEM) and plotted as a function of time, starting at the moment of cell death (red lines, PI+) (right panels). The light red area preceding cell death (30 minutes) corresponds to the disintegration phase (DP). The number of cells analyzed is reported on each graph (n). (**D**) The associated binary kymographs for ERK1/2 pulses detection are shown for each experimental condition. (**E-F**) Time-resolved quantitative analysis of EKAR4.0 signal in single cells was performed. Data are presented as histograms of ERK1/2 pulses amplitude (E) and ERK1/2 pulses number (F), when stimulated or not (control) with hFasL (apoptosis) or TNF (necroptosis). These parameters were calculated just after the time window corresponding to the DP (30 min) with a 2-hours-backtracked time interval for each cell and each condition (TF: Temporal frame). Statistical significance was determined using two-way ANOVA followed by a Tukey post hoc test. Significance between samples is indicated as follows: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; *ns, not significant.*

**Figure S9**

**A**

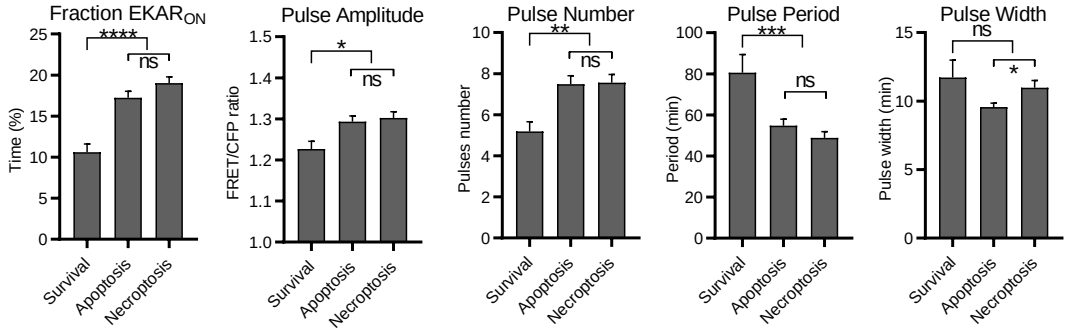

Fraction EKAR$_{ON}$
Time (%)
****
ns
Pulse Amplitude
FRET/CFP ratio
*
ns
Pulse Number
Pulses number
**
ns
Pulse Period
Period (min)
***
ns
Pulse Width
Pulse width (min)
ns
*
Survival
Apoptosis
Necroptosis

**Figure S9. Metrics from global analysis of ERK1/2 activity dynamics preceding death events, related to Figure 4 & 5.**

(**A**) Similarly to Fig S7B, global quantitative analysis of EKAR4.0 signal from data presented in Fig S8D was calculated just after the time window corresponding to the DP (30 minutes before PI positivity) until the end of TF4 (corresponding to 8 hours backtracked time interval), for each cell and each condition.

## Figure S10

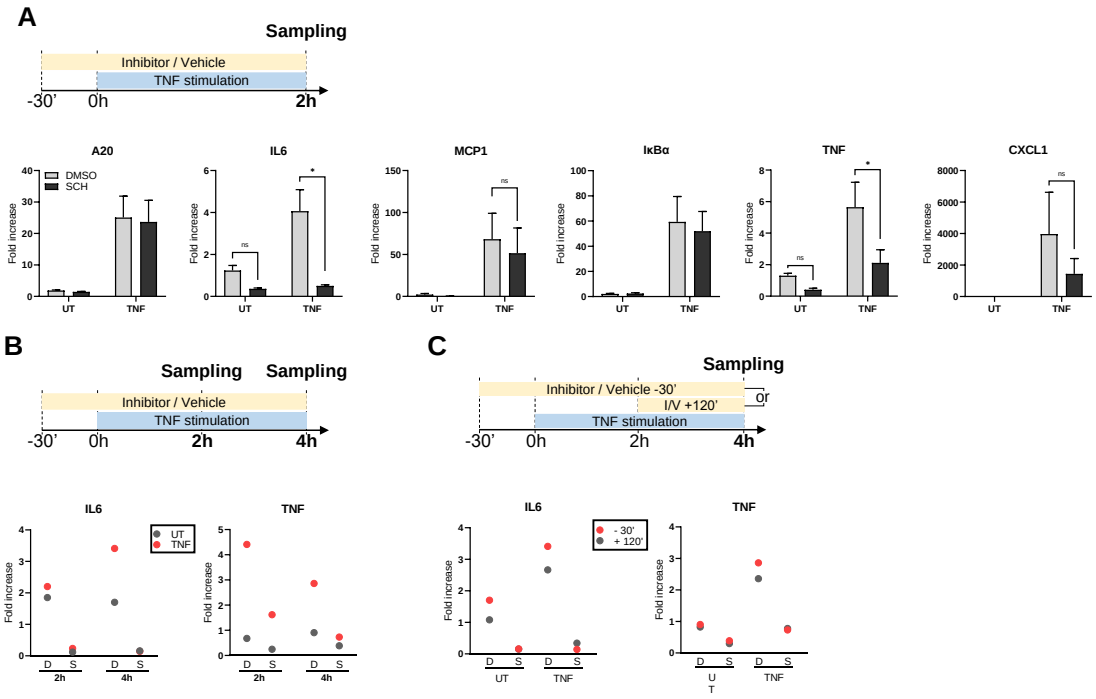

A
Sampling
Inhibitor / Vehicle
TNF stimulation
-30'
0h
2h
A20
IL6
MCP1
IκBα
TNF
CXCL1
DMSO
SCH
Fold increase
UT
TNF
ns
*
B
Sampling
Sampling
Inhibitor / Vehicle
TNF stimulation
-30'
0h
2h
4h
IL6
TNF
UT
TNF
D
S
2h
4h
C
Sampling
Inhibitor / Vehicle -30'
I/V +120'
or
TNF stimulation
-30'
0h
2h
4h
IL6
TNF
- 30'
+ 120'
UT
TNF

**Figure S10. Direct ERK1/2 inhibition before or after TNF treatment impairs inflammatory genes expression profiles in L929 during necroptosis, related to Figure 4, 5 & 6.**

(**A-B**) L929 cells were pretreated with 10 µM SCH772984 or vehicle control for 30 minutes and then stimulated or not with TNF for 2 hours (A) or 2 and 4 hours (B). Cells were then harvested and processed for RT-qPCR reactions. Key cytokines, chemokines gene products were tested at 2 hours post-induction. Only the ones showing differences at this time point were further studied at two different time points (2 and 4 hours post-stimulation). (**C**) L929 cells were treated with TNF for 4 hours. ERK1/2 inhibitor SCH772984 (10 µM) or vehicle control was added 30 min before or 2 hours after TNF stimulation. All data are presented as mean±SEM of two independent experiments (A) and a single experiment (B and C). Statistical significance was determined using an unpaired t-test between DMSO and SCH. Significance between samples is indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; *ns*, not significant. SCH, SCH772984, UT, untreated,

## Figure S11

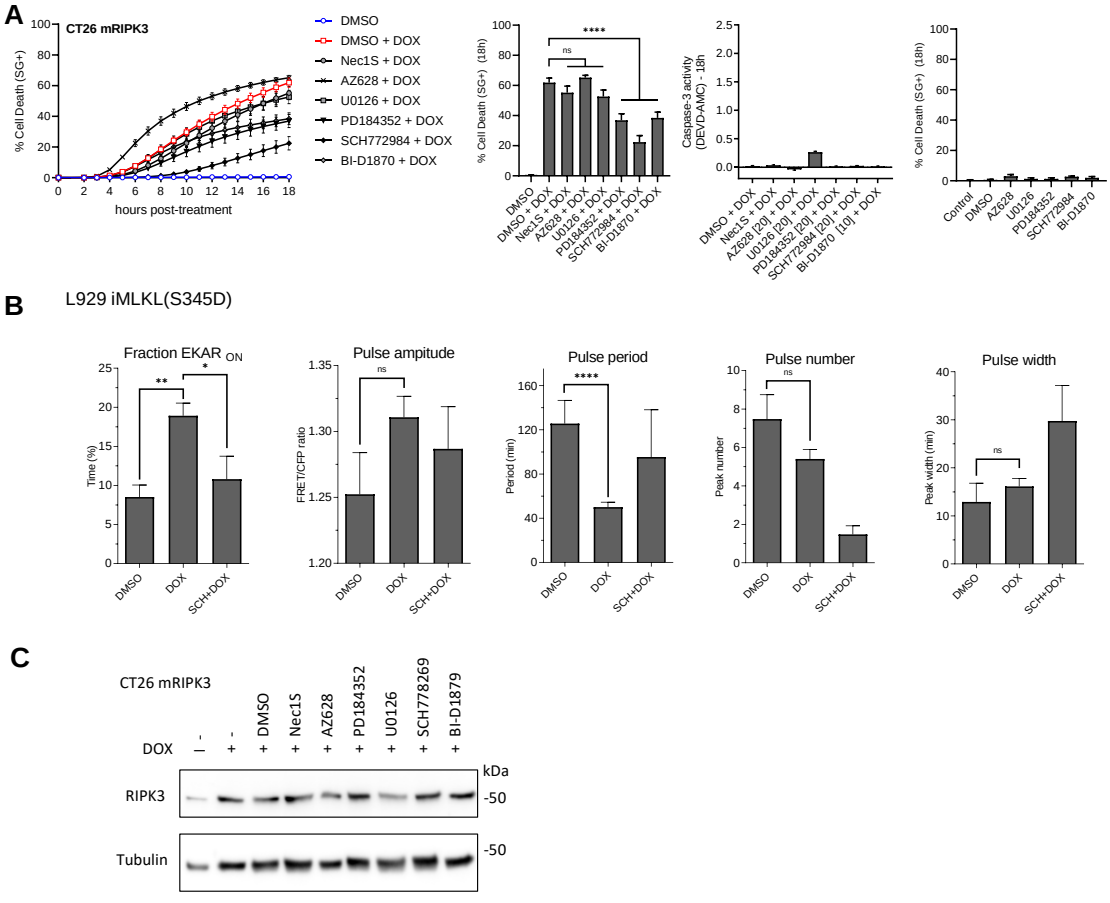

A
CT26 mRIPK3
% Cell Death (SG+)
hours post-treatment
DMSO
DMSO + DOX
Nec1S + DOX
AZ628 + DOX
U0126 + DOX
PD184352 + DOX
SCH772984 + DOX
BI-D1870 + DOX
% Cell Death (SG+) (18h)
Caspase-3 activity (DEVD-AMC) - 18h
B
L929 iMLKL(S345D)
Fraction EKAR ON
Time (%)
Pulse amplitude
FRET/CFP ratio
Pulse period
Period (min)
Pulse number
Peak number
Pulse width
Peak width (min)
DMSO
DOX
SCH+DOX
C
CT26 mRIPK3
DOX
DMSO
Nec1S
AZ628
PD184352
U0126
SCH778269
BI-D1879
RIPK3
Tubulin
kDa
-50

**Figure S11. Inhibition of the MAPK/ERK1&2 pathway downstream of MEK1/2 efficiently impairs necroptosis induced by RIPK3 overexpression, related to Figure 7.**

(**A**) RIPK3-deficient CT26 cells reconstituted with inducible wildtype RIPK3 were pretreated for 1 hour with the indicated compounds before Dox (red) or DMEM (blue) treatment (grey curves). Cell death was measured as a function of time by SytoxGreen ($SG^+$) positivity (kinetic experiment, left), 18 hours after Dox stimulation (A, left histogram), and caspase-3 activity was assessed by DEVD-AMC cleavage efficiency (A, middle histogram) 18 hours after Dox stimulation. Drugs-induced toxicity measurements ($SG^+$ positivity) 18 hours after DMEM stimulation (A, right histogram). All cell death data are presented as mean±SEM of at least four independent experiments. Statistical significance was determined using two-way ANOVA followed by a Tukey *post hoc* test. Significance between samples is indicated as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; *ns*, not significant. (**B**) Global quantitative analysis of EKAR4.0 pulse dynamics in L929 iMLKL (S345D) from Figure 7 were calculated from 1h after the start of imaging until the rest of the experiment for each cell. This global data analysis is presented as histograms like in Figure S6B and corresponds to the mean±SEM of at least two independent experiments. Statistical significance was determined using two-way ANOVA followed by a Tukey *post hoc* test. Significance between samples is indicated as follows:$^{*}p< 0.05$; $^{**}p< 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; *ns*, not significant. (**C**) CT26 mRIPK3 cells were pretreated or not for 1 hour with the indicated compounds and subsequently stimulated with Dox. Cells were then lysed and immunoblotted as indicated on the left of each blot. Corresponding molecular weights are reported on the right of each blot.

**Table S1. Protein sequences comparison between EKAR-EV and EKAR4.0, related to Figure 3.**

| **EKAR-EV** |
| --- |
| YPET |
| MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLLCTTGKLPVPWPTLVTTLGYGLQCFARYPDHMKQHDFFKSA<br>MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGG<br>VQLADHYQQNTPIGDGPVLLPDNHYLSYQSALFKDPNEKRDHMVLLEFLTAAGITEGMNELY |
| Linker |
| LE |
| WW Domain |
| MADEEKLPPGWEKRMSRSSGRVYYFNHITNASQWERPSGNSSSGGKNGQGEPAR |
| EV linker |
| GTSAGGSAGGSAGGSAGGSAGGSGSAGGSAGGSTSAGGSAGGSAGGSAGGSAGGSGSAGGSAGGSTSAGGSAGGSAGGSAGGSAGGSG<br>SAGGSAGGSTSAGGSAGGSAGGSAGGSAGGSG |
| Substrate + Docking site |
| PDVPRTPVDKAKLSFQFP |
| Linker |
| GGR |
| eCFP |
| MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTWGVQCFSRYPDHMKQHDFFKSA<br>MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGS<br>VQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK |
| **EKAR4.0** |
| YPET |
| MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLLCTTGKLPVPWPTLVTTLGYGLQCFARYPDHMKQHDFFKSA<br>MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYITADKQKNGIKANFKIRHNIEDGG<br>VQLADHYQQNTPIGDGPVLLPDNHYLSYQSALFKDPNEKRDHMVLLEFLTAAGITEGMNELY |
| Linker |
| ATLYTKVG |
| WW Domain |
| MADEEKLPPGWEKRMSRSSGRVYYFNHITNASQWERPSGNSSSGGKNGQGEPAR |
| EV linker |
| GTSAGGSAGGSAGGSAGGSAGGSGSAGGSAGGSTSAGGSAGGSAGGSAGGSAGGSGSAGGSAGGSTSAGGSAGGSAGGSAGGSAGGSG<br>SAGGSAGGSTSAGGSAGGSAGGSAGGSAGGSG |
| Linker |
| NPTFLYKVGPG |
| Substrate + Docking site |
| PDVPRTPVDKAKLSFQFP |
| Linker |
| LEATLYNKVG |
| eCFP |
| MVSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKFICTTGKLPVPWPTLVTTLTWGVQCFSRYPDHMKQHDFFKSA<br>MPEGYVQERTIFFKDDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYISHNVYITADKQKNGIKANFKIRHNIEDGS<br>VQLADHYQQNTPIGDGPVLLPDNHYLSTQSALSKDPNEKRDHMVLLEFVTAAGITLGMDELYK |

**Table S2. Primers used for assessing pro-inflammatory cytokines expression levels by Q-PCR, related to Figure S10.**

| *Mouse Gene* | *Forward primer* | *Reverse primer* |
|---|---|---|
| *Il-6* | TTCTCTGGGAAATCGTGGAAA | TCAGAATTGCCATTGCACAAC |
| *Mcp1* | GCATCTGCCCTAAGGTCTTCA | TGCTTGAGGTGGTTGTGGAA |
| *IκB-α* | ACGAGGAGTACGAGCAAATGGTG | TGATTGCCAAGTGCAGGAACGAG |
| *A20* | AGCACCCTTAAGGAGACAGACAC | TCCCATTCGTCATTCCAGTTCCG |
| *TNF-a* | ACCCTGGTATGAGCCCATATAC | ACACCCATTCCCTTCACAGAG |
| *Cxcl1* | CTATCGCCAATGAGCTGCG | TCTGAACCAAGGGAGCTTCA |