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Supplementary Materials for

NF-κB signaling dynamics is controlled by a dose-sensing autoregulatory loop

Mialy M. DeFelice, Helen R. Clark, Jacob J. Hughey, Inbal Maayan, Takamasa Kudo, Miriam V. Gutschow, Markus W. Covert*, Sergi Regot*

*Corresponding author. Email: mcovert@stanford.edu (M.W.C.); sregot@jhmi.edu (S.R.)

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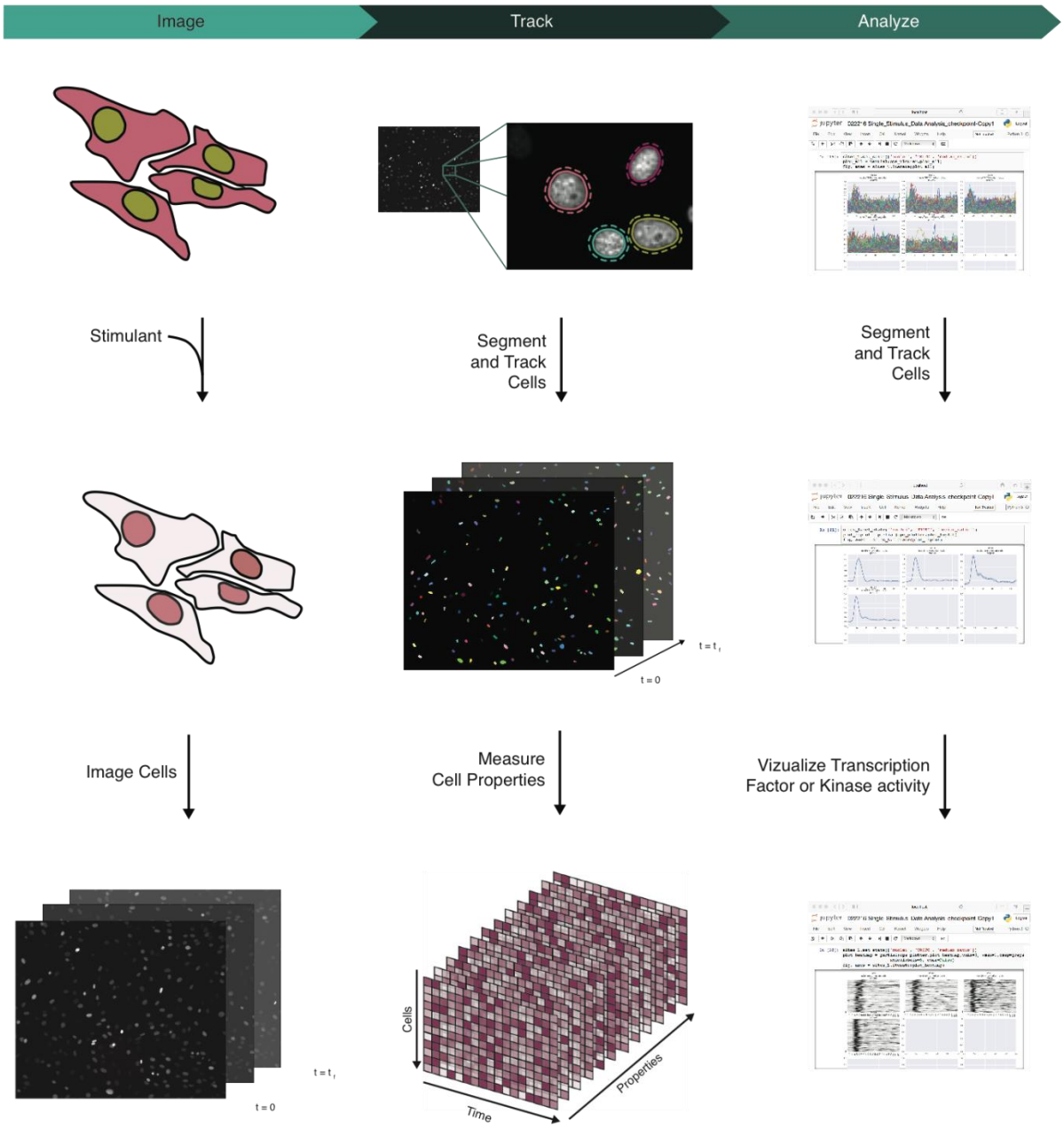

Fig. S1. Graphical summary of the image analysis pipeline. Adapted with permission from Kudo *et a*l. 2018 (*57)*. Schematic of the imaging and experimental pipeline. Cells are stimulated (for example, with TNFα), resulting in a spatial translocation of a fluorescent reporter (for example, NF-κB). Images of cells are segmented, objects are identified (for example, the nucleus and cytoplasm) and fluorescence intensity and localization data is extracted for every image. Cells are tracked and linked across frames in a movie. Data matrices contain information for every cell in a movie. Image processing is used to filter out data (for example, to exclude outliers) and to consolidate all of the data. For additional details, see the Materials and Methods.

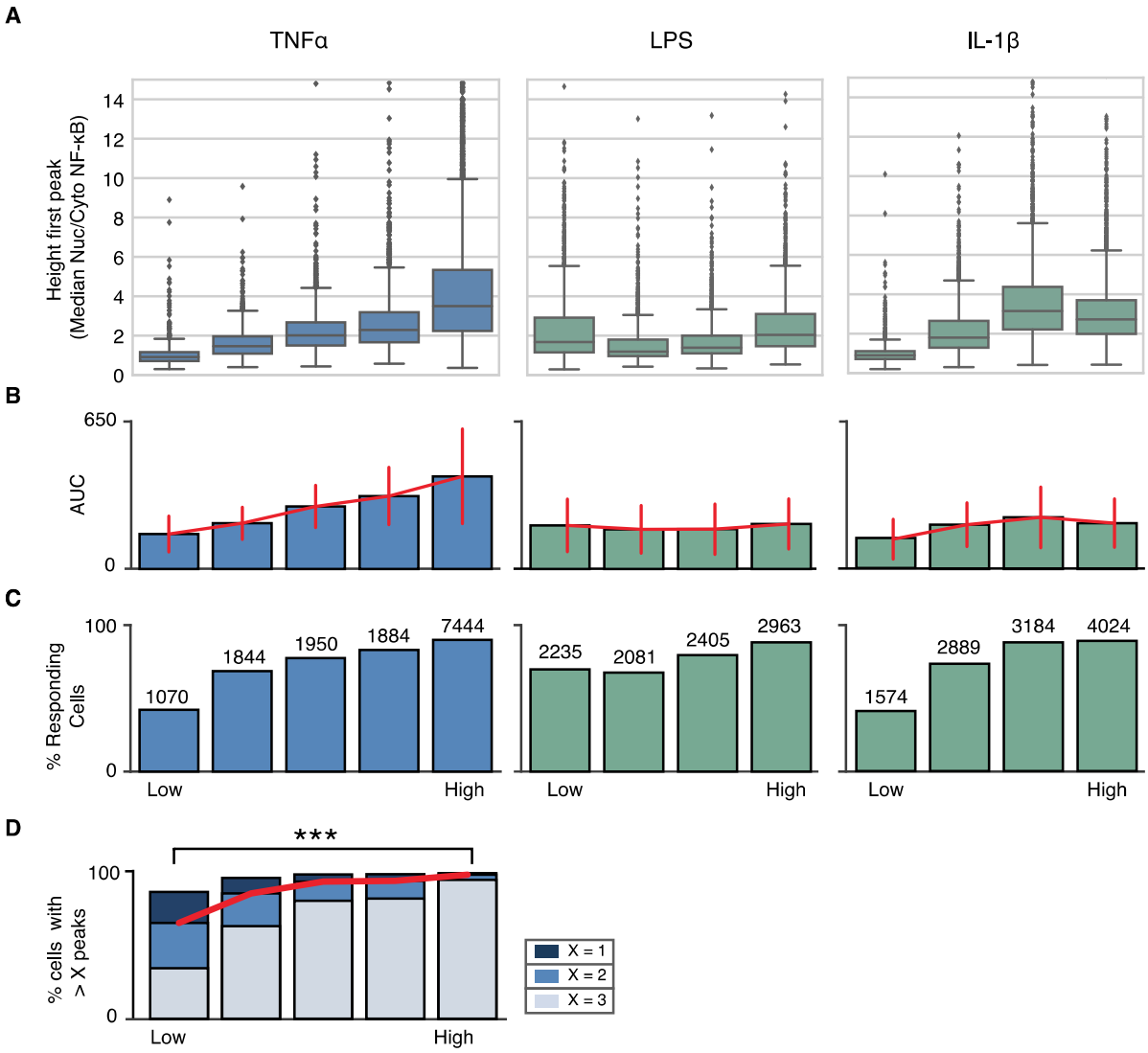

Fig. S2. Additional analysis and TNF concentration for data presented in Fig. 1. (A) Parental strain (PS) cells (NIH 3T3, *RelA*-/-, p65-dsRed, H2B-EGFP) were treated with increasing concentrations TNFα (0.01, 0.1, 1, 10 and 100 ng/ml); LPS (0.05, 0.5, 5 and 50 µg/ml); IL-1β (0.01, 0.1, 1 and 10 ng/ml) of indicated stimuli, imaged, and quantified as described in Materials and Methods. For all responding cells, the height of the first peak (median nuc/cyto NF-κB ratio), across stimuli and concentrations was measured. Boxplots show the distribution of peak heights. Traces were not normalized prior to this calculation. **(B)** The area under the curve for all the cell traces (median nuc/cyto NF-κB ratio) and standard deviation is plotted for all stimuli and concentrations as indicated. Traces were not normalized prior to this calculation. **(C)** The percentage of cells that responded to stimulation is plotted for all stimuli and concentrations as indicated. The number of cells this represents is indicated for each concentration as well. **(D)** Peak counting quantification (see Materials and Methods for additional details) was done as in Fig. 1. Fractions of cells with more than 1, 2, or 3 peaks are shown to highlight population distribution. High vs. Low, ****P*= 0.001 by chi-square test; data represent two independent experiments, with n > 7000 cells. The data for first four concentrations presented come from the data in Fig.1 and are included here for comparative purposes.

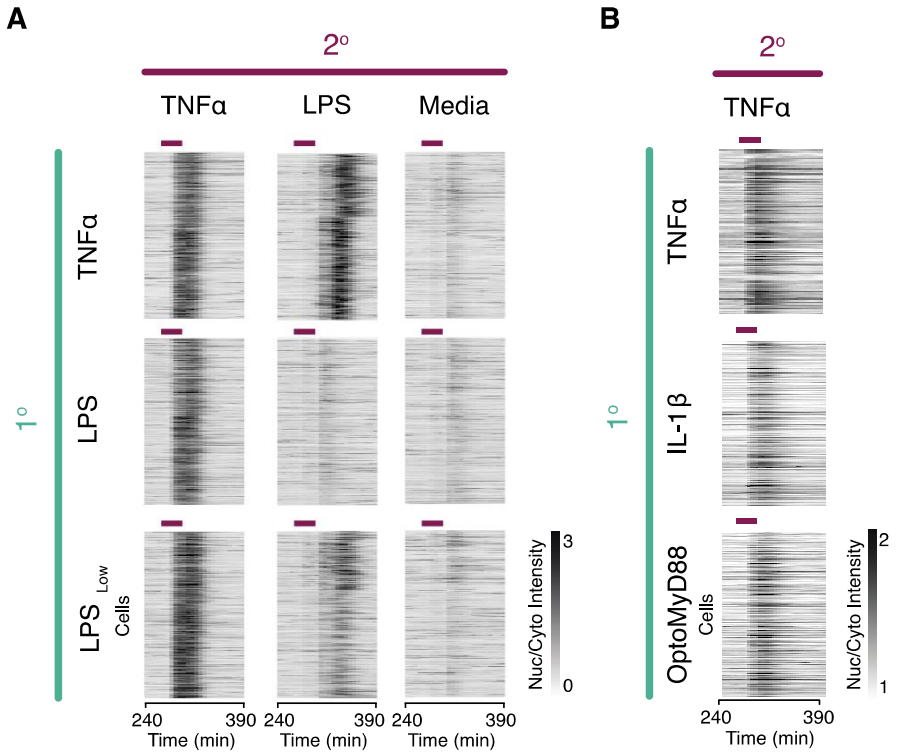

Fig. S3. Additional stimulus combinations for Figs. 2 and 3. (A) Parental Strain (PS) cells (*RelA*-/-, p65-DsRed, H2B-EGFP) were stimulated with different combinations of primary and secondary inputs as indicated (TNFα 10 ng/ml, LPS 5 μg/ml, LPS_{Low} 5 ng/ml), and monitored for NF-κB activity. Only the time period for secondary stimulation is shown for clarity. Purple lines over heatmaps indicate time period when cells were in secondary stimulus. Data represents two independent experiments (n > 300 cells per condition, n > 9500 cells total). Cells were filtered to include only those responding to the primary stimulus. **(B)** Cells (NIH 3T3, p65-mRuby, H2B-iRFP) were stimulated with different combinations of primary and secondary inputs (TNFα 10 ng/ml, IL-1β 1 ng/ml, and blue light five 250ms pulses). Heatmaps are shown in (A). Data represents three independent experiments n > 100 cells per condition.

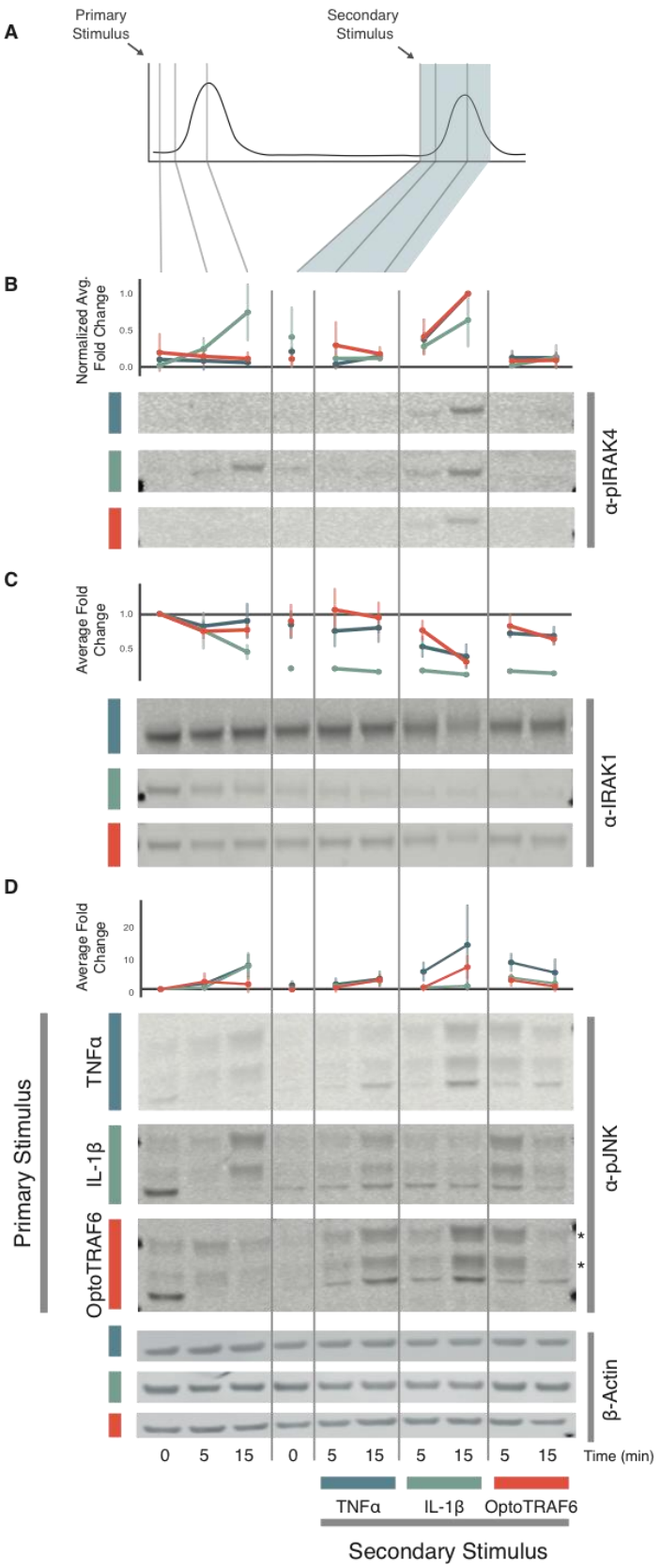
A
Primary Stimulus
Secondary Stimulus
B
Normalized Avg. Fold Change
1.0
0.5
0.0
α-pIRAK4
C
Average Fold Change
1.0
0.5
α-IRAK1
D
Average Fold Change
20
10
0
Primary Stimulus
TNFα
IL-1β
OptoTRAF6
α-pJNK
*
*
β-Actin
0 5 15 0 5 15 5 15 5 15
Time (min)
TNFα
IL-1β
OptoTRAF6
Secondary Stimulus

Fig. S4. Full gels and quantification of Western blots in Fig. 4. (A) Schematic detailing the timing of stimulation and sample collection, analogous to Fig. 4. **(B)** Cells were analyzed by western blotting to detect pIRAK4. The abundance of pIRAK4 was first normalized to that of the β-actin loading control, and then the normalized average fold-change in abundance compared to that in the unstimulated cells was calculated and is shown. Samples were collected at 0, 5, and 15 min after primary and secondary stimulations with TNF-a (10 ng/ml), IL-1β (1 ng/ml), and Light (OptoTRAF6), as indicated by the colored bars. Blots represent three independent experiments. **(C)** Cells were analyzed by western blotting to detect IRAK1. IRAK1 abundance was first normalized to that of the β-actin loading control, and then the average fold-change in abundance compared to that in the unstimulated cells was calculated and is shown. Cells were stimulated and collected as described for (B). Blots represent three independent experiments. **(D)** Cells were analyzed by western blotting to detect pJNK. The abundance of pJNK was first normalized to that of the β-actin loading control, and then the average fold-change in abundance compared to that of the unstimulated cells was calculated and is shown. Cells were stimulated and collected as described for (B). Blots represent three independent experiments.

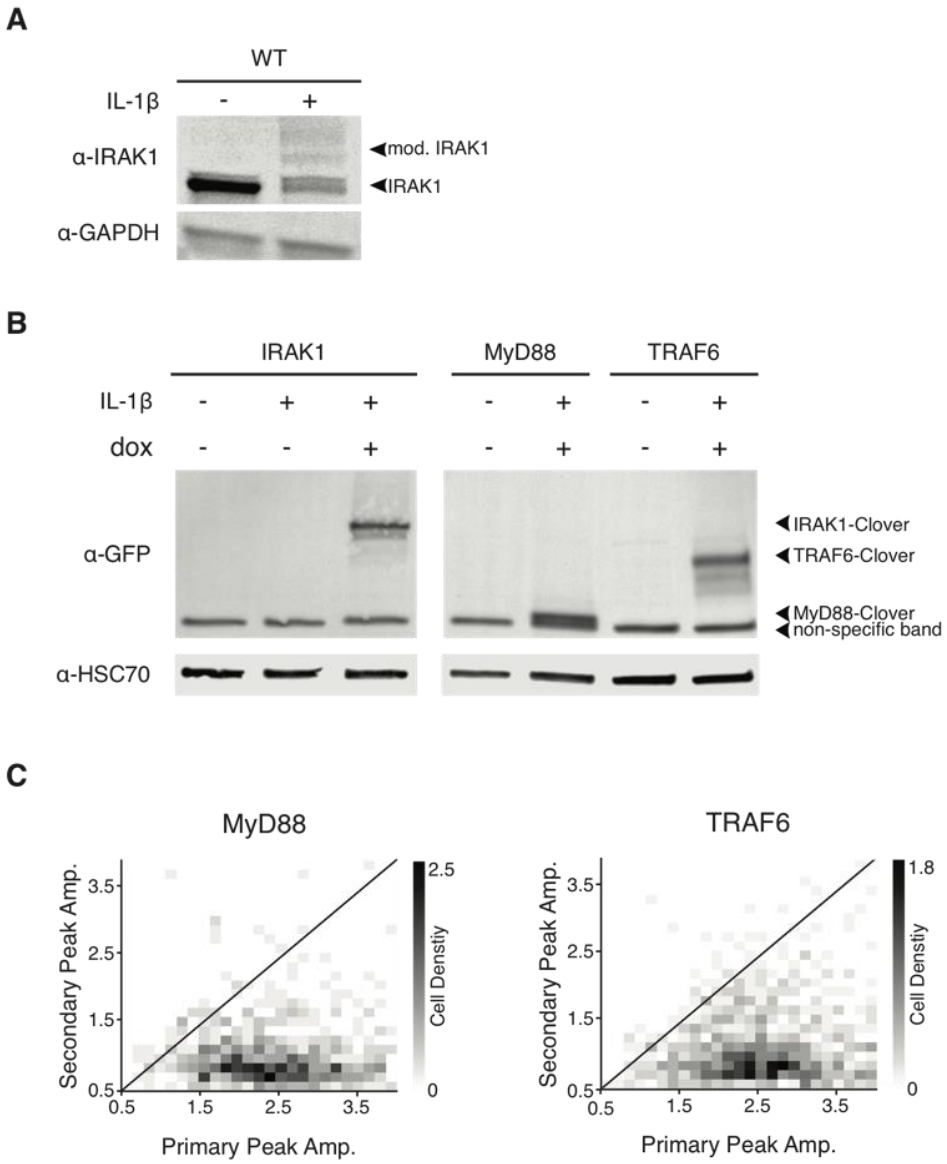

Fig. S5. Increased abundance of MyD88 or TRAF6 after primary stimulation cannot bypass tolerance. (A) Endogenous IRAK1 is modified following cytokine stimulation. WT cells were stimulated with IL-1β (1 ng/ml) for 30 minutes then harvested and analyzed by western blotting to detect IRAK1. Arrows indicate unmodified and modified IRAK1 protein. GAPDH was used as a loading control. **(B)** Cells (NIH 3T3, p65-mRuby, H2B-iRFP), were transduced with a IRAK1-Clover, MyD88-Clover, or TRAF6-Clover construct under the Tet Responsive Element Third Generation (TRE3G) promoter. Cells were harvested before or after IL-1β (1 ng/ml) stimulation and with or without a 3-hour incubation with doxycycline (dox) (2 μg/ml). Samples were analyzed by western blotting to detect GFP. IRAK1-Clover, as well as TRAF6-Clover, MyD88-Clover, and non-specific bands are indicated. HSC70 was used for loading control. Data is representative of three independent experiments. **(C)** 2D histograms of the maximum peaks during the primary and secondary responses of TRE3G::MyD88-Clover and TRE3G::TRAF6-Clover cells treated with dox and IL-1β simultaneously. Data represents three independent experiments with n > 100 cells.

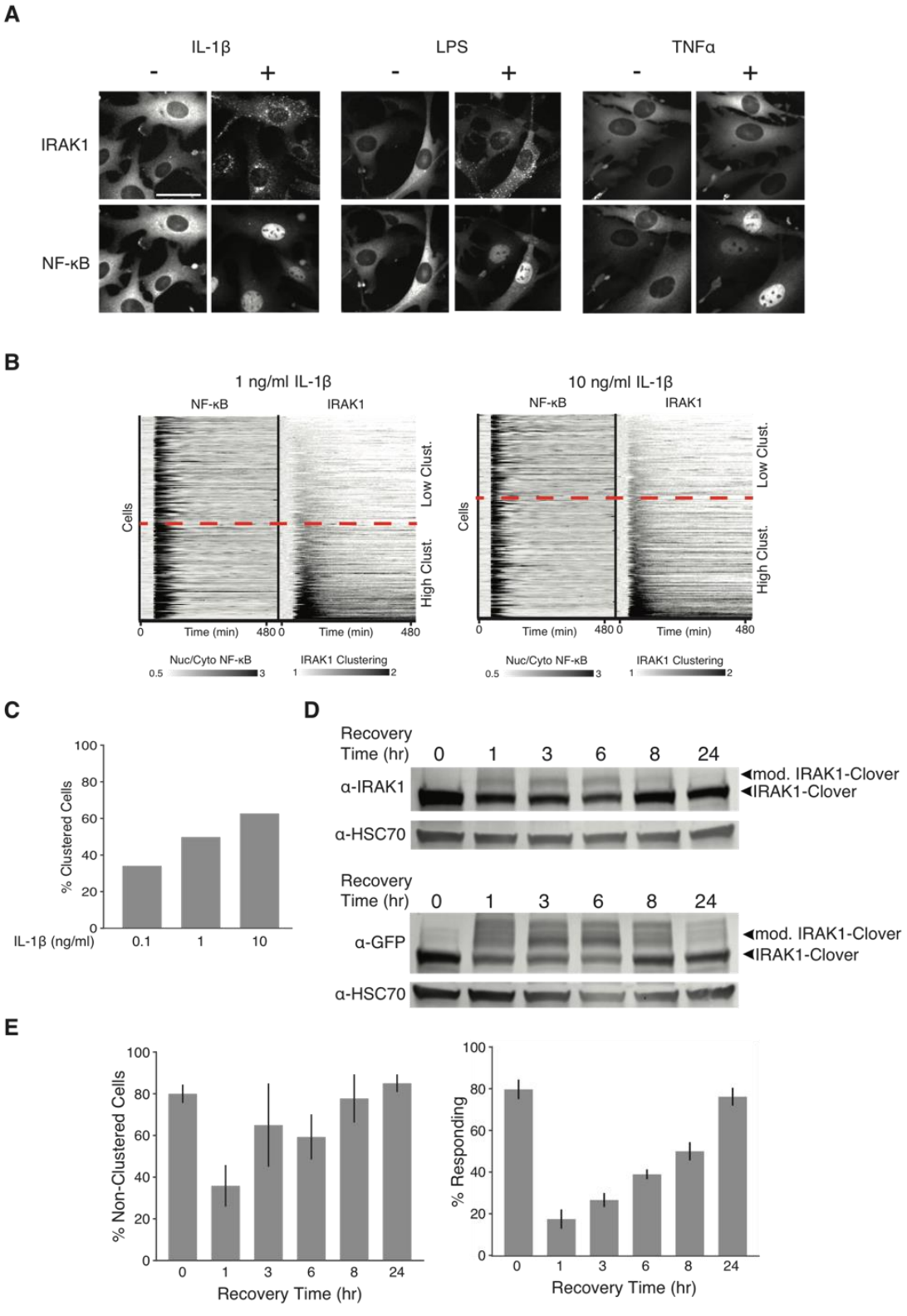
A
IL-1β
LPS
TNFα
-
+
IRAK1
NF-κB
B
1 ng/ml IL-1β
10 ng/ml IL-1β
NF-κB
IRAK1
Cells
Low Clust.
High Clust.
Time (min)
0
480
Nuc/Cyto NF-κB
0.5
3
IRAK1 Clustering
1
2
C
% Clustered Cells
IL-1β (ng/ml)
0.1
1
10
D
Recovery Time (hr)
0
1
3
6
8
24
α-IRAK1
α-GFP
α-HSC70
◀mod. IRAK1-Clover
◀IRAK1-Clover
E
% Non-Clustered Cells
% Responding
Recovery Time (hr)

Fig. S6. IRAK1 modification and clustering with varied recovery periods. (A) IL-1β and LPS induce the clustering of IRAK1-Clover whereas TNFα does not. Cells expressing IRAK1-Clover were imaged before and 40 min after stimulation with IL-1β (1 ng/ml), LPS (5 μg/ml) or TNFα (10 ng/ml). Images represent one experiment with five replicates per condition. Scalebar represents 50 μm. **(B)** Heatmaps show increased NF-κB oscillations in cells with less IRAK1-Clover clustering. NF-κB Nuclear/Cytoplasmic median intensity heatmap is displayed in tandem with IRAK1-Clover clustering (clustering quantification details are provided in the Materials and Methods). Red line represents an arbitrary IRAK1 clustering threshold (1.3-fold change) at which clustering was divided into "high" and "low" groupings for peak counting bar plots (see Fig. 5E). Heatmap cells were sorted based on IRAK1 clustering. Data represents three independent experiments. **(C)** Bar plot shows the percentage of clustered cells from data in (B) and Fig. 5D. **(D)** IRAK1-Clover cells were stimulated with IL-1β (1 ng/ml) for 30 min, washed, and collected at indicated hours after wash. Samples were analyzed by western blotting to detect for IRAK1 and GFP. HSC70 was used as a loading control. **(E)** Cells as in (D) were treated with 30 min IL-1β (1 ng/ml) stimulation, washed, and allowed to recover for the indicated time periods. Cells were then imaged every 5 minutes and stimulated with IL-1β (1 ng/ml). The 'zero' time point image was used to determine the percent non-clustered cells prior to stimulation. Standard Deviation Filter analysis was used to quantify clustering (See Materials and Methods), and in each condition, values greater than the 80^{th} percentile of the 'zero' time point were considered clustered. The image 20 min following IL-1β stimulation was used to quantify the percent responding cells, responders were defined by a nuc/cyto ratio of NF-kB greater than 1.5. Data represents three independent treatments.

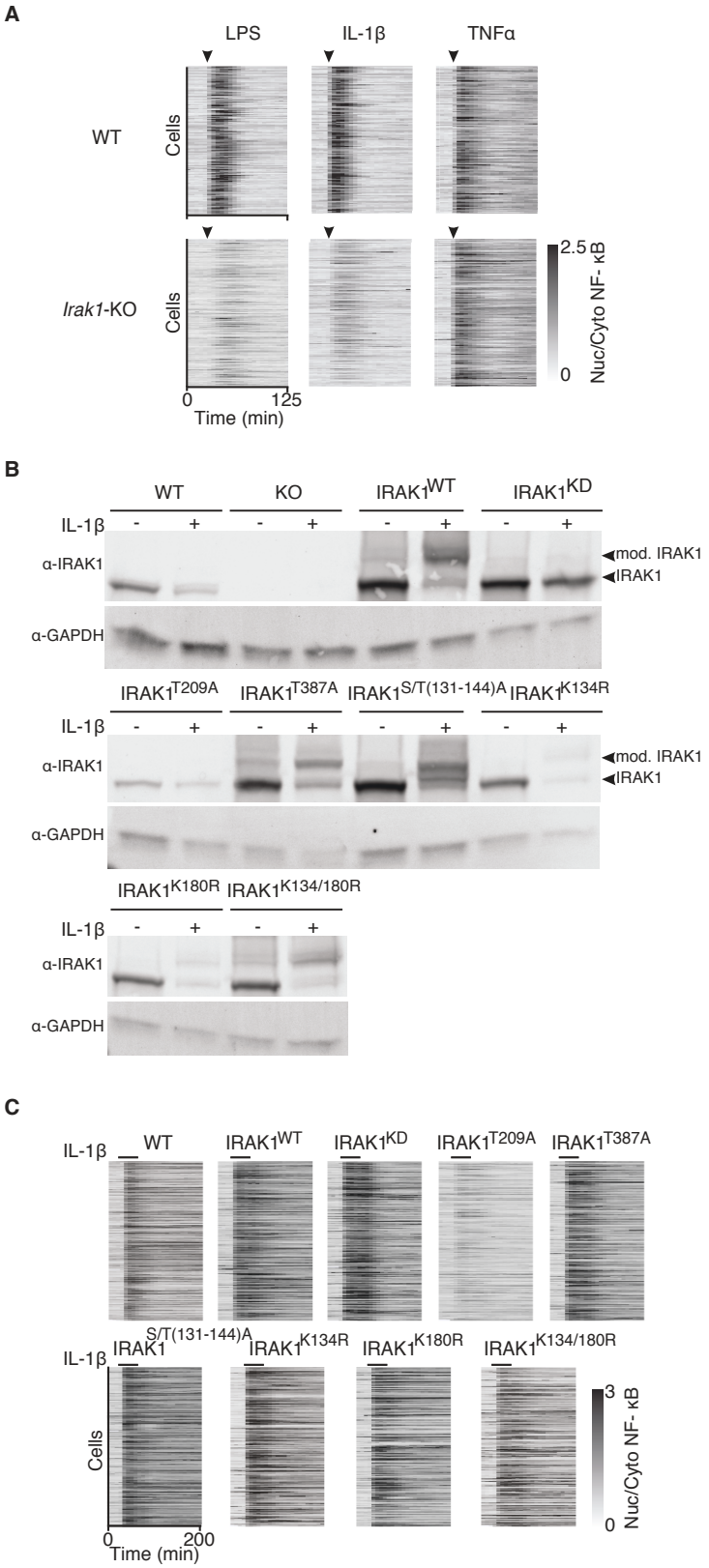
A
LPS
IL-1β
TNFα
WT
Cells
Irak1-KO
Cells
0
125
Time (min)
2.5
0
Nuc/Cyto NF- κB
B
WT
KO
IRAK1WT
IRAK1KD
IL-1β
- + - + - + - +
α-IRAK1
mod. IRAK1
IRAK1
α-GAPDH
IRAK1T209A
IRAK1T387A
IRAK1S/T(131-144)A
IRAK1K134R
IL-1β
- + - + - + - +
α-IRAK1
mod. IRAK1
IRAK1
α-GAPDH
IRAK1K180R
IRAK1K134/180R
IL-1β
- + - +
α-IRAK1
α-GAPDH
C
IL-1β
WT
IRAK1WT
IRAK1KD
IRAK1T209A
IRAK1T387A
IRAK1S/T(131-144)A
IRAK1K134R
IRAK1K180R
IRAK1K134/180R
IL-1β
Cells
0
200
Time (min)
3
0
Nuc/Cyto NF- κB

Fig. S7. IRAK1 mutant screen and KO characterization. (A) *Irak1*-KO cells do not respond to LPS or IL-1β stimulation. Heatmaps are shown of WT and *Irak1*-KO cells treated with LPS (5 μg/ml), IL-1β (1 ng/ml), or TNF (10 ng/ml) imaged and quantified as described in the Methods. Data represents two independent experiments with n > 100 cells. **(B)** Immunoblot of IRAK1 mutant panel. *Irak1*-KO cells were reconstituted with the indicated panel of mutants. Cells were incubated with media or IL-1β (1 ng/ml) for 3 hours. Arrows indicate unmodified and modified IRAK1 protein. GAPDH was used as a loading control. Blot represents two independent experiments. **(C)** NF-κB nuclear/cytoplasmic median intensity heatmaps of IRAK1 mutant panel treated with IL-1β. Indicated IRAK1-mutant cell lines were stimulated with IL-1β (1 ng/ml), imaged, and quantified as described in the Methods. Data represents two independent experiments with n > 100 cells.

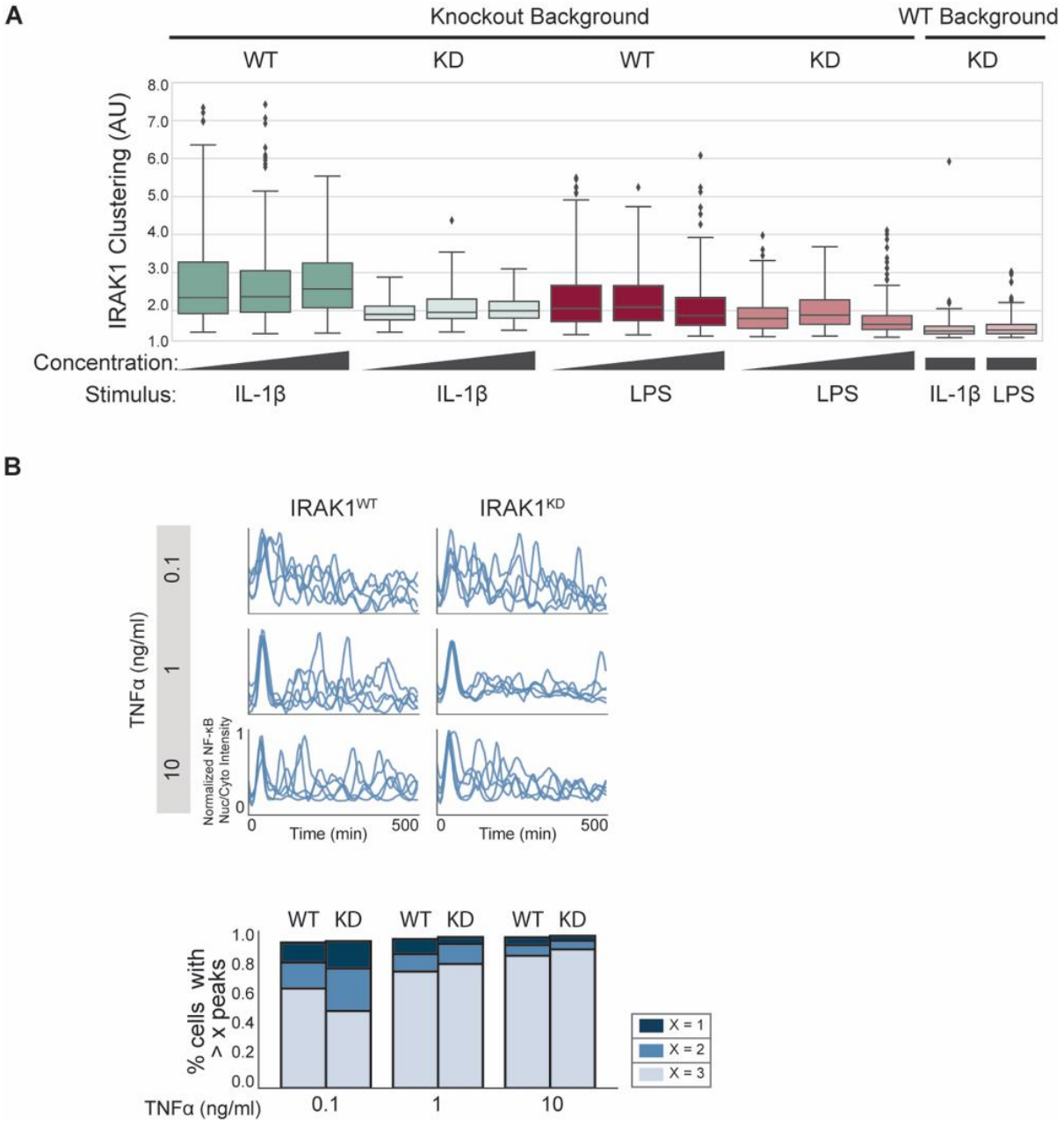

Fig. S8. Additional IRAK1 WT and KD-Clover characterization. (A) IRAK1^{WT} cells show more clustering than IRAK1^{KD} across different concentrations and stimuli. Boxplots show IRAK1^{WT} or IRAK1^{KD} clustering in *Irak1*-KO and wild-type cell line backgrounds. *Irak1*-KO cells reconstituted with IRAK1^{WT} or IRAK1^{KD} were stimulated with IL-1β (0.1, 1, and 10 ng/ml), or LPS (0.5, 5, 50 μg/ml). IRAK1^{KD} in a WT cell background (containing endogenous IRAK1) were stimulated with IL-1β and LPS (1 ng/ml and 5 μg/ml). Boxplots show the distribution of the 95th percentile of the cytoplasmic Standard Deviation Filter (described in Materials and Methods) in each trace. **(B)** Randomly selected traces and peak counting (as in Fig. 1, B and C, and Fig. 6E) of PS cells expressing IRAK1^{WT} or IRAK1^{KD} in response to three concentrations of TNFα (0.1, 1, and 10 ng/ml). Data represents three independent experiments.