Figure S1

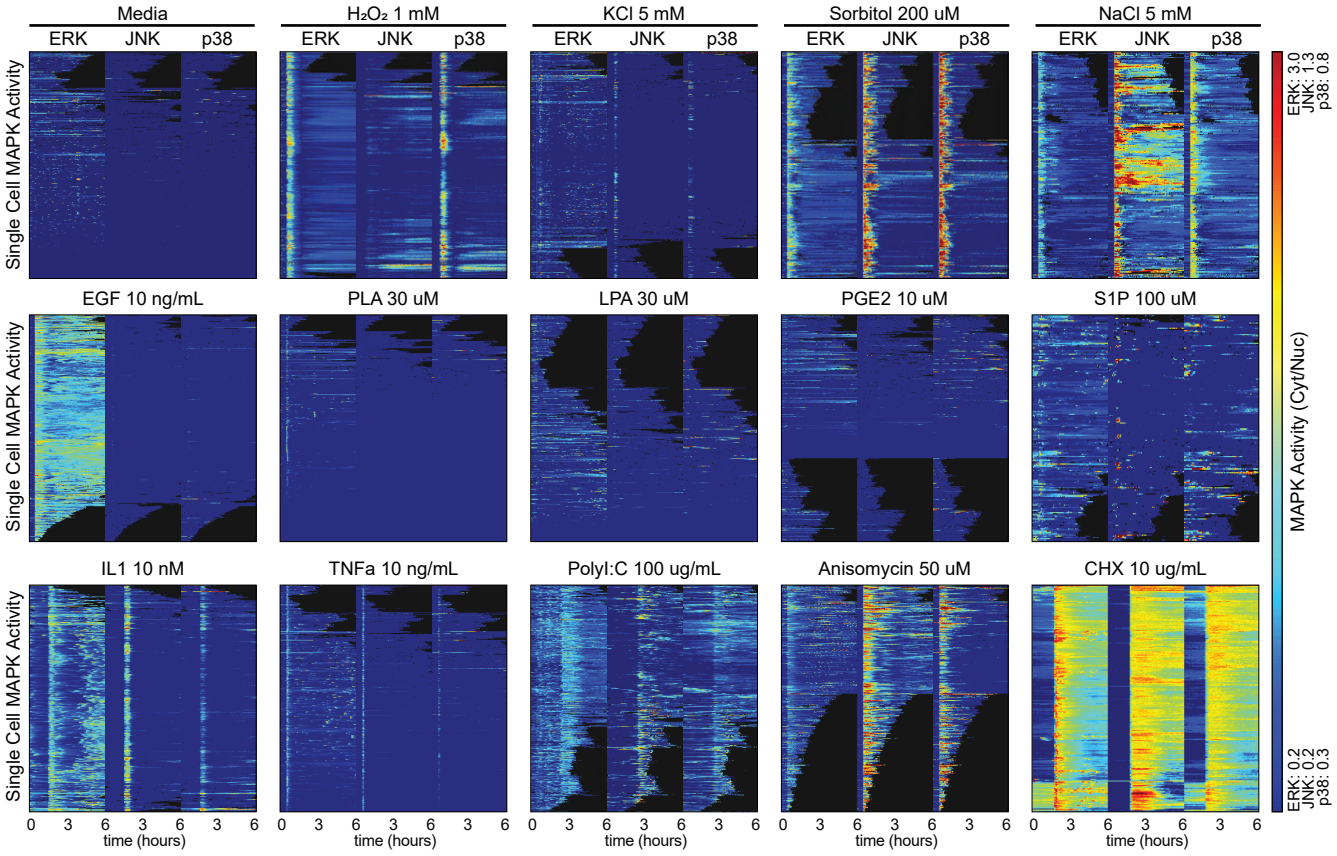

Figure S1. MAPK activity heatmaps of MCF10a reporter cells in response to natural stimuli (full panel).

Heatmap clustergrams wherein rows indicate individual cells, columns indicate time, and the jetmap colormap represents the nuclear to cytoplasmic median intensity ratio of ERK-KTR (left), JNK-KTR (middle), or p38-KTR (right) in response to indicated stimuli.

Figure S2

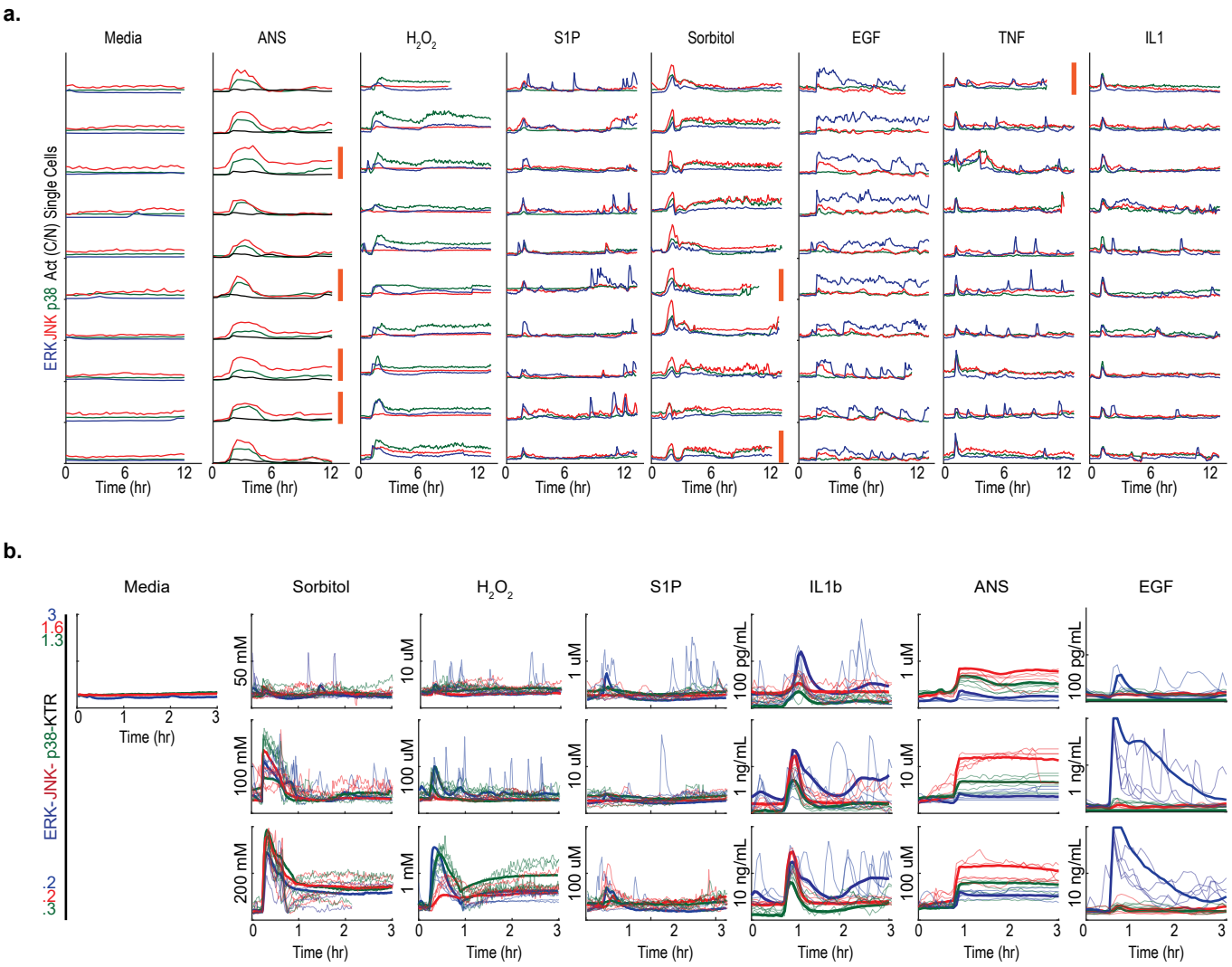

Figure S2: MAPK responses to natural stimuli.
a. Single cell ERK, JNK, and p38 traces of MCF10a reporter cells were treated with media control, (10 ng/mL), hydrogen peroxide (10 μM), sorbitol (100 μM), EGF (10 ng/mL), anisomycin (100 ng/mL), TNFα (10 ng/mL), or sphingosine-1-phosphate (100 μM), imaged every 5 minutes for 6 hours and quantified as described in methods. **b.** Serum-starved MCF10a MAPK reporter cells were stimulated with indictaed stimuli, imaged every 5 minutes for 3 hours and quantified as described in methods. Five representative single cell traces of the cytoplasmic to nuclear ratio of each KTR are plotted over time and overlaid with the average traces (>200 cells per condition).

Figure S3

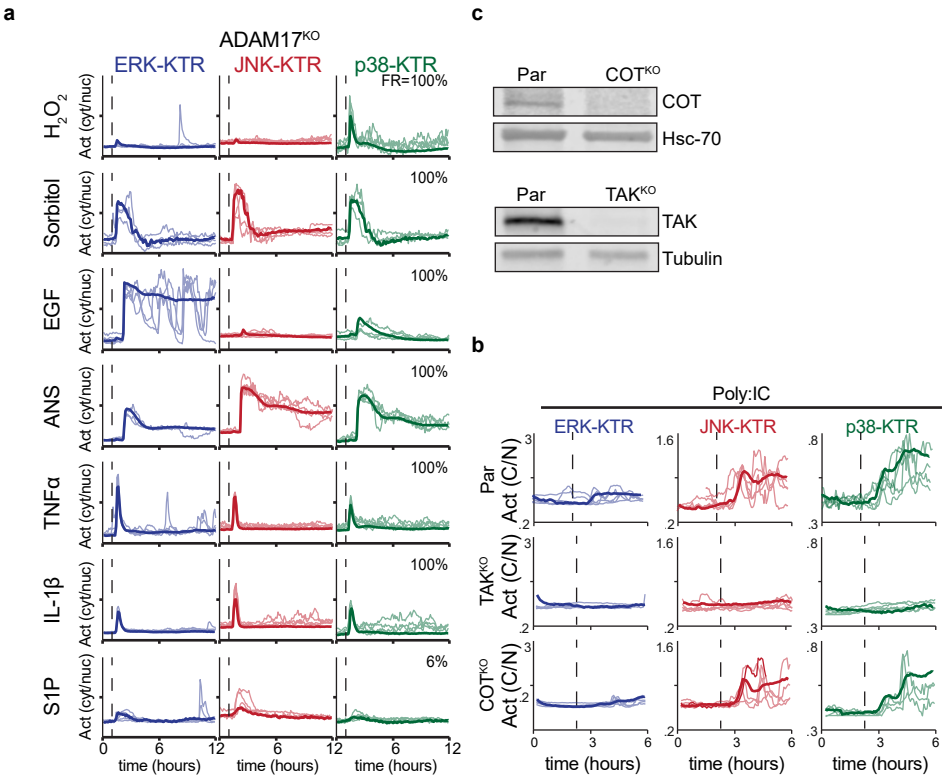

Figure S3. Complex signaling patterns through natural stimuli. **a.** ADAM-17^{KO} serum-starved MCF10a MAPK reporter cells were stimulated with media control, (10 ng/mL), hydro-gen peroxide (10 μM), sorbitol (100 μM), EGF (10 ng/mL), anisomycin (100 ng/mL), TNFα (10 ng/mL), or sphingosine-1-phosphate (100 μM), imaged every 5 minutes for 12 hours and quantified as described in methods. Five representative single cell traces of the cytoplasmic to nuclear ratio of each KTR are plotted over time and overlaid with the average traces (>350 cells per condition). Fraction of responders (FR) is indicated. **b.** Representative immunoblots showing protein levels of COT (left) or TAK (right) in the parental or clonal knock-out cell lines. **c.** Parental, TAKKO, or COTKO serum starved MCF10a reporter cell lines were treated with PolyI:C (100 μg/mL) and imaged every 5-minutes over 6 hours. Five representative single cell traces of KTR activity (cytoplasmic/nuclear ratio) were plotted over time and overlaid with the average traces (n>500 cells per conditions).

Figure S4

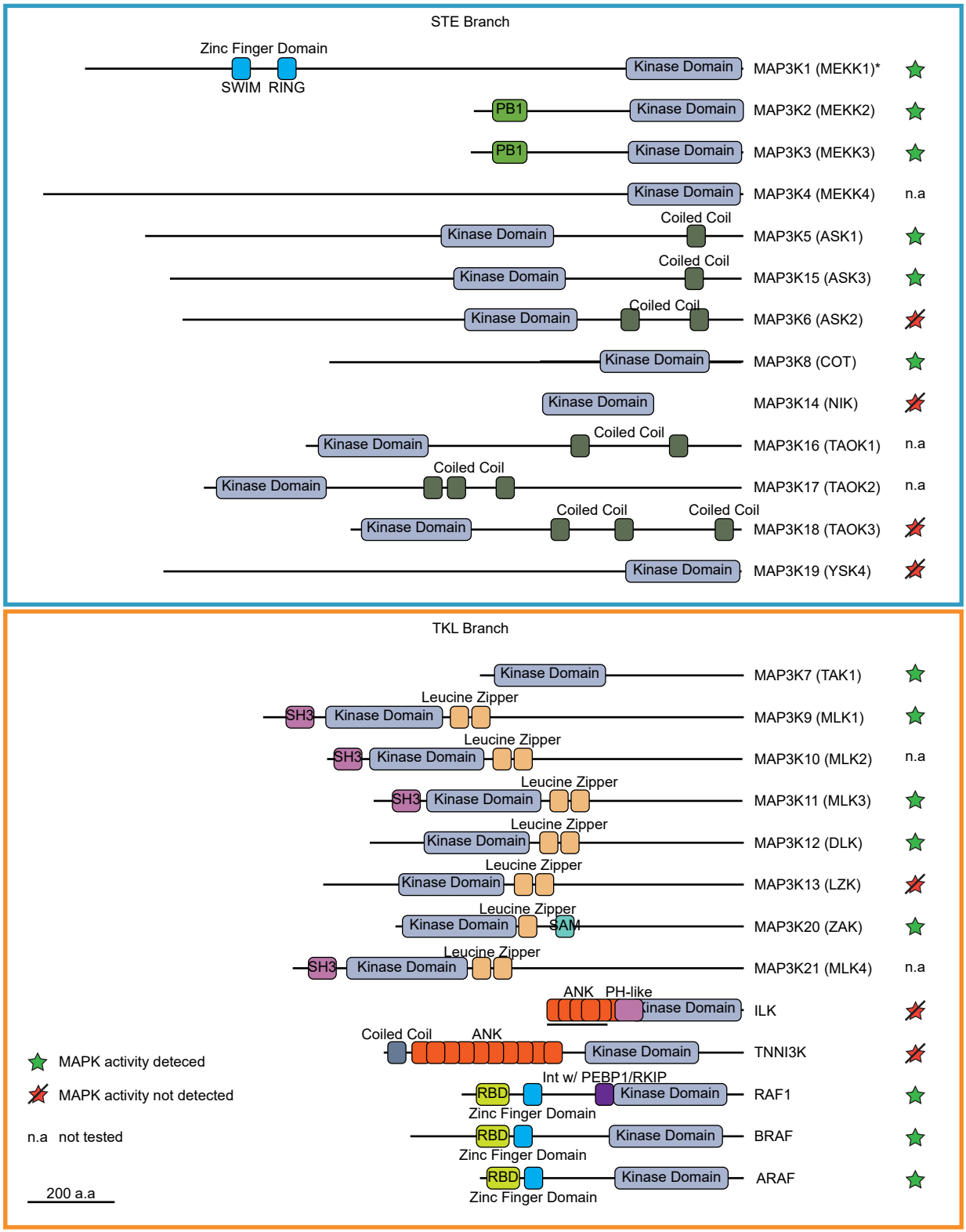

Figure S4. Domain architecture of human MAP3Ks.
Schematic of each MAP3K, separated by STE or TKL branch, with known domains labeled. Green star indicates that 4CTet inductions activated downstream MAPK. Red star indicates that 4CTet inductions had no change on downstream MAPK activity. Asterisk indicates special case: MEKK1 was only expressed as kinase domain, n.a. indicates MAP3K not tested.

Figure S5

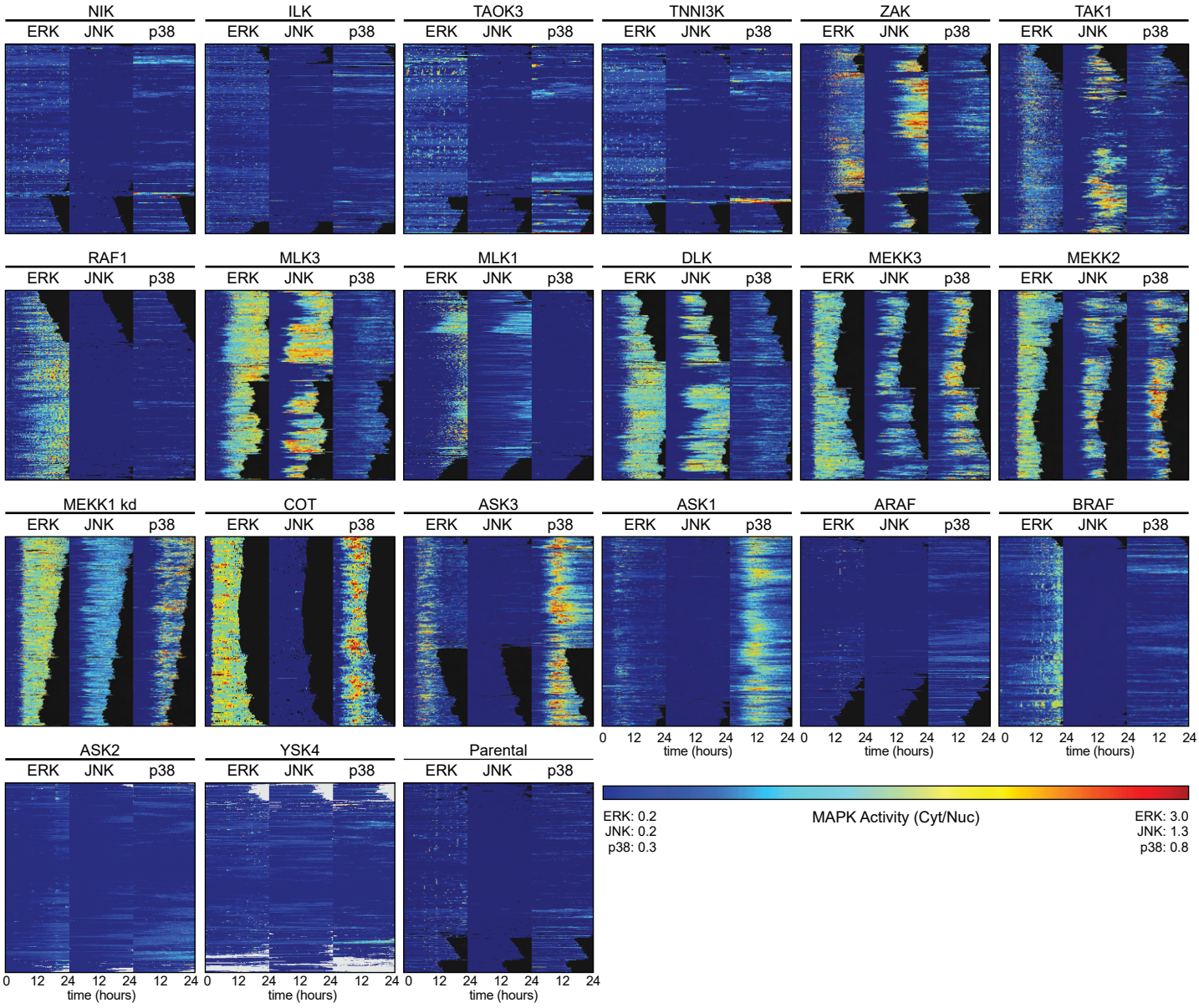

Figure S5. MAPK activity heatmaps of MCF10a reporter cells in response to MAP3K induc-tions (full panel)

Heatmap clustergrams wherein rows indicate individual cells, columns indicate time, and the jetmap colormap represents the nuclear to cytoplasmic median intensity ratio of ERK-KTR (left), JNK-KTR (middle), or p38-KTR (right). Indicated 4CTet cells were treated with doxycycline (2 μg/ml)

Figure S6

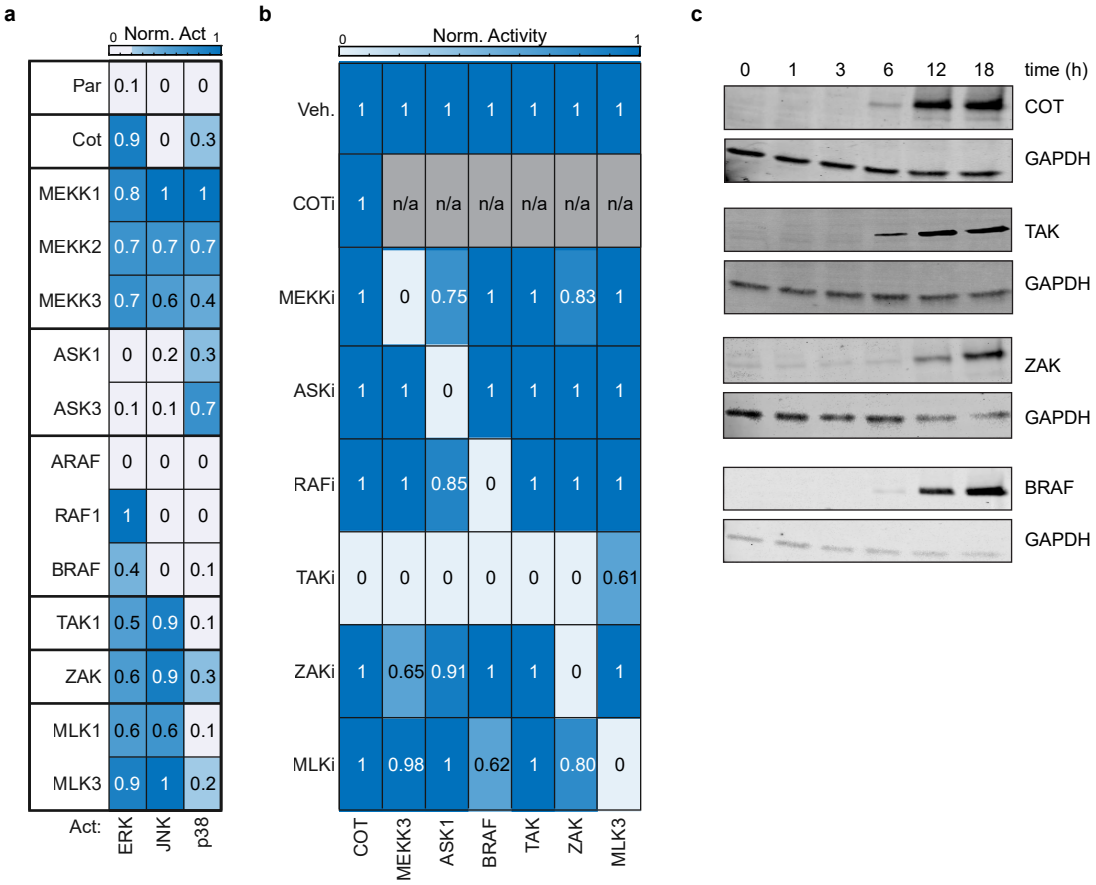

Figure S6: Homologous MAP3Ks have similar patterns of MAPK activity.
a. Heatmap illustrating ERK, JNK, or p38 activity ratios in each MAP3K induction. **b.** Indicated 4CTet cells were treated with doxycycline (2 μg/ml) and indicated MAP3K inhibitor, imaged for 24-hours over 5-min intervals. MAPK activities were then between AUC activity of the parental and AUC MAPK activity with doxycycline as described in methods. Heatmap illustrating the specificity of each indicated inhibitor in combination with each MAP3K induction (n>300 cells per conditions). **c.** Cells were harvested for immunoblotting at indicated timpoints post induction. Representative immunoblots showing total MAP3K levels in indicate cell lines

Figure S7

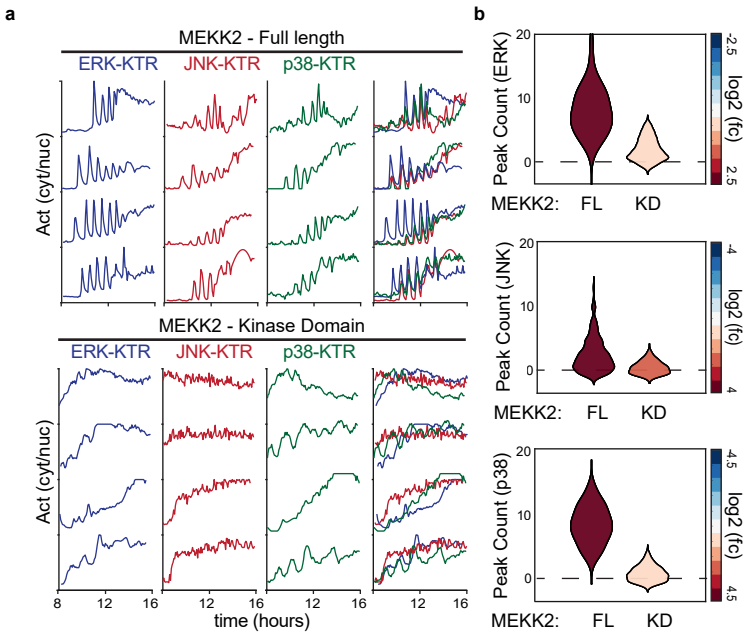

Figure S7: MEKKs trigger synchronous pulses of ERK, JNK and p38

a-b. 4CTet-MEKK2 full-length or kinase domain only cell lines were serum-starved, treated with doxycycline (2 μg/ml), and imaged every 5 min for 24 hours. **a.** Four representative single cell traces of the cytoplasmic to nuclear ratio of each KTR are plotted over time. **b.** Violin plots representing the number of independent peaks of KTR activity (cytoplasmic to nuclear ratio).

Figure S8

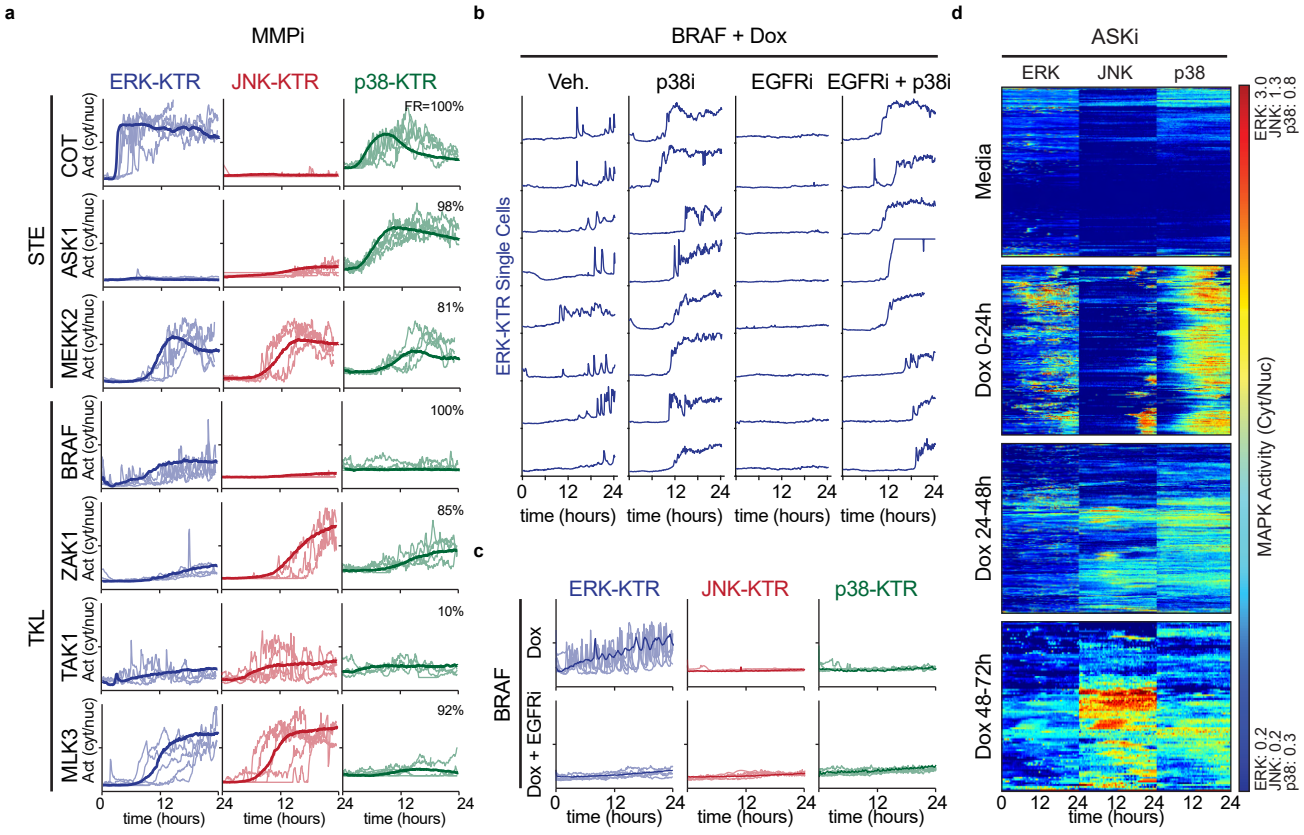

Figure S8. Non-cell autonomous activity following MAP3K induction. a. Serum-starved 4C cells containing indicated TRE3G::MAP3K constructs (4CTet) were treated with doxycycline (2 μg/ml), imaged for 24-hours over 5-min intervals, and quantified as the ratio cytoplasmic over nuclear KTR intensity. Five representative single cell activity traces are overlaid with the average (>350 cells per condition). **b.** Representative ERK single cell traces of 4CTet BRAF cells over the first 24 hours post induction in the presence of vehicle control, p38 inhibitor (SB203580) and/or EGFR inhibitor (Gefitinib). **c.** Average traces with single cell traces overlaid of BRAF inductions in 4CTet cells with vehicle control or EGFR inhibitor (Gefitinib). **d.** Dox was added to TRE3G::ASK1 cells at the beginning, 24 hr, or 48 hr prior to imaging for another 24h. Heatmap clustergrams wherein rows indicate individual cells, columns indicate time, and the jetmap colormap represents the nuclear to cytoplasmic median intensity ratio of ERK-KTR (left), JNK-KTR (middle), or p38-KTR (right) in response to indicated stimuli.

Figure S9

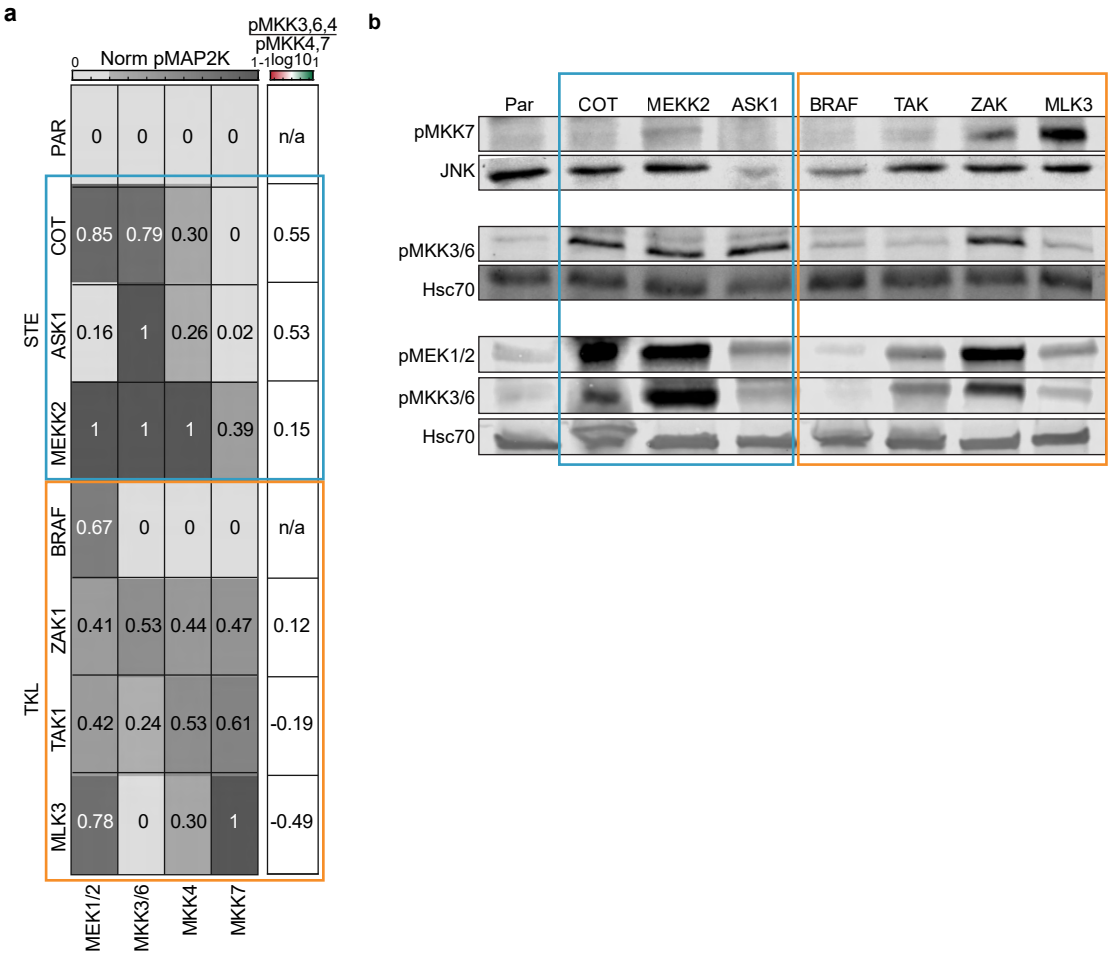

Figure S9: MAP3K have unique patterns of MAP2K-MAPK activation.
a. Cells were harvested for immunoblotting at 18h post induction. Phosphorylated MAP2K relative levels were calculated and normalized between media control and the upper 90th percentile of quantified phosphorylation (left). Ratio of p38 to JNK activity through MAP2K phosphorylation or MAPK-KTR activity was calculated by dividing (pMKK3/6 + pMKK4)/(pMKK7 + pMKK4) or P38/JNK biosensor activity. Heatmap illustrating the (pMKK3/6 + pMKK4)/(pMKK7 + pMKK4) ratios in each MAP3K induction (right). All experiments have two technical replicates and figures depict one of >3 independent experimental replicates. **b.** Representative immunoblots showing phosphorylated MAP2K levels at 18 hours post induction of indicated 4CTet cell line.

Figure S10

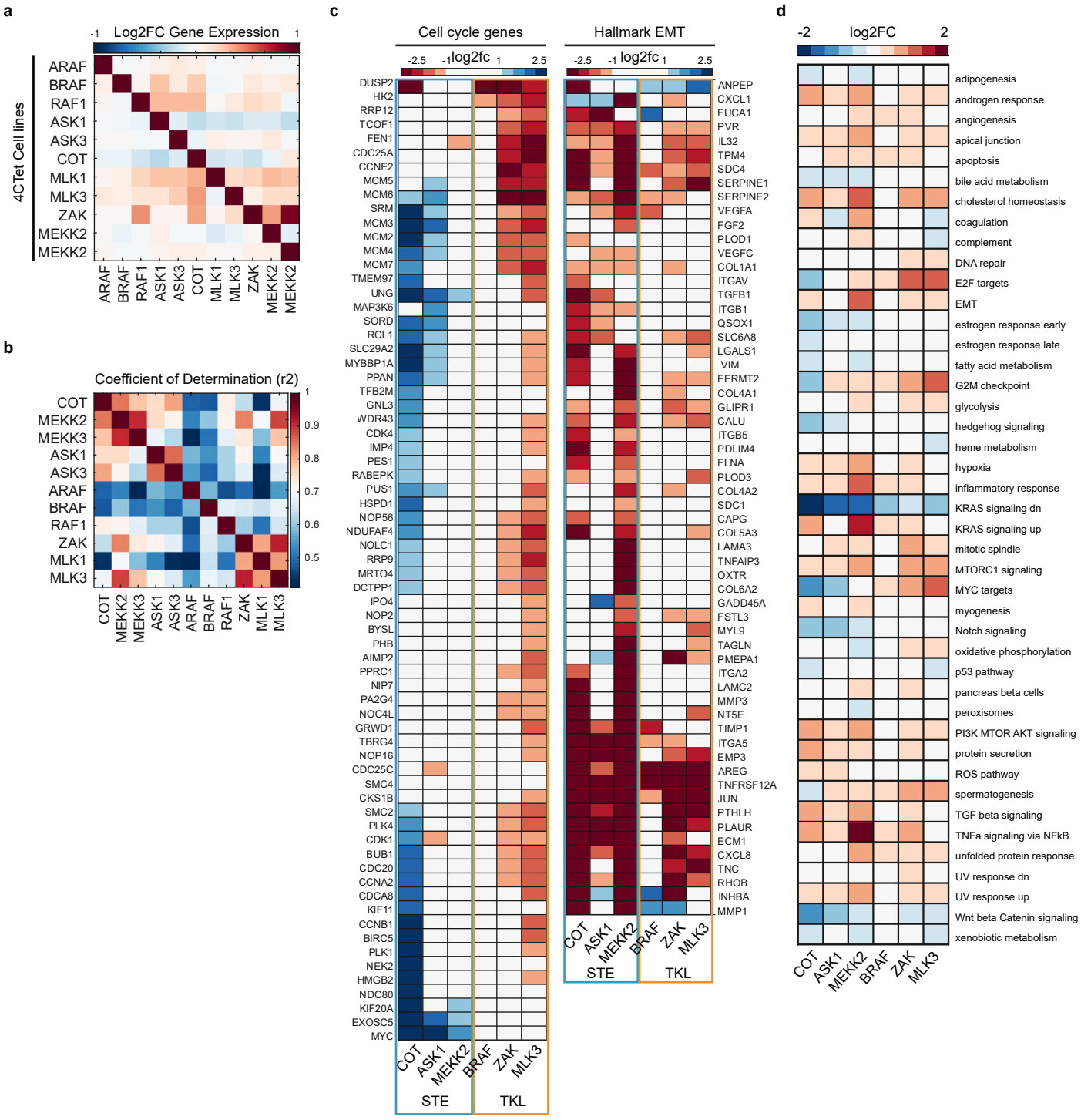

Figure S10: RNA-seq analysis indicates that STE kinases promote single cell migration while TKL kinases promote cell cycle progression.

a. 4CTet cells were serum-starved and treated with doxycycline (2 µg/ml) for 18 hours. RNA transcriptome was then harvested and sent for RNA-sequencing. Heatmap represents the log2 fold change gene expression of indicated MAP3Ks in each indicated 4CTet cell line. **b.** PCA analysis of the gene expression in each indicated 4CTet cell line. **c.** Heatmap representing the coefficient of determination (r2) of each indicated 4CTet cell line. **d.** Heatmap indicates the fold change gene expression of indicated cell cycle genes (left) or EMT genes (right). Each condition has two independent replicates. **b.** 4CTet cells were serum-starved and treated with doxycycline (2 µg/ml) for 18 hours. RNA transcriptome was then harvested and sent for RNA-sequencing. Heatmap represents the meanlog2 fold change gene expression of genes listed in indicated Hallmark GSEA gene lists.

Figure S11

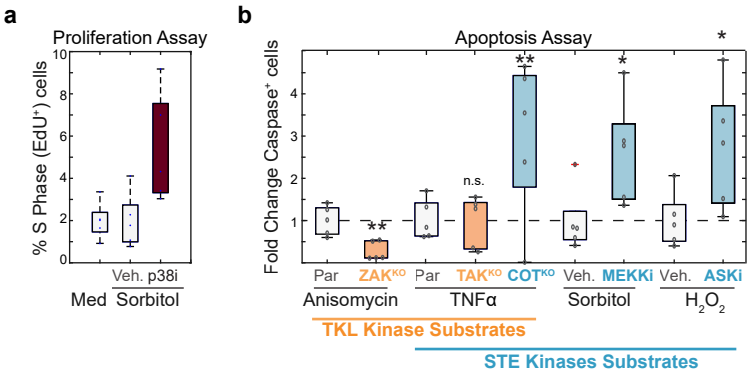

Figure S11. TKL kinases coordinate single cell fates in multicellular contexts. a. Serum starved cells were treated with indicated inhibitors or vehicle control. Cells were treated with Sorbitol (100 μM). After 20 hours, cells were incubated with EdU for 4 hours before fixation. Box plots represent relative % cells in S phase, as normalized to the vehicle control. **b.** Serum starved 4C cells were incubated with caspase dye as described in Fig. 6 and treated with JNK inhibitor or vehicle control. Cells were then treated with anisomycin (100 ng/mL), TNFα (10 ng/mL), sorbitol (100 μM), or H_2O_2 (1 mM). Relative apoptotic rates are quantified as described in methods. P-values of large data sets were quantified using 2-way Anova ('ns', not significant, *p<0.05, **p<0.01, ***p<0.001).

Figure S12

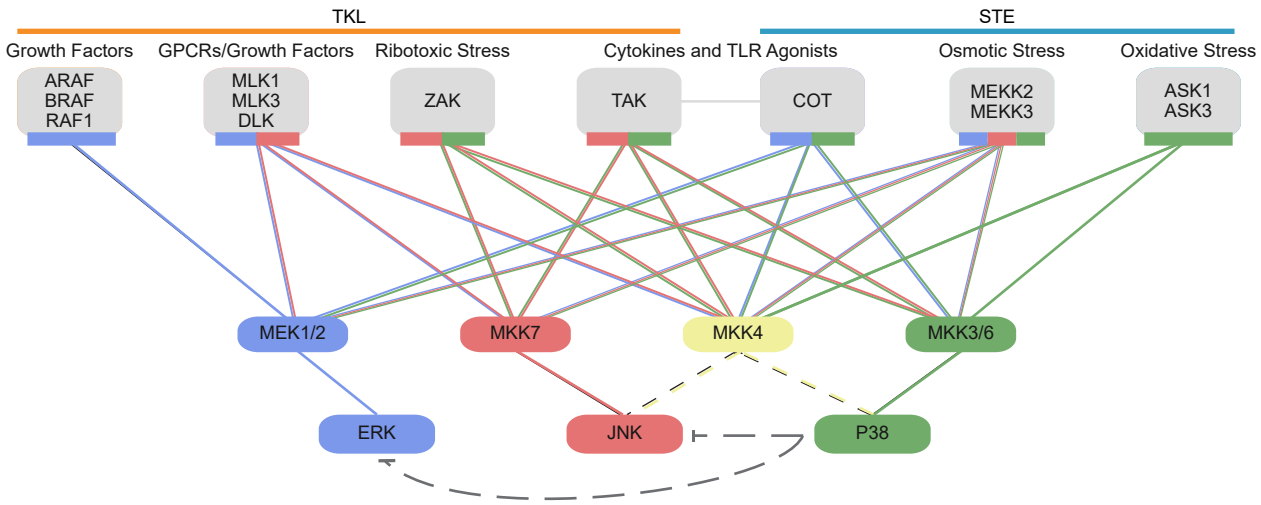

Figure S12: MAPK Network Map.
Schematic representing connectivity within the MAPK signaling network.

Table S1: Oligonucleotides		
Name	**Sequence**	**Source**
oAP6 F ZAKb STOP	GATAATAGTGAATGATGCccaactttcttgtacaaagttgg	this paper
oAP7 R ZAKb STOP	agaaagttggGCATCATTCACTATTATCCATGTCATTATCCTC	this paper
oAP10 F ZAK gBLOCK	CCTTCCTTTGAGATTGGTGCATGGACGG	this paper
oAP11 R ZAK gBLOCK	AAAGTTTCTCCATCCACGGTGGTCCC	this paper
oAP12 F ZAKcomp	GAGGGGACCACCGTGGATGGAGAAACTTTTGAttgccaactttcttgtacaaagttggcattataagaaagcattg	this paper
oAP24 R MAP3K2 STOP	TCACaAGTGATAATGCACAAACATGTGCCTTAAGAGTTCATCAGCTGA	this paper
oAP25 F MAP3K2 STOP	ATGTTTGTGCATTATCACTtGTGAccaactttcttgtacaaagttggcattataagaaag	this paper
oAP26 F MAP3K2 KinDom	ATGGACATCAGCCCACCCAGCCGTTCA	this paper
oAP27 R MAP3K2 KinDom	GGGTGGGCTGATGTCCATggtgccaact	this paper
oAP52 F MKK4ee	GAAattgccaagacaagaGAAgctggctgtaggccatacatggc	this paper
oAP54 R MKK4ee	TTCtcttgtcttggcaatTTCgtccacaagctgtccactgatgcc	this paper
oAP55 R MKK7eee	CTCCCGCTCCTTGGCTTTCTCGTCCACCAGGC	this paper
oAP56 F MKK7eee	GAGAAAGCCAAGGAGCGGGAGGCCGGCT	this paper
oAP81 R YSK4 STOP	aagttggTCACCTGGTCAGGCACATGCG	this paper
oAP82 F YSK4 STOP	CCTGACCAGGTGAccaactttcttgtacaaagttggc	this paper
oAP83 F MAP3K15 STOP	AGACAAGGCTTGAccaactttcttgtacaaagttggcat	this paper
oAP84 R MAP3K15 STOP	gaaagttggTCAAGCCTTGTCTTTGGTTTCTGAGGC	this paper
oAP85 F MAP3K8 STOP	AACGCTTGAATATGCCTGAccaactttcttgtacaaagttggc	this paper
oAP86 R MAP3K8 STOP	ggTCAGGCATATTCAAGCGTTGGTGGTCCC	this paper
oAP87 F TNNI3K STOP	ACAGCAGCTGAccaactttcttgtacaaagttggc	this paper
oAP88 R TNNI3K STOP	gaaagttggTCAGCTGCTGTCCTCAAAGCTGC	this paper
oAP100 F MLK1 MET	aagCAggCTTcATGGAGCCCTC	this paper
oAP90 R MLK1 MET	GAGGGCTCCATgAAGccTGctttttgtacaaagt	this paper
oAP93 F TAK1	atgtcgacagcctccgccgcctcgtcctcctc	this paper
oAP94 R TAK1	gcggaggctgtcgacatGGTGGAGCCTGCTTTTTTGTACA	this paper
oAP95 R RAF-pENTR	AGTGCGGCCGCttagaagactggtagccttggggatgtagtcagcg	this paper
oAP96 F backbone	AACCCAGCTTTCTTGTACAAAGTTGGCATTATAAGAAAGCATTGCTTATCAATTTGTTG	this paper
oAP97 R MEKK1bb	TGCCAACTTTGTACAAGAAAGCTGGGTTctaccacgtggtacggaagaccggatgtttcagc	this paper
oAP98 R backbone	GGTGCCTGCTTTTTTGTACAAAGTTGGCATTATAAAAAAGCATTGCTCA	this paper
oAP99 F MEKK1 backbone	CCAACTTTGTACAAAAAAGCAGGCACCctctccccagtcttcactcagtcaagaccccca	this paper
oAP101 R MEKK3 backbone	GCCAACTTTGTACAAGAAAGCTGGGTTTCAGTACATGAGCTGTGCAAAGTGGTGTGTGA	this paper
oAP102 F MEKK3 backbone	CCAACTTTGTACAAAAAAGCAGGCACCATGGACGAACAGGAGGCATTGAACTCAATCATGAACGATCT	this paper

oAP103 R MEKK4bb	AACTTTGTACAAGAAAGCTGGGTTTCATTCTTCATCTGTGCAAACCTTGACAAACGAATG	this paper
oAP104 F MEKK4bb	CTTTGTACAAAAAAGCAGGCACCATGAGAGAAGCCGCTGCCGCGC	this paper
oAP105 F MEKK3A-K	CTTGCTTCCAAGCAGGTCCAATTTGATCCAGACAGTCC	this paper
oAP106 R MEKK3A-K	GGACCTGCTTGGAAGCAAGTTCACGTCCCG	this paper
oAP113 F M3K6 STOP	AAAGGCAGCTTCTGAcccaactttcttgtacaaagttggcatt	this paper
oAP114 R M3K6 STOP	ttgggTCAGAAGCTGCCTTTGTCCTCTCCATTCATCC	this paper
oAP115 F MAP3K14 STOP	GAACAGGCCCTAAgccaactttcttgtacaaagttggcat	this paper
oAP116 R MAP3K14 STOP	gttggcTTAGGGCCTGTTCTCCAGCTGGC	this paper
oAP117 F TAOK3 STOP	GACTACAGATGACCTTgccaactttcttgtacaaagttggcattataagaaagc	this paper
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oAP157 F MKK4 deltaN	actttgtacaaaaaagttggcaccatgcagggtaaacgcaaagcactgaagttgaatttt	this paper
oAP158 R MKK4 deltaN	ctttgcgtttaccctgcatggtgccaacttttttgtacaaagttggcattataaaaaagc	this paper
oAP161 F MKK7dd deltaN	ATGCAGCGGCCCAGGCCCACCCTGCAGCTC	this paper
oAP162 MKK7dd deltaN	CCTGGGCCGCTGCATgccaacttttttgtacaaagttggcattataaaaaagcattg	this paper
Fwd MAP3K3dd	GACGTGGCCAAGGACATGGATGCCGGCTGC	this paper
Rev MAP2K3dd	GTCCTTGGCCACGTCGTCCACCAAGTAGCCACTGATGC	this paper
Fwd MAP2K4dd	GACattgccaagacaagaGACgctggctgtaggccatacatggc	this paper
Rev MAP2K4dd	GTCtcttgtcttggcaatGTCgtccacaagctgtccactgatgcc	this paper
Fwd MAP2K7dd	GACAAAGCCAAGGACCGGAGCGCCGGCT	this paper
Rev MAP2K7dd	GTCCTTGGCTTTGTCGTCCACCAGGCGGCC	this paper
oTA158 R MEK2dd	CGAAGTCGTTGGCCATGTCGTCTATGAGCTGGCCGCTCACCC	Aikin et al., 2020
oTA159 F MEK2dd	GACATGGCCAACGACTTCGTGGGCACGCGCTCCTA	Aikin et al., 2020
Rev MAP2K7 STOP	gcaaCTACCTGAAGAAGGGCAGGTGGGG	this paper
Fwd MAP2K7 STOP	CTGCCCTTCTTCAGGTAGttgccaactttcttgt	this paper
FWD ADAM17_KO_1	CACCGCTACAGATACATGGGCAGAG	Aikin et al., 2020
REV ADAM17_KO_1	aaacCTCTGCCCATGTATCTGTAGC	Aikin et al., 2020
MAP3K8 (ENSRNOG00000016378) Assembly 1 FWD	CACCGTATCTGACAGACGACAACCA	this paper
MAP3K8 (ENSRNOG00000016378) Assembly 1 REV	aaacTGGTTGTCGTCTGTCAGATAC	this paper
oAP194 papx MAP3K7-002 (ENST00000369329, CCDS5028) Assembly 2 FWD	CACCGCTGTAGACATGATCCCTCG	this paper

oap195 papx MAP3K7-002 (ENST00000369329, CCDS5028) Assembly 2 REV	aaacCGAGGGATCATGTCTACAGC	this paper