

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: 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 μ M), sorbitol (100 μ M), EGF (10 ng/mL), anisomycin (100 ng/mL), TNF α (10 ng/mL), or sphingosine-1-phosphate (100 μ M), 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. **b.** Serum-starves for 3 hours and quantified as described in methods. The 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 S2



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 μ M), sorbitol (100 μ M), EGF (10 ng/mL), anisomycin (100 ng/mL), TNF α (10 ng/mL), or sphingosine-1-phosphate (100 μ M), 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 μ g/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.

NIK	NIK ILK			TAOK3			TNNI3K			ZAK			TAK1			
ERK JNK	p38 El	RK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38
					AUDIO AND											
RAF1		Ν	MLK3			MLK1			DLK			MEKK3			MEKK2	
ERK JNK	p38 El	RK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38
MEKK1 kd			СОТ			ASK3			ASK1			ARAF			BRAF	
ERK JNK	p38 El	RK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38	ERK	JNK	p38
ASK2		١	YSK4		F	Parental		0 12 24 ti	l 12 24 me (hours	4 12 24)	0 12 2	4 12 2 time (hours	24 12 24 5)	10 12 2	4 12 2 time (hours	4 12 24 5)
ERK JNK	p38 El	RK	JNK	p38	ERK	JNK	p38									
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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)



0 1 3 6 12 18 time (h) COT GAPDH TAK GAPDH ZAK GAPDH ZAK GAPDH BRAF GAPDH

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: 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 μ g/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. 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: 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: 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 μ g/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 μ g/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. 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 μ M). 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), TNF α (10 ng/mL), sorbitol (100 μ M), or H₂O₂ (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: MAPK Network Map.

Schematic representing connectivity within the MAPK signaling network.

Name Sequence Source oAP6 F ZAKb STOP GATAATAGTGAATGATGCccaacttictigtacaaagtigg paper oAP6 F ZAKb STOP agaaagtiggGCATCATTCACTATTATCCATGTCATTATCCTC paper oAP10 FZAK CCTTCCTTTGAGATTGGTGCATGGAGCGG this oAP10 FZAK CCTTCCTTTGAGATTGGTGCATGGAGGAGACTTTTGATGCCATGATGAGGAGAACTTTTGATGCCATGAGGAGGAGAACTTTTGATGCCATGAGGAGGAGAACTTTTGATGCCATGAGGAGGAGAACTTTTGATGCCATGAGGAGGAGAACTTTTGATGCCATGAGGAGGAGAACTTTTGATGCCATGAGGAGGAGAGCTATCACGGGGA paper oAP212 F ZAKcomp attg paper paper oAP22 F AMPSK2 TCACCAGGTGATAATGCACAAACATGTGCCTTAAGGGTTCATCACGGGA paper STOP TCACCAGGGACTACGCCCCCCGCGCCTTCA this oAP25 F MAPSK2 ATGGTGGCGATGTCCCATGGCCGTTCA this MDDm GGGTGGGCGATGGCCATGGCCCATGGC paper MAPSK2 GGGTGGGCTGATGTCCCATGgQGCcatGatggC paper MAPSK2 TTCCttrgtgCtggCaggGGGAGCCGGCT this AP52 F MKK4ee GAAatgcCaAGGAGCGGGAGGCGGGCT paper oAP54 R MKK2ee CCTCACCAGGCTGAGGCACATGCG paper oAP54 R MKK2ee CCTCGCCTCTGGCTGGCTGCCCGGCGC paper oAP55 F MKK4ee GAGAAtgCCAAGGAGCGGGAGGCGGGCT <th colspan="7">Table S1: Oligonucleotides</th>	Table S1: Oligonucleotides						
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oAP113 F M3K6		this
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oAP115 F MAP3K14		paper this
OAP116 R MAP3K14	GAACAGGCCCTAAgccaactttcttgtacaaagttggcat	paper this
STOP 0AP117 F TAOK3	gttggcTTAGGGCCTGTTCTCCAGCTGGC	paper this
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STOP	gttggcAAGGTCATCTGTAGTCCTCCTTAGGAAAATCTAATGTAACCAAATTCC	paper
deltaN	actttgtacaaaaaagttggcaccatgcagggtaaacgcaaagcactgaagttgaatttt	paper
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oAP162 MKK7dd deltaN		this paper
Fwd MAP3K3dd		this
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Fwd MAP2K4dd	GACattgccaagacaagaGACgctggctgtaggccatacatggc	paper this
Rev MAP2K4dd	GTCtcttgtcttggcaatGTCgtccacaagctgtccactgatgcc	paper this
Fwd MAP2K7dd	GACAAAGCCAAGGACCGGAGCGCCGGCT	paper this
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