
Figures and figure supplements

MAPK activity dynamics regulate non-cell autonomous effects of oncogene expression

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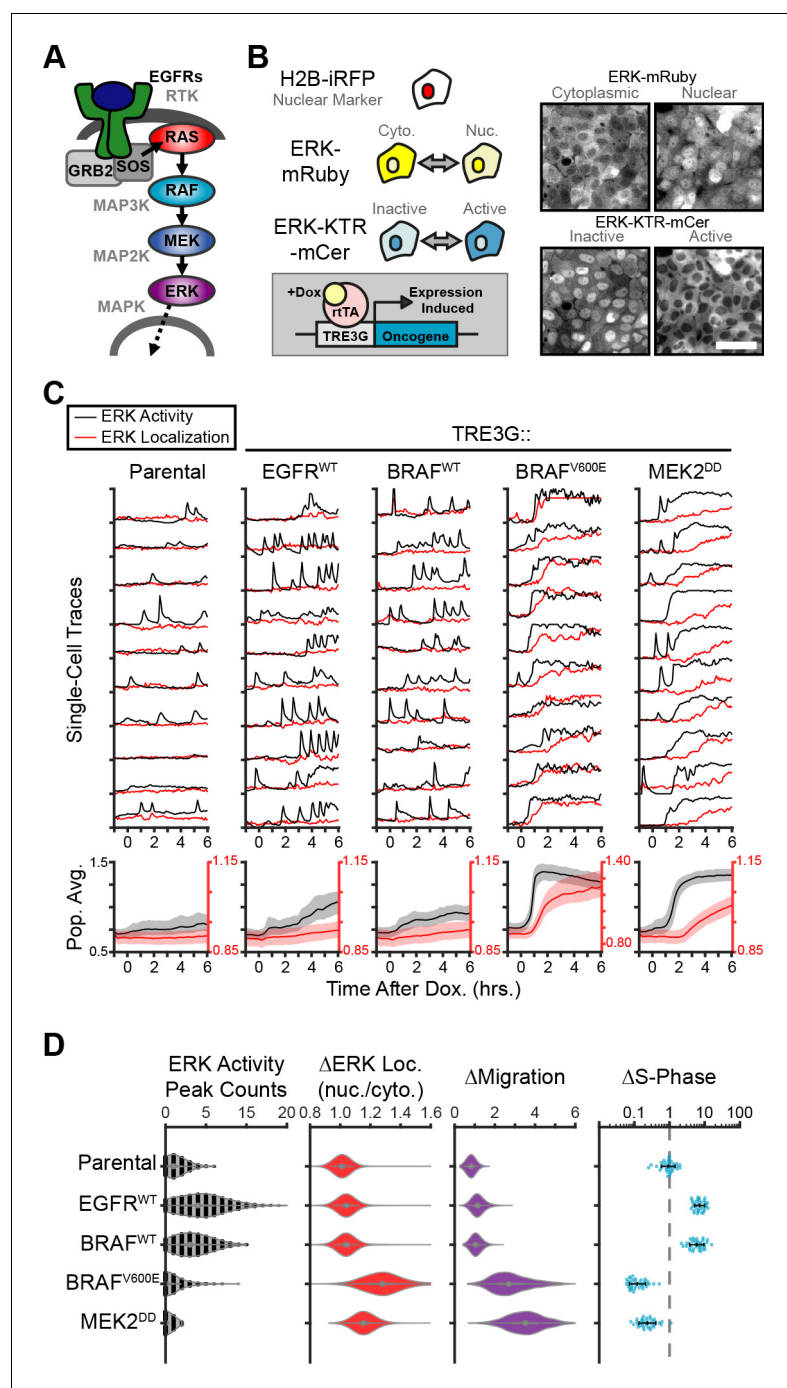


Figure 1. Oncogenic ERK signaling dynamics promote qualitatively different cell behaviors. (A) Schematic representation of the RTK/RAS/ERK signaling pathway. (B) MCF10A cells were transduced with lentiviral vectors expressing ERK KTR-mCerulean3 and ERK-mRuby2. The doxycycline inducible system (rTA and TRE3G) was used to drive the expression of oncogenes during live imaging. Representative images of cytoplasmic and nuclear ERK-mRuby2 (top) and inactive or active ERK as reported by ERK KTR-mCerulean3 (bottom). Scale bar = 50 μ m. (C) Cells described in B with indicated inducible oncogenes were imaged every 5 min for 6 hr upon doxycycline induction (2 μ g/ml) at $t = 0$. Single cells were analyzed as described in methods. Population averages represent more than 1000 cells per condition. Shaded regions indicate the 25th-75th percentiles. (D) Quantification of data obtained in C. Single-cell counts of ERK activity peaks after induction (6–12 hr), ERK kinase localization fold change (final N/C ratio over basal N/C ratio per cell), and cell migration (final over basal distance traveled per cell) were extracted as described in methods. For proliferation analysis the fraction of S phase cells was measured using Edu

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incorporation and the change over the no dox control was calculated and normalized to the mean of parental cells (dashed line) (see Materials and methods). Data represents 36 independent observations.

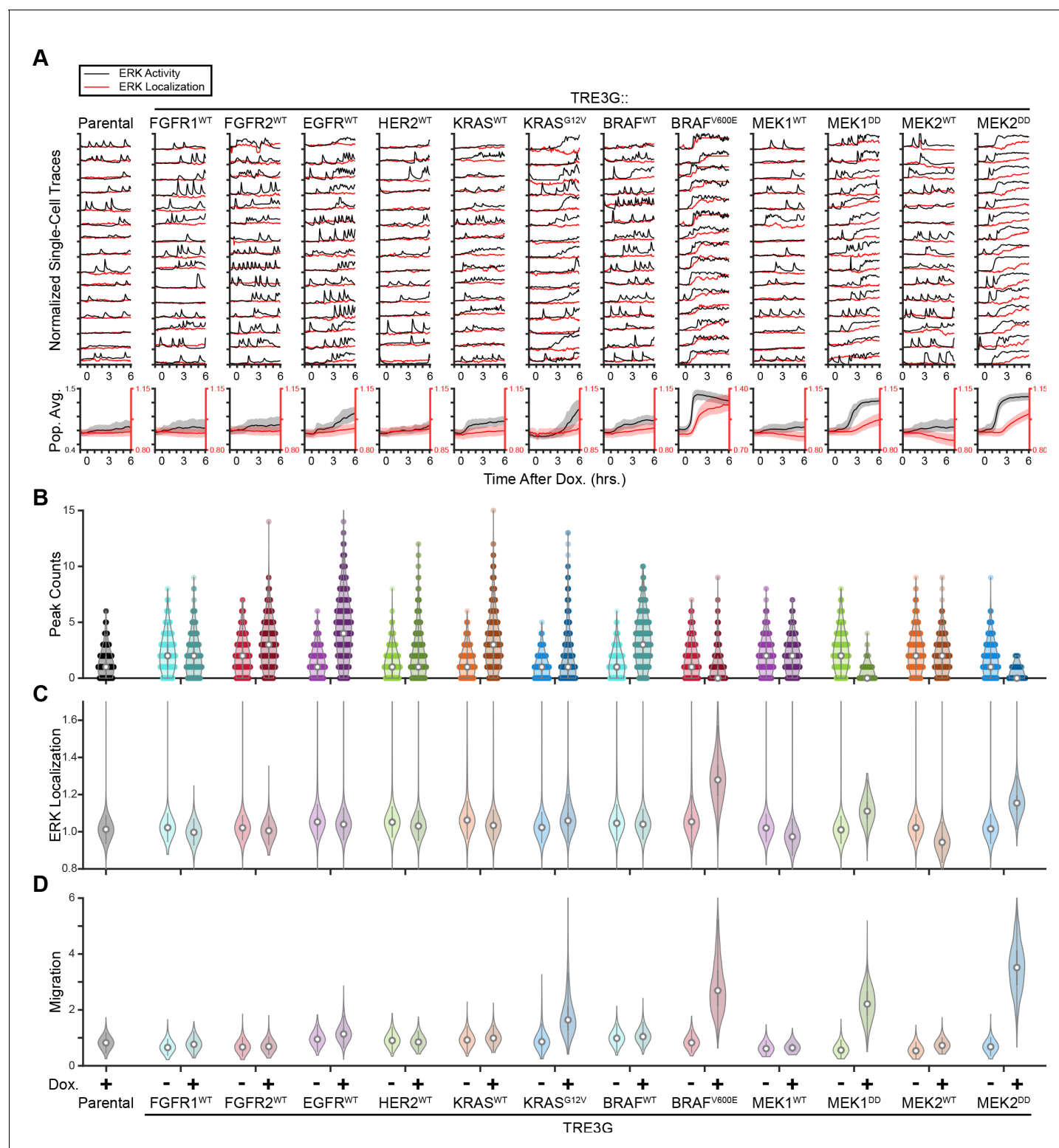


Figure 1—figure supplement 1. A screen for oncogenic effects on ERK dynamics and cell behavior. (A) Inducible cells expressing indicated genes treated and plotted as in 1C. Single traces and population data are reproduced for oncogenes appearing in **Figure 1**. (B–D) Single-cell peaks, ERK nuclear localization, and migration are quantified directly from imaging data as in 1D for indicated inducible cells in the presence or absence of doxycycline (2 μ g/ml). Data appearing in **Figure 1** is reproduced.

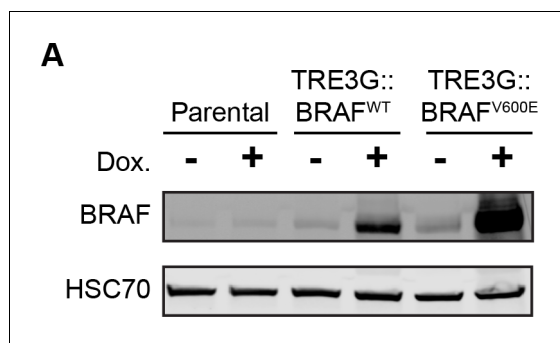


Figure 1—figure supplement 2. Relative expression of inducible genes. (A) Parental, BRAF^{WT}, and BRAF^{V600E} inducible cells were grown in the presence or absence of Dox (2 μ g/ml) for 24 hr before sample collection and western blot for BRAF and HSC70 (see Materials and methods).

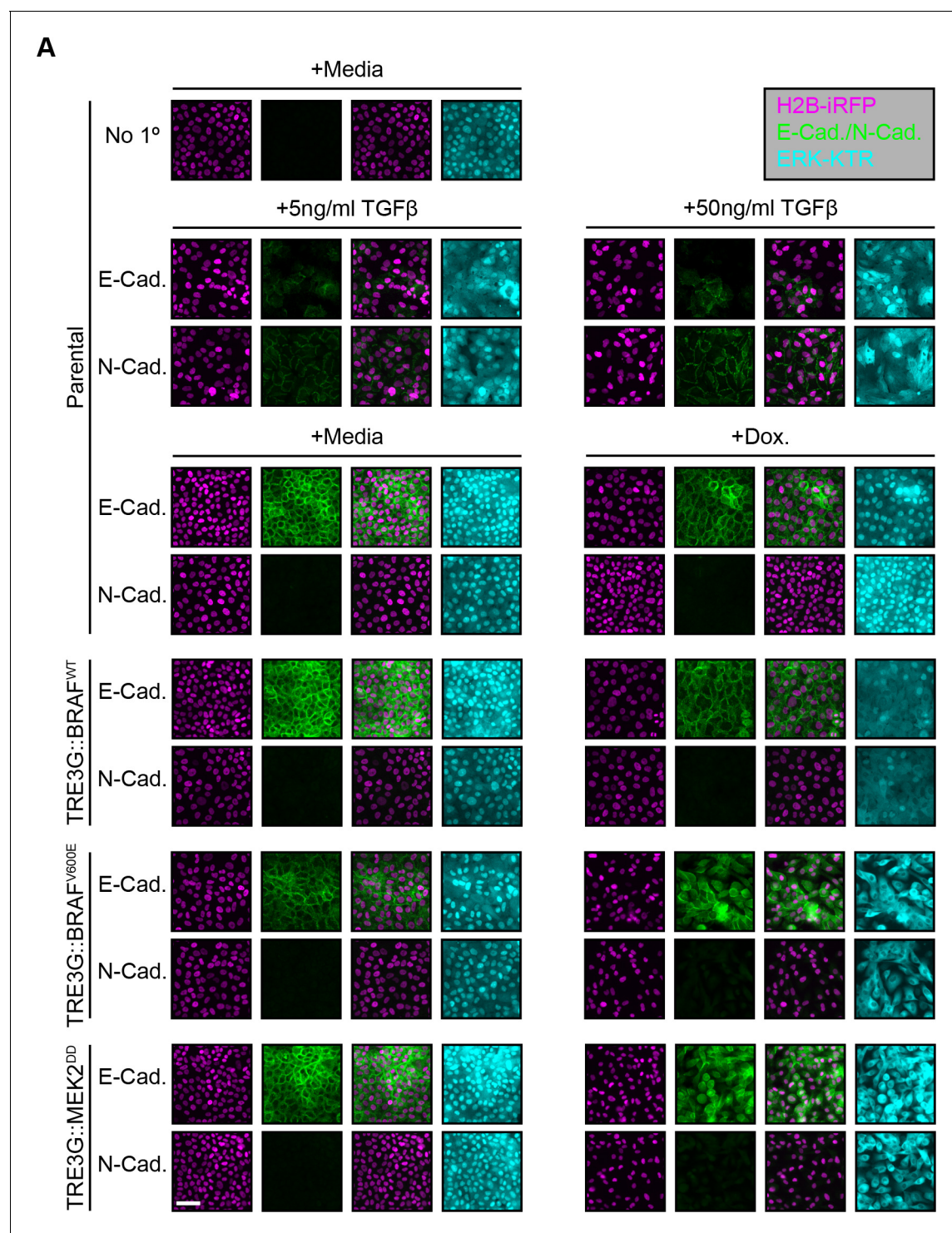


Figure 1—figure supplement 3. Oncogene-induced cell behaviors are distinct from epithelial-to-mesenchymal transition (EMT). (A) Indicated parental or inducible MCF10As were grown in the presence or absence of doxycycline (+Dox., 2 μ g/ml) for 24 hr before fixing for immunofluorescence using primary antibodies for E-Cadherin (E-Cad) or N-Cadherin (N-Cad), see Materials and methods. Staining is compared to parental cells treated with TGF β continuously for several passages over 10 days to induce EMT (5 ng/ml or 50 ng/ml). Nuclear marker (H2B-iRFP, magenta), IF staining (green), an overlay of nuclear marker with IF staining, and ERK activity (ERK-KTR, cyan) are shown. Scale bar = 100 μ m.

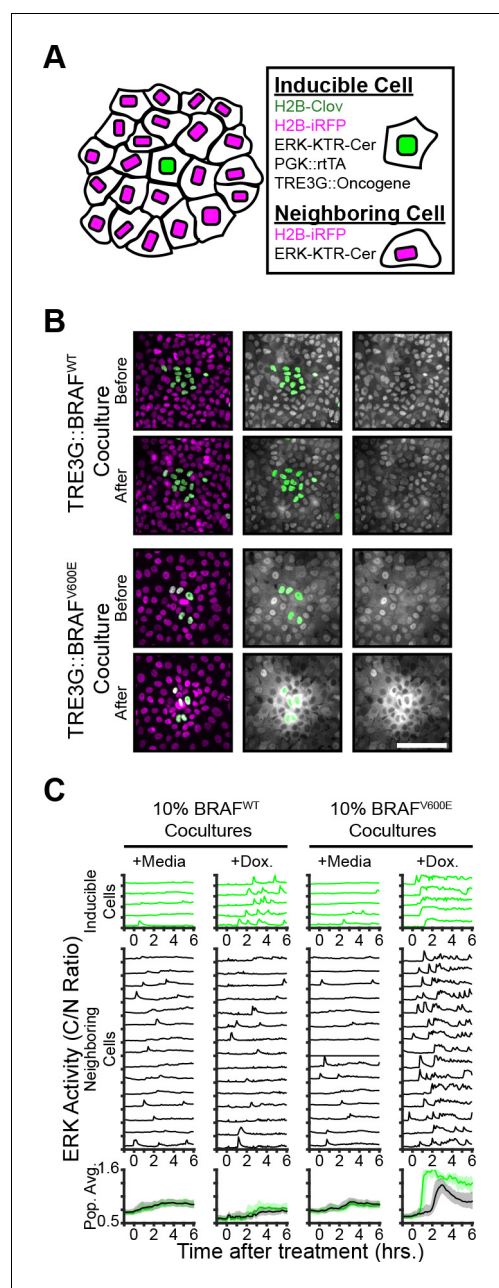


Figure 2. Oncogene induction results in dynamics-dependent paracrine ERK signaling. (A) Schematic representation of coculture assay. H2B-iRFP (magenta) and ERK KTR are expressed in all cells for segmentation and quantification. H2B-mClover (green) was used to label inducible cells. (B) BRAF^{WT} or BRAF^{V600E} inducible cells were cocultured at 10% with ERK KTR cells and treated with doxycycline (2 μ g/ml). Representative images are shown. Scale bar = 100 μ m. (C) BRAF^{WT} or BRAF^{V600E} cocultures, as in B, were treated with vehicle (+Media) or with doxycycline (+Dox, 2 μ g/ml). Single cells were quantified as described in methods. ERK activity traces in inducible (top, green) and neighboring cells (bottom, black) are shown. Population averages and 25th-75th percentiles

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(shaded) are shown for $n > 450$ cells per coculture condition.

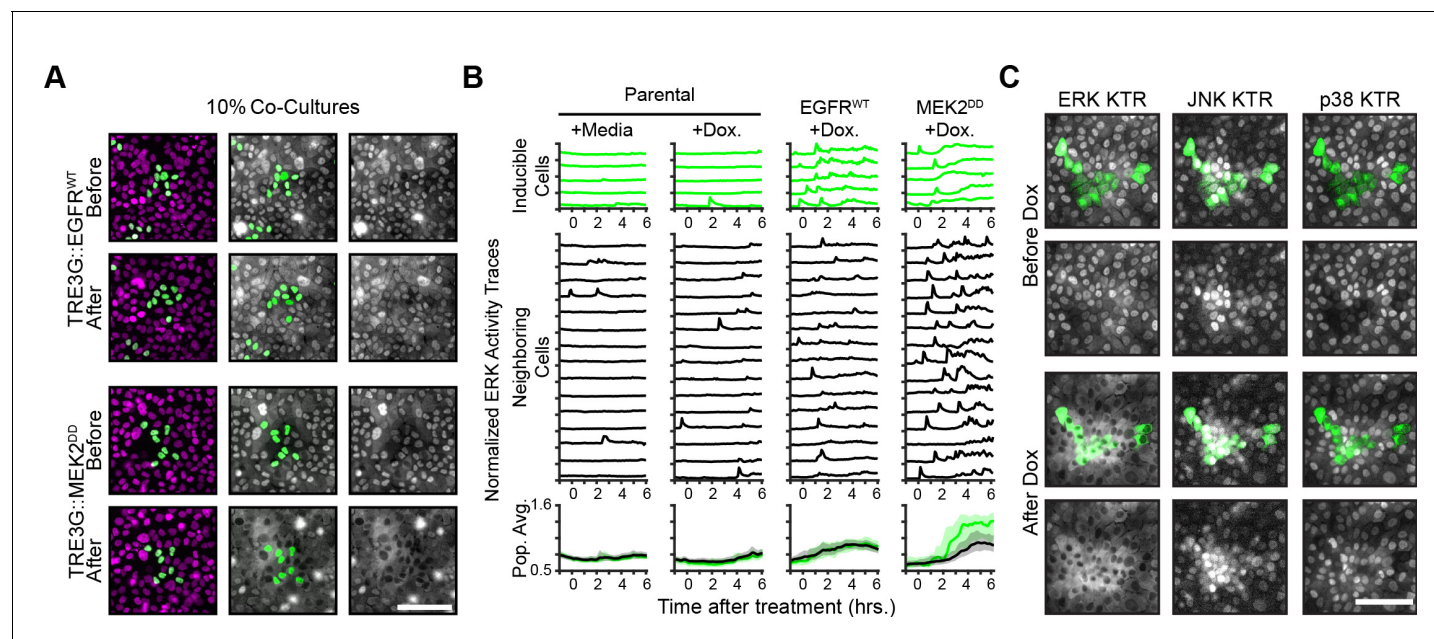


Figure 2—figure supplement 1. ERK dynamics-dependency of paracrine ERK activation. (A) Images from cocultures of inducible EGFR^{WT} and MEK2^{DD} cells presented as in 2B. Scale bar = 100 μ m. (B) ERK activity traces from cocultures of indicated cells treated as in 2C. Parental cells have an H2B-mClover nuclear marker without any inducible gene system. Population averages and 25th-75th percentiles (shaded region) shown for $n > 1000$ cells. (C) The TRE3G-BRAF^{V600E} construct was expressed in a cell line containing ERK KTR-mCerulean3, p38 KTR-mClover, and JNK KTR-mRuby2. These cells were incubated with CellTracker Deep Red dye (green, Invitrogen) and cocultured with unlabeled neighboring cells. Images display the activity of ERK, p38, and JNK before and 6 hr after addition of doxycycline (2 μ g/ml). Scale bar = 100 μ m.

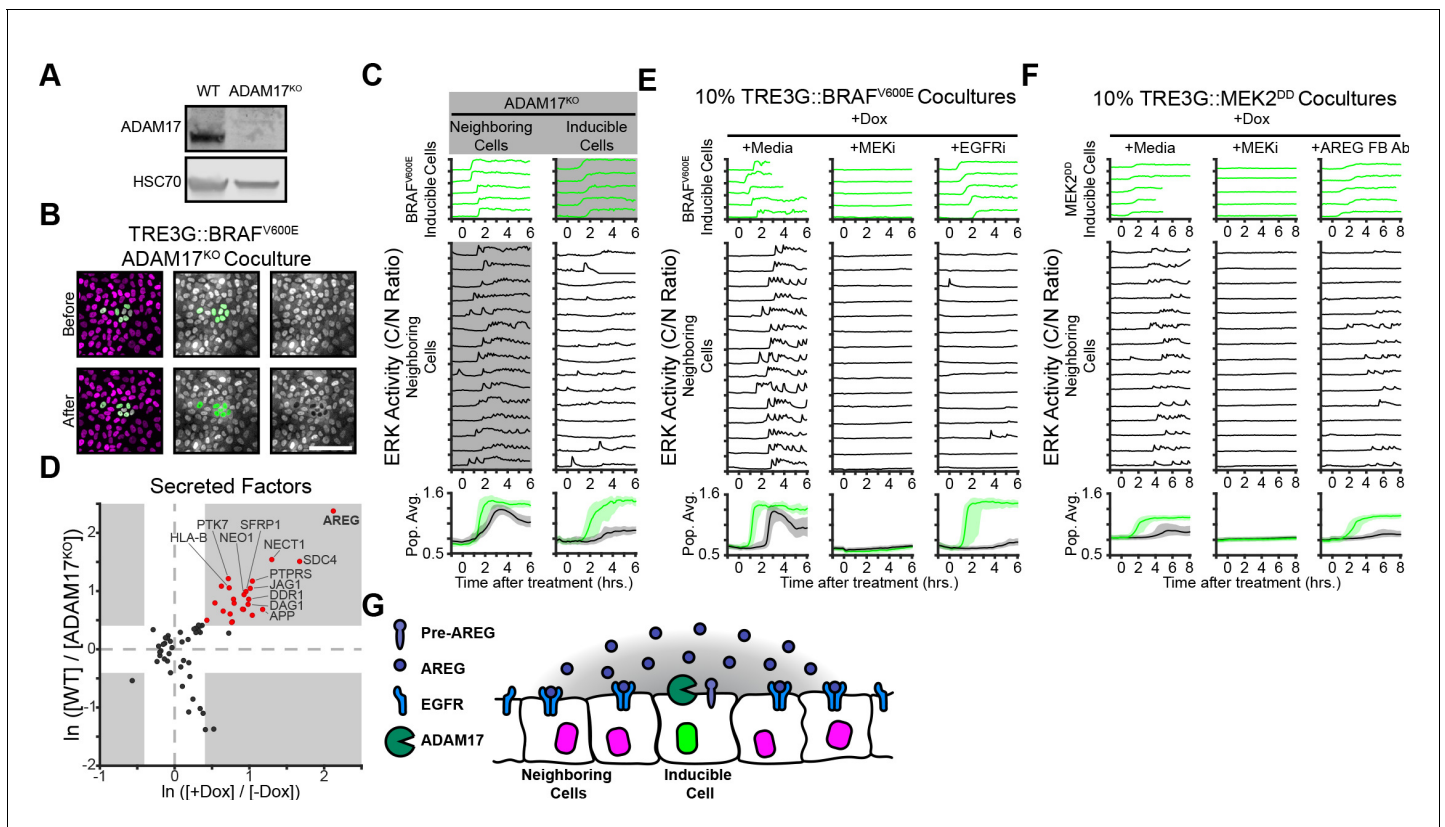


Figure 3. ERK activity waves require ADAM17 release of AREG and neighboring cell EGFRs. (A) Immunoblot against ADAM17 and HSC70 in WT and ADAM17^{KO} cells generated by CRISPR-Cas9 editing (see Materials and methods for details). (B) Representative images of ADAM17^{KO} BRAF^{V600E} inducible cells cocultured and treated as in **Figure 2B**. (C) ADAM17^{KO} cells (gray boxed traces) were used as inducible cells (right) or neighboring cells (left) in cocultures. Data for $n > 1100$ cells is presented as in **Figure 2C**. (D) ADAM17 substrates profiled by TMT mass spectrometry. Supernatants from ADAM17^{KO} or WT cells expressing (+Dox) or not expressing (-Dox) BRAF^{V600E} were collected and analyzed by Tandem-Mass-Tag (TMT) mass spectrometry as described in methods. Scatter plots show the natural log of fold change values of all statistically significant ($p < 0.05$) proteins in both WT vs. ADAM17^{KO} and +Dox vs. -Dox comparisons. Grey boxes indicate > 1.5 fold change. (E) BRAF^{V600E} co-cultured monolayers were plated as in **Figure 2C** and pretreated with indicated inhibitors (MEKi, 5 μ M PD0325901; EGFRi, 5 μ M Gefitinib) for one hour before induction with doxycycline (2 μ g/ml). Representative single cell traces and population averages for $n > 1000$ cells are shown as in **Figure 2C**. (F) MEK2^{DD} co-cultured monolayers were plated as in **Figure 2C** and pretreated with indicated inhibitors (MEKi, 5 μ M PD0325901; AREG FB Ab, 50 μ g/ml function-blocking antibody) for one hour before induction with doxycycline (2 μ g/ml). Representative single cell traces and population averages for $n > 1000$ cells are shown as in **Figure 2C**. (G) Schematic representation of ADAM17-AREG-EGFR paracrine signaling.

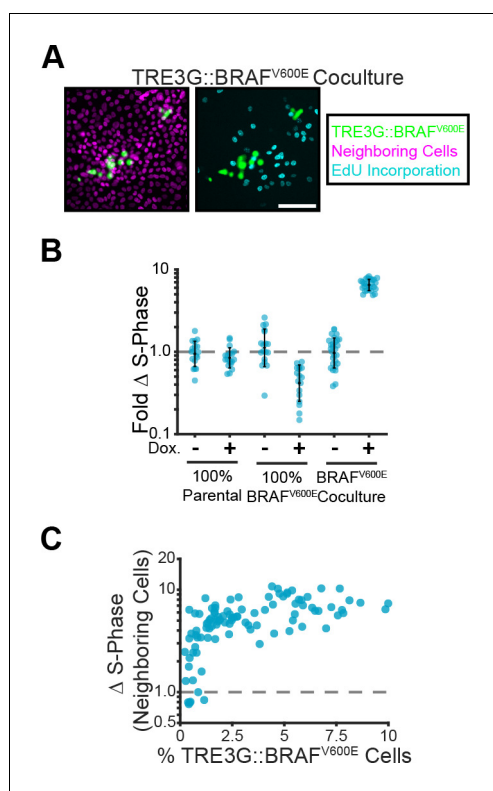


Figure 4. Paracrine ERK signaling leads to non-cell autonomous proliferation. **(A)** Representative images of BRAF^{V600E} cocultures treated with doxycycline and EdU as described in methods. Inducible cell nuclei (H2B-mClover, green), all nuclei (H2B-iRFP, magenta) and EdU staining (cyan) are shown. Scale bar = 100 μ m. **(B)** Indicated monolayers were cultured and incubated with or without doxycycline for 24 hr. The change in S-phase cell fractions was determined by EdU incorporation as described in methods and normalized to parental mean (dashed line). Bar represents mean and standard deviation for $n \geq 16$ observations. **(C)** Inducible BRAF^{V600E} cocultures were plated at different proportions and labelled with EdU as in A. The fold-change in S-phase cell fractions is plotted against the percent of BRAF^{V600E}-expressing cells for each position. 98 total observations shown.

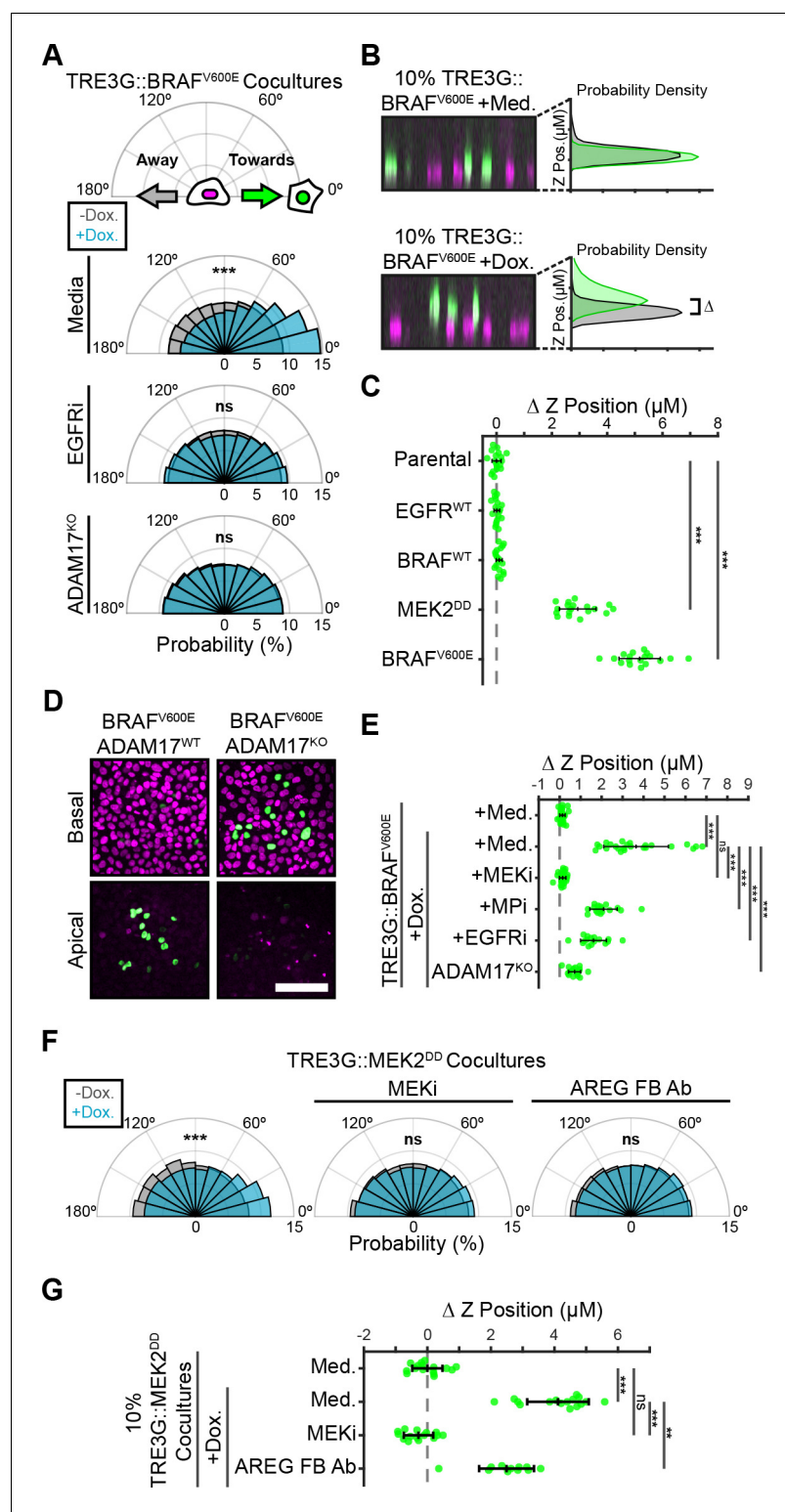


Figure 5. Paracrine ERK activation coordinates extrusion of aberrantly signaling cells through directed migration of the neighboring epithelium. (A) Inducible BRAF^{V600E} cells (WT or ADAM17^{KO}) were plated in 1% cocultures and treated with doxycycline (2 μg/ml) in the presence or absence of EGFR inhibitor gefitinib (5 μM) as indicated. Radial histograms represent migration angle distributions of neighboring cells before (grey) and 2–6 hr after (cyan) induction (see Materials and methods). Data represents angles from $n > 1000$ cells from 10 independent observations per condition. Data was assessed using subsampling and a two-sample KS test with 'ns' not

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significant, $***p < 0.001$ (see Materials and methods). (B) 10% BRAF^{V600E} cocultured monolayers were seeded as described in methods. After 24 hr with doxycycline (2 $\mu\text{g/ml}$), monolayers were imaged by spinning disk confocal. Representative orthogonal Z projections and probability densities for nuclear height of inducible (green) and neighboring (grey) cells are shown (see methods). Extrusion (ΔZ) is calculated as the height difference between gaussian-fitted maxima of the green and black distributions. (C) 10% cocultures of indicated parental or inducible cells were treated with 24 hr doxycycline (2 $\mu\text{g/ml}$), imaged, and analyzed as in B. Data represents difference in nuclear height (ΔZ) for $n = 18$ observations normalized to the mean height of parental cells (dashed line), with mean and \pm standard deviation (black bars). Significance was calculated by two-sample t-test with 'ns' indicating no significance, $***p < 0.001$. (D) Representative basal and apical images (+6 μm) of WT or ADAM17^{KO}, BRAF^{V600E} inducible cells (green) in WT monolayers (red) after 24 hr of doxycycline treatment. (E) 10% BRAF^{V600E} cocultures were pretreated with inhibitors (MEKi, 5 μM PD0325901, MPi, 5 μM Batimastat, EGFRi, 5 μM Gefitinib) and 24 hr doxycycline (2 $\mu\text{g/ml}$) or media, imaged and analyzed as in B. Data represents difference in nuclear height (ΔZ) for $n \geq 16$ independent observations presented as in C. (F) Inducible MEK2^{DD} cells were plated in 1% cocultures and treated with doxycycline (2 $\mu\text{g/ml}$) in the presence of MEK inhibitor (MEKi, 5 μM PD0325901) or amphiregulin function-blocking antibody (AREG FB Ab, 50 ng/ml) as indicated. Radial histograms are presented as in A for angles of $n > 100$ cells from two to three independent observations per condition. Data was assessed using subsampling and a two-sample KS test with 'ns' not significant, $***p < 0.001$ (see methods). (G) 10% MEK2^{DD} cocultures were pretreated with MEK inhibitor (MEKi, 5 μM PD0325901) or Amphiregulin function-blocking antibody (AREG FB Ab, 50 ng/ml) and 24 hr doxycycline (2 $\mu\text{g/ml}$) or media, as indicated, then imaged and analyzed as in B-C. Data represents difference in nuclear height (ΔZ) for $n \geq 11$ independent observations normalized to the mean height of media-treated MEK2^{DD} cells (dashed line), with mean and \pm standard deviation (black bars). Significance was calculated by two-sample t-test with 'ns' indicating no significance, $**p < 0.01$, and $***p < 0.001$.

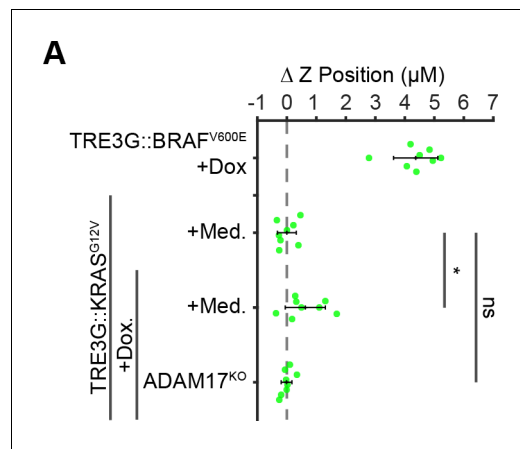


Figure 5—figure supplement 1. KRAS^{G12V} expressing cells do not extrude at 24 hr. (A) 10% BRAF^{V600E} or KRAS^{G12V} (WT or ADAM17^{KO}) cocultures were treated with 24 hr doxycycline (2 $\mu\text{g/ml}$) or media, imaged and analyzed as in **Figure 5B**. Data represents difference in nuclear height (ΔZ) for $n = 8$ independent observations normalized to the mean height of un-induced KRAS^{G12V} cells (dashed line), with mean and +/- standard deviation (black bars). Significance was calculated by two-sample t-test with 'ns' indicating no significance, * $p < 0.05$.

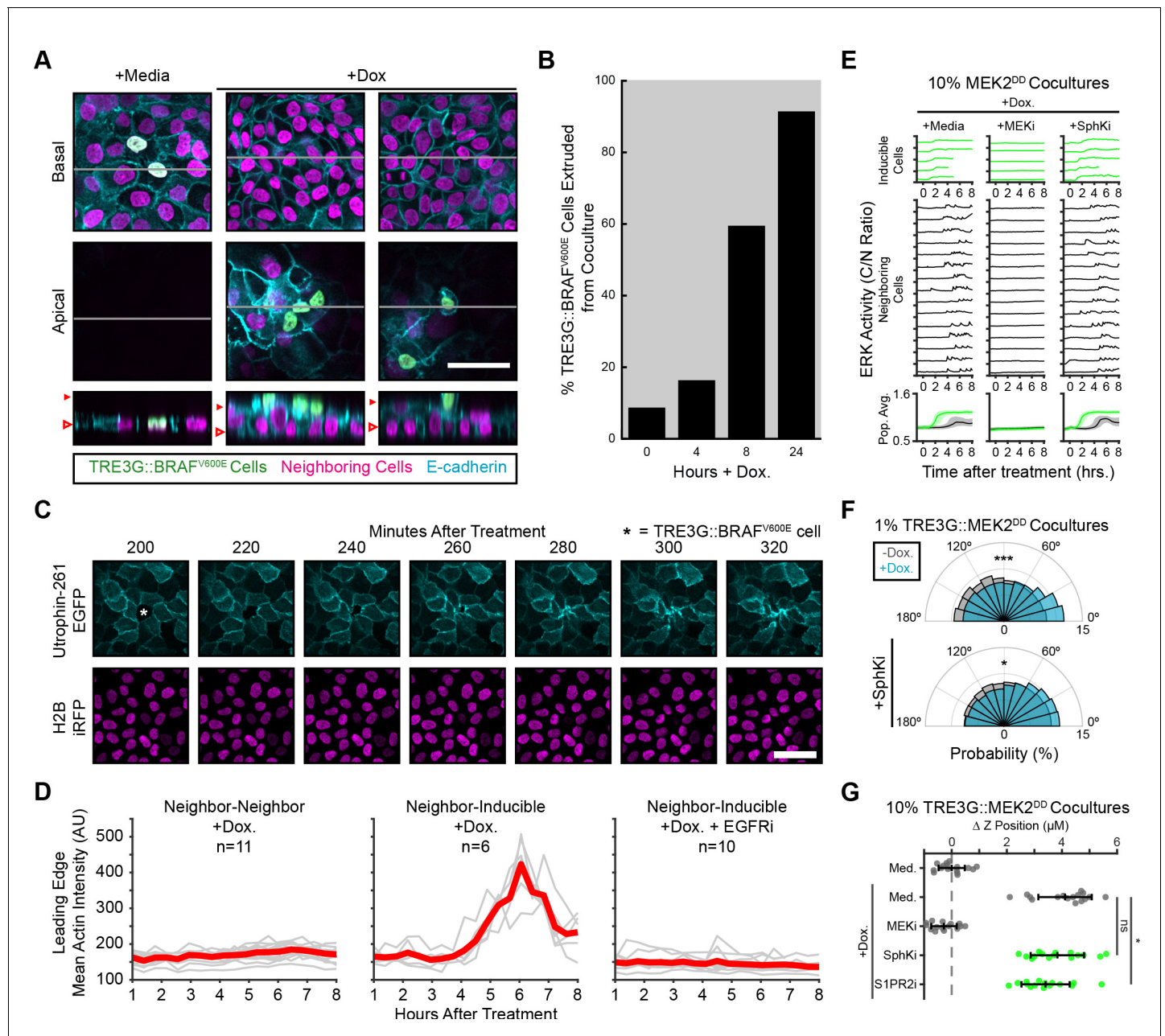


Figure 5—figure supplement 2. Actin dynamics and sphingosine kinase requirements during oncogenic cell extrusion. (A) Representative images of immunofluorescence for E-cadherin (cyan) from 1% BRAF^{V600E} cocultures treated for 24 hr with doxycycline (2 µg/ml) or media imaged by confocal. TRE3G::BRAF^{V600E} cells carry H2B-mClover nuclear markers (green), while neighboring cells have only H2B-iRFP (magenta). A basal and an apical plane are shown for each position, while the bottom shows an XZ orthogonal slice from the indicated position (grey line) in the above images. Orthogonal view shows positions of basal (hollow triangle) and apical planes (solid triangle). Scale bar = 50 µm. (B) Manual quantification of fully extruded TRE3G::BRAF^{V600E} cells from cocultures induced for indicated times (0, 4, 8, 24 hr) with doxycycline (2 µg/ml) after membrane immunofluorescence staining of E-Cadherin. In order to be considered fully extruded rather than elongated, cells must have no evidence of basal attachment. Data represents $n \geq 35$ cells. (C) Time-course of actin dynamics and basal protrusions of neighboring cells in 1% BRAF^{V600E} coculture imaged with Utrophin-261-EGFP (cyan, top, see **Figure 5—Video 2**). TRE3G::BRAF^{V600E} cell is indicated by bold asterisk (*), and H2B-iRFP (bottom, magenta) from cells in the plane of the monolayer are shown to observe timing of extrusion. Scale bar = 50 µm. (D) Quantification of actin enrichment from BRAF^{V600E} cocultures. Median actin intensities (AUs) from randomly selected neighbor-neighbor cell borders are compared to the leading edges of all neighboring cells adjacent to TRE3G::BRAF^{V600E} cells that were treated with doxycycline alone (2 µg/ml, from images in C) or with doxycycline plus EGFR inhibitor (Gefitinib, 5 µM). See Materials and methods for details. (E) MEK2^{DD} cocultures were plated as in 2B, and treated with doxycycline (+Dox, 2 µg/ml) in the presence of media or inhibitors (MEKi, 5 µM PD0325901; SphKi, 10 µM SKI2). Single-cell ERK activity traces are presented as in 2C with population averages and 25th-75th percentiles (shaded) shown for $n > 1000$ cells per coculture condition. (F) Neighboring cell migration angles from 1% Inducible MEK2^{DD} cells. (G) 10% TRE3G::MEK2^{DD} Cocultures. Scatter plot showing the Δ Z Position (µm) for +Dox. conditions: Med., MEKi, SphKi, and S1PR2i. The SphKi condition shows a significant increase in Δ Z Position compared to the other conditions.

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cocultures treated with doxycycline (2 $\mu\text{g/ml}$) in the presence or absence of Sphingosine Kinase inhibitor (5 μM SKI). Data represents angles from $n > 100$ cells from two independent observations per condition presented as in 5A, and Dox condition reproduced from **Figure 5F** for comparison. Data was assessed using subsampling and a two-sample KS test with $*p < 0.05$, $***p < 0.001$ (see Materials and methods). (G) Extrusion from 10% MEK2^{DD} cocultures pretreated with Sphingosine-1-Phosphate pathway inhibitors (SphKi, 10 μM SKI; S1PR2i, 10 μM JTE-013) and 24 hr doxycycline (2 $\mu\text{g/ml}$) or media, imaged and analyzed as in 5B-D (green). Some conditions reproduced from **Figure 5** for comparison (grey). Data represents difference in nuclear height (ΔZ) for $n \geq 15$ independent observations normalized to the mean height of parental cells (dashed line), with mean and \pm standard deviation (black bars). Significance was calculated by two-sample t-test with 'ns' indicating no significance, $*p < 0.05$.

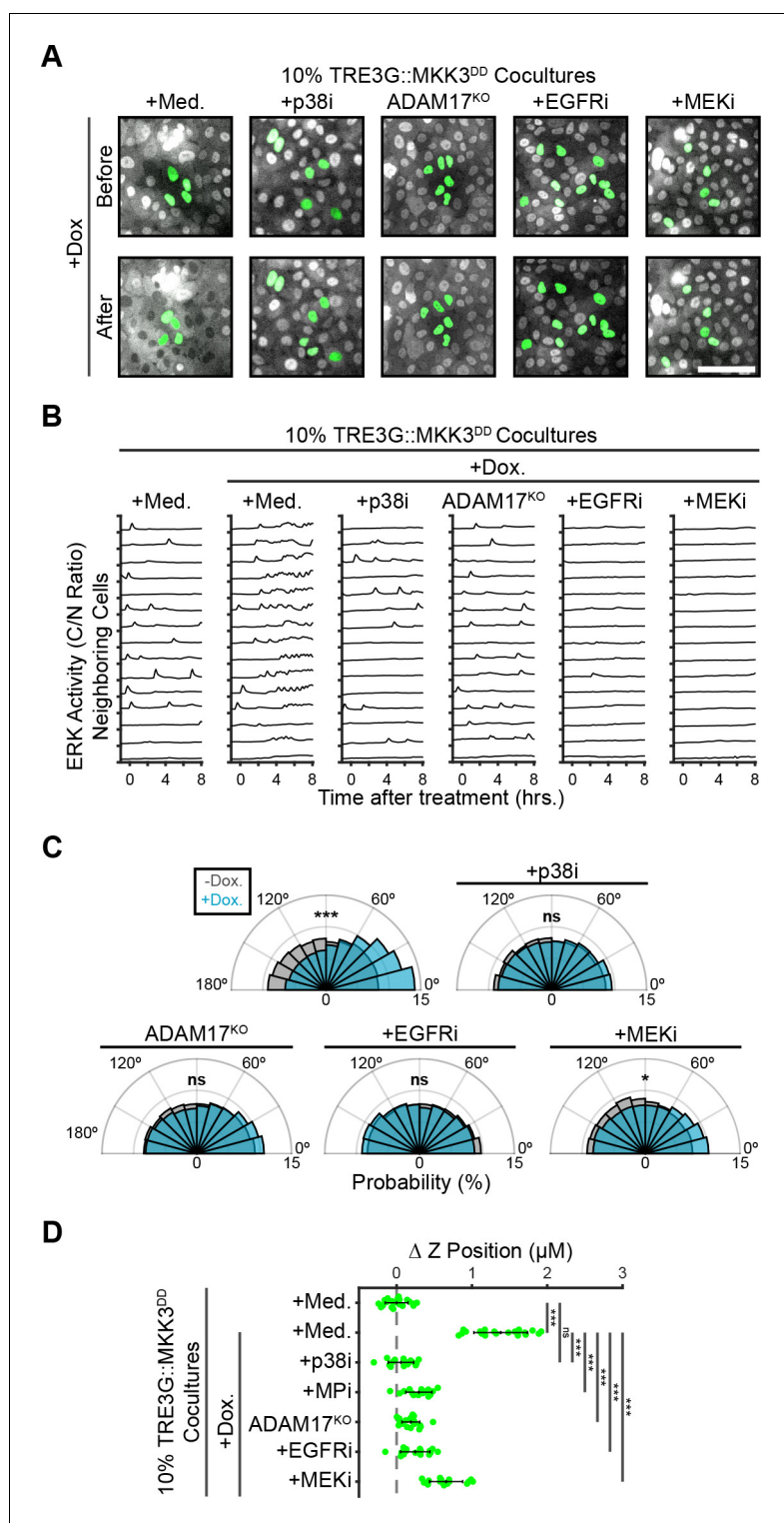


Figure 6. ERK activity in neighboring cells is required for coordinating extrusion. (A) Representative images showing WT or ADAM17^{KO} cells with inducible MKK3^{DD} (green), cocultured at 10% with neighboring ERK-KTR cells (grey). Cocultures were treated with doxycycline (2 μg/ml) in the presence of media, p38 inhibitor (5 μM BIRB-796), EGFR inhibitor (5 μM Gefitinib), or MEK inhibitor (5 μM PD 0325901). Scale bar = 100 μm. (B) ERK activity traces of neighboring cells in coculture with MKK3^{DD}-inducible cells (WT or ADAM17^{KO}) plated at 10%, pretreated with inhibitors (p38i, 5 μM BIRB 796; EGFRi, 5 μM Gefitinib; MEKi, 5 μM PD 0325901) and doxycycline (2 μg/ml) or media, and imaged as in **Figure 2C**. 15 representative neighboring cell ERK activity traces are shown for each

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condition. (C) Inducible MKK3^{DD} cells (WT or ADAM17^{KO}) were plated in 1% cocultures and treated with doxycycline (2 µg/ml) in the presence or absence of inhibitors. Radial histograms of migration angles before (grey) and 6–9 hr after (cyan) induction presented as in **Figure 5A**. Data represents angles from $n > 900$ cells from ≥ 6 observations per condition assessed using subsampling and a two-sample KS test with 'ns' not significant, * $p < 0.05$, *** $p < 0.001$ (see Materials and methods). (D) 10% MKK3^{DD} cocultures were pretreated with inhibitors (p38i, 5 µM BIRB 796; EGFRi, 5 µM Gefitinib; MEKi, 5 µM PD 0325901) and 24 hr doxycycline (2 µg/ml) or media, imaged and analyzed as in **Figure 5B–D**. Data represents difference in nuclear height (ΔZ) for $n \geq 16$ observations normalized to the mean height of parental cells (dashed line), with mean and +/- standard deviation (black bars). Significance was calculated by two-sample t-test with 'ns' indicating no significance, *** $p < 0.001$.

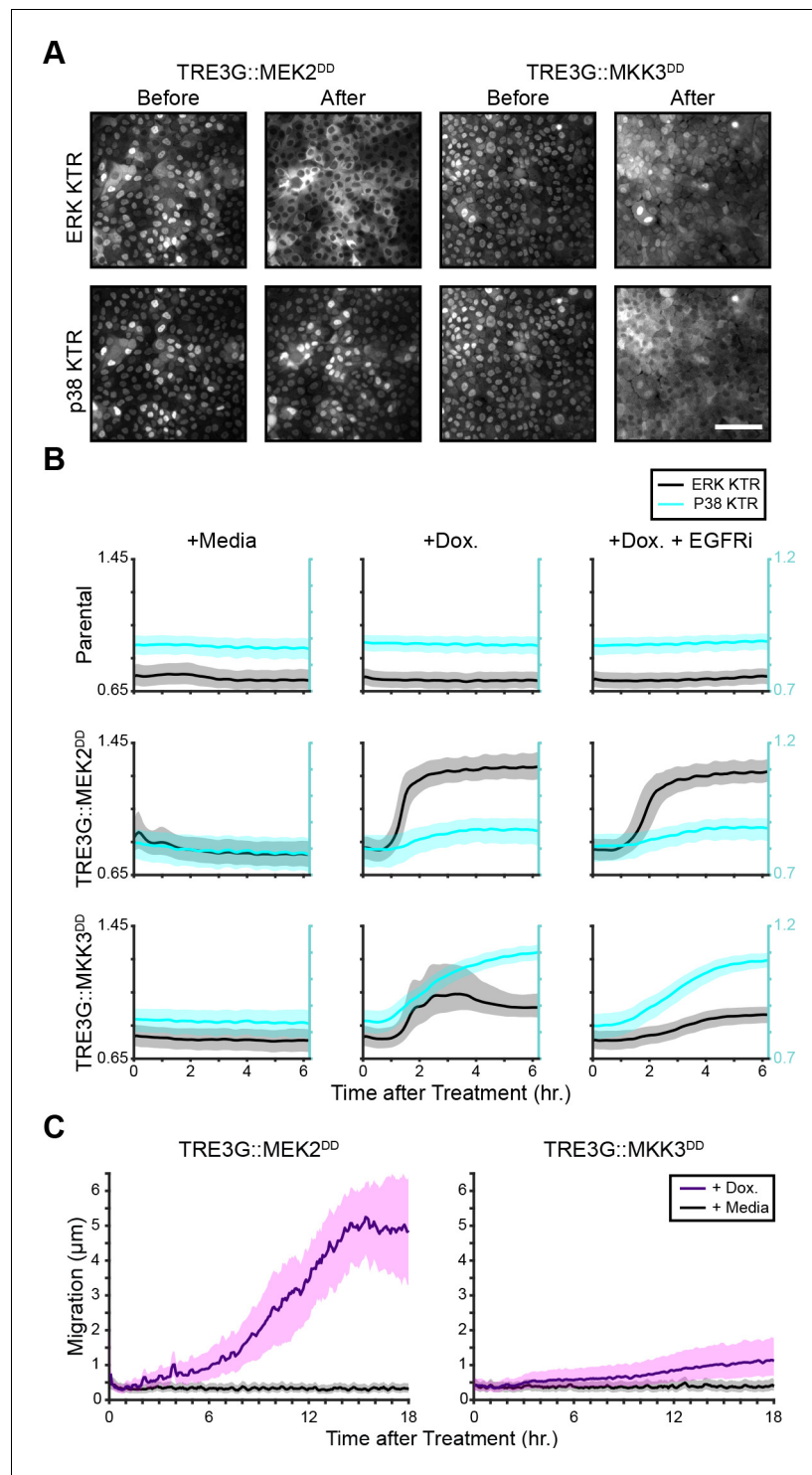


Figure 6—figure supplement 1. MAPK specificity of MEK2^{DD} and MKK3^{DD}. (A) Representative images from inducible MEK2DD and MKK3DD cells expressing H2B-iRFP, ERK KTR-mCerv3 and p38 KTR-mClov before and after induction with doxycycline (2 μg/ml). Scale bar = 100 μm. (B) Population average traces of ERK (black) and p38 (cyan) activity for indicated parental and inducible ERK/p38 KTR cells from A. Cocultures were treated with media, doxycycline, or doxycycline with EGFR inhibitor (5 μM gefitinib) to limit paracrine ERK activation. Population averages and 25th-75th percentiles (shaded regions) are shown for n > 2000 cells. (C) Migration of inducible cells from B plotted as μm change for every 5 min timepoint.

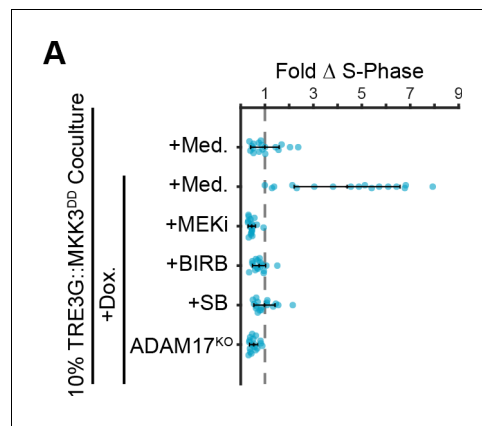


Figure 6—figure supplement 2. Mosaic p38 activation leads to ADAM17-EGFR -ependent proliferation of neighboring cells. (A) EdU of 10% MKK3^{DD} inducible cell cocultures treated for 24 hr with vehicle (+Media) or doxycycline (2 μ g/ml) in the presence of MEK inhibitor (MEKi, 5 μ M PD 0325901), p38 inhibitors (5 μ M BIRB 796 or 25 μ M SB 203580) or in ADAM17^{KO} cells. Data presented as in 4B for $n \geq 16$ observations. See Materials and methods for details about EdU incorporation.

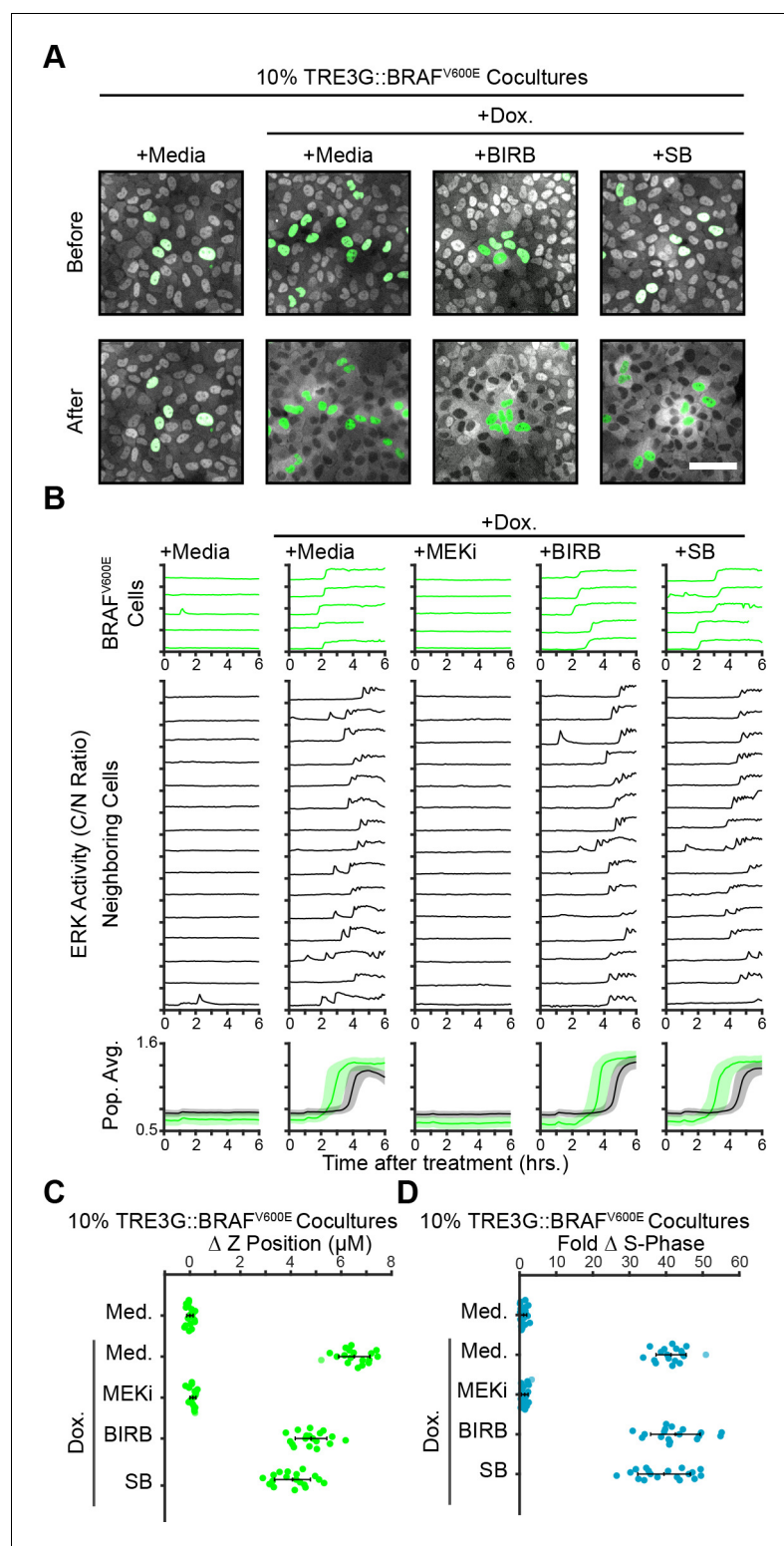


Figure 6—figure supplement 3. Oncogene-induced paracrine ERK activity and resulting cell behaviors are p38-independent. (A) Representative images from 10% TRE3G::BRAF^{V600E} cocultures plated as in 2B before and after treatment with media or doxycycline (2 μ g/ml) alone or with p38 inhibitors (5 μ M BIRB 796 or 25 μ M SB 203580). Scale bar = 100 μ m. (B) ERK activity traces, averages and 25th-75th percentiles from inducible BRAF^{V600E} cocultures from a represented as in 2C for $n > 1600$ cells. (C) Extrusion of BRAF^{V600E} inducible cells from 10% cocultures treated as indicated and presented as in 5C for $n = 18$ observations. (D) EdU incorporation in 10% BRAF^{V600E} cocultures treated as indicated and presented as in 5D for $n = 18$ observations. Figure 6—figure supplement 3 continued on next page

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cocultures treated as indicated and presented as in **Figure 4B** for $n = 18$ observations (see Materials and methods).

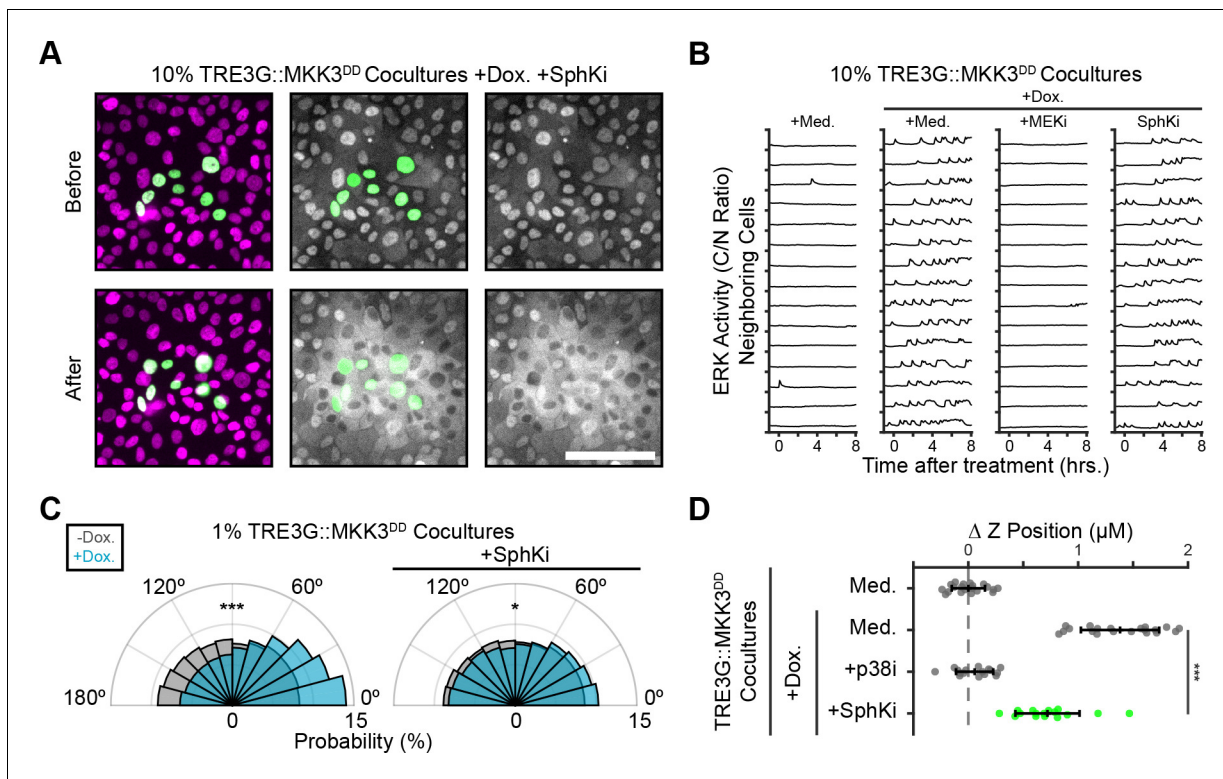


Figure 6—figure supplement 4. Partial involvement of S1P signaling in extrusion of p38-active cells. (A) Representative images from MKK3^{DD} cocultures pretreated with sphingosine kinase inhibitor (SphKi, 10 μM SKI) before induction with doxycycline (2 $\mu\text{g}/\text{ml}$). Scale bar = 100 μm . (B) ERK activity traces of neighboring cells in coculture with MKK3^{DD} inducible cells plated at 10%, pretreated with inhibitors (MEKi, 5 μM PD 0325901; SphKi, 10 μM SKI) and doxycycline (2 $\mu\text{g}/\text{ml}$) or media, imaged as in 2C and presented as in 6B. 15 representative neighboring cell ERK activity traces are shown for each condition. (C) Inducible MKK3^{DD} cells were plated in 1% cocultures and treated with doxycycline (2 $\mu\text{g}/\text{ml}$) in the presence of Sphingosine Kinase inhibitor (10 μM SKI). Radial histograms of migration angles before (grey) and after (cyan) induction presented as in **Figure 6C**, and the +Dox alone condition (left) is reproduced from **Figure 6C** for comparison. Data represents angles from $n > 900$ cells from ≥ 6 observations per condition assessed using subsampling and a two-sample KS test with $*p < 0.05$, $***p < 0.001$ (see methods). (D) 10% MKK3^{DD} cocultures were pretreated with inhibitors (p38i, 5 μM BIRB 796; SphKi, 10 μM SKI) and 24 hr doxycycline (2 $\mu\text{g}/\text{ml}$) or media, imaged and analyzed as in 5B-D. Data represents difference in nuclear height (ΔZ) for $n \geq 16$ observations normalized to the mean height of parental cells (dashed line), with mean and \pm standard deviation (black bars). Some conditions are reproduced from **Figure 6D** for comparison (grey). Significance was calculated by two-sample t-test with $***p < 0.001$.

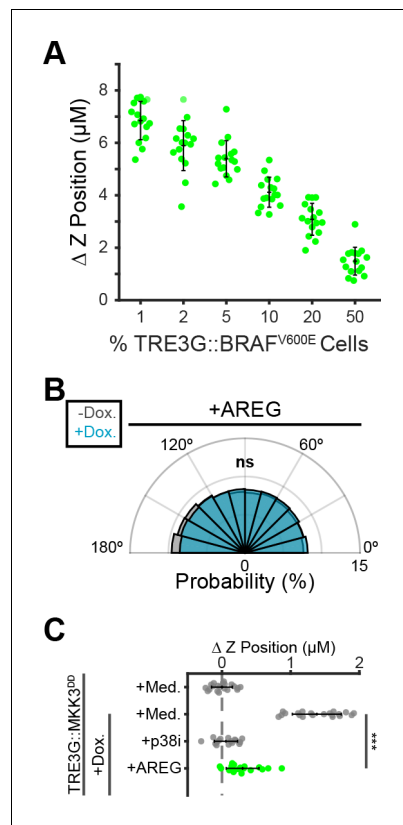


Figure 7. Localized paracrine signals coordinate directed migration and extrusion. (A) Inverse relationship between fraction of oncogenic cells in coculture and extrusion efficiency. Inducible BRAF^{V600E} cells were cocultured at indicated proportions, treated 24 hr with doxycycline (2 μg/ml), imaged, and analyzed as in Figure 5B. Data represents difference in nuclear height (ΔZ) for $n \geq 15$ observations presented as in 5C. (B) Inducible MKK3^{DD} cells were plated in 1% cocultures and treated with doxycycline (2 μg/ml) in the presence or absence of Amphiregulin (20 ng/ml). Radial histograms of migration angles before (grey) and after (cyan) induction presented as in Figure 6C. Data represents angles of $n > 900$ cells from ≥ 6 observations assessed using subsampling and a two-sample KS test with 'ns' not significant, *** $p < 0.001$ (see Materials and methods). (C) 10% MKK3^{DD} cocultures were pretreated with Amphiregulin (20 ng/ml, green) and 24 hr doxycycline (2 μg/ml) or media, imaged and analyzed as in Figure 5B–D, and compared to selected conditions reproduced from Figure 6D (grey). Data represents difference in nuclear height (ΔZ) for $n \geq 16$ observations normalized to the mean height of parental cells (dashed line), with mean and \pm standard deviation (black bars). Significance was calculated by two-sample t-test with 'ns' indicating no significance, *** $p < 0.001$.

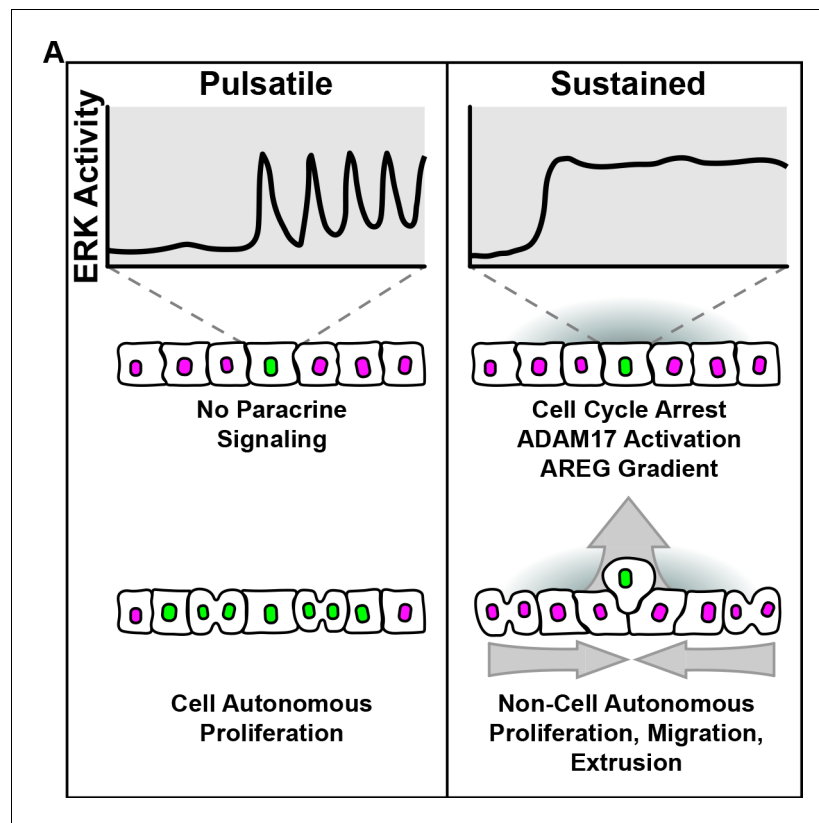


Figure 8. Graphical summary. (A) Model summarizing cell autonomous and non-cell autonomous effects resulting from pulsatile and sustained ERK signaling dynamics.