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Short-term Cellular Memory Tunes CXCR4 Signaling Responses

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Abstract

Chemokine receptor CXCR4 regulates fundamental processes in development, normal physiology, and numerous diseases including cancer. Small subpopulations of CXCR4-positive cells drive local invasion and dissemination of malignant cells in metastasis, emphasizing the need to understand mechanisms controlling responses of single cells to receptor activation by chemokine ligand CXCL12. Using single cell imaging, we discovered that short-term cellular memory of changes in environmental conditions tuned CXCR4 signaling to Akt and ERK, two major kinases activated by this receptor. Conditioning cells with growth stimuli prior to adding CXCL12 increased numbers of cells initiating CXCR4 signaling and amplitude of activation of Akt and ERK. Data-driven, single-cell computational modeling revealed that growth factor conditioning modulates CXCR4-dependent activation of Akt and ERK by shifting extrinsic noise in three key molecules: phosphatidylinositol-3-kinase (PI3K), Ras, and mTORC1. Modeling established mTORC1 as a central control point tuning responses of single cells to CXCL12-CXCR4 signaling. Our single-cell model predicted how combinations of extrinsic noise in PI3K, Ras, and mTORC1 superimpose on different driver mutations in ERK and/or Akt pathways to bias CXCR4 signaling. Computational experiments correctly predicted that selected kinase inhibitors used for cancer therapy would shift subsets of cells to states more permissive to activation of CXCR4, suggesting such drugs may inadvertently potentiate pro-metastatic signaling through CXCR4. Our work establishes how changing environmental inputs modulate CXCR4 signaling in single cells,

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providing a mechanistic framework to optimize develop and use of drugs targeting this signaling pathway.

One Sentence Summary:

Dynamic changes in environmental conditions shift extrinsic noise states of single cells to regulate the fraction of responding cells and amplitude of signaling outputs from chemokine receptor CXCR4.

Introduction

Recent research demonstrates that pre-existing cellular states, rather than stochasticity, dictate the ability of individual cells to signal in response to an input stimulus (8). Since variations in pre-existing states, individual cells within a population exhibit heterogeneous activation of signaling pathways, and subsets of cells expressing the target receptor fail to signal at all in response to uniform input of a specific ligand (1-7). The fact that extracellular ligand may not activate signaling through a target receptor confounds reliability of biomarkers based on protein expression instead of function for selection of targeted drugs. Additional heterogeneity in signaling outputs arises because cells adapt signaling responses based on changes in environmental conditions over time, indicating that context shapes plasticity in pre-existing cellular states. Context-dependent flexibility and intercellular heterogeneity in signaling allows single cells to survive under stressful conditions, hampering the ability to treat cancer and other diseases in which subpopulations of cells drive critical steps in pathogenesis. Discovering mechanisms that shift cells to states more or less responsive to receptor signaling promises to improve the ability to control cell behaviors for therapy and optimize responses to molecularly-targeted drugs.

We focused on identifying mechanisms underlying responsiveness of cells to signal through chemokine receptor CXCR4 and its ligand, CXCL12. CXCL12-CXCR4 Chemokine are essential for normal development and promote cancer initiation and metastasis (9-11). We previously observed that only a small subset of CXCR4-positive cells migrates toward a uniform gradient of CXCL12, making this ligand-receptor pair an ideal model to investigate cellular states controlling heterogeneous signaling. An inhibitor of CXCR4, balixafortide, recently showed promising results in a Phase I clinical trial as adjuvant therapy for advanced metastatic breast cancer, reinforcing the need to understand signaling through this receptor. CXCR4 activates downstream effector kinases, Akt and ERK, that mediate cell proliferation, survival, and chemotaxis. Akt and ERK are components of the most commonly activated oncogenic signaling pathways (phosphatidyl-inositol-3-kinase (PI3K)/Akt/mTOR and mitogen activated protein kinase (MAPK)) in cancer (12,13). Thus, understanding how cells edit responsiveness to CXCR4 signaling to Akt and ERK will advance fundamental knowledge of cell signaling and inform clinical applications of CXCR4-targeted therapies.

We combined single-cell fluorescent reporters and single-cell computational modeling to identify mechanisms through which changes in environmental conditions modulate CXCL12-CXCR4 signaling. Recent signaling inputs shift intracellular state based on extrinsic noise in PI3K, Ras, and mTORC1, generating a short-term cellular memory that

regulates subsequent CXCR4-mediated signaling to Akt and ERK. The computational model predicted how intersections among genetic mutations in pathway components, growth factor-induced cellular memory, and kinase inhibitors tune the ability of cells to signal through CXCR4. These data provide new insights into how cells adapt to dynamic changes in environmental conditions and how clinical treatments alter cell states and signaling by CXCR4.

Results

Growth factor conditioning potentiates subsequent CXCR4 signaling

CXCL12 signaling through CXCR4 activates the mitogen activated protein kinase (MAPK) and phosphatidylinositol-3-kinase (PI3K) pathways (Fig 1A). These pathways activate ERK and Akt, respectively. To capture CXCR4 signaling to ERK and Akt in single cells, we stably expressed fluorescent reporters for activities of these kinases (kinase translocation reporters, KTRs) (14,15). KTRs reversibly translocate from nucleus to cytoplasm based on phosphorylation of a specific substrate for each kinase. Quantifying ratios of fluorescence intensities in cytoplasm to nucleus provides analog, independent measurements of kinase activity for ERK and Akt in single cells. All reporter breast cancer cells stably expressed histone 2B fused to mCherry (H2B-mCherry) to mark the cell nuclei for purposes of image segmentation and analysis (fig S1A). MDA-MB-231 and SUM-159 reporter breast cancer cells also stably expressed CXCR4 fused to a blue fluorescent protein (CXCR4-mTagBFP), allowing us to identify levels of tagged CXCR4 in each cell.

Using live-cell imaging to quantify dynamics of Akt and ERK KTRs in single cells, we observed heterogeneous CXCR4 signaling in MDA-MB-231 breast cancer cells treated with 10 ng/ml CXCL12 as the only stimulus (Fig. 1A, fig. S1B). Single-cell responses ranged from strong activation of both Akt and ERK to absence of signaling despite expression of CXCR4 (Fig. 1B). MDA-MB-231 cells typically exhibited greater activation of Akt than ERK because mutant KRas and BRAf (16) in these cells constitutively drive ERK signaling, reducing the dynamic range for activation by CXCR4.

Since cells under physiologic conditions signal in the context of multiple signaling inputs, we hypothesized that treatment of cells with a different growth factor would generate a short-term memory (17) that modified subsequent CXCL12-CXCR4 signaling. To test this hypothesis, we conditioned cell for four hours with or without fetal bovine serum (FBS) before adding CXCL12. Conditioning with FBS produced a transient increase in Akt activity that resolved essentially to baseline within four hours, returning cells to an imaging appearance indistinguishable from control (fig. S1C). Single-cell time-tracks showed that FBS conditioning increased by four-fold the number of MDA-MB-231 cells with strong activation of Akt in response to CXCL12 (Fig. 1B-D). FBS conditioning also reduced the number of non-responding cells by 50%. Conditioning with FBS did not significantly alter activation of ERK by CXCL12-CXCR4 signaling likely because of constitutive activation of this kinase in MDA-MB-231 cells (Fig. 1B-D). Extending FBS conditioning to seven hours before adding CXCL12 produced only a two-fold increase in cells with strong activation of Akt and did not change the number of nonresponding cells, establishing time-dependence to cellular memory of prior signaling inputs (Fig. 1D). We next examined to what extent

conditioning with another growth factor, epidermal growth factor (EGF), modified subsequent CXCL12-CXCR4 signaling responses. Similar to FBS, conditioning with various concentrations of EGF transiently activated Akt and ERK, but activities of these kinases returned to baseline within four hours. Subsequent addition of CXCL12 increased cells with strong CXCR4-mediated activation of Akt, proportionate to concentrations of EGF used for conditioning (Fig. 1E). Regardless of experimental condition, CXCL12 did not activate Akt or ERK signaling in cells lacking CXCR4-BFP (fig. S1D). The amount of CXCR4-BFP on single cells did not account for intercellular heterogeneity in signaling, and conditioning did not alter expression or localization of the fluorescent receptor (fig. S1D). These data demonstrate that prior growth stimuli tune responses of cells to CXCR4 signaling through a mechanism downstream of the receptor.

Computational modeling predicts single-cell signaling dynamics

We hypothesized that conditioning with growth factors changed the intracellular state, thereby altering subsequent CXCR4 signaling. To uncover mechanisms controlling responsiveness of cells to CXCL12-CXCR4, we used ordinary differential equations to construct a computational single-cell Conditional Signaling Model (CSM) of CXCR4-mediated activation of Akt and ERK. Two key features of experimental signaling data informed construction of the CSM. First, the lack of correlation between basal activity and CXCL12-mediated activation of either kinase in single cells (fig. S2) indicated that different regulators controlled basal kinase activity versus responses of single cells to CXCL12. Second, the high correlation between activation of Akt and ERK by CXCR4 in single cells (fig. S2) suggested that a component common to both PI3K and MAPK pathways regulated responsiveness of both kinases. We constructed the framework of the CSM based on these experimental observations and literature data (Fig. 2A, fig. S3).

In addition to CXCR4 signaling outputs shown in Fig. 2A, the CSM includes extrinsic noise to account for signaling heterogeneity in a cell population (1,4,18-20). In this context, extrinsic noise refers to pre-existing cell-to-cell differences in kinase activity. We used the two observations in the experimental data described above to determine which components of the signaling pathways needed to contain extrinsic noise. PI3K, Ras, and mTORC1 constituted the main sources of heterogeneity in CXCR4 signaling because our data suggested that basal levels of upstream activators of Akt (PI3K) and ERK (Ras), as well as a downstream regulator common to both pathways (mTORC1), varied from cell-to-cell. Additionally, PI3K, Ras, and mTORC1 have known roles external to CXCR4 signaling relating to confluency, metabolism, or local mitogenic signals (19,21-23). Heterogeneity is mathematically incorporated in the CSM in the form of a conditional term on these three pathway components (fig. S4) that set baseline activities of Akt and ERK in each cell in the absence of any stimulation. We used the CSM with various combinations of PI3K, Ras, and mTORC1 extrinsic noise parameters, hereby referred to as conditional signaling state, to generate a library of predicted Akt and ERK signaling responses to CXCL12 independent of the presence and type of conditioning stimulus (Fig. 2B). We used this library of predicted signaling behavior as a framework for understanding the heterogeneous signaling data seen in experiments.

Maps of the signaling landscape reveal that conditional signaling states control CXCR4 responsiveness

The CSM captured the paired signaling behavior of Akt and ERK in single cells across the range of responses measured experimentally in the population (Fig. 3A). We used the CSM to generate a map of the signaling landscape displaying the conditional signaling states that permit CXCR4 activation of Akt and ERK (fig. S4). The signaling landscape reflects individual CSM simulations at all combinations of conditional signaling states. The Akt and ERK signaling landscape predicted by the CSM contains areas in which cells can activate one, both, or neither kinase (Fig. 3B). Generally, highest CXCR4 activation of Akt occurred in conditional signaling states with low PI3K and low mTORC1. By comparison, conditional signaling states with high PI3K, low Ras, and low mTORC1 showed greatest CXCR4 signaling to ERK (Fig. 3B). The CSM predicts which cellular states are permissive for CXCR4 signaling.

MDA-MB-231 cells occupy tunable conditional signaling states

The CSM provides a framework to organize complex signaling behavior and extract conditional information from cell populations. We constructed occupancy maps to illustrate distributions of experimental cells through the CSM signaling landscape. Under control conditioning, MDA-MB-231 cells occupy a region of the signaling landscape with moderate PI3K, Ras, and mTORC1 activities (Fig. 4A). Akt and ERK signaling responsiveness from Fig. 3B is shown as the underlay on the occupancy maps and illustrates regions of cells where CXCR4 activates Akt and/or ERK. When conditioned with FBS for four hours prior to CXCL12 stimulation, MDA-MB-231 cells shift to a region of the signaling landscape with lower PI3K and mTORC1, which favors CXCR4-mediated activation of Akt (Fig. 4B). Using the CSM-predicted Akt and ERK signaling behaviors that matched experimental data, the percentage of cells activating Akt in response to CXCL12 increased after FBS conditioning compared to control (Fig. 4C). Conditioning with three different concentrations of EGF also decreased PI3K and mTORC1 in a dose-dependent manner compared to control (fig. S6A, B). We conclude that conditioning cells with growth factors alters the conditional signaling states consistent with a decrease in PI3K and mTORC1 activity. These shifts in conditional signaling state provide a mechanism for effects of growth factor conditioning to enhance numbers of cells responding to CXCL12 and amplitude of signaling to Akt.

Vari-068 and SUM-159 cells occupy tunable conditional signaling states distinct from MDA-MB-231 cells

In contrast to MDA-MB-231 cells, many breast cancers harbor mutations in upstream activators of Akt (24). We tested the CSM on cells with constitutive activation of signaling to Akt, which we expected to occupy different conditional states in the signaling landscape than MDA-MB-231 cells. In Vari-068 patient-derived cells with mutant PTEN, CXCR4 signals primarily through ERK rather than Akt (fig. S6C), a behavior distinct from MDA-MB-231 cells. Vari-068 cells occupy a region of the signaling landscape with high PI3K, and moderate Ras (Fig. 5A). Because PTEN negatively regulates phosphatidylinositol-3,4,5-phosphate (PIP3), the model represents the PTEN mutation in Vari-068 cells as high PI3K activity. When conditioned with FBS for four hours prior to adding CXCL12, Vari-068 cells

shift to a region of the signaling landscape with lower mTORC1 but similar PI3K and Ras (Fig. 5B). Cells in this state show potentiated ERK signaling (Fig. 5C, fig. S6C). These results suggest that mTORC1 controls overall permissiveness of cells to signal through CXCR4, and conditioning with growth factors lessens mTORC1-mediated restraint mechanisms on ERK and Akt signaling. Conditioning breast cancer cells with growth factors decreases PI3K and mTORC1 activity to potentiate subsequent CXCR4-mediated signaling to Akt and ERK but cannot overcome activating mutations in upstream components of these pathways. Genetic mutations define the subset of conditional signaling states available to cells, but conditioning with growth factors further tunes the signaling state of any single cell.

We next investigated to what extent CXCR4 signals to Akt and ERK in cells with activating mutations in both PI3K and MAPK pathways. Similar to Vari-068 cells, SUM-159 cells with constitutively active HRas (an upstream activator of ERK) and PI3K signal primarily to ERK rather than Akt (fig. S6C), indicating that the ERK pathway remains inducible in the presence of an upstream activating mutation. SUM-159 cells occupy a regime with high PI3K due to the activating mutation in this kinase and moderate Ras and mTORC1 (Fig. 6A). The CSM reveals that despite activating mutations in both MAPK and PI3K pathways, PI3K/Akt pathway generally dominates and remains almost uninducible by CXCL12 in these cells. Signaling in breast cancer cells with genetic mutations in Akt, ERK, or both is tuned both by these mutations and growth factor availability. A summary cartoon illustrates the conditional states of the breast cancer cell types we tested, showing how genetic mutations and growth factor conditioning stimuli shift cell signaling states to various regimes of the signaling landscape (Fig. 6C). Genetic mutations dictate the subset of conditional signaling states available from the set of all possible states predicted from the CSM, and growth factor availability further tunes which states within that subset single cells will occupy.

MEK inhibition potentiates subsequent CXCR4-mediated Akt signaling in a subset of cells

We applied the CSM to predict responsiveness of cells treated with two therapeutic agents relevant to CXCR4 signaling, trametinib (MEK inhibitor) and ridaforolimus (mTORC1 inhibitor). The CSM predicted that conditioning with trametinib would block ERK signaling but potentiate CXCR4 signaling to Akt in a subset of cells (fig. S7A, B). Inhibiting MEK decreased the flux of ERK-mediated mTORC1 activation and released restraint on mTORC2 to activate Akt. A simulated dose response of trametinib conditioning revealed that larger doses of trametinib increased activation of Akt in MDA-MB-231 cells (Fig. 7A). We confirmed predictions of the CSM experimentally, demonstrating that conditioning MDA-MB-231 cells with trametinib for four hours heterogeneously potentiated CXCR4 signaling to Akt (Fig. 7B). Difference maps illustrate areas of change of peak activation with inhibitor conditioning compared to control (Fig. 7C). The CSM revealed that cells exhibiting enhanced Akt signaling with trametinib conditioning were those with low PI3K and mTORC1, corresponding to cells in states predisposed to be highly responsive to CXCL12 with control conditioning. By comparison, CXCR4 signaling in other, non-responsive states remained unaffected by trametinib (Fig. 7C). Notably, cells occupy similar regions of the signaling landscape as compared to control conditioning, confirming that the simulated

inhibitor treatment did not shift conditional signaling states and only affected responsiveness at each state.

mTORC1 inhibition potentiates subsequent CXCR4-mediated Akt and ERK signaling

A simulated dose response of ridaforolimus conditioning showed dose-dependent potentiation of CXCR4 signaling to both Akt and ERK in MDA-MB-231 cells (Fig. 8A, fig. S7C). Inhibition of mTORC1 releases restraint on both mTORC2 and Ras, thereby activating both Akt and ERK. Difference maps indicated enhanced CXCR4 signaling to Akt in cells with low PI3K, and all cells exhibited enhanced ERK signaling (Fig. 8B). We experimentally confirmed predictions of the CSM, demonstrating that conditioning MDA-MB-231 cells for four hours with ridaforolimus potentiated CXCL12-dependent activation of both Akt and ERK (fig. S7D). Again, conditional signaling states of cells remained similar to control conditioning. These data establish that targeted kinase inhibitors can potentiate CXCR4 signaling in subpopulations of cells.

Discussion

Rather than representing hard-wired pathways always generating the same output, signaling networks in single cells produce heterogeneous responses shaped by changing environmental conditions and signaling inputs. Our work demonstrates that while cells adapt signaling based on short-term memories of prior inputs, these adaptations are predictable based on a specific set of rules. We used one parameter set to simulate single-cell paired Akt and ERK signaling dynamics for entire populations of cells and introduced heterogeneity by adding extrinsic noise to only three pathway components: PI3K, Ras, and mTORC1. The ability of the CSM to predict heterogeneous basal states and responsiveness of single cells in multiple breast cancer cell types with only extrinsic noise in three pathway components suggests the model captures the major drivers of CXCR4 signaling to Akt and ERK.

Local intracellular and extracellular conditions tune signaling responses in individual cells. As a consequence, gradients of growth factors or kinase inhibitors *in vivo* may drive heterogeneous signaling outcomes. We showed that conditional effects such as genetic mutations, growth factors, and kinase inhibitors all collectively tune responsiveness of cells to CXCL12-CXCR4 signaling and activation of Akt and ERK. We propose that cells exist on a signaling landscape based on extrinsic noise states. The signaling landscape, which accounts for CXCR4 signaling dynamics at all possible cellular conditional states, defines the output of the CSM. Genetic mutations force cells into distinct regimes within the signaling landscape. Conditioning cells with growth factors allows cells to shift within these regimes to potentiate signaling, while conditioning with kinase inhibitors modifies cell signaling potential at each state but preferentially affects cells already existing in states poised to signal. We show that trametinib only potentiates subsequent CXCR4-mediated Akt signaling in cells with low PI3K and mTORC1, a small fraction of cells in the population. However, signaling and function in small numbers of cells drives pathogenesis of processes such as metastasis in cancer, making behaviors of “outlier” single cells relevant for disease and therapy.

We built the CSM to explore the entire design space (the single-cell signaling landscape) potentially occupied by experimental cells. By mapping experimental cells onto the signaling landscape, we assigned mechanisms for heterogeneous CXCR4 signaling responses observed in experiments and discovered how conditioning tuned these responses. The CSM predicted that trametinib not only would produce the expected outcome of suppressing ERK but also produce the off-target consequence of enhancing CXCR4 activation of Akt. Similarly, we predicted that ridaforolimus would potentiate both Akt and ERK signaling, two pathways widely appreciated as promoting cancer growth and metastasis. These results highlight how targeted cancer therapies may potentiate CXCR4 signaling, a known driver of tumor growth and metastasis, and how our computational model can predict such outcomes.

Cell signaling networks contain central nodes that store integrated information about multiple inputs and use this information to regulate responses to new signaling inputs. Our data indicate that mTORC1 functions as one of these central nodes, holding information about prior signaling to control subsequent activation of Akt and ERK. While negative regulation of ERK by mTORC1 remains poorly characterized in literature, our CSM and single-cell imaging experiments demonstrate this as a crucial mechanism driving coordinate regulation of Akt and ERK.

While robust and predictive, the computational model we present here points to additional research opportunities relevant to heterogeneity in single cell signaling. To establish heterogeneity among cells, the CSM incorporates extrinsic noise in three pathway components, PI3K, Ras, and mTORC1. However, specific molecular mechanisms driving noise in these pathway components remain to be identified. Mathematically, the source of the noise is embedded in a first-order kinetic rate constant. Understanding causes of extrinsic noise in these molecules will highlight potential approaches to tune CXCR4 signaling for therapy. In integrated 3D environments with mixed cellular composition, we predict cells will occupy some signaling states in the CSM-predicted signaling landscape that remain unoccupied in 2D monocultures. Since the mathematics in the CSM unrelated to extrinsic noise are kinetic reactions independent of cellular geometry, we expect the model will predict behaviors in mixed cell environments and complex tissues. Our future studies will advance into 3D environments and mouse models of cancer, where heterogeneous CXCR4 signaling responses are critical to mechanisms of metastasis and response to targeted therapies.

Materials and Methods

Cell Culture

We cultured breast cancer cell lines MDA-MB-231, which expresses constitutively active KRAS and BRAF (16), and SUM-159, which expresses constitutively active PI3K and HRAS (16), as described previously (25). Vari-068 cells (gift of Sofia Merajver, University of Michigan) are patient-derived, triple-negative breast cancer cells adapted to cell culture. These cells have an inactivating mutation in PTEN. We cultured these cells as described (26).

Fluorescent reporter construction

We constructed the kinase translocation reporter plasmid, pHAEP, in a PiggyBac transposon vector with CAG promoter based in part on plasmid pHGEA (gift of K. Aoki) (27). To optimize two-photon imaging of KTR reporters for Akt and ERK we fused the kinase substrates to fluorescent proteins Aquamarine (28) and mCitrine (29), respectively and replaced the histone-2B marker with mCherry to improve brightness and photostability. We also replaced the IRES to blasticidin resistance marker with a P2A sequence followed by a puromycin resistance marker. We assembled the plasmid using HiFi assembly (NEB, Ipswich, MA, USA) with synthetic double stranded DNA fragments (GenBlocks, IDT, Coralville, IA, USA) or double stranded DNA amplified from pHGEA as illustrated in fig. S1A. We constructed the CXCR4-mTagBFP2 (Evrogen, Moscow, Russia) in lentiviral expression vector pLVX-Ef1 α (Clontech/Takara, Kusatsu, Shiga, Japan).

Cell engineering

To generate cells stably expressing the pHAEP construct, we co-transfected each cell line with the pHAEP transposon and Super PiggyBac transposase vector (System Biosciences, Palo Alto, CA, USA) using FuGene HD (Promega, Milwaukee, WI, USA). We selected batch populations of stable cells with 4 μ g/ml puromycin. For MDA-MB-231 and SUM-159 cells, we transduced cells stably expressing the pHAEP reporter with lentiviral vector for CXCR4-mTagBFP and sorted BFP-positive cells by flow cytometry.

Time-lapse two photon microscopy and image processing

To prepare cells for time-lapse microscopy, we seeded cells (1.2×10^5 MDA-MB-231, 6.5×10^4 SUM159, or 2.0×10^5 Vari-068) in 35 mm dishes with a 20 mm glass bottom (Cellvis, Mountain View, CA, USA) in 2 ml of imaging base media (FluoroBrite DMEM media (A1896701, ThermoFisher Scientific, Waltham, MA USA), 1% GlutaMax, 1% PenStrep and 1% sodium pyruvate) supplemented with 10% FBS (HyClone). For SUM-159 cells, we also added 0.05% insulin (Sigma I9278) and 0.01% hydrocortisone (10mg/ml, 70% ethanol/water). Forty-eight hours after seeding, we changed to 1% FBS in imaging base media for all cell types. On the next day, four hours prior to imaging we conditioned cells by adding 200 μ l FBS (final concentration 10%), EGF (final concentration 1, 10 or 30 ng/ml) (R&D Systems, Minneapolis, MN, USA), ridaforolimus (Selleck Chemicals, Houston, TX, USA) (final concentration 10 nM), or trametinib (Selleck Chemicals) (final concentration 100 nM) to their existing media. For extended conditioning, we added 200 μ l FBS seven hours prior to imaging.

We imaged cells with an Olympus FVMPE-RS upright microscope, 25x NIR-corrected objective, and four channel detection (blue, cyan, yellow, red) with a live cell imaging chamber (Okolab, San Bruno, CA, USA). Laser settings were: mTagBFP2 excitation at 800 nm, laser power 6%, Aquamarine and mCitrine excitation 920 nm, laser power 6%, and mCherry excitation 1040, laser power 11%. We acquired images as a multi-area time lapse scanned every two minutes for 4 images prior to addition of CXCL12 (10 ng/ml final concentration) and every two minutes thereafter for a total of 1 hour. We developed custom MATLAB code to automatically segment cells; calculate the KTR cytoplasmic to nuclear ratio in each cell; measure intensity of CXCR4-mTagBFP; and track individual cells. The

segmentation algorithm identifies nuclei with adaptive thresholding followed by watershed segmentation. The extended minima from the nuclear watershed are used to seed watershed segmentation of a mask of the combined KTR channels, which yields cytoplasmic segmentation in good agreement with contours of individual cells in confluent monolayers. Nuclei are used for tracking individual cells during the time lapse imaging. For KTR reporters, we calculate the ratio of median fluorescence intensities in cytoplasm to the nucleus (CNR), expressed as the $\log(2)$ of the CNR, and output data as pairs of Akt and ERK KTR measurements for each cell with a complete time track (generally 300 to 500 cells per image). For cells engineered to express CXCR4-mTagBFP, we used only cells with detectable blue fluorescence for computational modeling.

Computational model: receptor dynamics

We constructed a computational conditional signaling model (CSM) of CXCR4-mediated Akt and ERK signaling using ordinary differential equations to generate predicted signaling outcomes. The CSM contains receptor, signaling, and reporter dynamics. A schematic including all connectivity in the CSM is drawn in fig. S2. All equations, parameters, and initial conditions can be found in Supplementary Information.

Receptor dynamics (CXCR4 trafficking following CXCL12 stimulation) are as described previously (30-32). Briefly, CXCL12 in the extracellular space binds to CXCR4. Upon receptor phosphorylation and β -arrestin recruitment to the plasma membrane, the receptor-ligand complex is internalized, trafficked to endosomes, and destined for degradation. Because β -arrestin is an adapter protein ubiquitously involved in desensitizing many different GPCRs (33,34), we assume it is in large excess and do not model it explicitly. CXCR4 not bound to CXCL12 can be internalized upon phosphorylation and β -arrestin recruitment, but the receptor is recycled to the cell surface rather than degraded.

Computational model: signaling dynamics

The CXCR4-CXCL12 complex promotes signaling through Akt and ERK in a mechanism involving feedback loops and crosstalk that restrain signaling. The model includes a cascade of events leading to phosphorylation of ERK and both the Thr-308 (T308) and Ser-473 (S473) sites on Akt needed for full activation (35-37). The PI3K/Akt pathway is initiated as CXCL12-CXCR4 complexes, whether phosphorylated or not, promote G-protein activation (38). To account for both ligand-independent and non-CXCL12 induced G-protein activation, we incorporate a basal rate of G-protein activation. Activated G-proteins organize subunits of phosphoinositide 3-kinase (PI3K) into their active state (39). Activated PI3K phosphorylates the membrane lipid PIP₂ to form PIP₃ (39,40). PIP₃ has two major roles in Akt signaling. First, it activates PDK1 by binding and forming a complex. The active form of PDK1 recruits and phosphorylates Akt and pAktS473 on the T308 site (40,41). The Akt S473 site is activated by a separate kinase, mTORC2. We assume that phosphorylation of either site on Akt is independent of the phosphorylation of the other, consistent with Pezze et. al (41). While there are many proposed mechanisms of mTORC2 activation, the literature is in agreement that it is PI3K-dependent (42,43). In our model, we propose that mTORC2 is activated and recruited to the plasma membrane by PIP₃, the second role for this lipid in the model and consistent with a recent study by Gan et. al (44). mTORC1 opposes mTORC2

formation (45). While many studies have emphasized the importance of mTORC1 opposing PI3K formation through IRS-1 and thus halting mTORC2 formation (46,47), recent studies demonstrate a more direct mechanism for mTORC1 to inhibit mTORC2 formation. The subunit on mTORC2 that promotes docking to PIP₃ and thus mTORC2 activation, mSIN1, is phosphorylated and inactivated by a product of mTORC1, activated (phosphorylated) S6K (48,49). This phosphorylation detaches mSIN1 from mTORC2, preventing mTORC2 from attaching to the plasma membrane and becoming activated (50). These dynamics closely follow uncompetitive inhibition. Therefore, we model the activation of mTORC2 with PIP₃ acting as the enzyme, inactive mTORC2 as the substrate, and mTORC1 as an uncompetitive inhibitor. ppAkt and pERK promote mTORC1 activation (23,51,52). This activation involves many species, including TSC1/2 and RHEB, which are not modeled here explicitly for simplicity. Instead, we assume ppAkt and pERK promote activation of mTORC1.

The MAPK signaling pathway is initiated with activation of Ras by active G-proteins (53). Recent evidence suggests that mTORC1 can oppose activation of Ras, and we incorporate this in our model (54). Ras promotes activation of the Raf/MEK complex (55). MDA-MB-231 cells also have a Raf mutation, and we account for this with a GPCR-independent Raf activation reaction and set this parameter to 0 when modeling cells without this mutation. Additionally, Raf is inhibited by active Akt (56). Activated Raf/MEK promotes phosphorylation of ERK (57).

Computational model: reporter dynamics

To connect active kinase concentrations (pERK and ppAkt) to their respective reporters in the CSM, we use a set of published ordinary differential equations (14). Briefly, the reporters exist in two locations, the nucleus or cytoplasm, and have two states in both locations, phosphorylated or unphosphorylated. The reporters are phosphorylated and dephosphorylated according to Michaelis-Menten kinetics and are transported between nucleus and cytoplasm by mass action kinetics. To determine the CNR in individual cells in our model, we calculate the ratio of each reporter (phosphorylated and unphosphorylated) in the cytoplasm to the nucleus, and we express this variable in log₂ format. The single-cell cytoplasmic to nuclear ratio (CNR) of each reporter is the output of our model.

Computational model: extrinsic noise

Extrinsic noise is now appreciated as a major driver of cell signaling heterogeneity (19,21,23,58). In the CSM, extrinsic noise encompasses cellular conditions driven by mutations, metabolism, mitogenic signals, or any other external force acting on signaling components. The model incorporates extrinsic noise in three molecules, PI3K, Ras, and mTORC1, to predict the heterogeneous single-cell CXCR4-mediated Akt and ERK responses. A first-order rate constant for each of these molecules promotes their activation independent of CXCR4 signaling. One important assumption of this approach is that the extrinsic noise rate parameters are constant over the time frame of our experiments.

Computational model: solution and calibration

The CSM is solved using MATLAB function *ode15s*. At the start of a simulation, the model is run in the absence of CXCL12 to calculate the steady-state concentrations of all model

species. Next, a dose of CXCL12 is given and downstream signaling dynamics occur as described above and by the differential equations given in Supplementary Information.

First-pass model parameters were taken from literature as documented in Supplementary Information. We performed Latin Hypercube sampling (LHS) (59) as a search strategy for efficient parameter selection using the first-pass model parameters and a $\pm 50\%$ variation to find a suitable baseline parameter set. We used the same baseline parameter set for every cell in all of our simulations, and varied only the three extrinsic noise rate parameters to generate signaling heterogeneity. The model is calibrated to control conditioned MDA-MB-231 cell responses to 10 ng/mL CXCL12.

Determining the conditional signaling state of experimental cells

By varying the extrinsic noise parameters for PI3K, Ras, and mTORC1, the CSM generates over 12,000 possible CXCR4-mediated Akt and ERK responses. To determine the conditional state of cells in our experiments, we calculated the residuals of each paired Akt and ERK experimental cell response to each of the predicted paired Akt and ERK responses from the CSM. We determine the conditional state of each experimental cell from the predicted cell to which it shared the minimum squared residual. Occupancy maps in Fig. 4-6 and fig. S6B are an illustration of the probability of experimental cells occupying a conditional state in the CSM. For each cell in an experiment, we calculated a fit score which is the reciprocal of the sum of the squared residuals for experimental Akt and ERK KTRs compared with the simulated Akt and ERK KTR at each conditional state in the CSM. We normalized fit scores for each experimental cell to their sum over all CSM conditional states. We set a lower bound (0.0005) below which fit scores are set to 0. We calculated the probability of occupancy of each CSM conditional state as the sum of the fit scores for all cells at that condition, normalized to the sum of fit scores for all cells to all CSM conditional states. For illustration purposes, these values were multiplied by one million cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Kim E, Kim J, Smith MA, Haura EB, Alexander R, Anderson A. Cell signaling heterogeneity is modulated by both cell-intrinsic and -extrinsic mechanisms : An integrated approach to understanding targeted therapy. *PLoS Biol.* 2018;16(3):1–29.
2. Altschuler SJ, Wu LF. Cellular Heterogeneity: Do Differences Make a Difference? *Cell.* 2010;141:559–63. [PubMed: 20478246]

3. Irish JM, Hovland R, Krutzik PO, Perez OD, Bruserud Ø, Gjertsen BT, et al. Single Cell Profiling of Potentiated Phospho-Protein Networks in Cancer Cells. *Cell*. 2004;118:217–28. [PubMed: 15260991]
4. Snijder B, Pelkmans L. Origins of regulated cell-to-cell variability *Nat Rev Mol Cell Biol* [Internet]. Nature Publishing Group; 2011;12(2):119–25. Available from: 10.1038/nrm3044
5. Hughey JJ, Lee TK, Lipniacki T, Quake SR, Covert MW. Single-cell NF- κ B dynamics reveal digital activation and analogue information processing. *Nature*. 2010;466:267–72. [PubMed: 20581820]
6. Gaudet S, Miller-Jensen K. Redefining signaling pathways with an expanding single-cell toolbox. *Trends Biotechnol*. 2016;34(6):458–69. [PubMed: 26968612]
7. Gross SM, Rotwein P. Akt signaling dynamics in individual cells. *J Cell Sci* [Internet]. 2015;128(14):2509–19. Available from: <http://jcs.biologists.org/cgi/doi/10.1242/jcs.168773>
8. Yao J, Pilko A, Wollman R. Distinct cellular states determine calcium signaling response. *Mol Syst Biol* [Internet]. 2016;12(12):894 Available from: <http://msb.embopress.org/lookup/doi/10.15252/msb.20167137>
9. Cojoc M, Peitzsch C, Polishchuk L, Telegeev G, Dubrovskaya A. Emerging targets in cancer management: role of the CXCL12/CXCR4 axis. *Onco Targets Ther* [Internet]. 2013;6:1347–61. Available from: <http://www.dovepress.com/permissions.php>
10. Sobolik T, Su Y, Wells S, Ayers GD, Cook RS. CXCR4 drives the metastatic phenotype in breast cancer through induction of CXCR2 and activation of MEK and PI3K pathways. *Mol Biol Cell*. 2013;25.
11. Ozawa P, Ariza C, Ishibashi C, Fujita T, Banin-Hirata B, Oda J, et al. Role of CXCL12 and CXCR4 in normal cerebellar development and medulloblastoma. *Int J Cancer*. 2014;138:10–3. [PubMed: 25400097]
12. Sun X, Cheng G, Hao M, Zheng J, Zhou X, Zhang J, et al. CXCL12/CXCR4/CXCR7 chemokine axis and cancer progression. *Cancer Metastasis Rev* [Internet]. 2010;29(4):709–22. Available from: <http://link.springer.com/10.1007/s10555-010-9256-x>
13. Domanska UM, Kruizinga RC, Nagengast WB, Timmer-Bosscha H, Huls G, De Vries EGE, et al. A review on CXCR4/CXCL12 axis in oncology: No place to hide *Eur J Cancer* [Internet]. Elsevier Ltd; 2013;49(1):219–30. Available from: 10.1016/j.ejca.2012.05.005
14. Regot S, Hughey JJ, Bajar BT, Carrasco S, Covert MW. High-sensitivity measurements of multiple kinase activities in live single cells *Cell* [Internet]. Elsevier Inc.; 2014;157(7):1724–34. Available from: 10.1016/j.cell.2014.04.039
15. Kudo T, Jeknic S, Macklin DN, Akhter S, Hughey JJ, Regot S, et al. Live-cell measurements of kinase activity in single cells using translocation reporters. *Nat Protoc*. 2017;13(1):155–69. [PubMed: 29266096]
16. Hollestelle A, Elstrodt F, Nagel JHA, Kallemeijn WW. Phosphatidylinositol-3-OH Kinase or RAS Pathway Mutations in Human Breast Cancer Cell Lines. *Mol Cancer Res*. 2007;5(2):195–201. [PubMed: 17314276]
17. Mitra T, Menon SN, Sinha S. Emergent memory in cell signaling: Persistent adaptive dynamics in cascades can arise from the diversity of relaxation time-scales. *Sci Rep*. 2018;8(13230):1–21. [PubMed: 29311619]
18. Kaschek D, Hahn B, Wrangborg D, Karlsson J, Kvarnström M. Heterogeneous kinetics of AKT signaling in individual cells are accounted for by variable protein concentration. *Front Physiol*. 2012;3(45):1–14. [PubMed: 22275902]
19. Yuan TL, Wulf G, Burga L, Cantley LC. Cell-to-cell variability in PI3K protein level regulates PI3K-AKT pathway activity in cell populations *Curr Biol* [Internet]. Elsevier Ltd; 2011;21(3):173–83. Available from: 10.1016/j.cub.2010.12.047
20. Iwamoto K, Shindo Y, Takahashi K. Modeling Cellular Noise Underlying Heterogeneous Cell Responses in the Epidermal Growth Factor Signaling Pathway. *PLoS Comput Biol*. 2016;12(11):1–18.
21. Cintas C, Guillermet-Guibert J. Heterogeneity of Phosphatidylinositol-3-Kinase (PI3K)/AKT/Mammalian Target of Rapamycin Activation in Cancer: Is PI3K Isoform Specificity Important? *Front Oncol* [Internet]. 2018;7 Available from: <http://journal.frontiersin.org/article/10.3389/fonc.2017.00330/full>

22. Jeantet M, Tougeron D, Tachon G, Cortes U, Archambaut C, Fromont G, et al. High intra- and inter-tumoral heterogeneity of RAS mutations in colorectal cancer. *Int J Mol Sci.* 2016;17(12).
23. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease Cell [Internet]. Elsevier Inc.; 2017;168(6):960–76. Available from: 10.1016/j.cell.2017.02.004
24. Millis S, Ikeda S, Reddy S, Gatalica Z, Kurzrock R. Landscape of Phosphatidylinositol-3-Kinase Pathway Alterations Across 19 784 Diverse Solid Tumors. *JAMA Oncol.* 2016;2(12):1565–73. [PubMed: 27388585]
25. Chen Y, Humphries B, Brien R, Gibbons AE, Chen Y, Qyli T, et al. Functional Isolation of Tumor-Initiating Cells using Microfluidic-Based Migration Identifies Phosphatidylserine Decarboxylase as a Key Regulator Sci Rep [Internet]. Springer US; 2018;8(244):1–13. Available from: 10.1038/s41598-017-18610-5
26. Gilani RA, Phadke S, Bao LW, Lachacz EJ, Dziubinski ML, Brandvold KR, et al. UM-164: a potent c-Src/p38 kinase inhibitor with in vivo activity against triple negative breast cancer. *Clin Cancer Res.* 2016;
27. Maryu G, Matsuda M, Aoki K. Multiplexed Fluorescence Imaging of ERK and Akt Activities and Cell-cycle Progression. *Cell Struct Funct.* 2016;41:81–92. [PubMed: 27247077]
28. Vincent P, Merola F, Bousmah Y. Minimum set of mutations needed to optimize cyan fluorescent proteins for live cell imaging. *Mol Biosyst.* 2013;9:258–67. [PubMed: 23192565]
29. Griesbeck O, Baird GS, Campbell RE, Zacharias DA, Tsien RY. Reducing the Environmental Sensitivity of Yellow Fluorescent. *J Biol Chem.* 2001;276(31):29188–94. [PubMed: 11387331]
30. Coggins NL, Trakimas D, Chang SL, Ehrlich A, Ray P, Luker KE, et al. CXCR7 Controls Competition for Recruitment of β -Arrestin 2 in Cells Expressing Both CXCR4 and CXCR7. *PLoS One* [Internet]. 2014;9(6):e98328 Available from: <http://dx.plos.org/10.1371/journal.pone.0098328>
31. Chang SL, Cavnar SP, Takayama S, Luker GD, Linderman JJ. Cell, Isoform, and Environment Factors Shape Gradients and Modulate Chemotaxis. *PLoS One* [Internet]. 2015;10(4):e0123450 Available from: <http://dx.plos.org/10.1371/journal.pone.0123450>
32. Spinosa PC, Luker KE, Luker GD, Linderman JJ. The CXCL12/CXCR7 signaling axis, isoforms, circadian rhythms, and tumor cellular composition dictate gradients in tissue. *PLoS One.* 2017;12(11).
33. Smith JS, Rajagopal S. The β -Arrestins: Multifunctional regulators of G protein-coupled receptors. *J Biol Chem.* 2016;291(17):8969–77. [PubMed: 26984408]
34. DeWire SM, Ahn S, Lefkowitz RJ, Shenoy SK. β -Arrestins and Cell Signaling. *Annu Rev Physiol.* 2007;69(1):483–510. [PubMed: 17305471]
35. Manning B, Cantley L. AKT/PKB Signalling: Navigating Downstream. *Cell.* 2017;169:1261–74.
36. Song G, Ouyang G, Bao S. The activation of Akt/PKB signaling pathway and cell survival. *J Cell Mol Med* [Internet]. 2005;9(1):59–71. Available from: <http://doi.wiley.com/10.1111/j.1582-4934.2005.tb00337.x>
37. Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, et al. Mechanism of activation of protein kinase B by insulin and IGF-1. *EMBO J* [Internet]. 1996;15(23):6541–51. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/8978681>
38. Lamb TD. Stochastic simulation of activation in the G-protein cascade of phototransduction *Biophys J* [Internet]. Elsevier; 1994;67(4):1439–54. Available from: 10.1016/S0006-3495(94)80617-4
39. Hatakeyama M, Kimura S, Naka T, Kawasaki T, Yumoto N, ICHIKAWA M, et al. A computational model on the modulation of mitogen-activated protein kinase (MAPK) and Akt pathways in heregulin-induced ErbB signalling. *Biochem J* [Internet]. 2003;373(2):451–63. Available from: <http://biochemj.org/lookup/doi/10.1042/bj20021824> [PubMed: 12691603]
40. Jain P, Bhalla US. Signaling logic of activity-triggered dendritic protein synthesis: An mTOR gate but not a feedback switch. *PLoS Comput Biol.* 2009;5(2).
41. Pezze PD, Ruf S, Sonntag AG, Langelaar-Makkinje M, Hall P, Heberle AM, et al. A systems study reveals concurrent activation of AMPK and mTOR by amino acids. *Nat Commun.* 2016;7:1–19.
42. Mendoza M, Emrah Er E, Blenis J. The Ras-ERK and PI3K-mTOR Pathways: Cross-talk and Compensation. *Trends Biochem Sci.* 2011;36(6):320–8. [PubMed: 21531565]

43. Yang G, Murashige DS, Humphrey SJ, James DE. A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation. *Cell Rep* [Internet]. The Authors; 2015;12(6):937–43. Available from: [10.1016/j.celrep.2015.07.016](https://doi.org/10.1016/j.celrep.2015.07.016)
44. Gan X, Wang J, Su B, Wu D. Evidence for direct activation of mTORC2 kinase activity by phosphatidylinositol 3,4,5-trisphosphate. *J Biol Chem*. 2011;286(13):10998–1002. [PubMed: 21310961]
45. Julien L-A, Carriere A, Moreau J, Roux PP. mTORC1-Activated S6K1 Phosphorylates Rictor on Threonine 1135 and Regulates mTORC2 Signaling. *Mol Cell Biol* [Internet]. 2010;30(4):908–21. Available from: <http://mcb.asm.org/cgi/doi/10.1128/MCB.00601-09>
46. Yoon M-S. The Role of Mammalian Target of Rapamycin (mTOR) in Insulin Signaling. *Nutrients* [Internet]. 2017;9(11):1176 Available from: <http://www.mdpi.com/2072-6643/9/11/1176>
47. Yoneyama Y, Inamitsu T, Chida K, Iemura S-I, Natsume T, Tatsuya M, et al. Serine phosphorylation by mTORC1 promotes IRS-1 degradation through SCF β -TRCP E3 ubiquitin ligase iScience [Internet]. Elsevier Inc.; 2018;5:20487 Available from: <http://www.springerlink.com/index/10.1007/978-3-642-20487-6%0Ahttps://linkinghub.elsevier.com/retrieve/pii/S2589004218300828>
48. Liu P, Gan W, Inuzuka H, Lazorchak AS, Gao D, Arojo O, et al. Sin1 phosphorylation impairs mTORC2 complex integrity and inhibits downstream Akt signalling to suppress tumorigenesis *Nat Cell Biol* [Internet]. Nature Publishing Group; 2013;15(11):1340–50. Available from: [10.1038/ncb2860](https://doi.org/10.1038/ncb2860)
49. Liu P, Guo J, Gan W, Wei W. Dual phosphorylation of Sin1 at T86 and T398 negatively regulates mTORC2 complex integrity and activity. *Protein Cell*. 2014;5(3):171–7. [PubMed: 24481632]
50. Ebner M, Sinkovics B, Szczygieł M, Ribeiro DW, Yudushkin I. Localization of mTORC2 activity inside cells. *J Cell Biol*. 2017;216(2):343–53. [PubMed: 28143890]
51. Sulaimanov N, Klose M, Busch H BM. Understanding the mTOR signaling pathway via mathematical modeling. *WIREs Syst Biol Med*. 2017;9.
52. Pezzer PD, Sonntag AG, Thien A, Prentzell MT. A dynamic network model of mTOR signalling reveals TSC independent mTORC2 regulation. *Syst Biol (Stevenage)*. 2012;5(217):1–18.
53. Della Rocca GJ, Van Biesen T, Daaka Y, Luttrell DK, Luttrell LM, Lefkowitz RJ. Ras-dependent Mitogen-activated Protein Kinase Activation by G Protein-coupled Receptors. *J Biol Chem*. 1997;272(31):19125–32. [PubMed: 9235901]
54. Posada IMD, Lectez B, Siddiqui FA, Oetken-Lindholm C, Sharma M, Abankwa D. Opposite feedback from mTORC1 to H-ras and K-ras4B downstream of SREBP1 *Sci Rep* [Internet]. Springer US; 2017;7(1):1–14. Available from: [10.1038/s41598-017-09387-8](https://doi.org/10.1038/s41598-017-09387-8)
55. Karoulia Z, Gavathiotis E, Poulikakos PI. New perspectives for targeting RAF kinase in human cancer *Nat Rev Cancer* [Internet]. Nature Publishing Group; 2017;17(11):676–91. Available from: [10.1038/nrc.2017.79](https://doi.org/10.1038/nrc.2017.79)
56. Moelling K, Schad K, Bosse M, Zimmermann S, Schweneker M. Regulation of Raf-Akt cross-talk. *J Biol Chem*. 2002;277(34):31099–106. [PubMed: 12048182]
57. Zhang W, Liu HT, Tu LIU H. MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Res* [Internet]. 2002;12(1):9–18. Available from: <http://www.cell-research.com>
58. Martínez-Revollar G, Garay E, Martín-Tapia D, Nava P, Huerta M, Lopez-Bayghen E, et al. Heterogeneity between triple negative breast cancer cells due to differential activation of Wnt and PI3K/AKT pathways. *Exp Cell Res*. 2015;339(1):67–80. [PubMed: 26453937]
59. Marino S, Hogue IB, Ray CJ, Kirschner DE. A Methodology for Performing Global Uncertainty and Sensitivity Analysis in Systems Biology. *Journal of Theoretical Biology*. 2009 178–196 p.
60. Fallahi-Sichani M, Linderman JJ. Lipid raft-mediated regulation of G-protein coupled receptor signaling by ligands which influence receptor dimerization: A computational study. *PLoS One*. 2009;4(8).
61. Tan WH, Popel AS, Mac Gabhann F. Computational Model of Gab1/2-Dependent VEGFR2 Pathway to Akt Activation. *PLoS One*. 2013;8(6):1–17.
62. Adlung L, Kar S, Wagner M, She B, Chakraborty S, Bao J, et al. Protein abundance of AKT and ERK pathway components governs cell type-specific regulation of proliferation. *Mol Syst Biol*

[Internet]. 2017;13(1):904 Available from: <http://msb.embopress.org/lookup/doi/10.15252/msb.20167258>

63. Borisov N, Aksamitiene E, Kiyatkin A, Legewie S, Berkhout J, Maiwald T, et al. Systems-level interactions between insulin-EGF networks amplify mitogenic signaling. *Mol Syst Biol*. 2009;5(256):1–15.
64. Spinosa PC, Luker KE, Luker GD, Linderman JJ. The CXCL12/CXCR7 signaling axis, isoforms, circadian rhythms, and tumor cellular composition dictate gradients in tissue. *PLoS One*. 2017;12(11):1–17.
65. Chang SL. Mechanistic and statistical models to understand CXCL12/CXCR4/CXCR7 in breast cancer. 2015.
66. Koh G, Fern H, Teong C, Hsu D, Thiagarajan PS. A compositional approach to parameter estimation in pathway modeling: a case study of the Akt and MAPK pathways and their crosstalk. *Bioinformatics*. 2006;22(14):271–80.
67. Sedaghat AR, Sherman A, Quon MJ. A mathematical model of metabolic insulin signaling pathways. *AmJPhysiol EndocrinolMetab* [Internet]. 2002;283(5):E1084–101. Available from: <http://www.ncbi.nlm.nih.gov/pubmed/12376338>
68. Sonntag A, Pezze Dalle P, Shanley DP TK. A modelling–experimental approach reveals insulinreceptor substrate (IRS)-dependent regulation of adenosinemonosphosphate-dependent kinase (AMPK) by [insulin.pdf](#). *FEBS J*. 2012;
69. Fujita KA, Toyoshima Y, Uda S, Ozaki YI, Kubota H, Kuroda S. Decoupling of receptor and downstream signals in the Akt pathway by its low-pass filter characteristics. *Sci Signal*. 2010;3(132):1–11.
70. Rahman A, Haugh JM, Toker A. Kinetic Modeling and Analysis of the Akt/Mechanistic Target of Rapamycin Complex 1 (mTORC1) Signaling Axis Reveals Cooperative, Feedforward Regulation. *J Biol Chem*. 2017;292(7):2866–72. [PubMed: 28069808]
71. Krishnan P, Vinod U, Viswanath K. Quantification of the effect of amino acids on an integrated mTOR and insulin signaling pathway w. *Mol Biosyst*. 2009;5:1163–73. [PubMed: 19756306]
72. Faratian D, Goltsov A, Lebedeva G, Sorokin A, Moodie S, Mullen P, et al. Systems Biology Reveals New Strategies for Personalizing Cancer Medicine and Confirms the Role of PTEN in Resistance to Trastuzumab. *Cancer Res*. 2009;69(16):6713–21. [PubMed: 19638581]

Fig. 1. Conditioning cells with a growth stimulus potentiates subsequent CXCR4 signaling. **(A)** CXCL12 binds to CXCR4 and elicits downstream Akt and ERK activation. Separate kinase translocation reporters (KTRs) for Akt (aquamarine) and ERK (citrine) were stably expressed in breast cancer cells. Phosphorylation and dephosphorylation of the kinase substrate drives the reporter into the cytoplasm or nucleus, respectively. **(B)** Imaging experiments involved conditioning cells for 4 hours with or without growth stimuli or kinase inhibitors followed by single-cell, time-lapse imaging for 10 minutes before and 50 minutes after addition of 10 ng/ml CXCL12. Single cell time tracks show CXCR4-dependent

activation of Akt and ERK in MDA-MB-231 breast cancer cells quantified as $\log_2$ of cytoplasmic to nuclear ratio (C/N) of fluorescence intensities for each KTR in individual cells and displayed on a pseudocolor scale. Conditioning MDA-MB-231 with 10% fetal bovine serum (FBS) prior to adding CXCL12 potentiated Akt signaling compared to control conditioned cells. **(C)** FBS conditioning increased the number of cells responding to CXCL12 with strong activation of Akt as defined by ≥ 1 increase in $\log_2$ (C/N) unit. FBS conditioning minimally affected ERK signaling. **(D)** Relative to control, FBS conditioning for 4 hours produced a 4-fold increase in cells with strong activation of Akt. Extending FBS conditioning to 7 hours diminished strong responses in Akt to 2-fold above control. FBS conditioning for 4 hours, but not 7 hours, decreased the number of MDA-MB-231 cells with no detectable response in the Akt KTR. **(E)** Epidermal growth factor (EGF) conditioning for 4 hours produced concentration-dependent increases in cells with strong Akt responses with the highest concentration producing effects comparable to 10% FBS.

Fig. 2.

The computational Conditional Signaling Model (CSM) predicts CXCR4-mediated Akt and ERK signaling responses, establishing a framework for understanding the range of heterogeneous signaling data. **(A)** The CXCL12-CXCR4 interaction elicits G-protein signaling to activate Akt and ERK but can be restrained by negative feedback and crosstalk mechanisms. mTORC1 functions as a central regulator of signaling as it can inhibit activation of both Akt and ERK. Extrinsic noise in phosphatidylinositol-3-kinase (PI3K), Ras, and mTORC1 promotes activation of Akt and/or ERK in the absence of CXCR4-mediated signaling. Signaling kinetics cover a range of time scales with thicker arrows and lines qualitatively indicating faster reaction rates. A complete list of differential equations, initial conditions, and parameters is available in Supplementary Materials. **(B)** To encompass heterogeneous signaling responses of single cells in both Akt and ERK, we varied extrinsic noise parameters for PI3K, Ras, and mTORC1 in the CSM. By running combinations of these three parameters, we generated a model library of >12,000 predicted paired Akt and ERK responses. We performed a least-square fit of experimentally-determined Akt and ERK responses from the KTRs to predicted responses to derive the PI3K, Ras, and mTORC1 extrinsic noise parameters that best describe each single cell in the experiments.

Fig. 3.

The CSM captures heterogeneous single-cell signaling responses seen in experiments and reveals the conditional signaling states controlling responsiveness to CXCR4 signaling. **(A)** Responses from the model library match experimentally determined control and FBS conditioned single-cell CXCR4 signaling to Akt and ERK. Greater than 95% of cells fit the matching criteria, detailed in Methods. **(B)** Low extrinsic noise in PI3K and mTORC1 maximizes Akt responsiveness through CXCR4, whereas high PI3K, low mTORC1, and low Ras maximize responsiveness of ERK. The PI3K (orange), Ras (green), and mTORC1 (blue) axes define the extrinsic noise parameters from each simulation. Green and orange dotted lines denote where each 2D plane originated in the 3D signaling landscape.

Fig. 4. FBS conditioning shifted the conditional signaling state of MDA-MB-231 breast cancer cells to a region more permissive to CXCL12-CXCR4 signaling to Akt. **(A)** Occupancy maps illustrate combinations of extrinsic noise parameters (conditional signaling state) corresponding to regions of 3D signaling landscape (CSM output) where experimental cells most frequently match. Contour lines display numbers of cells out of 1×10^6 . Occupancies were summed in the third dimension for purposes of viewing the map in 2D. Cyan and yellow underlays illustrate regions of responsiveness for Akt and ERK, respectively. Green and orange lines illustrate the specific Ras and PI3K planes, respectively, corresponding to the responsiveness underlays. All occupancy maps were normalized to contain 1×10^6 cells. When unconditioned prior to CXCR4 stimulation, MDA-MB-231 cells occupy a regime of moderate PI3K, Ras, and mTORC1. **(B)** When conditioned with FBS prior to CXCR4 stimulation, the conditional signaling state of MDA-MB-231 cells shifts to a regime of lower PI3K and lower mTORC1. **(C)** FBS conditioning increased the number of cells responding through CXCR4 in Akt from 51% (control) to 71%. Responses were defined by computational modeling as >5 nM increases in kinase activity for Akt or ERK.

Fig. 5.

Genetic mutations determine the subset of conditional states available to cells, but these states can be tuned to further edit signaling behavior. **(A)** Vari-068 patient derived cells signal mostly in ERK, not Akt, and occupy a regime of high PI3K, due to an inactivating mutation in PTEN in these cells, and low mTORC1. **(B)** FBS conditioning shifts Vari-068 cells into regimes of lower mTORC1, a regime with greater ERK responsiveness to CXCR4 signaling. **(C)** FBS conditioning increased the percentage of cells responding in ERK from 87% to 94%.

Fig. 6. SUM-159 cells occupy conditional signaling states with ERK responsiveness. **(A)** SUM-159 cells occupy a regime of high PI3K and moderate Ras and mTORC1. **(B)** For SUM-159 cells, 61% and 39% respond through CXCR4 in ERK and Akt, respectively, despite activating mutations in both of these pathways. **(C)** Panels summarize interactions of genetic mutations and growth factor conditioning to tune cellular responsiveness in Akt and ERK by shifting the conditional signaling state at the single-cell scale. CA: constitutive activation; lof: loss of function.

Fig. 7.

Computational modeling correctly predicts that trametinib conditioning potentiates subsequent CXCR4-mediated Akt signaling in a subset of MDA-MB-231 cells. **(A)** The CSM predicts Akt (left) and ERK (right) signaling dynamics at simulated concentrations of trametinib conditioning that inhibit 50% or 90% of MEK activity relative to control prior to CXCR4 stimulation. The predicted signaling dynamics denoted by the red and blue dots correspond to different conditional signaling states and correspond to the dots in C. Trametinib conditioning drives concentration-dependent increases in Akt responsiveness in cells heterogeneously depending on the conditional signaling state and concentration-

dependent reductions in basal ERK activity and responses to CXCR4 stimulation. **(B)** The CSM accurately predicts single-cell experimental CXCR4-mediated Akt and ERK signaling dynamics for trametinib conditioning on MDA-MB-231 cells. Trametinib conditioning was modeled as a 50% decrease in the rate of MEK phosphorylation of ERK, consistent with noncompetitive inhibition kinetics. **(C)** Difference maps show the CSM-predicted change in peak Akt activation between the control conditioned and trametinib conditioned cells at each conditional signaling state. Shaded gray surface contours show regions in signaling landscape with conditional signaling states that position trametinib-conditioned cells for the listed increases in peak Akt activation (nM). Contour lines display numbers of cells out of one million occupying conditional signaling states after matching experimental trametinib-conditioned cells to the CSM. Green and orange lines illustrate the specific Ras and PI3K planes, respectively, of the CSM corresponding to the responsiveness underlays. Trametinib conditioning prior to CXCR4 stimulation drives increases in Akt responsiveness in cells occupying conditional signaling states that were permissive to Akt signaling under control conditioning (red dot). By comparison, trametinib has little effect on cells in conditional states that were not permissive to Akt signaling in control conditioning (blue dot). The red and blue dots from C correspond with those in A.

Fig. 8.

Computational modeling correctly predicts that conditioning with the mTORC1 inhibitor ridaforolimus potentiates subsequent CXCR4-mediated Akt and ERK signaling in MDA-MB-231 cells. **(A)** The CSM predicts Akt (left) and ERK (right) signaling dynamics at simulated concentrations of ridaforolimus conditioning that inhibit mTORC1 activity by 50% or 90% relative to control prior to CXCR4 stimulation. The predicted signaling dynamics denoted by the red and blue dots correspond to different conditional signaling states. Ridaforolimus conditioning drives concentration-dependent increases in Akt and ERK responsiveness in cells heterogeneously depending on the conditional signaling state. The red and blue dots denote cells able to respond and not respond in Akt under control conditioning, respectively. **(B)** Difference maps show the CSM-predicted change in peak Akt (left) and peak ERK (right) activation between the control conditioned and ridaforolimus conditioned cells at each conditional signaling state. Shaded gray surface contours show regions in signaling landscape with conditional signaling states that position ridaforolimus-conditioned cells for the listed increases in peak activation (nM). Contour lines display numbers of cells out of one million occupying conditional signaling states after matching experimental ridaforolimus-conditioned cells to the CSM. Green and orange lines illustrate the specific Ras and PI3K planes, respectively, of the CSM corresponding to the responsiveness underlays. Ridaforolimus conditioning prior to CXCR4 stimulation drives the largest increases in Akt responsiveness in cells with low PI3K (red dot only) and drives increases in ERK responsiveness at most conditional signaling states (red and blue dots). The red and blue dots from C correspond to those in A. The CSM-predicted signaling dynamics are from 50% inhibition of mTORC1.