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Abstract

Understanding cell state transitions and purposefully controlling them is a longstanding challenge in biology. Here, we present cell State Transition Assessment and Regulation (cSTAR), an approach to map cell states, model transitions between them, and predict targeted interventions to convert cell fate decisions. cSTAR uses omics data as input, classifies cell states, and develops a workflow that transforms the input data into mechanistic models that identify a core signaling network, which controls cell fate transitions by influencing whole-cell networks. By integrating signaling and phenotypic data, cSTAR models how cells maneuver in Waddington's landscape[1] and make decisions about which cell fate to adopt. Importantly, cSTAR devises interventions to control the movement of cells in Waddington's landscape. Testing cSTAR in a cellular model of differentiation and proliferation shows a high correlation between quantitative predictions and experimental data. Applying cSTAR to different types of perturbation and omics datasets including single cell data demonstrates its flexibility and scalability and provides new biological insights. The ability of cSTAR to identify targeted perturbations that interconvert cell fates will allow designer approaches for manipulating cellular development pathways and mechanistically underpinned therapeutic interventions.

The concept of cell states is a useful lens to view and understand the organization of tissues and organisms, their development, and responses to exogenous and endogenous changes. While initially based on phenotypical descriptions, global analysis methods now can connect phenotypes with underlying molecular processes. These methods characterize cell states with fine molecular resolution and open the door to understand how cell states can evolve and transition into each other. In 1940, Waddington suggested that cells move through a landscape of mountains and valleys as rolling marbles from one (meta)stable state to another[1]. This now famous model appeals through its intuitive nature but leaves open why the marbles roll into certain valleys and whether they can revert to an initial state. Recent efforts have applied computational models to understand cell state transitions, generated by

stochastic molecular processes[2,3], or they used lineage analysis to characterize and infer cell state transitions[4]. These efforts showed that cell states are interconvertible and that this involves changes in dynamic molecular processes, such as gene expression and signal transduction networks. However, a critical gap is the lack of a mechanistic understanding of how cellular networks drive cell state transitions that would allow us to purposefully manipulate and control cell states.

Here, we present cell State Transition Assessment and Regulation (cSTAR), which distinguishes cell states, quantifies their determining elements, reconstructs a mechanistic network that controls cell state transitions, and identifies pathway manipulations that allow us to convert one cell state into another. cSTAR can use different types of omics data, is scalable to different data sizes and flexible in terms of granularity of the analyses. We validate cSTAR with experimental data and identify precision interventions for controlling cell fate decisions.

Concept of cSTAR

cSTAR uses molecular data as input that contains enough information to distinguish different cell states. Initially, we use RPPA phosphoproteomic data and later show that other omics data are suitable, if they contain perturbations and reflect different cell states. cSTAR integrates the following steps (Fig. 1A): (1) data clustering and construction of a hyperplane separating the molecular features which characterize a cell state. We use support vector machines (SVMs), as they exploit high dimensional space to efficiently separate data by maximizing the distance between data points belonging to different cell states; (2) construction of a State Transition Vector (STV) that in the molecular dataspace indicates a path leading from the centroid of a point cloud of one cell state to the centroid of another cell state. The STV identifies the components of a core signaling network that governs cell state transitions; (3) a Dynamic Phenotype Descriptor (DPD) that quantifies cell phenotypic changes in response to a perturbation by measuring whether the perturbation moves the centroid towards or away from the separating hyperplane; (4) a Bayesian formulation of Modular Response Analysis[5] (BMRA), which reconstructs the topology, directions and strengths of causal connections between nodes of the core network created from the components specified by the STV. The DPD is an additional node in this core network representing the remainder of the global network upon which the core network acts to drive cell fate transitions; and (5) a resulting mechanistic model based on ordinary/stochastic differential equations (ODE/SDE) that calculates the quality and quantity of changes which are needed to convert one cell state into another. This model quantifies the forces that move a cell along Waddington's landscape and provides direct instructions for experimental perturbations that can convert one cell state into another.

Experimental system and datasets

To develop cSTAR we chose an experimental system that features robust cell fate decisions based on subtle molecular differences. The SH-SY5Y human neuroblastoma cell line is a well-established model for neuronal differentiation, neurodegeneration, and therapeutic target discovery[6]. Expression of the TrkA or TrkB receptor tyrosine kinases specifies different cell fates. TrkA stimulates differentiation marked by neurite outgrowth, whereas TrkB drives proliferation[7] (Fig. 1B). Differentiated TrkA cells

continue to proliferate, albeit slower than TrkB cells (Extended Data Fig. 1). These diverse phenotypes correlate with clinical outcomes in neuroblastoma. TrkA expression is associated with good prognosis, while TrkB expression correlates with aggressive tumor behavior[8]. TrkA and TrkB activate similar signaling pathways, and it is unclear how they cause these distinct cell fate decisions[7]. We stimulated isogenic SH-SY5Y cell lines stably expressing TrkA or TrkB receptors with their cognate ligands and used a custom made reverse phase protein arrays (RPPA) with 115 validated antibodies (Table S1). We measured the activities of pathways involved in TrkA/B signaling[7] in untreated cells and cells treated with NGF (TrkA ligand) or BDNF (TrkB ligand) for 10 or 45 minutes (Table S1, Extended Data Fig. 2). After normalization, we calculated the fold changes in protein phosphorylation levels or abundances, producing a data point for each protein. In addition, TrkA and TrkB activities were measured by Western blotting (Table S2).

Separating distinct physiological states

The individual data points for TrkA and TrkB cells can be perceived as points in the molecular data space of 115 dimensions (corresponding to the measurement of 115 protein features) that describe the cell states. However, phenotypically SH-SY5Y cells exhibit only three different states, a common 'ground' state with no growth factor (GF) stimulation, a differentiation state following TrkA cell stimulation with NGF, and a proliferation state following TrkB cell stimulation with BDNF. This suggests that distinct states might be determined by a handful of patterns hidden in the molecular data. Consequently, transitions between different cell states can be described by a few critical parameters, termed order parameters for complex systems[9,10]. While in physics the order parameters are found by modeling state transitions[11,12], no mechanistic models can determine the dynamic changes in whole-cell signaling patterns distinguishing cell states[13]. To address this gap we developed the STV, which informs us how signaling data patterns of a given cell state must change to allow one cell state transitioning into another.

The first step distinguishes and separates distinct cell states in protein phosphorylation and/or expression data space, using machine learning (ML) methods to cluster and classify signaling patterns (Methods, Extended Data Fig. 3A). Using a SVM[14] we built a hyperplane that maximizes the separation between distinct phenotypic states in the multidimensional RPPA dataspace. Principal component analysis (PCA) visualizes this hyperplane that separates TrkA/B cell states (Fig. 2A).

Building a State Transition Vector (STV)

The second step builds a vector which connects the centroids of the point clouds that represent the differentiation and proliferation states. The components of this vector are the differences of fold changes in the phosphorylation levels or abundances of each protein between the centroids of the TrkA/B point clouds. Dividing this centroid-connecting vector by its length gives the STV (Fig. 2A). Hence, the STV is a unit length vector that characterizes each molecule's contribution to the difference between cell states and determines the direction of cell state transitions (here from differentiation to proliferation). The absolute values of these contributions directly rank individual proteins according to their importance in switching cell states. Thus, we can identify the components of a core signaling

network that controls a larger network of cellular responses (Extended Data Fig. 3B). Including more components increases the granularity but also the number of perturbations needed for subsequent network reconstruction. Hence, the cutoff for components to include depends on the data and desired granularity. Importantly, cSTAR robustly separates cell states and determines core network components for different types of noisy omics data (Supplementary Information and Tables S14 and S15).

In the TrkA/B system the highest ranked proteins are receptor tyrosine kinases (RTKs), cytosolic kinases (AKT, ERK), and the ribosomal S6 protein that is phosphorylated by RSK and S6K, which themselves are targets of ERK and AKT signaling pathways (Table S3). We also included the JNK stress kinase, because we previously showed that JNK activation dynamics in neuroblastoma predicts clinical response[15]. The ERK and AKT pathways are main downstream effectors of TrkA/B receptor signaling[16]. This indicates that the differential integration of ERK and AKT activities may be key to determining different cell fates in these cells. Next, we tested whether this knowledge can be used to design interventions that switch cell fates.

The Dynamic Phenotype Descriptor (DPD)

A logical strategy is perturbing the STV-defined core components experimentally and test whether these perturbations can change the cell states. The STV contains information about the contributions of all signaling network components measured by RPPA. Removing the core components from the STV renders it a representation of the overall signaling network downstream of the core components. It also eliminates potentially confounding effects resulting from the perturbations indirectly affecting the activity of upstream network components through feedback loops. For instance, ERK inhibition abolishes negative feedbacks to TrkA/B mediated RAS activation, which would register as change in ERK signaling. However, this is inconsequential for ERK downstream signaling, as ERK is blocked by the inhibitor. Thus, this reduced STV can estimate the network effects and biological outcomes of experimental perturbations.

For each perturbation we determine a perturbation vector that connects the centroids of the point clouds before and after the perturbation. This vector changes the phenotype when it pushes the centroid of the point cloud across the hyperplane that separates different cell states, stabilizes a cell state when moving the centroid away from the separating hyperplane, or has no effect when it moves (nearly) parallel to this hyperplane (Fig. 2B). The perturbation outcomes are defined by the DPD. Its absolute value ($|S|$) quantifies the distance from the separating hyperplane to a point cloud centroid, while its sign indicates the direction relative to the STV. Here, S is positive if the point cloud centroid is on the same side of the separating plane as the proliferation cloud, and S is negative at the differentiation side. Any perturbation that drives the cellular response from differentiation to proliferation increases S and can change its sign to positive, whereas moving from proliferation to differentiation decreases S and can make it negative.

For experimental testing we targeted core components with small molecule inhibitors. The effects of the inhibitors on the DPD correlated well with their experimentally observed phenotypical effects (Table S4). As predicted, Trk and p70S6K inhibition changed the DPD from positive to negative values and strongly increased differentiation of TrkB cells. The RSK inhibitor decreased differentiation in

TrkA cells and weakly increased differentiation in TrkB cells. These correlations show that the DPD accurately predicts which perturbations can move cells into the differentiation state. However, the DPD changes do not necessarily correlate with the proliferation rate (Extended Data Fig. 1), as some inhibitors can lead to cell cycle arrest and apoptosis, and because proliferation and differentiation may not be mutually exclusive in neuroblastoma.

Non-linear dynamic models

In Waddington's terms, the DPD predicts how we can steer a marble into a valley without revealing why that steer works. The only means to precisely predict *and* explain the outcome of these experimental manipulations, which maneuver a cell through a Waddington landscape, is to explicitly model the nonlinear signaling dynamics that determine cell state transitions. This mechanistic model needs to comprise (i) a faithfully reconstructed topology of the core network components deduced from the STV with interaction signs and strengths; and (ii) a network node, which summarizes the remainder of the global network controlled by the core network and links signaling changes to phenotypic changes; this node output is the DPD described above.

Reconstructing a mechanistic core network. We previously developed a physics-based method, Modular Response Analysis (MRA), to exactly reconstruct and quantify causal, local connections between network nodes, including feedback loops, from perturbation data[5,17-19]. Each node is a reaction module, which can be a single protein or gene, a pathway, or any object defined in terms of input-output relations. For instance, in our core network the ERK module is a three-tier pathway that includes all RAF/MEK/ERK isoforms. The network topology is quantified by connection coefficients, aka local responses, or connection strengths[20]. They cannot be directly measured as perturbations propagate. MRA infers network connections from systems-level responses at steady states or at time instances when a signaling response approaches its maximum or minimum, because in both cases the time derivative is zero[21,22]. Whereas the overall topology does not markedly change between early peak and steady-state responses, the connection strengths are highly dynamic[22,23]. The original MRA requires as many perturbations as there are network nodes and is sensitive to noise in the data[24]. To overcome these limitations we developed a Bayesian MRA (BMRA) that requires fewer perturbations, is tolerant to noise, and allows to incorporate existing knowledge as a prior network to improve inference precision[25]. Even when this information is inaccurate for half of the network edges, BMRA recovers a nearly perfect network topology[26].

Mapping the core components specified by the STV onto known signaling pathways, we obtained a prior topology of a core network, which was identical for the TrkA/B expressing cells (Extended Data Fig. 4A). To reconstruct the posterior network, we used drug perturbations (Table S4) and measured 10- and 45-minute timepoints in TrkA and TrkB cells stimulated with NGF or BDNF. TrkA, TrkB, EGFR, ERBB2, AKT, and ERK activation peaked around 10 minutes and attained steady-state levels at ~45 minutes (Extended Data Fig. 4B). BMRA network reconstruction showed that connection strengths were different between the peak and steady-state levels (Table S5), but a consensus network can readily be derived for each cell line (Extended Data Fig. 5). These signaling networks feature major differences, e.g. the activation of the ERBB module by strong positive feedback from RSK to ERBB in

the TrkB network. The ERBB->ERK->RSK->ERBB loop acts as autocatalytic amplifier of the ERBB module. The strong activation of p70S6K by ERK in TrkB cells is subverted into a strong inhibition of ERK by p70S6K in TrkA cells. Overall, the TrkA network has more inhibitory connections, while the TrkB network comprises more stimulatory interactions and positive feedback loops.

The DPD describes the phenotype as summary of molecular features of all other components of the cell-wide network (proteins, mRNAs, etc), which are outside of the core network. Using BMRA to include the DPD as a node in our core network allowed us to systematically examine the influence of all core network pathways on cell state transitions alone and in combination. We used BMRA to determine connections to the DPD, as a network node/module, for each core pathway (Figs. 3A,B). As the DPD links the network to cell fate decisions, a connection to the DPD node indicates how a signaling change influences the phenotype. A positive connection means that the cell is pushed towards proliferation, whereas a negative coefficient indicates a push to differentiation. Because the changes in the DPD are downstream of the core network and therefore require more time, we assessed the DPD responses after 45 minutes of GF stimulation. Measuring fold changes in the outputs of signaling pathways and the DPD module, we obtained the global, systems-level responses to perturbations and inferred the influence of each signaling pathway on the DPD and cell phenotype (Figs. 3C,D, Table S5). In this analysis, the ERK and S6K modules have positive connection coefficients to the DPD promoting cell proliferation in both TrkA and TrkB networks. However, the influence of the RSK and JNK modules on cell phenotypes is drastically different. In the TrkA network, RSK and JNK suppress proliferation and induce differentiation, whereas in the TrkB network these pathways do not influence the DPD and the phenotype. Thus, ERK-induced activation of JNK and RSK modules in TrkB-expressing cells does not suppress proliferation of these cells.

Predicting signaling dynamics

The BMRA-quantified core network topologies and their inferred influences on the DPD allow us to directly derive mechanistic models for TrkA/B cells, which predict both the dynamics of core pathway outputs and associated changes in cellular phenotypes (see Methods for a detailed model description). The TrkA/B core networks contain the same nodes but differ in connections and their strengths (Extended Data Fig. 5). The model predicts that these differences cause distinct signaling patterns, which is supported by experimental data (Figs. 4A and 4B). Based on the inferred activation of ERBB by TrkB and amplifying autocatalytic loops, ERBB->ERK->ERBB and ERBB->ERK->RSK->ERBB, the model predicts higher and sustained levels of active RTKs, ERK, AKT, S6K and RSK in TrkB compared to TrkA cells (Figs. 4A,B). The model also correctly predicts responses of core network pathways of TrkA/B cells to NGF and BDNF stimulation and their responses to different drug perturbations (Extended Data Figs. 6-8). For example, S6K inhibition increases ERK and AKT activation due to downregulation of S6K-induced negative feedback loops, which are stronger in TrkA than in TrkB cells (Extended Data Fig. 6A). In both TrkA and TrkB cells, inhibition of Trk receptors suppresses signaling by all core pathways confirming that they are driven by Trk receptors (Extended Data Fig. 6B). Even a moderate inhibition of ERK substantially downregulates phosphorylation of ERBB, AKT, and their downstream effectors in TrkB cells, whereas these effects are minute in TrkA

cells (Extended Data Fig. 7A). This is explained by the positive feedback from the ERK module to ERBB via RSK in TrkB cells (Figs. 3A,B). Also, AKT inhibition suppresses core pathways in TrkB more efficiently than in TrkA cells due to positive feedback from AKT to ERBB in TrkB cells (Extended Data Fig. 7B). Thus, self-amplifying positive feedback loops from ERK and AKT to ERBB receptors drive the sustained proliferation of TrkB cells.

Cell maneuvering in Waddington's landscape

cSTAR integrates cell state transitions into a mechanistic model, following both the output kinetics of the core network and cellular phenotype changes. It allows us to map how a cell maneuvers in Waddington's landscape and how external perturbations influence a cell's journey in this landscape. In the molecular dataspace, centroids of data point clouds present population-averaged cell states and phenotypes. Before ligand stimulations or drug perturbations, cells reside in (meta)stable states. Following a perturbation, centroid movements are governed by two forces: a signaling driving force emerging from the changes in core network activities, and a restoring force that pushes the centroid back to its original (meta)stable state (Extended Data Fig. 9). Only pathways with non-zero connections to the DPD node generate a driving force that affects the DPD (Table S5 and Fig. 3). The restoring force is a gradient force that increases in the vicinity of the stable steady state but then decreases to zero at the cell state separation surface (Extended Data Fig. 9A). A combination of signaling and restoring forces determines the shape of Waddington's landscape (Extended Data Fig. 9C). While internal or external noise can stochastically induce the crossing of landscape peaks and subsequent cell fate changes, the signaling force directs cell fate decisions in a controlled way. Summarizing, cSTAR can calculate the driving signaling force and predict the corresponding interventions allowing us to steer cell fate decisions in Waddington's landscape (Extended Data Fig. 9).

Our simulations show that ligand stimulation moves TrkA and TrkB cells from the ground state through Waddington's landscape along different trajectories towards differentiation or proliferation (Figs. 4C-E). These predictions are supported by experimental data (Fig. 4F). Calculating the DPD trajectories after inhibitor perturbations (Figs. 5A-L) showed that TrkB or S6K inhibition promotes differentiation, whereas RSK inhibition in TrkA cells interferes with differentiation. All simulations, except DPD responses to MEK inhibition in TrkB cells, were corroborated by quantifying cell differentiation by imaging. MEK inhibition features an abrupt transition between differentiation and proliferation. Thus, a small inaccuracy in the calculated DPD response to MEK inhibitor might have led to an incorrect phenotypic prediction.

Nevertheless, the model captures both direct and network-mediated effects of drugs on cellular phenotypes. It predicted that ramping up AKT activity in TrkA cell converts differentiation into proliferation (Fig. 5M). This was experimentally confirmed by transfecting TrkA cells with constitutively active myristolated AKT (Figs. 5N,O). Simulations predicted that inhibition of TrkB, AKT, S6K, or RSK, converts TrkB signaling into differentiation (Figs. 5A,B,G,I), as supported by experimental observations (Figs. 5D,E,J,L). Representative TrkA/B cell images for all inhibitor perturbations are shown in Extended Data Figs. 10 and 11.

Using the model, we calculated cell state responses for drug combinations. The efficiency of a two drug combination was assessed by the DPD response across a two-dimensional plane of drug doses, similar as pathway responses are assessed in pharmacology and therapeutics[27]. The lines of constant DPD are termed Loewe isoboles. For non-interacting drugs Loewe isoboles are straight lines, for synergizing inhibitors they are concave, whereas convex isoboles indicate antagonism. Predictive simulations suggested that combining ERBB and MEK inhibition synergizes to change the DPD and induce differentiation in TrkB cells (Fig. 6A) without impacting cell states in TrkA cells (Extended Data Fig. 12A). Experimentally, combination treatment with Gefitinib (ERBB inhibitor) and Trametinib (MEK inhibitor) markedly induced TrkB cell differentiation, despite individual treatments at double doses being ineffective (Fig. 6B and Extended Data Fig. 13). This inhibitor combination synergistically induced FAK phosphorylation (Fig. 6C), which is a well-established differentiation marker[28]. As predicted by the model, TrkA cell states were not affected (Extended Data Figs. 12B,C).

cSTAR flexibility and scalability

Next, we tested cSTAR's performance with data of different type and scale. Using the same conditions as in the RPPA dataset, we acquired quantitative phosphoproteomics MS datasets for TrkA and TrkB cells (Table S6). Calculating the STV and DPD changes for cell-wide signaling pattern of ca. 5000 phosphosites (Tables S7 and S8 and Extended Data Fig. 14) resulted in similar core network components (Extended Data Fig. 15) and a key prediction of synergy between ERBB and MEK inhibitors in inducing TrkB cell differentiation without affecting the TrkA cell phenotype (Fig. 6D, Supplementary Information), which was experimentally validated. Thus, cSTAR produces robust and reproducible results even when the input data differ vastly in scale and bias.

RAF inhibitor-resistant melanoma. To map drug resistance mechanisms, we applied cSTAR to an extensive RPPA dataset of 238 proteins measured under 89 perturbations of RAF inhibitor resistant SKMEL-133 cells[29]. As different phenotypic states we selected proliferation (untreated cells) and apoptosis induced by combination treatment with MEK and PI3K/AKT/mTOR inhibitors (Extended Data Fig. 16). The STV ranked the MEK/ERK, AKT, mTOR/S6K, SRC, CDK4/6, PKC, and IRS modules as the components of a core network that controlled these states (Table S9 and Methods). Next, we applied BMRA to single-drug perturbation data, inferring the core network circuitry and its connections to the DPD module (Fig. 7A and Table S10). The reconstructed network included known signaling routes, including the IRS-mediated activation of the ERK and AKT modules, AKT activation of mTOR, CDK4/6 activation by ERK and mTOR, and negative feedback from mTOR to IRS[26]. However, BMRA also uncovered activating connections from PKC to AKT, mTOR, SRC and CDK4/6, a negative connection from PKC to IRS, and CDK4/6-induced positive and negative feedback loops to the AKT and SRC modules (Fig. 7A). Based on their direct connections to the DPD, mTOR and PKC drive proliferation, while the phenotypical effect of other nodes is indirect. For instance, ERK activates mTOR through SRC and CDK4/6 to stimulate proliferation, partially counteracted by CDK4/6-mediated feedback inhibition of ERK. Although SRC directly inhibits the DPD, it stimulates proliferation on the systems level by activating mTOR.

The original publication[29] showed that MYC inhibition synergized with BRAF or MEK inhibition to suppress proliferation and induce apoptosis. Thus, we added MYC to our core network and re-inferred network connections. This extended network was very similar to the original network except that CDK inhibited SRC not directly but via MYC (Fig. 7B, Table S10). The equivalence of these networks illustrates that BMRA allows zooming-in/out on the inferred connections by adding nodes of interest or deleting unimportant nodes[5].

Informed by the BMRA network reconstruction, we built a nonlinear dynamical model of SKMEL-133 cell signaling and phenotypic behavior. Because cSTAR enables building models of different granularities, we tested the effects of including or omitting MYC. Adding MYC only changed parameters of modules directly interacting with MYC without changing any model predictions. Thus, the ODE description of each network module can be extended to include additional mechanistic knowledge (see Methods for successive steps of model refinement).

The model predicted that an mTOR inhibitor was the most efficient single drug to induce apoptosis in SKMEL-133 cells, whereas PI3K/AKT inhibition was less effective (Extended Data Fig. 17A). This differential sensitivity is explained by the double-positive feedback between CDK4/6 and mTOR (Fig. 7A,B), which greatly increases the stimulation of proliferation by mTOR and CDK4/6. PKC inhibition also markedly reduced proliferation, as PKC directly influences the DPD, whereas inhibition of other nodes, including MEK/ERK signaling, was less effective.

The cSTAR model recapitulated the results by Korkut et al[29] including the synergy between MEK and MYC inhibitors (Fig. 7C). Furthermore, the model predicted that combining Insulin/IGF1 receptor and PI3K/AKT inhibition enhances synergy (see Fig. 7D). This result is supported by calculating the Talalay-Chou combination index (Methods) and simulating SKMEL-133 cell maneuvering in Waddington's landscape following inhibitor treatments. PI3K/AKT or Insulin/IGF1 receptor inhibitors given separately do not switch the DPD to negative, apoptotic region (Figs. 7E and 7F). However, given in a combination at twice lower doses, they shift the DPD to apoptosis (Fig. 7G). We also found that combining MEK/ERK and PI3K/AKT inhibitors was highly synergistic[30] (Extended Data Fig. 17B). This example shows that cSTAR is a powerful tool to analyze drug responses and predict synergistic combinations.

Epithelial-Mesenchymal Transition (EMT). cSTAR quantifies phenotypic changes via the DPD, opening the possibility to integrate different omics datasets by comparing the normalized DPD changes following perturbations. Testing this, we applied cSTAR to two datasets that analyzed EMT suppression by kinase inhibitors. One study used single-cell RNA sequencing (scRNA-seq) of four cancer cell lines stimulated with three different ligands, TGFβ, EGF and TNFα[31]. The other used single-cell resolution mass cytometry of phosphoproteomic responses in Py2T breast cancer cells stimulated with TGFβ[32].

The results of the cSTAR analysis (Supplementary Information, Extended Data Figure 18) correspond well to the original phenomenological observations and conclusions drawn in these papers[31,32] (Extended Data Fig. 19 and Table S11). They show that cSTAR correctly captures the relationships between phenotypical and underlying molecular states. Moreover, cSTAR adds new insights. Interestingly, the DPD analysis of scRNAseq data demonstrated that at single-cell resolution the

observed partial EMT states comprise a continuum of intermediate states between fully epithelial and fully mesenchymal states (Extended Data Figs. 20 and 21). To underpin these states with mechanistic interpretations, which was previously not possible, we applied BMRA to reconstruct the twelve signaling networks (four cell lines, three ligands), underlying these phenotypes in each cell type under each condition. These networks show how differential network topologies and connection strengths cause cell type and stimulation-specific responses (Table S12). These reconstructions of different network topologies will help designing the most informative experiments to disentangle the relationships between these multiple EMT states.

Discussion

A grand challenge of modern biology is to interpret the vast amount of different data types we generate. We need tools that can progress accurate classifications into an actionable and causal analysis of cell states, enabling us to control transitions between them. cSTAR bridges the current gap between classification and mechanistic understanding of cell states and produces actionable predictions. This is enabled by three key features.

First, the SVM accurately determines the maximal margin hyperplane[14] that separates different cell states in the data space, whereas the STV identifies the molecular network components that control cell state transitions. Generally, several sequential or alternative cell transitions between several state states are possible. The STV can be built between any of two selected states, pointing to the overall change in the molecular features, required for the transition between these two states. However, biologically not all transitions are possible, and this is an exciting topic for further cSTAR applications.

Second, the DPD allows us to connect phenotypical to molecular changes and the movement in Waddington's landscape. The idea behind the DPD is based on two pillars: (*i*) non-equilibrium thermodynamics suggests that different physicochemical systems can behave similarly in the vicinity of a critical state, and the system dynamics can be parametrized by the distance to the critical point[9], and (*ii*) our results suggest that not only the binary SVM classification is informative, but the distance to the separating hyperplane determines when the variety of small quantitative changes in the signaling patterns can result in the qualitative change in the cell state. This distance is determined by the absolute DPD value as a key parameter that describes the dynamics of cell state transitions. The DPD connects signaling dynamics to the intuitively attractive picture of Waddington's landscape. Despite many attempts to quantify cell movements in Waddington's landscape, cell state transitions were never linked to external cues and downstream signaling networks that drive these transitions[3]. Integrating biochemistry and physics, cSTAR determines how multiple pathway activities dynamically control cell state transitions in an evolving Waddington's landscape, making cell fate decisions tractable and manipulatable.

Third, the BRMA guided network reconstruction enables a full mechanistic understanding and dynamic response analysis of the biological system. Notably, core network circuitries substantially differ in different cell lines, isogenic cells, and even in the same cell line stimulated with different ligands. The inferred networks and resulting mechanistic models are used for identifying mechanisms of biological

decision making and designing targeted interventions that eliminate an undesired phenotype, e.g., drug resistance.

Our application examples show that cSTAR can utilize and integrate diverse omics data including targeted and unbiased data of different scales as well as single cell data. This universality and scalability distinguishes cSTAR from other approaches that are more specialized in terms of input data, e.g, approaches relying on mRNA velocity input[33,34]. Summarizing, cSTAR offers a cell-specific, mechanistic approach to describe, understand and purposefully manipulate cell fate decisions. As such it has numerous applications across biology that go beyond the use for interconverting proliferation and differentiation shown here as examples.

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METHODS

Computational Methods

Normalization of Reversed Phase Protein Array (RPPA) and Western Blot data

Each analyte measurement was first normalized by the GAPDH level, and then on the value of the same analyte in the absence of inhibitors and ligand stimulation to obtain fold changes (Tables S1 and S2).

Data clustering and Principal Component Analysis (PCA)

To cluster RPPA signaling patterns two different unsupervised ML methods, Ward's hierarchical clustering and the K-means clustering, were used. These methods generated identical results and determined two distinct sets of data points that corresponded to two different cell states, NGF-stimulated TrkA differentiation state and BDNF-stimulated TrkB proliferation state (Extended Data Figures 2 and 3A).

The Pandas python library was used for RPPA data analysis and manipulation. For PCA compression and K-means data clustering we used the scikit-learn python library[35]. Crucially, PCA was used solely for visualization purposes, while all cluster analysis, the SVM, STV and DPD calculations were performed in the original data space. R base functions[36]. The pheatmap R package were used for Ward's hierarchical clustering and building a heatmap.

Separation of distinct physiological states using Support Vector Machines (SVMs)

Following stimulation with GFs or drug perturbations, the fold changes in the phosphorylation levels or protein abundances were depicted in a molecular dataspace with the Cartesian coordinates. The SVM with a linear kernel from the scikit-learn python library was applied to build a maximum margin hyperplane that distinguishes different cell states in the molecular dataspace.

The SVM maximizes the separating margin using the data points that are closer to the hyperplane and are termed the support vectors. The separating hyperplane is defined as,

$$\left(\vec{x}, \vec{n}\right) = h \qquad (1)$$

.

Here $\vec{x}$ is a radius vector from the origin of the coordinates to any point on the separating hyperplane, $\vec{n}$ is the vector of unit length that is orthogonal to the separating hyperplane, and h is a constant.

Derivation of the STV

Let A be the centroid of a cloud of points A_i (i = 1,2 …) that corresponds to state 1, and B be the centroid of the point cloud B_i corresponding to state 2. A state transition vector (STV) from state 1 to state 2 is defined as a vector $\vec{s}$ of unit length that has the same direction as the vector $\vec{AB}$ connecting the centroids A and B,

$$\vec{s} = \vec{AB} / \left|\vec{AB}\right| \qquad (2).$$

Eq. 2 shows that the STV is initially built in the full molecular dataspace of 115 dimensions.

Ranking each network protein by its contribution to the STV

The vector $\vec{s}$ determined by Eq. 2 in the Cartesian coordinates has the components s_k, $k = 1, ..., N$. Each STV component s_k corresponds to an analyte k, measured by an antibody detecting a specific phosphosite on a protein or the abundance of a specific protein. The absolute value $|s_k|$ determines the STV rank of the analyte k informing us about its importance for the switching of cell states. These STV ranks for all analytes are presented in Table S3. The highest ranked proteins and some of their immediate effectors were selected as core signaling network components.

Derivation of perturbation vectors

For a correct interpretation of perturbation data using the STV, we have to exclude the analytes that composed the modules of our core signaling network. Accordingly, the dimensionality of the molecular dataspace where the STV and perturbation vectors are calculated is reduced from 115 to 70.

Let A with the radius-vector $\vec{x}_A$ be the centroid of the point cloud A_i, corresponding to the unperturbed state 1. Let A_{pert} with the radius-vector $\vec{x}_{A_{pert}}$ be the centroid of the point cloud (A_i^{pert}), corresponding to the perturbed state 1. Then the perturbation vector is defined as,

$$\vec{x}_{A_{pert}} - \vec{x}_A = \vec{AA}_{pert} = \vec{P} \quad (3)$$

.

Distance from a data point to the separating plane along the STV

Starting from a data point A in the molecular dataspace we build a vector ($\vec{AA}_s$), which is parallel or antiparallel to the STV ($\vec{s}$) and crosses the separating hyperplane at a point, A_s. Thus, we have,

$$\vec{AA}_s = S \cdot \vec{s} \quad (4)$$

.

If the vector $\vec{AA}_s$ has the same direction as the STV, the value of S is positive, while S is negative if the vector $\vec{AA}_s$ has the opposite direction to the STV. In either scenario, the length ($|S|$) of the vector $\vec{AA}_s$ is the distance from the separating hyperplane to the point A along the STV.

The vectors $\vec{x}_{A_s}$ and $\vec{x}_A$ connecting the origin of the coordinates and the points A_s and A, respectively, are related by the following equation,

$$\vec{x}_{A_s} = \vec{x}_A + S \cdot \vec{s} \quad (5)$$

.

Using Eqs. 1, 4 and 5, we obtain,

$$\left(\vec{x}_{A_s}, \vec{n}\right) = \left(\left(\vec{x}_A + S{\cdot}\vec{s}\right), \vec{n}\right) = \left(\vec{x}_A, \vec{n}\right) + S{\cdot}\left(\vec{s}, \vec{n}\right) = h \quad (6)$$

.

Eq. 6 allows us to calculate the distance $|S|$ from a point A in the molecular dataspace to the separating hyperplane between two different cell states, as follows

$$|S| = \left|\left(h - \left(\vec{x}_A, \vec{n}\right)\right)/\left(\vec{s}, \vec{n}\right)\right| \quad (7)$$

.

If $\vec{s} = \vec{n}$ then $|S|$ is the shortest distance to the separating hyperplane. If $\vec{s} \neq \vec{n}$ then $|S|$ is larger than the shortest distance to the hyperplane, because vectors $\vec{s}$ and $\vec{n}$ have unit lengths. If point A is a centroid of a point cloud that corresponds to a distinguishable cell state, than Eq. 7 determines the distance of this centroid to the separating hyperplane.

Calculation of the Dynamic Phenotype Descriptor (DPD) module output (*S*) using experimental data

In the molecular dataspace, we consider the STV as a vector $\vec{s}$ of unit length directed from a centroid of a differentiation TrkA point cloud to a centroid of a proliferation TrkB point cloud. We now define the output of the DPD module as the *S* value,

$$DPD = S = \left(h - \left(\vec{x}_A, \vec{n}\right)\right)/\left(\vec{s}, \vec{n}\right) \quad (8)$$

.

The direction of the vector $\vec{n}$, which is orthogonal to the separating hyperplane, points from the TrkB cloud to the TrkA cloud. In this case, the DPD value (S) is positive for proliferation TrkB points and negative for differentiation TrkA points. The DPD values for TrkA and TrkB cells after GF stimulations and inhibitor treatments are given in Table S4.

Notably, the results of our analyses of all datasets presented in this work will practically be the same, if we define STV as the normal vector of the separating hyperplane ($\vec{n}$), rather than the vector ($\vec{s}$) connecting the centroids of two different cell states. Thus, both $\vec{n}$ and $\vec{s}$ can be defined as STV depending on the situation or preference.

Calculations of the DPD changes upon perturbations. Using the STV ($\vec{s}$), a perturbation vector ($\vec{P}$) and the unit length vector ($\vec{n}$) orthogonal to the separating hyperplane, we can calculate how the DPD changes following each perturbation by inhibitors. The DPD values, determined for the centroids of unperturbed (A) and perturbed (A_{pert}) states, S and S_{pert}, respectively, are the following (see Eq. 8),

$$S = \left(h - \left(\vec{x}_A, \vec{n}\right)\right)/\left(\vec{s}, \vec{n}\right) \quad (9).$$

$$S_{pert} = \left(h - \left(\vec{x}_{A_{pert}}, \vec{n}\right)\right) / \left(\vec{s}, \vec{n}\right) \quad (10)$$

Using Eqs. 3, 9 and 10, the change in the DPD upon a perturbation is expressed as follows,

$$\Delta S = S_{pert} - S = \left(\vec{x}_A - \vec{x}_{A_{pert}}, \vec{n}\right) / \left(\vec{s}, \vec{n}\right) = - \left(\vec{P}, \vec{n}\right) / \left(\vec{s}, \vec{n}\right) \quad (11)$$

From Eq. 11 it follows that if $\vec{s} = \vec{n}$, then $\Delta S = - \left(\vec{P}, \vec{n}\right)$.

Bayesian Modular Response Analysis (BMRA) network inference

To reconstruct the topology and strengths of causal connections of the core network, including the influence of each pathway module on the DPD module, we have used a modified version of BMRA. A family of Modular Response Analysis (MRA) methods, including BMRA[26], allows both (*i*) predicting systems-level network responses to different perturbations and (*ii*) reconstructing the topology and strengths of causal network connections based on experimentally measured responses to perturbations[19,25,37].

Each core network module has a single quantitative output (x_i), termed communicating species in the MRA framework. The temporal dynamics of the module outputs is given by a system of ordinary differential equations (ODE),

$$\frac{dx_i}{dt} = f_i\left(x_1, ..., x_n, p\right),\ i = 1, ..., n \quad (12)$$

Here the functions f_i describe how the rate of change of independent variables x_i depends on the activities of other network modules. The parameters, $p_i \in \boldsymbol{P}$, represent kinetic constants and any external or internal conditions, such as the conserved moieties and external concentrations that are maintained constant.

For each network module x_i, the connection coefficient (r_{ij}) quantifies the fractional change ($\Delta x_i/x_i$) in its output brought about by a change in the output of another module ($\Delta x_j/x_j$), while keeping the remaining nodes (x_k, $k \neq i,j$) unchanged to prevent the spread of this perturbation over the network[5,20].

$$r_{ij} = \partial \log log\, x_i \,/ \partial \log log\, x_j ;\ \ x_k = const\ (k \neq i,\ j) \quad (13)$$

Positive and negative r_{ij} quantify direct activation and inhibition, respectively, whereas zero values show that there are no direct connections. The coefficients r_{ij} are expressed in terms of the elements of the Jacobian matrix ($\partial f_i/\partial x_j$) of the ODE system at the steady state ($st.\ st.$), as follows[5],

$$r_{ij} = \frac{\partial \log log\ x_i}{\partial \log log\ x_j} = \frac{\partial x_i}{\partial x_j}\left(\frac{x_j}{x_i}\right) = -\frac{\frac{\partial f_i(x_1,\ldots,x_n,p)}{\partial x_j}}{\frac{\partial f_i(x_1,\ldots,x_n,p)}{\partial x_i}}\left(\frac{x_j}{x_i}\right)\Big|_{st.st.} \quad i,\ j = 1,\ldots n \qquad (14).$$

The connection coefficients cannot be directly measured and are inferred using the systems-level, global network responses to perturbations. Following a change (Δp_j) in a parameter (p_j) that affects node j, the global response (R_{ij}) to this perturbation is determined as,

$$R_{ij} = \frac{d\log log\ x_i}{d\log log\ p_j}\Big|_{st.st.} \qquad (15)$$

.

To infer the connection coefficients r_{ij} based on the experimentally measured, global responses R_{ij}, the entire network is initially divided into n subnetworks, each containing only edges directed to a particular node (i). To determine the connection coefficients for all x_k ($k \neq i$), n - 1 independent parameters p_j (j = 1,…,n-1) must be perturbed, neither of which can *directly* influence node i, whereas any other node k ($k \neq i$) is affected by at least one of these parameters p_j. Formally, for each x_i (i = 1, …, n), we choose a subset $\boldsymbol{P_i}$ of n-1 parameters p_j known to have the property that the function f_i for node i in Eq. 12 *does not* explicitly depend upon p_j, whereas each of the remaining nodes k ($k \neq i$) is perturbed by at least one $p_j \in \boldsymbol{P_I}$. This condition is described as follows,

$$\frac{\partial f_i(x_1,\ldots,x_n,p)}{\partial p_j} = 0,\ if\ k \neq i,\ then\ \frac{\partial f_k(x_1,\ldots,x_n,p)}{\partial p_j} \neq 0\ at\ least\ for\ one\ p_j \qquad (16)$$

.

Taken into consideration that $r_{ii} = -1$ (Eq. 14), all connections to the node i can be found by solving the following system of linear equations,

$$R_{ij} = \sum_{k=1,k\neq i}^{n} r_{ik}R_{kj},\quad \frac{\partial f_i(x_1,\ldots,x_n,p)}{\partial p_j} = 0,\quad i = 1,\ldots,n;\ j = 1,\ldots,n-1; \qquad (17)$$

.

Repeating this procedure for all n subnetworks, the entire network is reconstructed.

This standard MRA procedure fails, if the data are too noisy or some module responses were not detected[24]. BMRA overcomes these limitations by explicitly incorporating noise in Eq. 17[26],

$$\sum_{k=1,k\neq i}^{n} A_{ik}r_{ik}R_{kj} + \epsilon_{ij} = R_{ij} \qquad (18).$$

Here, A_{ik} are the elements of the adjacency matrix, which are equal to 1 if the connection coefficient r_{ik} is non-zero, or equal to 0 otherwise; ϵ_{ij} are the error variables assumed to be independently and identically distributed Gaussian random variables with the 0 mean and the variance σ^2, i.e. $\epsilon_{ik} \sim N(0, \sigma^2)$. The error variance ($\sigma^2$) is assumed to be a random variable with the inverse Gamma

distribution, i.e. $\sigma^2 \sim IG(a, b)$, where a and b are the location and scale parameters. Following common practice we chose $a = 1, b = 1$. Further, for brevity we refer to this distribution $P(\sigma^2)$.

BMRA uses prior knowledge that is formulated in the form of the prior probability distributions. Based on the existing knowledge[16,38] we derived the reference network $A_i^0 = \{A_{ik}^0\}$. The prior distribution $P(A_i)$, $A_i = \{A_{ik}\}$, has the maximum at the reference network A_i^0 and penalizes for the deviation from this network as follows, $P(A_i) \propto exp(-\psi \cdot d_H(A_i, A_i^0))$, where $d_H(A_i, A_i^0)$ is the Hamming distance between the network A_i and the reference network A_i^0, ψ is a constant. The prior distribution of r_i is dependent on A_i, and σ^2 and is denoted by $P(r_i|A_i, \sigma^2)$. If there is no direct connection from x_j to x_i, i.e. $A_{ij} = 0$, the corresponding connection strength (r_{ij}) is assumed to have 0 value with probability 1, whereas the connection strengths representing direct interactions ($r_i = \{r_{ij} : A_{ij} = 1, j \neq i\}$) are assumed to have a Gaussian prior $P(r_i|A_i) \sim N(0, V_i)$, where $V_i = c\sigma^2(R_i^* R_i^{*T} + \lambda I)$. Here, R_i^* is the global response matrix of the nodes ($n_j, j \neq i$) which directly regulate n_i (i.e. $n_j, j \neq i : A_{ij} = 1$), c is the proportionality constant which is also known as the Zellner's constant. As previously[26,39], we chose $c = N_p^i$, where N_p^i is the number of perturbations other than those directly affecting node x_i, and $\lambda = 0.2$.

Bayesian statistics is applied to update prior estimates of the binary vector $A_i = \{A_{ik}\}$, $k = 1, \ldots, n$, and the vector of connection coefficients $r_i = \{r_{ik}\}$, $k = 1, \ldots, n$, to obtain posterior estimates of these variables using the experimental data, i.e., the global response matrix $R = R_{ik}$ (Eq. 15),

$$P(R) = \frac{P(r_i, A_i, \sigma^2) P(A_i, \sigma^2) P(A_i) P(\sigma^2)}{P(R)} \quad (19)$$

.

Here, $P(r_i, A_i, \sigma^2)$ is the likelihood function of the global response matrix $\boldsymbol{R}$, given a connection coefficient vector r_i and a binary vector A_i. $P(A_i, \sigma^2)$ and $P(A_i)$ are the prior distributions of r_i and A_i, respectively. The denominator $P(R)$ is defined as follows,

$$P(R) = \iiint P(r_i, A_i, \sigma^2) P(A_i, \sigma^2) P(A_i) P(\sigma^2) dr_i dA_i d\sigma^2 \quad (20)$$

.

A key to BMRA is that the likelihood function for the observed global response matrix $\boldsymbol{R}$ is derived from the MRA equations (Eq. 17-19),

$$P\left(r_i, A_i, \sigma^2\right) = N(R_i | R_i^{*T} r, \sigma^2 I) \quad (21)$$

.

Here, $R_i = \{R_{ik}, k \neq i\}$ is the global response of node x_i to perturbations that do not directly affect x_i, R_i^{*T} is the global response matrix of the nodes (x_j, $j \neq i$) which directly regulate node x_i (i.e. x_j, $j \neq i$: $A_{ij} = 1$, and $r = \left\{r_{ij} : A_{ij} = 1,\ j \neq i\right\}$. $N(R_i | R_i^{*T} r, \sigma^2 I)$ designates the normal distribution for R_i where the mean equals $R_i^{*T} r$ and the variance $\sigma^2 I$.

The denominator in Eq. 19 that normalizes the probability $P(R)$ cannot be obtained analytically. Therefore, its posterior distributions were estimated using Markov Chain Monte Carlo (MCMC) sampling algorithm. The posterior probability of $\boldsymbol{A}_i$ provides a quantitative measure of how well a certain configuration of $\boldsymbol{A}_i$ is supported by both prior knowledge and experimental data.

The values and confidence intervals for the corresponding connection coefficients are obtained from the posterior probability of r_i. To increase the accuracy of the method, we have modified the previously published algorithm[26] and applied Occam's razor approach by calculating the mean and STD of r_i using not the entire posterior distribution of r_i, but only a part that has the highest posterior likelihood (5,000 networks from 200,000 sample networks).

Preparation of the RPPA perturbation dataset for the BMRA network inference

Table S5 presents a list of analytes that are outputs of signaling modules (TRK, ERBB, ERK, AKT, JNK, S6K and RSK) of our core network. The output of the DPD module is determined using Eqs. 8-11 in the 70-dimensional molecular dataspace. To calculate the global response coefficients for the signaling modules, x_i, we used central fractional differences to approximate the logarithmic derivatives,

$$R_{ij} = 2 \cdot \frac{x_{i1} - x_{i0}}{x_{i1} + x_{i0}} \quad (22)$$

.

Here x_{i0} and x_{i1} are the i-th module outputs before and after a perturbation to the parameter p_j. Because the sign of the DPD value (S) could change for large perturbations, we used either left or right fractional differences,

$$R_{Sj} = \frac{S_1 - S_0}{S_0} \quad (23)$$

.

A feature of the BMRA formalism is that it can infer the network topology without the need to perturb all modules. In a core network we have not perturbed a module consisting of the ERBB family of RTKs, which can crosstalk with Trk receptors either directly or through downstream signaling pathways and feedback loops. The output of this additional RTK module (termed ERBB) is determined as the sum of EGFR and ERBB2 phosphorylation, measured with the corresponding antibody that does not distinguish between these two ERBB family receptors. Having determined the global response coefficients of all modules using Eqs. 22 and 23, BMRA inferred the connection strengths and confidence intervals that are given in Table S5.

Nonlinear model of the core signaling network and cell state transitions

Using the quantified core network topologies and the inferred pathway influences on the DPD, a nonlinear ODE model was built for TrkA and TrkB cells using the rule based approach[40]. The signaling variables are the protein phosphorylation levels normalized by the protein abundances, and the phenotypic variable is the output of the DPD module (Eq. 8). Below we describe the fundamentals of the model.

The activation of Trk and ERBB receptors by ligand binding and dimerization is modeled mechanistically. Briefly, NGF/BDNF binding to TrkA/TrkB is followed by receptor dimerization and phosphorylation, whereas a basal rate of ERBB dimerization is maintained by diverse growth factors present in serum. The homodimerization of TrkA, TrkB and ERBB, and heterodimerization of TrkB and ERBB[41,42] is modeled using the thermodynamic approach developed previously[43]. The binding of the first and second molecules of the ligand and the subsequent homo- and hetero-dimerization of RTKs satisfy so-called "detailed balance" constraints[44,45]. These thermodynamic restrictions require the product of the equilibrium dissociation constants (K_d's) along a cycle to be equal to 1, as at equilibrium the net flux through any cycle vanishes, since the overall free energy change is zero. Because ligand binding facilitates the RTK dimerization, following the thermodynamic approach[43], we introduce three thermodynamic factors, describing how the K_d's of homo- and heterodimerization of RTKs change upon ligand binding. When Trk receptor inhibitor is added, an inhibitor-free protomer can still cross-phosphorylate the other protomer in a dimer.

The core network dynamics was modeled up to 45 minutes, and therefore the total moieties of ERK, AKT, JNK, S6K and RSK were assumed to be conserved. However, internalization of RTKs that is occurring on this timescale is included in the model. Following internalization some receptor molecules are subsequently degraded, whereas the others are recycled back to the membrane. The disappearance of RTKs from the plasma membrane depends on the dimer composition. In the model the rate of internalization of TrkB-ERBB heterodimers is slower than the internalization rate of TrkA and TrkB homodimers, based on the literature[46,47]. The BMRA-inferred connections show that there are multiple feedback loops to the ERBB module from downstream kinase modules (Table S5). The influence of these feedbacks on the ERBB module activity is modeled as hyperbolic multipliers that modify the rate of activating ERBB phosphorylation, defined as follows[48],

$$\alpha_Y^X = \frac{1+\gamma_Y^X \bullet Y_a / K_Y^X}{1+Y_a / K_Y^X} \quad (24)$$

.

Here Y_a is an active form of protein *Y* that influences protein *X*. The coefficient $\gamma_Y^X > 1$ indicates activation; $\gamma_Y^X < 1$ inhibition; and $\gamma_Y^X = 1$ denotes the absence of regulatory interactions, in which case the modifying multiplier α_Y^X equals 1. K_Y^X is the activation or inhibition constant.

The RTK dephosphorylation is catalyzed by phosphatases. The activation and deactivation dynamics of the downstream signaling modules is modeled using Michaelis-Menten kinetics and the hyperbolic multipliers that account for signaling crosstalk between the pathways.

The BMRA network reconstruction constrains parameters of the dynamical model by maximum likelihood values of the inferred connection strengths (Table S5). In particular, only interactions between modules, where the connection coefficients have statistically significant non-zero values, are included in the model. Additional constraints on the parameter values occur, because the inferred connection coefficients are normalized Jacobian elements[5], which are functions of the model parameters (Eq. 14 and the next section).

DPD time trajectories. The model includes the DPD module whose output summarizes the contributions of all individual proteins (minus the core network constituents) to the global network responses. This module describes cell-wide signaling, and the DPD output (S) is defined by Eq. 8. The DPD maps the network-wide changes, which occur in the multidimensional molecular dataspace upon perturbations, into a one-dimensional (S) space. Our model allows to determine the dynamics of S following any drug perturbation to core network pathways. If the data point clouds before and after a particular perturbation are measured by experiments, ΔS is calculated using Eq. 11, which can be used to test the model.

The DPD trajectory is a one-dimensional, time-dependent trajectory of a cell maneuvering in Waddington's landscape governed by a signaling driving force and a restoring force. The signaling driving force, $\sigma(t)$, is determined by the outputs of signaling modules of a core network and their connection coefficients to the DPD module,

$$\sigma(t) = \sum_j r_{Sj} \left(\frac{S_{st.st.}}{x_j^{st.st}} \right) x_j(t) \quad (25)$$

.

Here, $x_j(t)$ are the outputs of signaling modules, r_{Sj} are the corresponding BMRA-inferred connection coefficients to the DPD (see Table S5), and $S_{st.st.}$ and $x_j^{st.st}$ are the initial steady-state values of S and x_j before perturbations.

The restoring force $f(S)$ is a gradient force given by the derivative of the potential (U), as follows

$$f(S) = -\frac{dU}{dS} \quad (26).$$

The potential (U) has 3 minimums. These minimums correspond to three stable steady states, S_0, S_1 and S_2. There are two unstable steady states at the borders between the basins of attraction of two neighboring steady states (Extended Data Figure 9).

Assuming the quadratic potential U in the vicinity of each stable state, which is widely used in physics[49], the restoring force $f(S)$ is modeled using a piece-wise linear approximation. This force $f(S)$ is set to zero at the borders between the basins of attraction, and $f(S)$ reaches its maximum at the half distance between the border and the stable steady state (Eq. 27 and Extended Data Figure 9A).

$$f(S) = \{-\alpha_0\left(S - S_0\right), \quad S < \frac{3S_0+S_1}{4}\ \alpha_0\left(S - \frac{S_0+S_1}{2}\right),\ \frac{3S_0+S_1}{4} < S < \frac{2S}{} \quad (27).$$

The DPD trajectory is calculated, as follows,

$$\frac{dS}{dt} = f(S) + \sigma(t) \quad (28)$$

.

This equation allows for an interpretation of a cell progressing through the molecular dataspace as a particle that moves in the gradient force field and the field of external forces exerted by responses of core signaling pathways,

$$\frac{dS}{dt} = -\frac{dU}{dS} + \sum_j r_{Sj}\left(\frac{S_{st.st.}}{x_j^{st.st}}\right)x_j(t) \quad (29)$$

.

This gradient force field and the field of external forces shape the evolving Waddington's landscape (W), as follows,

$$W = U - \sum_j r_{Sj}\left(\frac{S_{st.st.}}{x_j^{st.st}}\right)x_j(t)\cdot S(t) \quad (30)$$

.

In the vicinity of the steady state $S_i \in \{S_0,\ S_1,\ S_2\}$, the solution of Eq. 28 is expressed analytically as follows,

$$S(t) = S_i + e^{-\alpha t}\int_0^t\left(\sum_j r_{Sj}\left(\frac{S_{st.st.}}{x_j^{st.st}}\right)x_j(\tau)\right)e^{\alpha\tau}\,d\tau \quad (31)$$

.

Here $\alpha \in \{\alpha_0, \alpha_1, \alpha_2\}$ is the slope parameter defined in Eq. 27. Eq. 31 illustrates the system has a characteristic memory time, $t_m \sim 1/\alpha$. At timescales much smaller than the memory time, $t \ll t_m$, the entire change in S is determined by the time integral over the signaling driving force.

Refining parameters of the dynamic model

To decrease the number of parameters to fit, the concentrations of different protein forms and the parameters with the concentration dimensionality, such as, the Michaelis' constants, were normalized

by the conserved total protein concentrations. Only time was left as dimensional variable (measured in seconds) to readily interpret model simulations.

To refine the parameters of pathway interactions of our core network inferred by BMRA, the data were split into a training set and a validation set. The training set included the time course of TrkA and TrkB phosphorylation measured by Western Blot and 10 min RPPA data for the remaining signaling modules. The model-generated time courses were fitted to these training set data with the objective function defined as the sum of squares of deviations. A feature of our parameter refinement is that in addition to the training dataset, we constrained the parameters using the BMRA inferred connection coefficients within their confidence intervals. Implicit constraints on the parameter values occur, because the connection coefficients defined in Eq. 14 must be within the confidence intervals of the BMRA inferred connections. Then, we used a unique feature of the pyBioNetFit software, which allows adding parameter constraints in the forms of inequalities to the parameter fitting process[50]. A combination of scatter search and simplex methods and pyBioNetFit software were used to fit the model simulations to the training dataset. Scatter search with a population size 20 was used to obtain the initial parameter set, and the simplex algorithm was used for the local refinement of the initial set. The validation set consisted of 45 min RPPA data for signaling modules of the core network. Figs. 4A, 4B, and Extended Data Figures 6-8 show the simulated time courses with the experimental data points imposed on the model predictions.

Eq. 28 determines the DPD dynamics when a cell's progression through the molecular dataspace is directed by the signaling driving force and the restoring force. For the signaling driving force we fit the coefficients, $\beta_j = r_{Sj}\left(\frac{S_{st.st.}}{x_j^{st.st}}\right)$, in the ranges constrained by the confidence intervals of the BMRA-inferred connection coefficients (r_{Sj}), while the signaling module outputs (x_j) are calculated by the model. For the restoring force, Eq. 27, slope parameters $\{\alpha_0, \alpha_1, \alpha_2\}$ and stable steady state positions $\{S_0,\ S_1,\ S_2\}$ were fitted.

Model Simulations

In total, the rule-based nonlinear model of the core signaling network and cell state transitions consists of 82 species and 405 reactions. The SBML files of the TrkA and TrkB models with all equations and parameters can be found at https://github.com/OleksiiR/cSTAR_Nature. The simulations of the models were run using the BioNetGen software[40], which used the CVODE routine from the SUNDIALS software package for solving ordinary differential equations (ODE). The Matplotlib python package was used for plotting experimental and modeling results.

Exploration of different omics datasets shows cSTAR flexibility and scalability

STV analysis and core network reconstruction using RAF inhibitor-resistant melanoma datasets

To build STV and separating hyperplane we labeled samples from non-treated melanoma SKMEL-133 cells as proliferation state, and samples from cells treated with a combination of MEK and PI3K, AKT or mTOR inhibitors, which stopped proliferation and induced apoptosis, as non-proliferating, apoptotic state. We also tested that the STV that was built using a subset of datapoints from non-proliferating, apoptotic state, which corresponded to any of these drug combinations, did not change the composition of a core network and yielded similar DPD values for two cell states and the rest of inhibitor perturbations.

The STV ranking for the SKMEL-133 cells is presented in Table S9 (the sheet "full_STV"). The top STV components include IRS, the PI3K/AKT/mTOR and ERK signaling modules and their downstream targets, such as the cell cycle proteins, RB, CycB1 and CycD. Based on this ranking, the core network contains ERK, AKT, mTOR, SRC, CDK4/6, PKC, IRS, and DPD modules. To analyze and predict the effects of inhibitor perturbations, the core components were removed from the STV. Table S9 (the sheet "reduced_STV") presents the ranking of components for the reduced STV.

Next, we inferred the connections between core network signaling proteins and their influence on the DPD phenotypic module using BMRA. The global response coefficients were calculated using Eqn. 22 and 23. The BMRA code was run twice, for the network without and with MYC module. The matrices of the prior and posterior networks are presented in the Table S10.

Building a nonlinear model of RAF inhibitor-resistant melanoma

A nonlinear dynamic model was derived based on the quantified core signaling network topology and signaling connections to the DPD module, using rule based approach[40]. As in the model of neuroblastoma SH-SY5Y cells, the concentrations of active and inactive protein forms were normalized by the protein abundances. The phenotypic variable S represents the output of the DPD module.

First, a coarse-grained model was built solely based on the inferred core network connections that did not include the MYC module (Fig. 7A and Table S10, sheet "noMYC"). In this model, core network proteins had only two, active and inactive, states, and all pathway cross-talks were described by hyperbolic multipliers (given in Eq. 24). The only exception was IRS, which had two groups of phosphorylation sites, activating and inhibitory. Accordingly, negative feedback connections to the IRS module were described through IRS phosphorylation on inhibitory sites by Michaelis-Menten equations. This phosphorylation resulted in enhanced IRS degradation, which correlated with the original data[51,52]. Therefore, the model also included the IRS synthesis and degradation. Next, we added the MYC module to this model based on the corresponding inferred network (Fig. 7B and Table S10, sheet "withMYC"). These simplified models were named "SKMEL-133-1.bngl" and "SKMEL-133-2.bngl", respectively.

Further model refinement included a more mechanistic description of IRS activation by Insulin and IGF1 receptors and the CDK module. To account for the measured changes in the Cyclin D abundance, we incorporated its synthesis and degradation and CDK4/6 activation by binding Cyclin D. To describe IRS activation, we introduced IGF1 and Insulin receptors into the model. This model was named "SKMEL-133-3.bngl". Although this model can be further mechanistically detailed by incorporating

the previously developed models of IRS1-ERK-AKT interplay through the GAB scaffold[53] and RAF dimerization[54], this was not required to describe the data obtained in the original publication[29]. We used the "SKMEL-133-3.bngl" model to generate predictions presented in the Results section.

Model parameters were refined by dividing the data[29] into a training set that included only single drug perturbations data and a validation set consisting of perturbations by drug combinations. The model-generated dose responses were fitted to these training set data with the objective function defined as the sum of squares of deviations. Similarly as above, we constrained the parameters using the BMRA inferred connection coefficients within their confidence intervals, and used the pyBioNetFit software, which allows adding parameter constraints in the forms of inequalities to the parameter fitting process[50]. A combination of scatter search and simplex methods in pyBioNetFit software were used to fit the model simulations to the training dataset. Scatter search with a population size 12 and 50 iterations was used to obtain the initial parameter set, and the simplex algorithm was used for the local refinement of the initial set. Extended Data Figure 15A shows the simulated dose responses with the experimental data points imposed on the model predictions.

Estimating synergy strength for drug combinations from model predicted DPD responses

Simulations of the developed nonlinear dynamic model allowed predicting DPD responses to different inhibitors and their combinations. To estimate the synergy strength to move the DPD towards apoptotic cell states we used Loewe isoboles (Figs. 7C,D and Extended Data Figure 15B) and the Talalay-Chou combination index (CI)[27,55]. These criteria require much more datapoints than Bliss independence criterion, and thus were applied only to computational data, which unlike experimental data can be generated for large numbers of different doses.

Let ICZ_1 and ICZ_2 be the concentrations of inhibitors 1 and 2 that produce the same effect (Z) given separately. Here, Z is an arbitrary inhibition level, which can be, for example, 10, 20, 30, 50, 70 or 90%. Let I_1 and I_2 be the concentrations of inhibitors 1 and 2 that produce the effect Z given in a combination. The Talalay-Chou combination index (CI) is defined as,

$$CI = \frac{I_1}{ICZ_1} + \frac{I_2}{ICZ_2} \quad (32).$$

For any particular drug combination and a dose ratio, the CI defined in Eq. 36 allows detecting whether the Loewe isoboles are concave or convex, corresponding to either synergy or antagonism, respectively. The CI indicates synergy[55] when CI < 1 and antagonism when CI > 1. Because the CI is a function of inhibitor doses and effects, we have determined the minimal value of CI across dose-response plane. This minimal CI value corresponds to the optimal ratio of drugs in a combination. It determines the maximal synergy strength that can be potentially achieved for a specific drug combination. These minimal CI levels were 0.48 for a combination of MEK and MYC inhibitors, and 0.4 for a combination of PI3K/AKT and Insulin/IGF1 receptor inhibitors, demonstrating the higher synergy strength for a combination of PI3K/AKT and Insulin/IGF1 receptor inhibitors.

Experimental Methods

Inhibitors and Reagents

As there are no highly selective TrkA and TrkB inhibitors available, we used SP600125 (Selleckchem, #S1460), which inhibits both TrkA and TrkB[56]. Because SP600125 also inhibits the cJun N-terminal Kinase (JNK)[57], we used JNK-IN-8 (Selleckchem, #S4901), which targets JNK but not the Trk receptors[58], to dissect the impact of the JNK inhibition. AKT was blocked by the AKT inhibitor IV (Merck Millipore, #124011). To inhibit p70S6 kinase, which phosphorylates the ribosomal RPS6 protein, we used LY2584702 (Selleckchem, #S7698). To perturb the ERK pathway, we used the MEK inhibitor Trametinib (Selleckchem, GSK1120212, #S2673), which inhibits the kinase that activates ERK, and BI-D1870 (Selleckchem, #S2843), which inhibits p90RSK, a kinase downstream of ERK. To inhibit ERBB module we used Gefitinib (Selleckchem, ZD-1839, #S1025).

Antibodies

Antibodies against phospho-TrkA$^{Tyr674/675}$/TrkB$^{Tyr706/707}$ (#4621), total TrkA (#2505), total TrkB (#4603), phospho-FAKY397(#8556), total GAPDH (#2118), as well as anti-rabbit (#7074) and anti-mouse (#7076) IgG HRP–linked antibodies were from Cell Signaling Technology. Antibodies against phospho-ERK1/2 (#8159) and total ERK1/2 (#5670) were from Sigma- Aldrich. Antibody against total FAK (sc-558) was from Santa Cruz. Mouse monoclonal anti-V5-HRP (R961-25) was from Life Technologies.

Plasmids

To generate the pLX302/NTRK1 and pLX302/NTRK2 expression vectors, pENTR223 entry vectors containing the NTRK1 or NTRK2 ORF (Addgene, 23891 and 23883, respectively) were used for LR recombination reaction together with the pLX302 destination vector (Addgene 25896) according to the manufacturer's instructions (Gateway Cloning system, Life Technologies). The myristoylated AKT plasmid, Myr-AKT1, was described previously[15].

Generation of isogenic cell lines

The SH-SY5Y neuroblastoma cell line was a generous gift from Dr Frank Westermann (DKFZ, Heidelberg). Dr Frank Westermann has obtained these cells from DSMZ. The cells were validated using STR profiling. These cells have no MYCN gene amplification and only 19 mutations according to the CCLE and COSMIC databases, and they are genetically stable in culture[6,59]. The cells were cultured in RPMI 1640 (Gibco) supplemented with 10% FBS (Gibco), 2 mM L-glutamine (Gibco), and 1% penicillin-streptomycin (Gibco) under standard tissue culture conditions (5% CO2). The cells were regularly mycoplasma tested.

To generate the SH-SY5Y/pLX302/NTRK1 ('SY5Y/TrkA') and SH-SY5Y/pLX302/NTRK2 ('SY5Y-TrkB') cell lines, stably expressing TrkA or TrkB, respectively, the expression vectors were transfected into SH-SY5Y cells using the JetPrime transfection reagent as instructed by the manufacturer (Polypus). Puromycin (Sigma-Aldrich) selection (2 μg/ml) was initiated 48 hours after

transfection and continued for 2 weeks with culture media refreshed in every two days. TrkA/B expression was confirmed by Western blotting.

Stimulation of TrkA and TrkB-expressing SH-SY5Y cells was carried out 30 minutes following inhibitor addition, using 100 ng/mL of recombinant NGF (#450-01) and BDNF (#450-02), respectively (both from Peprotech).

TrkA and TrkB receptors activate very similar signaling pathways, which include PLCγ, ERK and AKT as main effectors, and it is unclear what particular changes in signaling and expression patterns cause distinct TrkA/TrkB cell fate decisions[7]. Therefore, SH-SY5Y cells are an ideal system to test cSTAR.

Imaging experiments and drug interaction calculations

Differentiation was assessed visually at 72 hours following treatment, using the EVOS FL Imaging System (Life Technologies). Cells with neurites ≥ 2 cell body diameters were scored as differentiated. For each treatment condition 3 biological replicates were used to quantify percentage of differentiated cells. Table S13 represents the total number of cells and the number of differentiated cells for each image. Statistically significant differences were determined using unpaired one-tailed t-test.

The phenotypic effect of drugs was assessed using the changes in the percentage of differentiated cells calculated from imaging experiments. For each drug (Trametinib, Gefitinib or their combination), the drug-induced differentiation effect (Y) was defined as follows,

$$Y = 1 - \frac{P_{drug} - P_B}{P_A - P_B} \quad (33)$$

.

Here P_{drug} is the percentage of differentiated TrkB cells stimulated with BDNF and treated with the drug, P_A is the percentage of differentiated TrkA cells stimulated with NGF, and it is always greater than P_B, which is the percentage of differentiated TrkB cells stimulated with BDNF. Eq. 33 shows that Y is 1 if the drug has no effect, Y is 0, if the drug drives TrkB cell differentiation to the same percentage as observed for TrkA cells, and Y is between 0 and 1 for a partial induction of TrkB cell differentiation by the drug. The definition of the drug effect by Eq. 33 allows us to calculate the Bliss synergy score for a combination of drugs 1 and 2 using Eq. S4 in Supplementary Information. The calculated synergy score between Gefitinib and Trametinib is 51% ± 7%, demonstrating high synergy[60].

Western Blotting

Cells were lysed in ice-cold 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% (v/v) NP-40 (Calbiochem), complemented with COMPLETE Mini protease and PhosSTOP phosphatase inhibitor cocktails (Roche). Lysates were cleared by centrifugation at 10,000 x g for 10 minutes at 4°C and adjusted to equal protein concentrations following protein quantification using the Pierce™ BCA Protein Assay Kit (Thermo Scientific). SDS-PAGE (10% PAA) and Western Blotting were performed using the Mini

Protean Tetra system (Bio-Rad). Protein bands were visualized using the enhanced chemiluminescence system (GE Healthcare) by the ChemoStar Imager (INTAS Science Imaging Instruments GmbH). Blots were quantified using the Image J software.

Proliferation assays

To assess cellular proliferation and viability, we used two different assays and compared their results. For the first assay, 20,000 cells per well were seeded into Nunc™ F96 MicroWell™ White Polystyrene Plates (Thermo Scientific™ #136101). Cell Proliferation was assessed at 72 hours post treatment, using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega), according to manufacturer's instructions, and the SpectraMax® M3 Microplate Reader (Molecular Devices). For the second assay, cells were plated at 20,000 cells per well into 96-well F-bottom tissue culture plates (Greiner, #655180). Proliferation and viability of inhibitor- and control-treated cells was assayed after 72 hrs by CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS; Promega) according to manufacturer's instructions. In both cases, 3 biological replicates were performed for each condition, the results represent the mean ± SD of triplicate samples, expressed as a percentage of control.

Reverse Phase Protein Array (RPPA)

We used a custom made RPPA with 115 validated antibodies (Table S1) to measure the activities of signaling pathways involved in TrkA/B signaling[7], including MAPK (RAS-ERK, JNK, p38), PI3 kinase (AKT, mTORC1/2), JAK-STAT, PLCγ-PKC, TGFβ-SMAD, Wnt, cyclic-AMP, cell cycle (CDK1, Cyclins, Rb, p53, $p21^{WAF}$), apoptosis (BAX, BCL2, BCLx, Caspase 3), transcription factor (MYC, NFκB, JUN), and tyrosine kinase (SRC, EGFR, PDGFR, IGFR) pathways. These antibodies detect phosphorylation sites that change protein activities, or protein abundances.

RPPA analysis was carried out using established protocols for nitrocellulose-based arrays[61]. Briefly, cell lysates were prepared in 1% Triton X-100, 50 mM HEPES (pH 7.4), 150 mM sodium chloride, 1.5 mM magnesium chloride, 1 mM EGTA, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 10% glycerol, supplemented with COMPLETE Mini protease and PhosSTOP phosphatase inhibitor cocktails (Roche). After clearing by centrifugation at 13,300 rpm for 10 min at 4°C the protein concentration was determined using the Coomassie Plus Protein Assay (ThermoFisher Scientific). Protein concentrations were adjusted to a final concentration of 1mg/ml followed by denaturation upon addition of 4X sample buffer containing 10% β-mercaptoethanol and heating to 95°C for 5 minutes. A 4-step dilution series of each sample was prepared in PBS with 10% glycerol giving final concentrations of 0.75, 0.375, 0.1875 and 0.09375 mg/ml. Samples were printed onto nitrocellulose-coated slides (Grace Bio-Labs) across multiple sub-array areas under conditions of constant 70% humidity using an Aushon 2470 array platform (Quanterix). Printed slides were blocked using SuperBlock (TBS) blocking buffer (Thermo Fisher Scientific) and each sub-array was separately incubated with validated primary antibodies (all diluted 1:250 in SuperBlock). Bound antibodies were detected by incubation with anti-rabbit or anti-mouse DyLight 800-conjugated secondary antibodies

(New England BioLabs). Slide images were acquired using an InnoScan 710-IR scanner (Innopsys) with laser power and gain settings optimised for highest readout without saturation of the fluorescence signal. The relative fluorescence intensity of each array feature was quantified using Mapix software (Innopsys).

The linear fit of the dilution series of each sample was verified for each primary antibody and the median relative fluorescence intensity from the dilution series was calculated to represent relative abundances of total proteins and posttranslational epitopes across the sample set. Finally, signal intensities for each sample were normalized to total protein loading on the array slides by using the signal readout from a fast-green (total protein) stained array.

Sample Preparation for Mass Spectrometry Experiments

Cells were resuspended in 100 µl of ice cold 8M urea/50 mM Tris-HCL pH 8.0, supplemented with phosphatase and protease inhibitors (Roche). Each sample was sonicated (Syclon Ultrasonic Homogenizer) for 2 x 9 seconds at a power setting of 15%to disrupt the cell pellet. The protein samples were normalized to 550 µg. Each sample was reduced by adding 8mM dithiothreitol (DTT) and mixing (thermomixer 1200 rpm, 30°C) for 60 min and subsequently carboxylated by adding 20 mM iodoacetamide and mixing (thermomixer 1200rpm, 30°C) for 30 min in the dark. The solution was diluted with 50 mM Tris-HCL pH 8.0 to bring the urea concentration down to 2M. Sequencing Grade Modified Trypsin (Promega #V5111) was resuspended in 50 mM Tris-HCL at a concentration of 0.5 µg/µl and added to each solution. The samples were digested overnight (1:100 enzyme to protein ratio) with gentle shaking (thermomixer 850rpm, 37°C). The digestion was terminated by adding formic acid to 1% final concentration and cleaned up using c18 (HyperSep SpinTip P-20, BioBasic C18, Thermo Scientific).

Phosphopeptide enrichment was carried out with TiO_2 (Titansphere Phos-TiO Bulk 10 µm, (GL Sciences Inc, Tokyo, Japan) using an adapted method previously described[62]. In summary, each sample was incubated with TiO_2 beads for 30 minutes by rotation in 80% acetonitrile, 6% trifluoroacetic acid, 5mM monopotassium phosphate, 20mg/ml 2,5- dihydroxybenzoic acid, this step was carried out twice. The beads were washed 5 times in 80% acetonitrile/1% trifluoroacetic acid, before elution of the phosphopeptides with 50% acetonitrile, 7% ammonium hydroxide. The two eluents from each sample were then pooled and dried down.

LC/MSMS Method (Bruker timsTof Pro)

Samples were run on a Bruker timsTof Pro mass spectrometer connected to a Evosep One liquid chromatography system. Tryptic peptides were resuspended in 0.1% formic acid and each sample was loaded on to an Evosep tip. The Evosep tips were placed in position on the Evosep One in a 96-tip box. The autosampler picks up each tip, elutes and separates the peptides using a set chromatography method (30 samples a day) The mass spectrometer was operated in positive ion mode with a capillary

voltage of 1500 V, dry gas flow of 3 l/min and a dry temperature of 180°C. All data were acquired with the instrument operating in trapped ion mobility spectrometry (TIMS) mode. Trapped ions were selected for ms/ms using parallel accumulation serial fragmentation (PASEF). A scan range of (100-1700 m/z) was performed at a rate of 5 PASEF MS/MS frames to 1 MS scan with a cycle time of 1.03s. Due to a breakdown of an MS instrument, sample replicates were analyzed on another MS instrument using identical conditions.

Analysis of Mass Spectrometry Data

The mass spectrometer raw data was searched against the Homo sapiens subset of the Uniprot Swissprot database (reviewed) using the search engine Maxquant (release 2.0.1.0) In brief, specific parameters were used (Type: TIMS DDA, Variable mods; Phospho (STY)).

Each peptide used for protein identification met specific Maxquant parameters, i.e., only peptide scores that corresponded to a false discovery rate (FDR) of 0.01 were accepted from the Maxquant database search. Phospho (STY)Site intensities with localization scores > 0.75 were used for relative quantitation.

Data Availability

MS proteomics data are uploaded to the PRIDE database (accession number PXD028943). The RPPA data for SKMEL-133 cell line[29] are available at http://projects.sanderlab.org/pertbio/. The scRNAseq data for EMT in A549, DU145, MCF7 and OVCA420 cell lines[31] are available at the NCBI Gene Expression Omnibus (the accession number GSE147405). The CYTOF data for EMT in Py2T cell line[32] is available at https://community.cytobank.org/cytobank/experiments#project-id=1296.

Code Availability

All codes for the data analysis, network reconstruction and modeling are available at https://github.com/OleksiiR/cSTAR_Nature.

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Author contributions. BNK, OSR, and WK conceived the study, MH generated a biological model of TrkA and TrkB expressing cell lines, which specify different cell fate decisions, NR, MH, SM, KW, KML, NOC performed experiments and primary RPPA data analysis, VZ performed clustering analysis of RPPA data and kinase enrichment analysis of scRNAseq data, BNK and OSR derived equations and developed mathematical approach, EK analyzed robustness of the results with respect to noise, OSR and TP carried out numerical calculations, BNK, OSR and WK wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests. Patent application (No. UK2107576.7) related to this work was filed (O.S.R., V.Z., W.K. and B.N.K.). All other authors declare no competing interests.

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Figure legends

Figure 1. Overview of cSTAR and experimental system. (**A**) cSTAR approach workflow. Clockwise: (*i*) Acquisition of omics datasets; (*ii*) Cell state classification by clustering and SVM state separation; (*iii*) The STV indicates a path between centroids of cell state data point clouds; (*iv*) A core signaling network of high-ranked STV components is reconstructed by BMRA . The DPD summarizes the cell-wide network; (*v*) A mechanistic model of the core network and cell state transitions; ***x,*** the outputs of signaling modules; *S,* the DPD module output; *U*, the potential, cell state transitions interpreted as cell maneuvering in Waddington's landscape. (**B**) SH-SY5Y cells stably expressing TrkA or TrkB receptors were stimulated with 100 ng/ml NGF or BDNF resulting in differentiation or proliferation, which . were assessed 72 hours after GF treatment.

Figure 2. Separation of proliferation and differentiation signaling patterns and the STV projection into the PCA space. (**A**) Following GF stimulation, TrkA (blue triangles) and TrkB (red triangle) states were separated by SVM. Large triangles are point cloud centroids. Projections of data points, the separating hyperplane (grey) and the STV (dark red) are shown in the space of the first 3 principal components. (**B**) Decomposition of a perturbation vector (solid black line) into a vector collinear with the STV and a vector perpendicular to the STV (dotted lines). TrkB cells were treated with BDNF and the p90RSK inhibitor BI-D1870. Green triangles, TrkB data points after perturbation.

Figure 3. The Dynamic Phenotype Descriptor (DPD) module output. (**A, B**) BMRA reconstructed topologies of the core signaling networks and connections to the DPD module in TrkA (A) and TrkB (B) cell lines. Arrowheads indicate activation, blunt ends show inhibition, line widths indicate the absolute values of interaction strengths. Edges specific to TrkA and TrkB are shown in blue and red. (**C, D**) Blue, red and green triangles show PCA-compressed 45 min data points for TrkA cells, TrkB cells, and TrkB cells treated with p90RSK inhibitor. The distances of TrkB centroids to the separating hyperplane (grey) before and after perturbation are shown by black lines. The DPD module output is the distance of a centroid from the separation hyperplane determined along or opposite the STV direction taken with the plus sign if the centroid is located at the right side from the separation hyperplane (proliferation), and with the minus sign if the centroid is at the left side (differentiation).

Figure 4. Computing signaling patterns and modeling cell maneuvering in Waddington's landscape using the DPD. (**A, B**) Experimental data are imposed on model-predicted time-courses for NGF-stimulated TrkA (blue) and BDNF-stimulated TrkB (red) cells. (**C, D**) Waddington's landscape evolution predicted by the model for TrkA (**C**) and TrkB (**D**) cells following ligand stimulation. The Waddington landscape potential W is plotted against the DPD (S) and time (from 0 to 45 minutes). At t = 0, cells reside in stable ground state (S_0). Following stimulation by GFs, TrkA and TrkB cells maneuver to differentiation (C) and proliferation (D) states (model-predicted trajectories shown by black lines). (**E**) Experimentally measured (dots) and model-predicted (solid lines) DPD responses (S) to ligand stimulation in TrkA and TrkB cells. (**F**) Percentages of differentiated TrkA/B cells stimulated with GFs for 72 hours compared to unstimulated controls. The decrease in differentiation of BNDF-stimulated TrkB cells reflects the increase of proliferation. Data are presented as mean values +/- SEM for n=3 biologically independent experiments, Table S13 contains data. The asterisk * indicates $p<0.05$ calculated using unpaired one-sided t-test.

Figure 5. Model predicted DPD values and quantification of phenotypic responses of TrkA and TrkB cells to different inhibitors. (**A-C, G-I**) Model-predicted time courses (lines) and experimentally measured (dots) DPD responses of TrkA (blue) and TrkB (red) cells to inhibitor treatments (see legends to Extended Data Figures 6-8 for inhibitor concentrations). (**D-F, J-L**) Percentages of differentiated TrkA and TrkB cells quantified using live-cell images. (**M**) Simulated DPD time-course of the response of NGF-stimulated TrkA cells to a 10-fold increase in AKT activity predicts persistent proliferation (solid line), whereas simulated DPD time-course of NGF-stimulated control cells predicts differentiation (dashed line). (**N, O**) Live cell images of TrkA cells transfected with myristoylated AKT (**N**) and stimulated with NGF for 72 hours. Representative live images of 3 biological replicates are shown. (**O**) confirm modeling predictions. Data are presented as mean values +/- SEM for n=3 biologically independent experiments. The asterisk * indicates $p<0.05$ calculated using unpaired one-sided t-test.

Figure 6. Inhibition of ERBB and ERK modules synergistically induces differentiation of TrkB-expressing cells. (**A**) Model-predicted DPD responses of TrkB cells to ERBB and MEK inhibitors applied separately and in combinations are shown in a two-dimensional plane of the drug doses at 45 min BDNF stimulation. Constant DPD lines, Loewe isoboles, show the borders between the differently colored areas; concave isoboles demonstrate synergy. (**B**) Percentages of differentiated TrkB cells corroborate model-predicted synergy between ERBB and MEK inhibitors in inducing TrkB cell differentiation. Data are presented as mean values +/- SEM for n=3 biologically independent experiments. The asterisk * indicates that $p<0.05$ calculated using unpaired one-sided t-test. (**C**) Responses of FAK phosphorylation (cell differentiation marker[28]) to Geftitinib (2.5 and 5 μM), Trametinib (0.1 and 0.2 μM) and their combination (2.5 and 0.05 μM, and 1.25 and 0.1 μM) at 72 hours. For gel source data, see Supplementary Figure 1. Representative blot of 3 biological replicates is shown. (**D**) ERBB and MEK inhibitors synergistically induce TrkB cell differentiation. The DPD values calculated using MS phosphoproteomics data for TrkA and TrkB cells treated with Trametinib (0.5 μM), Gefitinib (2.5 μM), and their combination (0.25 μM and 1.25 μM) at 45-minute stimulation. Data are presented as mean values +/- SEM for n=3 biologically independent samples examined over 2 independent experiments. Dashed, red bar shows the expected DPD value for the Bliss independence of a combination treatment of TrkB cells with Trametinib and Gefitinib.

Figure 7. cSTAR analysis of RAF inhibitor resistant SKMEL-133 cells. (**A-B**) Inferred topologies of a core signaling network that (**A**) lacks or (**B**) includes c-MYC. Arrowheads indicate activation, blunt ends show inhibition, line widths indicate the absolute values of interaction strengths. (**C-D**) Model-predicted steady-state DPD responses to (**C**) MYC and MEK inhibitors and (**D**) Insulin/IGF1 receptor and PI3K/AKT inhibitors are shown by Loewe isoboles. Synergy is more pronounced in (**D**). (**E-G**) Model-predicted SKMEL-133 cell maneuvering (black lines) in Waddington's landscape following inhibitor treatments. The Waddington landscape potential (W) plotted against the DPD (S) and time. At $t = 0$ cells reside in a highly proliferating state (high positive values of DPD). PI3K/AKT and Insulin/IGF1 receptor inhibitors were added at $t = 30$ min at the $3K_d$ and $4K_d$ doses. When the inhibitors are applied separately (**E**, **F**), the decreasing DPD values remain in the proliferation region (positive DPD values). (**G**) Treated with a combination of inhibitors in twice lower doses ($1.5K_d$ for

PI3K/AKT inhibitor and $2K_d$ for Insulin/IGF1 receptor inhibitor), the cells maneuver to the apoptotic state manifested by negative DPD values. A threshold-like switch to negative DPD (black arrow) is a switch from proliferation to apoptosis.

Extended Data Figures

Extended Data Figure 1. Proliferation responses of SH-SY5Y-TrkA and SH-SY5Y-TrkB cells to NGF, BDNF, and kinase inhibitors. Proliferation of NGF-stimulated TrkA cells and BDNF-stimulated TrkB cells treated with different inhibitors was measured using the **(A)** ATP luminescence and **(B)** MTS assays following 72 hours after treatment. Concentrations of inhibitors: TRKi (SP600125) – 5 μM, AKTi (AKT inhibitor IV) – 1 μM, JNKi (JNK-IN-8) – 1 μM, S6Ki (LY2584702) – 1 μM, MEKi (Trametinib) – 0.5 μM, RSKi (BI-D1870) – 1 μM. Data are presented as mean values +/- SEM for n=3 biologically independent experiments.

Extended Data Figure 2. RPPA phospho-proteomics data. Heatmap of RPPA data obtained at 10 and 45 minutes stimulation of TrkA and TrkB cells with 100 ng/ml NGF and BDNF (the time and replicate numbers are indicated at the bottom, the proteins on the side). The data were clustered using Ward hierarchical clustering.

Extended Data Figure 3. PCA compression of RPPA data and selection of core network components. (A) PCA compressed RPPA data for TrkA and TrkB cells are plotted in the space of the first two principal components that are normalized by the data variance captured by these components. Following NGF or BDNF stimulation (100 ng/ml) the data points measured by RPPA were clustered in a 115-dimensional molecular dataspace using K-means clustering (K=2). All data points from NGF-stimulated TrkA cells constitute a single cluster shown in blue, and all data points from BDNF-stimulated TrkB cells form a cluster shown in red. Unstimulated control cells are shown in black. (**B**) High ranked STV components determine the components of a core signaling network. The changes in individual protein activities or abundances between the centroids of the data point clouds that characterize two different cells states were projected onto the STV to determine protein ranks. Resulting high rank proteins constitute the nodes of a core signaling network.

Extended Data Figure 4. Literature-based prior network and signaling responses to ligand stimulation. (**A**) Prior topology of core network connections based on the existing knowledge[16,38,63]. (**B**) Time courses of Trk and ERK activation (measured with phosphospecific antibodies, pTrk and ppERK) in TrkA and TrkB cells after stimulation with 100 ng/ml NGF or BDNF, respectively. GAPDH staining was used as loading control. For gel source data, see Supplementary Figure 1. Representative blot of 3 biological replicates is shown.

Extended Data Figure 5. Reconstruction of core signaling networks by BMRA. Inferred topologies of TrkA (**A**) and TrkB (**B**) core signaling networks. Edges that are specific to TrkA and TrkB are shown in blue and red, respectively. Arrowheads indicate activation, blunt ends indicate inhibition. Line widths indicate the absolute values of interaction strengths.

Extended Data Figure 6. Model predicted time courses of responses to p70S6K and Trk inhibitors. Experimental data (dots) are imposed on model predicted time course of signaling responses of TrkA and TrkB cells treated with (**A**) S6K inhibitor (LY2584702, 1 μM) or (**B**) Trk inhibitor (SP600125, 5 μM) and stimulated with 100 ng/ml NGF and BDNF, respectively. Dashed lines are the time courses in the absence of inhibitor. TrkA, blue; TrkB, red. Data are presented as mean values +/- SEM for n=3 biologically independent experiments.

Extended Data Figure 7. Model predicted time courses of responses to MEK and AKT inhibitors. Experimental data are imposed on model predicted time courses of signaling responses of TrkA and TrkB cells treated with (**A**) MEK inhibitor (Trametinib, 0.5 μM) or (**B**) AKT inhibitor (AKT inhibitor IV, 1 μM) and stimulated with 100 ng/ml NGF and BDNF, respectively. Dashed lines show the time courses in the absence of inhibitor. TrkA, blue; TrkB, red. Data are presented as mean values +/- SEM for n=3 biologically independent experiments.

Extended Data Figure 8. Model predicted time courses of responses to JNK and RSK inhibitors. Experimental data are imposed on model predicted time-courses of signaling responses of TrkA (blue) and TrkB (red) cells treated with (**A**) JNK inhibitor (1 μM) or (**B**) RSK inhibitor (1 μM) and stimulated with 100 ng/ml NGF and BDNF, respectively. Dashed lines show the time courses in the absence of inhibitor. TrkA, blue; TrkB, red. Data are presented as mean values +/- SEM for n=3 biologically independent experiments.

Extended Data Figure 9. (**A**) The restoring force *f(S)* is plotted versus the DPD output *S*. (**B**) Waddington's landscape in the absence of the signaling driving force. The basins of attraction for differentiation and proliferation are colored blue and red, respectively. (**C**) Schematic diagram of the generation of cell fate decisions by the driving signaling force, which drives cell state changes, and the restoring force, which stabilizes a given cell state.

Extended Data Figure 10. Live cell images of TrkA cells stimulated with NGF and treated with inhibitors. Inhibitor concentrations are given in the legend to Extended Data Figures 6-8. Representative images of 3 biological replicates are shown.

Extended Data Figure 11. **Live cell images of TrkB cells stimulated with BDNF and treated with inhibitors.** Inhibitor concentrations are given in the legend to Extended Data Figures 6-8. Representative images of 3 biological replicates are shown.

Extended Data Figure 12. Model predicted outcomes of TrkA cell inhibitor treatments are corroborated by cell images. (**A**) Model predicted DPD responses of TrkA cells to ERBB and MEK inhibitors are shown at 45 min 100 ng/ml NGF stimulation by Loewe isoboles. The ERBB inhibitor applied alone has a negligible effect. (**B**) The percentages of differentiated TrkA cells show that a combination of ERBB (Gefitinib, GEF) and MEK inhibitors (Trametinib, TRAM) does not change the cell state, as correctly predicted by the model. Data are presented as mean values +/- SEM for n=3 biologically independent experiments. (**C**) Live cell images of NGF-stimulated TrkA cells treated with 2.5 μM Gefitinib, 0.2 μM Trametinib and a combination of 1.25 μM Gefitinib and 0.1 μM Trametinib taken at 72 hours. Representative images of 3 biological replicates are shown.

Extended Data Figure 13. Model predicted outcomes of TrkB cell inhibitor treatments are corroborated by cell images. Live cell images of BDNF-stimulated TrkB cells treated with 2.5 μM Geftitinib, 0.2 μM Trametinib and a combination of 1.2 μM Geftitinib and 0.1 μM Trametinib at 72 hours. Representative images of 3 biological replicates are shown.

Extended Data Figure 14. Inhibition of p38 does not change the percentage of differentiated TrkA and TrkB cells. Live cell images of NGF-stimulated TrkA (**A**) cells and BDNF-stimulated TrkB

(**B**) cells treated with 10 μM p38 inhibitor SB203580 for 72 hours. Representative images of 3 biological replicates are shown.

Extended Data Figure 15. Separation of MS phosphoproteomic patterns of TrkA and TrkB cell states and the STV projection into the PCA space. Following GF stimulation, TrkA (blue) and TrkB (red) states were separated by a SVM. Projections of data points, the separating hyperplane (grey) and the STV (dark red) are shown in the space of the first three principal components. The text in red indicates the kinases that phosphorylate the top STV components.

Extended Data Figure 16. Separation of apoptotic and proliferation states of SKMEL-133 cells and a projection into the PCA space. SVM separation of phosphoproteomic patterns of proliferation states in growing SKMEL-133 cells and apoptotic states after treatment with a combination of PI3K/AKT/mTOR and MEK inhibitors. The data are taken from Korkut et al.[29] Projections of the separated data points, the separating hyperplane (black) and the STV (dark red) are shown in the space of the first two principal components.

Extended Data Figure 17. Model calculated and experimentally determined DPD responses of SKMEL-133 cells to different inhibitors. (**A**) The experimentally measured DPD values (dots) are calculated based on the data from the reference Korkut et al[29]. Model-predicted (blue curves) DPD responses to many inhibitors exhibit abrupt DPD decreases at certain inhibitor doses caused by the loss of stability of a proliferation state and the induction of apoptosis in a threshold manner. Mathematically, an abrupt DPD decrease relates to a saddle-node bifurcation[64] (a fold catastrophe) that occurs when a stable steady-state solution corresponding to a proliferation state disappears. Data are presented as mean values +/- SEM for n=3 biologically independent experiments. (**B**) Synergy between MEK/ERK and PI3K/AKT inhibitors is demonstrated by concave Loewe isoboles.

Extended Data Figure 18. Separation of scRNAseq transcriptomic patterns of epithelial and mesenchymal states and projections into the PCA states. Single cell RNAseq data[31] were separated by SVM in untreated (blue) and treated with TGFβ (red) A549 (**A**), DU145 (**B**), MCF7 (**C**) and OVCA420 (**D**) cells. Projections of data points, the separating hyperplane (grey) and the STV (dark red) are shown in the space of the first three principal components.

Extended Data Figure 19. DPD responses of Py2T, A549, DU145, MCF7 and OVCA420 cell lines to specific inhibitors of different signaling modules. The cell lines, ligands and inhibited modules are indicated on the horizontal and vertical axes. The normalized DPD value 1 corresponds to fully mesenchymal state, and normalized DPD value 0 corresponds to fully epithelial state.

Extended Data Figure 20. Single-cell DPD distributions for A549, DU145, MCF7, OVCA420 cells. Left panels show single-cell DPD distributions for cells stimulated with TGFβ, EGF or TNF for 7 days. Right panels show single-cell DPD distributions for TGFβ-stimulated cells treated with RIPK1 inhibitor for 7 days.

Extended Data Figure 21. Single-cell DPD distribution for Py2T cells treated with TGFβ for 7 days.