Visual Barcodes for Multiplexing Live Microscopy-Based Assays

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Visual barcodes for multiplexing live microscopy-based assays

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Abstract:

While multiplexing samples using DNA barcoding revolutionized the pace of biomedical discovery, multiplexing of live imaging-based applications has been limited by the number of fluorescent proteins that can be deconvoluted using common microscopy equipment. To address this limitation we developed visual barcodes that discriminate the clonal identity of single cells by targeting different fluorescent proteins to specific subcellular locations. We demonstrate that deconvolution of these barcodes is highly accurate and robust to many cellular perturbations. We then used visual barcodes to generate ‘Signalome’ cell-lines by multiplexing live reporters to monitor the simultaneous activity in 12 branches of signaling, in live cells, at single cell resolution, over time. Using the ‘Signalome’ we identified two distinct clusters of signaling pathways that balance growth and proliferation, emphasizing the importance of growth homeostasis as a central organizing principle in cancer signaling. The ability to multiplex samples in live imaging applications, both in vitro and in vivo may allow better high-content characterization of complex biological systems.

Introduction:

The ability to multiplex samples has revolutionized science as well as medical practice. Genetic barcoding applications enabled unprecedented multiplexing, followed by parallel processing and analysis of dozens to hundreds of thousands of samples in applications like scRNA-Seq or functional CRISPR/shRNA/Open reading frame (ORF) screens. In contrast, in the field of image-based screens, high order multiplexing is limited by the small number of channels that can be practically separated with common microscopy equipment. We developed visual barcodes that enable multiplexing of microscopy-based applications and used them to multiplex live-cell reporters for the study of signaling pathways dynamics in cancer cells.

To maintain growth homeostasis, individual cells must balance and coordinate numerous and sometimes competing demands. These requirements are answered by signaling networks that integrate information from numerous branches of signaling. In cancer, genetic and non-genetic alterations in major signaling pathways have been tightly linked to tumor initiation, progression and response to anti-cancer therapies. It was also demonstrated that many of these alterations can be classified into a dozen signaling pathways, which together regulate core cellular processes such as cell fate, cell survival, and genome maintenance.^{1,2}

To facilitate understanding of cancer signaling, previous studies have developed genetically tagged activity reporters, i.e. fluorescent-proteins that exhibit changes in abundance or localization in response to particular signaling activities.^{3,4} Unlike endpoint assays, which necessitate ending of the experiment in order to measure a phenotype, these reporters helped
5 reveal the intricate dynamics of individual branches of signal transduction pathways, in live cells, at single cell resolution. However, since it is difficult to multiplex fluorescence reporters, mutual dependencies between these separate branches of signaling remained less explored.

In the present study, we describe the development and application of visual barcodes, a
10 technology that enables multiplexing live cell imaging applications. The visual barcodes are used as labels of clonal identity of single cells in mixed populations. To advance the understanding of cancer signaling pathways and their crosstalk, we used visual barcodes to multiplex 12 live reporters of major signaling pathways, generating an experimental system that we term, the 'Signalome'.

15 Using the Signalome we investigated the coordinated (multiplexed) dynamics of 12 signal transduction pathways in cancer cells that were challenged with well characterized chemical perturbants. Our results show that multiplexing 12 reporter lines not only increases throughput but also eliminates noise associated with well to well variations in high throughput drug screens.
20 Surprisingly, our results also identify a previously undescribed binary partitioning of cancer signaling into two distinct clusters.

To maintain homeostasis, proliferating cells require mechanisms that coordinate rates of cell division with appropriate rates of biosynthesis. To sustain the proliferative demands of oncogenes,
25 cancer cells require precisely tuned rates of biosynthesis.⁵ If cells fail to double their mass between consecutive cell divisions, cell mass will progressively diminish. Conversely, rates of biosynthesis that exceed rates of cell division can result in cellular enlargement and senescence.⁶⁻⁸ We show that the two clusters of signaling pathways identified by the Signalome system represent a general stress response that correlates with the cells' need to balance
30 between growth and proliferation. Implementing Signalome in cancer cell lines thus revealed previously underappreciated importance of growth homeostasis as a central organizing principle in cancer signaling.

Our findings thus demonstrate that the Signalome is a robust technology that can help study the dynamics of signaling pathways in a single cell resolution and the interconnection between different signaling branches. As the system is highly modular, replacing reporters can be easily achieved to allow the study of many other questions across the different fields of biology.

- 5 Additionally, the visual barcodes can be used in numerous in-vitro and in-vivo applications in which visual deconvolution of multiplexed cell lines is of need.

Results:

10 Developing visual barcodes to allow multiplexing of live imaging applications.

To construct visual barcodes, we first stably infected the A375 melanoma cell line with a lentivirus containing the nuclear marker histone 2A fused to iRFP (iRFP-H2A) to accurately demarcate nuclear and cytoplasmic compartments (Fig. 1A). Next, iRFP-H2A cancer cells were used to generate five subclones, each labeled with a cyan fluorescent protein (CFP) tagged with unique
15 cellular localization sequences, targeting the CFP into one of five subcellular locations - nucleus, endoplasmic reticulum (ER), cytoplasm (NES), peroxisomes (Peroxi) and whole cell (WC) (Fig. 1B). CFP localization was thus used as a visual barcode that can discriminate clonal identity.

To test the accuracy of the visual barcodes, we imaged each of the five subclones separately with both phase-contrast as well as in iRFP and CFP channels. We then used
20 CellProfiler⁹ to (1) segment the cells, (2) identify both nucleus and cytoplasmic compartments and (3) extract texture, shape, and intensity-based features in the CFP channel for each cell (Sup code). CellProfiler Analyst¹⁰ was used to classify single cells based on visual barcode readouts. In our implementation, we used 70% of the cells as a training set, with the remaining 30% as a validation set. Average false detection rate (false positive barcode labeling) was 1.15%, while
25 miss rate (false negative rate) was 1.17%, representing very high precision and recall rates respectively (Fig 1C and Sup Fig. 1A). The highest false detection rate and miss rate were both detected between nuclear and ER localizations, which were thus deprioritized from additional follow up.

30 To scale up the dimensionality of visual barcodes to a 12-barcode system, we fused three of the localization signals to four fluorescent proteins (CFP, BFP, GFP, and YFP), resulting with 12 distinct visual barcodes (Fig. 1D). This increase in barcode number still maintained a very high accuracy with an average false detection rate of 1.45% and a miss rate of 1.34% (Fig. 1E and Sup fig 1B). Most detection errors were between subclones with the same fluorescence color,

with the highest false detection rate detected between whole-cell and peroxisomal BFP (Fig. 1E). To explore the robustness of the system under different types of perturbations, we treated each of the subclones separately with 75 drugs (Sup Table 1). We found that after 48 hours of drug treatment, both precision and recall were still very high in almost all drugs with only eight drugs (12%) having a miss rate of above 3%, five of which strongly affecting the cells proliferation and viability. (Fig. 1F, Sup figs 1B,C).

Lastly, we showed that the clones can also be separated when cells are in suspension, using the Imagestream high resolution microscopy and flow cytometry system (Fig 1G-J). As our system was lacking a laser to detect YFP, we only multiplexed nine of the subclones. To demonstrate that the visual barcodes can also be used in-vivo, we mixed the nine clones and implanted them subcutaneously in a nude mouse. When the tumor reached a diameter of 8mm it was excised, dissociated into single cells and analyzed by the imagestream system, demonstrating that all nine clones could be detected (Sup Fig. 1D-G).

Generating the ‘Signalome’ reporting cell lines

To generate the Signalome, we assembled 12 previously published and well characterized reporter constructs (Sup Fig 2A), each associated with the activity of a different cancer-related signaling pathway. To enable their multiplexing, we replaced the original tagged fluorescent protein in each of the 12 reporter vectors with the mStrawberry fluorescent protein. We used two different types of reporters: reporters that drive the expression of the fluorescent protein by a specific transcription response element (TRE) or kinase translocation reporters (KTRs) that translocate the fluorescent protein from the nucleus to the cytoplasm upon activation of upstream signaling (Sup Fig 2A).

We then infected each of the 12 A375 subclones that have visual barcodes with one of the 12 reporters (Fig 2A). Next, we validated that the proliferation rate of the single-cell derived reporter subclones is not different from that of the parental cell line (Sup Fig 2B). We also validated that all 12 subclones are as sensitive to the BRAF inhibitor, vemurafenib, as the parental A375 cell line (Sup fig. 2C). Lastly we pooled together all 12 subclones generating the A375 signalome cell line, and demonstrated that the proportions of the 12 different clones remained constant over 48 hours of culture (Figure 2B).

An advantage of single cell measurements is that perturbations can be characterized not only for their influence on population average but also on the full distribution (i.e. the frequency of cells with low, medium or high signaling activity). To quantify condition-dependent differences in the distribution of reporter activity, we used the Kolmogorov-Smirnov (KS) test as it is a non-parametric test that can also detect changes in the distribution that are not reflected by the mean of the distribution (Sup Figs. 2D,E).^{11,12} To add to the KS score, activity scores were assigned a positive or negative sign based on the change in direction of the mean of these distributions. To validate our readout, we used known positive or negative regulators for each of the reporters (Sup Fig 2F).

As an additional validation, we show that drug-dependent changes in the activity of all reporters of the multiplexed A375-Signalome cell line are strongly correlated with measurements performed on the single reporter subclones ($r=0.75$, $p<10^{-16}$) (Fig 2C). Reassuringly, we found that Vemurafenib and the MEK inhibitor Trametinib, both inhibitors of the MAPK pathway, exerted highly similar effects on the A375-Signalome cell line (figure 2D-F).

An advantage of multiplexed, time dependent measurements on signaling is the ability to differentiate direct and indirect drug influences. For example, while MAPK inhibitors (vemurafenib and trametinib) promoted detectable changes in measured ERK signaling that were observed 1.5 hours into drug treatment, an influence of these same drugs on other pathways was also observed, albeit at much later times (Fig 2D-F). This is in agreement with the direct effect of these drugs on the MAPK pathway and the subsequent adaptive response of the other pathways to the inhibition of the MAPK pathway. Indeed, previous reports already described activation of PKA¹³, NFkB¹⁴, HIF¹⁵, and YAP/TAZ¹⁶ in response to vemurafenib and suggested that these adaptations can contribute to resistance to MAPK inhibition. In addition, we observed a significant upregulation of retinoic acid receptor activity 48 hours after BRAF or MEK inhibition ($P\text{-value} < 6 \times 10^{-5}$). Interestingly, examination of three independent cohorts of melanoma patients demonstrated that patients with high activity of the RAR/RXR pathway, as calculated from expression data by PathOlogist¹⁷, had an overall better survival (Sup figure 2G-I). Therefore, it may be of interest to continue and better explore the role of RAR in melanoma and its response to therapy. These measurements also demonstrate the efficiency and throughput of the Signalome system: with one 384-well plate, we screened the influence of 75 drugs (in triplicates) and 39 DMSO controls on 12 branches of signaling, in multiple time points, and at single cell resolution.

A single signalome plate thus provided measurements on half a million cells from each of the different time points.

To demonstrate that that visual barcodes and a signalome system can be readily applied to other cell lines we generated two more signalome cell lines using the PC9 EGFR-mutated non-small cell lung cancer cell line and the SK-MEL-5 BRAF-mutated melanoma cell line. We were able to demonstrate that our barcode precision and recall rates are also very high in these cell lines (Sup. figure 3A-D) and that an early inhibition of ERK by both inhibitors can be detected followed by cell-line specific adaptive mechanisms. For example - while inhibition of BRAF or MEK in A375 melanoma cell line resulted in activation of the YAP/TAZ pathway, the effect of BRAF/MEK inhibition in the SK-MEL-5 cell line resulted in inhibition of the YAP/TAZ pathway (Sup figure 3E-H). Here again we found that EGFR inhibition in the PC9 cells also drives upregulation of RAR activity.

Large scale correlations in signaling suggest a generalized response that is compound independent.

To investigate interdependencies in the cancer signaling, we treated the pooled A375 signalome cell line with a library of 422 well characterized chemical perturbants (Table 2, Sup Figure 4A). Of all tested compounds, 122 (28.9%) promoted significant changes in at least one of the reported pathways (KS absolute score > 0.25). As expected, different drugs with similar targets displayed similar patterns of reporters changes in response to these drugs (Sup figure 4B-G).

Surprisingly, in addition to target-specific signatures, unsupervised clustering of the reporters activity scores also suggested a higher structure that partitions the signal transduction signatures into three clusters, two of which seem anticorrelated (Fig 3A). Cluster A groups compounds that seem to all activate the pathways PKA, AKT, ERK, p38 and JNK while inhibiting WNT, p53, NFkB, RAR, HIF and YAP\TAZ; while cluster B contains drugs that orchestrate the opposite response. Cluster C contained drugs that did not follow this dichotomy. The large number of drugs with varying mechanisms of action that result in signaling clusters A and B, suggests a coordinated response to drug treatment that involves all of our measured branches of signaling and is surprisingly independent of drug target. As a case in point, Fig 3B shows a negative correlation between the activities of p53 and p38 which is persistent across a wide diversity of chemical perturbations. Drugs that diminished p38 activity correlate with equivalent increase in the activity of p53 and vice versa (Pearson's $r = -0.517$, $p < 2.2 \times 10^{-16}$) (Fig. 3B). More generally, drugs promoted positive correlations among pathways within cluster A or B (intra-cluster

correlations) and negative correlations when comparing pathways from cluster A to pathways from cluster B (inter-cluster correlations). For simplicity, we will refer to these two clusters as the *p38 signaling state (cluster A)* and the *p53 signaling state (Cluster B)* (Fig 3A).

Chemical perturbations can generate correlated influences by simultaneously affecting more than one target. Such correlated influences, however, should be compound specific, relating to target affinities that differ from one compound to another. By contrast, we found that the same pairs of pathways are positively, or negatively correlated, across a large number of drugs (n=122) that have multiple and highly different targets. (Fig 3B, 3C). To demonstrate that this partition is not cell line specific we treated the PC9 signalome cell line with 247 drugs and found very similar bifurcation into two anti-correlated clusters of pathways (Sup Figure 4H). The question, therefore, is as follows: how can such a wide variety of perturbations, each associated with different targets, converge onto only two main outcomes? We reasoned that the partitioning of the signaling pathways into two clusters suggests a regulatory process that is common and upstream of all of our measured branches of signaling Fig 3D.

Large scale correlations in signaling are present pre-treatment and increase over time by multiple drugs

To gain insight into the nature of this upstream regulatory process, we first performed time course measurements to ask, how soon after drug treatments do the pairwise correlations become apparent? We found that pairwise correlations in pathway activity became more and more prominent throughout the 48 hours following drug treatments (Fig 4A, 4B). The segregation of the two signaling states post drug treatment is also apparent from visual inspection of time course measurements (Fig 4C-H). Trajectories of activity of the reporters in response to six representative drugs that are chemically distinct and associated with different targets, demonstrated that while three drugs promoted the p38 signaling state, the other three activated the p53 signaling state. Altogether, these results demonstrate the binary partitioning of the signaling pathways by showing that a variety of different drugs, each associated with different targets, converge to promote two main signaling states outcomes.

These results suggest that the p38- and p53- signaling states are mediated by a process that, in response to drug treatments, gradually increases its influence, or activity, over time. Since it is likely that the drug treatments only promoted the activity of an already existing process, we were curious as to why the correlations shown in Fig 3C seem absent in unperturbed cells (Fig 4B)? One possibility is that, prior to drug treatments, signaling pathways are subjected to the

simultaneous influence of several competing regulatory demands, each pulling in a different direction. According to this interpretation, drug treatments promote correlated signaling by increasing the relative weight of one particular regulatory process – most likely a stress response – such that its affect is no longer averaged by competing influences. This model suggests a testable hypothesis: the binary partitioning of the 12 pathways should become apparent in untreated cells if the measurements are normalized for independently existing correlations.

To this end we used Principal component analysis (PCA), a technique that transforms a dataset into a linear combination of independently existing multivariate correlations. As expected, PCA confirmed the high degree of correlations by identifying a single principle component (PC1) that explains almost 50% of the variance after 48h of treatment (Fig 5A). Further, the first principle component clearly identified the two signaling states; drugs that promote the *p38-signaling* or *p53-signaling* states are characterized by positive or negative values of PC1, respectively (Fig 5B). Next, we repeated the PCA, but this time on measurements collected prior to drug treatments (time zero). Note that in this latter implementation of PCA, variation in measured activities did not reflect differences in drug response, as no drugs were yet applied, but rather, small well-to-well variations like differences in evaporation rate or oxygen concentrations that are usually referred to as noise. Since the signalome provides measurements on 12 pathways in each well, it can test whether these small variations will lead to a well-specific shift in the signaling states that can be detected by PCA. Indeed, PCA significantly identified both p38- and p53-signaling states also in the unperturbed cells (Fig 5C).

To further explore whether the p38- and p53- signaling states precede drug treatments, we tested whether measurements on cells that were not exposed to drug treatments can predict the specific correlations observed post drug treatments (Sup figure 5). This analysis identified dynamics that are classically characteristic of homeostasis (Fig 5D). In the first hour post drug treatment, chemical perturbations effectively eliminated correlated activities that linked the different branches of signaling in the unperturbed cells. Several hours into drug treatment, however, correlated activities resumed and, in fact, gained more prominence. These results suggest a general stress program that: (A) had functioned in cells that were not subject to drug treatment (B) was effectively eliminated in the first hour of drug treatment and (C) had resumed activity in the hours following drug treatment. This observation can also be visualized in Fig 4C-H, in which the two signaling states are apparent at time zero (pre drug treatment), lost at 1 hour, and gains significance at the latter time points.

The p38- and p53- signaling states are linked to perturbations in cell size

The constancy of the two signaling states, in the face of diverse chemical perturbations, suggests that the large-scale correlations described by these signaling states function to support some process that is critical in our cell lines. Since our measurements were performed on cancer cells, we further reasoned that the process in question may relate to demands imposed by continuous cell divisions. To maintain homeostasis, proliferating cells must double their mass between consecutive cell divisions. In cancer, this requirement may be more critical.^{18–20} If cancer cells fail to match the proliferative demands of an oncogene with equivalent increases in biosynthesis, cell size will decrease over time.²¹ We therefore wondered whether the observed pattern of coordinated signaling results from stress-sensing systems that respond to changes in cell size resulting from imbalances in cell growth and cell division (Fig 6A).

As a first step, we asked whether drugs that selectively interfere with rates of biosynthesis trigger compensatory mechanisms that will help the cell to reach a new steady state between growth and proliferation rates. To inhibit cell division rate, we used various chemical inhibitors of cyclin dependent kinases (CDK) like SNS-032²¹ while to lower biosynthetic activity (cell growth), we either inhibited protein synthesis by cycloheximide or mTOR activity by rapamycin and torin. In all cases, drug doses were carefully optimized to ensure that cells are still proliferating and are not undergoing complete cell cycle arrest. For quantitative measurements of cell growth (protein synthesis per unit time), we followed a previously described protocol for single cell measurements of total macromolecular protein mass using fluorescently labeled succinimidyl ester (SE) that label all proteins (Sup methods)^{21–23}.

Our results demonstrate that during the initial hours of rapamycin treatment, while cell growth was rapidly inhibited, the rates of cell division were relatively unaffected (Fig 6B). Conversely, the CDK2 inhibitor SNS-032 lowered rates of cell division but did not affect cell growth (Fig 6B). At the later time points, however, a coordination of growth and proliferation was re-established, but at a slightly different setpoint. Cells with inhibited rates of biosynthesis adapted by promoting longer periods of biosynthetic activity (longer cell cycles). Yet, this lengthening of the cell cycle fell short of a perfect adaptation, resulting in paired values of growth and division that fell slightly below the proportionality line. Similarly, to adapt to the longer growth periods imposed by CDK2 inhibitors, cells lowered the amount of protein synthesized per unit time. Yet, here too, the compensation was incomplete, resulting in paired values that lay above the line. Extending these results to multiple cell cycle or cell growth inhibitors across five cell lines

demonstrated that incomplete compensation of the growth and proliferation rate is a general phenomena (Fig 6C). In conclusion our results suggest that: (A) Drugs that perturb rates of biosynthesis trigger compensatory changes in division rates, and vice versa (B) The adaptation of growth rates and division rates to drug treatments is typically incomplete, resulting in paired values of growth and division rates that lie above and below the proportionality line.

To investigate the possibility that the p38- and p53-signaling states that we observed are related to the homeostasis of cell size, we asked whether drugs that induce these two states differ in their influence on cell size. To that end, we scored each compound for the extent that it promoted the p38-signaling vs p53-signaling states (Sup methods). We then used the signalome single-cell resolution measurements to calculate the influence of each drug on cell division rate and cell size. Consistent with our hypothesis, we found that drug treatments that promote the p38 state correlated with a smaller cell size while drugs that promote the p53 state correlated with an increased cell size (Pearson's $r = -0.625$, $p = 1.68 \times 10^{-23}$) (Fig 6D).

Next, we used the signalome to test whether the two signaling states correlate with imbalances in cell growth and division rates. We found that drugs that disproportionately decrease growth rates (i.e. data points below the diagonal) were associated with the p38-signaling state, while drugs that disproportionately decrease proliferation (i.e. points above the diagonal) induced the p53-signaling state (Fig 6E,F).

In cancer, disproportional changes in growth and division can spontaneously result from intracellular genetic changes or from external stresses, including nutrient or growth factor deprivation. To test if the p38 and p53 signaling states are represented in human cancers, we mined the TCGA (<https://www.cancer.gov/tcga>) to retrieve proteomic measurements from 8,167 human tumors that span 32 different types of cancer.^{24,25} To compare signalome reporters with TCGA, we assembled a list of 8 proteins or phosphoproteins that are known to correlate with the activity of pathways included in the signalome. Using these proteins as a surrogate for pathways activity we separately analyzed each of the 32 cancers for correlated signaling. Specifically, for each cancer, we calculated all pairwise correlation coefficients related to the signalome pathways. Our results demonstrated that, with the exception of cholangiocarcinoma, similar signaling bifurcation was present in all of the other cancer types as demonstrated in Sup Fig 5F. This further indicates a common regulatory process upstream of all of our measured branches of signaling (Fig 3D).

Discussion

Live reporters are widely used to study signaling dynamics in cells. However, currently, the ability to multiplex live reporters together is limited. The integration of information from multiple signaling branches is critical for the understanding of complex biological processes. In this study we introduced visual barcodes, a fluorescent protein coupled to a specific subcellular localization peptide, which allows multiplexing cells in live imaging applications. We demonstrate that visual barcodes are robust to perturbations, have a high precision and recall rates and are applicable for multiplexing both *in vitro* and *in vivo*. Multiplexing of different subclones not only increases the throughput of experiments but also reduces cost and well-to-well or animal-to-animal variation.²⁶ Adding more fluorescent proteins or cellular localizations to the system can augment its multiplexing potential, and we predict that the system can be easily expanded to 20-plex combinations. Deconvolution of the visual barcodes was done using freeware, thus enabling the use of the system without licensing limitations. The visual barcodes system can be used for a very wide variety of applications such as competition assays between clones with different perturbations *in vitro* or *in vivo*, live tracking of cells with reduced risk of switching between subclones, as well as multiplexing of live reporters, as we demonstrated by the Signalome cell lines.

For generating the Signalome cell lines we added a different fluorescent reporter for each of our 12 validated visual barcodes subclones, reporting for major signaling pathways in cancer cells. While we generated each of the Signalome subclones using three consecutive round of infections (nuclear marker, visual barcode, fluorescent reporter), we envision that a visual barcode and a reporter could be integrated into a single plasmid thus allowing one round of infection with a mix of plasmids on a cell line with a nuclear marker, allowing the generation of additional Signalome cell lines in days rather than weeks.

To better understand the interdependencies of signaling pathways we treated the A375 and PC9 signalome cell lines with hundreds of characterized chemical perturbants. Altogether, our results suggest an explanation as to how a chemically diverse collection of drugs converged onto a much smaller number of signaling states. Growth and division are fundamental processes that are subject to multiple mechanisms of homeostasis. While different drugs affect different intracellular mechanisms, an influence on growth or division is a common denominator of many different drug targets. According to this model, the question of whether a drug promotes the p38 or p53 signaling

states is not answered by the affected drug targets but rather, by how that drug targets relates to growth and division, i.e. to cell size.

The association of p38 with changes in cell size is consistent with previous reports^{23,27} that show that p38 MAPK is selectively activated in cells that are smaller than their target size. These
5 aforementioned studies, however, failed to identify why size sensing may be critical in continuously proliferating populations. The present work links size sensing in proliferating cells with an adaptation to homeostasis of growth and cell division. It is also interesting to note that, while both p38 and p53 are well established stress proteins, their physiological response to stress conditions is very distinct. Stress conditions that activate p38 typically promote inflammatory
10 programs which promote growth and suppress apoptosis.²⁸ By contrast, the activation of p53 is both pro-apoptotic and functions to suppress mTORC1-mediated biosynthesis.²⁹

Overall, the visual barcodes are an easy to implement system that can help researchers to multiplex cells for a very wide variety of applications. The system is highly modular and can serve to generate Signalome cell lines with different reporters and thus may be useful in the
15 research of a very wide variety of biological fields.

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Figure 1. Developing visual barcodes for multiplexing live imaging applications. **A.** Representative image of the A375 cell line with an iRFP-H2A nuclear marker. **B.** Representative images of five CFP localizations in the A375 cell line: Whole Cell (WC), Nuclear export signal (NES), Nuclei, Peroxisome (Peroxi), and endoplasmic reticulum (ER). **C.** Heatmap of false detection rate (FDR) for barcode calling for the five CFP localizations in the A375 cell line. **D.** Representative images of all 12 visual barcodes used in A375 cell line. **E.** Average miss rate of barcode calling for all 12 visual barcodes of the A375 cell line, treated with DMSO controls (n = 39). Numbers in the diagonal represent the sensitivity for each barcode. **F.** Violin plots showing miss rate for all 12 visual barcodes of the A375 cell line, treated with DMSO controls (n = 39) or with drugs (n=75). **G-I.** Scatter plot showing the separation of nine A375 clones with visual barcodes by the ImageStream system according to their fluorescent color and localization. The separation by localization is only demonstrated for GFP positive clones (**I**). **J.** Representative images from ImageStream of all nine clones. Two cells from each clone are presented. Scale bar in **A** and **B** is 50 μ m, in **D** 100 μ m and in **J** 7 μ m.

A **B** **C** **D** **E** **F**

Figure 2. Generating the A375 ‘Signalome’ reporter cell line. **A.** Illustration of the 12 clones that were used to generate the A375 Signalome cell line. A375 cells were first infected with iRFP-H2A to mark the cell nucleus. Then, 12 clones were generated with 12 visual barcodes. Lastly, a different live reporter was added to each of the clones. Transcription activating reporters are represented by gold while translocation reporters are represented in red. Binding partners in the nucleus are represented in purple. **B.** Relative number of cells from each clone in a DMSO control wells over time. **C.** Scatter plot showing the correlation between the reporter activity scores, for all 12 clones when grown separately or as part of the Signalome cell line, in response to 75 drugs. **D-F.** Reporter activity plots of the A375 signalome cell line in response to DMSO, vemurafenib (1 μ M) or trametinib (0.125 μ M) over time. Blue and red backgrounds represent activation or inhibition scores above 0.2 or below -0.2 respectively.

A

B

C

D

Figure 3. Large scale correlations in signaling suggest a generalized response that is compound independent. **A.** Unsupervised hierarchical clustering of A375 signalome cells treated by 122 active drugs according to their activity scores. Due to technical error, Geminin reporter was not measured for all drugs and was thus discarded. **B.** Scatter plot showing the correlation between the activities of the p38 and p53 reporters after 48 hours of treatment with 422 drugs. **C.** Heatmap showing the pairwise Pearson correlations between the different A375 Signalome clones, after 48 hours of treatment with 122 active drugs. **D.** A model proposing how drugs with different mechanisms may converge into two major signaling states. While each of the drugs has different targets, many of the targets affect the same sensing mechanism that later governs the p53 vs p38 signaling states.

— p38 state — p53 state

Figure 4. Drug treatments increase the correlations between the activity of pathways

A. Scatter plots of the activity scores of p38 and p53 reporters in the A375 signalome cell line before and at multiple time points after treatment with 422 drugs. Each dot represents a different drug. Pearson's r is depicted for each of the time points

B. Pearson correlation coefficients were calculated for each pair of pathways in the A375 signalome cell line before and at different time points after treatment with 422 drugs. Each datapoint represents a correlation value for one given pair of pathways over all 422 drugs. The correlation between the activity score of p38 and p53 pathways is marked by a red circle

C-H. locally weighted smoothing (Lowess) regression of all reporters in each of the two signaling states is shown for 6 representative drug treatments, each associated with different drug targets. While three of the drugs drive the p53 signaling state (**C-E**), the other three drugs drive the p38 signaling state (**F-H**). Gray sleeves represent the confidence interval for each of the signaling states.

A **B** **C** **D**

Figure 5. PCA suggests that the p38 and p53 signaling states exist pretreatment and increase in weight over time. A. PCA of the activity scores of 11 signaling pathways after 48 hours of treatment with 122 drugs. The color of each drug is indicating its cluster in figure 3A. **B, C.** bar plots representing the PC1 loading of each pathway after 48 hours of drug treatment (**B**) or pre-treatment (**C**). **D.** Variance of pathway activity, when projected on the principle components calculated from measurements on cells that were not exposed to drug treatment.

A

B

C

D

E

F

Figure 6. Cell growth and proliferation are tightly regulated and correlate with p38 and p53 signaling states. **A.** A model demonstrating how sensing of cell size can affect both cell growth and proliferation to keep homeostasis of cell size. **B.** Scatter plot showing the initial and long-term effects of rapamycin or SNS-032 on the average cellular growth rate and division rate of Rpe1 cells. Data points indicate the average growth and division rates measured: (1) during the first 24 hours of drug treatment and (2) during 24-60 hours of drug treatment. **C.** Average growth rate vs. division rate in five cell lines (Rpe1, HeLa, U2OS, SAOS2, 16HBE) treated with either growth inhibitors (red) or cdk1/2 inhibitors (blue). Measurements in each cell line were normalized by the values measured for untreated control samples (gray) of the same cell line. The growth inhibitors used were cycloheximide, Torin-2, and rapamycin, at varying doses (detailed in methods section). The cdk1/2 inhibitors used were SNS-032, PHA848125, Cdk2 Inhibitor III, and Dinaciclib, at varying doses (detailed in methods section). **D.** The average cell size in a given condition is negatively correlated with its PC1 value. **E.** Average growth rate vs. division rate for A375 cells in Signalome screen. Each circle represents one screened condition (drug treatment). The circle's color indicates the value of PC1 in that condition. Contour lines show the average value of PC1 as a function of growth rate and division rate. **F.** The average level of p38 (top) and p53 (bottom) activity as a function of growth rate and cell cycle length.

A

B

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G

Supp Figure 1. Multiplexing cell lines for live imaging applications using visual barcodes. **A.** Barcode detection miss rate for the five CFP localizations in A375 cells. **B.** Miss rate percentage for all 12 A375 clones with visual barcodes treated with DMSO (Blue, n = 39) or drugs (Red, n = 75). **C.** Average Barcode calling false detection rate for all 12 visual barcoded clones of the A375 cell line treated with a library of 75 drugs. Numbers in the diagonal represent the average precision (%) for each barcode. **D-F.** Scatter plots showing the separation of nine A375 clones with visual barcodes by the ImageStream system according to their fluorescent color and localization. The separation by localization is only demonstrated for GFP positive clones (**F**). **G.** Representative images from ImageStream of all nine clones. Two cells from each clone are presented. Scale bar in (**G**) 7 μ M.

A

Reporter	Reporter type	addgene source
ERK	KTR	https://www.addgene.org/59150/
JNK	KTR	https://www.addgene.org/59151/
p38	KTR	https://www.addgene.org/59152/
PKA	KTR	https://www.addgene.org/59153/
HIF	TRE	https://www.addgene.org/42621/
p53	TRE	https://www.addgene.org/16593/
YAP/TAZ	TRE	https://www.addgene.org/34615/
RAR	TRE	https://www.addgene.org/13458/
NFKB	TRE	https://www.addgene.org/26699/
WNT	TRE	https://www.addgene.org/24304/
AKT	Translocation	https://www.addgene.org/67759/
GEMININ	TRE	https://www.addgene.org/62451/

B **C** **D** **E** **F**

Reporter	Molecule	Action	Activity score
RAR	BMS-806	Inhibitor	-0.9
AKT	MK-2206	Inhibitor	-0.76
ERK	Trametinib	Inhibitor	-0.69
GEMININ	Vemurafenib	Inhibitor	-0.6
p38	Nutlin 3a	Inhibitor	-0.42
WNT	LiCl	Activator	0.35
PKA	Forskolin	Activator	0.42
p53	Nutlin 3a	Activator	0.5
NFKB	TNF	Activator	0.55
YAP/TAZ	Trametinib	Activator	0.67
HIF	DFO	Activator	0.72
JNK	Sorbitol	Activator	0.95

G **H** **I**

Supp Figure 2. Generating the 'Signalome' reporter cell lines. **A.** Reporter type and addgene source for each of the 12 reporters used for the Signalome cell lines. **B.** Growth rate of all 12 visual barcode clones and their parental A375 cell line. The parental cell line was labeled with GFP positive lentivirus to facilitate a more accurate cell counting. **C.** Dose response curve of all 12 visual barcoded A375 clones and the parental A375 population (GFP) to vemurafenib. **D.** Density plots of ERK reporter activity depicted by the cytoplasm to nuclei ratio of the ERK translocation reporter after 72h of treatment with DMSO (Blue) or trametinib (Red). **E.** Cumulative distribution of ERK reporter activity of the same cells as in (D). KS statistics was used to calculate the activity score. The negative sign of the score reflects the lower mean of the trametinib density plot in (D) as compared to the mean of the DMSO plot. **F.** Validation of the reporters activity by using known activators/inhibitors of all 12 pathways. **G-I.** Kaplan-Meier plots showing patient survival stratified by RAR/RXR pathway activity as calculated by PathOlogist, based on RNA-Seq data. TCGA (n = 465 patients), Cirenajwis (n = 210 patients), Budden (n = 122 patients).

Supp Figure 3. Creating additional signalome cell lines. **A, E.** Barcode detection miss rate for PC9 and SK-MEL-5 cell lines respectively. **B, F.** Average barcode false detection rate for all 12 visual clones in both PC9 and SK-MEL-5 cell lines treated with DMSO. **C, D.** PC9 Signalome reporters' activity plot for gefitinib and vemurafenib respectively. **G, H.** SK-MEL-5 Signalome reporters' activity plot for vemurafenib and trametinib respectively.

Glucocorticoids

ROCK inhibitors

Supp Figure 4. Drugs with similar mechanisms of action cluster together by their effect of signaling pathways. **A.** Composition of all 422 drugs used in the screen, based on their mechanism of action or target. **B-D.** Signalome reporter activity plots for the glucocorticoids (GCs) dexamethasone, budesonide and beclomethasone respectively. All GCs showed a similar pattern: inhibition of both JNK and PKA reporters. **E-G.** Signalome reporter activity plots for the ROCK inhibitors: GSK269962A, GSK429286A and thiazovivin respectively. All three drugs have a similar pattern which includes mild activation of ERK and PKA together with significant inhibition of YAP/TAZ. **H.** Unsupervised clustered heatmap showing the pairwise correlations (Pearson) between PC9 reporter clones activity scores under 49 active drugs after 48 hours of treatment.

A

B

C

D

E

$$\Pi \quad S = Var(erk) \times Var(p38)$$

$$\Pi \quad \Psi = Var(ax1) \times Var(ax2)$$

$$\Phi = \frac{\Pi \Psi}{\Pi S}$$

F

Supp Figure 5. Calculating the extent to which principle components of cells that were not exposed to drug treatments represent drug-induced correlations. **A.** Fluorescence microscopy image of Rpe1 cells stained with AlexaFluor-647-succinimidyl ester. **B.** Heatmap showing intensity of fluorescence signal in **(A)**, indicating concentration of cellular proteins. **C, D.** An illustration depicting the correlation of p38 and ERK as represented in two different coordinate systems. In the first coordinate system **C**, axes are defined by the measured activities of p38 and ERK. With the second coordinate system **D**, axes are defined by performing PCA on cells that were not exposed to drug treatment. The yellow and blue shaded regions represent the area (or volume) that encloses the data in the alternate coordinate systems, which is calculated as the product of the variances. As shown in **E**. The volume enclosing the data is smaller when the coordinates are aligned with linear trends with the dataset. To calculate the extent to which the principle components calculated from measurements on cells that were not exposed to drug treatments represent drug-induced correlations, we compare the product of the variances in the alternate coordinate systems. **F.** To calculate a similarity score for each TCGA cancer, we calculated the pairwise correlation coefficients (Pearson) between measured activities in the tumor samples and the signalome. The similarity scores were then grouped together in a boxplot to show the distribution of similarities in every pathway for each cancer. The box blots represent the top and bottom quartiles for the distribution with the whiskers showing the extent of the distribution barring outliers (marked by diamonds). A pathway which scores 1 by this measure has the same pattern of correlations and anti-correlations between the given cancer and the signalome. Similarity scores of 1 indicate that all the pathways in the cancer have an identical grouping of correlations and anti-correlations as observed in the signalome. By contrast, scores of zero indicate there is no similarity in the pattern, while negative scores indicate some pathways exhibit inverse patterns in correlations to those observed in the signalome. Median similarity scores indicate the degree to which the pattern of signaling in that cancer was similar to those observed in the signalome. Those above 0 (marked by the red line) indicate that most of the TCGA cancers shared a pattern of correlations with those observed in the signalome. Cholangiocarcinoma (CHOL), Adrenocortical carcinoma (ACC), Skin Cutaneous Melanoma (SKCM), Lung squamous cell carcinoma (LUSC), Thymoma (THYM), Testicular Germ Cell Tumors (TGCT), Head and Neck squamous cell carcinoma (HNSC), Lymphoid Neoplasm Diffuse Large B-cell Lymphoma, Kidney renal papillary cell carcinoma (KIRP), Uterine Carcinosarcoma (UCS), Cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC), Liver hepatocellular carcinoma (LIHC), Thyroid carcinoma (THCA), Pancreatic adenocarcinoma (PAAD), Mesothelioma (MESO), Sarcoma (SARC), Stomach adenocarcinoma (STAD), Kidney renal clear cell carcinoma (KIRC), Lung adenocarcinoma (LUAD), Esophageal carcinoma (ESCA), Prostate adenocarcinoma (PRAD), Kidney Chromophobe (KICH), Breast invasive carcinoma (BRCA), Bladder Urothelial Carcinoma (BLCA), Colon adenocarcinoma (COAD), Rectum adenocarcinoma (READ), Pheochromocytoma and Paraganglioma (PCPG), Uterine Corpus Endometrial Carcinoma (UCEC), Ovarian serous cystadenocarcinoma (OV), Brain Lower Grade Glioma (LGG), Glioblastoma multiforme (GBM).

Methods:

Cell lines and reagents:

Experiments were performed using the BRAF mutated melanoma A375 (ATCC CRL-1619) and SK-Mel-5 (ATCC HTB-70) cell lines and the non small-cell lung cancer EGFR-mutated PC9 cell lines. PC9 was a gift from Dr. Channing Yu of the Broad Institute of Harvard and MIT. All cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, #10569-010). Growing media were supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, pyruvate and glutamine (Invitrogen, #15140-122).

PCR for detection of Mycoplasma:

The protocol is based on the Takara Kit (#6601). The buffer used, deoxynucleotide triphosphates (dNTPs) and Taq polymerase were obtained from the Takara Ex Taq kit (#RR001A). The following forward and reverse Mycoplasma-specific primers were used for PCR: 5'-ACACCATGGGAGCTGGTAAT-3', 5'-CTTCWATCGACTTYCAGACCCAAGGCAT-3'.

PCR reactions contained 13.9 µl of Invitrogen UltraPure DDW (10977-015), 2 µl of buffer, 1.6 µl of dNTPs, 0.1 µl of TaKaRa Ex Taq™, 0.5 µl of both forward and reverse primers (final concentration of 20 µM), and 2 µl of genomic DNA or 2 µl of CM. CM was collected after an incubation time of three days on cells, at which point the cells had reached at least 80% confluence. Reactions were held at 94°C for 30 s to denature the DNA, with amplification proceeding for 40 cycles at 94°C for 30 s, 55°C for 2 min, and 72°C for 1 min

Visual barcodes and Signalome plasmids construction:

All of the plasmids described in this paper to generate nuclear marker, visual barcodes and live reporters have been submitted to Addgene and are available. We have also submitted the backbones used to create the visual barcodes and reporters to assist generation of additional visual barcodes or reporter cell lines. The complete list of the plasmids that we have submitted is the following:

Addgene ID	Plasmid	PURPOSE
158665	Barcode backbone	This plasmid serves as the backbone for the visual barcodes described in our paper. It has a CMV-Puro-F2A backbone to which we insert a fluorescent protein cellular localization combination
158666	mTAGBFP2 WC visual barcode	tagging clonal populations with whole cell mTAGBFP2 fluorescent protein
158667	mTAGBFP2 NES visual barcode	Tagging clonal populations with NES mTAGBFP2 fluorescent protein. Adding NES to mTAGBFP2 WC
158668	mTAGBFP2 Peroxisome visual barcode	Tagging clonal populations with Peroxisomal mTAGBFP2 fluorescent protein. Adding Peroxisome localization signal to mTAGBFP2 WC
158669	mTurquoise2 WC visual barcode	Tagging clonal populations with whole cell mTurquoise2 fluorescent protein
158670	mTurquoise2 NES visual barcode	Tagging clonal populations with NES mTurquoise2 fluorescent protein.
158671	mTurquoise2 Peroxisome visual barcode	Tagging clonal populations with Peroxisomal mTurquoise2 fluorescent protein.
158672	acGFP WC visual barcode	Tagging clonal populations with whole cell acGFP fluorescent protein
158673	acGFP NES visual barcode	Tagging clonal populations with NES acGFP fluorescent protein. Adding NES to acGFP WC
158674	acGFP Peroxisome visual barcode	Tagging clonal populations with Peroxisomal acGFP fluorescent protein. Adding Peroxisome localization signal to acGFP WC
158675	EYFP WC visual barcode	Tagging clonal populations with whole cell EYFP fluorescent protein
158676	EYFP NES visual barcode	Tagging clonal populations with NES EYFP fluorescent protein. Adding NES to EYFP WC
158677	EYFP Peroxisome visual barcode	Tagging clonal populations with Peroxisomal EYFP fluorescent protein. Adding Peroxisome localization signal to EYFP WC
158678	TRE reporter backbone	This plasmid serves as the backbone for the TRE reporters described in our paper. It's a promoterless-mStrawberry-pGK-BSD backbone to which we insert a pathway specific promoter before the mStrawberry
158679	WNT-TRE-mStrawberry reporter	This plasmid is a WNT pathway reporter. It has a 7xTcf promoter driving the expression of mStrawberry-pGK-BSD
158680	NFKB-TRE-mStrawberry reporter	This plasmid is a NFKB pathway reporter. It has a 3X-KB-L promoter driving the expression of mStrawberry-pGK-BSD
158681	HIF-TRE-mStrawberry reporter	This plasmid is a HIF1A pathway reporter. It has a 6 x HIF binding element promoter driving the expression of mStrawberry-pGK-BSD
158682	YAP/TAZ-TRE-mStrawberry reporter	This plasmid is a YAP/TAZ pathway reporter. It has a YAP/TAZ-responsive synthetic promoter driving the expression of mStrawberry-pGK-BSD
158683	RAR-TRE-mStrawberry reporter	This plasmid is a RAR pathway reporter. It has a Retinoic Acid Receptor Response Element promoter driving the expression of mStrawberry-pGK-BSD
158684	p53-TRE-mStrawberry reporter	This plasmid is a p53 pathway reporter. It has two copies of wild-type p53 binding sites promoter driving the expression of mStrawberry-pGK-BSD
158685	AKT-translocation-mStrawberry reporter	This plasmid is an AKT pathway reporter. It has 1EF1a promoter driving the expression of truncated FoxO1 fused to mStrawberry-pGK-BSD
158686	ERK-KTR-mStrawberry reporter	This plasmid is a ERK pathway reporter. It has CMV promoter driving the expression of ERK-KTR fused to mStrawberry-pGK-BSD
158687	p38-KTR-mStrawberry reporter	This plasmid is a p38 pathway reporter. It has CMV promoter driving the expression of p38-KTR fused to mStrawberry-pGK-BSD
158688	PKA-KTR-mStrawberry reporter	This plasmid is a PKA pathway reporter. It has CMV promoter driving the expression of PKA-KTR fused to mStrawberry-pGK-BSD

158689	JNK-KTR-mStrawberry reporter	This plasmid is a JNK pathway reporter. It has CMV promoter driving the expression of JNK-KTR fused to mStrawberry-pGK-BSD
158690	Geminin-mStrawberry reporter	This plasmid is a Geminin cell cycle reporter. It has CMV promoter driving the expression of Geminin fused to mStrawberry-pGK-BSD
158691	iRFP-H2A	This plasmid encodes an iRFP fused to H2A histone nuclear marker. It has a CMV promoter driving the expression of iRFP fused to H2A. It has no selection markers

To generate the plasmids for the visual barcode clones we used a CMV-Puromycin-F2A construct on the backbone of pLKO.1 containing a multiple cloning site following the F2A. First, the plasmid was linearized using NheI and MluI right after the F2A sequence. Next, we used Gibson assembly (New England Biolabs, Inc. #E2611) to add the fluorescent protein and localization peptide right after the F2A sequence. All plasmid sequences were verified by Sanger sequencing.

To generate the transcription response element (TRE) type of reporter plasmids as well as the AKT reporter plasmid we first created a promoterless-mStrawberry plasmid (TRE backbone plasmid on backbone of pLKO.1) with a SanDI recognition site before the mStrawberry. First, the plasmid was linearized using SanDI. Next, PCR products containing the promoter region of the plasmids from Sup figure 2a were fused by Gibson assembly into the TRE backbone plasmid to generate the mStrawberry reporter plasmids.

To generate the translocation reporters, we used the KTR reporters created by Regot et al 2014.⁴ Using Gibson assembly, we introduced these reporters to our TRE backbone plasmid by adding CMV promoter driving the expression of the KTRs fused to mStrawberry. GEMININ reporter was constructed in an identical fashion to the KTR reporters.

Generating visual barcode reporter clones:

We constructed our visual barcode signalome reporter clones in three steps: 1) for visual demarcation of the nuclear region we infected the cancer cell-lines with lentiviruses containing an iRFP-H2A plasmid that we generated. We then generated a single cell-derived parent clone (see below); 2) we infected the parent clone with a lenti-virus containing the visual barcode plasmids and selected for positive cells using puromycin; 3) the puromycin positive cells were then infected

with a lentiviruses containing a mStrawberry Signalome reporter and positive cells were selected using blasticidin. Next, we derived single cell clones from the puromycin-blasticidin positive cells and tested the clones for their visual barcode and reporter activity using known activator/inhibitors of the signaling pathway (Sup Figure 2f).

- 5 For generating the lentiviruses, plasmids were transfected into the 293T cells-2nd generation lentivirus system using jetPEI (Polyplus transfection) according to the manufacturer's protocol. 12 hours post transfection the 293T cells media was replaced with a fresh media containing 30% FBS to increase the 293T cells population doubling. 24 hours later, lentivirus containing supernatant was collected from the 293T cells growing plates and filtered through a 0.45μM filter. Next, we
10 removed the target cells (A375, PC9, SK-Mel-5) media and replaced it with the virus containing filtered media for a period of 24 hours. Finally, the virus containing media was washed and the cells were subjected to positive selection as detailed above.

Generation of single-cell derived clones:

- Cells (0.5 cells/well in 150μl) were seeded on a Corning 96-well plate (Cat. Number 3595). After
15 6-8 hours, wells were manually screened for the existence of a single cell in each well. Wells with more than one cell were excluded from further handling. After 2-3 weeks clones were propagated to bigger wells to generate cell lines that were validated for their reporter activity as indicated in sup figure 2f.

Mice experiments using visual barcodes:

- 20 Nine subclones (BFF, CFP and GFP in WC, NES and peroxisome) were mixed in equal proportions and injected (2.5 million cells in total) into the flanks of nude mice (Harlan, Israel). Following four weeks of growth, the mice were sacrificed and the tumors were extracted.

- The extracted tumors were broken down into a single-cell suspension using the cold protease
25 method described by Adam et al., 2017 (PMID: 28851704). In short: Tumors were incubated at 6°C for 7 min in a dissociation buffer containing Bacillus Licheniformis protease (10mg/ml final concentration), PBS and DNaseI(125U/ml). Next, the tumors were transferred to GentleMACS C-tubes (miltenyibiotec) and placed in the gentleMACS Dissociator (brain_03 program,

miltenyibiotec). Following dissociation, the cells were sequentially filtered on 70 and 40 μ m strainers and spun-down at 500G for 5 minutes at 4°C and resuspended in 50 μ L cold PBS.

ImageStream analysis:

- 5 Cells were imaged by an Imaging Flow Cytometer (ImageStreamX Mark II, AMNIS corp. - part of Luminex, TX, USA). Data was acquired using a 60X lens, and lasers used were 405nm (30mW), 488nm (30mW), 561nm (200mW), 642mW (150) and 785nm (5mW). Data was analyzed using the manufacturer's software IDEAS 6.2 (AMNIS corp.). Images were compensated for spectral overlap using single stained controls. Cells were first gated according to their area (in μ m²) and
- 10 aspect ratio (the Minor Axis divided by the Major Axis of the best-fit ellipse) of the iRFP staining. Cells were further gated for focus using the Gradient RMS and contrast features (measures the sharpness quality of an image by detecting large changes of pixel values in the image). Cropped cells were eliminated using the bright-field Area and Centroid X (the number of pixels in the horizontal axis from the upper, left corner of the image to the center of the mask) features. Cells
- 15 were divided to CFP+, BFP+ and GFP+ according to their corresponding intensities. To identify the 3 cell morphologies, two features were calculated for each of the dyes used: Area of the highest intensity pixels using the Threshold mask, and the similarity feature (a measure of the degree to which two images are linearly correlated, calculated as log transformed Pearson's Correlation Coefficient) calculated between each staining and the iRFP signal. Plotting these features on a bi-
- 20 variate plot gave a clear distinction of the 3 morphologies.

Drug libraries:

- Three drug libraries were used in the screen. The first and second libraries contained 75 and 247 drugs respectively (Sup table 1) that were selected from the Selleck chemicals bioactive screening libraries and purchased from the G-INCPM at the Weizmann institute (<https://g-incpm.weizmann.ac.il/units/WohlDrugDiscovery/chemical-libraries>). The third library was a gift
- 25 from Pfizer and contained 175 drugs (Sup table 2). All libraries were screened at a final concentration of 0.5 μ M.

Screening and Imaging:

- For screens, clones were grown in 15 cm culture plates (ThermoScientific, #168381) overnight in
- 30 DMEM supplemented as above. Before seeding, cells were detached by trypsin (Trypsin EDTA

Solution A (0.25%), EDTA (0.02%), 03-050-1B Biological Industries) and resuspended in imaging media (DMEM without phenol red (01-053-1A, Biological Industries), supplemented as above). The number of cells/ml was counted by Vi-cell XR (Beckman Coulter) and all clones were brought to the same cellular concentration. Clones were then mixed in equal proportions and seeded using EL406 washer dispenser (BioTek) at 5,000 or 2500 cells/well onto clear bottom 96 or 384-well plates respectively (Greiner, product #60-655090, Greiner, product #781-091). Cells were seeded in 135µl/well in 96-well plates or in 45 µl/well in 384-well plates. We also seeded each of the clones in a separate well to allow training of the CellProfiler analyst software to identify the different clones (see below). Plates were then cultured for 24 hours at 37°C, 5% CO₂ and 100% humidity. After 24 hours, Cells were imaged with the Operetta CLSTM High Content Imaging system (Perkin Elmer) using 10x high NA objective before treating with 15/5µl of 10X drugs for 96/384-well plates using the CyBi-Well Vario 96/250 Simultaneous Pipettor (CyBio). Next, the cells were imaged in 12-hour intervals for a period of 48 to 72 hours. Temperature (37°C) and CO₂ (3%) were held constant during the imaging. All images were acquired with the same contrast and brightness parameters controlled by the Harmony software (Perkin Elmer). 4-9 fields were acquired from each well in a 384-well plate using a 10X objective in both digital phase-contrast (DPC) as well as in each of the six FP specific wavelengths.

Image analysis and feature extraction workflow:

We first used CellProfiler (Version 2.2.0) to detect nuclei (iRFP), segment cells (DPC), identify tertiary objects (cytoplasm, perinuclear, cell-specific background), and detect and quantify fluorescent proteins of visual barcodes (BFP, CFP, GFP, YFP) and reporters (mStrawberry) in all cellular compartments from all images. The resulting data, termed cytological profiles, consist of more than 200 features that describe the characteristics of each cell such as its size, shape, and the intensity and texture of all FPs expressed. Results of this pipeline were exported both to spreadsheet and sql-lite database.

Barcode deconvolution workflow:

To determine the visual barcode identity of each cell, we used CellProfiler Analyst (Version 2.0) classifier supervised machine learning software. We first trained the software with images from our control wells that were plated with only one subclone type per well (one visual barcode). We made sure that for each clone we trained on at least 200 cells, representing all timepoints in the

experiment. We instructed the CellProfiler to use 50 rules in order to differentiate between the clones. This type of training was done for each of our experiments as we noticed that using the same set of rules between experiments reduces the overall accuracy of barcode calling. Note that a feature could be used more than once in the classifier. Finally, we applied the rules to all cells in the experiment in order to determine the barcode of each cell.

Data analysis:

Data analysis and statistical tests were performed using R (R version 3.6.0) and RStudio (Version 1.2.5033). Briefly, a metadata file containing each well treatment is joined to the experiment raw data (per cell table with cell features and predicted barcode and reporter). Next, for each cell we calculated the mStrawberry (the FP used for our reporters) cytoplasmic to nuclei ratio and the mean intensity of the nuclei after subtracting the cell specific background intensity. Next, the combined data-frame is grouped by time-point, treatment and reporter and Kolmogorov-Smirnov (KS) test is being performed on these two new calculated features. For the translocation reporters the test is being calculated on the treatment's cytoplasm to nucleus ratio density plot and for the transcription reporters the test is being calculated on the nuclei after subtracting the cell specific background. The result is an activity score for each reporter at a given treatment and time-point (the score was not calculated in cases where the group contained less than 30 cells). These activity scores were used to perform Hierarchical clustering (distance = Euclidean, agglomeration method = Ward.D2) on the data using the pheatmap package (version 1.0.12). Plotting the activity score was done using the ggplot2 package (version 3.3.0). KS as well as other statistical tests were performed using the stats package (version 3.6.0).

Reporter activity score:

To measure the effect of treatments we used a modified KS test, comparing the treated population's intensity distributions with respect to the control (Supp Fig 2d). The test, which measures the biggest difference between the two CDFs (Cumulative Distribution Function) allows us to identify almost every significant effect the treatments had on the reporters' activity. However, since the KS statistic is the absolute value of the maximum difference in CDFs, we assigned a sign to the statistic based on the location of both populations' median values. Thus, our score is ranged between -1 for maximum inactivation, as shown for ERK translocation reporter treated with

trametinib (Supp Fig 2d) to 1, maximum activation. In general we noticed a high correlation between the KS score and the effect of drugs on the distribution mean (data not shown).

RAR/RXR pathway activity:

Three cohorts of melanoma patients that contain expression data were downloaded from the TCGA and the gene expression omnibus (GEO, GSE65904, GSE59455). To calculate pathway activity metrics, we used the PathOlogist tool, which uses gene expression levels and prior knowledge about the interactions within a pathway (as explained by Ben-Hamo et al., 2020³⁰).

Cancer Proteome Comparisons

To quantify signaling activities in clinical tumor cell data, we relied on the Cancer Proteome Atlas (<https://tcpaportal.org/tcpa/download.html>) to retrieve quantitative protein expression data collected from 8,167 human tumors that span 32 different types of cancer from the TCGA (<https://www.cancer.gov/tcga>). To compare signalome reporters with protein expression data from the TCGA, we assembled a list of 8 proteins or phospho-proteins that are known to correlate with the activity of pathways included in the signalome (AKTpS473, MAPKpT202Y204, JNKpT183Y185, p38pT180Y182, PKCALPHApS657, NFKBP65pS536, p53, YAP). TCGA protein-level data was correlated to generate the full pairwise correlation matrix for each type of cancer. Cancers with over 30 samples were further analysed for the similarity of their pattern of correlations between protein-levels and the corresponding pattern of correlations observed in the signalome. To do this, the correlation matrices were edited to remove the diagonal elements (as these are trivial). Then, each row of the cancer's correlation matrix was again correlated with the corresponding row of the signalome correlation matrix. This gave a similarity score for each pathway in each cancer. This data was grouped to find how similar the cancers were overall to the signalome in terms of correlations between pathways. Analysis was done with the pandas (version 1.01), numpy (version 1.18.1), matplotlib (version 3.1.3), and seaborn (0.10.0) libraries through the Anaconda Python distribution, and with ggplot (version 3.3.0) through R (version 3.6.2) for visualisation.

Measurements of cell growth:

To measure the average cellular growth rate (protein accumulation rate) in each condition, we used a previously-described method for quantification of total macromolecular protein mass in

individual cells.^{21–23} At intervals during drug treatment, samples were fixed and permeabilized, and cells were reacted with a succinimidyl ester that is covalently bound to a fluorescent dye (SE-A647). SE-A647 covalently binds to fixed proteins to produce a fluorescent signal that is proportional to cell mass as shown in Sup figure 5A and 5B. After fixation and staining with SE-A647 (protein) and DAPI (DNA), widefield fluorescence images were collected. The bulk protein content (total SE-A647 intensity of sample) and number of cells were measured in each sample. From these measurements, we calculated the average growth rate and cell cycle length of cells in each condition, by fitting all data points (from two replicates of each condition) to an exponential growth model. Prior to fixation, throughout the course of drug treatment, proliferation was independently monitored by periodic imaging of live cells via differential phase contrast imaging. These measurements were used to estimate the average cell cycle length in each condition by fitting data to an exponential proliferation model.

Cells were imaged using a Perkin Elmer Operetta high content microscope, controlled by Harmony software, with an incubated chamber kept at 37°C and 5% CO₂ during live-cell imaging. A Xenon lamp was used for fluorescence illumination, and a 740 nm LED light source was used for transmitted light. Differential phase contrast images were collected using a 10 × 0.4 NA objective lens. Widefield fluorescence images were collected with a 20 × 0.75 NA objective lens.

To slow growth rate the following drug treatments were used: cycloheximide (1, 0.6, and 0.06 μM, Sigma C4859), Torin-2 (10, 5, and 2.5 nM, Tocris 4248), rapamycin (7, 0.7, and 0.07 μM, CalBiochem 553211). To slow the cell cycle, the following drug treatments were used: BN82002 (25, 12.5, 6.2, 3.1, 1.6, and 0.78 μM, Calbiochem 217691), SNS-032 (39 and 9.8 nM, Selleckchem S1145), PHA848125 (175 nM, Selleckchem S2751), Cdk2 Inhibitor III (5, 1.5, 0.75, and 0.38 μM, Calbiochem 238803), Dinaciclib (10, 5, and 2.5 nM, Selleckchem S2768).

Metric to quantify PCA pre and post drug treatment:

We developed a metric that quantifies how well measurements of cells that were not exposed to any drug treatment can predict the correlated signaling activities that we observed post drug treatment. As shown in fig 4, correlated activities in untreated cells seem absent when calculated by pairwise correlations, but are identified by PCA with significance (Fig. 5). The reason for this discrepancy is that pairwise correlations fall short of representing multivariate dependencies. By examining the dataset as separate pairs of pathways, piecemeal comparisons of pairwise

correlations reduces the statistical power of any data analysis. On the other hand, performing independent PCA on treated versus untreated also falls short of answering our question. While PCA identified multivariate trends in measurements on both drug-treated and untreated cells, it is hard to say whether such independently identified dependencies are similar.

5 To circumvent this challenge, we quantified the persistence of the correlations with a different approach. In simple terms, we asked how well principle components calculated from measurements on untreated cells can explain trends that emerge post drug treatment. Intuitively, principal component analysis (PCA) is a method that calculates a new coordinate system that is optimally aligned with linear trends within the measured data (Sup Figure 5B). In the present study, any given drug is scored by measuring its influence on 12 branches of signaling (Sup Figure 5A). In Sup Fig 5A, for example, drugs are represented as points on a 2D coordinate system, where the ‘coordinates’ represent the drug’s influence on the measured pathways. In such case, PCA constructs a new (orthogonal) coordinate system (Sup Figure 5B) that is optimally aligned with linear dependencies in the raw data. Further, the new coordinates calculated by PCA are hierarchically ordered such that the first coordinate (the first principle component) is aligned in a direction that captures the highest variance in the dataset and so on.

To test whether measurements on untreated cells can predict the drug-induced multivariate correlations, we performed a PCA from measurements on cells that were not exposed to drug treatments. This resulted in a coordinate system that we call Ψ_0 , that has twelve axes in the directions $\Psi_{0,n}$. Since Ψ_0 results from performing PCA on untreated cells, these axes (coordinates) are trivially aligned with correlated signaling pre-drug treatment. What is not clear is whether Ψ_0 would also align with the correlations that are promoted by drugs. To test this, we compared the product of the variances of the original measurements to the product of the variance after measurements are projected onto Ψ_0 .

$$\phi = \frac{\prod_{n=1}^{12} \sigma_{\Psi_{0,n}}^2}{\prod_{p=1}^{12} \sigma_p^2} \quad \text{Eq. 1}$$

As is illustrated by supplementary Fig 5, the product in the variances describes a region that encloses the data points (in a given coordinate system). When two pathways (here we use the example of p38 and ERK) have activity that's correlated, the region enclosing the data will be smaller in a coordinate system that is aligned with those correlations. A useful aspect of ϕ is that it is naturally normalized to the range of (0,1). To begin, if measured activities are not correlated, the volume that encloses the data should not change no matter what coordinate system it is projected onto and the value of ϕ will be near one in all coordinates. By contrast, if we imagine perfectly correlated data, ϕ would tend to zero (e.g. a line has no volume).

In our work, this was used to see whether the linear combinations of pathway activity seen in unperturbed cells reflect correlations in drug-treated cells better than the pathway activities themselves: low values of ϕ indicate that these combined signaling groups do not reflect the coordination of signaling changes in response to drugs, while higher values indicate that they do.

Qualify drugs conformity to p38- or p53- signaling state:

To score the extent to which a given compound promotes the p38-signaling vs p53-signaling states, we used Eq. 2.

$$\psi = \frac{PC_1}{\sqrt{\sum_{i=1}^{12} PC_i^2}} \quad \text{Eq. 2}$$

Here, the value PC_i is the numerical value of the drug's effect in the i th axis of the principal coordinate system Ψ_0 . Intuitively, the extent by which a drug conforms with the p38- and p53-signaling clusters is represented by the extent to which its influence on the 12 pathways is aligned with the first principal component: the magnitude of which is PC_1 . Eq. 2 is the ratio of a drug's influence on PC_1 as compared to the size of the effect vector. This quantifies how closely aligned the drug's effect is with p38-p53 signaling.

Sup table 1

	Drug	Targets
1	2-Methoxyestradiol	HIF
2	ABT-199 (GDC-0199)	BCL2
3	ABT-263 (Navitoclax)	BCL2
4	Afatinib (BIBW2992)	EGFR;HER2
5	Alpelisib (BYL719)	PI3K
6	AT7867	S6 Kinase,Akt
7	Aurora A Inhibitor I	Aurora Kinase
8	AZ 3146	Kinesin
9	AZ 960	JAK
10	AZD5363	Akt
11	AZD6244 (Selumetinib)	MEK
12	AZD8055	mTOR
13	Bexarotene	RXRA;RXRB;RARβ;RARA
14	BIRB 796 (Doramapimod)	p38 MAPK
15	BIX 02188	MEK5
16	BKM120 (NVP-BKM120)	PI3K
17	BX-795	IκB/IKK;PDK-1
18	CHIR-99021 (CT99021) HCl	GSK3A;GSK3B;CD33
19	Chrysophanic acid (Chrysophanol)	GSK-3
20	CI-1040 (PD184352)	MAP3K1;RAF1;MAP2K1
21	Dasatinib (BMS-354825)	LCK;EPHA2;LYN;ABL1;FYN;SRC;DDR2;DDR1;YES1;BMX
22	Dovitinib Dilactic acid (TKI258 Dilactic aci	PDGFR;FGFR;c-Kit;FLT3;VEGFR
23	Enzastaurin (LY317615)	PKC
24	Everolimus (RAD001)	mTOR
25	Fasudil HCl (HA-1077)	ROCK
26	Forskolin	cAMP
27	GM6001	MMP
28	GSK1120212 (Trametinib)	MEK
29	GSK1904529A	IGF-1R
30	GSK2126458	PI3K;mTOR
31	GSK429286A	ROCK
32	H 89 2HCl	PKA
33	IKK-16	IκB/IKK
34	IMD0354	IκB;IKK
35	Iniparib (BSI-201)	PARP
36	INK 128 (MLN0128)	mTOR
37	Ipatasertib (GDC-0068)	PAN Akt
38	Ivacaftor (VX-770)	CFTR
39	JNJ 26854165 (Serdemetan)	p53
40	JNJ-7706621	CDK2;TYK2;AURKB;CDK1;JAK3;JAK1;AURKA;CDK5;JAK2
41	JNK inhibitor IX	JNK
42	JNK-IN-8	JNK
43	Losmapimod	p38 MAPK
44	LY2835219	CDK
45	NU7441(KU-57788)	DNA-PK;PI3K
46	Nutlin-3	p53
47	OSU-03012 (AR-12)	PDK-1
48	PF-00562271	FAK
49	PF-04691502	Akt,mTOR;PI3K
50	PF-562271	PTK2;NTRK2;PTK2B;INSR;CDK2;JAK2;ROS1;MUSK;TYK2;NTRK1;JK3;JAK1
51	PHA-793887	CDK

52	PLX-4720	Raf
53	Ponatinib (AP24534)	KIT;FLT3;LCK;ABL1;FGFR1;FGFR2;KDR;SRC;MAP4K1;LRRK2
54	PP-121	DNA-PK;PDGFR;mTOR
55	Pralatrexate(Folotyn)	DHFR
56	Roflumilast (Daxas)	PDE4D;PDE4B;PDE4A;TNF;PDE4C;IFNG;IL2;IL5;MC4R;IL10
57	Roxadustat (FG-4592)	HIF
58	S-Ruxolitinib	JAK
59	SC75741	NFkB
60	SGI-1776 free base	Pim
61	SP600125	JNK
62	Sunitinib Malate (Sutent)	VEGFR;PDGFR;c-Kit
63	TAK 715	p38 MAPK
64	TAK-733	MEK
65	Tandutinib (MLN518)	FLT3
66	TCS 359	FLT3
67	Thiazovivin	ROCK
68	TPCA-1	IkB/IKK
69	Triciribine (Triciribine phosphate)	Akt
70	Vemurafenib (PLX4032)	Raf
71	VX-680 (MK-0457, Tozasertib)	Aurora-KinaseA;B;C;c-ABL;PLK;NTRK;FLT3;HTR2C
72	VX-702	p38 MAPK
73	Wnt-C59 (C59)	WNT/b-catenin
74	WZ3146	EGFR
75	XAV-939	Wnt/beta-catenin

Supp table 2

	Drug name/PubChemID	Target
1	KU-55933	ATM inhibitor
2	Dabrafenib (GSK2118436)	BRAFV600
3	PLX-4720	BRAFV600
4	Vemurafenib (PLX4032)	BRAFV600
5	BML-190	cannabinoid CB2 receptor inverse agonist
6	Torcetrapib (CP-529414)	CETP inhibitor
7	Pramipexole (Mirapex)	D4 receptor agonist
8	WZ3146	EGFR(L858R) and EGFR(E746_A750)
9	Apixaban	Factor Xa
10	Rivaroxaban (Xarelto)	Factor Xa
11	Dapagliflozin	hSGLT2 inhibitor
12	MK-8245	inhibitor of stearyl-CoA desaturase
13	Costunolide	inhibits FPTase
14	PF-5274857	inhibits Hedgehog signaling
15	Pomalidomide	inhibits LPS-induced TNF- α release
16	S-Ruxolitinib	JAK1/2
17	Axitinib	multitargeted RTK inhibitor
18	Cabozantinib malate	multitargeted RTK inhibitor
19	Dovitinib Dilactate acid (TKI258 Dilactate aci	multitargeted RTK inhibitor
20	Pazopanib HCl	multitargeted RTK inhibitor
21	Tandutinib (MLN518)	multitargeted RTK inhibitor
22	VX-702	p38 α MAPK
23	Sotrastaurin (AEB071)	pan-PKC inhibitor
24	AZD2461	PARP inhibitor
25	TAK-700 (Orteronel)	17,20-lyase inhibitor
26	Dutasteride	5- α Reductase inhibitor
27	Finasteride	5- α Reductase inhibitor
28	Sertraline HCl	5-HT antagonist
29	SPIPERONE	5-HT inhibitor
30	Agomelatine	5-HT Receptor antagonist
31	OLANZAPINE	5-HT2 serotonin and D2 dopamine receptor antagonist.
32	DASATINIB	ABL, SRC
33	PNU-120596	AChR
34	Tropicamide	AChR
35	Tropium chloride (Sanctura)	AChR
36	Istradefylline (KW-6002)	adenosine A2A receptor (A2AR) antagonist
37	GW9508	agonist for FFA1
38	WAY 200070	AGONIST OF ESR2
39	PROGESTERONE	agonist of nuclear progesterone receptor
40	6603901	agonist of PPARD
41	TANAPROGET	agonist of the progesterone receptor
42	AT7867	AKT, S6K
43	A-674563	AKT1
44	Honokiol	AKT1
45	135565159	AKT1,PI3K
46	CCT128930	Akt2
47	CERITINIB	ALK inhibitor
48	SB 431542	ALK5
49	CLOTIRMAZOLE	alters the permeability of the fungal cell wall by inhibiting the biosynthesis of ergosterol
50	Aniracetam	AMPA Receptor-kainate modulator
51	BICALUTAMIDE	androgen receptor (AR) antagonist
52	PF-998425	androgen receptor antagonist
53	Losartan potassium	angiotensin II receptor antagonist
54	Captopril (Capoten)	angiotensin-converting enzyme (ACE) inhibitor
55	Levosulpiride (Levogastrol)	antagonist for D2 dopamine receptors
56	SB705498	antagonist for hTRPV1
57	SB-408124	antagonist for OX1 receptor
58	GW9662	antagonist for PPAR
59	(+)-Bicuculline	antagonist of GABAA receptors
60	PF-4455242	antagonist of the μ -opioid receptor
61	Pralatrexate(Folotylin)	Antifolate
62	53311136	Antimalarial activity
63	MDV3100 (Enzalutamide)	AR antagonist
64	Letrozole	Aromatase
65	Exemestane	aromatase inhibitor
66	Oligomycin A	ATP synthase
67	AZ20	ATR inhibitor
68	Aurora A Inhibitor I	Aurora A
69	MLN8237 (Alisertib)	Aurora A
70	AT-9283	Aurora A/B and JAK2/3 inhibitor

71	Barasertib (AZD1152-HQPA)	Aurora B
72	AT101	Bcl-2, Bcl-xL and Mcl-1
73	DCC-2036 (Rebastinib)	Bcr-Abl inhibitor
74	NILOTINIB	Bcr-Abl inhibitor
75	Carvedilol	beta blocker/alpha-1 blocker
76	btp2	blocker of store-operated Ca ²⁺ entry
77	TRIMETREXATE	blocks epithelial Na channel
78	DABRAFENIB	BRAFV600E
79	VEMURAFENIB	BRAFV600E
80	JQ1	BRD2,BRD3,BRD4
81	PFI-1(PF-6405761)	BRD4 inhibitor
82	PCI-32765 (Ibrutinib)	BTK
83	AMG458	c-Met
84	SGX-523	c-Met
85	MGCD-265	c-Met and VEGFR1/2/3
86	7299199	Calcium Channel
87	Amlodipine (Norvasc)	calcium channel blocker
88	Tioxolone	carbonic anhydrase I inhibitor
89	Brinzolamide	Carbonic anhydrase II inhibitor
90	Rebamipide	CCK1 receptor inhibitor
91	449087	CCNA1,CCNA2
92	449087_1	CCNA1,CCNA2
93	11149391	CDC7
94	BMS-265246	CDK1/2
95	Flavopiridol hydrochloride	CDK1/2/4/6
96	5329663	CDK2
97	PNU-292137	CDK2
98	5330949	CDK2,CCNA1,CDK5,CDK1
99	PHA-793887	CDK2/5/7
100	abemaciclib	CDK4/6
101	BS-181 HCl	CDK7
102	Anacetrapib (MK-0859)	CETP
103	Dalcetrapib (JTT-705)	CETP
104	Ivacaftor (VX-770)	CFTR
105	VX-809	CFTR
106	16065975	CFTR,TRPA1
107	11844347	CHEK1,WEE1
108	11844610	CHEK1,WEE1
109	LY2603618 (IC-83)	Chk1
110	PF 477736	Chk1 inhibitor
111	Entacapone	COMT inhibitor
112	HYDROCORTISONE	corticosteroid with anti-inflammatory and immunosuppressive properties
113	Tolfenamic acid	COX-2 inhibitor
114	Linifanib (ABT-869)	CSF-1R;PDGFR;VEGFR
115	709208	CSNK2A2
116	2-Methoxyestradiol	depolymerizes microtubules and blocks HIF-1_ nuclear accumulation
117	WP1130	deubiquitinase inhibitor
118	Pyrimethamine	DHFR inhibitor
119	PENFLURIDOL	diphenylbutylpiperidine antipsychotic
120	Triciribine (Triciribine phosphate)	DNA synthesis inhibitor, AKT
121	pp-121	DNA-PK,PDGFR,mTOR,SRC
122	PP-121	DNA-PK,PDGFR,mTOR,SRC
123	NU7441(KU-57788)	DNA-PK,PI3K
124	Rotundine	dopamine D1 receptor antagonist
125	Linagliptin (BI-1356)	DPP-4 inhibitor
126	PHA-767491	dual Cdc7/CDK9 inhibitor
127	Ku-0063794	dual-mTOR inhibitor
128	Erlotinib(OSI-744)	EGFR
129	Gefitinib (Iressa)	EGFR
130	PD168393	EGFR
131	PELITINIB	EGFR
132	WZ4002	EGFR(L858R)/(T790M)
133	Afatinib (BIBW2992)	EGFR/ErbB
134	WZ8040	EGFR790M inhibitor,
135	Bosentan Hydrate	Endothelin Receptor antagonist
136	Sitaxentan sodium (TBC-11251)	endothelin receptor-A antagonist
137	Fulvestrant (Faslodex)	ER antagonist
138	16065674	ERBB2
139	PROPYLPYRAZOLETRIOL	ESR1
140	10215241	ESR2
141	135494488	ESR2
142	10236407	ESR2, ESR1

143	10308176	ESR2, ESR1
144	10308197	ESR2, ESR1
145	135454802	ESR2, ESR1
146	10125290	ESR2,ESR1
147	11369558	ESR2,ESR1
148	76302	ESR2,ESR1
149	76302-1	ESR2,ESR1
150	ESTRADIOL	ESR2,ESR1
151	Toremifene Citrate (Fareston, Acapodene)	estrogen receptor modulator (SERM)
152	Tazemetostat	EZH2
153	PF-3845	FAAH
154	JNJ 1661010	FAAH inhibitor
155	PF-00562271	FAK
156	5328999	FGFR1
157	PD-166866	FGFR1
158	PD173074	FGFR1
159	AZD4547	FGFR1/2/3
160	FENOFIBRIC ACID	fibrate that acts as a lipid-lowering agent, decreasing low-density lipoprotein cholesterol and triglycerides.
161	TCS 359	FLT3
162	TUROFEXORATE ISOPROPYL	FXR agonist
163	Etomidate	GABAA receptor agonist
164	Semagacestat (LY450139)	Gamma-secretase blocker
165	YO-01027	Gamma-secretase inhibitor
166	24768019	GCK
167	10407399	GLI3
168	BECLOMETHASONE DIPROPIONATE	glucocorticoid steroid
169	BUDESONIDE	glucocorticoid steroid
170	DEXAMETHASONE	glucocorticoid, agonist of the glucocorticoid receptor (GR)
171	GW 4064	agonist of farnesoid X receptor
172	CHIR-99021	GSK-3
173	SB 216763	GSK-3
174	SB 415286	GSK-3
175	AR-A014418	GSK3
176	GSK J4 HCl	H3K27 histone demethylase
177	Belinostat (PXD101)	HDAC inhibitor
178	Mubritinib (TAK 165)	HER2
179	AEE788 (NVP-AEE788)	HER2,EGFR
180	BMS-599626 (AC480)	HER2,EGFR
181	Lapatinib Ditosylate (Tykerb)	HER2,EGFR
182	IOX2	HIF activator
183	5800609	HIF1A inhibitor
184	GENISTEIN	highly specific inhibitor of protein tyrosine kinase
185	Clemastine Fumarate	histamine H1 receptor antagonist
186	Lidocaine (Alphacaine)	histamine H1-receptor agonist
187	Ranitidine (Zantac)	histamine H2-receptor antagonist,
188	CIPRALISANT	histamine H3 antagonist
189	NELFINAVIR	HIV protease inhibitor
190	Fluvastatin sodium (Lescol)	HMG-CoA reductase
191	4284720	HTR2B
192	GSK1838705A	IGF-1R
193	GSK1904529A	IGF-1R
194	Linsitinib (OSI-906)	IGF-1R
195	NVP-ADW742	IGF-1R
196	PQ 401	IGF-1R
197	NVP-ADW742	IGF-1R inhibitor
198	TPCA-1_1	IKK-2
199	TPCA-1	IKK-2
200	118870	IL4R
201	PTC124 (Ataluren)	induces ribosomal read-through
202	Voriconazole	inhibiting fungal cytochrome P-450
203	HARMINE	Inhibition of the Wnt signaling pathway. It also selectively binds to MAO-A
204	Rotenone	inhibitor of mitochondrial electron transport
205	ML-161	inhibitor of PAR1
206	3-Deazaneplanocin	inhibitor of S-adenosylhomocysteine hydrolase
207	VANDETANIB	Inhibitor of VEGFR2/3 and EGFR
208	Vildagliptin (LAF-237)	inhibits DPP_4
209	Aminosalicilate Sodium	inhibits NF- κ B pathway
210	IKK-16	inhibits NF- κ B pathway,
211	IMD0354	inhibits NF- κ B pathway,
212	Parthenolide ((-)-Parthenolide)	inhibits NF- κ B pathway, activates p53
213	BMS-806 (BMS 378806)	inhibits the binding of HIV-1 gp120 to the CD4 receptor
214	52913874	IRAK4

215	BMS-509744	ITK
216	25190534	ITK,INSR
217	RUXOLITINIB	JAK
218	SOLCITINIB	JAK1
219	AZ 960	JAK2
220	AZD-1480	JAK2 inhibitor
221	BMS-911543	JAK2 inhibitor
222	12083227	JAK3
223	Tasocitinib	JAK3
224	Tofacitinib (CP-690550, Tasocitinib)	JAK3
225	JNK-IN-8	JNK1/2/3
226	17860295	JUN
227	6326407	KDR,FLT1
228	SB 743921	kinesin spindle protein
229	ORLISTAT	lipase inhibitor
230	DIBUCAINE	local anesthetics. reversibly binds to and inactivates sodium channels in the neuronal cell membrane
231	Ki16198	LPA antagonist
232	77068229	LRRK2
233	PF-06447475	LRRK2
234	T091317	LXR agonist
235	GW3965 HCl	LXR agonist
236	Moclobemide	MAO-A (5-HT) inhibitor
237	Rasagiline mesylate	MAO-B inhibitor
238	984148	MAPT
239	44433047	MBTPS1
240	10162188	MEK1/2
241	AZD6244 (Selumetinib)	MEK1/2
242	AZD8330	MEK1/2
243	CI-1040	MEK1/2
244	GSK1120212 (Trametinib)	MEK1/2
245	PD0325901	MEK1/2
246	TAK-733	MEK1/2
247	TRAMETINIB	MEK1/2 inhibitor
248	BIX 02188	MEK5
249	70677396	MET
250	VU 0364439	mGlu4 positive allosteric modulator
251	AZ 3146	MPS1
252	SB-747651A	MSK1
253	AZD8055	mTOR
254	Deforolimus (MK-8669)	mTOR
255	Everolimus (RAD001)	mTOR
256	INK 128 (MLN0128)	mTOR
257	PP242	mTOR
258	Rapamycin (Sirolimus)	mTOR
259	PONATINIB	multi-target inhibitor of Abl, PDGFR, VEGFR2, FGFR1 and Src
260	IMATINIB	multi-target inhibitor of tyrosine kinase with inhibition for v-Abl, c-Kit and PDGFR
261	BIBF1120 (Vargatef)	multitargeted RTK inhibitor
262	Pazopanib	multitargeted RTK inhibitor
263	Sunitinib Malate (Sutent)	multitargeted RTK inhibitor
264	SC75741	NF-kB inhibitor
265	Pancuronium (Pavulon)	nicotinic acetylcholine receptor antagonist
266	Memantine hydrochloride (Namenda)	NMDA receptor
267	(-)-MK 801 Maleate	NMDA receptor antagonist
268	IBUDILAST	non selective PDE
269	LG-190178	nonsecosteroidal agonist of the vitamin D receptor
270	44438485	Norepinephrine transporter b
271	44137303	NR1H2,NR1H3
272	44136213	NR1H3, NR1H2
273	44136417	NR1H3, NR1H2
274	44136902	NR1H3, NR1H2
275	9600409	NR1I3
276	334509	NR4A2
277	10126288	p38
278	VX-745	p38
279	SB 203580	p38 MAPK
280	SB 202190	p38 MAPK i
281	Losmapimod	p38 MAPK inhibitor
282	TAK 715	p38
283	JNJ 26854165 (Serdemetan)	p53 activation
284	Nutlin-3	p53 activation
285	YH239-EE	p53 activation
286	Pifithrin-A	p53i

287	ZM-447439	pan Aurora kinase
288	AZD5363	pan-Akt inhibitor
289	GSK690693	pan-Akt inhibitor
290	Ipatasertib (GDC-0068)	pan-Akt inhibitor
291	MK-2206 dihydrochloride	pan-Akt inhibitor
292	PF-03814735	pan-Aurora inhibitor
293	TOZASERTIB	pan-Aurora inhibitor
294	JNJ-7706621	pan-CDK inhibitor with the highest potency on CDK1/2
295	Dacomitinib (PF299804, PF-00299804)	pan-ErbB inhibitor
296	BIRB 796 (Doramapimod)	pan-p38 MAPK
297	Go 6983	pan-PKC inhibitor
298	AG14361	PARP1
299	Iniparib (BSI-201)	PARP1
300	ABT-888 (Veliparib)	PARP1/2
301	DIPYRIDAMOLE	PDE
302	TREQUINSIN	PDE
303	Tadalafil (Cialis)	PDE-5
304	135418212	PDE1, PDE9
305	ZAPRINAST	PDE10, PDE 11 also inhibits PDE5/6
306	TP-10	PDE10A
307	20146322	PDE1B,PDE1C
308	PF 05180999	PDE2A
309	PF-05085727	PDE2A
310	Pimobendan (Vetmedin)	PDE3
311	MILRINONE	PDE3 inhibitor
312	Cilomilast (SB-207499)	PDE4
313	ROFLUMILAST	PDE4
314	Rolipram	PDE4
315	APREMILAST	PDE4 inhibitor
316	15951010	PDE4B
317	PF-03049423 free base	PDE5A
318	6918799	PDE7A
319	70697189	PDE8B
320	5328649	PDGFR
321	BX-795	PK-1
322	BX-912	PK-1
323	GSK2334470	PK-1
324	OSU-03012 (AR-12)	PK-1
325	Varespladib (LY315920)	Phospholipase (e.g. PLA)
326	3-Methyladenine	PI3K
327	A66	PI3K
328	AS-252424	PI3K
329	BKM120 (NVP-BKM120)	PI3K
330	BUPARLISIB	PI3K
331	CAL-101 (GS-1101)	PI3K
332	GDC-0941	PI3K
333	IPI-145 (INK1197)	PI3K
334	PI-103	PI3K
335	TASELISIB	PI3K
336	TG 100713	PI3K
337	TG100-115	PI3K
338	TGX-221	PI3K
339	Wortmannin	PI3K
340	ZSTK474	PI3K
341	Alpelisib (BYL719)	PI3K_
342	PIK-75	PI3K,DNA-PK
343	58379551	PI3K,mTOR
344	GSK2126458	PI3K,mTOR
345	PF-04691502	PI3K,mTOR
346	AS-605240	PIK3CG
347	SGI-1776 free base	PIM1
348	H 89 2HCl	PKA
349	Enzastaurin (LY317615)	PKC
350	10290302	PLA2G4A - phospholipase A2
351	BI6727 (Volasertib)	PLK1
352	GSK461364	PLK1
353	BI-2536	Plk1 inhibitor
354	Gliclazide (Diamicon)	potassium currents blocker
355	GO-6976	potent PKC inhibitor
356	T0070907	PPAR
357	GW501516	PPARD
358	777903	PRKCE

359	49746	PTGS2
360	71454272	PTK2B
361	71461467	PYK2
362	2745738	RAB9A, NPC1
363	Sorafenib (Nexavar)	Raf-1, B-Raf and VEGFR-2
364	Isotretinoin	RAR activator
365	AM580	RAR agonist
366	TRETINOIN	RAR agonist
367	PF-562271	reversible inhibitor of FAK
368	Fasudil HCl (HA-1077)	ROCK
369	GSK-269962A	ROCK
370	Thiazovivin	ROCK
371	GSK429286A	ROCK1
372	GSK429286A-1	ROCK1
373	BEXAROTENE	RXR agonist
374	Oxymetazoline hydrochloride	selective __ adrenergic receptor agonist
375	Capeserod	selective 5-HT4 receptor partial agonist
376	CELECOXIB	selective COX-2 inhibitor
377	RALOXIFENE	selective estrogen receptor modulator
378	DEFACTINIB	selective FAK inhibitor
379	ESREBOXETINE	selective norepinephrine reuptake inhibitor
380	CP-724714	selective of HER2/ErbB2 inhibitor
381	S-119	SEROTONIN
382	Canagliflozin	SGLT2
383	44414722	SLC6A4,SLC6A2
384	Oxcarbazepine	Sodium Channel
385	MEXILETINE	Sodium channel inhibigtor
386	TIPIFARNIB	specific farnesyltransferase (FTase) inhibitor
387	AG 18 (Tyrphostin 23)	Src family kinase inhibitor
388	Roxadustat (FG-4592)	stabilizes HIF-2 and induces EPO production
389	10003041	STAT6
390	Quercetin (Sophoretin)	stimulator of SIRT1, PI3K inhibitor
391	Bazedoxifene HCl	estrogen receptor modulator (SERM)
392	Piceatannol	Syk
393	R406(free base)	Syk
394	R788 (Fostamatinib)	Syk
395	20899393	TDP1
396	LY-364947	TGFBR
397	70683591	TGFBR1
398	10473992	TGFBR2
399	10390904	THRB
400	PF-00277343	THRB
401	10027822	THRB,THRA
402	Ozagrel	thromboxane A(2)
403	Ozagrel HCl	thromboxane A(2)
404	LIOTHYRONINE	thyroid hormone receptor
405	SOBETIROME	thyroid hormone receptor isoform beta-1
406	Cepharanthine	TNFi
407	16049257	TNK2
408	Daunorubicin HCl	Topoisomerase
409	HC-030031	TRPA1 channel blocker
410	AMG-517	TRPV1 antagonist
411	GSK-319347A	TRPV4
412	PAPAVERINE	Tryptophan hydroxylase
413	10474090	TSPO
414	Asiatic acid	used in wound healing
415	DMXAA (ASA404)	vascular disrupting agents
416	DILAZEP	vasodilator that acts as an adenosine reuptake inhibitor
417	Tolvaptan (OPC-41061)	vasopressin V2 receptor antagonist
418	OSI-632	VEGFR angiogenesis inhibitor.
419	SAR131675	VEGFR3
420	10450912	WEE1
421	Wnt-C59 (C59)	WNT/b-catenin inhibitor
422	Embelin	XIAP

Figures

Figure 1

Developing visual barcodes for multiplexing live imaging applications. A. Representative image of the A375 cell line with an iRFP-H2A nuclear marker. B. Representative images of five CFP localizations in the A375 cell line: Whole Cell (WC), Nuclear export signal (NES), Nuclei, Peroxisome (Peroxi), and

endoplasmic reticulum (ER). C. Heatmap of false detection rate (FDR) for barcode calling for the five CFP localizations in the A375 cell line. D. Representative images of all 12 visual barcodes used in A375 cell line E. Average miss rate of barcode calling for all 12 visual barcodes of the A375 cell line, treated with DMSO controls (n = 39). Numbers in the diagonal represent the sensitivity for each barcode. F. Violin plots showing miss rate for all 12 visual barcodes of the A375 cell line, treated with DMSO controls (n = 39) or with drugs (n=75). G-I. Scatter plot showing the separation of nine A375 clones with visual barcodes by the ImageStream system according to their fluorescent color and localization. The separation by localization is only demonstrated for GFP positive clones (I). J. Representative images from ImageStream of all nine clones. Two cells from each clone are presented. Scale bar in A and B is 50 μ m, in D 100 μ m and in J 7 μ m.

Figure 2

Generating the A375 'Signalome' reporter cell line. A. Illustration of the 12 clones that were used to generate the A375 Signalome cell line. A375 cells were first infected with iRFP-H2A to mark the cell nucleus. Then, 12 clones were generated with 12 visual barcodes. Lastly, a different live reporter was added to each of the clones. Transcription activating reporters are represented by gold while translocation reporters are represented in red. Binding partners in the nucleus are represented in purple. B. Relative

the different A375 Signalome clones, after 48 hours of treatment with 122 active drugs. D. A model proposing how drugs with different mechanisms may converge into two major signaling states. While each of the drugs has different targets, many of the targets affect the same sensing mechanism that later governs the p53 vs p38 signaling states.

Figure 4

Drug treatments increase the correlations between the activity of pathways A. Scatter plots of the activity scores of p38 and p53 reporters in the A375 signalome cell line before and at multiple time points after treatment with 422 drugs. Each dot represents a different drug. Pearson's r is depicted for each of the time points B. Pearson correlation coefficients were calculated for each pair of pathways in the A375 signalome cell line before and at different time points after treatment with 422 drugs. Each datapoint represents a correlation value for one given pair of pathways over all 422 drugs. The correlation between the activity score of p38 and p53 pathways is marked by a red circle C-H. locally weighted smoothing (Lowess) regression of all reporters in each of the two signaling states is shown for 6 representative drug treatments, each associated with different drug targets. While three of the drugs drive the p53 signaling state (C-E), the other three drugs drive the p38 signaling state (F-H). Gray sleeves represent the confidence interval for each of the signaling states.

Figure 5

PCA suggests that the p38 and p53 signaling states exist pretreatment and increase in weight over time. A. PCA of the activity scores of 11 signaling pathways after 48 hours of treatment with 122 drugs. The color of each drug is indicating its cluster in figure 3A. B, C. bar plots representing the PC1 loading of each pathway after 48 hours of drug treatment (B) or pre-treatment (C). D. Variance of pathway activity, when projected on the principle components calculated from measurements on cells that were not exposed to drug treatment.

Figure 6

Cell growth and proliferation are tightly regulated and correlate with p38 and p53 signaling states. A. A model demonstrating how sensing of cell size can affect both cell growth and proliferation to keep homeostasis of cell size. B. Scatter plot showing the initial and long-term effects of rapamycin or SNS-032 on the average cellular growth rate and division rate of Rpe1 cells. Data points indicate the average growth and division rates measured: (1) during the first 24 hours of drug treatment and (2) during 24-60

hours of drug treatment. C. Average growth rate vs. division rate in five cell lines (Rpe1, HeLa, U2OS, SAOS2, 16HBE) treated with either growth inhibitors (red) or cdk1/2 inhibitors (blue). Measurements in each cell line were normalized by the values measured for untreated control samples (gray) of the same cell line. The growth inhibitors used were cycloheximide, Torin-2, and rapamycin, at varying doses (detailed in methods section). The cdk1/2 inhibitors used were SNS-032, PHA848125, Cdk2 Inhibitor III, and Dinaciclib, at varying doses (detailed in methods section). D. The average cell size in a given condition is negatively correlated with its PC1 value. E. Average growth rate vs. division rate for A375 cells in Signalome screen. Each circle represents one screened condition (drug treatment). The circle's color indicates the value of PC1 in that condition. Contour lines show the average value of PC1 as a function of growth rate and division rate. F. The average level of p38 (top) and p53 (bottom) activity as a function of growth rate and cell cycle length.