Visual barcodes for multiplexing live microscopy-based assays

Authors: Tom Kaufman¹, Erez Nitzan¹, Nir Firestein¹, Miriam Bracha Ginzberg², Seshu Iyengar³, Nish Patel², Rotem Ben-Hamo¹, Ziv Porat⁵, Andreas Hilfinger³, Ran Kafri²,⁴,*, Ravid Straussman¹,*

Affiliations:
¹Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel
²Programme in Cell Biology, The Hospital for Sick Children, Toronto, Canada
³Department of Chemical and Physical Sciences, University of Toronto, Toronto, Canada
⁴Department of Molecular Genetics, University of Toronto, Toronto, Canada
⁵Flow Cytometry Unit, Life Sciences Core Facilities, Weizmann Institute of Science, Rehovot, Israel

* Corresponding authors: ravidst@weizmann.ac.il, ran.kafri@sickkids.ca
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. Unlike endpoint assays, which necessitate ending of the experiment in order to measure a phenotype, these reporters helped 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 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'.

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. 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, cancer cells require precisely tuned rates of biosynthesis. 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. 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 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.

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:**

**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 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 CellProfiler 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 Analyst 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 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.

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).\textsuperscript{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 PKA\textsuperscript{13}, NFkB\textsuperscript{14}, HIF\textsuperscript{15}, and YAP/TAZ\textsuperscript{16} 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 ($P$-value $< 6x10^{-5}$). 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 PathOlogist\textsuperscript{17}, 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 < 2.2x10^{-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. If cancer cells fail to match the proliferative demands of an oncogene with equivalent increases in biosynthesis, cell size will decrease over time. 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-032 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).

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 = 1.68x10^{-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.\textsuperscript{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. 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\textsuperscript{23,27} that show that p38 MAPK is selectively activated in cells that are smaller than their target size. These 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 programs which promote growth and suppress apoptosis.\textsuperscript{28} By contrast, the activation of p53 is both pro-apoptotic and functions to suppress mTORC1-mediated biosynthesis.\textsuperscript{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 research of a very wide variety of biological fields.

References:

1. Vogelstein, B. \textit{et al.} Cancer Genome Landscapes. \textit{Science} \textbf{339}, 1546–1558 (2013).
2. Sanchez-Vega, F. \textit{et al.} Oncogenic Signaling Pathways in The Cancer Genome Atlas. \textit{Cell} \textbf{173}, 321-337.e10 (2018).
3. Zhou, W. \textit{et al.} Assessment of hypoxia inducible factor levels in cancer cell lines upon hypoxic induction using a novel reporter construct. \textit{PloS One} \textbf{6}, e27460 (2011).
4. Regot, S., Hughey, J. J., Bajar, B. T., Carrasco, S. & Covert, M. W. High-sensitivity measurements of multiple kinase activities in live single cells. \textit{Cell} \textbf{157}, 1724–1734 (2014).
5. DeBerardinis, R. J., Sayed, N., Ditsworth, D. & Thompson, C. B. Brick by brick: metabolism and tumor cell growth. \textit{Curr. Opin. Genet. Dev.} \textbf{18}, 54–61 (2008).
6. Demidenko, Z. N. & Blagosklonny, M. V. Growth stimulation leads to cellular senescence when the cell cycle is blocked. \textit{Cell Cycle Georget. Tex} \textbf{7}, 3355–3361 (2008).
7. Neurohr, G. E. \textit{et al.} Excessive Cell Growth Causes Cytoplasm Dilution And Contributes to Senescence. \textit{Cell} \textbf{176}, 1083-1097.e18 (2019).
8. Wright, J., Dungrawala, H., Bright, R. K. & Schneider, B. L. A growing role for hypertrophy in senescence. \textit{FEMS Yeast Res.} \textbf{13}, 2–6 (2013).
9. McQuin, C. et al. CellProfiler 3.0: Next-generation image processing for biology. *PLOS Biol.* **16**, e2005970 (2018).

10. Jones, T. R. et al. CellProfiler Analyst: data exploration and analysis software for complex image-based screens. *BMC Bioinformatics* **9**, 482 (2008).

11. Caicedo, J. C. et al. Data-analysis strategies for image-based cell profiling. *Nat. Methods* **14**, 849–863 (2017).

12. Kang, J. et al. Improving drug discovery with high-content phenotypic screens by systematic selection of reporter cell lines. *Nat. Biotechnol.* **34**, 70–77 (2016).

13. Johannessen, C. M. et al. A melanocyte lineage program confers resistance to MAP kinase pathway inhibition. *Nature* **504**, 138–142 (2013).

14. Lehraiki, A. et al. Increased CD271 expression by the NF-kB pathway promotes melanoma cell survival and drives acquired resistance to BRAF inhibitor vemurafenib. *Cell Discov.* **1**, 15030 (2015).

15. Vergani, E. et al. Overcoming melanoma resistance to vemurafenib by targeting CCL2-induced miR-34a, miR-100 and miR-125b. *Oncotarget* **7**, 4428–4441 (2016).

16. Lin, L. et al. The Hippo effector YAP promotes resistance to RAF- and MEK-targeted cancer therapies. *Nat. Genet.* **47**, 250–256 (2015).

17. Greenblum, S. I., Efroni, S., Schaefer, C. F. & Buetow, K. H. The PathOlogist: an automated tool for pathway-centric analysis. *BMC Bioinformatics* **12**, 133 (2011).

18. Qiu, B. & Simon, M. C. Oncogenes strike a balance between cellular growth and homeostasis. *Semin. Cell Dev. Biol.* **43**, 3–10 (2015).

19. Schulze, A. & Harris, A. L. How cancer metabolism is tuned for proliferation and vulnerable to disruption. *Nature* **491**, 364–373 (2012).

20. Vander Heiden, M. G. et al. Metabolic pathway alterations that support cell proliferation. *Cold Spring Harb. Symp. Quant. Biol.* **76**, 325–334 (2011).

21. Ginzberg, M. B. et al. Cell size sensing in animal cells coordinates anabolic growth rates and cell cycle progression to maintain cell size uniformity. *eLife* **7**, (2018).

22. Kafri, R. et al. Dynamics extracted from fixed cells reveal feedback linking cell growth to cell cycle. *Nature* **494**, 480–483 (2013).

23. Liu, S. et al. Size uniformity of animal cells is actively maintained by a p38 MAPK-dependent regulation of G1-length. *eLife* **7**, e26947 (2018).

24. Li, J. et al. TCPA: a resource for cancer functional proteomics data. *Nat. Methods* **10**, 1046–1047 (2013).

25. Li, J. et al. Explore, Visualize, and Analyze Functional Cancer Proteomic Data Using the
26. Guo, C. et al. CellTag Indexing: genetic barcode-based sample multiplexing for single-cell genomics. *Genome Biol.* **20**, 90 (2019).

27. Sellam, A. et al. The p38/HOG stress-activated protein kinase network couples growth to division in Candida albicans. *PLOS Genet.* **15**, e1008052 (2019).

28. Maik-Rachline, G., Zehorai, E., Hanoch, T., Blenis, J. & Seger, R. The nuclear translocation of the kinases p38 and JNK promotes inflammation-induced cancer. *Sci. Signal.* **11**, (2018).

29. Feng, Z., Zhang, H., Levine, A. J. & Jin, S. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 8204–8209 (2005).

30. Ben-Hamo, R. et al. Predicting and affecting response to cancer therapy based on pathway-level biomarkers. *Nat. Commun.* **11**, 3296 (2020).
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 50µm, in D 100µm and in J 7µm.
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 (1µM) or trametinib (0.125 µM) over time. Blue and red backgrounds represent activation or inhibition scores above 0.2 or below -0.2 respectively.
A

Cluster B
(p53 signaling state)

Cluster A
(p38 signaling state)

Cluster C

B

Effect on growth

No
Yes

p38 activity score

p38 activity score

C

Pearson (r)

D

422 drugs

Hundreds of direct targets

2 clusters
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.
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.
A

B

C

D

E

F

G

### Figure A

Bar chart comparing miss rate across different cell compartments:
- **Nuclei**
- **NES**
- **ER**
- **Peroxi**
- **Whole cell**

**Miss rate [%]**

### Figure B

Graph showing treatment effect:
- **DMSO**
- **Drugs (n = 75)**

### Figure C

Heatmap illustrating true barcode versus called barcode:
- **Drugs**
- **n = 75**

### Figure D

Scatter plots of intensity distributions:
- **Intensity_MC_CFP**
- **Intensity_MC_BFP**

### Figure E

Scatter plots comparing different fluorescence markers:
- **GFP**
- **CFP**

### Figure F

Graph showing area threshold:
- **GFP**
- **CFP**

### Figure G

Images comparing Peroxi, WC, NES expression:
- **Peroxi**
- **WC**
- **NES**
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) 7µM.
### Table A

| Reporter | Reporter type | addgene source |
|----------|---------------|----------------|
| ERK      | KTR           | [https://www.addgene.org/59150/](https://www.addgene.org/59150/) |
| JNK      | KTR           | [https://www.addgene.org/59150/](https://www.addgene.org/59150/) |
| p38      | KTR           | [https://www.addgene.org/59150/](https://www.addgene.org/59150/) |
| PKA      | HIF TRE       | [https://www.addgene.org/82921/](https://www.addgene.org/82921/) |
| p53      | TRE           | [https://www.addgene.org/16888/](https://www.addgene.org/16888/) |
| YAP/TAZ  | TRE           | [https://www.addgene.org/13458/](https://www.addgene.org/13458/) |
| RAR      | TRE           | [https://www.addgene.org/13458/](https://www.addgene.org/13458/) |
| NFKB     | TRE           | [https://www.addgene.org/16593/](https://www.addgene.org/16593/) |
| WNT      | TRE           | [https://www.addgene.org/16593/](https://www.addgene.org/16593/) |
| AKT      | Translocation | [https://www.addgene.org/83461/](https://www.addgene.org/83461/) |
| GEMININ  | TRE           | [https://www.addgene.org/83461/](https://www.addgene.org/83461/) |

### Figure B

*Normalized cell count over time for different barcode treatments.*

### Figure C

*Normalized cell count as a function of Vemurafenib concentration.*

### Figure D

*Density plot of cytoplasm/nuclei ratio for different treatments.*

### Figure E

*Cumulative distribution of cytoplasm/nuclei ratio for DMSO and Trametinib.*

### Figure 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           |

### Figure G

*Survival probability for the TCGA cohort.*

### Figure H

*Survival probability for the Cirenaakis et al. cohort.*

### Figure I

*Survival probability for the Budden et al. cohort.*
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.
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.
$$\Pi S = \text{Var(erk)} \times \text{Var(p38)}$$

$$\Pi \Psi = \text{Var(ax1)} \times \text{Var(ax2)}$$

$$\Phi = \frac{\Pi \Psi}{\Pi S}$$
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 plots 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 (SARCO), 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 TM, 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 an 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. |
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. 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

| **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 |
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).

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 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 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:**

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

**ImageStream analysis:**

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 µm²) and 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 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-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](https://g-incpm.weizmann.ac.il/units/WohlDrugDiscovery/chemical-libraries)). The third library was a gift from Pfizer and contained 175 drugs (Sup table 2). All libraries were screened at a final concentration of 0.5µM.

**Screening and Imaging:**

For screens, clones were grown in 15 cm culture plates (ThermoScientific, #168381) overnight in 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., 2020).

**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. 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 uM, Sigma C4859), Torin-2 (10, 5, and 2.5 nM, Tocris 4248), rapamycin (7, 0.7, and 0.07 uM, 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 uM, 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 uM, 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.

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 $\Psi_0$, that has twelve axes in the directions $\Psi_{0,n}$. Since $\Psi_0$ 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 $\Psi_0$ 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 $\Psi_0$.

$$\phi = \frac{\prod_{n=1}^{12} \sigma_{\Psi_{0,n}}^2}{\prod_{p=1}^{12} \sigma_p^2} \text{ Eq. 1}$$
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 $\phi$ 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 $\phi$ will be near one in all coordinates. By contrast, if we imagine perfectly correlated data, $\phi$ 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 $\phi$ 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.

$$\phi = \frac{PC_i}{\sqrt{\sum_{i=1}^{12} PC_i^2}}$$  \hspace{1cm} \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 $\Psi$. 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.
| Drug                        | Targets                               |
|-----------------------------|---------------------------------------|
| 2-Methoxyestradiol          | HIF                                   |
| ABT-199 (GDC-0199)          | BCL2                                  |
| ABT-263 (Navitoclax)        | BCL2                                  |
| Afatinib (BIBW2992)         | EGFR;HER2                             |
| Alpelisib (BYL719)          | PI3K                                  |
| AT7867                      | S6 Kinase,Akt                         |
| Aurora A Inhibitor I        | Aurora Kinase                         |
| AZ 3146                     | Kinesin                               |
| AZ 960                      | JAK                                   |
| AZD5363                     | Akt                                   |
| AZD6244 (Selumetinib)       | MEK                                   |
| AZD8055                     | mTOR                                 |
| Bexarotene                  | RXRA;RXRB;RARB;RARA                   |
| BIRB 796 (Doramapimod)      | p38 MAPK                              |
| BIX 02188                   | MEK5                                  |
| BKM120 (NVP-BKM120)         | PI3K                                  |
| BX-795                      | IKB;IKK;PDK-1                         |
| CHIR-99021 (CT99021) HCl    | GSK3A;GSK3B;CD33                      |
| Chrysophanic acid (Chrysophanol) | GSK-3                               |
| CI-1040 (PD184352)         | MAP3K1;RAF1;MAP2K1                    |
| Dasatinib (BMS-354825)      | LCK;EPHA2;LYN;ABL1;FY0;SRC;DDR2;DDR1;YES1;BMX |
| Dovitinib Dilactic acid (TKI258 Dilactic aci | PDGFR;FGFR;c-KIT;FL3;VEGFR |
| Enzastaurin (LY317615)      | PKC                                   |
| Everolimus (RAD001)         | mTOR                                  |
| Fusudil HCI (HA-1077)       | ROCK                                  |
| Forskolin                   | cAMP                                  |
| GM6001                      | MMP                                   |
| GSK1120212 (Trametinib)     | MEK                                   |
| GSK1904529A                 | IGF-1R                                |
| GSK2126458                  | PI3K;mTOR                             |
| GSK429286A                  | ROCK                                  |
| H 89 2HCl                   | PKA                                   |
| IKK-16                      | IKB;IKK                               |
| IMD0354                     | IKB;IKK                               |
| Iniparib (BSI-201)          | PARP                                  |
| INK 128 (MLN0128)           | mTOR                                  |
| Ipatasertib (GDC-0068)      | PAN Akt                               |
| Ivacaftor (VX-770)          | CFTR                                  |
| JNJ 26854165 (Serdemetan)   | p53                                   |
| JNJ-7706621                 | CDK2;TYK2;AURKB;CDK1;JAK3;JAK1;AURKA;CDK5;JAK2 |
| JNK inhibitor IX            | JNK                                   |
| JNK-IN-8                    | JNK                                   |
| Losmapimod                  | p38 MAPK                              |
| LY2835219                   | CDK                                   |
| NU7441(KU-57788)            | DNA-PK;PI3K                          |
| Nutlin-3                    | p53                                   |
| OSU-03012 (AR-12)           | PDK-1                                 |
| PF-00562271                 | FAK                                   |
| PF-04691502                 | Akt,mTOR;PI3K                         |
| PF-562271                   | PTK2;NTRK2;PTK2B;INSR;CDK2;JAK2;ROS1;MUSK;TYK2;NTRK1;JK3;JAK1 |
| 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 | Prolactin (Gilotyn) | 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 stearoyl-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 Dilactic acid (TKI258 Dilactic acid) | multitargeted RTK inhibitor |
| 20          | Pazopanib HCl       | multitargeted RTK inhibitor |
| 21          | Tandutinib (MLN518) | multitargeted RTK inhibitor |
| 22          | VX-702              | p38_MAPK |
| 23          | Sotrastaurin (AE8071) | pan-PKC inhibitor |
| 24          | AZD2461             | PARP inhibitor |
| 25          | TAK-700 (Orteronel) | 17,20-lyase inhibitor |
| 26          | Dutasteride         | 5-alpha Reductase inhibitor |
| 27          | Finasteride         | 5-alpha 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          | Tropicaamide        | AChR |
| 35          | Trosipium 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          | AT7987              | Akt, S6K |
| 43          | A-674563            | Akt1 |
| 44          | Honokiol            | Akt1 |
| 45          | 135565159           | Akt1_P13K |
| 46          | CCT128930           | Akt2 |
| 47          | CERTITINIB          | ALK inhibitor |
| 48          | SB 431542           | ALK5 |
| 49          | CLOTRIMAZOLE        | 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 DX1 receptor |
| 58          | GW9662              | antagonist for PPAR |
| 59          | (+)-Bicuculline     | antagonist of GABAA receptors |
| 60          | PF-4455242          | antagonist of the _-opioid receptor |
| 61          | Pralatrexate(Folotyn) | Antifolate |
| 62          | S3311136            | 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 |
| No. | Drug Name          | Action or Target                        |
|-----|--------------------|-----------------------------------------|
| 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 Ca2+ 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  | Brinzolamide       | Carbonic anhydrase I inhibitor          |
| 89  | Rebamipide         | CCK1 receptor inhibitor                 |
| 90  | 449087             | CCNA1, CCNA2                           |
| 91  | 11149391           | CDC7                                   |
| 92  | BMS-265246         | CDK1/2                                 |
| 93  | Flavopiridol hydrochloride | CDK1/2/4/6                           |
| 94  | S329663            | CDK2                                   |
| 95  | PNU-292137         | CDK2                                   |
| 96  | 533049             | CDK2, CCNA1, CDK5, CDK1                |
| 97  | PHA-793887         | CDK2/5/7                               |
| 98  | Abemaciclib        | CDK4/6                                 |
| 99  | BS-181 HCl         | CDK7                                   |
| 100 | Anacetrapib (MK-0859) | CETP                                 |
| 101 | Daclatrapib (JTT-705) | CETP                                 |
| 102 | Ivacaftor (VX-770) | CFTR                                   |
| 103 | VX-809             | CFTR                                   |
| 104 | 16065975           | CFTR, TRPA1                            |
| 105 | 11844347           | CHEK1, WEE1                           |
| 106 | 11844610           | CHEK1, WEE1                           |
| 107 | LY2603618 (IC-83)  | Chk1                                   |
| 108 | PF 477736          | Chk1 inhibitor                         |
| 109 | Entacapone         | COMT inhibitor                         |
| 110 | Hydrocortisone     | corticosteroid with anti-inflammatory and immunosuppressive properties |
| 111 | Toltenamic acid    | COX-2 inhibitor                        |
| 112 | Linfanib (ABT-869) | CSF-1R, PDGFR, VEGFR                   |
| 113 | 705208             | CSNK2A2                                |
| 114 | 2-Methoxyestradiol | depolymerizes microtubules and blocks HIF-1 nuclear accumulation |
| 115 | WP1130             | deubiquitinase inhibitor              |
| 116 | Pyrimethamine      | DHFR inhibitor                         |
| 117 | Penfluridol        | diphenylbutylpiperidine antipsychotic |
| 118 | Triciribine (Triciribine phosphate) | DNA synthesis inhibitor, AKT          |
| 119 | pp-121             | DNA-PK, PDGFR, mTOR, SRC               |
| 120 | PP-121             | DNA-PK, PDGFR, mTOR, SRC               |
| 121 | NU7441(KU-57788)   | DNA-PK, P3K                           |
| 122 | Rotundine          | dopamine D1 receptor antagonist        |
| 123 | Linagliptin (BI-1356) | DPP-4 inhibitor                      |
| 124 | PHA-767491         | dual Cdc7/Cdk9 inhibitor               |
| 125 | Ku-0063794         | dual-mTOR inhibitor                    |
| 126 | Erlotinib(OSI-744) | EGFR                                   |
| 127 | Gefitinib (Iressa) | EGFR                                   |
| 128 | PD168393           | EGFR                                   |
| 129 | Pemetinib          | EGFR                                   |
| 130 | WZ4002             | EGFR(L858R)/(T790M)                    |
| 131 | Afatinib (BIBW2992) | EGFR/Erbb                            |
| 132 | W28040             | EGFR7790M inhibitor,                   |
| 133 | Bosentan Hydrate   | Endothelin Receptor antagonist         |
| 134 | Sitaxentan sodium (TBC-11251) | endothelin receptor-A antagonist       |
| 135 | Fulvestrant (Faslodex) | ER antagonist                     |
| 136 | 16065674           | ERBB2                                  |
| 137 | Propylpyrazoletriol | ESR1                                  |
| 138 | Propylpyrazoletriol | ESR2                                  |
| 139 | 10215241           | ESR2                                  |
| 140 | 1354944488         | ESR2                                  |
| 141 | 10236407           | ESR2, ESR1                            |
| Compound | Description |
|----------|-------------|
| Toremifene Citrate (Fareston, Acapodene) | estrogen receptor modulator (SERM) |
| Tazemetostat | EZH2 |
| PF-3845 | FAAH |
| JNJ 1661010 | FAAH inhibitor |
| PF-00562271 | FAK |
| 5328999 | FGFR1 |
| PD-166866 | FGFR1 |
| PD173074 | FGFR1 |
| AZD4547 | FGFR1/2/3 |
| ESTRADIOL | |
| Toremifene Citrate (Fareston, Acapodene) | estrogen receptor modulator (SERM) |
| Tazemetostat | EZH2 |
| PF-3845 | FAAH |
| JNJ 1661010 | FAAH inhibitor |
| PF-00562271 | FAK |
| 5328999 | FGFR1 |
| PD-166866 | FGFR1 |
| PD173074 | FGFR1 |
| AZD4547 | FGFR1/2/3 |
| FENOFIBRIC ACID | fibrate that acts as a lipid-lowering agent, decreasing low-density lipoprotein cholesterol and triglycerides. |
| TCS 359 | FLT3 |
| TUFOEXORATE ISOPROPYL | FXR agonist |
| Etoximide | GABAA receptor agonist |
| Semagacestat (LY450139) | Gamma-secretase blocker |
| YO-01027 | Gamma-secretase inhibitor |
| 24768019 | GCK |
| 10407399 | GLI3 |
| BECLOMETHASONE DIPROPIONATE | glucocorticoid steroid |
| BUDERONIDE | glucocorticoid steroid |
| DEXAMETHASONE | glucocorticoid, agonist of the glucocorticoid receptor (GR) |
| GW 4064 | agonist of farnesoid X receptor |
| CHIR-99021 | GSK-3 |
| SB 216763 | GSK-3 |
| SB 415286 | GSK-3 |
| AR-A014418 | GSK3 |
| GSK J4 HCl | H3K27 histone demethylase |
| Belinostat (PXD101) | HDAC inhibitor |
| Mubritinib (TAK 165) | HER2 |
| AEE788 (NVP-AEE788) | HER2,EGFR |
| BMS-596626 (AC480) | HER2,EGFR |
| Laptatinib Ditosylate (Tykerb) | HER2,EGFR |
| IOX2 | HIF activator |
| 5B00609 | HIF1A inhibitor |
| GENISTEIN | highly specific inhibitor of protein tyrosine kinase |
| Clemastine Fumarate | histamine H1 receptor antagonist |
| Lidocaine (Alphacaine) | histamine H1-receptor agonist |
| Ranitidine (Zantac) | histamine H2-receptor antagonist, |
| CIPRALISANT | histamine H3 antagonist |
| NELFINAVIR | HIV protease inhibitor |
| Fluvasatin sodium (Lescol) | HMG-CoA reductase |
| 4284720 | HTR2B |
| GSK1838705A | IGF-1R |
| GSK1904529A | IGF-1R |
| Linsitinib (OSI-906) | IGF-1R |
| NVP-ADW742 | IGF-1R |
| PQ 401 | IGF-1R |
| NVP-ADW742 | IGF-1R inhibitor |
| TP-CA-1 | IKK-2 |
| TP-CA 1 | IKK-2 |
| 3-Deazaneplanocin | inhibitor of S-adenosylhomocysteine hydrolase |
| BMS-806 (BMS 378806) | inhibits the binding of HIV-1 gp120 to the CD4 receptor |
|     | Name                          | Targets               |
|-----|-------------------------------|-----------------------|
| 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 | DIBUCAINS                      | 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                        | LXRX agonist          |
| 235 | GW3965 HCI                     | LXRX agonist          |
| 236 | Moclobemide                    | MAO-A (5-HT) inhibitor |
| 237 | Rasagiline mesylate            | MAO-B inhibitor       |
| 238 | 98414                          | 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 | PD035901                       | 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-κB 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                      | nonselectosteroidal 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                        | NR1J3                 |
| 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 (Seremedetan)     | p53 activation        |
| 284 | Nutlin-3                       | p53 activation        |
| 285 | YM239-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  | MC-2206 ditydrochloride | 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 (Doraminapimod) | 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  | S328649  | PDGFR                        |
| 321  | BX-795  | PDK-1                        |
| 322  | BX-912  | PDK-1                        |
| 323  | GSK2334470  | PDK-1 |
| 324  | OSU-03012 (AR-12) | PDK-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  | BUPARUSIB  | PI3K                         |
| 331  | CAL-101 (GS-1101) | PI3K |
| 332  | GDC-0941  | PI3K                         |
| 333  | IPI-145 (JNK1197) | PI3K |
| 334  | Pi-103  | PI3K                         |
| 335  | TASELSIB  | 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  | S8379551  | PI3K,mTOR                   |
| 344  | GSK2126458  | PI3K,mTOR                   |
| 345  | PF-0469102  | PI3K,mTOR                   |
| 346  | AS-605240  | PI3KCG                       |
| 347  | SGI-1776 free base | PIM1 |
| 348  | H 89 2HCl  | PKA                          |
| 349  | Enzastaurin (LY317615) | PKC |
| 350  | 10290302  | PLAZG4A - phospholipase A2   |
| 351  | BI6727 (Volasertib) | PLK1 |
| 352  | GSK461364  | PLK1                         |
| 353  | Bi-2536  | PLK1 inhibitor              |
| 354  | Gliclazide (Diamicron) | 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 GS429286A  ROCK1
372 GS429286A-1  ROCK1
373 BEXAROTENE  RXR agonist
374 Oxymetazoline hydrochloride  selective alpha-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 inhibitor
386 TIPFFARNIB  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 (Postamatinib)  Syk
395 20899393  TDP1
396 LY-364947  TGFB
397 70683591  TGFB1
398 10473992  TGFB2
399 10390904  THR8
400 PF-00277343  THR8
401 10027822  THR8, THR9
402 Ozagrel  thromboxane A(2)
403 Ozagrel HCl  thromboxane A(2)
404 IOUHYRONINE  thyroid hormone receptor
405 SOBETIROME  thyroid hormone receptor isoform beta-1
406 Cepharanthine  TNF
407 16049257  TNK2
408 Daunorubicin HCl  Topoisomerase
409 HC-O30031  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 DILAPEP  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