Supplementary Methods

I. Testing the sensitivity of S2 cells to the MEK inhibitor PD-0325901 (PD)

Prior to large scale RNAi screening and RNA-Seq the sensitivity of S2 cells towards small molecule MEK inhibitor PD-0325901 (Cayman chemical, Ref: CAY-13034-5, Biomol GmbH, Germany) was tested under 3 different conditions: (i) normal treatment regimen, (ii) recovery after drug wash-out, (iii) treatment with used drug medium. All cells used in this project were from the same culture of the, serum free medium adapted, Drosophila *melanogaster* S2 cell line (S2) and will be referred to as cells (Schneider's Drosophila Line 2 [D. Mel. (2), SL2] (ATCC® CRL1963™) from ThermoFisher (Waltham, MA))[1–4].

1. Experimental setup and drug treatment regimen

Cells were seeded in 2 Greiner µClear plates (Ref: 781073, Greiner Bio One International GmbH, Frickenhausen, Germany), pre-spotted with 250 ng/well anti-GFP dsRNA and left to incubate for 24 h at 25 °C. After incubation, the cells were treated with a gradient of PD-0325901 in medium (Express V, serum free cell culture medium + 10 % Glutamax, Gibco, Ref: 10486-025 & Ref: 35050-061, Life Technologies GmbH, Darmstadt, Germany) using robotic liquid handling. The gradient was pipetted as a 2-fold serial dilution in 100 % DMSO from a 10 mM stock of PD-0325901 using a Beckmann NX-P pipetting robot (Beckman Coulter, Brea, California, USA). The prepared gradient was then diluted in 384 well format 1:20 in medium reaching a pre-assay concentration of 5 % DMSO in medium. Addition of 5 µl gradient dilution mix to the cell culture adds another 1:10 dilution step yielding an in-assay concentration gradient ranging from 50 µM to 0.024 nM PD-0325901 in 0.5 % DMSO in medium. Thus, each column was treated with one concentration of PD-0325901. Column 1 and 24 were left mock treated with 0.5 % DMSO. Plates were sealed again and incubated at 25 °C for 96 h. After completed incubation one plate was subjected to fixation and staining while the medium of the other plate was carefully removed and dispensed on a plate containing cells freshly seeded 24 h in advance. 50 µl of fresh medium was then added to the emptied plate. Both, the plate with fresh medium and the plate with fresh cells and used medium were then incubated for additional 72 h prior to fixation and staining. This results in 3 dose response analyses. 1) with normal assay protocol, 2) where the cells could recover from the treatment to test if the phenotypes are temporal and 3) where the remaining activity of PD-0325901 was tested after 96 h of incubation on cells. All experiments in this setup have been replicated twice.

2. Data normalization

All plates were subjected to standard fixation, staining and imaging protocols and analyzed using automated image analysis as described in the main methods. Data were triaged by removing NA value containing wells and single cell data was aggregated by calculating the mean per field of view. The data were scaled per feature on a scale from 0 to 1 and subjected to dose-response modelling.

3. Dose-Response modeling and statistical analysis

Dose-response-modelling was carried out using the drm function for dose response models from R package drc[10]. For modelling, the replicates and fields per well were used as independent measurements to inform the model. Dose response data was fitted to a four-

parameter log-logistic function estimating the parameters slope, lower limit, upper limit and ED_{50}. We used this model to accurately estimate the ED_{50} concentration of cell growth inhibition. There the model provided us with a fixed value plus and minus a confidence interval in which the coefficient was estimated. The resulting model was assessed via plotting the dose response curves for different features, including all measured points with their mean and s.e.m. and the ED_{50} was further used in all subsequent experiments.

4. Western Blot analysis of in vitro PD-0325901 activity

Inhibition of Dsor1 kinase activity by PD-0325901 was tested by assaying levels of phosphorylated rl. Therefore, S2 cells were seeded as 4.5 * 10^5 cells per well in a 24-well cell Culture plate (Greiner, Ref: 2511, Greiner Bio One International GmbH, Frickenhausen, Germany) in 700 µl Express Five cell culture medium (Gibco, Ref: 10486-025, Life Technologies GmbH, Darmstadt, Germany) supplemented with 10 % Glutamax (Gibco) in 4 identical wells. Cells were incubated at 25 °C for 24 hours prior to compound addition (150 nM PD-0325901, 0.5 % in assay) in well A. After another 24 hours, well B was treated. This procedure was repeated for well C and D. After additional 24 hours of incubation, cells in all wells were lysed and western-blot analysis was carried out as follows. After washing the cells once with 500 µl PBS (Life Technologies, Ref: 10010015, Gibco), 50 µl of ice-cold RIPA-lysis buffer (50 mM Tris HCl, pH 8.0, 150 mM NaCl, 1 % NP-40, 2 mM EDTA,1 x Protease Inhibitor, 2 % Phosphatase inhibitor cocktail II & III, Sigma, Merck KGaA, Darmstadt, Germany) were added and cells left to lyse for 30 sec on ice. The lysate was collected in fresh 1.5 ml tubes (Eppendorf, Eppendorf AG, Hamburg, Germany) and samples were centrifuged (21000 rpm, 5 min, 4 °C). 20 µl of supernatant were mixed with 5 µl 5x Laemmli buffer and incubated 10 min at 95 °C. Another 2 µl of each sample were used to measure protein content after manufacturer's instructions for BCA assays 96-well plates (Pierce, Ref: 10741395, Thermo Scientific, Ref: H1399, Life Technologies GmbH, Darmstadt, Germany). Samples were then loaded on a 12-well NuPAGE 4-12 % Bis-Tris Gel (Novex, Ref: NP0323BOX, Life Technologies GmbH, Darmstadt, Germany) and left for electrophoresis for 30 min at 80 V constant voltage followed by 60 min at 120 V constant voltage in 1 x 3-(N-morpholino) propanesulfonic acid (MOPS) buffer (40 mM MOPS, 10 mM NaAc, 1 mM EDTA). Following electrophoresis, proteins were transferred onto a methanol activated PVDF-membrane (Immobilon-P, Millipore, Ref: IPVH00010, Merck KGaA, Darmstadt, Germany) by tank-blotting in 1 x Transfer buffer (25 mM Tris base, 192 mM glycine, 10 % methanol) at 35 V for 90 min. After transfer, the membrane was shortly washed in 1 x TBST (137 mM NaCl, 2.7 mM KCl, 19 mM Tris Base) and incubated for 60 min with constant shaking at room temperature (RT) in 5 % skimmed milk (Sigma, Ref: 70166-500G, Merck KGaA, Darmstadt, Germany). After washing the membrane 5 times 5 min at RT with 1 X TBST, the membrane was incubated overnight in a 1:2000 dilution of anti pp-p44/42 rabbit monoclonal antibody (Cell-Signaling, Ref: 4370, Cell Signaling Technology Europe, B.V., Frankfurt a.M., Germnay) in 5 % BSA (Sigma, Ref: A9085-25G). Following washing the membrane 5 times, 5 min in 1 x TBST it was incubated 60 min in 1:10000 anti-rabbit IgG-HRP conjugate (from Donkey) (Amersham ECL igG-HRP Conjugate, GE Healthcare). After an additional washing step, the membrane was developed on Hyperfilm ECL (Sigma Aldrich, Ref: GE28-9068-36) using Immobilon ECL substrate (Merck Millipore, Ref: WBKLS0100). The membrane was then stripped in 1 x Re-

Blot Plus strong (Merck Millipore, Ref: 2504) for 15 min, RT followed by washing once 5 min in 1 x TBST. The membrane was again blocked 60 min in 5 % skimmed milk in TBST, stained with 1:1000 anti p44/42 rabbit monoclonal antibody (Cell-Signaling, Ref: 4695), developed the same way as with the first antibody and stripped again. The same procedure was repeated using a 1:2000 dilution of anti α-tubulin monoclonal rabbit antibody (Cell-Signaling Ref: 2144).

II. Transcriptional Profiling of S2 cells

RNAi most effectively induces a visible phenotype when targeting highly expressed genes [1,2]. Thus, prior to image based screening and to gain information about ongoing cellular processes, differential expression analysis was performed under different treatment conditions using conventional bulk RNA-seq analysis.

1. Plate preparation

10 x 96-well plates (cell culture grade, flat bottom, transparent, Greiner, Ref: 655083) were prepared by dispensing 18 µl of a 50 ng/µl solution (equaling ~900 ng dsRNA per well) of anti-RLUC dsRNA in culture medium (Express V, serum free cell culture medium + 10 % Glutamax, both Gibco) into each well using a multichannel manual pipette. Additional 95 µl pre-warmed (25 °C) medium were added to each plate using a MultiDrop dispenser (Thermo Scientific, low speed, 96-well standard plate, full plate, 20 µl pre-dispense and 16 mm offset). All further MultiDrop dispensing steps have been carried out using the same settings if not denoted different. The same cell culture medium was used in all the following experiments and is referred to as medium. All cells used in this project were from the same culture of the, serum free medium adapted, Drosophila *melanogaster* S2 cell line (S2) [3,4] and will be referred to as cells.

5. Cell culture

S2 cells (at passage 21, counted via a Nexcelom Cellometer Auto 1000 for 90 % viability, Ref: CETHT4SD100002, Cenibra GmbH) were seeded in all plates at 40000 cells/well in 35 µl medium using a MultiDrop dispenser. Plates were sealed using a heat plate sealer (PlateLoc, Agilent Technologies Deutschland GmbH & Co. KG, Waldbronn, Germany) and centrifuged at 140 g for 60 sec. Cells were left to incubate for 24 h at 25 °C in a cell culture incubator with no CO_2 adjustment. After incubation, plates were opened and 15 µl of 150 nM solution of PD-0325901 (MEK1/2 inhibitor, CAS: 391210-10-9, Cayman Chemical, 15 nM in assay) dissolved in medium with 5 % DMSO (Sigma Aldrich, Ref: 41644-1l, Merck KGaA, Darmstadt, Germany) or 5 % DMSO in medium were added using MultiDrop dispensing. Column 1-6 of each plate were filled with the DMSO control medium and columns 7-12 of each plate were treated with the inhibitor solution. Plates were heat sealed again and left to incubate for an additional 48 h, 72 h and 96 h at 25 °C without CO_2 adjustments prior to cell lysis and RNA extraction.

6. Cell lysis and RNA extraction

Cell lysis of 3 plates from each time point was carried out after 48 h, 72 h and 96 h on a Beckman FX pipetting robot using a custom protocol with reagents supplied from the Agencourt

RNAdvance tissue kit (Beckman Coulter, Ref: A32649). RNA extraction was performed on a Beckman NX-P robot with a custom protocol using lab ware and the reagents from an Agencourt RNAdvance tissue kit. In brief, medium was removed and the cells were washed carefully using 200 µl PBS before 50 µl freshly prepared lysis buffer were added (5 % Proteinase K in 30 ml of lysis buffer). Lysis was left to incubate for 30 min at room temperature (RT). The solution was then mixed, transferred to a new plate and stored at -20 °C. Cell lysates were mixed vigorously in a 1:1 mass ratio with the magnetic bead solution (320 µl 70 % 2-Propanol + 80 µl magnetic beads) and incubated for 5 min at RT prior to 5 min of incubation at RT on a plate magnet. The supernatant was removed and the plate removed from the magnet. The samples were re-suspended in 800 µl of washing buffer before another 4 min of incubation at RT on the magnet. The remaining supernatant was removed, the plate removed from the magnet and the samples dissolved in 800 µl of 70 % ethanol. After gentle mixing of the solution, samples were left to incubate for 4 min at RT on the magnet and the supernatant was removed. Prior to addition of 100 µl DNase mix (80 µl H_2O, 10 µl DNase buffer, 10 µl DNase) sample were incubated for 5 min at 37 °C on a pre-warmed heating block. Samples with DNase were then incubated another 15 min at 37 °C on the heating block before 500 µl of washing buffer were added and the samples were mixed gently by pipetting. Samples were left to rest for 4 min at RT before the plate was transferred to the magnet and incubated for 4 min at RT. The supernatant was discarded and the samples washed three times with 200 µl 70 % ethanol. The supernatant was removed and the samples allowed to dry 5 min at 37 °C before they were vigorously re-suspended in 30 µl RNase, DNase free water. RNA was allowed to dissolve from the beads by incubating the mixture 5 min at RT before the plate was transferred to the magnet. After 30 sec, the DNase treated total RNA containing eluate was transferred to a fresh plate, pooled per sample and stored in conventional Eppendorf tubes at -20 °C.

7. *Sample preparation and sequencing*

Sample preparation and sequencing were carried out by the DKFZ high throughput sequencing core facility using standard protocols. Sample libraries were prepared using an Illumina RNA TruSeq protocol and sequenced on a HiSeq V4 using 125 bp paired end mode. All samples passed quality controls and provided on average 10 mio. reads to be mapped.

8. *Fixation, staining and imaging of a plate for visual inspection*

A plate for visual inspection of experimental outcome was prepared by fixation and fluorescent staining of cells after 72 hours of post treatment incubation. All steps were carried out manually. The cell culture medium was discarded and the cells were washed twice with 100 µl 1xPBS. The PBS was removed and 100 µl fixation buffer (4 % PFA (Roth, Ref: 0335.3, Carl Roth GmbH + Co. KG, Karlsruhe, Germany), 0.3 % Triton-X (Sigma Aldrich, Ref: T8787-250ml), 0.1 % Tween20 (Sigma Aldrich, Ref: P1379-100ML), 1 % BSA (GERBU, Ref: 1507.0100, GERBU Biotechnik Biotechnik GmbH, Heidelberg, Germany) in 1xPBS were added prior to incubation for 45 minutes at RT in the dark. Prior to another washing with 100 µl PBS, the fixation buffer was removed and after washing the plate was stored filled with 100 µl of PBS. For staining the PBS was removed and the cells blocked by incubating 20 min at RT with 100 µl blocking solution (0.3 % Triton-X, 0.1 % Tween 20, 4 % BSA in 1xPBS). After blocking, the blocking solution was removed and the samples were incubated 30 min at RT in the dark in

50 µl the staining solution (1:4000 Hoechst (Thermo Scientific, Ref: H1399, Life Technologies GmbH, Darmstadt, Germany), 1:1500 primary FITC labelled anti α-tubulin antibody (Sigma, F2168-.5ml), 1:6000 Phalloidin-TRITC conjugate (Sigma, P1951) in 1x blocking solution). The stained cells were washed twice in 50 µl PBS and stored in 50 µl PBS at 4 °C. The plate was imaged on an IncellAnalyzer 2200 (GE Healthcare GmbH, Solingen, Germany) automated microscope (Channels: DAPI (ex/em: 390/435) at 400 ms, Cy3 (ex/em: 542/597) at 400 ms, FITC (ex/em: 475/511) at 300 ms). Images were analyzed using the same R/Bioconductor pipeline as is described below.

9. Data analysis and statistical testing

Data were analyzed using the DeSeq2 R/Bioconductor package according to the protocol published by Anders *et al.* [5]. In short, reads were subjected to FASTQC based quality assessment and all samples with good quality were mapped against the BDGP6 *Drosophila melanogaster* reference genome using the RNA-STAR aligner with pre-set default parameters for paired end RNA sequences. Resulting BAM files were then subjected to normalized feature counting using htseq-tool by Anders *et al.* DeSeq2 was used for normalization of count data and statistical testing of differential expression between different conditions. During the statistical analysis default parameters were chosen as supposed in the methods of Anders *et al.*. Quality was assessed by replicate correlation using spearman rank correlation. Fold changes of read counts between conditions were calculated as log2 normalized ratio of quantile normalized read counts between two conditions. Significance of differential expression was assessed using linear mixed effect modelling and corrected for multiple testing by p-value adjustment after Benjamini Hochberg (FDR)[6]. Cut-off for significantly dysregulated genes was an FDR of 10 %.

III. The genome wide HD3 dsRNA library

The "HD3 genome wide Drosophila dsRNA library" maintained by our laboratory was established prior to this study [7,8]. It contains 28941 sequence unique dsRNA reagents targeting 14242 unique gene IDs and comprising two sequence independent reagents targeting each gene, where possible. The reagents were optimized for efficient targeting of the most recent (BDGP5) mRNA annotations in *Drosophila melanogaster* by for example avoiding CAN repeats and non-unique sequences (off-targets). Target genes in the library are organized into the categories of chromatin biology, human homologs, non-human homologs, human homologs II and non-human homologs II. The library contains primer pairs against exonic regions of target genes that are amplified by PCR and processed to double stranded RNA by *in vitro* transcription (IVT) the following way. Starting from purified genomic DNA of S2 cells, a first PCR (0.28 µM fwd./rev. primer, H_2O, 10x Buffer (Qiagen, Ref: 203209, Qiagen GmbH, Hilden, Germnay), 0,28 mM dNTPs (Fermentas, Ref: R0182, Life Technologies), 0.03 U Hotstar Taq (Qiagen), 2 ng genomic DNA) with genomic sequence specific primers was performed, which also adds 4 different TAGs for later re-amplification of the product. Using TAG specific primers, a second PCR (same conditions as first one) was performed adding an additional T7-promotor sequence to either end of the resulting amplicon. A list of primers can be found at **Supplementary File 4**. The product of this reaction was then input for *in vitro* transcription at 37 °C, incubated overnight (10 µl buffer (200 mM Tris-HCl, 100 mM $MgCl_2$, 50 mM DTT, 10 mM Spermidine), 20 µl NTPs (Sigma), 5 µl Isopentenyl pyrophosphate (IPP, Sigma), 0,25 µl RNA lock (40 U/µl,

Fermentas), 5 µl T7 Polymerase (20 U/µl)). The IVT product (dsRNA) was normalized to 250 ng/µl and diluted to a final concentration of 50 ng/µl in DNase, RNase free water. All products were subjected to E-GEL (E-Gel 96 2 % 8-PAK, Life Technologies) quality control using the QIAXEL automated gel electrophoresis (Qiagen). Samples, which did not pass quality control (no band, band of the wrong size or smear) were processed again or flagged in the annotation. The final solution was stored in 384-well stock plates and aliquoted to 384 Greiner µ-clear plates prior to the screening assay at a mass of 250 ng/well. A table containing all HD3 library IDs that were used in the combinatorial RNAi screen can be found in **Supplementary File 5**.

IV. Measuring RNAi and/or drug induced phenotypes by high-content imaging

All the following experiments have been carried out using the same protocols for cell culture, fixation, staining, imaging and image analysis. All reagents were from the same vendors, though LOTs may vary and treatment and incubation times were subject to experimental design. Thus, the general workflow for automated assaying of cellular reactions by a high-content readout will be introduced first followed by a detailed description of the different experiments that were performed.

1. Plate preparation

dsRNA reagents dissolved in water were spotted into barcoded 384-well plates for microscopy (Greiner µClear, black, flat-transparent-bottom, Ref: 781073) to reach a final mass of 250 ng dsRNA in each well (5 µl of a 50 ng/µl solution). Express V medium (Gibco, Ref: 10486-025) with 10 % Glutamax (Gibco, Ref: 35050-061) were pre-warmed to 25 °C and 30 µl dispensed on top of the spotted dsRNA using a MultiDrop dispenser (high speed, 384-well standard plate, full plate, 20 µl pre-disp. and 16mm offset).

2. Cell culture

Cultured S2 cells were detached from cell culture flasks (T175, Greiner, Ref: 12668) by rough rinsing with pre-warmed medium and counted via a Nexcelom Cellometer Auto 1000 using Trypan blue viability staining. 10 µl of pre-diluted S2 cell solution were then seeded to a final concentration of 9000 cells/well into the prepared medium using MultiDrop dispensing (low speed) under constant steering in a sterile Corning spinner flask (half maximal power on Variomag Biosystem magnet stirrer). Cell containing plates were heat sealed using the PlateLoc (Agilent, 2.5 sec, 182 °C) and centrifuged at 140 g for 60 sec. Cells were then left to incubate for 24 h at 25 °C without CO_2 adjustment in a Binder cell culture incubator. A culture was kept in stock for maintenance (60 x 10^6 cells in 20 ml ExpressFive + 10 % Glutamax, splitted every 4 days and expanded for assays if needed).

3. Treatment

After 24 h of incubation, plates with growing cells were opened again and small molecule treatment was added. The concentration of applied compound is outlined with the separate experiments. Each time 5 µl of a solution containing 5 % DMSO (Sigma) in medium, or PD-0325901 dissolved in 5 % DMSO in medium, was added to an assay concentration of 0.5 % DMSO and varying concentrations of compound. After compound addition, plates were closed

again using heat sealing (2.5 sec, 182 °C) and incubated for another 48 h to 96 h depending on the experiment at 25 °C without CO_2 surveillance.

4. Fixation

After treatment, cells were subjected to fixation using a robotics procedure on a CyBiWell Vario with 384-pipette head (Analytic Jena AG, Jena, Germany). There, medium was removed and cells were washed with 50 µl PBS (Sigma Aldrich, Ref: P3813-10PAK). After addition of 40 µl of Fix-Perm solution (4% Para-formaldehyde (Roth, Ref: 0335.3); 0,3% Triton X-100 (Sigma Aldrich, Ref: T8787-250ml); 0,1% Tween20 (Sigma Aldrich, P1379-100ML); 1% BSA (GERBU, Ref: 1507.0100)), plates were left to incubate for 60 min at RT and then washed twice with 50 µl of PBS. 50 µl of PBS were added again and plates could be stored at 4 °C prior to staining.

5. Staining

Fixed cells were first blocked by adding 30 µl of blocking solution (4% BSA; 0,1% Triton X-100, 0,1% Tween20) and incubated for 30 minutes at RT. Next, the blocking buffer was removed and 10 µl of staining solution (1:4000 Hoechst (Invitrogen, H1399), 1:1500 primary FITC labelled anti α-tubulin antibody (Sigma, P1951), 1:6000 Phalloidin-TRITC conjugate (Sigma, F2168-.5ml) in 1x blocking buffer) were added. The plates were left for staining for 60 min at RT. After staining, 30 µl of PBS were added and the staining solution was removed. After two additional washing steps in 50 µl PBS, the plates were filled with 50 µl of fresh PBS and stored until imaging.

6. Imaging cells on the InCell-Analyzer 2200

All plates were imaged using the same protocol. There, the InCell-Analyzer 2200 (GE-healthcare, hereof short InCell) was calibrated to scan Greiner µClear plates setting the bottom height to 2850 µm and the bottom thickness to 200 µm so that the laser autofocus function can find the bottom of the cells at 10 % laser power. This function measures the refraction peaks of the laser while moving the focus in Z-direction. Two peaks are detected, where the second peak marks the position where the focal plane hits the inner side of the well bottom. This Z-position was used for image acquisition in three channels: DAPI (excitation: 390±18, emission: 435±48) at 400 ms exposure (100 ms in dose response experiments and genome wide screens), Cy3 (ex: 475±28, em: 511±23) at 300 ms exposure (200 ms in dose response experiments and genome wide screens) and FITC (ex: 542±27, em: 597±45) at 300 ms exposure (300 ms in dose response experiments and genome wide screens). Four tiles per well were imaged as fields of view each representing a 665.60 µm x 665.60 µm area covered by 2048 x 2048 pixels. The fields were centered around the well's center in a layout touching each other's margins. A KinedX robot (PAA Scara, Peak Analysis & Automation Ltd, Hampshire, UK) was used to automate feeding of plates into the microscope. For efficient data handling, the microscope's workstation was directly connected to a server cluster storage node via X520 optical fiber network adapter (Intel).

7. Image processing and feature extraction

On a 96 CPU server cluster, a script scheduled the automated processing of image files through the following analysis workflow, representative for each field of each well on each plate. First, the images were read in and, before each image is duplicated, each dye was assigned to the cell organelle it is supposed to stain (DAPI: DNA, Cy3: actin, FITC: α-tubulin). The duplicate image was log2 transformed, scaled from 0 to 1 and smoothened by a Gaussian filter using a sigma of one and a radius of 7 pixels. This reduces optical noise and smoothens the image gradients for further segmentation by thresholding. Afterwards the normalized actin and tubulin images were binarized by global thresholding (thresholds of 0.2 and 0.35, respectively). If the tubulin channel was bright enough (*diff (range (raw image tubulin))>0.1*) the binary cell body mask was defined as the sum of the actin and tubulin binary masks, else the tubulin was left out of the cell body segmentation. Second, the cell nuclei were identified by applying a local adaptive average threshold to binarize the nuclei image and assigning objects after morphological smoothing of the binary image. Each normalized and smoothened DAPI channel image was binarized using an average thresholding method with a 21x21 pixel wide filter. Every Pixel above the local average plus the given offset is defined as foreground and every other pixel is defined as background. The resulting binary image was then subjected to morphological operations of opening and hull filling such that filled objects with smoothly roundish outlines result. The function *bwlabel* numbers each area of connected foreground pixels to be an object (here nuclei). Offsets for segmentation were varied if the channels surpassed certain thresholds. If more than 30 nuclei were counted per field, the objects were subjected to further propagation of nuclei objects into the *á priori* defined cell body mask. Starting from the nucleus objects as seed regions, the cell bodies are segmented by propagating the nuclei objects into foreground area. Allowed foreground area is defined by the cell body binary mask. The direction of propagation is defined by the image gradient of the normalized actin channel image, together with the lambda factor. The extend of lambda defines how strong the image gradient is considered. If lambda is 1 the half distance between two nuclei defines the cell border and the cell bodies equal a Voronoi map between the nuclei. Here a relatively small lambda was chosen to allow also for cell shapes other than roundish cells. There, the lambda factor defines to which extend the propagation algorithm follows the image gradient of the normalized actin image while the body binary provides the borders of the propagation. Using the segmented object outlines as masks on the original image, cell features have been extracted using EBImage's *computeFeatures* function on each object and original non-normalized channel of the image. Specifically numeric descriptors for 4 feature classes are defined in this function (**Supplementary File 6**): (i) shape features describing how the object outline forms, (ii) basic features that describe pixel intensity based summary statistics, such as 5 % quantiles, of pixel intensity within the borders of the object, (iii) moment features that describe the spatial orientation of the objects, (iv) Haralick features derived from a pixel intensity co-occurrence matrix as texture descriptors and (v) social features such as distance to the first 20 nearest neighboring cells [9]. Cell body shape and moment features (dependent on the cell outlines) were only computed once using the actin channel. The tubulin channel, because of having the same outlines as the actin channel, was only measured for basic, intensity and texture features. All categories giving the nuclear shape, moment, intensity and texture features were measured in the DAPI channel. Social features were derived by a k-nearest-neighbor search based on the geometric centers of segmented nuclei. Taken together, we extracted 78

features for every single cell. Single cell data were stored and aggregated to well averaged data by calculating the trimmed mean (q=0.01) of all cells belonging to all fields of one well and its standard deviation.

V. Genome-wide chemo-genetic interaction screen

We performed genome-wide RNAi screens in combination with differential drug treatment to test dsRNA reagents, identify interesting candidates for combinatorial screening and to find which genes react most differentially as resistance mediators or sensitizers to the MEK inhibitor (PD-0325901) treatment.

1. Genome wide high content RNAi vs drug screen

Four sets of 88 x 384-well Greiner µClear plates were spotted with the HD3 library, 5 µl of 50 ng/µl dsRNA in each well. This library targets one gene with one dsRNA per well. Most genes were targeted with two separate designs per gene, where possible. Each plate contained the same controls. Two additional plates, containing only controls were added for controlling assay stability. Controls were chosen to spread over the complete dynamic range of cell fitness. dsRNAs against *RLUC* and *GFP* expressing plasmids serve as non-targeting negative controls, such that we could control for unspecific dsRNA induced phenotypes.

2. Assay workflow

Briefly, dsRNA containing plates were thawed, seeded with cells and left 24 h for incubation prior to drug treatment. Plates were opened and 5 µl of 15 nM PD-0325901 in 5 % DMSO were added resulting in a final concentration of 1.5 nM PD-0325901 in 0.5 % DMSO in medium using a MultiDrop dispenser. The cells were left to incubate for another 72 h at 25 °C prior to fixation, staining and imaging. Images were acquired using the standard protocol with low illumination timings (DAPI: 100 ms, Cy3: 200 ms, FITC: 300 ms). The resulting images were analyzed in line with the acquisition using the standard image analysis pipeline.

3. Data analysis workflow

Resulting data were collected to a data frame containing feature values aggregated per well as trimmed mean and standard deviation. This data frame was then reformatted to a 4-dimensional data cube featuring the dimensions: feature, plate, well, screen. Each feature's minimum was added to the feature prior to $\log_n(x+1)$ transforming to adjust each feature's histogram to a more normal distribution. Following transformation, each plate in each screen was normalized separately for each feature by B-Score normalization[11,12]. There, a median polish was performed across the plate to remove spatial column and row biases from the plate. Additionally, the B-Score normalization centers and scales the data to be the residuals of the median polish divided by the median absolute deviation (mad) across all values of the plate and thus be symmetrically centered around zero and scaled in units of mad. An artifact resulting from the dispensing pattern of the MultiDrop dispenser is a Chess-board like pattern biasing all values spatially. Under the assumption that the values of the black panels and the white panels should both follow the same distribution, we performed the following normalization. We subtract the absolute difference of the means of the two groups from the group with the higher

mean, thus equaling the two group means ($X_{high,1} = X_{high,0} - |mean(X_{high}) - mean(X_{low})|$). This ensures that all features, plates and screens are on the same normal scale and can thus be compared statistically. Here, 38 representative features were chosen manually based on their biological significance (our ability to refer them back to cellular phenotypes) and their technical reproducibility between the two mock (DMSO) treated replicate screens and their information content, as measured by added variance (**Supplementary File 6**).

4. Statistical Methods

From the screening data, we derived metrics to judge whether a gene is a suitable candidate for gene-gene-drug combinatorial screening. For this purpose, several metrics have been deployed based on the Euclidean distances ($\sqrt{\sum_{i=1}^{n}(x_i - y_i)^2}$) between samples and controls under different conditions, Pearson correlation coefficients between independent design replicates and effect size compared to control. They are described comprehensively in the methods part and further summarized in **Supplementary File 1**.

VI. Combinatorial RNAi screening under differential time and treatment conditions

During this work, we conducted an experiment were genes were knocked down in a pairwise manner and under differential treatment conditions. After treatment, the process of phenotype development was followed over time. All lab ware and reagents, which are not further detailed here, were equivalent to previous experiments. The dsRNA sequences that were used in the combinatorial library can be found in **Supplementary File 4**.

1. Gene combinatorial library design and synthesis

168 genes were chosen for combinatorial library design. The library contained 12 batches for screening, each comprising 80 384-well Greiner µ-clear plates spotted with 250 ng dsRNA/well dissolved in 5 µl of DNase, RNase free water. dsRNAs were obtained from the HD3-library templates and synthesized accordingly. To avoid contaminations, all dsRNAs were sterile filtered using Steriflips-0.22µm for the query dsRNAs and MultiScreenHTS-GV 0.22 µm filter plates for the target dsRNAs. Here, genes were divided into target and query genes and screened a matrix of 76 genes combined with 168 genes. We defined the larger group as target genes and chose 76 of them to be the query genes. We screened each target gene in two sequence independent designs and each query gene in one design. This way, we screened 25536 dsRNA combinations (12768 gene pairs) in each batch. Combinatorial dsRNA spotting was achieved with combining the query and target master plates such that 2.5 µl of each query dsRNA were spotted onto 2.5 µl target dsRNA by a Beckman FX liquid handling robot using a 384-well pipetting head. This results in 76 x 384-well plates where each well, except the control wells in column 12 and 13, contains 250 ng of dsRNA targeting two different genes. Controls in columns 12 and 13 contained only 125 ng of dsRNA in the same volume of water. Control plates containing the uncombined target gene dsRNA matrix with 250 ng dsRNA per well complete one screening batch of 80 plates. To achieve differential treatment and time resolution, 12 screening batches were prepared. They were divided into two groups of six batches, which then were treated under different conditions in duplicate. 6 library batches are needed to screen two conditions (1.5 nM PD-0325901 and 0.5 % DMSO) at three time points

(48 h, 72 h, 96 h), all in all comprising 480 screened plates. The entire experiment was repeated twice and we screened 960 x 384-well plates.

2. *Assay workflow*

The assay workflow follows the same procedures as outlined above. Briefly, 9000 cells per well were seeded onto 384-well Greiner µClear plates for microscopy, which were pre-spotted with a combinatorial dsRNA library. After centrifugation, plates were sealed and left to incubate for 24 h at 25 °C prior to compound addition. Therefore, a PD-0325901 solution, (15 nM in medium with 5 % DMSO), and a 5 % DMSO only dilution in medium were prepared and added to the opened plates. This resulted in either 1.5 nM PD-0325901 or 0.5 % DMSO in-assay concentrations. Plates were closed again by heat sealing and left to incubate until the experiment was stopped by fixation after 48 h, 72 h and 96 h, respectively. Fixation with 4 % PFA and staining with Hoechst, Phalloidin-TRITC and anti-α-tubulin-FITC conjugate antibody were carried out using standard protocols on a CybiWell Vario liquid handling workstation. Stained plates were imaged on an InCell Analyzer 2200 automated high-throughput microscope according to the protocol above with 20x magnification, 3 channels per field and 4 fields per well.

3. *Data analysis workflow*

Resulting images were analyzed using the R/EBImage based pipeline described above. As a result, we obtained a data frame containing one row for each well that was screened and columns containing the measured features and well, plate and batch identifier for later trace back. The resulting raw data contained 368640 rows and 164 columns. To this end, data acquired from single cells was aggregated by calculating the trimmed mean (q=0.01) for each feature extracted in the respective well together with its standard deviation. This way, outliers, produced by over or under segmentation of cells, should be excluded from further analysis. Here, data were either kept as a table or transformed into a multidimensional data cube depending on the analysis. Data were normalized by dividing each feature in each plate by the median of the negative control wells (if that was not zero). Further, the values of each feature were glog transformed with c being the 3 % quantile of the features value distribution over all values. Glog transformation works effectively in smoothening the histogram of each feature without the anomaly observed by conventional log transformation [13]. For each feature, data were then scaled and centered around 0 by using the robust Z-transform, where the feature median is subtracted from each value and the result was divided by the median absolute deviation (x=(x-med(x))/mad(x)). After that, all feature data were in normalized units of median absolute deviation from the median of that feature normalized to each plate. The normalized feature vector provides the basis to all further analyses. Screening quality was assessed per plate such that plates with a replicate Pearson correlation smaller than 0.6 and a Z'-factor (between Diap1 and RasGAP1) smaller than 0.3 were masked by the NA_real_ constant, so all experimental dimensions remain complete while the values are masked from downstream analyses.

4. Statistical Methods

From normalized features per well, the data frame is reformatted to fit the experimental design into a 5-dimensional data cube. The dimensions are: target dsRNA (2 entries for each gene), query gene, time, treatment and feature. The data cube is further subjected to genetic interaction inference following the protocol established by Bernd Fischer[13]. There, genetic interactions are defined as the residuals of a modified median polish over the double perturbation matrix of one replicate, feature, treatment and time point. In the median polish a robust linear fit ($\boldsymbol{\delta_{ij} = m_i + n_j + \pi_{ij} + \varepsilon}$) is used to lift the main effect (m, n) of each query such that it resembles the value of a single gene knockdown. The residuals of this fit scaled by their median absolute deviation are then defined as π-scores. π-scores further provide us with a quantitative measure of genetic interaction following the multiplicative model plus an error term (ε) estimating the experimental noise. There, the interaction of two genes is defined as the deviation of the measured combined phenotype (δ) from the expected. The expected phenotype would then be a multiple of the two independent single knockdowns. The resulting π-scores were collected for all replicates (dsRNA and experimental) and the significance of their mean over all measured scores was estimated by a moderated students t-test as is implemented in the R-package limma. There, the t-test was adapted for situations where a small amount of observations was tested in many tests, normally causing large test variability, using an empirical Bayes variance estimator. P-values were adjusted using the methods of Benjamini Hochberg method [9]. From there on, adjusted p-values were used to control false discovery rates. The FDR estimates the chance that the finding was observed by chance given the entire dataset.

5. qPCR analysis

Quantitative real time PCR (qPCR) was used to analyze the transcriptional response following Rel/pnt co-RNAi. To this end, as $5*10^5$ cells / well were seeded in 630 µl ExpressFive (Gibco) culture medium (supplemented with 10 % Glutamax, Gibco) and reverse transfected with 14 µg dsRNA (0,2 µg/µl solution in sterile DNase/RNase free water), at the time of seeding, without transfection reagents. For this experiment, all dsRNAs denoted with #2 were used in 3 biological replicates. Combinatorial RNAi was achieved by mixing 7 µg of dsRNA targeting each gene (**Supplementary File 5**). After 72 h incubation at (25 °C), cells were washed once in 750 µl PBS (Gibco) and lysed in 350 µl RLT buffer shipped with the RNAeasy-mini Kit (Qiagen). RNA was then purified from all samples according to manufacturer's standard instructions for spin column purification. An optional DNase digestion step was performed using the RNase-Free DNase Set (Qiagen). RNA was diluted from the spin columns using 40 µl RNase/DNase-free water. The concentration was measured using a NanaDrop spectrometer (Thermo scientific). Samples were prepared for qPCR by reverse transcription of 1 µg of RNA using RevertAid H minus First strand cDNA Synthesis kit (Thermo scientific) according the manufactures standard protocol. A qPCR reaction was prepared using PrimaQuant 2x qPCR-Mastermix (Steinbrenner, Steinbrenner Laborsysteme GmbH, Wiesenbach, Germany) by mixing 5 µl of sample (1:10 diluted cDNA) with 5 µl of Mastermix (including 0,3 µM of forward and reverse primer) on a 384-well qPCR plate (LightCycler 480 Multiwell Plate 384, white, Roche, Ref: 4729749001, Roche Molecular Systems Inc., Pleasanton, California, USA). The plate was then centrifuged (2 min, 2000 rpm) and processed for qPCR in a Roche 480 LightCycler using the following PCR program: (i) 10 min at 95 °C, (ii) 15 sec at 95 °C, (iii) 60

sec at 60 °C, repeat step ii and iii 40 times and measure fluorescence at 494 nm-521 nm during step. Melting curve analysis of each sample was performed to assess reaction quality. Relative expression of each gene in each sample (normalized to rps7 expression) was analysis as log_2-foldchange over RLUC dsRNA treated samples [15,16]. qPCR primers were designed using the GETprime web service (**Supplementary File 8,9**) [17].

6. qPCR and Western Blot analysis of Rel/pnt crosstalk

Since phosphorylation levels of rl were already equalized after 96 hours of different dsRNA treatments (*Sos* k.d., MEK inhibition and rl k.d. showed no or little reduction in pp-rl, data not shown) we examined the molecular response also 48 hours after dsRNA transfection. To this end, we seeded $1*10^6$ S2 cells per well into a 12-well cell culture plate (Greiner) and transfected them with 28 µg of each dsRNA (14 µg + 14 µg in case of co-RNAi). Cells were then harvested after 48 hours and proceeded to qPCR analysis as outlined above. In this experiment sequence, independent dsRNAs targeting each gene were used (#1 and #2 of each gene) as replicates. Phenotypic analysis during image-based screenings confirmed high reproducibility (PCC>0.8) between either dsRNA reagents targeting the same gene.

Another replicate of these same samples was proceeded to Western Blot analysis of pp-rl levels according to the same protocol as outlined previously.

VII. References

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