# ▪ Supplementary Information

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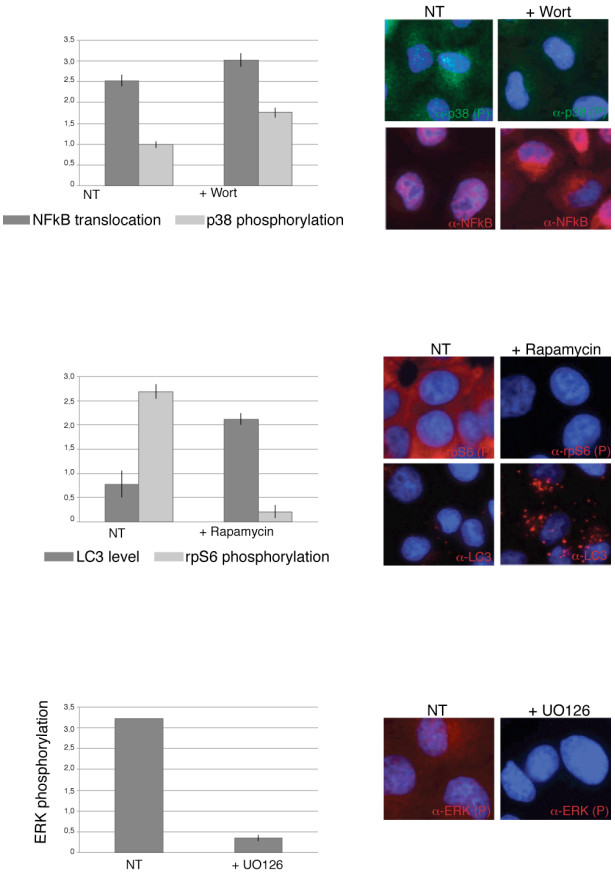



**Fig. S1. Inhibition of specific upstream kinases affects the activity of the analyzed readouts.** The NFkB and p38 activity was monitored in HeLa cells treated with 200 μM wortamannin (PI3K inhibitor) per 2 hours and then stimulated with TNFα for 10 minutes. The mTOR as well as autophagy activation were analyzed in HeLa cells incubated with medium containing 100 μM rapamycin (mTOR inhibitor) per 1 hour. While the ERK phosphorylation level was measured in cells treated with 10 μM UO126 (MEK inihibitor) per 1 hour. Cells were fixed and stained with anti-phospho p38, anti-NFkB (p65), anti-LC3, anti-phospho rpS6 and anti-phospho ERK antibodies. Four images per samples were acquired by indirect immunofluorescence on Delta Vision microscope with a 20X objective. Images were automatically analyzed by Cell Profiler and the signal intensity of each antibody was measured in both the nuclear and the cytosolic compartments. NFkB activation was measured as the ratio between the nuclear and the cytosolic fraction in each cell. For each sample, the mean value was measured and plotted in the bar graph. ERK as well as p38 activation were measured as the phosphorylation level of such antibodies in the nuclear region. For each image, the mean values and standard deviation were measured and plotted in the bar graph. rpS6 phosphorylation as well as LC3 intensity were measured in the cytosolic region. For each image, the mean value and the standard deviation were calculated and plotted in the bar graph.

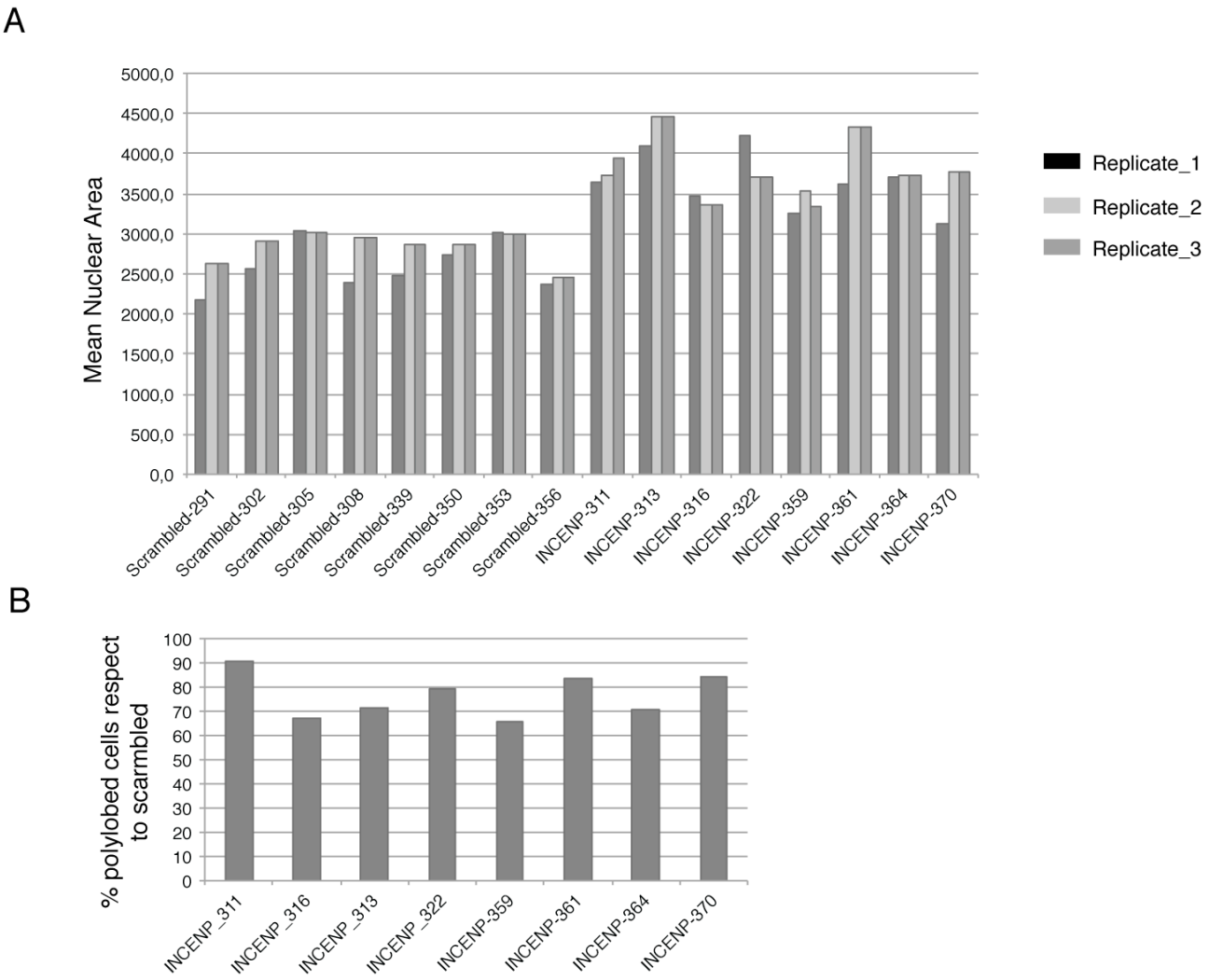



**Fig. S2. Down-regulation of INCENP gene induces the formation of polylobed nuclei. A)** Each LabTek, used in the primary screening, contains 8 control spots, in which the siRNA oligo targeting the INCENP gene was spotted. As previously demonstrated, the down-regulation of the INCENP gene induces the formation of polylobed nuclei [1]. To assess the transfection efficiency variability across the different spots of the different labTeks, the area of the nuclei was measured and plotted as bar graph. The three biological replicas are shown as different grey tones. **B)** The percentage of polylobed nuclei has been measured and plotted by counting the nuclei, whose area is larger than twice the mean nuclear area in scrambled controls. For a few images we have checked that this estimates match the frequency of polylobed cells as estimated visually.

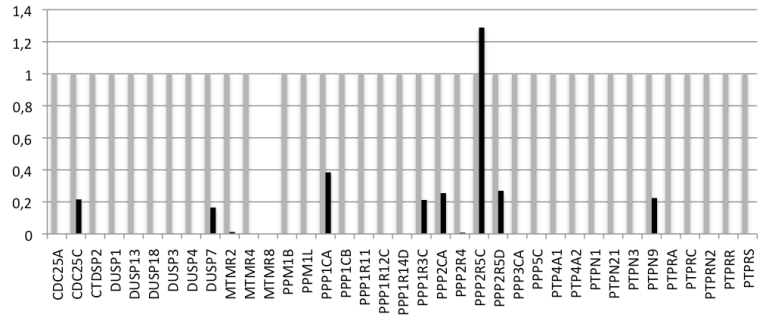



**Fig. S3. Knock-down analysis of specific phosphatase mRNA by siRNA.** Total RNA was isolated from HeLa cells and primers designed to specifically amplify the indicated phosphatases and a reference gene (HPRT) were used in RT-PCR reactions. Data were quantified and the relative mRNA expression was calculated applying the comparative CT method [2] and then plotted. mRNA levels were measured after introduction of three different siRNA oligos (black bar) or of a scrambled sequence, as negative control (grey bars).

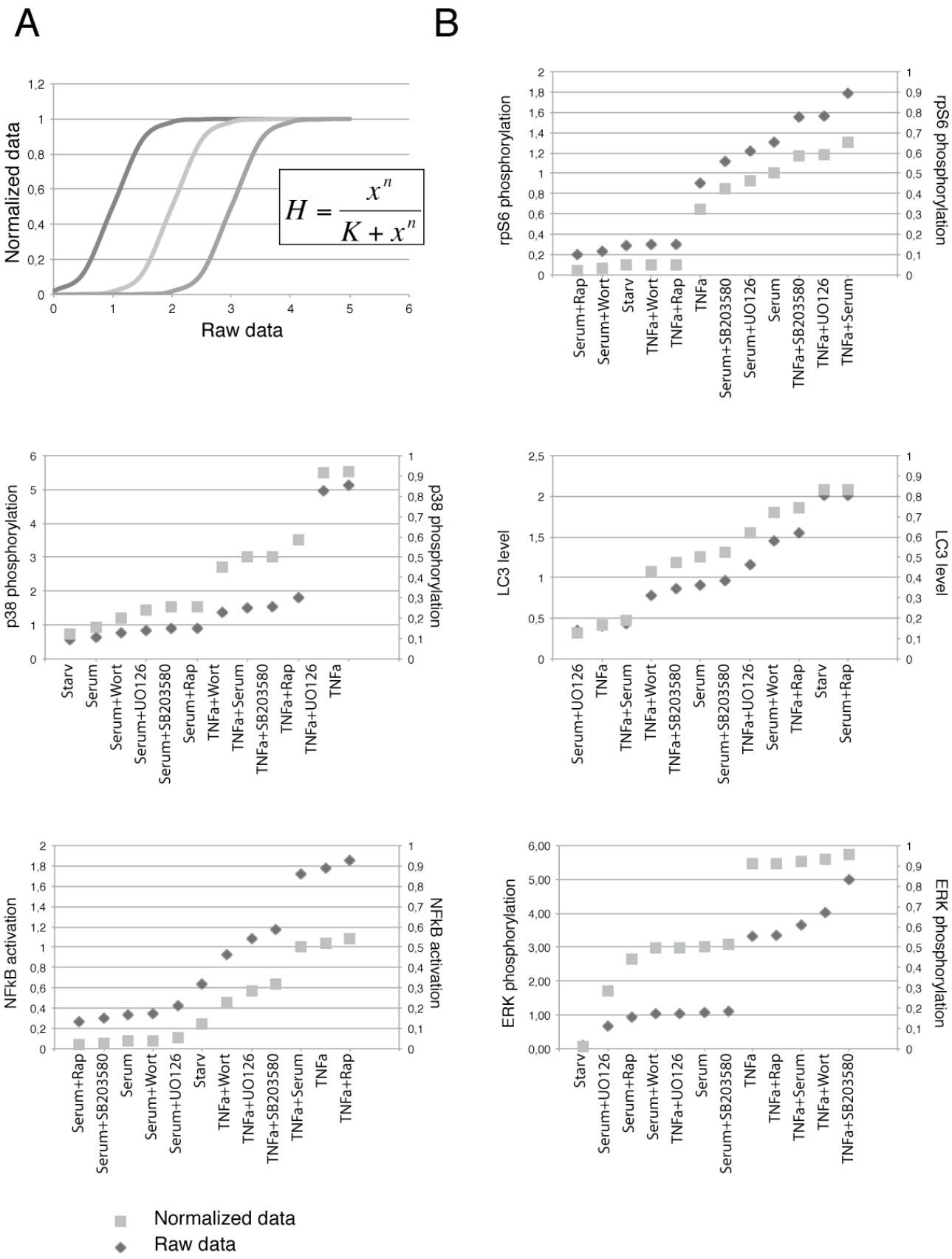
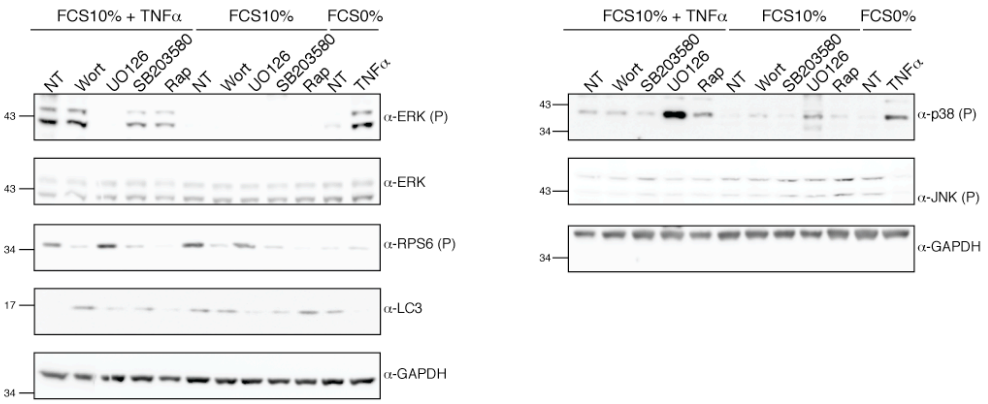


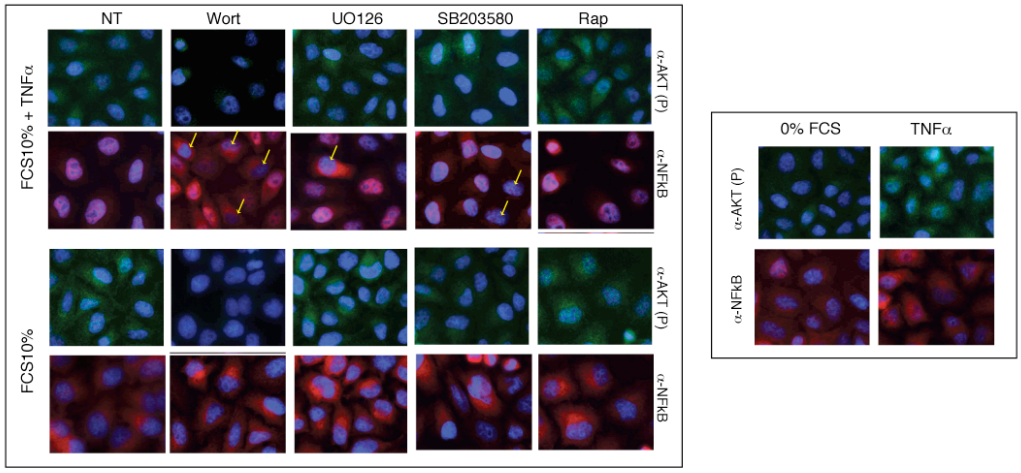

**Fig. S4. Hill normalization of experimental data. A) Experimental data were normalized in the 0-1 range by the Hill function.** Three different Hill functions have been plotted in the upper left panel. From left to right, the Hill functions have an inflection point at 1, 2, 3 respectively. For

each function, the Hill coefficient K was set to 2. **B to F**) For each analyzed phenotype, each measurement was normalized in the 0-1 range, by applying the Hill function. Dark grey points represent raw data, while light grey points represent normalized data. The different left and right y-scales represent raw and normalized values respectively. The points were sorted by raw value.

A




B




C

| | ERK | LC3 | RPS6 | NFkB | p38 | JNK | AKT | ERK | LC3 | RPS6 | NFkB | p38 | JNK | AKT |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| TNFα+Serum | 3.66 | 0.44 | 1.79 | 1.72 | 1.51 | 1.16 | 1.48 | 0.69 | 0.05 | 1.00 | 0.91 | 0.74 | 0.09 | 0.60 |
| TNFα+Wort | 4.01 | 0.79 | 0.30 | 0.93 | 1.36 | 1.06 | 0.30 | 0.77 | 0.26 | 0.06 | 0.42 | 0.70 | 0.01 | 0.00 |
| TNFα+UO126 | 1.05 | 1.16 | 1.57 | 1.08 | 4.95 | 1.34 | 1.34 | 0.09 | 0.49 | 0.86 | 0.51 | 1.00 | 0.25 | 0.53 |
| TNFα+SB203580 | 4.99 | 0.87 | 1.56 | 1.18 | 1.52 | 1.05 | 1.46 | 1.00 | 0.31 | 0.86 | 0.57 | 0.75 | 0.00 | 0.59 |
| TNFα+Rap | 3.34 | 1.54 | 0.30 | 1.86 | 1.79 | 1.14 | 2.12 | 0.62 | 0.72 | 0.07 | 1.00 | 0.80 | 0.08 | 0.92 |
| Serum | 1.05 | 0.91 | 1.30 | 0.34 | 0.64 | 1.07 | 2.09 | 0.09 | 0.34 | 0.70 | 0.04 | 0.17 | 0.01 | 0.91 |
| Serum+Wort | 1.04 | 1.45 | 0.23 | 0.35 | 0.76 | 1.11 | 0.50 | 0.09 | 0.66 | 0.02 | 0.05 | 0.33 | 0.05 | 0.10 |
| Serum+UO126 | 0.66 | 0.35 | 1.22 | 0.42 | 0.85 | 1.27 | 2.08 | 0.00 | 0.00 | 0.64 | 0.10 | 0.42 | 0.20 | 0.90 |
| Serum+SB203580 | 1.09 | 0.96 | 1.12 | 0.30 | 0.89 | 1.16 | 1.86 | 0.10 | 0.36 | 0.58 | 0.02 | 0.46 | 0.10 | 0.79 |
| Serum+Rap | 0.94 | 2.01 | 0.19 | 0.27 | 0.89 | 1.91 | 2.27 | 0.06 | 1.00 | 0.00 | 0.00 | 0.46 | 0.76 | 1.00 |
| Starv | 1.12 | 2.01 | 0.29 | 0.64 | 0.56 | 2.19 | 0.84 | 0.11 | 1.00 | 0.06 | 0.23 | 0.10 | 1.00 | 0.28 |
| TNFα | 3.31 | 0.41 | 0.90 | 1.78 | 5.14 | 1.30 | 2.14 | 0.61 | 0.03 | 0.44 | 0.95 | 1.00 | 0.22 | 0.93 |

**Fig. S5. Training of the HeLa Boolean model.** HeLa cells treated, as indicated, were analyzed by SDS-PAGE or by Immuno-fluorescence. **A)** Protein extracts were revealed with anti-phospho ERK, anti-ERK, anti-LC3, anti-phospho rpS6, anti-phospho JNK, anti-phospho p38, anti-Tubulin and anti-GAPDH antibodies. **B)** Cells were fixed and stained with anti-NFkB (TRITC), anti-phospho AKT (FITC) and DAPI, to visualize nuclei. Four fields containing approximately

400 cells were acquired for each sample by indirect immunofluorescence (20X objective) and automatically analyzed by the Cell Profiler software. **C)** Intracellular signaling protein activity was quantified (raw data) and then, normalized by the Hill function, as described in Material and Method section. The red or green box color indicates respectively low and high values.

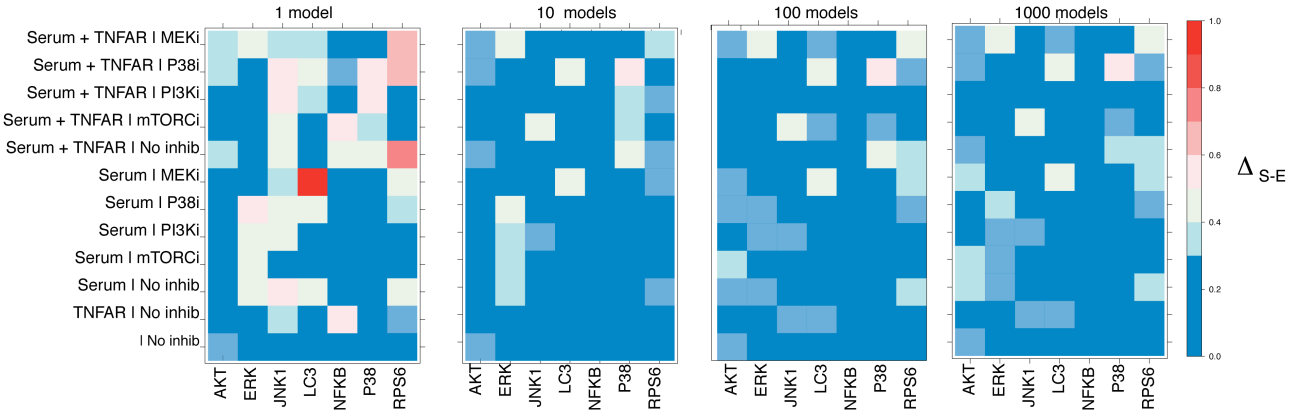



**Fig. S6. Performance of the optimized model.** The absolute value of the difference (Δ) between the model simulation results (S) to normalized experimental data (E), is represented with a color code. If $\Delta_{S-E}$ =1 (strongest disagree) or $\Delta_{S-E}$= 0 (strongest agree) the box color is red or blue respectively. Intermediate values of $\Delta_{S-E}$ are mapped to shades of red and blue. Simulation results of the average of respectively 1, 10, 100, 1000 models are shown.

## ▪ Supplementary Tables

**Table S1. siRNA screening results per cell.** For each analyzed image, the mean intensity value of each cell has been reported.

See Supplementary Table S1.

**Table S2. siRNA primary screening results.** HeLa cells were transfected with three different oligos targeting 300 human phosphatases or phosphatase related genes and 84 positive (transfection control) and negative control oligos. For each phosphatase interfered by siRNA screening, Uniprot ID, gene name, protein name and Ambion Oligo ID have been reported. In addition according to substrate specificity, phosphatases were classified as Protein Tyrosine Phosphatase (PTP), Phosphoserine Proteine Phosphatases (PPP), Protein Phosphatase Metal dependent (PPM), Carboxy Terminal Domain phosphatases (CTD), Regulatory Subunits (RS), Sugar Phosphatase (SP), Lipid Phosphatase (LP), Nucleotide Phosphatase (NP) and Alkaline Phosphatase (ALP). As final value for each data point, the median of the three biological replicates was used. For each analyzed phenotype, a robust Z-score was calculated and reported (from column F to G).

See Supplementary Table S2.

| Accession | Entry name | Phenotype | Readout ratio with respect to the scrambled control | Fraction of mRNA expression levels after interferencel | Validated |
|---|---|---|---|---|---|
| P30304 | CDC25A | p38 phosphorylation | 0,67 | 0,00 | No |
| P30307 | CDC25C | rpS6 phosphorylation | 1,89 | 0,22 | Yes |
| P30307 | CDC25C | ERK phosphorylation | 1,82 | 0,22 | Yes |
| P30307 | CDC25C | NFkB translocation | 1,74 | 0,22 | Yes |
| O14595 | CTDSP2 | ERK phosphorylation | 1,19 | 0,00 | No |
| O14595 | CTDSP2 | p38 phosphorylation | 0,96 | 0,00 | No |
| P28562 | DUSP1 | LC3-II level | 1,73 | 0,00 | Yes |
| P28562 | DUSP1 | rpS6 phosphorylation | 0,41 | 0,00 | Yes |
| Q9UII6 | DUSP13 | rpS6 phosphorylation | 0,65 | 0,00 | No |
| Q8NEJ0 | DUSP18 | rpS6 phosphorylation | 2,18 | 0,00 | Yes |
| Q8NEJ0 | DUSP18 | ERK phosphorylation | 1,77 | 0,00 | Yes |
| P51452 | DUSP3 | NFkB translocation | 1,98 | 0,00 | Yes |
| P51452 | DUSP3 | rpS6 phosphorylation | 1,00 | 0,00 | No |
| Q13115 | DUSP4 | rpS6 phosphorylation | 0,49 | 0,00 | Yes |
| Q13115 | DUSP4 | p38 phosphorylation | 0,78 | 0,00 | No |
| Q13115 | DUSP4 | NFkB translocation | 0,49 | 0,00 | Yes |
| Q16829 | DUSP7 | rpS6 phosphorylation | 0,48 | 0,16 | Yes |
| Q16829 | DUSP7 | p38 phosphorylation | 1,44 | 0,16 | Yes |
| Q13614 | MTMR2 | rpS6 phosphorylation | 1,01 | 0,01 | No |
| Q13614 | MTMR2 | ERK phosphorylation | 1,55 | 0,01 | Yes |
| Q9NYA4 | MTMR4 | LC3-II level | 1,65 | 0,00 | Yes |
| Q96EF0 | MTMR8 | ERK phosphorylation | 1,04 | 0,00 | No |
| O75688 | PPM1B | rpS6 phosphorylation | 0,98 | 0,00 | No |
| O75688 | PPM1B | NFkB translocation | 1,64 | 0,00 | Yes |
| Q5SGD2 | PPM1L | LC3-II level | 1,55 | 0,00 | Yes |
| Q5SGD2 | PPM1L | NFkB translocation | 1,25 | 0,00 | No |
| P62136 | PPP1CA | rpS6 phosphorylation | 1,89 | 0,38 | Yes |
| P62140 | PPP1CB | rpS6 phosphorylation | 1,54 | 0,00 | Yes |
| O60927 | PPP1R11 | LC3-II level | 1,85 | 0,00 | Yes |
| O60927 | PPP1R11 | ERK phosphorylation | 2,18 | 0,00 | Yes |
| Q9BZL4 | PPP1R12C | LC3-II level | 1,14 | 0,00 | No |
| Q9BZL4 | PPP1R12C | rpS6 phosphorylation | 0,67 | 0,00 | No |
| Q9NXH3 | PPP1R14D | rpS6 phosphorylation | 1,67 | 0,00 | Yes |
| Q9NXH3 | PPP1R14D | p38 phosphorylation | 2,02 | 0,00 | Yes |
| Q9NXH3 | PPP1R14D | NFkB translocation | 1,62 | 0,00 | Yes |
| Q9UQK1 | PPP1R3C | LC3-II level | 1,57 | 0,21 | Yes |
| Q9UQK1 | PPP1R3C | rpS6 phosphorylation | 0,39 | 0,21 | Yes |
| P67775 | PPP2CA | rpS6 phosphorylation | 2,03 | 0,25 | Yes |
| P30154 | PPP2R1B | rpS6 phosphorylation | 0,44 | NA | Yes |
| Q15257 | PPP2R4 | ERK phosphorylation | 1,93 | 0,01 | Yes |
| Q13362 | PPP2R5C | rpS6 phosphorylation | 1,36 | 1,29 | No |
| Q13362 | PPP2R5C | ERK phosphorylation | 1,23 | 1,29 | No |
| Q14738 | PPP2R5D | rpS6 phosphorylation | 1,07 | 0,27 | No |
| Q08209 | PPP3CA | LC3-II level | 2,02 | 0,00 | Yes |
| Q08209 | PPP3CA | ERK phosphorylation | 1,85 | 0,00 | Yes |
| Q08209 | PPP3CA | rpS6 phosphorylation | 1,79 | 0,00 | Yes |
| P53041 | PPP5C | LC3-II level | 0,33 | 0,00 | Yes |
| Q93096 | PTP4A1 | NFkB translocation | 1,76 | 0,00 | Yes |
| Q12974 | PTP4A2 | p38 phosphorylation | 1,79 | 0,00 | Yes |
| P18031 | PTPN1 | rpS6 phosphorylation | 1,89 | 0,00 | Yes |
| Q16825 | PTPN21 | LC3-II level | 0,39 | 0,00 | Yes |
| Q16825 | PTPN21 | ERK phosphorylation | 0,35 | 0,00 | Yes |
| Q16825 | PTPN21 | p38 phosphorylation | 0,50 | 0,00 | Yes |
| Q16825 | PTPN21 | NFkB translocation | 0,49 | 0,00 | Yes |
| P26045 | PTPN3 | rpS6 phosphorylation | 1,54 | 0,00 | Yes |
| P43378 | PTPN9 | rpS6 phosphorylation | 1,79 | 0,22 | Yes |
| P18433 | PTPRA | p38 phosphorylation | 1,80 | 0,00 | Yes |
| P18433 | PTPRA | NFkB translocation | 1,67 | 0,00 | Yes |
| P08575 | PTPRC | LC3-II level | 1,96 | 0,00 | Yes |
| Q92932 | PTPRN2 | p38 phosphorylation | 1,81 | 0,00 | Yes |
| Q15256 | PTPRR | LC3-II level | 2,00 | 0,00 | Yes |
| Q13332 | PTPRS | rpS6 phosphorylation | 1,84 | 0,00 | Yes |

**Table S3. siRNA secondary screening results.** HeLa cells were transfected with a pool of three plasmids (Mission® shRNA phosphatase library, Sigma) directing the synthesis of three different siRNA targeting each phosphatase hit and scrambled sequences. 48 hours after transfection, the cell cultures were stimulated with TNFα for 10 minutes or left untreated, as in the primary

screening. Upon phosphatase down-regulation, the activation level of ERK, p38 as well as autophagy was monitored by western-blot, whereas rpS6 phosphorylation and NFkB nuclear translocation were analyzed by indirect immunofluorescence. For each phosphatase hits, Uniprot ID and gene name have been reported in the first two columns. The phenotype affected by phosphatase interference is reported in the third column. Results are expressed as ratios with respect to the scrambled control (fourth column). Hits having a ratio lower than 0.5 or higher than 1.5 were classified as confirmed. The success in gene down regulation was monitored by quantitative PCR. The ratio of mRNA levels after and before shRNA treatment is reported in the fifth column. The final result of secondary screening is summarized in last column.

| Uniprot A | Protein A | Effect | Uniprot B | Protein B | Pmid |
|---|---|---|---|---|---|
| P31749 | AKT | -1 | P04049 | RAF | 11443134 |
| P31749 | AKT | 1 | O14920 | IKK | 11259436 |
| P31749 | AKT | -1 | Q92574 | TSC | 16636147 |
| Q9C0C7 | AMBRA1 | 1 | Q14457 | BCN1 | 17589504 |
| Q9Y478 | AMPK | 1 | Q92574 | TSC | 14651849/19584320 |
| Q9Y478 | AMPK | 1 | O75385 | ULK | 21205641/19584320 |
| P10415 | BCL2 | -1 | Q14457 | BCN1 | 18570871 |
| Q14457 | BCN1 | 1 | 92/Q9GZQ8/Q9B | LC3 | 18843052 |
| P53355 | DAPK | 1 | Q14457 | BCN1 | 19180116 |
| P53355 | DAPK | 1 | P28482 | ERK | 15616583 |
| P28482 | ERK | 1 | O75582 | MSK | 15568999/9687510/18267068 |
| P28482 | ERK | -1 | Q16539 | P38 | 11842088/18481201 |
| P28482 | ERK | -1 | P01112 | RAS | 8626428 |
| P28482 | ERK | -1 | Q92574 | TSC | 17671177 |
| P28482 | ERK | 1 | P53355 | DAPK | 15616583 |
| O14920 | IKK | 1 | Q04206 | NFKB | 9346241/18267068 |
| O14920 | IKK | 1 | Q16539 | P38 | 11259436/18267068 |
| P45983 | JNK1 | -1 | P10415 | BCL2 | 18570871 |
| Q02750 | MEK | 1 | P28482 | ERK | 9677429 |
| Q02750 | MEK | 1 | P28482 | ERK | 9677429 |
| O75582 | MSK | 1 | Q04206 | NFKB | 12628924/18267068 |
| P42345 | mTORC | 1 | Q15418 | S6K | 15809305 |
| P42345 | mTORC | 1 | O75385 | ULK | 19690328 |
| Q16539 | P38 | 1 | O75582 | MSK | 15568999/ 9687510/18267068 |
| Q16539 | P38 | -1 | P28482 | ERK | 18481201 |
| P42336 | PI3K | 1 | P31749 | AKT | 12167717 |
| P01112 | RAS | 1 | P04049 | RAF | 8622647 |
| Q15418 | S6K | 1 | P62753 | RPS6 | 15809305 |
| Q15418 | S6K | -1 | P45983 | JNK1 | 17181399 |
| na | Serum | -1 | P45983 | JNK1 | 15899879 |
| na | Serum | -1 | Q9Y478 | AMPK | 19584320 |
| P19438 | TNFAR | 1 | Q16539 | P38 | 16813528 |
| P19438 | TNFAR | 1 | O14920 | IKK | 10795740 |
| P19438 | TNFAR | 1 | P45983 | JNK1 | 15837794 |
| Q92574 | TSC | -1 | P42345 | mTORC | 12271141 |
| O75385 | ULK | 1 | Q9C0C7 | AMBRA1 | 20921139 |
| P01112 | RAS | 1 | P42336 | PI3K | 8052307 |
| P19438 | TNFAR | 1 | P62993 | GRB2 | 10359574 |
| P62993 | GRB2 | 1 | Q07889 | SOS | 8479541 |
| Q07889 | SOS | 1 | P01112 | RAS | 8493579 |
| P62993 | GRB2 | 1 | Q13480 | GAB1 | 12766170 |
| Q13480 | GAB1 | 1 | P42336 | PI3K | 11606067 |
| P04049 | RAF | 1 | Q02750 | MEK | 8621729 |
| na | Serum | 1 | P62993 | GRB2 | 9356464 |
| P42345 | mTORC | 1 | O14920 | IKK | 18519641 |
| P19438 | TNFAR | 1 | O43318 | TAK1 | 16260783 |
| O43318 | TAK1 | 1 | P45985 | MKK4 | 17875933 |
| P45985 | MKK4 | 1 | P45983 | JNK1 | 11062067 |
| O43318 | TAK1 | 1 | P46734 | MKK3 | 15837794 |
| P46734 | MKK3 | 1 | Q16539 | P38 | 7535770 |
| O43318 | TAK1 | 1 | O14920 | IKK | 11460167 |
| P19438 | TNFAR | 1 | P19174 | PLC | 12645577 |
| P19174 | PLC | 1 | P17252 | PKC | 12645577 |
| P17252 | PKC | 1 | P12931 | SRC | 12645577 |
| P12931 | SRC | 1 | O14920 | IKK | 12645577 |
| P12931 | SRC | 1 | P42336 | PI3K | 7797556 |
| P12931 | SRC | 1 | P04049 | RAF1 | 7517401 |
| P12931 | SRC | 1 | P29353 | SHC | 10523831 |
| P29353 | SHC | 1 | P62993 | GRB2 | 10523831 |

**Table S4. Literature-derived directed network.** Experimental data describing the functional relationships between signaling proteins in the pathways of interest were collected from the literature (Pmid column). This information is represented as a signed directed model, where edges have sign (activating, indicated as 1, or inhibitory, indicated as -1) and directionality (enzyme–substrate relationships). For each protein, Uniprot ID and gene name have been reported.

| Phosphatase | Observed phenotype in siRNA screening | Literature experimental evidence | Pmid |
|---|---|---|---|
| PPP3CA | LC3 dots increase | Calcineurin (PPP3CA) defective strains exhibit enhanced autophagy | 19279398 |
| MTMR2 | rpS6 (P) decrease | MTMR2 over-expression increase AKT activation | 19912440 |
| PTPN9 | rpS6 (P) increase | PTPN9 inhibition augmented the phosphorylation of AKT in response to insulin | 16679294 |
| PPP2R5C | rpS6 (P) increase | PPP2R5C counteracts p70S6K phosphorylation | 20444422 |
| PPP2CA | rpS6 (P) increase | Inhibition of PP2A, by okadaic acid, partially reverses inactivation of mTORC1 substrates | 20639120 |
| PTPN1 | rpS6 (P) increase | PTP-1B-/- adipose shows an increased basal activity of p70S6K | 17664276 |
| PTPN21 | ERK (P) decrease | PTPN21 activates SRC tyrosine kinase and increases the ERK phosphorylation | 15143158 |
| DUSP4 | p38 (P) decrease | mice DUSP4-/- show a decreased phosphorylation of p38 | 20351138 |
| PPM1B | NFkB activity increase | Overexpression of PPM1B results in dephosphorylation of IKKbeta and termination of NF-kappaB activation | 18930133 |
| PPP2R1A | NFkB activity increase | PPP2R1A binds the CBM complex, inactivating the NFkB signaling | 21157432 |
| DUPD1 | ERK (P) increase | DUPD1 is a MAPK phosphatase | 21199871 |

**Table S5. Literature reported phosphatase-pathway relationship.** An extensive literature curation was carried out and phosphatase-pathway relationships already described were annotated.

## ▪ References


1. Neumann, B. et al. Phenotypic profiling of the human genome by time-lapse microscopy reveals cell division genes. *Nature* **464**, 721-727 (2010).
2. Yuan, J.S., Reed, A., Chen, F. & Stewart, C.N., Jr. Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**, 85 (2006).