A high-throughput integrated microfluidics method enables tyrosine autophosphorylation discovery

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Autophosphorylation of receptor and non-receptor tyrosine kinases is a common molecular switch with broad implications for pathogeneses and therapy of cancer and other human diseases. Technologies for large-scale discovery and analysis of autophosphorylation are limited by the inherent difficulty to distinguish between phosphorylation and autophosphorylation in vivo and by the complexity associated with functional assays of receptors kinases in vitro. Here, we report a method for the direct detection and analysis of tyrosine autophosphorylation using integrated microfluidics and freshly synthesized protein arrays. We demonstrate the efficacy of our platform in detecting autophosphorylation activity of soluble and transmembrane tyrosine kinases, and the dependency of in vitro autophosphorylation assays on membranes. Our method, Integrated Microfluidics for Autophosphorylation Discovery (IMAD), is high-throughput, requires low reaction volumes and can be applied in basic and translational research settings. To our knowledge, it is the first demonstration of posttranslational modification analysis of membrane protein arrays.
Protein arrays complement mass-spectrometry in proteomic research. Much like DNA microarrays, standard protein arrays are essentially a matrix spotted with thousands of proteins. Each protein is equally represented and virtually the only one in its spot, thereby circumventing the main challenge in mass-spectrometry-based analyses, i.e., protein/peptide relative abundance. This challenge is much heightened in the context of protein posttranslational modification (PTM) discovery. First, PTMs are reversible, highly dynamic, and often occupying only a small fraction of the target protein. Second, PTMs are identified on their unique target peptides, which can be low abundant by themselves. Standard protein arrays, however, rely on purified recombinant proteins and thus, incompatible with insoluble and other biochemically challenging proteins. Moreover, the spotted proteins are aged in non-physiological conditions for weeks if not months before use, raising concerns about protein folding and functionality.

Integrated microfluidics paved the way to freshly expressed protein arrays. The microfluidic platform enables expression of thousands of proteins in reticulocyte lysates. A set of pneumatic valves, allow compartmentalization of each target protein in individual unit cells, overriding major limitations and caveats of open protein arrays. This technology was originally developed for screening direct protein–protein interactions. Interaction between proteins and nucleic acids was also showed. More recently, the platform was proven to be compatible also with protein PTM analyses. In that study, we applied recombinant enzymes or active cell extracts to the chip to promote PTM of fresh proteins in quasi-cellular environments. Both the target protein and the protein modifier were then quantified colorimetrically to derive a normalized PTM signal. Tyrosine (Tyr) phosphorylation, ubiquitination, and ubiquitin chain preference was demonstrated. Although functional as substrates for protein interactions and PTMs, it is still unclear whether the arrayed proteins maintain intrinsic catalytic activity. This is not a marginal distinction because enzymes are expected to be considerably more demanding in terms of folding and functionality. Arrays of functional enzymes for high-throughput activity assays are valuable for basic and translational research; in fact, targeting enzymes is a major strategy in drug design.

Autophosphorylation is a biochemical process in which a phosphate (P) group is added to a protein kinase by itself. This molecular node is a ubiquitous mediator between extracellular cues and signal transduction pathways associated with a great variety of normal and pathological processes ranging from cancer to complex developmental disorders. We evaluated the potency of integrated microfluidic as an enzymatic array, focusing on autophosphorylation of soluble and membrane Tyr kinases.

**Results**

On-chip autophosphorylation of soluble p-Tyr. The shift from Tyr phosphorylation to Tyr autophosphorylation assay on our microfluidic platform is conceptually simple (Fig. 1). In brief, a device combining a microarray spotted with a double-tagged cDNA library of interest, and bilayer microfluidics that are based on polydimethylsiloxane (PDMS) lithography, is assembled. This design generates a set of channels regulated by pneumatic valves capturing each of the spotted cDNA molecules in an individual chamber, i.e., DNA chamber (Fig. 1b). Reticulocyte lysate is then applied to all DNA chambers for in vitro transcription and translation. The resulted in vitro transcription and translation products with intrinsic phosphorylation activity potentially undergo autophosphorylation during the expression process (Fig. 1d). Following washing, each unit cell is enriched with a single kind of protein whose level and P-Tyr level are both quantified in situ by immunofluorescence to determine total or net autophosphorylation on chip (Fig. 1e). We hypothesized that this course of events has the potential to specifically detect autophosphorylation if the arrayed proteins are catalytically active.

We base this hypothesis on the assumption that little or no Tyr kinase activity is present in the reticulocyte lysate. To test for specific autocatalytic activity, we first measured the intrinsic P-

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**Fig. 1** Device and strategy. Integrated microfluidic device combined with His/Myc-double-tagged ORF library spotted on glass (the observed DNA in the picture encodes for FRK protein), allow parallel expressions of thousands of proteins ready for biochemical assays. Each unit cell comprises DNA and protein chambers isolated by valves. a The entire microfluidics device. b Target proteins are expressed in DNA chambers following incubation with reticulocyte lysate, diffused, and immobilized in protein chambers via His tag. Overall, 10 μl reagents are sufficient to cover a chip of thousands unit cells. c Proteins are expressed in mammalian cell lysates. Thus, enzymes with inherent autophosphorylation activity are expected to be functional and undergo autophosphorylation during expression. d, e Arrayed proteins and phosphorylated Tyr (P-Tyr) are both quantified in situ using Cy3-coupled anti-Myc antibodies and Cy5-coupled anti-phosphorylated-Tyr antibodies, respectively. Finally, a net autophosphorylation signal is determined.
Tyr signals of 882 human proteins on chip, underrepresented for Tyr kinases (see Supplementary Table 1). We used FRK and HCK, known non-receptor Tyr kinases, as positive controls. After normalization to protein levels, mean P-Tyr-to-protein ratios were plotted (Fig. 2a; Supplementary Figure 1). Only the following three proteins: Hck, Frk, and Rcl1 (in that order), exhibited noticeable, P-Tyr signal observed for inactive Hck. Owing to the majority of Hck signals of the two mutant kinases dropped by 75 to 90%, nearly reaching a background signal. Noteworthy, a faint, albeit noticeable, P-Tyr signal observed for inactive Hck. Owing to the overall low Tyr kinase activity in reticulocyte lysates, as demonstrated for hundreds of proteins (Fig. 2a), it is easy to speculate that Hck carrying a Lys to Glu mutation at position 290 can still maintain a weak catalytic activity. Regardless, the majority of Hck’s P-Tyr signal resulted from autophosphorylation per se. We independently validated the autophosphorylation of Frk and Hck by immunoprecipitation of these two proteins with anti-P-Tyr antibody (Fig. 2c–e). We further validated Frk’s and Hck’s autophosphorylation using a mobility shift assay by SDS–PAGE (Fig. 2f). Unfortunately, this assay was not informative for Hck. Together, the results presented in Fig. 2d–f validated the potential of our platform to specifically detect Tyr autophosphorylation.

Sensitivity and specificity of the microfluidic platform. In order to determine the sensitivity and specificity of the assay numerically, we arrayed 17 non-receptor Tyr kinases with known autophosphorylation activity alongside 11 negative control

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**Fig. 2** Large-scale Tyr autophosphorylation analysis of freshly expressed protein arrays. **a** An array of 882 human proteins was expressed on chip in quadruplicates. Protein and P-Tyr signals were quantified as described in Fig. 1. Each bar represents an average P-Tyr-to-Protein ratio of a single protein (n = 4). Top 3 hits are indicated. Source data is provided in Supplementary Figure 2. **b** Assay validation. Hck and Frk kinases, their inactive variants (K290E, K262R) and the non-kinase protein Securin, were expressed in tube, deposited on chip, and assayed for autophosphorylation activity as described in Fig. 1. Average P-Tyr normalized to protein levels are shown (n = 17–36; *P < 0.05). Data are normalized to maximal activity for each kinase. Absolute P-Tyr and protein levels, and representative raw data are shown for wild type and inactive Hck to clarify the methodology. Source data is provided in Supplementary Figures 4 and 5. **c** Schematic presentation of anti-P-Tyr immunoprecipitation. **d, e** Immunoprecipitation of autophosphorylated proteins on chip. **d** Frk wt or e Hck wt and their kinase dead derivatives (K262R, K290E respectively) were immobilized on chip using biotinylated anti-P-Tyr antibody. Proteins expression value was evaluated using anti-His antibody for immobilization and anti-C-myc. Representative raw data is shown below (n = 34 for (d) and n = 63 (e); *P < 0.01). Results were normalized to each protein expression level (P-Tyr/Protein) as well as to maximum activity (Frk or Hck phosphorylation level). Source data is provided in Supplementary Figures 6 and 7. F 35S-labeled Frk wt, Hck wt and their kinase dead derivatives were in vitro transcribed and expressed using rabbit reticulocyte lysate and incubated for 30 min, 37 °C in parallel with HEK293 cell extracts supplemented with sodium orthovanadate (1 mM), as depicted in the plot. Protein’s mobility shift was assayed by SDS–PAGE and autoradiography. Source data is provided in Supplementary Figures 8 and 9.
including Ser/Thr kinases, protein-O-mannose kinase, and inactive Tyr kinases. Mean P-Tyr levels normalized to protein levels were plotted and a cutoff value for autophosphorylation activity was calculated using receiver operating characteristic analysis. Normalized P-Tyr levels of 12 out of 17 Tyr kinases were above the cutoff value (Fig. 3). On the other hand, only 2 out of 11 negative controls exhibited P-Tyr signals above the threshold. Overall, sensitivity and specificity of the assay were calculated to be 0.7 and 0.8, respectively (Table 1). Taken together, results in Figs. 2 and 3 demonstrate an on chip assay for autophosphorylation of soluble Tyr kinases. These results also provide definite evidence that our freshly expressed protein arrays are catalytically active.

On chip autophosphorylation of membrane protein. Membrane proteins mediate essential cellular processes, first and foremost cell signaling and communication. They are the key for host–pathogen interactions, and profoundly linked to human disease and disorders. Located at the cell surface, receptor Tyr kinases (RTKs) are also considered attractive targets for drug-based therapy. This highlights the need for systematic approaches for studying RTKs and membrane proteins in general. The technological challenge, however, is high; arrays of pre-purified proteins are restricted to soluble proteins. Even if freshly expressed, most membrane proteins cannot fully fold in an aqueous solution and are dysfunctional (illustrated in Fig. 4a, b). This limitation can, in principle, be overcome by applying microsomal membranes to the protein translation solution (Fig. 4c). Relying on this technique, we have recently reported on chip autophosphorylation of Fgfr1 by immunoprecipitation with anti-p-Tyr antibody (Fig. 4e).

Next, a library of 17 RTKs, all of which known to undergo autophosphorylation, were expressed in quadruplicates on chip using reticulocyte lysate supplemented with microsomal membranes (+MM) or mock (−MM). Eleven soluble Tyr kinases, 4 of which are inactive mutants, were also arrayed as negative controls and to determine non-specific background signals. P-Tyr signals were quantified for the 28 proteins expressed in the two conditions (Supplementary Figure 13). Mean P-Tyr(+MM) levels were subtracted from the matching P-Tyr(−MM) signals, and the ΔP-Tyr(+MM)−(−MM) values were plotted (Fig. 4f). Proteins within upper and lower cutoff lines (red zone) presented no significant changes in autophosphorylation levels whether or not membranes were present. Thresholds were determined by receiver operating characteristic analysis. B-lymphocyte kinase (Blk) was the only soluble kinase for which we noticed a significant impact of microsomal membranes on autophosphorylation. Interestingly, this effect was negative; P-Tyr signal of Blk1 was reduced in the presence of membranes (Fig. 4f), suggesting that membranes might have an inhibitory impact on Blk catalytic activity in our assay. More importantly, microsomal membranes

![Table 1: Sensitivity and specificity of the mobility shift assay](https://example.com/table1.png)

| P-Tyr(+MM) − P-Tyr(−MM) | True positive: 12 | False negative: 5 | Sensitivity: 0.7 |
|--------------------------|-------------------|-------------------|----------------|
| False positive: 2        | False negative: 9 | Specificity: 0.8  |               |

![Fig. 3: Autophosphorylation analysis of Tyr kinase array.](https://example.com/fig3.png)

An ORF library comprising 17 soluble Tyr kinases and 11 negative controls (5 inactive Tyr kinases and 6 non-Tyr kinases) was generated by assembly PCR. ORFs were spotted in quadruplicates. A freshly expressed protein array was generated and assayed for autophosphorylation as described in Fig. 1. Bars represent an average and standard deviation values of P-Tyr signals. A cutoff value of 0.05 was calculated by receiver operating characteristic analysis.
increased autophosphorylation activity in 15 out of 17 arrayed RTKs (88%), affirming the indispensability of this reagent for RTK activity assays in vitro. Altogether, the data presented in Fig. 4 demonstrate the power of our platform in studying Tyr autophosphorylation of RTKs on chip in a physiological-relevant context, as well as validate the functionality of our membrane protein array by showing a specific, well-characterized, enzymatic activity.

**Autophosphorylation of Ror2 a controversial pseudokinase.** Receptor Tyr kinase-like orphan receptor (Ror) subfamily of RTKs includes two related proteins, Ror1 and Ror2, both functioning in the Wnt signaling pathway. Mutations in Ror2 associate with human disease including Robinow and Brachydactyls type B skeletal syndromes. Overexpression of the protein has been linked to cancer development and prognosis. Ror2 intrinsic activity is controversial. In vitro studies combined with phylogenetic analyses showed that Ror2, different from its C. elegans homologue CAM-1, nearly lost its innate catalytic activity, classifying it as a catalytically deficient RTK-like pseudokinase. This notion, however, is challenged by contradicting evidence. Intrigued by this debate, we utilized our platform to investigate the potential of Ror2 to undergo autophosphorylation in vitro. To this end, we tested Ror2.
autophosphorylation on chip in the presence of microsomal membranes or mock. As shown in Fig. 5, a profound P-Tyr signal was observed for Ror2 in a membranous environment. This signal dropped by nearly 75% in Ror2 mutant lacking ATP-binding site (Ror2 K507E), thus, confirming exclusive detection of autophosphorylation. The dependency of Ror2 activity on membranes was remarkable; in fact, the impact of membranes was more dramatic than Lys to Glu substitution at the ATP-binding site. This signal was remarkable; in fact, the impact of membranes was more dramatic than Lys to Glu substitution at the ATP-binding site. This is a challenging task by all means and beyond the capacity of our current apparatus. On the other hand, our platform is optimal for distinguishing Tyr phosphorylation from autophosphorylation. Relying on spotted cDNA molecules, arraying a library of inactive kinases is straightforward. Because kinase dead mutants can still be targeted by other active kinases, P-Tyr signal in this case represents phosphorylation. An example highlighting this concept is shown for Btk (Supplementary Figure 17).

Detection of autophosphorylation on chip is based on immunolabeling. The method is currently limited to Tyr because of the poor specificity of global anti-phospho Ser/Thr antibodies. In view of mammalian Ser/Thr kinases being ~80% of the kinome, the motivation to mitigate this limitation in the future is high. Further we note that autophosphorylation, although widespread, is not the only known autophosphorylation domain. Distinguishing between these two forms is important for understanding the mechanism by which the kinase is activated. This is a challenging task by all means and beyond the capacity of our current apparatus. On the other hand, our platform is optimal for distinguishing Tyr phosphorylation from autophosphorylation. Relying on spotted cDNA molecules, arraying a library of inactive kinases is straightforward. Because kinase dead mutants can still be targeted by other active kinases, P-Tyr signal in this case represents phosphorylation. An example highlighting this concept is shown for Btk (Supplementary Figure 17).

**Methods**

**Microfluidics device fabrication.** Integrated two-layer microfluidic devices were designed in AutoCAD2013 (Autodesk, Inc., Mill Valley, CA). Mold fabrication was performed using soft lithography and chrome mask, as previously detailed. These molds were used for fabricating microfluidic device by casting silicone elastomer polydimethylsiloxane (PDMS: SYLGARD 184® Dow Corning). Each microfluidic device consists of two aligned PDMS layers, the flow and the control layers.

**Surface chemistry.** Biotinylated-BSA (1 μg/μl, Thermo) was flowed for 30 min through the device, binding the BSA to the epoxy surface. On top of the biotinylated-BSA, 0.5 μg/μl of Neuravidin (Pierce, Rockford, IL) was added for 30 min.
The button valve was then closed, and biotinylated-PEG (1 µg/µl, Nanocs) was flowed over for 30 min, thus passivating the rest of the flow layer. Following passivation, the button was released a flow of 0.2 µg µl of a biotinylated (Qiagen, Venlo, Netherlands) or 0.01 µg/µl anti-Myc biotinylated anti-bodies (Cell Signaling, Danvers, MA, USA) were applied. The antibodies bound to the exposed Neutravidin, specifically to the area under the button, creating an array of anti-His- or anti-Myc tag. PBS buffer was used for washing between each surface chemistry step.

Generating expression library. A library of Tyk kinase and controls open reading frames (ORFs) was generated. Most of the genes were cherry picked from the Open BioSystem’s library of human ORFome, while others were purchased from Addgene (Cambridge USA). The ORF’s library used to create synthetic linear genes by two steps PCR. The ORF’s were used as a template. ORF’s were then double tagged using a 5′ primer with-Myc tag, and a 3′ primer with a His tag. The second PCR step was performed with extension primers containing T7 promoter (5′) and T7 terminator (3′). The products were then double digested with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and eluted with 40 µl DDW. All PCR reactions were performed with high-fidelity hot start DNA polymerase KAPA (KAPA Biosystems, Wilmingon, USA). DNA point mutations, for creating inactive kinases, were produced using QuikChange Lightning kit (Agilent, Santa Clara, USA). The following point was set: T7 primer and labeled with FluroTect Green Lysine (Promega, Madison, USA). Primer with-Myc tag, and a 3′ primer with a His tag. The template products were filtered with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, USA) and eluted with 40 µl DDW. All PCR reactions were performed with high-fidelity hot start DNA polymerase KAPA (KAPA Biosystems, Wilmingon, USA). DNA point mutations, for creating inactive kinases, were produced using QuikChange Lightning kit (Agilent, Santa Clara, USA). The following point mutations were made to generate inactive kinases: Hck K290E; Btk K430E; Ror2 using QuikChange Lightning kit (Agilent, Santa Clara, USA). The following point mutations were made to generate inactive kinases: Hck K290E; Btk K430E; Ror2 using QuikChange Lightning kit (Agilent, Santa Clara, USA). The following point mutations were made to generate inactive kinases: Hck K290E; Btk K430E; Ror2 using QuikChange Lightning kit (Agilent, Santa Clara, USA).

DNA arraying and device alignment. Synthetic linear DNA samples were mixed with 1.25% D-trehalose dehydrate and 0.125% of polyethylene glycol (Sigma, Israel) in 384-well plates (Greiner bio-one) and spotted in quadruplicates on epoxy coated glass slides (CEL Associates) using a MicroGrid 610 microarrayer (Bio Robotics) equipped with SMT-575 silicone pins (Parallel Synthesis). Next, the DNA array was aligned using PDMS using a µDAS semiautomatic aligner.

Protein expression and immobilization. For on-chip expression, a pre-mixed reticulocyte lysate supporting protein expression by T7 promoter (12.5 µl) with or without microosomal membranes was flowed into the DNA chambers, following surface chemistry. For more details see ref. \(^{8,21}\). Alternatively, in vitro translation took place in tube, and the lysate was flowed directly into the protein chambers for immobilization. Detection of the immobilized proteins was based on immunofluorescence with either Cy3-coupled anti-Myc antibodies (1:100 dilution; Sigma Israel) or Alexa-Fluor 647-coupled anti-His antibodies (Qiagen, Venlo, Netherlands). The detection antibodies were flowed into the device, and incubated with the immobilized proteins under the button for 30 min at RT, followed by a wash with PBS buffer. Protein expression levels were determined with a microarray scanner (LS Reloaded, Tecan) using a 532 nm laser and labeled with 535/25 nm filter for Alexa 647 anti-His antibodies. The button valve was then closed, and biotinylated-PEG (1 µg/µl, Nanocs) was flowed over for 30 min, thus passivating the rest of the flow layer. Following passivation, the button was released a flow of 0.2 µg µl of a biotinylated (Qiagen, Venlo, Netherlands) or 0.01 µg/µl anti-Myc biotinylated anti-bodies (Cell Signaling, Danvers, MA, USA) were applied. The antibodies bound to the exposed Neutravidin, specifically to the area under the button, creating an array of anti-His- or anti-Myc tag. PBS buffer was used for washing between each surface chemistry step.

Immuno precipitation of autophosphorylated proteins on chip. Kinases were expressed by in vitro transcription and translation kit (Promega, Madison, USA) and labeled with FluoroTect Green Lysine (Promega, Madison, USA). The kinases were flowed into the device and immobilized on the surface under the button within the protein chamber with either anti-His (Qiagen, Venlo, Netherlands) or phospho-biotin antibody (Cell Signaling, Danvers, MA, USA). After washing, autophosphorylation or expression levels were determined with the microarray scanner using a 488 nm laser (emission filter: 535/25).

Mobility shift assay. Kinases were expressed by in vitro transcription and translation kit (Promega, Madison, USA) and labeled with Methionin 35S (Perkin Elmer, Israel). The kinases and their derivate mutants were incubated with HEK293 cell extract, supplemented with mock or 1 mM Sodium Orthovanadate for 30 min at 37 °C. Samples were resolved by SDS-PAGE and visualized by autoradiography using Fuji phosphorimagery BAS-2500.

Image and data analysis. The signal of Tyk phosphorylation and kinase expression were measured using the button. Each experiment was performed on at least two separate devices, with four technical repetitions for each protein in each device. LS Reloaded microarray scanner (Tecan) and GenePix 7.0 (Molecular Devices) image analysis software were used for all experiments analysis. We consider the signal measured around the button valve as the background since no protein immobilization occurs, therefore, some background is observed. The background signal in our microfluidic protein arrays results from non-specific binding of antibodies to the surfaces. For each wavelength scanned, we subtracted the corresponding background signal around the buttons in a ring the size of 2R with 2-pixel spacing (see supplementary material in Noach-Hirsh et al.\(^{8}\)). MCP. Protein expression levels and autophosphorylation were detected using fluorescent antibodies: 532 and 635 nm emission, respectively. Scanning in the two wavelengths was performed in a serial manner. No crossing signals were observed between the two wavelengths in control experiments. The uniformity of the immobilized protein spots was analyzed and also manually curated. The level of background (e.g., the DNA was spotted outside the DNA chamber and no protein was expressed in one of the four repeats).

Cell culture. HEK293 cells were maintained in tissue culture plates containing Dulbecco’s Modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin (100 U)/mL streptomycin (0.1 mg/ml) (all reagents were purchased from Biological Industries, Kibbutz Beit Haemek, Israel). Cells were maintained at 37 °C in a humidified 5% CO2 environment.

Active cell extract preparation. HEK293 cells were lysed in swelling buffer, containing: 20 mM Hapes pH 7.5, 2 mM MgCl2, 5 mM KCl, 1 mM DTT, and protease inhibitor cocktail (Roche, Israel), supplemented with energy regeneration mixture (1 mM ATP, 7.5 mM creatine phosphate, 70 µM creatine phosphokinase, 0.1 mM EGTA). Cells were incubated in swelling buffer on ice for 30 min and homogenized by freeze-thawing in liquid nitrogen and passage through a 22 G needle. Extracts were cleared by subsequent centrifugations (14,000 RPM, 10 min; 14,000 RPM for 40 min), quick frozen in liquid nitrogen, and stored at ~80 °C.

Ligand-dependent c-Kit autophosphorylation assay. Double-tagged proteins were expressed off chip using linear DNA and TNT T7 mix, in the presence or absence of microsomal membranes. After expression, the proteins were incubated with different concentrations of Stem Cell Factor (SCF), for 30 min at RT and were then immobilized under the button via anti-Myc-biotinylated antibody. Next, a 10 min wash with PBS buffer was performed. Protein expression levels were evaluated following incubation (30 min) with anti-His-Alexa Fluor 633 antibody (Qiagen, Venlo, Netherlands) and wash (10 min). Autophosphorylation levels were determined following incubation (30 min) with anti-P-Tyr fluorescently labeled with Alexa-Fluor 647 or Alexa-Fluor 488 (Cell Signalling, Danvers, MA, USA) and wash (10 min). Net autophosphorylation levels were normalized to protein expression levels.

Data availability. The data that support the findings of this study are attached as source data.

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Additional information
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