Rationale for experimental design

All phosphoproteomics experiments in this manuscript are performed in a qualitative rather quantitative manner. While the use of TMT isobaric mass labelling could have provided further insight into the dynamic changes occurring during cell response to deformation by adding a quantitative dimension to our datasets, there are multiple caveats to this approach which led us to pursue qualitative analysis instead.

First, the use of quantitative methods would have required deciding at which point should peptide labelling be applied. Labelling samples before TiO_2 enrichment introduces a problem regarding the amount of protein used for analysis, since the ubiquitous presence of hemoglobin in the samples would quench the labelling of other peptides and decrease the amount of protein which can be analyzed. In our system, TMT labelled samples comprise a maximum equivalent amount of 2 million cells whereas qualitative analysis allows for the use of 10 million cells, maximizing the probability of detecting low-abundance phosphopeptides.

While hemoglobin depletion constitutes a possible solution to this problem, its use would have multiple detrimental effects, in that protocol time would be extended (resulting in loss of phosphorylation over time, if we consider that any phosphorylation unique to deformation must be active for a relatively short period) and that any proteins in direct interaction with hemoglobin would also be removed. Furthermore, the hemoglobin removal step does not strongly contribute towards the remainder of the experiment, given that a large proportion of the hemoglobin present will already be removed during TiO_2 enrichment (where most non-phosphorylated peptides will be removed).

On the other hand, labelling samples for quantitative analysis after TiO_2 enrichment would introduce the risk of assaying differences resulting from the peptide isolation step rather than between the different samples. Through the 1% false discovery rate (FDR) used in this study and the extensive filtering steps for determining the presence of phosphorylations exclusive to samples which have undergone deformation, we are confident that our approach results in a more efficient filtering step while side-stepping the experimental constrains of quantitative analysis.

Supplemental Methods

Phosphoproteomics Sample Processing

The samples were separated using SDS-PAGE, allowing the dye front to run approximately 1cm into the separating gel and the gel piece was then subjected to in-gel tryptic digestion using a DigestPro automated digestion unit (Intavis Ltd). The resulting peptides were then subjected to TiO2-based phosphopeptide enrichment according to the manufacturer's instructions (Pierce).

Enriched phosphopeptides were fractionated using an Ultimate 3000 nano-LC system in line with an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific). In brief, peptides in 1% (v/v) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Fisher Scientific). After washing with 0.5% (v/v) acetonitrile 0.1% (v/v) formic acid peptides were resolved on a 250 mm × 75 μm Acclaim PepMap C18 reverse phase analytical column (Thermo Fisher Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1 min., 6-15% B over 58 min., 15-32%B over 58 min., 32-40%B over 5 min., 40-90%B over 1 min., held at 90%B for 6 min and then reduced to 1%B over 1 min.) with a flow rate of 300 nl min−1. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were ionized by nano-electrospray ionization at 2.2 kV using a stainless-steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 250°C.

All spectra were acquired using an Orbitrap Fusion Tribrid mass spectrometer controlled by Xcalibur 2.0 software (Thermo Fisher Scientific) and operated in data-dependent acquisition mode. FTMS1 spectra were collected at a resolution of 120,000 over a scan range (m/z) of 350-1,550, with an automatic gain control (AGC) target of 400,000 and a max injection time of 100ms. The Data Dependent mode was set to Cycle Time with 3s between master scans. Precursors were filtered according to charge state (to include charge states 2-7) and with monoisotopic precursor selection. Previously interrogated precursors were excluded using a dynamic window (40s +/-10ppm). The MS2 precursors were isolated with a quadrupole mass filter set to a width of 1.6m/z. ITMS2 spectra were collected with an AGC target of 5,000, max injection time of 50ms and HCD collision energy of 35%.

Proteomics Raw Data Processing

The raw proteomic mass spectrometry data files were processed using Proteome Discoverer software v1.4 (Thermo Scientific) and searched against the UniProt Human database (134,169 entries) using the SEQUEST algorithm. Peptide precursor mass tolerance was set at 10ppm, and MS/MS tolerance was set at 0.6Da. Search criteria included carbamidomethylation of cysteine (+57.0214) as a fixed modification and oxidation of methionine (+15.9949) and phosphorylation of serine, threonine and tyrosine (+79.966Da) as variable modifications. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled, and all peptide data was filtered to satisfy false discovery rate (FDR) of 1%. Conversion of msf files (result files of Proteome Discoverer) into the mzIdentML standard format was conducted using ProCon - PROteomics CONversion tool (ver. 0.9.718)[1].

Proteomic Data Analysis

Resulting proteomics data from the processing step was analysed in order to search for differentially present phosphopeptides among the samples. The first filtration step was based on retaining only differentially phosphorylated proteins that had been detected in at least 50% or more of all samples in any given condition or cell type. At this point, a prediction for a protein-protein interaction network was created with the use of the STRING[2] database based on the joint dataset of mid and post-deformation exclusive phosphoproteins in reticulocytes, due to the larger quantity of phosphopeptides present in those samples. The data was further filtered to only include phosphorylations that fulfilled the previous criterion, were also exclusively present in at least 2 samples per condition and were only found in the mid- or post-deformation subsets. The same analysis was performed for the microfluidics-related proteomics data. Finally, a list of significantly enriched or exclusive phosphopeptides in reticulocytes after deformation was produced by including only phosphopeptides that were present in at least 4 out of the 7 total samples (4 microsphiltration + 3 microfluidics) and not present in at least 4 out of 7 pre-deformation samples. The exception to this analysis consists of the phosphopeptides marked as 7* in **Table 1**, which were present in all post-deformation samples but also in 4 out of 7 of the pre-deformation samples. A prediction of the most probable phosphosites to be modified in the phosphopeptides was included according to a combination of the prediction given by SEQUEST and the count of occurrences in previous low throughput and high throughput studies, as assayed through PhosphoSitePlus[3].

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE[4] partner repository with the dataset identifiers PXD013652 and PXD013960, with corresponding DOIs 10.6019/PXD013652 and 10.6019/PXD013960.

Haemoglobin Estimation via Drabkin's Reagent

Drabkin's solution was prepared by reconstitution of one vial of the Drabkin's Reagent (Sigma Aldrich) with 1 L of water. 0.5 mL of a 30% Brij L23 solution (Alfa Aesar) were then added to the reconstituted Drabkin's reagent. The resulting solution was mixed and filtered.

Lysates were diluted 1:10 in Drabkin's solution and absorbance was measured at 540 nm. The standard curve for comparison was built through use of varied known concentrations of lysed cells.

High-speed video analysis

Images were processed in Fiji v. 2.0.0-rc-54/1.51h[5] as follows: A copy of the image was created, and the brightness/contrast sliders were adjusted in order to maximize the contrast of microfluidics channel limits. The wand tracing tool was then used for joint selection of all the inter-channel segments. Using this selection, a mask image was created and then inverted to define the regions of interest to analyse. The mask image was then subjected to binary erosion three times to further define the region of interest as not including any channel walls. The colours of the original image were inverted, and the Image Calculator was used to multiply the original image by the mask image with a 32-bit float result. The TrackMate plugin[6] was used for the remaining image processing steps: The Z and T dimensions were swapped. The Laplacian of Gaussian (LoG) detector was used, with an estimated blob diameter of 8-10 μm and an appropriate threshold for cell detection (varying by image set). The hyperstack displayer was used, without any filters set. The linear motion Linear Assignment Problem (LAP) tracker was used for cell tracking, with initial search radius of 15 μm, search radius of 10 μm and maximum frame gap of 2 frames. The detected tracks were then filtered so that only tracks with a total horizontal displacement ≥ 700 μm were kept for statistical analysis. The track data was then exported to .csv format and analysed with Microsoft Excel.

Supplemental Legends

Sup. Figure 1 – Lyn and Gsk3 inhibition does not disrupt the reticulocyte's intrinsic capacity to deform

A) Mean deformability index for reticulocytes treated with DMSO, anti-GPA antibody (BRIC256) as a positive control for loss of cell deformability, Lyn inhibitor (Bafetinib), Gsk3 inhibitor (CHIR98014) or both Lyn and Gsk3 inhibitors (Baf+CHIR). Error bars correspond to the standard deviation (n = 3). A minimum of 1000 cells was analysed per sample.

B) Deformability index profiles for reticulocytes treated with DMSO and with the anti-GPA antibody, showing disruption of cell deformability upon treatment with the antibody. A minimum of 1000 cells was analysed per sample.

C) Deformability index profiles for reticulocytes treated with DMSO, Lyn inhibitor (Bafetinib), Gsk3 inhibitor (CHIR98014) or both Lyn and Gsk3 inhibitors (Baf+CHIR). A minimum of 1000 cells was analysed per sample.

Sup. Table 1 – Phosphoproteins and phosphopeptides identified in erythrocytes and reticulocytes pre-, mid- and post- exposure to deformation through microsphiltration

Columns in the table include: Phosphopeptide sequence, predicted phosphosite location, gene name, UniProt accession number, count of occurrences per sample type and protein name. The data was processed using FDR = 1%. The data are filtered to include only differentially phosphorylated peptides that have been detected in at least 50% or more of all samples in any given condition or cell type, removing peptides that are found in all samples. Pre-processed data may be found in the supplement available with the online version of this article.

Sup. Table 2 – Phosphoproteins and phosphopeptides identified in reticulocytes pre- and post-exposure to deformation through the microfluidic biochip

Columns in the table include: UniProt ID (accession number), phosphopeptide sequence, count of occurrences per sample type, protein name and gene name. The data was processed using FDR = 1%. The data are filtered to include only differentially phosphorylated peptides that have been detected in at least 50% or more of all samples in any given condition or cell type. Pre-processed data may be found in the supplement available with the online version of this article.

Sup. Video 1 – Reticulocyte traversal through the microfluidic biochip

Reticulocytes are shown traversing the microcapillary channels and undergoing multiple rounds of deformation in the microfluidic biochip. The original frame rate for capture was 900 frames per second. The video is set at 30 frames per second (30x slower than real time).

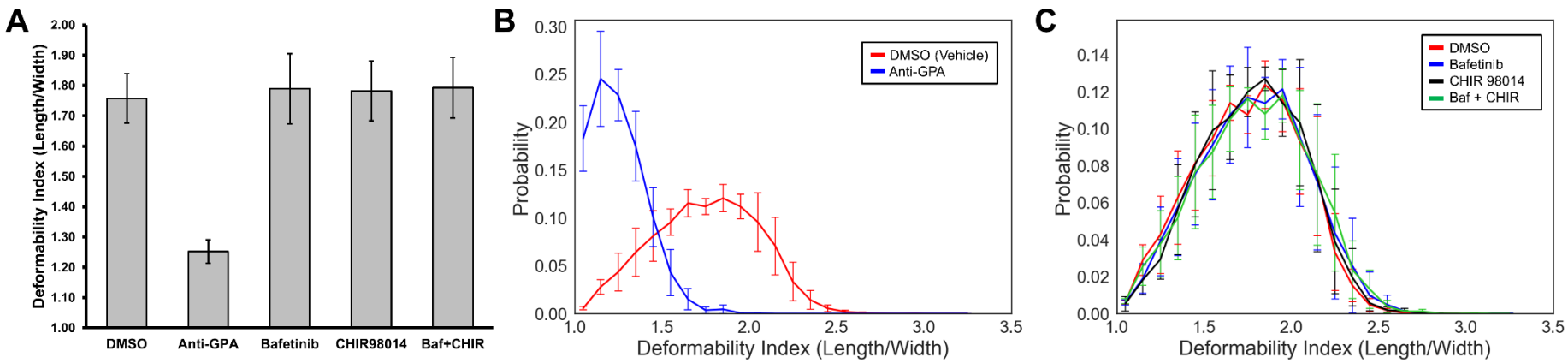
Sup. Video 2 – Cell tracking analysis using the microfluidic biochip

The same sample from Video 1 is shown, having undergone multiple steps of processing and automated tracking analysis (with the use of TrackMate[6]). Cell tracks are shown including the cell's position 10 frames before and after its current position.

Sup. Video 3 – Concurrent inhibition of Lyn and GSK3 disrupts reticulocyte traversal through the microcapillaries

Reticulocytes treated with Bafetinib and CHIR-98014 are shown, unable to traverse the microcapillary channels due to blockage of the system. The original frame rate for capture was 900 frames per second. The video is set at 30 frames per second (30x slower than real time).

Sup. Figure 1 - Lyn and Gsk3 inhibition does not disrupt the reticulocyte's intrinsic capacity to deform



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