Weir *et al*. Supplementary Document 1

Materials and Methods

Plasmids and site-directed mutagenesis

The plasmid for the expression of human SFKs in yeast was pASZ-DM and its construction has been described [1]. For the expression of human Fyn in mammalian cells, we acquired through Addgene (Cambridge, MA, USA) full-length human Fyn wild type (plasmid 16032), SH2 mutant Arg176Glu (plasmid 16035) and kinase dead mutant Lys299Met (plasmid 16033). These plasmids were originally constructed in the lab of F. Giancotti using pRK5 [2] and the wild type construct was used with QuikChange methodolgy (Stratagene, La Jolla, CA, USA) to construct the individual or combination Tyr-to-Phe and Tyr-to-Asp mutant alleles. For the expression of GST-SH2 domain fusions in *E. coli* we PCR-amplified the SH2 domain of Fyn from the various pRK5-Fyn constructs and cloned them into pGEX-4T-1 using primers and restriction sites as described previously [3]. All mutations were sequence-verified using the University of Vermont/Vermont Cancer Center DNA Analysis Facility. In the case of the GST-SH2 domain alleles, their protein products were also verified by protein mass spectrometry in our laboratory. The expression construct for ESDN/DCBLD2-Myc-Flag in pCMV6-Entry was described previously [3].

Anti-phosphotyrosine peptide immunoprecipitation from yeast expressing human SFKs

S. cervisiae strain L40c was transformed [4] with the various SFK constructs and were grown in selective media (SD5:-Leu-Trp-Ura-His-Ade), and induced with 20 μM Cu^{2+} as described previously [1]. Two yeast pellets (each 1 ml dry volume) were frozen in liquid nitrogen and stored at -80°C. Preparation and enrichment essentially followed the phospho-scan procedure (Cell signaling #7900, PhosphoScan ® Kit (P-Tyr-100)). An equal volume of zirconia beads (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was added followed by the addition of 500 μl freshly prepared lysis buffer (20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate). The pellets were lysed in a FastPrep24 (MP Biomedicals, Santa Ana, CA, USA) for 20 seconds at its highest speed (6.5 Ms^{-1}). Lysates were centrifuged on a cooled (4°C) table-top centrifuge for 15 min at 15,000 rpm and the supernatants were transferred to a 50 ml centrifugation tube. The lysis was

repeated twice more by adding each time 500 µl lysis buffer to the cell debris/pellets. 1/10th volume of 45 mM DTT was added to the combined supernatants and incubated for 20 min in a 60°C water bath. After cooling the solution to room temperature (RT) for 10 min, 1/10th volume of 110 mM iodoacetamide was added and the solution was incubated for 10 min at RT in the dark. For Trypsin digestion, the solution was increased in volume 4 times and diluted with HEPES buffer such that the final concentration was 1 M urea and 10 mM HEPES, pH 8.0. Finally, 1/100th volume of 1 mg/ml trypsin-TPCK solution (Worthington Biochemical Corporation, Lakewood, NJ, USA / Roche Diagnostics GmbH, Mannheim, Germany) was added and the proteins in solution were digested overnight at RT.

The tryptic digest was acidified by the addition of 1/20th volume of 20% tri-fluoroacetic acid (TFA) solution and an incubation for 10 min at RT. In order to remove precipitant the acidified peptide solution was centrifuged for 5 min at 4,000 rpm and the supernatant decanted into a fresh tube. Peptides were desalted using a reversed-phase Sep-Pak solid phase extraction column (Waters, Milford, MA, USA). After the column was pre-wetted with 5 ml 100% acetonitrile (MeCN) and washed twice with 3.5 ml 0.1% TFA, the entire acidified peptide solution was passed through the column by gravity flow or by the use of a plunger. Subsequently, the column was washed by applying 1 ml, then 5 ml and finally 6 ml of 0.1% TFA before eluting the peptides into a polypropylene tube by applying 2 ml 0.1 % TFA, 40% MeCN thrice. The eluate was then frozen and lyophilized. For anti-phosphotyrosine immunoaffinity enrichment we built on the protocol first established by Rush *et al.* [5] and also on our previous work [6,7]. The final protocol and reagents used were from the P-Tyr-100 PhosphoScan Kit (Cell Signaling Technology, Danvers, MA, USA). Briefly, lyophilized peptides were spun down and resuspended in 1.4 ml IAP buffer, left at RT for 5 min and briefly sonicated in an ultrasound bath. The pH was controlled to be neutral or not lower than 5 to 6 by adjustments using using 1 M Tris Base. All of the following steps were conducted at 4°C. The peptide solution was clarified via centrifugation at full-speed for 15 min and transferred directly onto P-Tyr-100-conjugated beads. The tubes were sealed with parafilm and incubated on a rotator for 2 hours. After subsequent centrifugation at 2,700 x g for 1 minute the beads were washed two times by the addition of 1 ml “IAP buffer plus detergent” and inverting the tube 5 times. The supernatant was then removed after 1 min centrifugation at 2,700 x g. The beads were again washed 5 times by applying 1ml purified water, inverting the tube 5 times, and removing the supernatant after 1

min centrifugation at 2,700 x g. After the final wash, the supernatant was carefully and completely drained. Finally, peptides were eluted from the beads by the addition of 55 µl of 0.15% TFA, tapping the bottom of the tube, and letting the tubes stand for 10 minutes at RT. After tapping the bottom of the tube again and after brief centrifugation, the supernatant was transferred to a fresh low-peptide-binding tube. Another 45 µl of 0.15% TFA were added to the beads and after tapping the tube and brief centrifugation the supernatants were combined. The eluate from the peptide immunoprecipitation was centrifuged briefly to collect residual beads on the bottom of the tube. Then, the supernatant was transferred to a fresh tube while ensuring no residual beads were present in the solution which could block the ZipTip C18 column (Milipore, Billerica, MA, USA) column. The eluate was divided into two aliquots of 50 µl each in 1.5 ml low-binding reaction tubes. By attaching a cut 200 µl pipet tip as an adaptor to the pipette, the ZipTip was installed while the adaptor was fit tightly into the upper "ring" of the ZipTip, but not further. Setting the pipette to 70 µl, up to 55 µl could be picked up with the tip. The ZipTips were processed using solvent A (0.1% TFA) and solvent B (0.1 % TFA, 40 % MeCN). For each step, a fresh low-peptide-binding tube was used with the appropriate solvent. First, 40 µl solvent B was aspirated slowly twice into the ZipTip while avoiding air. The ZipTips were equilibrated twice using two separate tubes by pipetting 50 µl solvent A up and down. The first aliquot was aspirated and expelled carefully 10 times into the tip and the loading repeated with the second aliquot but using the same ZipTip. Unabsorbed sample was left in the tubes. Subsequently, the tip was washed three times by pipetting up and down 55 µl solvent A. After the last washing step the ZipTip was dabbed well on a lint-free tissue. In quick succession, the ZipTip was transferred without adaptor to a 10 µl pipette (set to 10 µl) and peptides were eluted in 4 µl solvent B by pipetting three times up and down and one final aspiration into a 0.5 ml non-peptide-binding tube. Finally, the sample was dried in a vacuum concentrator for 60 min.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS), synthetic standard peptides, and data analysis

LC-MS/MS analyses were set up and conducted as described previously [8] using a MicroAs autosampler, a Surveyor PumpPlus HPLC and a linear ion trap-orbitrap (LTQ-Orbitrap) platform (Thermo Electron, Waltham, MA, USA). Synthetic standards were run on the same platform using the same methodology except that MS/MS scans were not data-dependent but

rather were targeted on the average mass (+/- 1.6 *m/z*) of the doubly-charged precursor ions. Stable Isotope-containing peptide standards were synthesized at Cell Signaling Technology (Danvers, MA, USA). All peptides contain one $^{13}C_6$, $^{15}N_1$-leucine (+7.01714 Da) residue enabling them to be distinguished from the native peptides in the mass spectrometer while retaining the same overall fragmentation patterns. For the peptide harboring phosphorylated Tyr214 an additional heavy residue, $^{13}C_2$, $^{15}N_1$ -glycine (+3.00374), replaced the second glycine in the peptide in order to distinguish it from the same peptide but phosphorylated on Tyr 213. To identify tyrosine phosphorylated peptides, we performed a SEQUEST search of the MS/MS data using the forward and reverse [9] concatenated human kinome database. The search parameters required a precursor mass tolerance of 10 PPM, required peptides to be tryptic, and allowed dynamic modification of methionine (+15.99491 Da for oxidation), cysteine (+57.02146 Da for carbamidomethylation) and serines, threonines and tyrosines (+79.9663 Da for phosphorylation). Filtering to a less than 1% false discovery rate yielded peptide hits exclusively to tyrosine phosphorylated Fyn, except when Fyn shared an identical tryptic peptide with other SFKs. Phosphorylation site localization and associated metrics were performed as described [10].

Production of GST-Fyn-SH2 fusion proteins, pulldowns and in vitro kinase assays

E. coli harboring plasmids encoding various alleles of GST-Fyn-SH2 or GST alone were induced and fusion proteins were captured on glutathione agarose as described previously [11]. Fusion proteins were eluted with 20 mM free glutathione, 100 mM NaCl and 50 mM Tris pH 8 (the final pH was kept between 7.7 and 8.0 by adding a small amount of Tris Base as needed). Eluted proteins were dialyzed against PBS at 4 °C overnight, normalized by total protein amount and stored at 4 °C until use. *In vitro* kinase reactions were performed in 25 mM Tris pH 7.5, 400 μM ATP, 10 mM $MgCl_2$, 100 μM Na_3VO_4, 5 mM β-glycerophosphate, and 1mM DTT and included the indicated substrates and either water or purified Fyn (ThermoFisher Scientific, P3042). 0.04 μg of recombinant Fyn was used for each assay and 4-12 μg of substrate in a reaction volume of 25 μl. For direct phosphorylation assessment, kinase reactions were incubated at room temperature for two hours followed by SDS-PAGE using 12% gels (37.5 acrylamide:1 bis-acrylamide) and immunoblotting. For pulldowns after kinase assays, glutathione agarose was added and incubated at 4 °C for 2 hours in a volume of 500 μl of lysis buffer (25 mM Tris, pH 7.4, 137 mM NaCl, 1% Igepal, 10% glycerol, 25 mM NaF, 10 mM $Na4P_2O_7$, 1 mM Na_3VO_4, 5

mg/ml pepstatin A, 10 mg/ml leupeptin and 1 mM PMSF). The pulldowns were then washed twice with lysis buffer and then incubated with cell extract from human embryonic kidney 293 (HEK 293) cells that had been transfected with ESDN/DCBLD2-Flag and treated with 5mM H_2O_2 for 15 minutes prior to lysis. Straight pulldowns were performed using equal amounts of GST or GST-fusion proteins bound to glutathione agarose. HEK 293 cell extracts were incubated with the resins overnight and then washed three times with lysis buffer prior to SDS-PAGE and immunoblotting.

Cell culture, transfection, lysis, immunoblotting, antibodies, densitometry and statistics

For calcium phosphate-based transfections, conducted for Fig. 3A and Fig. 4A, E1A-transformed HEK 293 cells were grown to 70% of confluence prior to transfection. Six hours after transfection the cells were washed with warm PBS and grown for an additional 16-18 hours. When indicated, hydrogen peroxide stimulation was for 15 min at 8 mM. Just prior to lysis, cells were placed on ice, washed with ice-cold PBS and then lysed. Extracts were cleared of insoluble material by microcentrifugation at 15,000 x g and then heated to 95 °C for five min in protein sample buffer [8] and loaded for SDS-PAGE using 10% (37.5 acrylamide:1 bis-acrylamide). For polyethylenimine transfections [12], conducted for Fig. 3B, HEK 293 cells were grown to 70% of confluence. Plasmids were incubated with polyethylenimine for 20 minutes prior to the addition of the mixture to the cells. 16h after transfection the media was removed and replaced with fresh growth media and cells were grown for another 24 hours before lysis. Cells were lysed and extracts clarified as described above. Following SDS-PAGE immunoblotting was conducted using nitrocellulose membranes and conditions as described previously [3,8,11]. Primary antibodies were from the following sources: Upstate Biotechnology/Milipore (Billerica, MA, USA), α-GST (#06-332) and α-phosphotyrosine 4G10 (#05-321) ; Cell Signaling Technology, α-Fyn (#4023), α-Src pY416 (#6943), α-alpha tubulin (#3873), and α-actin (#4968); Sigma, α-Flag M2. Densitometry was conducted using ImageJ (Fig. 3B) or using Adobe Photoshop as an inverted histogram (Fig. 3A). After subtracting background, mutant Fyn levels were calculated as a percentage of wild type Fyn levels and mutant Src pY416 levels were calculated as a percentage of wild type Fyn pY416 levels. The resulting relative pY416 levels were then divided by the relative levels of Fyn for each sample type in each of the three independent experiments. Values for all experiments were then

transformed to Log2 scale and imported into JMP Pro10 where means were compared (ANOM Each-pair student's t 0.05).

References

[1] Grossmann, A., Benlasfer, N., Birth, P., Hegele, A., Wachsmuth, F., Apelt, L. and Stelzl, U. (2015). Phospho-tyrosine dependent protein-protein interaction network. Mol Syst Biol 11, 794.

[2] Mariotti, A., Kedeshian, P.A., Dans, M., Curatola, A.M., Gagnoux-Palacios, L. and Giancotti, F.G. (2001). EGF-R signaling through Fyn kinase disrupts the function of integrin alpha6beta4 at hemidesmosomes: role in epithelial cell migration and carcinoma invasion. J Cell Biol 155, 447-58.

[3] Aten, T.M. et al. (2013). Tyrosine phosphorylation of the orphan receptor ESDN/DCBLD2 serves as a scaffold for the signaling adaptor CrkL. FEBS Lett 587, 2313-8.

[4] Worseck, J.M., Grossmann, A., Weimann, M., Hegele, A. and Stelzl, U. (2012). A stringent yeast two-hybrid matrix screening approach for protein-protein interaction discovery. Methods Mol Biol 812, 63-87.

[5] Rush, J. et al. (2005). Immunoaffinity profiling of tyrosine phosphorylation in cancer cells. Nat Biotechnol 23, 94-101.

[6] Ballif, B.A., Carey, G.R., Sunyaev, S.R. and Gygi, S.P. (2008). Large-scale identification and evolution indexing of tyrosine phosphorylation sites from murine brain. J Proteome Res 7, 311-8.

[7] Doubleday, P.F. and Ballif, B.A. (2014). Developmentally-Dynamic Murine Brain Proteomes and Phosphoproteomes Revealed by Quantitative Proteomics. Proteomes 2, 197-207.

[8] Cheerathodi, M., Vincent, J.J. and Ballif, B.A. (2015). Quantitative comparison of CrkL-SH3 binding proteins from embryonic murine brain and liver: Implications for developmental signaling and the quantification of protein species variants in bottom-up proteomics. J Proteomics 125, 104-11.

[9] Elias, J.E. and Gygi, S.P. (2007). Target-decoy search strategy for increased confidence in large-scale protein identifications by mass spectrometry. Nat Methods 4, 207-14.

[10] Beausoleil, S.A., Villen, J., Gerber, S.A., Rush, J. and Gygi, S.P. (2006). A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat Biotechnol 24, 1285-92.

[11] Saha, M., Carriere, A., Cheerathodi, M., Zhang, X., Lavoie, G., Rush, J., Roux, P.P. and Ballif, B.A. (2012). RSK phosphorylates SOS1 creating 14-3-3-docking sites and negatively regulating MAPK activation. Biochem J 447, 159-66.

[12] Ehrhardt, C., Schmolke, M., Matzke, A., Knoblauch, A., Will, C., Wixler, V. and Ludwig, S. (2006). Polyethylenimine, a cost-effective transfection reagent. Signal Transduction 6, 179-184.