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Decoding the signaling profile of hematopoietic progenitor kinase 1 (HPK1) in innate immunity: A proteomic approach

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Signaling via β_2 integrins (CD11/CD18) as well as TCRs and BCRs involves similar pathways. However, the activation of the same signaling molecule can result in opposing effects. One such example is the hematopoietic progenitor kinase 1 (HPK1), which negatively regulates T and B cell activation but enforces neutrophil adhesion via β_2 integrins. This difference may be defined by specific HPK1 interacting networks in different leukocyte subsets which have already been described in the adaptive immune system. Here, we set out to identify interacting proteins of HPK1 in neutrophil-like differentiated HL-60 cells exposed to immobilized fibrinogen and left nonactivated or Mn^{2+}-activated to allow β_2 integrin-dependent adhesion. Co-IP experiments followed by mass spectrometry led to the identification of 115 HPK1-interacting proteins. A total of 58 proteins were found only in nonactivated cells and 39 proteins only in Mn^{2+}-activated adherent cells. From these results, we decoded a pre-existing signaling cluster of HPK1 in nonactivated cells encompassing proteins essential for β_2 integrin-mediated signaling during neutrophil trafficking, namely DNAX-activation protein 12 (DAP12), spleen tyrosine kinase (Syk), and Rac1. Thus, our study provides novel insights into the complex architecture of the signaling processes during neutrophil activation and the complex signaling profile of HPK1 in leukocytes.

Keywords: HPK1 · neutrophils · interactome · interacting network · β_2 integrins

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

The fine-tuned spatiotemporal response of leukocytes to external stimuli is a hallmark of the immune system. Sensing of stimuli occurs through a wide variety of different cell surface receptors, including integrins, which transmit signals into the cell. Signaling of β_2 integrins (CD11/CD18), as well as TCRs and BCRs, involves similar pathways [1]. Outside-in signaling of β_2 integrins as well as signaling via TCRs and BCRs are initiated by phosphorylation of ITAMs in the side chains of transmembrane receptors such as DNAX-activation protein 12 (DAP12) or Fcγ receptor IIA by Src family kinases [1,2]. Phosphorylation recruits spleen tyrosine kinase (Syk) family kinases, which mediate downstream signaling via, among others, Src homology 2 domain-containing

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leukocyte protein of 76 kDa (SLP-76), adhesion, and degranulation promoting adapter protein (ADAP) and/or hematopoietic progenitor kinase 1 (HPK1) [3–8]. While most of these signaling molecules reinforce the initial signal along the cascade, leading to the execution of cell effector functions, such as antibody production, cell killing, and removal of pathogens, HPK1 has been reported, depending on the cell type, to positively or negatively regulate cell effector functions.

In general, HPK1 is regarded as a negative regulator of TCR and BCR signaling [6,9,10]. In T cells, HPK1 phosphorylates the adaptor proteins SLP-76 and growth factor receptor-bound protein 2 (Grb2)-related adaptor protein downstream of Shc (GADS), resulting in the interruption of TCR signaling [5,6,11]. Thus, HPK1 activity dampens T cell activation and T cell-mediated immune responses. Similarly, HPK1 phosphorylates the B cell linker protein (BLNK) upon BCR activation, inducing degradation of BLNK, thereby terminating BCR signaling [9]. Furthermore, β_2 integrin signaling in T and B cells is negatively regulated by HPK1 [8,12]. Here, HPK1 competes for the SLP-76 binding site with the adaptor protein complex ADAP-Src kinase-associated phosphoprotein of 55 kDa (SKAP55) that is critical for β_2 integrin activation [7,13]. This leads to attenuated activation of the β_2 integrin lymphocyte function-associated antigen 1 (LFA-1, CD11a/CD18) and, thus, impaired binding to its ligand ICAM-1 [8,12]. In sharp contrast, HPK1 is a positive regulator of β_2 integrin signaling in neutrophils [14]. Here, HPK1 mediates the induction of the high affinity state of the β_2 integrin LFA-1 upon stimulation with chemokine (C-X-C motif) ligand 1 (CXCL-1). This effect depends on inside-out activation of LFA-1 since bypassing inside-out signaling with Mn^{2+} completely restores the ICAM-1 binding ability of HPK1-deficient neutrophils. Accordingly, HPK1-deficient neutrophils show reduced CXCL-1-mediated adhesion to immobilized ICAM-1 under flow conditions in vitro and neutrophil adhesion is impaired in postcapillary venules of the cremaster muscle upon stimulation with TNF-α in HPK1-deficient mice compared to WT mice [14].

The exact signaling pathways and/or binding partners of HPK1 in neutrophils are unknown to date, while the interacting proteins of HPK1 in lymphocytes have already been analyzed in detail [10,15]. As the function of HPK1 may be defined by its signaling network, we set out to decipher the interacting proteins of HPK1 in myeloid cells by co-IP using GFP Nano-Trap technology and subsequent mass spectrometry (MS).

Results

Stable expression of HPK1-EGFP in HL-60 cells

To decipher the interactome of HPK1 in neutrophils, we generated human HL-60 cells stably expressing the HPK1-enhanced GFP (EGFP) fusion protein (Fig. 1A). Proper expression of endogenous, nonlabeled HPK1, as well as the HPK1-EGFP fusion protein, was detected in differentiated HL-60 (dHL-60) HPK1-EGFP cells (Fig. 1B). In dHL-60 EGFP control cells, EGFP and endogenous HPK1 but no HPK1-EGFP was found as expected. Similar to dHL-60 cells, HPK1 was expressed in human neutrophils freshly isolated from the blood of healthy donors (Supporting Information Fig. S1). Confocal microscopy revealed that HPK1-EGFP was specifically localized at the lamellipodium of *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-stimulated dHL-60 HPK1-EGFP cells upon exposure to immobilized fibrinogen and colocalized with F-actin and mammalian actin-binding protein 1 (mAbp1) as previously described for endogenous HPK1 in dHL-60 cells (Fig. 1C) [14].

Identification of the HPK1 interacting partners in dHL-60 cells

Next, dHL-60 HPK1-EGFP cells and dHL-60 EGFP control cells were used to identify HPK1-interacting proteins (Fig. 2A). Co-IP experiments using the GFP Nano-Trap technology were performed with whole-cell lysates of dHL-60 HPK1-EGFP cells and dHL-60 EGFP control cells exposed to immobilized fibrinogen and either left nonactivated for control or activated with Mn^{2+} to allow β_2 integrin-mediated adhesion. Subsequently, interacting proteins were subjected to MS analysis. The successful and highly specific IP of HPK1-EGFP in dHL-60 HPK1-EGFP cells but not in dHL-60 EGFP control cells was confirmed in Western blot studies (Fig. 2B). Here, the HPK1-EGFP fusion protein was pulled down in dHL-60 HPK1-EGFP cell lysate precipitates as well as endogenous HPK1 as HPK1 can form dimers [16]. As expected, no HPK1-EGFP fusion protein or HPK1 was detectable in dHL-60 EGFP control cell lysates.

Following MS, the identified peptides were further analyzed with MaxQuant software (Supporting Information Table S1) [17]. Here, proteins in dHL-60 HPK1-EGFP cells with a $\log_2$ fold change >2 compared to dHL-60 EGFP control cells were defined as HPK1-specific interactors. Eight nonspecific protein hits were excluded by CRAPome database analysis [18]. In total, 115 proteins interacting with HPK1-EGFP were found. Here, 58 proteins were only detected in nonactivated, suspended cells and 39 proteins were found only in Mn^{2+}-activated, adherent cells (Fig. 2C, Supporting Information Table S2). Moreover, 18 proteins were coimmunoprecipitated with HPK1 in both conditions. HPK1 (gene *MAP4K1*) itself was detected in both samples as expected.

Interacting network of HPK1 in dHL-60 cells

To decipher the HPK1 signaling profile, we focused on proteins with a known function in signaling including transmembrane, membrane-associated, adaptor, cytoplasmic, and cytoskeleton-associated proteins (Table 1). Among the resulting 39 signaling proteins, 21 proteins were found in nonactivated cells only (Fig. 3A), 12 proteins were detected in Mn^{2+}-activated cells only (Fig. 3B), and six proteins were found in both samples. From these 39 HPK1-interacting proteins, we constructed the interacting network of HPK1 by analyzing them for known interactions with each

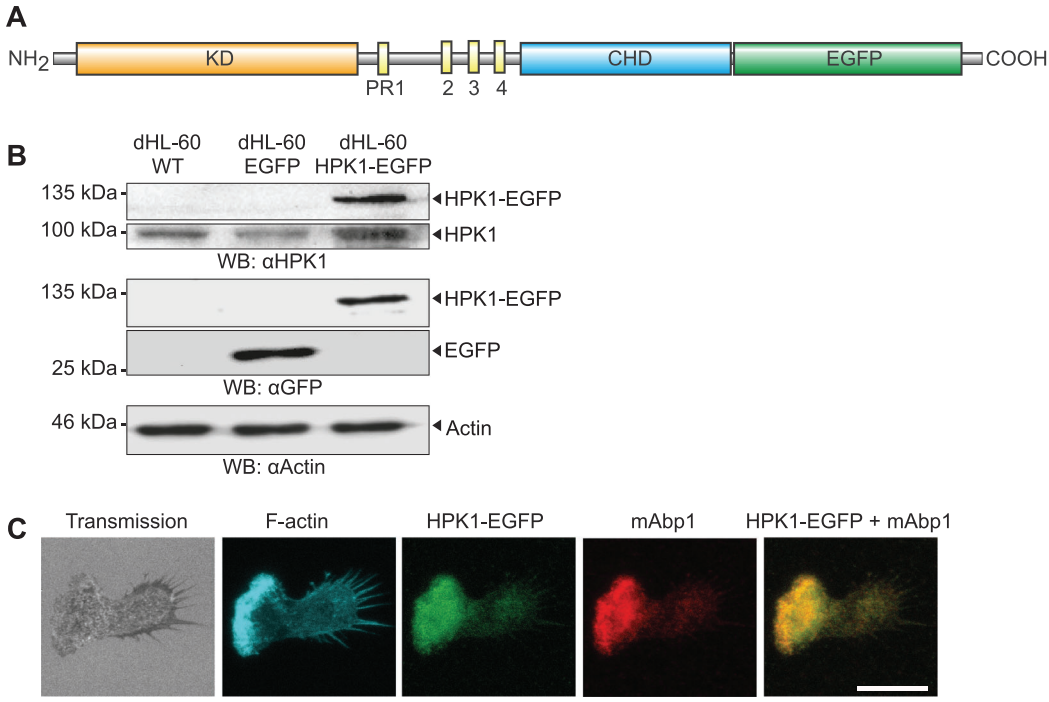

Figure 1. (A) Schematic presentation of the HPK1-EGFP fusion protein used for subsequent co-IP assays with the GFP Nano-Trap technology. Kinase domain (KD), proline-rich motifs (PR) 1–4, citron homology domain (CHD), and C-terminally fused EGFP. (B) Western blot of whole-cell lysates of dHL-60 WT, dHL-60 EGFP, and dHL-60 HPK1-EGFP cells. Endogenous HPK1, HPK1-EGFP, EGFP, and actin were detected by immunoblotting using specific antibodies. Representative image of three independent experiments with three biological replicates. (C) Confocal microscopic images of dHL-60 HPK1-EGFP cells expressing HPK1-EGFP (green) stimulated with fMLP exposed to immobilized fibrinogen and stained for mAbp1 (red) and F-actin (cyan) using a specific antibody and phalloidin, respectively. Scale bar, 10 μm. Representative image of three independent experiments with three biological replicates.

other and grouping them accordingly, using the STRING database with stringent parameter settings to only include protein-protein interactions with the highest confidence [19] (Fig. 3A and B). Next, we defined clusters of proteins that interact not only with HPK1 but also with an interactor of HPK1 in nonactivated and activated dHL-60 HPK1-EGFP cells. Interestingly, we found in nonactivated cells a network of 11 proteins (cluster 1) and a second, smaller network with four proteins (cluster 2) (Fig. 3A). In contrast, in activated cells, only two small clusters with known protein-protein interactions were identified, containing five and three proteins, respectively (Fig. 3B, clusters 3 and 4), suggesting that HPK1 is integrated into different signaling complexes in nonactivated and activated cells.

In neutrophils, HPK1 is required for β_2 integrin activation, allowing firm adhesion of the neutrophil to the inflamed endothelial cells lining the postcapillary venules [14]. This process occurs within seconds after the induction of leukocyte rolling [20]. Strikingly, we identified in cluster 1, in nonactivated, suspended dHL-60 HPK1-EGFP cells, three proteins well-known for their critical roles during β_2 integrin-dependent neutrophil adhesion and postarrest functions, namely DAP12 (gene *TYROBP*), Syk (gene *SYK*), and Rac1 (gene *RAC1*) (Fig. 3A). DAP12 and Syk mediate signal transduction functionally linking β_2 integrins to the actin cytoskeleton during neutrophil adhesion and postadhesion events [1,3,21]. Actin cytoskeleton remodeling during neutrophil adhesion is mediated by Rac1, which is activated by the Rho guanine nucleotide exchange factor 6 (ARHGEF6, gene *ARHGEF6*), another HPK1-interacting protein identified in cluster 1 [22]. The Rho GTPase dissociation inhibitor alpha (ARGHDIA, gene *ARGHDIA*), which can inhibit Rac1 activity, was found in our screen in activated, adherent cells (Fig. 3B). Furthermore, the cell surface molecule CD157 (gene *BST1*), the urokinase plasminogen activator surface receptor (uPAR, gene *PLAUR*), and integrin α5 (gene *ITGA5*) were also included in cluster 1 and have been reported to mediate different neutrophil functions [23–25]. Interestingly, the composition of the signalosome completely changed upon induction of adhesion. Proteins involved in β_2 integrin signaling including DAP12, Syk, Rac1, and ARHGEF6 were no longer part of the HPK1 interacting network. The only protein in activated, adherent dHL-60 HPK1-EGFP cells with a reported function for neutrophil activation was ADP-ribosylation factor 6 (Arf6, gene *ARF6*) [26]. Arf6 forms cluster 3 in Mn^{2+}-activated, adherent dHL-60 HPK1-EGFP cells together with CD157, uPAR, and integrin α5.

In summary, we decoded the signaling profile of HPK1 in myeloid cells and identified a pre-existing signaling cluster in nonactivated cells containing DAP12, Syk, and Rac1. However, upon induction of adhesion, HPK1 leaves this preformed signaling environment critically involved in rapid β_2 integrin-dependent

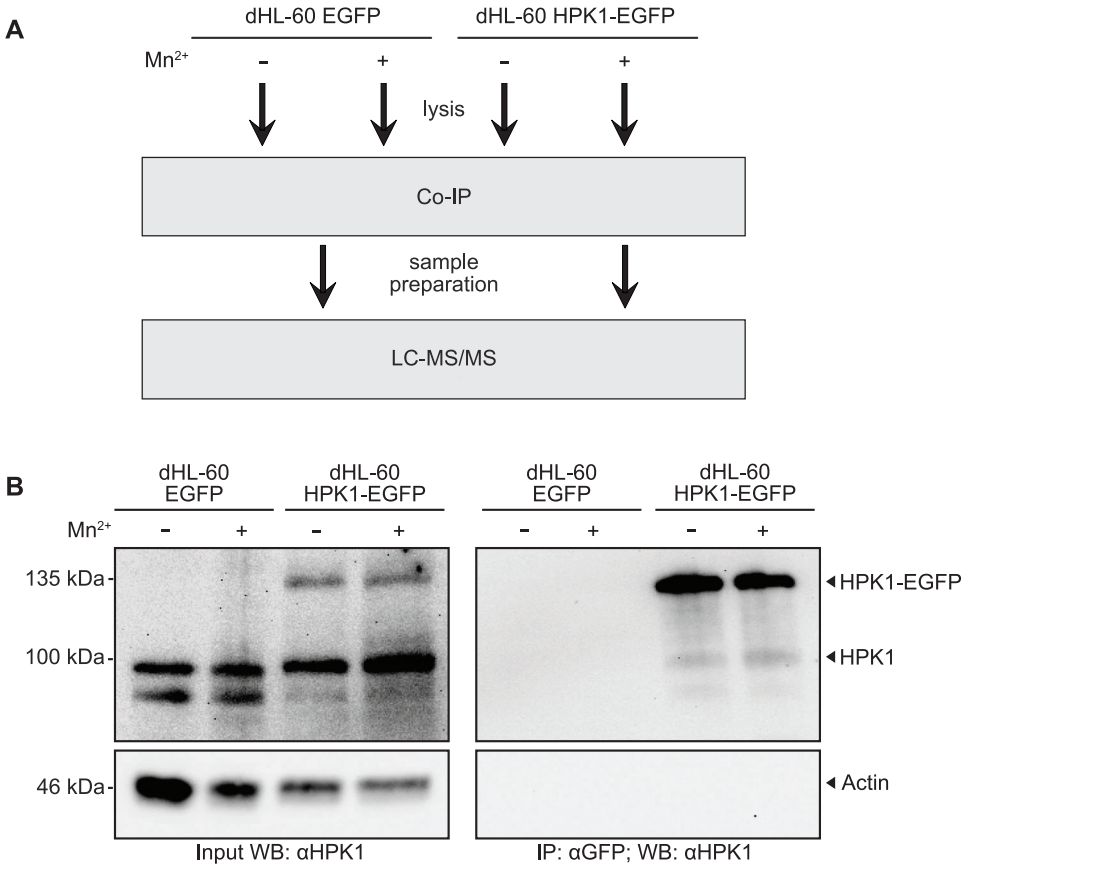

Figure 2. (A) Workflow of co-IP experiments to identify the interactome of HPK1 in dHL-60 HPK1-EGFP cells. dHL-60 HPK1-EGFP and dHL-60 EGFP control cells were left nonactivated or activated with Mn^{2+} for 15 min, lysed, and HPK1-EGFP-interacting proteins were coprecipitated and prepared for subsequent LC-MS/MS analysis. (B) Representative Western blot of co-immunoprecipitated samples of dHL-60 HPK1-EGFP cells and dHL-60 EGFP control cells. Endogenous HPK1 and HPK1-EGFP were detected with a specific antibody. Actin was used as loading control. Representative image of three independent experiments with three biological replicates. (C) Venn diagram of HPK1-interacting proteins in dHL-60 HPK1-EGFP cells were left nonactivated (grey) or were activated with Mn^{2+} (orange) upon exposure to immobilized fibrinogen. Gene names of identified interacting proteins are based on the accession number of the UniProt database. Data are pooled from three independent experiments from three biological replicates. Primary data are deposited at PRIDE database, please see Methods section for data access.

Table 1. Signaling proteins interacting with HPK1 in nonactivated (grey) or Mn^{2+}-activated (orange) dHL-60 HPK1-EGFP cells. Proteins detected in both conditions are shown in yellow. Protein categories were transmembrane (TM), membrane-associated (MA), adaptor (AD), cytoplasmic (CY), and cytoskeleton-associated (CA)

Gene name	Protein name	Category
ANXA1	Annexin A1	MA
ANXA7	Annexin A7	MA
ARHGEF6	Rho guanine nucleotide exchange factor 6	CY
BAK1	Bcl-2 homologous antagonist/killer	TM
CTSC	Dipeptidyl peptidase 1	CY
DNPEP	Aspartyl aminopeptidase	CY
DYNLRB1	Dynein light chain roadblock-type 1	CA
FAM120A	Constitutive coactivator of PPAR-gamma-like protein 1	CY
GNG5	Guanine nucleotide-binding protein subunit gamma-5	MA
GRB2	Growth factor receptor-bound protein 2	AD
HRH4	Histamine H4 receptor	TM
MOB1A	MOB kinase activator 1A	CY
PECAM1	Platelet endothelial cell adhesion molecule	TM
PLGRKT	Plasminogen receptor (KT)	TM
POF1B	Protein POF1B	CA
PTPN11	Tyrosine-protein phosphatase non-receptor type 11	CY
RAC1	Ras-related C3 botulinum toxin substrate 1	CY
S100A6	Protein S100-A6	CY
SRI	Sorcin	CY
SYK	Tyrosine-protein kinase SYK	CY
TYROBP	TYRO protein tyrosine kinase-binding protein	TM
BST1	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase 2	MA
HINT1	Histidine triad nucleotide-binding protein 1	CY
ITGA5	Integrin alpha-5	TM
MAP4K1	Mitogen-activated protein kinase kinase kinase kinase 1	CY
PLAUR	Urokinase plasminogen activator surface receptor	MA
RGS11	Regulator of G-protein signaling 11	CY
ANXA2	Annexin A2	MA
APIP	Methylthioribulose-1-phosphate dehydratase	CY
ARF6	ADP-ribosylation factor 6	CY
ARHGDIA	Rho GDP-dissociation inhibitor 1	CY
CPPED1	Serine/threonine-protein phosphatase CPPED1	CY
EPB41L3	Band 4.1-like protein 3	MA
GMIP	GEM-interacting protein	CY
LASP1	LIM and SH3 domain protein 1	CY
LGALS3	Galectin-3	CY
RANBP1	Ran-specific GTPase-activating protein	CY
TAGLN2	Transgelin-2	CA
YWHAH	14-3-3 protein eta	CY

signaling and neutrophil activation and now associates with novel partners in a different signaling network.

Discussion

In the present study, we identified the interacting proteins of HPK1 in nonactivated, suspended, as well as in Mn^{2+}-activated, adherent dHL-60 HPK1-EGFP cells. Among the identified 115 proteins interacting with HPK1, we focused on those proteins with known functions in signaling and constructed the interacting network of HPK1. Within the interacting network, a subset of proteins formed a signaling cluster with HPK1 specifically in nonactivated cells, including the proteins DAP12 and Syk, known to be involved in β_2 integrin signaling in neutrophils [1,3,21,27]. Syk and the related tyrosine kinase zeta-chain-associated protein kinase 70 (ZAP-70) expressed in T cells are well-known interactors of HPK1 in leukocytes, inducing HPK1 activation [28–30]. However, in T cells, the activation of HPK1 leads to termination of TCR signaling, whereas in neutrophils, HPK1 is necessary to activate the β_2 integrin LFA-1, leading to neutrophil adhesion [6,14]. In nonactivated dHL-60 cells, Rac1 was identified as

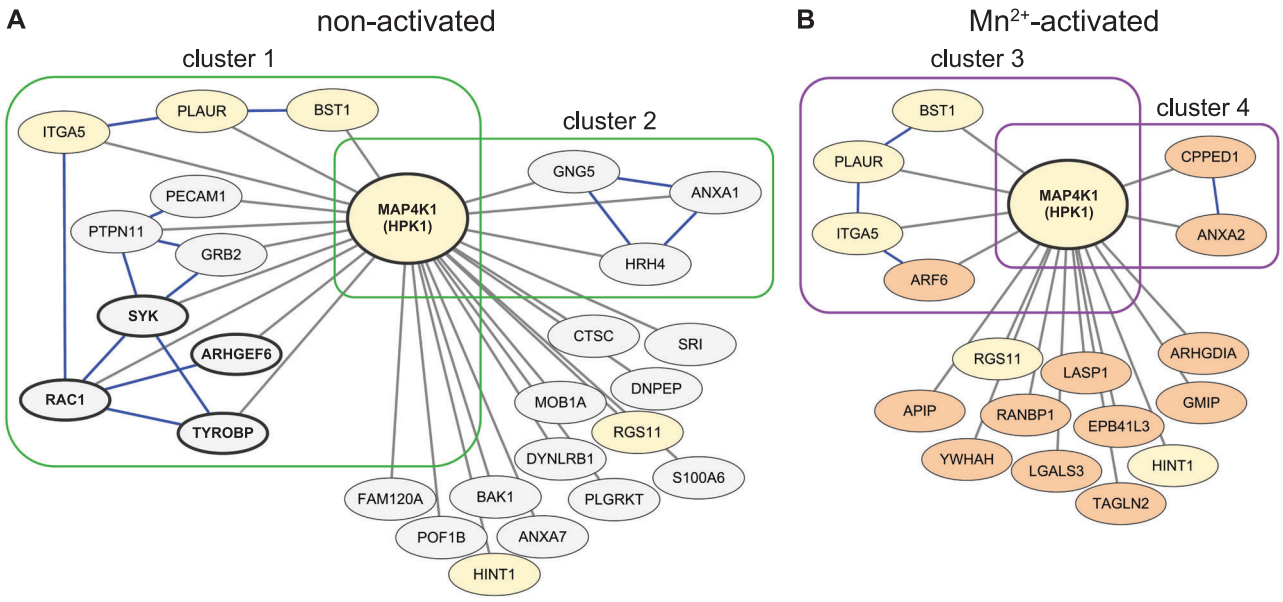

Figure 3. Interacting network of HPK1 in (A) nonactivated and (B) Mn^{2+}-activated dHL-60 HPK1-EGFP cells. Proteins identified in nonactivated cells are depicted in grey, proteins found only in Mn^{2+}-activated cells are depicted in orange and proteins found in both conditions are depicted in yellow. Protein interactions extracted from STRING network are shown in blue. (A) Cluster 1 in nonactivated cells included the proteins DAP12 (gene *TYROBP*), Syk (gene *SYK*), and Rac1 (gene *RAC1*) which are crucial for β_2 integrin signaling in neutrophils suggesting HPK1 functions as scaffold protein providing the molecular machinery for rapid β_2 integrin signaling.

HPK1 interactor together with its activator, ARHGEF6. Rac1 is an important regulator of neutrophil adhesion by inducing cytoskeleton remodeling [22,31,32]. Interestingly, Rac1 activity has been shown to be downregulated upon β_2 integrin-dependent adhesion of human neutrophils [33]. The Rho GTPase dissociation inhibitor alpha (ARGHDIA, gene *ARGHDIA*), which can inhibit Rac1 activity, was found in activated, adherent cells. This suggests that HPK1 represents a functional switch which supports Rac1 activation in non-adherent cells by recruitment of ARHGEF6 and enforces Rac1 deactivation by dissociation from ARHGEF6 and association with ARGHDIA upon induction of adhesion. Association of HPK1 with DAP12 and Syk as well as Rac1 and ARHGEF6, therefore, suggests that HPK1 composes or is at least part of a preformed protein complex necessary for rapid induction of β_2 integrin signaling in adherent neutrophils.

A considerable difference between the roles of HPK1 in neutrophil recruitment and T cell activation may be due to the different time course and context of these two processes. Free-flowing neutrophils have to be prepared for the rapid induction of β_2 integrin-dependent adhesion and subsequent arrest on inflamed endothelial cells. Therefore, the proteins necessary for β_2 integrin signaling may exist in a preformed signaling complex in which HPK1 may play a central role as an adaptor protein, providing the molecular machinery for rapid neutrophil activation while the cell is still inactive [34]. In this context, HPK1 may act in analogy to lymphocytes as a negative regulator which keeps its interacting partners in an inactive state until the induction of adhesion. Thus, HPK1 may be important for sustained signaling processes, promoting an inactive state. This is in line with the finding that the absence of HPK1 promotes hyperinflammation [6].

To date, it is unclear whether the kinase activity of HPK1 is necessary for β_2 integrin signaling in neutrophils, as in previous studies neutrophils were used from HPK1 KO mice [14]. Since HPK1 is harboring proline-rich motifs as well as a citron homology domain enabling protein-protein interactions, HPK1 could at least partly exert its functions as scaffold molecule [35,36]. The use of HPK1 kinase-dead mouse strains showed that dampening TCR signaling by HPK1-induced destabilization of SLP-76 complexes depends on HPK1 kinase activity in T cells [36]. In contrast, HPK1 interactions with Grb2, GADS, and mAbp1, among others, apparently do not involve phosphorylation of these adaptors by HPK1 [29,30,36–39]. Similarly, a scaffold function has also been shown for the kinase Syk linking the B cell-specific adaptor BLNK to Ca^{2+} mobilization [40]. Accordingly, HPK1 may recruit and/or stabilize a protein complex including all components necessary for rapid β_2 integrin signaling and neutrophil arrest independent from its kinase activity [36]. However, substrates of HPK1 will probably not be identified by co-IP and subsequent MS as the interaction between kinases and their substrates is often not stable enough to survive this procedure. Here, phosphoproteomic or HPK1 kinase-dead co-IP approaches would be helpful to distinguish signaling processes dependent on HPK1 kinase function and/or a proposed HPK1 scaffold function.

In conclusion, our data indicate that HPK1 may function as a potential scaffold protein providing a pre-existing protein cluster for rapid β_2 integrin signaling and neutrophil postarrest functions. Surprisingly, the majority of the identified interacting proteins of HPK1 in neutrophils were not previously described as HPK1 interactors in T and B cells which may explain the different functions of HPK1 in neutrophils versus T and B cells [10,15]. Some of

the proteins found to interact with HPK1 in neutrophils, such as CD157, platelet endothelial cell adhesion molecule 1 (PECAM-1), uPAR, and Arf6, have already been reported to regulate neutrophil adhesion, spreading, chemotactic migration, and extravasation as well as postadhesion functions [23,25,26,41]. Recently, HPK1 has emerged as potent target for cancer therapy [42,43]. Here, suppression of HPK1 function enhances antitumorigenic behavior of T cells and DCs and HPK1 inhibitors specifically targeting HPK1 kinase activity are currently being designed as therapeutic molecules. In this context, the effect of HPK1 inhibition on neutrophil function has to be carefully considered.

Material and methods

Cell culture

HL-60 cells (ACC 3, German Collection of Microorganisms and Cell Cultures, Germany) and human embryonic kidney (HEK)-293T/17 cells (CRL-11268, American Type Culture Collection, USA) were cultured in RPMI-1640 (Biochrom, Germany) supplemented with 10% FCS (Biochrom) and 100 μg/mL penicillin/streptomycin (Biochrom). For differentiation towards neutrophil-like dHL-60 cells, the cell culture medium was supplemented with 1.3% DMSO for 6 days. Cells were kept at 37°C and 5% CO_2 in a humidified incubator.

Generation of HL-60 HPK1-EGFP cells

The coding sequence of human HPK1 (Source BioScience, United Kingdom) was cloned into a pEGFP-N1 vector to generate the HPK1-EGFP fusion sequence which was subcloned into the FuVal lentiviral vector pFu-P2A-Puro using the In-Fusion cloning kit (Takara Bio Europe, France). The resulting pFu-HPK1-EGFP-P2A-Puro vector was used to transfect HEK-293T/1744]. Virus-containing supernatant was harvested 48 h post-transfection and used to transduce HL-60 cells, which were subsequently cultured in the presence of 1 μg/mL puromycin (Sigma-Aldrich, Germany) for 3 weeks. Cells were sorted according to EGFP fluorescence. Expression of the HPK1-EGFP fusion protein was detected by Western blotting.

Western blotting

For preparation of whole-cell lysates, refer to the Supporting Information. For protein detection, mouse anti-HPK1 antibody (sc-376169, Santa Cruz Biotechnology, USA), rabbit anti-GFP antibody (sc-8334, Santa Cruz Biotechnology), mouse anti-actin antibody (sc-47778, Santa Cruz Biotechnology), and secondary HRP-conjugated goat anti-mouse (sc-2005, Santa Cruz Biotechnology) or goat anti-rabbit (sc-2004, Santa Cruz Biotechnology) antibodies were used. Detection was performed with Pierce ECL Western blotting substrate (Thermo Fisher Scientific, USA).

Immunofluorescence staining and confocal microscopy

For localization studies, dHL-60 HPK1-EGFP cells were exposed to immobilized fibrinogen (250 μg/mL, Sigma-Aldrich) and stimulated with 100 nM fMLP (Sigma-Aldrich) in adhesion medium for 15 min at 37°C [44]. Antibody staining was performed with a primary rabbit anti-mAbp1 antibody (sc-366772, Santa Cruz Biotechnology) and a secondary AlexaFluor647-conjugated anti-rabbit antibody (Thermo Fisher Scientific). The actin cytoskeleton was visualized using AlexaFluor546-labeled phalloidin (Thermo Fisher Scientific). Samples were mounted in ProLong Diamond (Thermo Fisher Scientific).

Confocal microscopy was performed at the Core Facility Bioimaging of the Biomedical Center of the Ludwig-Maximilians-Universität München using an upright confocal Leica SP8X WLL microscope (Leica, Germany).

Co-immunoprecipitation

Co-IP experiments were conducted with GFP Nano-Trap beads (Chromotek, Germany) and performed as described before [44]. In brief, dHL-60 HPK1-EGFP or dHL-60 EGFP control cells were exposed to immobilized fibrinogen (250 μg/mL, Sigma-Aldrich) for 15 min and either left nonactivated as control or activated with 1 mM Mn^{2+} to allow β_2 integrin-mediated adhesion. Successful performance of the co-IP was analyzed via Western blot technique and the immunoprecipitates were subjected to MS.

Mass spectrometry

GFP Nano-Trap beads with bound proteins were washed with 50 mM NH_4HCO_3 and digested with 10 ng/μL trypsin in 1 M urea and 50 mM NH_4HCO_3. The overnight digestion was performed in the presence of 1 mM DTT. Digested peptides were alkylated and desalted before LC. For LC-MS purposes, desalted peptides were injected in an Ultimate 3000 RSLCnano system (Thermo Fisher Scientific), separated in a 15 cm analytical column (75 μm ID with ReproSil-Pur C18-AQ 2.4 μm, Dr. Maisch GmbH, Germany) with a 50 min gradient from 5 to 60% acetonitrile in 0.1% formic acid. The effluent from the HPLC was directly electrosprayed into a QExactive HF (Thermo Fisher Scientific) operated in a data-dependent mode to automatically switch between full-scan MS and MS/MS acquisition. Survey full-scan MS spectra (from m/z 375 to 1600) were acquired with resolution $R = 60{,}000$ at m/z 400 (AGC target of 3×10^6). The ten most intense peptide ions with charge states between 2 and 5 were sequentially isolated to a target value of 1×10^5, and fragmented at 27% nor-

malized collision energy. Typical mass spectrometric conditions were: spray voltage, 1.5 kV; no sheath and auxiliary gas flow; heated capillary temperature, 250°C; ion selection threshold, 33.000 counts.

MaxQuant version 1.5.2.8 software was used for protein identification and quantification using the intensity-based absolute quantification (iBAQ) value [17]. Proteins were considered as relevant interaction partners of HPK1 if the difference of the iBAQ value ($\log_2$ fold-change) between HPK1-EGFP and EGFP co-IP experiments was higher than 2. To identify and eliminate non-specific interacting proteins, a CRAPome analysis was conducted as described previously [18]. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021136 [45].

Generation of the HPK1 interacting network

The interacting network of HPK1 was analyzed and visualized using Cytoscape 3.8.0 software [46]. All interacting proteins of HPK1 identified in our screen were manually categorized into transmembrane, membrane-associated, adaptor, cytoplasmic, and cytoskeleton-associated proteins based on gene ontology terms according to The Human Protein Atlas and those with known functions in signaling were further considered [47]. These HPK1-interacting proteins were merged with known interactions extracted from the STRING database [19]. The shown interactions were limited to those found in experiments or databases, and only those interactions with highest confidence according to STRING parameters (score > 0.9) were considered.

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Author Contributions

AB carried out research, analyzed data, and wrote the manuscript. MW carried out research and analyzed data. IF performed mass spectrometry and analyzed data. BW and DM-B designed the research, analyzed data, and wrote the manuscript.

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Conflict of Interest: The authors declare no commercial or financial conflict of interest.

Ethics Approval Statement: All volunteers signed a written informed consent prior to blood donation. The blood collection was performed according to the Declaration of Helsinki and was approved by the Ethics Committee of the LMU Munich.

Data Availability Statement: The datasets generated for this study can be found in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD021136.

Peer review: The peer review history for this article is available at https://publons.com/publon/10.1002/eji.202149283.

References

1 **Mocsai, A., Abram, C. L., Jakus, Z., Hu, Y., Lanier, L. L. and Lowell, C. A.**, Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat. Immunol.* 2006. **7**: 1326–1333.

2 **Jakus, Z., Fodor, S., Abram, C. L., Lowell, C. A. and Mocsai, A.** Immunoreceptor-like signaling by beta 2 and beta 3 integrins. *Trends Cell Biol.* 2007. **17**: 493–501.

3 **Mocsai, A., Zhou, M., Meng, F., Tybulewicz, V. L. and Lowell, C. A.**, Syk is required for integrin signaling in neutrophils. *Immunity* 2002. **16**: 547–558.

4 **Newbrough, S. A., Mocsai, A., Clemens, R. A., Wu, J. N., Silverman, M. A., Singer, A. L., Lowell, C. A. et al.**, SLP-76 regulates Fcgamma receptor and integrin signaling in neutrophils. *Immunity* 2003. **19**: 761–769.

5 **Di Bartolo, V., Montagne, B., Salek, M., Jungwirth, B., Carrette, F., Fourtane, J., Sol-Foulon, N. et al.**, A novel pathway down-modulating T cell activation involves HPK-1-dependent recruitment of 14-3–3 proteins on SLP-76. *J. Exp. Med.* 2007. **204**: 681–691.

6 **Shui, J. W., Boomer, J. S., Han, J., Xu, J., Dement, G. A., Zhou, G. and Tan, T. H.**, Hematopoietic progenitor kinase 1 negatively regulates T cell receptor signaling and T cell-mediated immune responses. *Nat. Immunol.* 2007. **8**: 84–91.

7 **Baker, R. G., Hsu, C. J., Lee, D., Jordan, M. S., Maltzman, J. S., Hammer, D. A., Baumgart, T. et al.**, The adapter protein SLP-76 mediates "outside-in" integrin signaling and function in T cells. *Mol. Cell. Biol.* 2009. **29**: 5578–5589.

8 **Patzak, I. M., Königsberger, S., Suzuki, A., Mak, T. W. and Kiefer, F.**, HPK1 competes with ADAP for SLP-76 binding and via Rap1 negatively affects T-cell adhesion. *Eur. J. Immunol.* 2010. **40**: 3220–3225.

9 **Wang, X., Li, J. P., Kuo, H. K., Chiu, L. L., Dement, G. A., Lan, J. L., Chen, D. Y. et al.**, Down-regulation of B cell receptor signaling by hematopoietic progenitor kinase 1 (HPK1)-mediated phosphorylation and ubiquitination of activated B cell linker protein (BLNK). *J. Biol. Chem.* 2012. **287**: 11037–11048.

10 **Chuang, H. C., Wang, X. and Tan, T. H.**, MAP4K Family Kinases in Immunity and Inflammation. *Adv. Immunol.* 2016. **129**: 277–314.

11 **Wang, X., Li, J. P., Chiu, L. L., Lan, J. L., Chen, D. Y., Boomer, J. and Tan, T. H.**, Attenuation of T cell receptor signaling by serine phosphorylation-mediated lysine 30 ubiquitination of SLP-76 protein. *J. Biol. Chem.* 2012. **287**: 34091–34100.

12 **Königsberger, S., Peckl-Schmid, D., Zaborsky, N., Patzak, I., Kiefer, F. and Achatz, G.**, HPK1 associates with SKAP-HOM to negatively regulate Rap1-mediated B-lymphocyte adhesion. *PLoS One* 2010. **5**: e12468.

13 **Wang, H., McCann, F. E., Gordan, J. D., Wu, X., Raab, M., Malik, T. H., Davis, D. M. et al.**, ADAP-SLP-76 binding differentially regulates supramolecular activation cluster (SMAC) formation relative to T cell-APC conjugation. *J. Exp. Med.* 2004. **200**: 1063–1074.

14 **Jakob, S. M., Pick, R., Brechtefeld, D., Nussbaum, C., Kiefer, F., Sperandio, M. and Walzog, B.**, Hematopoietic progenitor kinase 1 (HPK1) is required for LFA-1-mediated neutrophil recruitment during the acute inflammatory response. *Blood* 2013. **121**: 4184–4194.

15 **Zhang, Q., Ding, S. and Zhang, H.**, Interactions between hematopoietic progenitor kinase 1 and its adaptor proteins (Review). *Mol. Med. Rep.* 2017. **16**: 6472–6482.

16 **Wu, P., Sneeringer, C. J., Pitts, K. E., Day, E. S., Chan, B. K., Wei, B., Lehoux, I. et al.**, Hematopoietic progenitor kinase-1 structure in a domain-swapped dimer. *Structure* 2019. **27**: 125–133.

17 **Tyanova, S., Temu, T. and Cox, J.**, The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. *Nat. Protoc.* 2016. **11**: 2301–2319.

18 **Mellacheruvu, D., Wright, Z., Couzens, A. L., Lambert, J. P., St-Denis, N. A., Li, T., Miteva, Y. V. et al.**, The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. *Nat. Methods.* 2013. **10**: 730–736.

19 **Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., Simonovic, M. et al.**, STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic. Acids Res.* 2019. **47**: D607–D13.

20 **von Andrian, U. H., Chambers, J. D., McEvoy, L. M., Bargatze, R. F., Arfors, K. E. and Butcher, E. C.**, Two-step model of leukocyte-endothelial cell interaction in inflammation: distinct roles for LECAM-1 and the leukocyte beta 2 integrins in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 1991. **88**: 7538–7542.

21 **Schymeinsky, J., Then, C. and Walzog, B.**, The non-receptor tyrosine kinase Syk regulates lamellipodium formation and site-directed migration of human leukocytes. *J. Cell. Physiol.* 2005. **204**: 614–622.

22 **Glogauer, M., Marchal, C. C., Zhu, F., Worku, A., Clausen, B. E., Foerster, I., Marks, P. et al.**, Rac1 deletion in mouse neutrophils has selective effects on neutrophil functions. *J. Immunol.* 2003. **170**: 5652–5657.

23 **Funaro, A., Ortolan, E., Ferranti, B., Gargiulo, L., Notaro, R., Luzzatto, L. and Malavasi, F.**, CD157 is an important mediator of neutrophil adhesion and migration. *Blood* 2004. **104**: 4269–4278.

24 **Gao, J. X., Wilkins, J. and Issekutz, A. C.**, Migration of human polymorphonuclear leukocytes through a synovial fibroblast barrier is mediated by both beta 2 (CD11/CD18) integrins and the beta 1 (CD29) integrins VLA-5 and VLA-6. *Cell. Immunol.* 1995. **163**: 178–186.

25 **Gyetko, M. R., Aizenberg, D. and Mayo-Bond, L.**, Urokinase-deficient and urokinase receptor-deficient mice have impaired neutrophil antimicrobial activation in vitro. *J. Leukoc. Biol.* 2004. **76**: 648–656.

26 **Gamara, J., Davis, L., Rollet-Labelle, E., Hongu, T., Funakoshi, Y., Kanaho, Y., Aoudjit, F. et al.**, Assessment of Arf6 deletion in PLB-985 differentiated in neutrophil-like cells and in mouse neutrophils: impact on adhesion and migration. *Mediators Inflamm.* 2020. **2020**: 2713074.

27 **Frommhold, D., Mannigel, I., Schymeinsky, J., Mocsai, A., Poeschl, J., Walzog, B. and Sperandio, M.**, Spleen tyrosine kinase Syk is critical for sustained leukocyte adhesion during inflammation in vivo. *BMC Immunol.* 2007. **8**: 31.

28 **Liou, J., Kiefer, F., Dang, A., Hashimoto, A., Cobb, M. H., Kurosaki, T. and Weiss, A.**, HPK1 is activated by lymphocyte antigen receptors and negatively regulates AP-1. *Immunity*. 2000. **12**: 399–408.

29 **Ling, P., Meyer, C. F., Redmond, L. P., Shui, J. W., Davis, B., Rich, R. R., Hu, M. C. et al.**, Involvement of hematopoietic progenitor kinase 1 in T cell receptor signaling. *J. Biol. Chem.* 2001. **276**: 18908–18914.

30 **Sauer, K., Liou, J., Singh, S. B., Yablonski, D., Weiss, A. and Perlmutter, R. M.**, Hematopoietic progenitor kinase 1 associates physically and functionally with the adaptor proteins B cell linker protein and SLP-76 in lymphocytes. *J. Biol. Chem.* 2001. **276**: 45207–45216.

31 **Block, H., Stadtmann, A., Riad, D., Rossaint, J., Sohlbach, C., Germena, G., Wu, D. et al.**, Gnb isoforms control a signaling pathway comprising Rac1, Plcbeta2, and Plcbeta3 leading to LFA-1 activation and neutrophil arrest in vivo. *Blood* 2016. **127**: 314–324.

32 **Sun, C. X., Downey, G. P., Zhu, F., Koh, A. L., Thang, H. and Glogauer, M.**, Rac1 is the small GTPase responsible for regulating the neutrophil chemotaxis compass. *Blood* 2004. **104**: 3758–3765.

33 **Dib, K., Melander, F., Axelsson, L., Dagher, M. C., Aspenström, P. and Andersson, T.**, Down-regulation of Rac activity during beta 2 integrin-mediated adhesion of human neutrophils. *J. Biol. Chem.* 2003. **278**: 24181–24188.

34 **Lefort, C. T. and Ley, K.**, Neutrophil arrest by LFA-1 activation. *Front. Immunol.* 2012. **3**: 157.

35 **Kiefer, F., Tibbles, L. A., Anafi, M., Janssen, A., Zanke, B. W., Lassam, N., Pawson, T. et al.**, HPK1, a hematopoietic protein kinase activating the SAPK/JNK pathway. *EMBO J.* 1996. **15**: 7013–7025.

36 **Hernandez, S., Qing, J., Thibodeau, R. H., Du, X., Park, S., Lee, H. M., Xu, M. et al.**, The kinase activity of hematopoietic progenitor kinase 1 is essential for the regulation of T cell function. *Cell Rep.* 2018. **25**: 80–94.

37 **Anafi, M., Kiefer, F., Gish, G. D., Mbamalu, G., Iscove, N. N. and Pawson, T.**, SH2/SH3 adaptor proteins can link tyrosine kinases to a Ste20-related protein kinase, HPK1. *J. Biol. Chem.* 1997. **272**: 27804–27811.

38 **Liu, S. K., Smith, C. A., Arnold, R., Kiefer, F. and McGlade, C. J.**, The adaptor protein Gads (Grb2-related adaptor downstream of Shc) is implicated in coupling hemopoietic progenitor kinase-1 to the activated TCR. *J. Immunol.* 2000. **165**: 1417–1426.

39 **Ensenat, D., Yao, Z., Wang, X. S., Kori, R., Zhou, G., Lee, S. C. and Tan, T. H.**, A novel src homology 3 domain-containing adaptor protein, HIP-55, that interacts with hematopoietic progenitor kinase 1. *J. Biol. Chem.* 1999. **274**: 33945–33950.

40 **Abudula, A., Grabbe, A., Brechmann, M., Polaschegg, C., Herrmann, N., Goldbeck, I., Dittmann, K. et al.**, SLP-65 signal transduction requires Src homology 2 domain-mediated membrane anchoring and a kinase-independent adaptor function of Syk. *J. Biol. Chem.* 2007. **282**: 29059–29066.

41 **Wu, Y., Stabach, P., Michaud, M. and Madri, J. A.**, Neutrophils lacking platelet-endothelial cell adhesion molecule-1 exhibit loss of directionality and motility in CXCR2-mediated chemotaxis. *J. Immunol.* 2005. **175**: 3484–3491.

42 **Sawasdikosol, S. and Burakoff, S.**, A perspective on HPK1 as a novel immuno-oncology drug target. *Elife* 2020. **9**: e55122.

43 **Si, J., Shi, X., Sun, S., Zou, B., Li, Y., An, D., Lin, X. et al.**, Hematopoietic progenitor kinase1 (HPK1) mediates T cell dysfunction and is a druggable target for T cell-based immunotherapies. *Cancer Cell* 2020. **38**: 551–566.

44 **Hepper, I., Schymeinsky, J., Weckbach, L. T., Jakob, S. M., Frommhold, D., Sixt, M., Laschinger, M. et al.**, The mammalian actin-binding protein 1 is critical for spreading and intraluminal crawling of neutrophils under flow conditions. *J. Immunol.* 2012. **188**: 4590–4601.

45 **Perez-Riverol, Y., Csordas, A., Bai, J., Bernal-Llinares, M., Hewapathirana, S., Kundu, D. J., Inuganti, A. et al.**, The PRIDE database and related tools and resources in 2019: improving support for quantification data. *Nucleic Acids Res.* 2019. **47**: D442–D50.

46 **Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., Amin, N. et al.**, Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res*. 2003. **13**: 2498–2504.

47 **Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, A. et al.**, Tissue-based map of the human proteome. *Science* 2015. **347**: 1260419.

Abbreviations: **BLNK**: B cell linker protein · **DAP12**: DNAX-activation protein 12 · **EGFP**: enhanced green fluorescent protein · **fMLP**: *N*-formyl-methionyl-leucyl-phenylalanine · **HPK1**: hematopoietic progenitor kinase 1 · **LFA-1**: lymphocyte function-associated antigen 1 · **mAbp1**: mammalian actin-binding protein 1 · **MS**: mass spectrometry · **SLP-76**: Src homology 2 domain-containing leukocyte protein of 76 kDa · **Syk**: spleen tyrosine kinase · **uPAR**: urokinase plasminogen activator surface receptor

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