An Integrated Phosphoproteomics Work Flow Reveals Extensive Network Regulation in Early Lysophosphatidic Acid Signaling*§

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Lysophosphatidic acid (LPA) induces a variety of cellular signaling pathways through the activation of its cognate G protein-coupled receptors. To investigate early LPA responses and assess the contribution of epidermal growth factor (EGF) receptor transactivation in LPA signaling, we performed phosphoproteomics analyses of both total cell lysate and protein kinase-enriched fractions as complementary strategies to monitor phosphorylation changes in A498 kidney carcinoma cells. Our integrated work flow enabled the identification and quantification of more than 5,300 phosphorylation sites of which 224 were consistently regulated by LPA. In addition to induced phosphorylation events, we also obtained evidence for early dephosphorylation reactions due to rapid phosphatase regulation upon LPA treatment. Phosphorylation changes induced by direct heparin-binding EGF-like growth factor-mediated EGF receptor activation were typically weaker and only detected on a subset of LPA-regulated sites, indicating signal integration among EGF receptor transactivation and other LPA-triggered pathways. Our results reveal rapid phosphoregulation of many proteins not yet implicated in G protein-coupled receptor signaling and point to various additional mechanisms by which LPA might regulate cell survival and migration as well as gene transcription on the molecular level. Moreover, our phosphoproteomics analysis of both total lysate and kinase-enriched fractions provided highly complementary parts of the LPA-regulated signaling network and thus represents a useful and generic strategy toward comprehensive signaling studies on a system-wide level. Molecular & Cellular Proteomics 9:1047–1062, 2010.

Lysoosphosphatidic acid (LPA) is a simple bioactive lipid that activates G protein-coupled receptors (GPCRs) and elicits a variety of biological responses, such as cell proliferation, migration, and survival (1, 2). In addition to important physiological functions in, for example, vascular biology and wound healing, LPA signaling has also been implicated in pathophysiological processes, such as the onset and progression of human cancers (3, 4). The lipid mediator LPA is primarily generated upon extracellular hydrolysis of larger lysophospholipids by the secreted enzyme autotaxin, a lysophospholipase D that is up-regulated in several cancers and was initially identified as an autocrine motility factor for tumor cells (5). To date, seven high affinity receptors for LPA (LPA1–5, GPR 87, and P2Y5) are known with distinct but overlapping expression patterns. The well characterized LPA receptors LPA1–3, which belong to the endothelial differentiation gene family of lysophospholipid-recognizing GPCRs, activate multiple signaling pathways through heterotrimeric G proteins from the Gα, Gq, and G12/13 families (2, 6). Activation of Gq inhibits adenylyl cyclase-mediated production of cyclic AMP and has been implicated in the activation of the ERK and phosphatidylinositol 3-kinase signaling (1, 2). LPA also triggers Gq-dependent signals through phospholipase C-mediated generation of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, which induce protein kinase C

1 The abbreviations used are: LPA, lysophosphatidic acid; GPCR, G protein-coupled receptor; EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; ERK, extracellular-signal regulated kinase; PKD, protein kinase D; EGFR, epidermal growth factor receptor; MAPK, mitogen-activated protein kinase; ADAM, a disintegrin and metalloproteinase; SILAC, stable isotope labeling by amino acids in cell culture; LDS, lithium dodecyl sulfate; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; SCX, strong cation exchange; nano-LC, nanoscale capillary LC; LTQ, linear trap quadrupole; IPI, International Protein Index; GO, gene ontology; DAVID, Database for Annotation, Visualization and Integrated Discovery; STRING, Search Tool for the Retrieval of Interacting Genes/Proteins; AAK1, AP2-associated protein kinase 1; FAK, focal adhesion kinase; MAP, mitogen-activated protein; GAP, GTPase-activating protein; GRLF1, glucocorticoid receptor DNA-binding factor 1; ARHGAP29, Rho GTPase-activating protein 29; GEF, guanine nucleotide exchange factor; PEA15, phosphoprotein enriched in astrocytes 15; PDCD4, programmed cell death protein 4; HDAC, histone deacetylase; BMPR2, bone morphogenic protein receptor 2; DHCR7, 7-dehydrocholesterol reductase.
activity and elevate cytosolic calcium levels (2, 7). Moreover, LPA induces rapid cytoskeletal rearrangements, such as stress fiber formation via G12/13-mediated activation of the small GTPase Rho (7–9). Early LPA signaling can cooperatively regulate downstream effectors as for example demonstrated for the convergence of Gαi, Gαq, and G12/13-triggered pathways on protein kinase D (PKD) (7, 10).

In many cell types, LPA stimulation leads to the rapid trans-activation of the epidermal growth factor receptor (EGFR) tyrosine kinase, which relays mitogenic downstream signaling through ERK MAPK- and Akt/protein kinase B-dependent pathways (4, 7, 11–13). This GPCR-EGFR cross-talk is mediated by ADAMs, which proteolytically cleave transmembrane precursors to generate mature EGFR ligands, such as heparin-binding EGF-like growth factor (HB-EGF) or amphiregulin (13–17). The GPCR-induced mechanisms underlying cellular metalloproteinase regulation are still incompletely understood. Potential control elements include Gi and Gq (18, 19), Src family and phosphatidylinositol 3-kinases (20, 21), and Rac and Ras GTPase effector pathways.

Taken together, extensive previous research unveiled LPA as a rapid inducer of multiple pathways that form interdependent networks and utilize protein kinases and phosphorylation-based signaling to drive key biological processes, such as cell proliferation, survival, and migration. However, as previous studies have not analyzed LPA-triggered kinase signaling in an unbiased manner, it is unclear to what extent our current knowledge is still incomplete.

Notably, recent developments in mass spectrometry-based proteomics have set the stage for global approaches toward GPCR-mediated signaling to address these issues. In particular, stable isotope labeling by amino acids in cell culture (SILAC) (22), optimized phosphopeptide fractionation and enrichment protocols (23, 24), high resolution MS on ion trap/orbitrap hybrid instruments (25), and breakthroughs in MS data processing (26) can now be integrated in efficient phosphoproteomics work flows to quantify thousands of phosphorylation events in a cellular system. Furthermore, new enrichment techniques based on affinity purification with immobilized inhibitors provide a valuable tool to selectively increase the analytical sensitivity for protein kinases, which are often underrepresented in total cell lysate analysis because of their low cellular abundance (27–29). The detection of regulated phosphorylation events on protein kinases can be particularly informative as these enzymes are located at critical nodes of signaling cascades and networks.

Here, we applied SILAC-based, quantitative phosphoproteomics in a differential analysis of phosphorylation events triggered by either LPA or HB-EGF stimulation. To assess the overall contribution of EGFR transactivation to early phosphorylation changes upon LPA treatment, human A498 kidney carcinoma cells were used as a cancer-relevant cell line in which LPA rapidly elicits EGFR-dependent signaling by ADAM-mediated processing of pro-HB-EGF ligand precursors (13). Our quantitative analysis revealed numerous new phosphorylation events that are likely associated with either known biological responses or point to previously unrecognized signaling elements in the context of LPA-triggered GPCR signaling. In this study, we further demonstrate that parallel phosphoproteomics of total cell lysate and kinase-enriched fractions provides highly complementary sets of data and therefore represents a general strategy to assess phosphorylation-based signaling networks in a comprehensive manner.

**Experimental Procedures**

**Cell Culture and Cell Lysis—** A498 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen), glutamine, non-essential amino acids, and sodium pyruvate. For SILAC, cells were grown for 8 days in Dulbecco’s modified Eagle’s medium containing either normal L-arginine (Arg0) at 42 mg/liter and L-lysine (Lys0) at 71 mg/liter or equimolar amounts of L-[U-13C6,14N4]arginine (Arg6) and L-[1,2,6,15N4]lysine (Lys4) or L-[U-13C6,14N4]arginine (Arg10) and L-[U-13C6,14N4]lysine (Lys8) (from Sigma Isotec or Cambridge Isotope Laboratories). Cells were serum-starved for 2 days prior to growth factor stimulation. In the first experiment, Arg0/Lys0-labeled cells were left untreated, Arg6/Lys4-labeled cells were stimulated with 0.5 ng/ml HB-EGF (Sigma-Aldrich) for 3 min, and Arg10/Lys8-labeled cells were treated with 10 μM LPA (Sigma-Aldrich or Biomeri) for 3 min. In the second experiment, Arg0/Lys0-labeled cells were left untreated, and Arg10/Lys8-labeled cells were stimulated with HB-EGF. For each condition, labeled cells from 35 dishes (15-cm diameter) were lysed with ice-cold lysis buffer containing 50 mM HEPES-NaOH, pH 7.5, 150 mM NaCl, 0.5% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM PMSF, 10 mM NaF, 2.5 mM Na3VO4, 50 μg/ml calyculin A, 1% phosphatase inhibitor mixture 2 (Sigma-Aldrich or Biomeri) and 0.45-μm PVDF membrane. Protein concentrations were determined by the BCA assay (Pierce), and lysates were pooled at equal protein amounts. Stimulation was controlled by immunoblot analysis with HB-EGF.

**Kinase Affinity Resins and Enrichment—** The kinase inhibitors VI16832, AX14596, and SU6688 were prepared as described elsewhere (27, 30, 31). VI16741 was synthesized as VI16832 except that 8-ethyl-2-methanesulfonyl-6H-pyrido[2,3-d]pyrimidin-7-one was used as starting material instead of 8-bicyclo[2.2.1]hept-2-yl-2-methanesulfonyl-6H-pyrido[2,3-d]pyrimidin-7-one. Commercially available inhibitors were bisindolylmaleimide X (Alexis Biochemicals), purvalanol B (Tocris), and dasatinib (LC Laboratories). For immobilization, 2 volumes of 5 mM bisindolylmaleimide X, 10 mM AX14596, 1.5 mM VI16832, 5 mM VI16741, or 5 mM dasatinib were coupled to 1 g/ml leupeptin, 1 mg/ml aprotinin, 1 μg/ml calyculin A, 1% phosphatase inhibitor mixture 2 (Sigma-Aldrich or Biomeri) and 0.45-μm PVDF membrane. Protein concentrations were determined by the BCA assay (Pierce), and lysates were pooled at equal protein amounts. Stimulation was controlled by immunoblot analysis with HB-EGF.
Phosphoproteome Quantification in GPCR Signaling

MS Analysis—Tryptic peptides were separated by on-line reverse phase nanoscale capillary LC (nano-LC; Agilent 1100) coupled to ESI-MS/MS. Using the nano-LC system, samples were injected onto a 15-cm reverse phase, fused silica capillary column (inner diameter, 75 μm; packed in-house with 3-μm ReproSil-Pur C₁₈-AQ medium; Dr. Maisch GmbH) kept at 31 °C. The nano-LC system was connected to an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with a nanoelectrospray ion source (Proxeon Biosystems). Loaded peptides were eluted with 140-min gradients ranging from 5 to 40% ACN in 0.5% acetic acid with a flow rate of 250 nl/min. Data-dependent acquisition was performed on the LTQ-Orbitrap using the Xcalibur 2.0 software in the positive ion mode as described (27). Briefly, the instrument was recalibrated in real time by co-injection of an internal standard from ambient air into the C-trap ("lock mass option") (25). Survey spectra were acquired in the orbitrap with a resolution of 60,000 at m/z 400. Up to five of the most intense multiply charged ions were sequentially isolated, fragmented, and analyzed in the LTQ part of the instrument. To improve phosphopeptide analysis, multistage activation was enabled, and the neutral loss species at 97.97, 48.99, and 32.66 m/z below the precursor ion were activated for 30 ms during fragmentation (pseudo-MS²) (34). All raw data files from this study have been uploaded to the Tranche file-sharing system (https://proteomecommons.org/; hash 4n6ku2WIuVPMWrWBPJnvYjMR0KcgaGWOF6GQasC2CPSVt1JA3Afuxl1cSrOv3dYN2OJdJ8erLq5KLR6GLTzalZYPuAAAAAAAAn98g=).

Data Processing—Raw MS data were processed using the in-house software MaxQuant (version 1.0.12.16) (26). MaxQuant-generated peak lists were searched with the Mascot search engine (version 2.2.04; Matrix Science, London, UK) against an in-house curated International Protein Index (IPI) human protein database (version 3.37 containing 69,141 entries) and 175 commonly observed contaminants (such as human keratins, porcine trypsin, and endoprotease Lys-C) to which reversed versions of all sequences had been added. The maximum mass deviations allowed for MS and MS² peaks were 5 ppm and 0.5 Da, respectively. Carbamidomethylation was set as fixed modification, and oxidized methionine, phosphorylation on serine, threonine, or tyrosine and protein N-acetylation were searched as variable modifications. In addition, the SILAC labels Lys4, Lys8, Arg6, and Arg10 were searched as modifications. Full tryptic specificity was required, and up to three missed cleavages were allowed. Searches against a concatenated target/decoy database allowed us to filter peptide and protein identifications for a false discovery rate of less than 1% (35).

Identified peptides were automatically quantified, and phosphorylation sites assigned by post-translational modification score-based localization in MaxQuant (24, 26). For phosphorylation site analysis, only class I sites with a localization probability of at least 0.75 and a localization probability score difference of 5 or higher were considered (24). For identified class I sites, annotated phosphopeptide spectra have been uploaded to the Tranche file-sharing system (https://proteomecommons.org/; hash: m2s2D5EBC5mrnrGszhY-50b1bEC94X2Sfgfnb0Y7gCoBqhmnn0QH9ftFjoEvjRh0ynYyEkht-J3ot7t0KnMeE8SaoKudUQAAAAAAA8xg===). All phosphopeptide ratios were normalized for unequal protein amounts and log₂-transformed. In the case of singly and multiply phosphorylated peptides harboring the same site, ratios were separately calculated for singly, doubly, and multiply phosphorylated species to detect possible priming phosphorylation events. To identify significantly regulated phosphopeptides on the basis of biological reproducibility, the ratios of the individual peptide ratios determined in the two experiments were calculated. Gaussian regression analysis on the histogram plot of the log₂-transformed ratios of ratios was performed using SigmaPlot (version 10.0; Systat Software Inc.). The obtained values
for the mean and S.D. across the whole quantitative data set were used to determine thresholds for significant regulation of at least ±2.5 \( \sigma \). Furthermore, only phosphosites conforming to this criterion and showing consistent regulation in biological replicates were considered as regulated for further analysis.

**Gene Ontology (GO) and STRING Network Analyses**—Significantly overrepresented GO terms were identified with the DAVID gene functional classification tool (36). To identify significant enrichment of GO terms, the Expression Analysis Systematic Explorer (EASE) score threshold in DAVID was set to \( p \leq 0.05 \). Kinase-enriched fractions were analyzed for overrepresented GO molecular function terms using the complete human proteome as the background data set. To analyze regulated phosphoproteins from total cell lysate experiments for enrichment of GO biological process terms, all identified phosphoproteins were used as reference data.

All proteins with confidently identified LPA-regulated phosphorylation sites were uploaded to the STRING protein-protein interaction database (37). The interaction network was generated on the basis of experimental and database knowledge with seven external interactors and visualized with Cytoscape (38).

**RESULTS**

**Integrated Work Flow for Comprehensive Signaling Analysis**—The GPCR ligand LPA rapidly triggers phosphorylation-dependent signaling in many mammalian cell types. In addition to capturing these changes in A498 kidney cancer cells on a proteome-wide level, our goal was to compare EGFR transactivation in the context of GPCR signaling with a similar activation of the EGFR by exogenous ligand. Therefore, we initially monitored time- and dose-dependent EGFR autophosphorylation at Tyr(P)\(^{1173}\) upon addition of HB-EGF to define which concentration induced EGFR activation similar to that observed upon LPA-induced processing of endogenous HB-EGF precursors (supplemental Fig. 1). These test experiments revealed that 10 \( \mu \)M LPA or 0.5 ng/ml HB-EGF induced comparable EGFR Tyr(P)\(^{1173}\) phosphorylation, which peaked after 3 min of stimulation. Thus, these treatment conditions were deemed as suitable for further SILAC-based proteomics analyses (Fig. 1A). To enable quantitative comparisons, populations of A498 cells were differentially labeled with three combinations of isotopic lysine and arginine variants (Lys0/Arg0, Lys4/Arg6, and Lys8/Arg10) as indicated in Fig. 1B. After five cell doublings in SILAC media to ensure near complete incorporation into cellular proteomes, A498 cells were stimulated with growth factors and lysed, and equal protein amounts of total cell extract were pooled. Furthermore, we performed two independent experiments with different labeling schemes to assess the reproducibility and reliability of SILAC-based quantifications in biological replicates.

Total cellular protein extracts were digested with trypsin, and the resulting peptide mixture was resolved by SCX chromatography into eight fractions followed by phosphopeptide enrichment with TiO\(_2\) beads. As the TiO\(_2\) resin only captured a fraction of the phosphopeptides present in the initial flow-through of the SCX column, the supernatant was consecu-
tively applied to further phosphopeptide enrichment steps to capture remaining phosphopeptide species. All phosphopeptide fractions were analyzed by LC-MS on an LTQ-Orbitrap mass spectrometer.

In addition, we enriched for protein kinases to enable their analysis with considerably higher sensitivity than achievable from total cell extracts. Protein kinases are often difficult to detect in total phosphoproteomes because of their relatively low expression compared with their more abundant substrates. However, as kinases represent the key elements of phosphorylation-based signal transmission, knowledge about their site-specific phosphorylations can provide important insights into the architecture of signaling networks and their regulation by external stimuli. We therefore incubated total cell extract with a mixture of seven kinase inhibitor resins with distinct binding characteristics to enable the affinity purification of a maximum number of kinases from A498 cells. After extensive washing, 70% of the kinase-enriched fraction was resolved by gel electrophoresis prior to in-gel digestions with trypsin followed by phosphopeptide enrichment. We further prepared total peptide fractions from gel slices to monitor whether protein changes occur in kinase-enriched fractions upon growth factor treatment. The remaining 30% of the inhibitor resin eluate was digested in solution and subjected to consecutive enrichments with TiO2 beads as a complementary strategy for phosphopeptide fractionation. All resulting peptide fractions were analyzed on an LTQ-Orbitrap mass spectrometer. An overview of our phosphoproteomics strategy for the parallel analysis of both total cell lysate and kinase-enriched fractions is shown in Fig. 1B. All raw MS data from two independent experiments were collectively processed using the MaxQuant software package, which automatically performed peptide to protein assignment, SILAC-based quantification of proteins and phosphorylation events, and phosphorylation site localization in identified phosphopeptides (26).

Qualitative and Quantitative Phosphoproteomics Analysis of A498 Cells—The combined analysis of kinase-enriched and total lysate fractions led to the identification of 3,914 and 5,869 distinct phosphopeptides (supplemental Table 1) in the first and second biological replicate experiments, respectively (Table I), with an overlap of 1,967 phosphopeptides detected in both experiments. In total, the identified phosphopeptides were derived from 2,115 distinct proteins (supplemental Table 2), and 5,332 class I phosphorylation sites (supplemental Table 3) could be assigned with high confidence ($p \geq 0.75$).

Moreover, of the 2,414 distinct phosphopeptides detected upon kinase enrichment, only 516 were found among the 5,918 phosphopeptides identified from total cell lysates (Table II). Thus, the two phosphoproteomics strategies yield highly complementary data sets, and this was also evident on the level of identified protein kinases. Notably, 73 of the 219 distinct protein kinases identified with phosphopeptides were only found upon prefraccionation. The estimated kinome coverage of the inhibitor resin mixture was about 70% based on our data that 107 of the 146 phosphorylated protein kinases identified from total cell lysates were also found in the affinity-purified fractions. Moreover, our analysis of total peptide fractions upon kinase enrichment revealed an additional 43 kinases identified exclusively with non-phosphorylated peptides. In total, as many as 262 distinct protein kinases according to nomenclature by Manning et al. (39) were detected; this to the best of our knowledge, represents the highest number of these key enzymes identified from a single biological source to date. The overall relative abundances of Ser(P), Thr(P), and Tyr(P) were 88.9, 10.3, and 0.8% in total lysate and 69.2, 22.1, and 8.7% in kinase-enriched fractions, consistent with a higher prevalence of Tyr(P) on protein kinases reported earlier (27). We further used GO analysis to identify significantly overrepresented molecular functions in the identified phosphoproteins. As expected, protein kinase activity was highly overrepresented upon kinase prefractionation (supplemental Fig. 2 and Table 4).

Quantification was possible for about 97% of the more than 29,000 uniquely modified peptides (supplemental Table 1) identified in this study. Overall, identified peptides harbored 5,155 or 5,169 distinct class I ($p \geq 0.75$) phosphorylation sites.
measured ratios were log2-transformed and normalized on all measured peptide ratios. To determine the interexperimental variance and define threshold values for significantly regulated sites, corresponding normalized phosphopeptide ratios from both experiments were divided by each other to calculate “ratios of ratios.” After binning and plotting a histogram chart, the mean and σ of the ratio distribution were determined by Gaussian fourth order regression (Fig. 2, A and B). Based on this analysis, we applied stringent filter criteria to our data set and considered only sites for further analysis if they were consistently up- or down-regulated by more than 2.5 times σ in both experiments (Fig. 2C and supplemental Table 5).

As cells were stimulated with either LPA or HB-EGF for only 3 min before lysis, protein changes were highly unlikely to occur within this short treatment period. Therefore, we only analyzed phosphopeptide-enriched fractions from total lysate. However, as it cannot be formally excluded that rapidly induced post-translational modifications might affect inhibitor binding of some kinases, we also analyzed total peptide fractions upon kinase enrichment in addition to phosphopeptide fractions. Notably, none of the identified phosphoproteins exhibited stimulation-dependent binding. Consequently, the ratios of regulated phosphorylations were highly similar with or without normalization for protein abundance (Fig. 2D). Because of this high concordance, we did not normalize these phosphorylation changes for protein levels. This further ensured consistency of phosphorylation data from kinase-enriched fractions with our results from total lysate phosphoproteome analysis, which we considered as important for further bioinformatics processing of merged data sets.

In total, about 5% of all quantified phosphorylation sites were significantly and reproducibly regulated upon growth factor treatment. LPA stimulation led to significant and reproducible changes at 224 confidently assigned phosphorylation sites (supplemental Table 6) of which 150 were up-regulated and 74 were down-regulated. By comparison, with only 23 phosphorylation sites up-regulated and 21 down-regulated, HB-EGF treatment resulted in a considerably smaller number of phosphorylation changes (Fig. 4 and supplemental Table 6). Notably, 43 of all regulated sites have not been identified previously according to the phosphorylation site database PhosphoSitePlus®. Selected phosphorylation sites are compiled in Table III.

LPA-triggered Dephosphorylation Events—Although the majority of phosphorylations were induced upon treatment, we found down-regulation for a considerable number of sites. This raises the question whether these signaling events could be due to rapid, growth factor-induced dephosphorylation. In the case of a dephosphorylation event, the relative abundance of the non-phosphorylated counterpart of a phosphopeptide would increase to an extent determined by the change in phosphorylation site stoichiometry. Remarkably, we could identify a small number of dephosphorylation events based on such observations, for example for a peptide derived from the protein-tyrosine kinase Abl. The peptide 574-GQGESDLPDHAPVSPLLPR593, which encompasses Ser(P)588, was reduced upon LPA stimulation by a factor of 2.5, whereas a more than 3-fold up-regulation was observed for its unphosphorylated counterpart (Fig. 3A). Similarly, dephosphorylation events were also detected on a multiply phosphorylated peptide of the AP2-associated protein kinase 1 (AAK1) encompassing Ser(P)676 and Ser(P)678 as evident from a decrease of phosphorylated species accompanied by a 4-fold increase of the non-phosphorylated counterpart. To our knowledge, these results provide the first experimental evidence of early dephosphorylation events in LPA signaling.

Alternative to dephosphorylation, the phosphorylation site ratio determined from a singly phosphorylated peptide species can decrease in the case of an induced secondary phosphorylation mapping to the same peptide as illustrated by a set of peptides harboring the adjacent phosphorylation sites Ser(P)205 and Ser(P)208 from the serine/threonine PKD1 (Fig. 3B). In response to LPA, both the unphosphorylated and the Ser(P)205 phosphorylated peptide species were down-regulated by about 2-fold, whereas the corresponding singly and doubly phosphorylated Ser(P)208-containing peptides exhibited reciprocal ratios. These results demonstrate LPA-induced phosphorylation on Ser(P)208 of PKD1 and further suggest that phosphorylation on Ser(P)205 did neither change nor prime phosphoryl transfer on the adjacent serine residue. Notably, phosphorylation on Ser(P)205 and Ser(P)208 has been implicated in the molecular association of PKD1 with 14-3-3β in response to oxidative stress (40) and might therefore have regulatory functions in the context of LPA-triggered signaling.

Comparison of Phosphorylation Changes upon LPA and HB-EGF Treatment—In our experiments, we used an HB-EGF concentration titrated to induce similar EGFR phosphorylation on Tyr(P)1173 as triggered by the LPA-induced transactivation process. This was also evident from the MS-based quantification of EGFR Tyr(P)1173, which was identified in one of the two replicate experiments and quantified with ratios of 2.5 and 1.9 upon HB-EGF and LPA incubation, respectively. HB-EGF induced fewer phosphorylation changes than LPA as expected because of the LPA-selective activation of Rho GTPase and other GPCR-triggered signaling pathways. Furthermore, nearly all HB-EGF-regulated phosphorylation sites exhibited regulation in the same direction upon LPA in concordance with the EGFR transactivation component of LPA signaling. The only exceptions were found for Ser(P)548 of PKD1, which was 4.6-fold induced by LPA in contrast to a 5.7-fold down-regulation upon HB-EGF treatment, and for the Ser(P) residues 4476, 4485, and 4489 on plectin-1, which were found to be significantly reduced by HB-EGF but not by LPA treatment. In contrast, the majority of all other HB-EGF-
regulated sites showed more pronounced changes upon LPA treatment despite similar EGFR activation (Fig. 4).

**Bioinformatics Analysis of LPA-induced Phosphoproteome Changes**—Next, we performed a GO analysis to identify significantly overrepresented biological process categories in the subset of LPA-regulated phosphoproteins using all identified proteins with phosphorylation sites as the background data set (supplemental Table 7). We found LPA-regulated phos-
TABLE III

Selected LPA-regulated phosphorylation sites

Remarks were deduced from GO and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotations or directly from the literature where indicated. Ratios only measured in one experiment are indicated by asterisks.

| Protein | IPI       | Position | Sequence window | Mean Ratio HB-EGF/Control | Mean Ratio LPA/Control | Remarks                       |
|---------|-----------|----------|-----------------|----------------------------|------------------------|-------------------------------|
| Receptors                                      |           |           |                 |                            |                        |                               |
| RP65/BLNK | IPI00783156 | 757       | LPKRPTPQQLNTK   | 1.32                      | 2.60                   |                               |
| EGFR     | IPI0018274 | 1166      | KGSHQPWLDNPY    | 1.13                      | 1.86                   |                               |
| EGFR     | IPI0018274 | 1110      | SVJDNPVQWWQPLN  | 3.12*                     | 1.95*                  | Autophosphorylation site      |
| Ephrin-5B | IPI00252979 | 586       | KLOQHTSGHMTPG   | 2.83                      | 10.33                  |                               |
| Hepatocyte growth factor receptor               | IPI00029273 | 977       | YDARVHFLDLRL    | 0.63                      | 0.44                   |                               |
| Non-receptor kinases / phosphatases            |           |           |                 |                            |                        |                               |
| Proto-oncogene tyrosine-protein kinase ABL1    | IPI00022117 | 16        | GDQRRPRLALHF    | 0.96                      | 2.14                   | Novel site                    |
| Proto-oncogene tyrosine-protein kinase ABL1    | IPI00022117 | 588       | DHEAVPQLPRK     | 0.86                      | 0.40                   |                               |
| Tyrosine-protein kinase ABL2                   | IPI000328488 | 820       | RTVSTSGQPEENV   | 1.14                      | 2.50                   |                               |
| Focal adhesion kinase 1                        | IPI00413961 | 386       | EKQGMRTHAWSVS   | 1.40                      | 2.64                   | Activation loop               |
| Focal adhesion kinase 1                        | IPI00413961 | 577       | MEDSTYQASKGK    | 1.40                      | 2.08                   |                               |
| Serine/threonine-protein kinase D3             | IPI00015538 | 731       | RIIEKSRFRSV     | 2.08                      | 13.55                  | Activation loop               |
| Serine/threonine-protein kinase D3             | IPI00015538 | 735       | EKSFRAAVGFLPA   | 2.31                      | 6.77                   | Activation loop               |
| Protein tyrosine kinase 2 beta                 | IPI00029702 | 579       | YIEDEDYKASVT    | 3.39                      | 15.03                  | Activation loop               |
| Protein tyrosine kinase 2 beta                 | IPI00029702 | 580       | IEDEDYKASVT     | 2.22                      | 8.75                   | Activation loop               |
| Protein tyrosine kinase 2 beta                 | IPI00029702 | 583       | EDYYKAVTRLP     | 2.45                      | 9.58                   | Activation loop               |
| Ribosomal protein S6 kinase alpha-1            | IPI00017305 | 573       | ENGLMIPCYTAN    | 1.25                      | 3.03                   | Activation loop               |
| Tyrosine-protein kinase Tec                    | IPI0000878 | 519       | YLVDQVSSSQA     | 1.63                      | 6.99                   | Phosphorylation activates enzyme (62) |
| Proto-oncogene tyrosine-protein kinase Yes     | IPI00013981 | 425       | LIEDEYKAROGA    | 1.21                      | 1.84                   | Activation loop               |
| Cyclin-dependent kinase-like 5                 | IPI00746301 | 407       | NIPHLAPKIEAKS   | 1.58                      | 2.91                   |                               |
| Tyrosine-protein phosphatase non-receptor type 12 | IPI00289082 | 435       | KLERNLIFKIKV    | 2.02                      | 2.89                   | Protein tyrosine phosphatase  |
| Tyrosine-protein phosphatase non-receptor type 12 | IPI00018914 | 578       | RPRPAPAAPDLAS   | 0.82                      | 0.49                   | Protein tyrosine phosphatase  |
| Cell cycle / Apoptosis                         |           |           |                 |                            |                        |                               |
| Serine/threonine-protein kinase Chk1           | IPI00022164 | 280       | KRPPVTGGQSVSE   | 1.52                      | 2.09                   | Involved in G2/M transition   |
| Astronomic phosphoprotein PEA-15               | IPI00064332 | 116       | DIIQGRKDLEEIK   | 1.17                      | 2.03                   | Site stabilizes anti-apoptotic effect (42-43) |
| STE20-like serine/threonine-protein kinase     | IPI00247439 | 340       | PASKRAGDLSSA    | 1.10                      | 2.17                   | Implicated in the regulation of apoptosis (63) |
| Serine/threonine-protein kinase 17B            | IPI00014934 | 10        | RRRDCRQSGSLTL  | 1.58                      | 5.89                   | Implicated in the regulation of apoptosis |
| Endo-/Exocytosis                               |           |           |                 |                            |                        |                               |
| AP2-associated protein kinase 1                | IPI00479760 | 620       | QKVSSLTPPSSIPK  | 1.54                      | 2.86                   | Endocytosis                   |
| AP2-associated protein kinase 1                | IPI00479760 | 623       | GSLTPPSTKTPQR   | 1.23                      | 3.50                   | Endocytosis                   |
| AP2-associated protein kinase 1                | IPI00479760 | 624       | SLTPPSTKTPQR    | 1.51                      | 2.80                   | Endocytosis                   |
| AP2-associated protein kinase 1                | IPI00479760 | 637       | GHRRILGDVTHSA  | 0.68                      | 0.43                   | Endocytosis                   |
| AP2-associated protein kinase 1                | IPI00479760 | 676       | SATTPGSPSRTS    | 0.49                      | 0.17                   | Endocytosis                   |
| AP2-associated protein kinase 1                | IPI00479760 | 731       | PEKLLGSQALELIP | 0.92                      | 0.33                   | Endocytosis                   |
| AP-2 complex subunit beta-1                    | IPI00783966 | 4         | _MTDQKYFTTN    | 2.73                      | 8.40                   | Endocytosis                   |
| GTase-activating protein and VPS9 domain-containing protein 1 | IPI00292753 | 929       | LRVSRSQSDIVS   | 1.23                      | 1.90                   | Endocytosis                   |
| Dynamin-1                                      | IPI00431340 | 774       | VPAGRRSPSSPT    | 1.38                      | 2.55                   | Endocytosis, downstream of EGFR (20) |
| Dynamin-1                                      | IPI00431340 | 776       | AGRRSPLSPYFPQ   | 1.47                      | 2.47                   | Endocytosis, downstream of EGFR |
| Exocyst complex component 1                    | IPI00251762 | 486       | MGNMAGLQDLVAD  | 0.55                      | 0.58                   | Exocytosis, CDC42 interactor (64) |
| Kinesin-like protein KIF21A                    | IPI00425409 | 855       | TRKLLSSQAPAOD  | 0.58                      | 3.74                   | Intracellular transport       |
| Intersectin-1                                   | IPI00304740 | 904       | ATGSSSP7VLGQG  | 0.29                      | 0.46                   | Endocytosis, Rho signaling    |
| **G protein signaling** | **IP** | **Gene** | **Name** | **Description** | **Ratio** |
|-------------------------|--------|----------|----------|----------------|----------|
| Rab GEF activity, GTPase regulator | IP000290110 | 457 | GRKPIVAGDDGGK | Site de-represses AP-1 elements | 1.60 1.83 |
| Elongation factor 1 X-type | IP00014263 | 265 | VTLGGGSTPSPS | Transcription factor | 1.57 1.32 |
| Histone deacetylase 7 2-like | IP00014842 | 486 | PLRSAQPSIANLAA | Histone deacetylase, site triggers nuclear export | 1.45 1.97 |
| Histone deacetylase 7 2-like | IP00014842 | 444 | RLRQIPSAEDELAT | Histone deacetylase | 0.81 3.42 |
| Gem-associated protein 1 | IP000291783 | 757 | TVPVLGTSIDNEE | EIF4-H interactors | 1.30 3.69 |
| Eukaryotic translation initiation factor 4H | IP00014263 | 21 | FGGRGGRGDGAGG | Translation initiation factor | 1.21 2.22 |
| Ubiquitin conjugation factor E4 B | IP00005715 | 103 | WSLSLRQGDMDID | Protein degradation | 1.14 1.90 |
| Ubiquitin-associated protein 2-like | IP00014856 | 454 | APPPSPLPSKS | Protein degradation | 0.81 0.49 |
| Ubiquitin-associated protein 2-like | IP00014856 | 609 | PSSSSSSQKDLT | Protein degradation | 0.93 0.48 |

| **DNA damage response** | **IP** | **Gene** | **Name** | **Description** | **Ratio** |
|-------------------------|--------|----------|----------|----------------|----------|
| Interferon-inducible double stranded RNA-dependent protein kinase activator A | IP000021167 | 18 | PLREDJTGTSLG | Protein activates interferon-inducible double stranded RNA-dependent protein kinase | 1.76 4.66 |

| **Adhesion / mobility** | **IP** | **Gene** | **Name** | **Description** | **Ratio** |
|-------------------------|--------|----------|----------|----------------|----------|
| CD44 antigen | IP000030064 | 697 | VEDRPKPSLNGEA | Site regulates mobility | 1.31 3.03 |
| Platelet F11 receptor | IP000001754 | 451 | VIYSPGRASSEGE | Cell adhesion | 2.47 5.69 |
| Tight junction protein ZO-1 | IP000018219 | 1366 | SYFDRRQENKPP | Adhesions junction | 1.39 2.86 |
| Plakophilin-3 | IP000035834 | 88 | QSNSVPGYYYYY | Cell adhesion | 1.65 2.20 |
| Plakophilin-3 | IP000026952 | 314 | TLQPSGFFODID | Cell adhesion | 2.62 18.39 |
| Liprin-beta-1 | IP000179172 | 532 | NLDFQKASAPITL | Cell adhesion | 1.87 3.67 |
| Filamin A | IP000049065 | 876 | AEPGSPFAEDEGA | Phosphorylation inhibits F-actin binding | 0.85 2.23 |

| **Other Proteins** | **IP** | **Gene** | **Name** | **Description** | **Ratio** |
|---------------------|--------|----------|----------|----------------|----------|
| ADAM 9 | IP0000208894 | 791 | SSTAAKSEPQDLT | Involved in ligand shedding | 0.81 0.53 |
| ADAM 17 | IP000049065 | 876 | PSRPGQPRHVS | Involved in ligand shedding | 2.05 3.72 |
| C-C chemokine receptor type 4 | IP00016445 | 328 | CTRVQGSLKLS | Receptor for stromal derived factor-1 | 0.76 3.43 |
| 7-dehydrocholesterol reductase | IP0000294501 | 14 | NIPKAKLGDGTVT | Cholesterol synthesis, activated by phosphorylation | 1.25 7.70 |
| Oxysterol-binding protein-related protein 11 | IP000032970 | 189 | ISORRPPSQNAISF | Sterol transport signaling | 1.10 1.95 |

**TABLE III—continued**

**Phosphoproteome Quantification in GPCR Signaling**

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various interactions among regulators and effectors of Rho small GTPase Rho, and the protein network further illustrates characterized signaling events upon LPA-induced activation of the receptor, and the protein-tyrosine phosphatase PTPN12. Tyrosine phosphorylations of FAK and paxillin are well characterized upon LPA-induced activation, and we could identify an LPA-induced phosphorylation site in one experiment. Collectively, our results indicate that LPA-triggered kinase activities modulate Rho GTPase signaling on multiple levels and suggest complex mechanisms of signal integration instead of linear signal transduction processes. The network analysis of LPA-modulated phosphoproteins further revealed that the RhoGTPase GAPs GRLF1 and ARHGAP29 are connected to the endocytosis regulators intersectin 1 (ITSN1) and dynamin 1 (DNM1). In addition to modulation of Rho GTPase signaling, our quantitative MS analysis identified LPA regulation of components involved in signaling through Ras, Rab, and Arf small GTPases, such as development and differentiation enhancing factor 1, neurofibromin, TBC1 domain family member 4, and others.

phosphoproteins enriched for proteins annotated to biological processes, such as cell proliferation, cell motility, and cell adhesion with highest enrichment found in the latter category (Fig. 5). To further analyze the functional relationships among LPA signaling components, we queried the STRING database to assemble a protein-protein interaction network based on the signaling components, we queried the STRING database to assemble a protein-protein interaction network based on the regulated phosphoproteins identified in this study (Fig. 6). We solely considered associations backed up by experimental data and allowed for a few external interactors to enhance network connectivity. Network analysis revealed multiple interactions between central kinase mediators of LPA signaling, such as the EGFR, focal adhesion kinase (FAK), and ERK MAP kinases, and LPA-regulated phosphoproteins implicated in migration and adhesion, such as CD44, paxillin, the myristoylated alanine-rich protein kinase C substrate MARCKS, F11 receptor, and the protein-tyrosine phosphatase PTPN12. Tyrosine phosphorylations of FAK and paxillin are well characterized signaling events upon LPA-induced activation of the small GTPase Rho, and the protein network further illustrates various interactions among regulators and effectors of Rho GTPases, such as the GTPase-activating proteins (GAPs) glucocorticoid receptor DNA-binding factor 1 (GRLF1) and Rho GTPase-activating protein 29 (ARHGAP29) as well as the guanine nucleotide exchange factor (GEF) protein kinase A anchor protein 13. Additionally, LPA regulated site-specific phosphorylations on the GEF SOLO and the Cdc42 effector protein 4 (41). The heterotrimeric G protein subunit Gα12 couples LPA1/2 receptors to Rho GTPase activation, and we could identify an LPA-induced phosphorylation site in one experiment. Collectively, our results indicate that LPA-triggered kinase activities modulate Rho GTPase signaling on multiple levels and suggest complex mechanisms of signal integration instead of linear signal transduction processes.

A major signaling response to LPA involves the phosphorylation and activation of the protein kinase Akt/protein kinase B (14). Although Akt does not bind to our kinase enrichment resin and was therefore not detected in our MS analyses, we added this known mediator in LPA signaling to gain better insights into cellular network regulation. Notably, this provided additional links involving the known Akt substrates phosphoprotein enriched in astrocytes 15 (PEA15) and Thiccin 1 (ITSN1) and dynamin 1 (DNM1). In addition to modulation of Rho GTPase signaling, our quantitative MS analysis identified LPA regulation of components involved in signaling through Ras, Rab, and Arf small GTPases, such as development and differentiation enhancing factor 1, neurofibromin, TBC1 domain family member 4, and others.

Remarkably, our data indicate potential mechanisms for rapid regulation of transcriptional activity upon LPA treatment.

**Fig. 3.** MS spectra from SILAC-based quantitative analysis. A, MS spectra of peptides derived from the tyrosine kinase Abl. The peptide with the amino acid sequence GGGESDPLDHEPAPSSIPLLPR was quantified in its non-phosphorylated and singly phosphorylated form harboring Ser(P)866. B, MS spectra of a set of peptides derived from the serine/threonine kinase PKD1. Peptides harboring the phosphorylation sites on Ser236 and Ser238 were quantified as non-, singly, or doubly phosphorylated forms with either phosphorylation detected in distinct singly modified peptide variants. A and B, isotopic variants originated from the differentially labeled and stimulated cells as indicated. Ratios obtained by SILAC-based quantification are shown. p(S), phosphoserine.
For instance, the tumor suppressor protein programmed cell death protein 4 (PDCD4) was phosphorylated at Ser\(^{457}\), a known Akt substrate site that regulates the repression of c-Jun-dependent transactivation of AP-1-responsive elements by PDCD4 (45). In addition, LPA induced phosphorylation on histone deacetylase 7 (HDAC7), likely through the upstream regulator PKD2, which has previously been characterized as cellular HDAC7 kinase and was activated in response to LPA as evident from increased activation loop phosphorylation (46). Notably, HDAC7 shuttles between the cytoplasm and the nucleus. Nuclear HDAC7 is recruited by the transcription factor MEF2D to its target gene promoters where it represses gene transcription. PKD-mediated phosphorylation leads to the accumulation of HDAC7 in the cytoplasm, thereby derepressing MEF2D/HDAC7-regulated genes (46–49). We observed LPA-induced HDAC7 phosphorylation on Ser\(^{486}\), which has been functionally implicated in T cell receptor-mediated transcriptional regulation. By extension, our identification of Ser\(^{486}\) as an LPA-regulated phosphorylation site could indicate a similar role of HDAC7 in GPCR-mediated gene regulation. In addition to PKD2, also the closely related kinase PKD1 was linked to HDAC7 regulation, and both PKD1 and PKD2 are activated by LPA according to our data. Literature data suggest that PKD1 can be activated by bone morphogenic protein receptor 2 (BMPR2) (50) and that the serine/threonine kinase receptor BMPR2 undergoes autophosphorylation at Ser\(^{757}\) upon activation. Surprisingly, we found this particular phosphorylation site to be up-regulated by a factor of 2.6 upon LPA stimulation, pointing to a previously unknown cross-talk mechanism involving a transmembrane receptor with serine/threonine kinase activity as a potential mediator of transcriptional regulation through PKD and HDAC7. In addition, LPA rapidly induced phosphorylation of several other factors involved in protein biosynthesis, such as the transcription factor nuclear factor \(1\) X-type and the transcription elongation factor 3 as well as eukaryotic translation initiation factor 4H and Gem-associated protein 5 involved in protein translation.

The protein-protein interaction network of LPA-regulated phosphoproteins illustrates a remarkable gain in connectivity by the combined analysis of both kinase-enriched and total lysate fraction (Fig. 6). Data about regulated phosphoproteins were highly complementary, and only the protein kinases PKD1, calcium/calmodulin-dependent protein kinase type II \(\delta\), and calcium/calmodulin-dependent protein kinase type I were identified by either analytical strategy, whereas the majority of protein kinases identified in the LPA signaling response were contributed by the analysis of kinase inhibitor-resin-purified fractions.

Most protein kinases with LPA-induced phosphorylations are involved in either MAPK (TRAF2 and NCK-interacting kinase, TAO kinases 1/2, ERK1/2, and ribosomal protein S6 kinase \(\alpha1/3\)), calcium-dependent (calcium/calmodulin-dependent protein kinases type II and PKDs), or tyrosine kinase signaling (Src, Yes, FAK, PYK2, and ABL1/2). It is noteworthy that several receptor tyrosine kinases such as Met, EGFR, and EPHB2 were phosphorylated at serine residues. These site-specific phosphorylations could influence their cellular activities and signaling capacities by, for example, modulating receptor trafficking and localization in conjunction with other signaling pathways.

![Fig. 5. Overrepresented GO biological process categories. LPA-regulated phosphoproteins from total cell lysate were compared with all phosphoproteins identified in the total cell lysate. Significantly overrepresented GO biological process terms (\(p < 0.05\)) are shown. Percentage values indicate the fractions of proteins annotated to the listed GO biological terms found in all LPA-regulated or all identified phosphoproteins with annotated GO biological process terms.](image-url)
with LPA-induced phosphorylations on several other factors implicated in endocytosis, such as AAK1, AP2 complex subunit β1, GTPase-activating protein and VPS9 domains 1, and dynamin 1.

**Kinase Activation Loop Phosphorylations**—Several LPA-induced phosphorylation events on protein kinases located to the conserved activation loop domain. As activation loop phosphorylation stabilizes the catalytically active conformation, these signaling events are of particular interest as they can provide a readout for cellular kinase activity. We found up-regulated activation loop phosphorylations on 11 distinct protein kinases (ERK1/2, FAK, PYK2, ribosomal protein S6...
kinase α1, PKD1/2/3, Yes/Src, and Tec), indicating rapid induction of their catalytic activities upon LPA stimulation. Interestingly, the tyrosine kinase Tec plays an important role in diverse biological processes, such as antigen receptor signaling, actin reorganization, and cell adhesion (51). Tec might be of particular interest for further studies. Although Tec activation has been reported upon expression of either Gα12/13 subunits or Src family kinases (52), our results provide the first evidence of Tec activation in the context of endogenous LPA signaling.

**DISCUSSION**

In our phosphoproteomics study, we analyzed early signaling events upon stimulation with the GPCR ligand LPA in comparison with HB-EGF treatment. HB-EGF was added at a concentration that induced EGFR tyrosine phosphorylation similar to that of the LPA-induced transactivation of the EGFR in A498 cells. Because of this experimental design, we could investigate how system-level effects, involving the EGFR and other components in LPA signaling, might affect downstream signal synthesis. DHCR7 activity is positively regulated by phosphorylation (54), and depletion of cellular cholesterol increased ADAM17-mediated ligand shedding (55). Therefore, phosphorylation of DHCR7 may control a negative feedback loop that down-regulates ligand shedding in addition to effects on EGFR internalization due to increased membrane cholesterol levels (56).

The parallel phosphoproteomics analysis of total lysate and kinase-enriched fractions provided highly complementary information on phosphoproteins and their regulation as evident from the respective numbers of quantified phosphorylation sites. Overall, we quantified more than 10 times as many phosphopeptides as in a previous study on LPA signaling (57). Moreover, the merged information of kinases and kinase substrates proved to be essential for the generation of an LPA-regulated phosphoprotein network. The network connectivity was dramatically reduced in the case from kinase enrichment experiments were omitted. Therefore, our integrated phosphoproteomics approach is particularly useful for signal transduction analysis on the systems level as evident, for example, for protein kinases and other LPA-regulated phosphoproteins with roles in cell migration and adhesion. Network visualization with the STRING program revealed multiple associations of regulated phosphoproteins identified from total cell lysate, such as F11R, paxillin, and the protein-tyrosine phosphatase PTPN12, with protein kinases identified in the enrichment experiments, such as FAK, PYK2, Src, ERK MAP kinases, and others. In addition, LPA, but not HB-EGF, induced an about 3-fold up-regulation of Ser(P)697 and Ser(P)704 on the type I transmembrane glycoprotein CD44. Phosphorylation of Ser697 has been functionally linked to cell motility upon phorbol ester treatment (58). Furthermore, CD44 co-localizes and co-immunoprecipitates with the EGFR (59), which has also been implicated in LPA-triggered A498 cell migration and invasion (13). These lines of evidence point to a potential cooperation of CD44 and EGFR in LPA-induced migration, which might further involve a modulation of pro-HB-EGF processing by CD44 (60, 61). Collectively, this evidence together with our phosphoproteomics data and previous knowledge highlights the role of multifactorial signal processing within complex networks to modulate cell behavior such as a migratory response upon LPA stimulation. Such knowledge might be particularly relevant in the context of pathophysiological processes, such as cancer cell migration and invasion, and help to define cooperating signal factors for multitargeted therapeutic intervention to account for compensatory mechanisms in disease-relevant signaling networks.

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