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Regulation of Platelet Derived Growth Factor Signalling by LAR Protein Tyrosine Phosphatase: A Quantitative Phosphoproteomics Study

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Abbreviations

LAR - leukocyte common antigen-related protein

PDGF - platelet derived growth factor

RPTP – receptor protein tyrosine phosphatase

SILAC - stable isotope labelling with amino acids in cell culture

WCL - whole cell lysate

WT - wild-type

SUMMARY

Intracellular signalling pathways are reliant on protein phosphorylation events that are controlled by a balance of kinase and phosphatase activity. Although kinases have been extensively studied, the role of phosphatases in controlling specific cell signalling pathways has been less so. Leukocyte common antigen-related protein (LAR) is a member of the LAR subfamily of receptor-like protein tyrosine phosphatases (RPTPs). LAR is known to regulate the activity of a number of receptor tyrosine kinases, including platelet-derived growth factor receptor (PDGFR). To gain insight into the signalling pathways regulated by LAR, including those that are PDGF-dependent, we have carried out the first systematic analysis of LAR-regulated signal transduction using SILAC-based quantitative proteomic and phosphoproteomic techniques. We have analysed differential phosphorylation between wild-type mouse embryo fibroblasts (MEFs) and MEFs in which the LAR cytoplasmic phosphatase domains had been deleted (LAR Δ P), and found a significant change in abundance of phosphorylation on 270 phosphosites from 205 proteins due to the absence of the phosphatase domains of LAR. Further investigation of specific LAR-dependent phosphorylation sites and enriched biological processes reveal that LAR phosphatase activity impacts on a variety of cellular processes, most notably regulation of the actin cytoskeleton. Analysis of putative upstream kinases that may play an intermediary role between LAR and the identified LAR-dependent phosphorylation events has revealed a role for LAR in regulating mTOR and JNK signalling.

INTRODUCTION

Phosphorylation is a key post-translational modification (PTM) involved in the regulation of cell signalling. Control of phosphorylation is vital in maintaining normal biological processes, and dysregulation is implicated in many diseases. Kinases and phosphatases have opposing roles in modulating levels of phosphorylation, acting in a coordinated manner within cells to maintain cellular homeostasis via their regulation of cell signalling pathways. Historically phosphatases were viewed as being promiscuous enzymes whose role was simply to dephosphorylate their substrates in order to terminate signal transduction pathways. It is now evident that phosphatases display selectivity and are not simply 'off switches' but can contribute to both deactivation and activation of signalling pathways (1). Whilst the role of kinases has been extensively studied, much less is known about phosphatases and their specific contributions to cell signalling.

Leukocyte common antigen-related protein (LAR) belongs to the LAR subfamily of receptor-like protein tyrosine phosphatases (RPTPs). It is composed of an extracellular domain containing three immunoglobulin domains (Ig), a fibronectin type III domain (FNIII), and cytoplasmic domains - D1 and D2 that are essential for phosphatase activity (2-4). LAR is widely expressed in a variety of cell types, such as neuronal cells, epithelial cells and fibroblasts (5). Several disorders are associated with LAR including defective development of mammary glands, abnormal neuronal development and function, diabetes and cancer (6, 7). Signal transduction regulated by LAR has thus far predominantly been studied in neuronal cells, where it participates in axonal outgrowth, nerve regeneration and orchestration of synapse development (6, 8). LAR regulates tyrosine kinase receptor growth factor signalling

by either dephosphorylating negative regulatory tyrosine residues to enhance receptor activation (9), or by dephosphorylating activating tyrosine residues to deactivate the receptor (10, 11). LAR localises to integrin-based focal adhesion complexes (12) and adherens junctions (13).

Platelet-derived growth factor (PDGF) signalling is involved in many cellular processes such as cell growth, survival and motility (14). Overexpression of the PDGF receptor is associated with diseases such as atherosclerosis and cancer, signifying it as a target for therapeutic interventions (15-17). PDGF isoforms act as dimers composed of interacting A, B, C and D polypeptide chains. These can be homodimeric or heterodimeric isoforms that can interact with PDGF α and PDGF β receptors leading to receptor dimerization and activation of kinase activity via autophosphorylation (18). This results in the recruitment and activation of signalling pathways that culminate in transcriptional responses and the promotion of cell proliferation and survival (18, 19).

Phosphatases are generally considered as negative regulators of signalling pathways. A number of protein tyrosine phosphatases (PTPs) have been reported to dephosphorylate tyrosine residues (Tyr) on PDGFR β thereby deactivating the receptor and inhibiting downstream signalling. For example, dephosphorylation of Tyr857 on PDGFR β by low molecular weight protein tyrosine phosphatase (LMW-PTP) inhibits the receptor kinase activity and subsequent downstream signalling via PI-3 kinase (20). T-cell protein tyrosine phosphatase (TC-PTP) has been shown to inhibit binding of phospholipase C γ 1 (PLC γ 1) through dephosphorylation of Tyr1021 that results in altered cell migration in response to PDGF (21). SHP-2 can

inhibit binding of Ras-GAP to PDGFR β by dephosphorylation of PDGFR β Tyr771, which results in enhanced activity of the Ras signalling pathway (22). By contrast, LAR promotes PDGF signalling by inhibiting activity of the cytoplasmic tyrosine kinase, c-Abl (23). In the absence of LAR phosphatase activity c-Abl inhibits PDGFR β signalling by phosphorylating and inhibiting the receptor (23).

In this study, we set out to gain insight into the landscape of cell signalling events regulated by LAR. In the first systematic analysis of LAR-regulated signal transduction we have used stable isotope labelling by amino acids in cell culture (SILAC) (24, 25) to analyse differential phosphorylation in wild-type (WT) mouse embryo fibroblasts (MEFs) and MEFs in which the LAR cytoplasmic phosphatase domains had been deleted (LAR Δ P) (26). Whilst LAR is known to promote PDGFR activation in fibroblasts (23), the signalling consequences of this regulation have not been fully studied, thus we carried out these studies in the absence and presence of PDGF. We identified 270 LAR-dependent phosphorylation events on 205 proteins, including known LAR interactors, kinases, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). Subsequent functional classification revealed an enrichment of LAR-mediated phosphorylation events on proteins involved in cytoskeletal organisation. Further kinase prediction analysis revealed a role for LAR in regulating both mTOR and JNK signalling pathways, both of which play a role in regulation of the actin cytoskeleton.

These results significantly expand our understanding of signalling events downstream of LAR. This approach has enabled us to identify LAR-dependent changes in

phosphorylation within the entire signalling network, highlighting the role of LAR as a key regulator of growth factor-dependent cell signalling pathways.

EXPERIMENTAL PROCEDURES

Reagents and antibodies

Antibodies were purchased from Cell Signalling Technologies (SAPK/JNK, SAPK/JNK Thr183/Tyr185, mTOR, mTOR Ser2448, P70S6K Thr389, c-Jun, c-Jun Ser63, MKK7, MKK7 Ser271/Thr275), Santa Cruz (ERK1/2 and ERK Thr202/Tyr204), and Sigma (Flag). Recombinant human PDGF-BB was obtained from Cell Signalling Technologies. Rabbit polyclonal Alix has been previously described (27). The secondary goat anti-mouse and goat anti-rabbit IgG IRDye conjugated antibodies were from LI-COR Biosciences.

Cell culture

Mouse embryonic fibroblasts (MEFs) from mice where the LAR phosphatase domains had been deleted (LAR Δ P) and from littermate wild-type (WT) (26), were used in the study (both cell types are kind gifts from Wiljan J.A.J. Hendriks, Radboud University Medical Center, Nijmegen, Netherlands). Cells were grown in DMEM supplemented with 10 % foetal bovine serum (FBS), 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 250 μ g/mL amphotericin B. For SILAC labelling, cells were cultured in SILAC DMEM (Thermo Fisher Scientific) supplemented with either “light” isotopically normal L-Lysine and L-Arginine (R0K0) (Sigma), “medium” $^{13}\text{C}_6$ L-Arginine and 4,4,5,5-D4 L-Lysine (R6K4), or “heavy” $^{13}\text{C}_6$ $^{15}\text{N}_4$ L-Arginine and $^{13}\text{C}_6$ $^{15}\text{N}_2$ L-Lysine (R10K8) (Goss Scientific), with 0.5 mg/mL proline (Sigma), 0.1 mg/mL streptomycin, 100 U/mL penicillin, 250 μ g/mL amphotericin B and 10 % v/v

dialysed fetal bovine serum (Labtech International). Cells were grown in SILAC media for 7 doubling times before being analysed for incorporation efficiency of the SILAC amino acids (see **supplemental Figure S1**).

Transfection

The FLAG-LAR expression vector was kindly provided by Ruey-Hwa Chen (National Taiwan University, Taipei, Taiwan). LAR Δ P cells were transfected using Lipofectamine 2000 (Thermo Fisher Scientific) according to manufacturer's instructions.

Cell stimulation, cell lysis and immunoblotting

Cells were serum starved for 16 hours prior to stimulation with 20 ng/mL PDGF-BB for the indicated times. Treated cells were placed on ice and washed twice with ice-cold phosphate buffered saline (PBS). Cells were then lysed with lysis buffer (20 mM Tris-HCl, pH 7.5, 0.5 % Triton X-100, 0.5 % deoxycholate, 150 mM NaCl, 10 mM EDTA, 0.5 mM Na₃VO₄ and 1 % Trasylol) for 15 minutes on ice. Lysed cells were centrifuged at 15000 x g for 15 minutes at 4 °C and the supernatant (WCL) was collected. Protein concentrations were determined using the BCA protein assay (Thermo Scientific) as per the manufacturer's instructions. An equal volume of 2X sample buffer (1.0 M Tris-HCl pH 8.8, 0.5 % Bromophenol blue, 43.5 % glycerol, 10 % SDS, 1.3 % β -mercaptoethanol) was added to the WCL, and the sample was boiled at 95 °C for 6 minutes. Samples were run on SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5 % bovine serum albumin (BSA) (Sigma Aldrich) at room temperature for one hour and incubated in 5 % BSA in TBS-T (20 mM Tris-HCl, pH 7.5, 0.1 % Tween 20, 150 mM NaCl)

containing primary antibody overnight at 4 °C. Following 3 x 10 minute washes in TBS-T, the membrane was incubated in TBS-T containing IRDye conjugated secondary antibody (LI-COR Biosciences) for 1 hour at room temperature. The membranes were washed again as above and proteins were visualized using fluorescence detection on the Odyssey Infrared Imaging System (LI-COR Biosciences). Following quantitation of immunoblots (n=3) statistical analysis was performed using a two-way ANOVA, Sidak's multiple comparison test.

Trypsin digestion, sample fractionation and phosphopeptide enrichment of samples

For the proteome analysis, 5 µg of “light”, “medium” and “heavy” lysates were mixed, run on a 10 % SDS-PAGE gel, and Coomassie stained. Each lane was cut into 10 bands. In-gel digestion using Trypsin Gold (Promega) was carried out as previously described (28). For the phosphoproteome analysis, 10 mg of “light”, “medium” and “heavy” lysates were pooled prior to trypsin digestion. Proteins were reduced with 8 mM DTT, alkylated with 20 mM iodoacetamide in 50 mM ammonium bicarbonate and digested with Trypsin Gold (1:100 enzyme:protein ratio) at 37 °C overnight. Digested samples were acidified by addition of 0.5 % TFA. Peptides were desalted using Sep-Pak C18 Cartridges (Waters, Milford, MA) according to manufacturer's instructions. Desalted and dried peptides were resuspended in 100 µL mobile phase A (10 mM KH₃PO₄, 20 % acetonitrile, pH 3) and loaded onto a 100 × 4.6 mm polysulfoethyl A column (5 µm particle size, 200 nm pore size, PolyLC). Separation used a gradient elution profile that started with 100 % mobile phase A, increased from 0 to 50 % mobile phase B (10 mM KH₃PO₄, 20 % acetonitrile, 500 mM KCl, pH 3) over 30 min, increased to 100 % B over 5 min, and then returned to 100 % A. Each of the 20 resulting fractions was desalted using a C8 macrotrap

cartridge (Michrom BioResources) according to manufacturer's instructions. Phosphopeptides were enriched using TiO₂ tips (Titansphere™ Phos-TiO kit, GL Sciences). Tips were washed in buffer A (0.5 % (v/v) TFA, 80 % (v/v) ACN) and equilibrated in buffer B (0.38 % (v/v) TFA, 60 % (v/v) ACN, 25 % (v/v) lactic acid). Phosphopeptides were resuspended in buffer B and loaded onto the tips, washed once in buffer B and twice in buffer A before being eluted sequentially in 5 % ammonia solution followed by 5 % pyrrolidine. Phosphopeptide-enriched samples were desalted on reverse-phase C18 ZipTips (Millipore). Peptides were eluted in 50 % (v/v) ACN, 0.1 % (v/v) formic acid (FA), dried to completion and resuspended in 0.1 % FA. All resulting peptide mixtures were analysed in duplicate by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Mass spectrometry

On-line liquid chromatography was performed by use of a Dionex Ultimate 3000 NSLC system (Thermo Fisher Scientific). Peptides were loaded onto an Acclaim PepMap 100 C18 resolving column (15 cm length; 75 µm internal diameter; LC Packings, USA) and separated over a 30 minute gradient from 3.2 % to 44 % acetonitrile (Baker, Holland). Peptides were eluted directly (350 nL/min) via a Triversa nanospray source (Advion Biosciences, NY, USA) into a LTQ Orbitrap Elite mass spectrometer (Thermo Fisher Scientific). The mass spectrometer alternated between a full FT-MS scan (m/z 380-1600) and subsequent CID MS/MS scans of the seven most abundant ions. Survey scans were acquired in the Orbitrap cell with a resolution of 60,000 at m/z 200. Precursor ions were isolated and subjected to CID in the linear ion trap. Isolation width was 2 Th and only multiply-charged precursor ions were selected for MS/MS. The MS1 maximum ion inject time was 1000 ms with an

AGC target of $1e^6$ charges. The MS2 ion inject time was 50ms with an AGC target of $2e^5$ charges. Dynamic exclusion was utilised, fragmented ions were excluded for 60 seconds with an exclusion list size of 500. CID was performed with helium gas at a normalised collision energy of 35 %. Precursor ions were activated for 10 ms. Data acquisition was controlled by Xcalibur 3.0.63 software.

Identification and quantification of peptide and proteins

Mass spectra were processed using the MaxQuant software (version 1.5.3.8) (29). Data were searched, using the Andromeda search engine within MaxQuant (30), against the mouse Swiss-Prot database (downloaded 6.10.15). The mouse database contained 16,719 reviewed protein entries. The search parameters were: minimum peptide length 7, peptide tolerance 20 ppm (first search) and 6 ppm (second search), mass tolerance 0.5 Da, cleavage enzyme trypsin/P, and 2 missed cleavages were allowed. Carbamidomethyl (C) was set as a fixed modification. Oxidation (M), acetylation (Protein N-term), and phospho (STY) were set as variable modifications. The appropriate SILAC labels were selected and the maximum labelled amino acids was set to 3. All experiments were filtered to have a peptide and protein false-discovery rate (FDR) below 1 % and the match between runs featured was enabled. All raw files from both the phosphoproteome and proteome pipeline were analysed together in MaxQuant. Within the MaxQuant output, phosphorylation sites were considered to be localised correctly if the localisation probability was at least 0.75 (75 %) and the score difference at least 5. Bioinformatics analysis was performed in the Perseus software environment, which is part of MaxQuant (Perseus version 1.5.0.15; www.perseus.framework.org). Significance testing was carried out using a Student's *t*-test on log 2 transformed ratios and controlled with a Benjamini-Hochberg FDR

threshold of 0.05. Peptides quantified in three or more experimental repeats were deemed significantly changed and regulated by LAR phosphatase activity if they had a p-value of < 0.05 and a ratio of < 0.667 or > 1.5 (at least a 1.5-fold change in abundance).

The mass spectrometry proteomics data, including the MaxQuant output, have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD002545 (31).

Cluster analysis, GO analysis, and Kinase motif analysis

GProX software (32) was used to perform clustering of log₂ transformed ratios from the MaxQuant output. Unsupervised fuzzy *c*-means clustering was used with an upper regulation threshold of 1 and a lower regulation threshold of -1. Overrepresentation of GO terms in the clusters was performed within GProX using a binomial statistical test with a Benjamini-Hochberg p-value adjustment, a p-value threshold of 0.05, and a minimum occurrence of 2. DAVID (Database for Annotation, Visualization and Integrated Discovery) (33) was used to identify over-represented GO terms in the phosphoproteome dataset. The background list comprised of all of the proteins identified across our experiments. The threshold count and EASE score were set to 2 and 0.05 respectively. Phosphopeptides containing well localised phosphosites were analysed for predicted kinase motifs using GPS (34) with a high stringency setting. Protein network visualisation was performed using Cytoscape (35) with WordCloud plugin (36).

RESULTS

Absence of LAR phosphatase activity leads to alterations in the global phosphoproteome and proteome

Our aim was to gain insight into the protein signalling networks downstream of LAR. We utilised SILAC (24, 25) to quantitatively compare levels of protein expression (proteome analysis) and phosphorylation (phosphoproteome analysis) in PDGF-stimulated wild-type (WT) and LAR Δ P (lacking cytoplasmic phosphatase domains) MEFs. Three populations of WT and LAR Δ P cells were SILAC-labelled by culturing them in ‘Light’ R0K0, ‘Medium’ R6K4, or ‘Heavy’ R10K8 SILAC media. Cells were left untreated or stimulated with PDGF-BB for 7 minutes as indicated in **Figure 1A**. Within the phosphoproteome dataset, 2559 unique phosphosites from 1311 proteins were identified with high localisation scores (localisation probability > 0.75; score difference > 5) in one or more experimental replicates. These phosphosites were comprised of 2125 (83 %) serine, 260 (10 %) threonine and 174 (7 %) tyrosine phosphorylation sites. Of these, 266 (10 %) are not listed in PhosphoSitePlus (37) and are considered novel.

To compare the phosphoproteome of PDGF stimulated WT and LAR Δ P cells four biological replicates, including a label swap control, were incorporated into the experimental design (**Figure 1A**). The overlap between the four biological replicates is shown in **supplemental Figure S2A**: 54 % of the phosphopeptides were identified in 2 or more replicates, and 27 % were identified in 3 or more replicates. The Pearson’s correlation coefficient for the peptide ratios measured across the four biological replicates, including the label swap experiment, ranged from 0.64 - 0.86 indicating good biological reproducibility (**supplemental Figure S2B**). In cells lacking LAR phosphatase activity, a total of 270 phosphopeptides from 205

proteins showed a significant change in abundance ($p < 0.05$; > 1.5 -fold change) (**Figure 1B; supplemental Table S1**). Of these, 255 (95 %) contained serine phosphorylation sites, 9 (3 %) threonine, and 6 (2 %) tyrosine. A total of 103 phosphosites were up-regulated and 167 down-regulated. Within our phosphoproteome dataset we identified serine, threonine and tyrosine phosphorylation events mediated by LAR allowing us to gain an understanding of the global signalling landscape. LAR could contribute to the regulation of phosphorylation on these sites via modulation of the activity of specific kinases and phosphatases, or in the case of tyrosine, via direct dephosphorylation, given that LAR is a tyrosine phosphatase.

These LAR-dependent changes in phosphopeptide abundance could be due to alterations in regulation of specific phosphorylation events or changes in protein abundance, hence our combined proteomic and phosphoproteomic approach. Within the proteomic dataset (comparing PDGF treated WT cells and LAR Δ P cells) a total of 2939 proteins were identified; 1150 with associated quantitation data in two or more biological replicates. Of these, 147 proteins (47 up-regulated; 100 down-regulated) showed a significant change ($p < 0.05$; > 1.5 -fold change) in abundance in the LAR Δ P cells compared to the WT cells (**Figure 1C; supplemental Table S2**). This is a significant finding as 13 % of the quantified proteome was changed due to the absence of LAR phosphatase activity, suggesting that LAR may be involved in regulating protein turnover. Merging the phosphoproteome and proteome datasets resulted in a measure of corresponding protein abundance for 23 % of the quantified phosphorylation sites. Of the 270 LAR-dependent phosphorylation events, 11% changed significantly at the level of the proteome indicating regulation at the protein level rather than the peptide level.

Tyrosine phosphorylation regulated by LAR

Considering potential direct LAR substrates, a loss of LAR phosphatase activity would lead to an increase in tyrosine phosphorylation of these proteins, hence we looked for tyrosine phosphorylated peptides within the dataset that increased in LARΔP cells. Only one tyrosine phosphorylated peptide, belonging to the protein Lcp2 (SLP76), increased in abundance in the absence of LAR activity (LARΔP cells) (**supplemental Table S1**). SLP76 is an adaptor protein, mostly studied in T cells, that relays signals from activated receptors to the cytoskeleton (38). We have identified an increase in Tyr465 phosphorylation which is a tyrosine residue located in the C-terminal SH2 domain of SLP76. The remainder of the tyrosine phosphopeptides decrease in abundance in LARΔP cells, which suggests that the regulation of phosphorylation on these sites is via an indirect LAR-regulated mechanism.

Biological processes regulated by LAR

Gene Ontology (GO) analysis of the phosphoproteins regulated by LAR revealed a number of enriched GO biological processes, molecular functions and cellular components. The most significantly enriched terms are shown in **Figures 2A, B and C** (for full DAVID output see **supplemental Table S3**). The dominant enriched GO terms were associated with cytoskeletal organisation and cell adhesion. LAR has been shown to regulate the cytoskeleton in conjunction with TRIO, a guanine nucleotide exchange factor for small GTPases (39). Here we have identified a 3-fold increase in the phosphorylation of TRIO on Ser2458 and Ser2462 in LARΔP cells, indicating that LAR dependent signalling networks are regulating its phosphorylation status. Despite this link to cytoskeletal organisation, the extent of the LAR-dependent cytoskeletal regulatory network has not been previously studied. It is evident from our phosphoproteome dataset that a large number of cytoskeletal proteins are dependent upon LAR phosphatase activity to regulate their phosphorylation (**Figure 2D**). LAR has also been

shown to interact with cadherin, β -catenin, and plakoglobin to regulate adherens junctions and desmosomes (40-42). Here, we have identified specific LAR-dependent phosphorylation sites on these proteins and discovered additional LAR regulated cell junction proteins (**Figure 2D**). In LAR Δ P cells we have identified a decrease in phosphorylation on cadherin-11 (Ser714), α -catenin (Ser641), β -catenin (Ser191; Ser675), δ -catenin (Ser864), and plakoglobin (Ser665), all proteins present at sites of cell-cell adhesion. β -catenin is a reported substrate for LAR, and tyrosine dephosphorylation has been linked to inhibition of epithelial cell migration (43). Here, we have not identified specific tyrosine phosphorylation sites on β -catenin that may be directly dephosphorylated by LAR, but instead we identified two serine residues with altered phosphorylation. Phosphorylation of one of these, Ser191, by JNK2 has been shown to be essential for nuclear accumulation of β -catenin in response to Wnt (44). It is possible that LAR is capable of regulating β -catenin phosphorylation indirectly by regulating the activity of kinases that phosphorylate β -catenin, such as JNK2, as well as directly by dephosphorylating specific tyrosine residues (43). In addition, we have evidence that LAR also regulates tight junctions with phosphorylation of two key proteins, ZO-1 (Tjp1) and ZO-2 (Tjp2), decreased in LAR Δ P cells (ZO-1 Ser1614; ZO-2 Ser107; Ser239; Ser1136).

LAR-regulated phosphorylation events downstream of PDGF

In order to identify differential changes in abundance within the phosphoproteome dataset, phosphopeptides were clustered according to their response to PDGF stimulation versus unstimulated cells. The comparison of PDGF stimulated WT and LAR Δ P cells versus unstimulated WT cells allowed the evaluation of the comparative endpoint of phosphopeptide abundance. This is the phosphorylation signal that the cells would ultimately respond to in the presence of PDGF. This may be due to a differential response to PDGF or constitutive

down- or up-regulation in unstimulated LAR Δ P cells, hence we also included a comparison of PDGF stimulated LAR Δ P cells versus unstimulated LAR Δ P cells. Our experimental design included three biological replicates for the ratio between PDGF treated and unstimulated WT cells, and two biological replicates for the ratios between PDGF treated LAR Δ P cells and unstimulated WT or LAR Δ P cells (**Figure 1A**). We obtained ratios for 375 peptides, each of which had been quantified in two biological replicates. Six clusters were identified (**Figure 3A and supplemental Table S4**). Clusters 2, 3, 4 and 6 contained those phosphopeptides that, in the presence of PDGF, showed a LAR phosphatase-dependent alteration in relative abundance when compared to basal levels in WT cells. This is not true for those phosphopeptides in clusters 1 and 5 where similar levels were observed in both PDGF stimulated WT and LAR Δ P cells when compared to unstimulated WT cells. Phosphopeptides in clusters 2 and 3 exhibited similar fold changes in phosphopeptide abundance due to PDGF stimulation in both WT and LAR Δ P cells compared to their basal levels, however, the abundance in PDGF stimulated LAR Δ P cells compared to unstimulated WT cells was significantly different. This indicated that the absence of LAR phosphatase activity causes changes in basal levels of phosphorylation on these phosphoproteins. Phosphopeptides in clusters 4 and 6 have a similar fold change in LAR Δ P cells in response to PDGF, whether this is compared to unstimulated WT or LAR Δ P cells. However, the fold change is different to that observed in WT cells.

Enrichment analysis for GO terms over-represented in each cluster showed a clear distinction between the biological roles regulated by these groups of phosphoproteins (**supplemental Figure S3; supplemental Table S4**). This is highlighted in **Figure 3B** which is focused on Cellular Component GO Terms. There is a clear distinction between the discrete cellular components within which the differentially regulated phosphopeptides reside. The majority

of the enriched terms are cytoskeletal and vesicular compartments. With regards to PDGF stimulation, cluster 4 is perhaps the most interesting as these proteins contain phosphosites that are rapidly phosphorylated in response to PDGF; however, this is not the case when LAR phosphatase activity is reduced. These responses are not due to constitutive down-regulation in LAR Δ P cells. One of the enriched components in this cluster is the late endosome compartment (GO:0005770), which contains a Rab7a peptide phosphorylated on Ser72. Phosphorylation of this residue on Rab7a plays a regulatory role in late endosome maturation (45) and our data revealed a 14-fold increase in response to PDGF in WT cells; however, this was reduced over two-fold in LAR Δ P cells (**supplemental Table S4**). Within cluster 4 there was also an enrichment of cytoskeletal proteins (GO:0005856) including Sorbs3 (vinexin), a protein involved in regulation of actin stress fibre formation (46), and Add3 (gamma-adducin), a protein that promotes assembly of the spectrin-actin network which plays a role in regulating both adherens and tight junctions (47). We have identified phosphorylation events on both Sorbs3 and Add3 that are reduced by up to 3-fold in the absence of LAR activity, hence this activity is a requirement for PDGF-regulated phosphorylation of these proteins (**supplemental Table S4**). These data highlight the interplay between LAR and PDGF in regulation of the cytoskeleton and protein transport.

Regulation of kinase activity by LAR

LAR-dependent phosphorylation of several kinases has been identified (**Figure 2D**). These include Braf (B-Raf) and Mapk1 (ERK2), both members of the Ras-MAPK signalling pathway. An increase in B-Raf Ser484 and a decrease in ERK2 Tyr185 was observed in LAR Δ P cells. Phosphorylation of ERK1 and 2 on Thr183 and Tyr185 (Thr202/Tyr204 in human) occurs during MAPK signalling and activates the ERK kinases, which in turn can phosphorylate their many substrates. PDGF-dependent ERK1/2 phosphorylation at these

activating sites has previously been shown to be reduced in the absence of LAR phosphatase activity (23) and this was verified here. Analysis of PDGF-dependent ERK phosphorylation in WT and LAR Δ P cells confirms that ERK activity is significantly reduced in LAR Δ P cells treated with PDGF when compared to WT cells (**Figures 4A and 4B**). Re-expression of WT LAR in LAR Δ P cells increased ERK phosphorylation to levels resembling those observed in WT cells, confirming that LAR phosphatase activity is required for ERK activation (**Figures 4C and 4D**).

With an aim to delineate further signalling pathways regulated by LAR we sought to identify those kinases which may be responsible for inducing phosphorylation of substrates within our phosphoproteomic dataset. The kinase prediction tool GPS (34) was used to identify predicted kinases upstream of substrate motifs containing a phosphorylation site showing differential abundance between WT and LAR Δ P (270 phosphopeptides). Our proteome dataset allowed the identification of instances where phosphopeptide abundance was a result of proteome regulation rather than control of specific phosphorylation sites by regulatory kinases and phosphatases. In order to control for these effects, any proteins found to have a similar fold change in expression to the change in phosphopeptide abundance were not included in our analysis. Of the remaining 240 LAR-regulated phosphorylation sites, 223 were identified as putative substrates for a particular kinase (**supplemental Table S5**). Members of the CMGC family (includes Cyclin-dependent kinases, Mitogen-activated protein kinases, Glycogen synthase kinases and CDK-like kinases) were predicted to phosphorylate the majority of sites (**Figure 5**). The most predominant predicted kinase subfamily was the CMGC/CDK family followed by the CMGC/MAPK subfamily, including ERK, JNK, and p38 kinases. Other predominant kinases were MAPKAPK and mTOR (**Figure 5**).

LAR regulates mTOR signalling

Our kinase prediction analysis revealed mTOR as a prominent node of regulation (**Figure 5**). The mTOR signalling pathway is known to regulate protein synthesis via the mTORC1 complex and cytoskeletal organisation via the mTORC2 complex (48). Considering the significant changes in protein abundance in LAR Δ P cells and also the number of LAR-regulated cytoskeletal proteins identified it was hypothesised that LAR may be regulating the mTOR pathway. In order to further analyse the role of LAR in regulating the activity of mTOR we used antibodies recognising Ser2448 phosphorylated mTOR and Thr389 phosphorylated P70S6 kinase, both of which are indicators of active mTOR signalling. In WT cells, phosphorylation of mTOR on Ser2448 increased following stimulation with PDGF (**Figures 4A and 4B**). However, the absence of LAR phosphatase activity in LAR Δ P cells resulted in a significant decrease in PDGF-dependent phosphorylation of this residue establishing a role for LAR in mTOR signalling (**Figures 4A and 4B**). Analysis of P70S6 kinase Thr389 phosphorylation revealed a similar response to PDGF to that seen with mTOR Ser2448 in WT cells and reduced phosphorylation in LAR Δ P cells (**Figures 4A and 4C**). Re-expression of WT LAR in LAR Δ P cells resulted in an increase in mTOR Ser2448 phosphorylation to levels resembling those observed in WT cells (**Figure 4D and 4E**). Taken together, these results confirm a novel role for LAR phosphatase in the regulation of mTOR signalling.

JNK is a key node of kinase regulation by LAR

JNK kinases are involved in regulation of the actin cytoskeleton, a role also played by LAR. Predicted substrates for JNK kinases were found enriched within our phosphoproteomic dataset (**Figure 5**). Using GPS (34), predicted JNK targets identified in our dataset of LAR-

dependent phosphosites included: Eps8, a highly phosphorylated signalling adaptor protein that regulates actin dynamics and architecture (49-53); Stathmin1 and Stathmin2, both involved in microtubule disassembly (54); Tjp1, involved in tight junction assembly (55); and Tenc1 (Tns2), a focal adhesion protein that binds actin filaments (56). JNK is known to phosphorylate Ser62 of Stathmin 2 (57) and phosphorylation of this residue was significantly reduced in LAR Δ P cells compared to WT. These data prompted us to investigate whether LAR phosphatase regulates JNK phosphorylation. In the absence of LAR phosphatase activity, we observed significantly reduced JNK activity upon stimulation with PDGF (**Figures 7A and 7B**). Consistent with this, we also observed a significant decrease in activity of MKK7, an upstream kinase known to activate JNK (**Figures 7A and 7C**), and also a JNK downstream effector, c-Jun (**Figure 7A and 7D**), in LAR Δ P cells. Re-expression of WT LAR in LAR Δ P cells restored JNK phosphorylation (**Figures 7E and 7F**) demonstrating that LAR phosphatase domains are required for regulation of JNK activity. These data show LAR plays a role in regulating PDGF-mediated activation of the JNK signalling pathway.

DISCUSSION

Phosphorylation events are crucial for the regulation of cell signalling networks and, consequently, the cells response to a biological outcome. The regulatory role of kinases in specific cell signalling pathways has been long established. In more recent years it has been realised that phosphatases can be viewed in a similar manner and can regulate specific cell signalling events rather than acting as generic dephosphorylation enzymes as was once thought (1). The breadth of cell signalling pathways regulated by LAR has not previously been investigated. Using combined global quantitative phosphoproteomics and proteomics we have provided a comprehensive analysis of signalling events regulated by LAR phosphatase. The phosphorylation of 270 sites on 205 proteins was significantly up or down-

regulated in LARΔP cells compared to WT cells. Our data establish that LAR phosphatase activity is essential for the regulation of many phosphorylation events within the cell that impact on a variety of cellular processes, particularly regulation of the cytoskeleton and cell-cell interactions. Our dataset significantly expands the number of proteins regulated by LAR that are involved in these biological functions, and identifies specific regulatory phosphosites for future scrutiny. It is likely that LAR regulates phosphorylation via a number of mechanisms; via direct dephosphorylation, via regulation of activity of other phosphatases or kinases that can directly modulate the specific site, or via alterations in protein abundance.

As well as regulation at the phosphoproteome level, the absence of LAR also caused considerable changes to the identified proteome. These results highlight a possible role for LAR phosphatase activity in maintaining levels of proteins within the cell, either via regulation of protein degradation or protein synthesis. We have evidence that LAR may be regulating both processes. Protein degradation is controlled via two major pathways: lysosomal proteolysis and the ubiquitin-proteasome pathway. A number of proteins with roles in these two pathways have significant changes in phosphorylation levels due to the inactivity of LAR. The phosphorylation of Ser72 on Rab7a, a small GTPase, was decreased in LARΔP cells. Dephosphorylation of this residue is necessary for late endosome maturation in preparation for lysosomal fusion and protein degradation (45). This is one example of LAR-dependent regulation of a serine residue that is likely to occur indirectly via modulation of the activity of critical serine/threonine kinases or phosphatases upstream of Rab7a phosphorylation. Evidence for LARs involvement in protein ubiquitination is the identification of LAR-regulated phosphorylation sites on three E3 ubiquitin-protein ligases: Rffl (Ser254), Rlim (Ser229), and Dtx3l (Ser9). It is possible that the ubiquitin ligase activity of these proteins is regulated via these phosphorylation events. We have also identified LAR

as a regulator of mTOR signalling. mTOR is a serine/threonine protein kinase that regulates numerous cellular functions including protein synthesis and consequently, cell growth (58). Additionally, LAR-regulated phosphoproteins include those involved in translation of mRNA and protein synthesis. Hence, LAR may contribute to the maintenance of protein levels via the regulation of protein synthesis and mTOR signalling.

Within the dataset are TRIO, and β -catenin, proteins known to interact with, and in the case of β -catenin be a substrate for LAR (13, 39, 43, 59-61). TRIO is a multi-domain protein that acts as a guanine-nucleotide exchange factor for Rac and Rho small GTPases (39) and β -catenin an important protein involved in regulation of cell-cell junctions (62). In addition to localising LAR-regulated sites of phosphorylation on these proteins, we have also expanded the protein networks around these two proteins that also contain LAR-regulated phosphosites. For each of these proteins we identified LAR-mediated changes in serine phosphorylation which could result from an alteration in activity of a serine/threonine kinase or a serine/threonine phosphatase. These proteins may need to be localised in the vicinity of LAR via a direct interaction with TRIO or β -catenin in order to be regulated by these intermediate regulatory kinases or phosphatases.

Also within our dataset are IRS1 and IRS2, adaptor proteins that bind to the insulin receptor and regulate insulin sensitivity (63). Both proteins are reported to interact with LAR (59, 60). LAR is known to regulate insulin dependent signalling, however, there is some debate in the literature as to whether this is due to direct dephosphorylation of the insulin receptor or a consequence of regulation of the pathway further downstream of the receptor (64, 65). IRS1 has been reported to be direct substrate for LAR, however, there is some controversy over whether this is the case (64, 65). Despite evidence that serine and threonine phosphorylation

of IRS1 and IRS2 is important for regulation of insulin sensitivity (63) previous work has concentrated on identifying LAR-dependent tyrosine phosphorylation of IRS proteins. To date there has been no analysis of indirect, LAR-mediated, phosphorylation events on IRS1 or IRS2 that contribute to modulation of the cells response to insulin. Here, we have identified a reduction in serine phosphorylation of both IRS1 (Ser265) and IRS2 (Ser362) in LAR Δ P cells. Significantly, both phosphorylation events are reported to be insulin dependent (63).

Grouping the phosphopeptides according to their relative abundance in PDGF stimulated cells resulted in six distinct clusters. These clusters can be differentiated on their response to PDGF and also on their functional subclasses. There are three possible scenarios that may cause relative changes in abundance of the phosphopeptides between PDGF-stimulated LAR Δ P cells and unstimulated WT cells: (1) the levels of phosphorylation in unstimulated WT and LAR Δ P are similar, however, the response to PDGF is altered; (2) the basal level of phosphorylation of the specific residue in unstimulated LAR Δ P cells has changed, coupled with an absence of PDGF response or a similar fold response to wild-type cells; or (3) there is a change at the level of the proteome, i.e. a change in protein abundance in LAR Δ P cells. In each case the result would still be differential phosphorylation in PDGF-stimulated cells due to the absence of the phosphatase domains of LAR, which would ultimately lead to changes in signalling pathways reliant on the specific phosphorylation events. Using cluster analysis we have identified those phosphoproteins regulated by both LAR and PDGF, and these include Rab7a and a number of cytoskeletal proteins.

c-Jun N-terminal kinase (JNK) is serine/threonine kinase which is activated by a broad range of external stimuli including PDGF, transforming growth factor- β , and environmental stress

(66). Signalling via JNK regulates cell migration and enhances chemotaxis in response to PDGF stimulation (67). Several strands of evidence supporting a role for LAR in regulating JNK signalling are present within our data. First, a member of the JNK signalling pathway, Zak, is present within the LAR-regulated phosphoproteomic dataset. JNK can be activated via phosphorylation of Thr183 and Tyr185 via the action of MKK4 and MKK7 kinases (68, 69). Zak is a stress-activated kinase upstream of both MKK4 and MKK7 (70) and phosphorylation of Ser638 of Zak was increased 3.9-fold in LAR Δ P cells. This is the first strand of evidence that links LAR to JNK signalling. The second piece of evidence is the fact that we have identified specific JNK regulated phosphosites within the data that are regulated by LAR, including Ser191 of β -catenin, and Ser62 of Stathmin 2 (44, 57). In addition to this, using kinase motif predictions, we have identified JNK as a key node of regulation of a number of additional phosphosites within the LAR-regulated phosphoproteomics dataset. LAR-regulated PDGF-dependent phosphorylation of JNK on Thr183 and Tyr185 has been verified by western blotting. This demonstrates the strength of our approach in identifying novel signalling pathways regulated by LAR and has highlighted a novel role for LAR in regulating JNK signalling.

CONCLUSIONS

We have employed a global quantitative phosphoproteomics approach for the interrogation of LAR-mediated cell signalling events. We have focused on obtaining information pertaining to both direct and indirect phosphorylation events to increase our knowledge of the complete landscape of LAR-regulated signalling. The study has identified LAR as a regulator of key signalling pathways, including mTOR and JNK, and has significantly expanded the number of proteins regulated downstream of LAR phosphatase activity.

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FIGURE LEGENDS

Figure 1. Global phosphoproteomics and proteomics to measure contribution of phosphatase activity of LAR to PDGF signalling. (A) Schematic overview of experimental design. Volcano plots showing the magnitude (log2 fold-change) and significance ($-\log_{10}$ Benjamini–Hochberg adjusted p-value) of differential phosphopeptide (B) and protein (C) abundance in PDGF-stimulated WT versus LAR Δ P cells. Significantly up-regulated phosphopeptides and proteins are marked in red (adjusted p-value < 0.05; > 1.5 fold-change).

Figure 2. LAR regulated distinct biological processes. Proteins containing the 270 LAR regulated phosphosites were analysed in DAVID to identify enriched GO Terms and keywords. The top (≤ 10) enriched categories for GO Biological Processes (A), GO Molecular Function (B) and GO Cellular Component (C) are plotted as bar charts. (D) Proteins were clustered according to their keywords. Blue indicates that the protein contained a phosphosite(s) that was down-regulated in LAR Δ P cells and red indicates up-regulation.

Figure 3. Phosphorylation events in WT and LAR Δ P cells show clusters of regulation correlated to distinct biological processes. (A) GProX clustering of phosphopeptide abundance changes. Ratios of PDGF (7 minutes) stimulated WT and LAR Δ P cells over WT unstimulated cells and PDGF (7 minutes) stimulated LAR Δ P cells over LAR Δ P unstimulated cells were subjected to unsupervised clustering using the fuzzy c means algorithm. The number of phosphopeptides in each cluster is indicated. (B) Overrepresentation of GO terms in the clusters was performed within GProX using a

binomial statistical test with a Benjamini-Hochberg p-value adjustment (p-value threshold 0.05). Enriched categories for GO Cellular Components are represented as a heat map.

Figure 4. LAR Regulates the ERK signalling pathway. (A) WT and LAR Δ P cells were stimulated with 20 ng/mL PDGF for differing periods of time as indicated. Levels of ERK 1/2 Thr202/Tyr204, total ERK and Alix in whole cell lysates were analysed by western blotting. (B) Western blots (n=3) were quantified and ratios of pERK to Alix were plotted over time (*** P < 0.001; * P < 0.05). (C) WT, LAR Δ P, and LAR Δ P cells transfected with Flag-WT-LAR were stimulated with 20 ng/mL PDGF for the indicated time periods. Levels of pERK 1/2, total ERK and Alix in whole cell lysates were analysed by western blotting. (D) Western blots (n=3) from the rescue experiments were quantified and ratios of pERK to Alix were plotted (** P < 0.01).

Figure 5. LAR Regulates Distinct Kinase Nodes. Phosphorylation sites regulated by LAR were searched using the kinase prediction tool GPS. All kinases predicted to phosphorylate at least five identified phosphorylation sites are displayed. Each node represents an individual kinase, and nodes are coloured according to kinase group (see key for details). An edge connecting two nodes indicates that the corresponding kinase groups were predicted to phosphorylate at least one common residue. Node size corresponds to the total number of LAR regulated phosphorylation sites that were predicted to be phosphorylated by the corresponding kinase.

Figure 6. LAR Regulates the mTOR signalling pathway. (A) WT and LAR Δ P cells were stimulated with 20 ng/mL PDGF for differing periods of time as indicated. Levels of mTOR

Ser2448, total mTOR, P70S6 Thr389, and Alix in whole cell lysates were analysed by western blotting. (B,C) Western blots (n=3) were quantified and ratios of phospho-proteins to Alix were plotted over time (*** P < 0.001; ** P < 0.01; * P < 0.05). (D) WT, LARΔP, and LARΔP cells transfected with Flag-WT-LAR were stimulated with 20 ng/mL PDGF for the indicated time periods. Levels of mTOR Ser2448, total mTOR, FLAG-LAR and Alix in whole cell lysates were analysed by western blotting. (E) Western blots (n=3) from the rescue experiments were quantified and ratios of mTOR Ser2448 to Alix were plotted (*** P < 0.001; ** P < 0.01; * P < 0.05).

Figure 7. LAR Regulates the JNK signalling pathway. (A) WT and LARΔP cells were stimulated with 20 ng/mL PDGF for differing periods of time as indicated. Levels of JNK Thr183/Tyr185, total JNK, MKK7 Ser271/Thr275, total MKK7, c-Jun Ser63, c-Jun and Alix in whole cell lysates were analysed by western blotting. (B-D) Western blots (n=3) were quantified and ratios of phospho-protein to total protein were plotted over time (*** P < 0.001; ** P < 0.01; * P < 0.05). (E) WT, LARΔP, and LARΔP cells transfected with Flag-WT-LAR were stimulated with 20 ng/mL PDGF for the indicated time periods. Levels of JNK Thr183/Tyr185, total JNK, Flag-LAR and Alix in whole cell lysates were analysed by western blotting. (F) Western blots (n=3) from the rescue experiments were quantified and ratios of JNK Thr183/Tyr185 to total JNK were plotted (** P < 0.01).

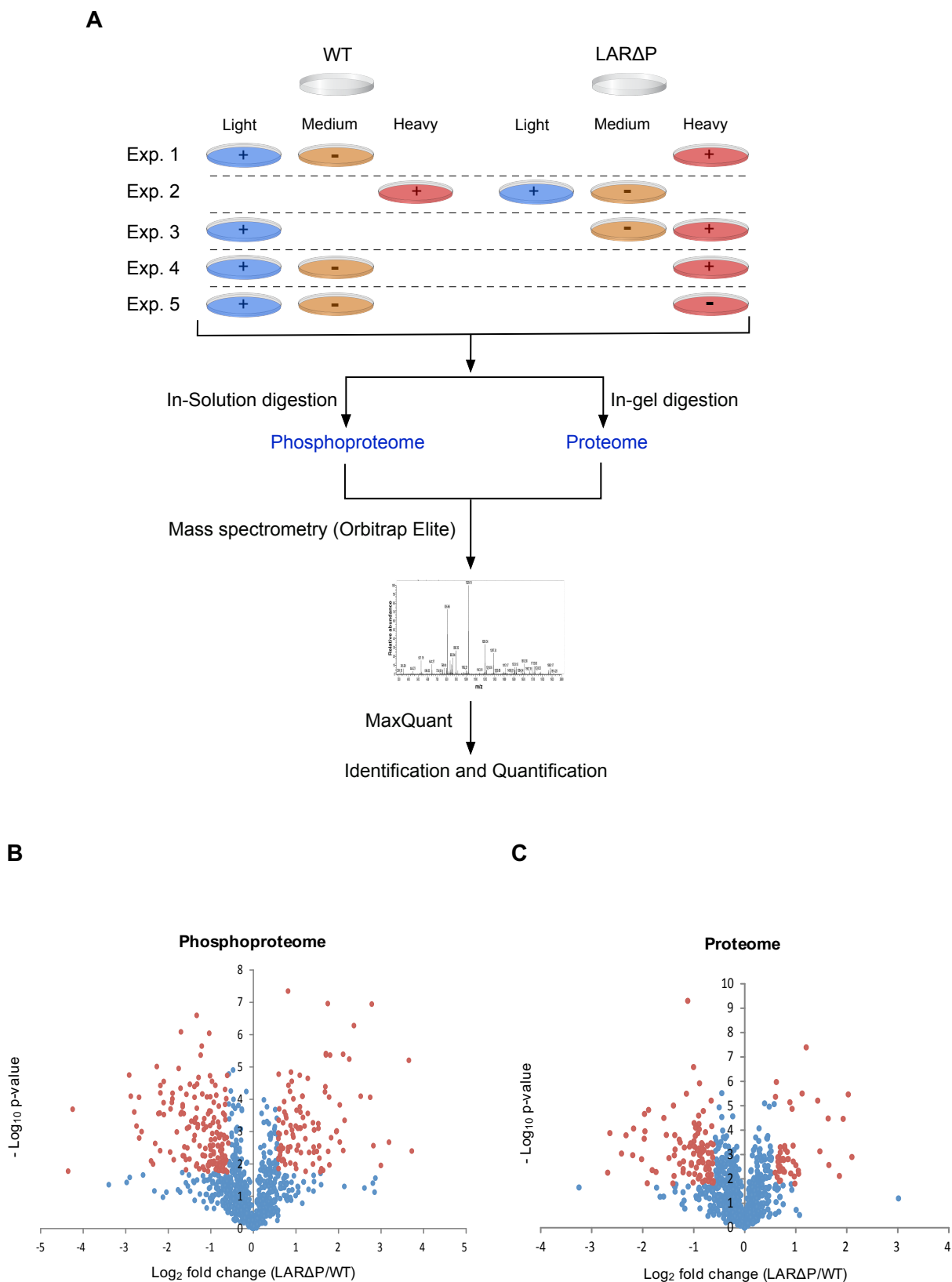


Figure 1

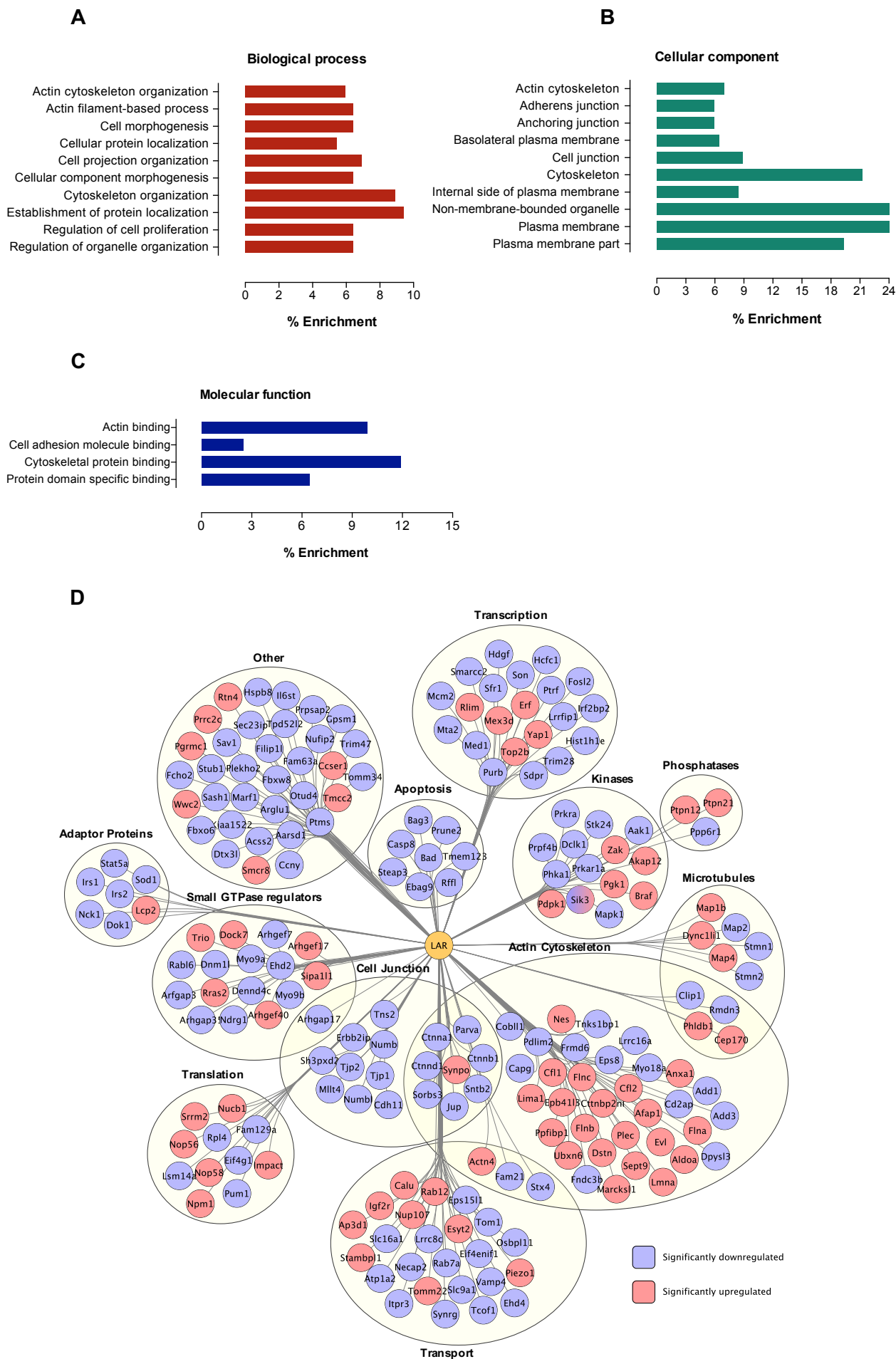
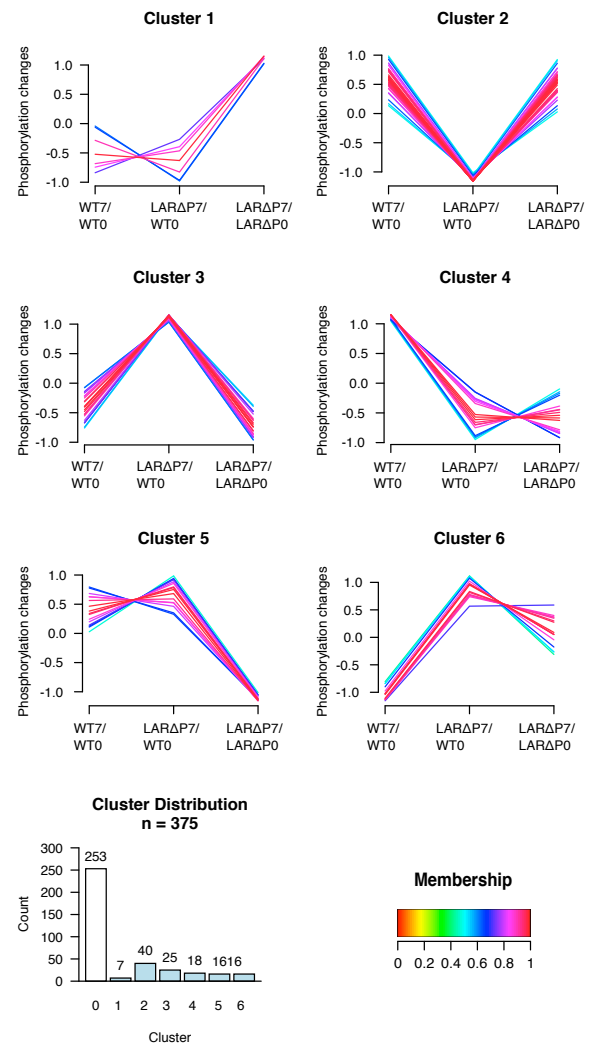


Figure 2

A



B

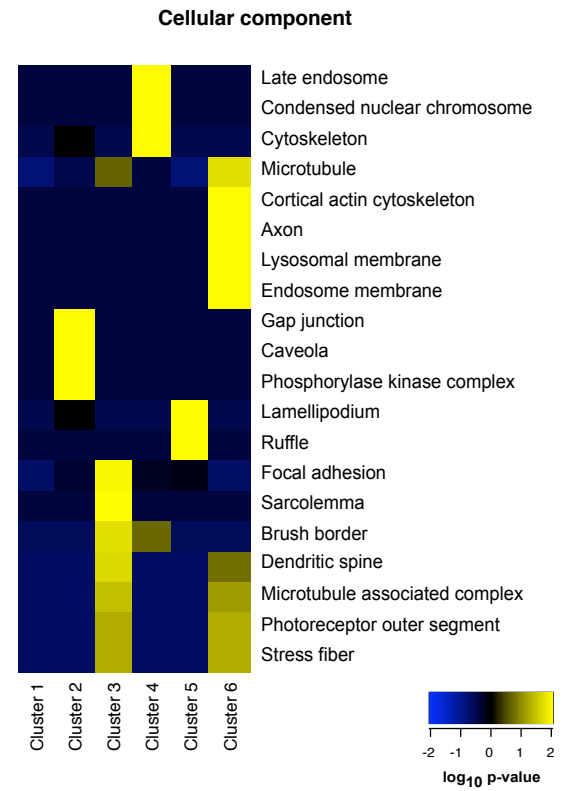


Figure 3

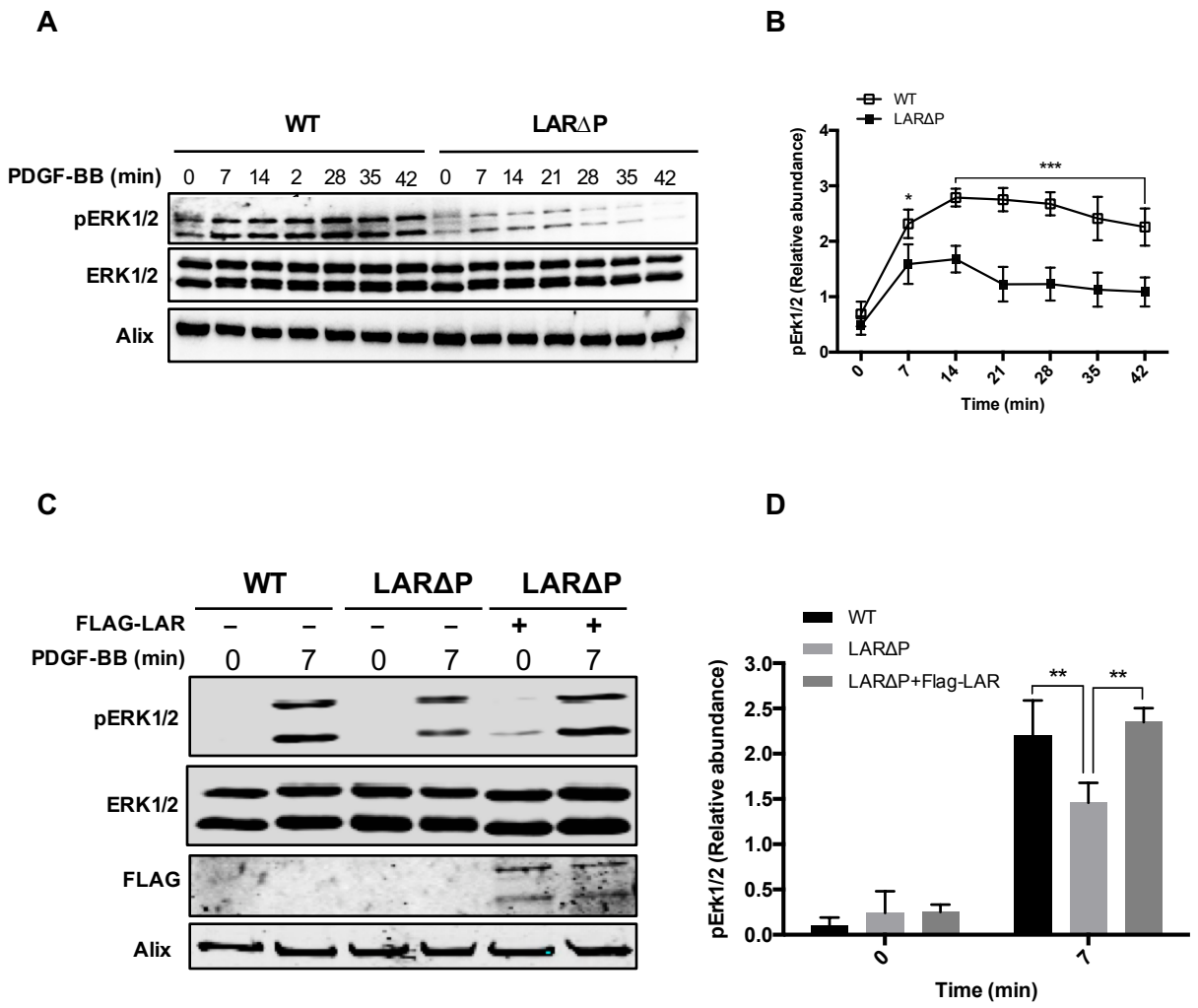


Figure 4

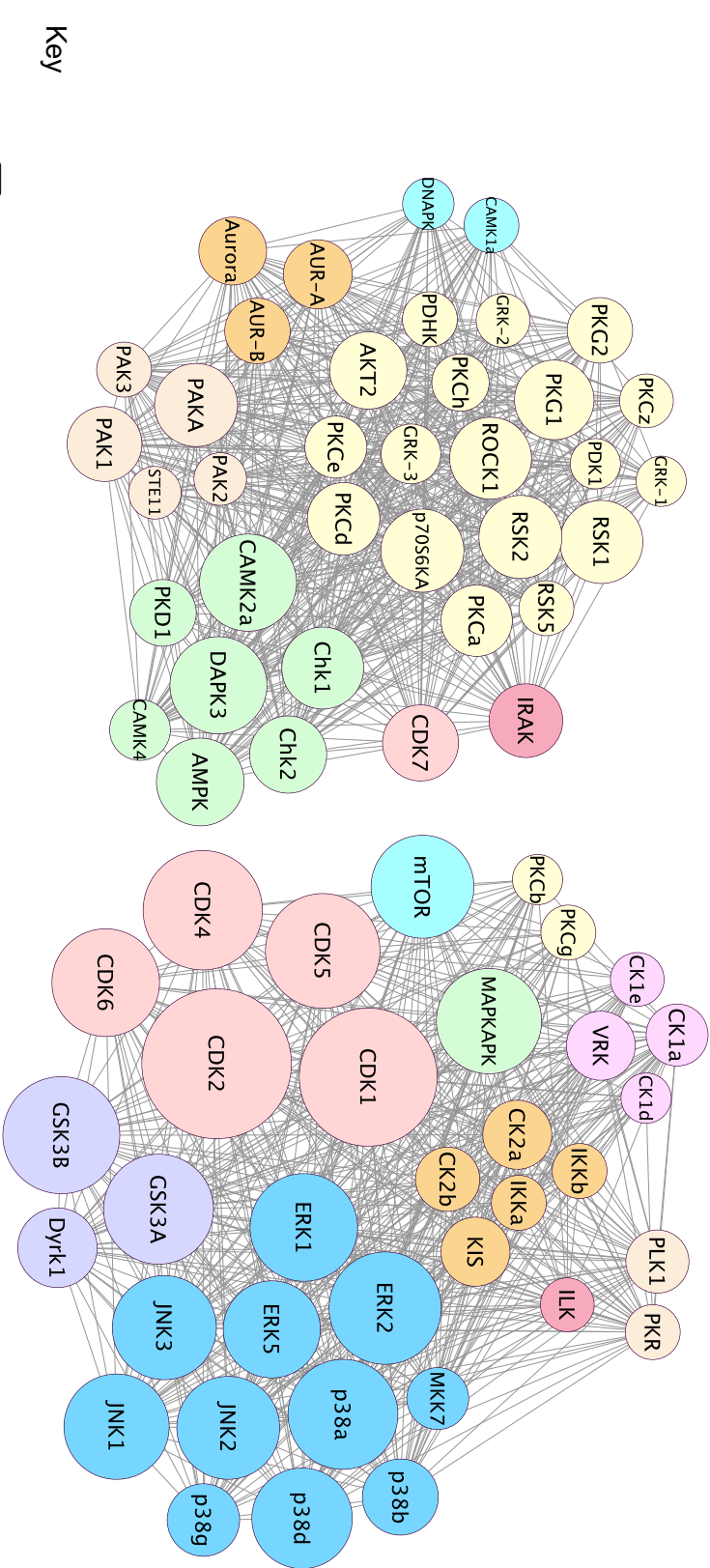


Figure 5

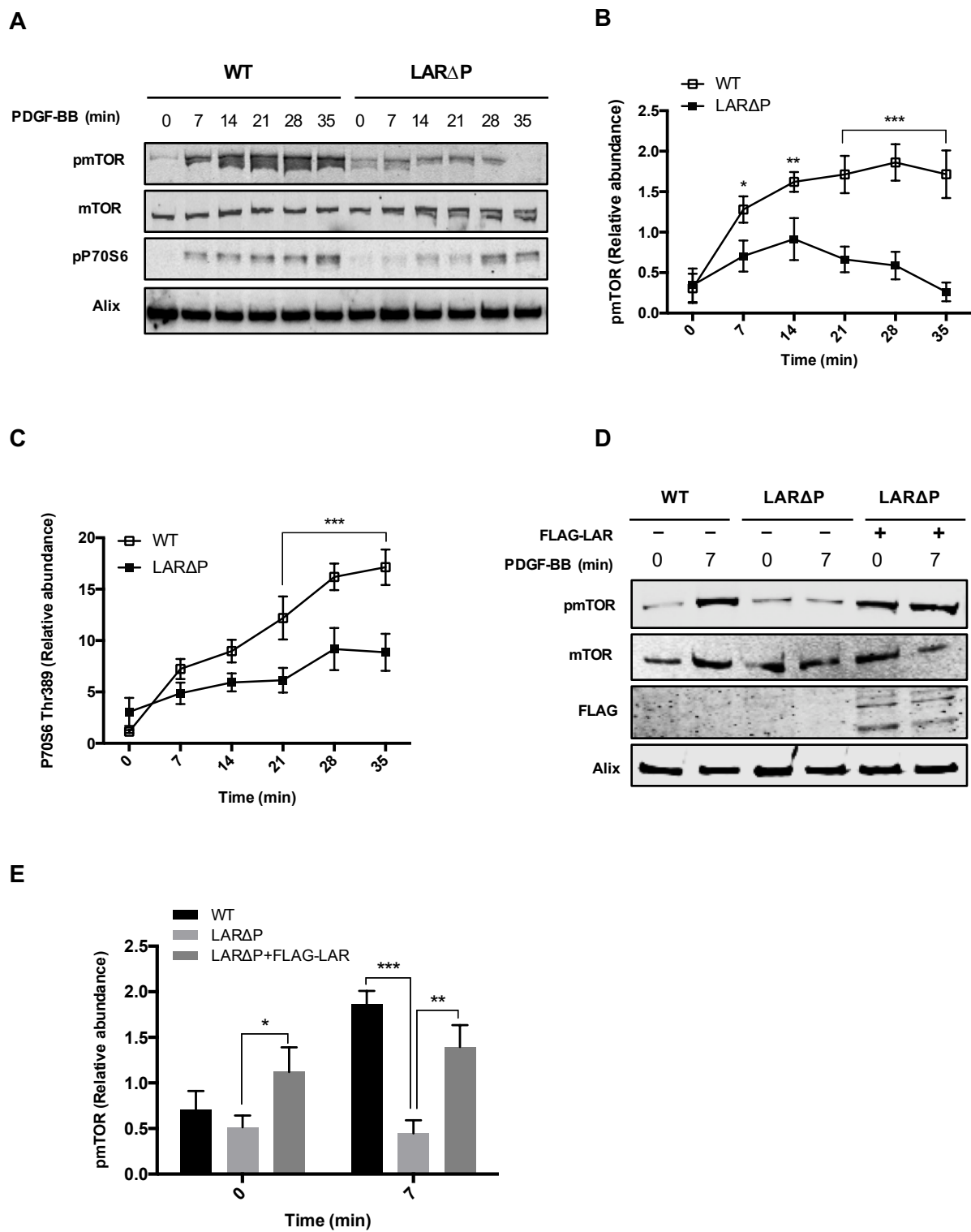


Figure 6

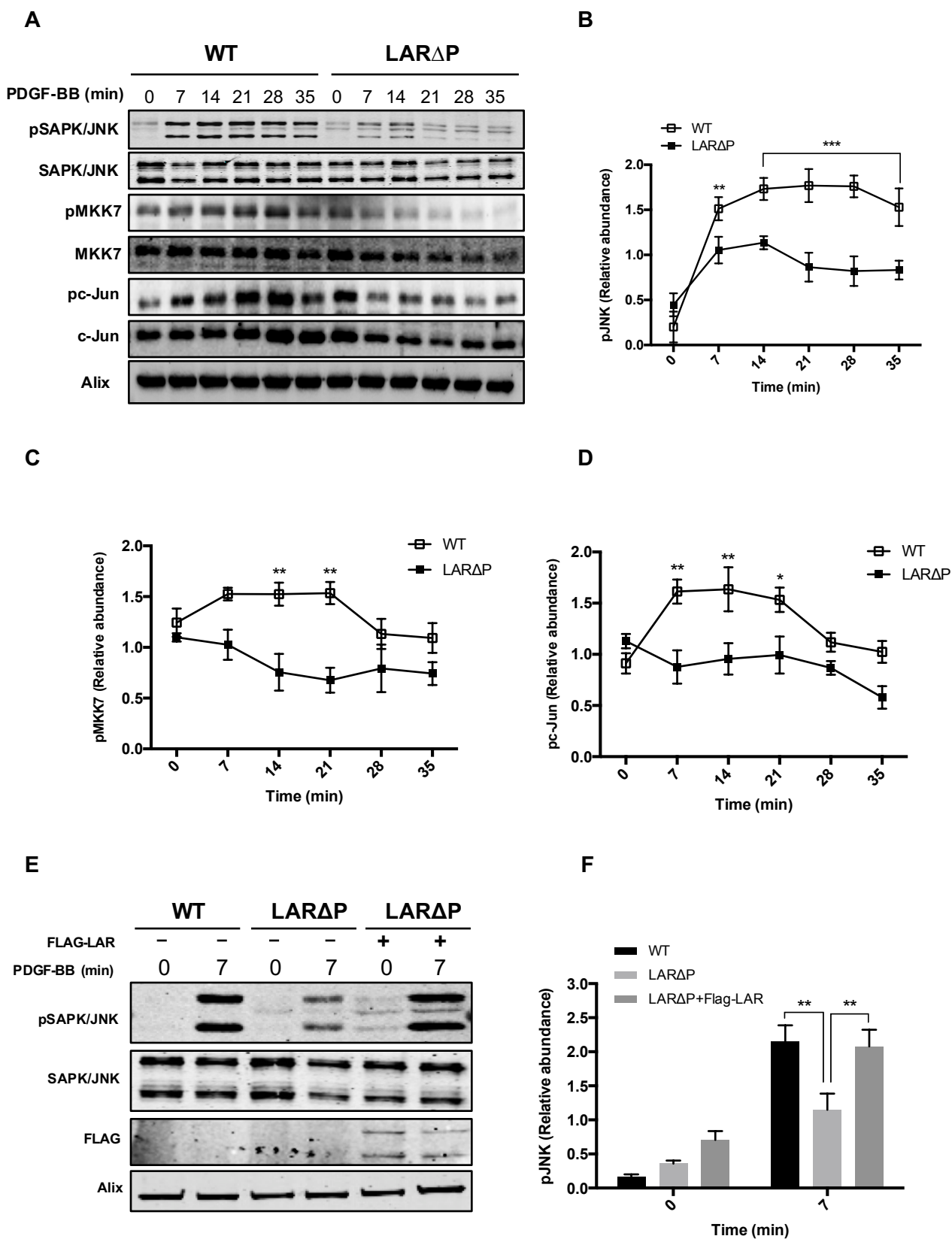


Figure 7