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LAR Protein Tyrosine Phosphatase Regulates Focal Adhesions via CDK1

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SUMMARY STATEMENT

We demonstrate that LAR, a receptor tyrosine phosphatase, signals via CDK1 to promote adhesion complex formation and adhesion to extracellular matrix.

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ABSTRACT

Focal adhesions are complex multi-molecular structures that link the actin cytoskeleton to the extracellular matrix via integrin adhesion receptors and play a key role in regulation of many cellular functions. LAR is a receptor protein tyrosine phosphatase that regulates PDGF signalling and localises to focal adhesions. We have observed that loss of LAR phosphatase activity in mouse embryonic fibroblasts results in reduced numbers of focal adhesions and decreased adhesion to fibronectin. To understand how LAR regulates cell adhesion we used phosphoproteomic data, comparing global phosphorylation events in wild type and LAR phosphatase-deficient cells, to analyse differential kinase activity. Kinase prediction analysis of LAR-regulated phosphosites identified a node of cytoskeleton- and adhesion-related proteins centred on cyclin-dependent kinase-1 (CDK1). We found that loss of LAR activity resulted in reduced activity of CDK1, and that CDK1 activity was required for LAR-mediated focal adhesion complex formation. We also established that LAR regulates CDK1 activity via c-Abl and PKB/Akt. In summary, we have identified a novel role for a receptor protein tyrosine phosphatase in regulating CDK1 activity and hence cell adhesion to the extracellular matrix.

INTRODUCTION

Traditionally, phosphatases have been considered relatively non-specific regulators of cellular signalling pathways, a view that has been increasingly challenged in recent years. It is now clear that phosphatases play very specific roles in both the positive and negative propagation of signals (Tonks, 2013). Leukocyte common antigen-related (LAR), also known as receptor type protein tyrosine phosphatase F (PTPRF), is a receptor protein tyrosine phosphatase. It is comprised of an extracellular domain that contains three immunoglobulin domains and eight fibronectin type III domains in addition to two cytoplasmic phosphatase domains, D1 and D2 (Chagnon et al., 2004). LAR is expressed in a number of human tissues including brain, heart and bladder, and LAR-deficient mice are characterised by axon guidance defects, diabetes, neuron degeneration, cancer and abnormal development of mammary glands (Chagnon et al., 2004; Van Lieshout et al., 2001). Previous work has identified roles for LAR in neuronal growth and regeneration, mammary gland development and in mitogenic signalling via the PDGF receptor β (PDGFRβ) (Schaapveld et al., 1997; Um and Ko, 2013; Zheng et al., 2011).

Cell interaction with the extracellular matrix is mediated in part through complex, dynamic multi-molecular structures called focal adhesions that link the actin cytoskeleton to the extracellular matrix via integrin adhesion receptors (Wehrle-Haller, 2012). Focal adhesions mediate transfer of signals from the extracellular matrix to the cytoplasm and nucleus, regulating many functions including proliferation, apoptosis, differentiation and cell migration (Wehrle-Haller, 2012). Proteins localizing to focal adhesions include structural proteins that link integrins to the actin cytoskeleton as well as many other proteins involved in signal transduction. These include the well-documented regulators of focal adhesions, Src and focal adhesion kinase (FAK) but also other kinases including members of the cyclin-dependent kinase (CDK) family (Horton et al., 2015; Robertson et al., 2015; Zaidel-Bar et al., 2007). The extracellular domain of LAR is known to interact with components of the extracellular matrix including the laminin–nidogen complex and syndecan (Johnson et al., 2006; O'Grady et al., 1998) and imaging and proteomic studies have established that LAR is a component of focal adhesions and can regulate the actin cytoskeleton (Serra-Pages et al., 1995; Zaidel-Bar et al., 2007).

However, whilst LAR is known to localise to focal adhesions and interact with components of the extracellular matrix, its role in cell adhesion has not been established. To analyse the role of LAR in cell adhesion we used wild-type (WT) mouse embryonic fibroblasts (MEFs) and MEFs in which the LAR phosphatase domains have been deleted (LARΔP) (Schaapveld et al., 1997). Our results reveal a novel signalling pathway in which LAR phosphatase signals via c-Abl, Akt and CDK1 to regulate adhesion complex assembly and optimal adhesion to fibronectin.

RESULTS

Focal adhesion formation is significantly decreased in cells lacking LAR phosphatase activity.

LAR localises to adhesion complexes and is known to regulate the actin cytoskeleton (Bateman et al., 2001; Serra-Pages et al., 1995). Therefore, we evaluated the role of LAR in adhesion complex formation and adhesion to extracellular matrix. To do this we used mouse embryo fibroblasts (MEFs) expressing either wild type LAR (WT) or a truncated form of LAR lacking phosphatase activity (LARΔP) (Schaapveld et al., 1997). Following serum starvation, cells were either unstimulated or stimulated with PDGF BB, a well-established regulator of adhesion complex formation (Ridley et al., 1992), for 14 minutes prior to fixation and staining using an anti-phospho-paxillin (Tyr^{118}) antibody to visualise adhesion complexes (Burridge et al., 1992). We observed a significant decrease in phospho-paxillin staining in unstimulated LARΔP cells when compared to WT cells (Figure 1A, B, E). Treatment with PDGF BB resulted in increased phospho-paxillin staining in both LARΔP and WT cells but again there was a significant decrease in adhesion complexes in LARΔP cells compared to WT cells (Figs 1C-E).

The extracellular matrix protein fibronectin is secreted by fibroblasts and plays a key role in cell-extracellular matrix interactions during development and in wound healing (Hynes, 1994). In order to investigate whether LAR phosphatase activity contributes to these cell-extracellular matrix interactions, we used a standard adhesion assay to analyse attachment of WT and LARΔP MEFs to fibronectin. Serum-starved WT and LARΔP cells were allowed to attach to fibronectin in the presence or absence of 20ng mL^{-1} PDGF BB for 30 minutes. LARΔP cells were substantially impaired in their ability to attach to fibronectin when compared to WT cells, and this was apparent over a range of fibronectin plating concentrations between 0.5 and 10μg mL^{-1} (Fig. 2A). Treatment with PDGF did not significantly affect attachment of either WT or LARΔP cells (Fig. 2A). To confirm these data, we used RNA interference to knockdown expression of LAR in WT cells (Fig. 2B). RNAi-mediated reduction of LAR expression in WT cells was accompanied by a significant decrease in cell attachment to 10μg mL^{-1} fibronectin when compared to WT cells transfected with a non-silencing

control (NSC) oligo (Fig. 2C). Having observed that LAR regulated adhesion complex formation and attachment to fibronectin we analysed cell migration of WT and LARΔP cells, using a standard scratch wound assay. We found that loss of LAR phosphatase activity did not affect cell migration, with both WT and LARΔP cells closing wounds at equivalent rates (Fig. 2D). We conclude that LAR phosphatase regulates adhesion complex formation and attachment to fibronectin in mouse embryonic fibroblasts but has no effect on cell migration.

Regulation of adhesion complexes by LAR is independent of focal adhesion kinase activity.

Focal adhesion kinase (FAK) and Src are non-receptor tyrosine kinases frequently linked to adhesion complex dynamics, cell adhesion and cell migration (Mitra and Schlaepfer, 2006), although recent data also report that adhesion complex composition is unaffected when Src and FAK are inhibited (Horton et al., 2016). Tyr^{118} of paxillin is a known FAK substrate (Bellis et al., 1995), and we tested whether activation of FAK was affected in the absence of LAR activity. Autophosphorylation of Tyr^{397} is a key stage in activation of FAK, creating a docking site for the recruitment and activation of the non-receptor tyrosine kinase Src which subsequently phosphorylates FAK on Tyr^{407} (Calalb et al., 1995). Consistent with immunostaining of adhesion complexes (Fig. 1) we observed substantially reduced paxillin Tyr^{118} phosphorylation in LARΔP cells when compared to WT cells, both in the absence and presence of PDGF (Fig. 3A). However, when we analysed FAK activity in LARΔP cells we observed no significant difference in phosphorylation of either Tyr^{397} or Tyr^{407} when compared to WT cells (Figs 3B-D). These data are consistent with the previous observation that activation of Src is not regulated by LAR (Zheng et al., 2011) and indicate that the decreased phosphorylation of paxillin and reduced adhesion complex formation seen in LARΔP cells are not a consequence of altered FAK signalling.

Phosphoproteomic analysis of LARΔP cell signalling identifies a role for LAR phosphatase in regulation of CDK1

To identify key signalling events regulated by LAR we analysed our comparative (WT versus LARΔP MEFs) global phosphoproteomic dataset (deposited in the ProteomeXchange Consortium via the PRIDE partner repository

(www.ebi.ac.uk/pride/archive dataset identifier PXD002545) (Sarhan et al., 2016). Within this dataset, we identified altered phosphorylation of 270 phosphorylation sites in 205 proteins in PDGF-BB stimulated LARΔP cells when compared to WT cells. To identify key nodes of regulation within the dataset, the kinase prediction tool GPS (Xue et al., 2008) was used to identify putative kinases upstream of the 270 identified phosphorylation motifs. A cluster of 38 proteins containing predicted cyclin dependent kinase 1 (CDK1) phosphorylation motifs were identified with significantly reduced phosphorylation in LARΔP cells. Of these, 19 (50%) form part of the recently characterised meta-adhesome (Horton et al., 2015) (Fig. 4A).

Phosphorylation of Thr^{161} in the activation loop of CDK1 is a key step in activation of the kinase (Krek and Nigg, 1992; Solomon et al., 1992), therefore we used a phospho-specific antibody to analyse activation of CDK1 in WT and LARΔP cells. In serum-starved WT cells, basal phosphorylation of CDK1 Thr^{161} was observed and this increased on addition of PDGF (Fig. 4B). In contrast, the basal phosphorylation of Thr^{161} in LARΔP cells was lower than in WT cells and no increase in phosphorylation of Thr^{161} was observed when cells were treated with PDGF (Fig. 4B). CDK activity requires interaction with a partner cyclin and CDK1 forms a complex with cyclin B1 during G2/M (Malumbres, 2014) but no difference in expression of CDK1 or cyclin B1, or association between CDK1 and cyclin B1, was observed when LARΔP cells were compared to WT cells (Fig. 4C). This suggests the decrease in CDK1 activity in LARΔP cells was not a consequence of cyclin availability.

In the absence of LAR phosphatase activity we have observed decreased phosphorylation of CDK1 consistent with decreased activity and have identified a cluster of potential adhesion-related CDK1 substrates. CDK1 has recently been linked to cell adhesion (Robertson et al., 2015), thus we next examined the role of CDK1 in LAR-dependent cell adhesion. Treatment of WT MEFs with the CDK1 inhibitor, RO-3306, resulted in significantly fewer focal adhesions, consistent with CDK1 regulating adhesion complex formation in this cell type (Figs 4D, E). To confirm that CDK1 regulates adhesion complexes in MEFs downstream of LAR we transiently expressed a constitutively active form of CDK1 (CDK1 AF) in LARΔP cells. This resulted in a significant increase in adhesion complex formation (Figs 4F, G). These data establish

CDK1 as a protein kinase acting downstream of LAR to regulate adhesion complex formation.

LAR signals to CDK1 via Akt

Previous work has shown that LAR regulates PDGF-mediated activation of Akt (Zheng et al., 2011) and loss of Akt activity is associated with decreased phosphorylation of CDK1 at Thr^{161} (Nogueira et al., 2012), suggesting that Akt may be part of the pathway downstream of LAR leading to activation of CDK1 and regulation of adhesion complex formation.

To investigate whether Akt is involved in LAR-dependent cell adhesion we analysed adhesion complex formation in WT cells treated with an Akt inhibitor (InSolution Akt Inhibitor VIII) and observed a significant ($p<0.001$) decrease in adhesion complex formation compared to control, vehicle (DMSO) treated cells (Fig. 5A). Activation of Akt requires phosphorylation of Thr^{308} in the activation loop (Alessi et al., 1996), and consistent with previously published data that PDGFRβ-induced activation of Akt is dependent on LAR (Zheng et al., 2011), we observed a rapid increase in phosphorylation of Akt Thr^{308} in control, serum-starved WT cells, treated with PDGF (Figs 5B, C) and significantly reduced phosphorylation of Thr^{308} in LARΔP cells at all time points (Figs 5B, C). Treatment of WT cells with the Akt inhibitor, in the absence or presence of PDGF, also resulted in a significant reduction in Thr^{161} phosphorylation of CDK1 (Fig. 5D) confirming Akt-dependent regulation of CDK1 activity in MEFs. This provides further evidence for a novel role of LAR in regulation of cell adhesion via CDK1.

Inhibition of c-Abl restores activation of Akt and CDK1 in cells lacking LAR phosphatase activity.

Previous work has reported hyper-phosphorylation of the tyrosine kinase c-Abl in LARΔP cells and Abl has been identified as a potential substrate for the Drosophila LAR ortholog, DLAR (Wills et al., 1999; Zheng et al., 2011). In mammalian cells a large number of c-Abl substrates have been identified, many of which are involved in regulation of cell adhesion and the actin cytoskeleton (Wang, 2014; Woodring et al., 2003). To establish whether c-Abl is a LAR substrate we transfected 293T cells with

cDNA constructs expressing either WT LAR or one of two LAR substrate trapping mutants - LAR C/S (C1548S) or LAR D/A (D1516A) (Fig. 6A) (Wang et al., 2007). These mutations within the intracellular phosphatase domain D1 allow LAR to interact with substrates but reduce its dephosphorylation activity (Wang et al., 2007). Using this approach, we identified that both the LAR trapping mutants and WT LAR interacted with c-Abl (Fig. 6B). When compared to cells transfected with either trapping mutant, a reduction in tyrosine phosphorylation was observed in c-Abl bound to WT LAR (Fig. 6B). These data identify c-Abl as a LAR substrate. To determine whether c-Abl is involved in LAR-mediated regulation of CDK1 we treated LARΔP cells with AG957, a selective inhibitor of c-Abl (Anafi et al., 1992), and examined the effect of inhibiting c-Abl on activation of Akt and CDK1. Inhibition of c-Abl in LARΔP cells resulted in significantly increased phosphorylation of both Akt Thr^{308} and CDK1 Thr^{161} (Figs 6C-F). These data indicate that c-Abl is a key component of the LAR pathway upstream of Akt and CDK1.

DISCUSSION

Much of what we currently know about LAR function focuses on its role in the nervous system and comes from work on the Drosophila ortholog, DLAR (Um and Ko, 2013). DLAR localises to synaptic junctions and regulates synaptic growth and axonal guidance through regulation of the actin cytoskeleton via Rho family GTPases (Bateman et al., 2000; Kaufmann et al., 2002; Pawson et al., 2008). Outside of the nervous system relatively little is known about LAR function, although DLAR has been reported to regulate the actin cytoskeleton in Drosophila epithelia (Bateman et al., 2001; Conder et al., 2007). In addition, in mammalian cells an interaction between LAR and EphA2 has been reported to be important for cell migration (Lee and Bennett, 2013). Our data, showing decreased adhesion complex formation and decreased cell adhesion to extracellular matrix in the absence of LAR phosphatase activity, identify LAR as a regulator of cell adhesion to extracellular matrix in mammalian cells. Furthermore we identify that LAR affects cell adhesion through regulation of CDK1 activity.

Kinase prediction analysis of LAR-regulated phosphosites identified a node of cytoskeleton- and adhesion-related proteins centred on CDK1 (Fig. 4), 50% of which form part of the recently characterised meta-adhesome (Horton et al., 2015). Most work has focused on nuclear functions for CDKs, and the role of CDK1 in regulating the G2/M cell cycle transition is well established (Malumbres, 2014). However, there is increasing evidence from both imaging and proteomic studies to support the hypothesis that CDKs have significant non-nuclear roles. CDK1 has been identified as being present in focal adhesions and recent data indicates that CDK1, along with other CDKs, plays a role in integrin-based cell adhesion and motility (Manes et al., 2003; Robertson et al., 2015). During G2/M transition in vertebrates, phosphorylation of CDK1 Thr^{161} is a function of CDK7, a CDK activating kinase (CAK) and one possibility is that LAR might also regulate CDK7 activity. (Desai et al., 1995; Fisher, 2005). However, analysis of our phosphoproteomics dataset (Sarhan et al., 2016) did not reveal any significant change in phosphorylation of Ser^{164} which is required for activation of CDK7 (Larochelle et al., 2001). This suggests LAR may be regulating phosphorylation of Thr^{161} and activation of CDK1 in a CDK7-independent manner.

We have identified that the tyrosine kinase c-Abl is a direct substrate for LAR in mammalian cells and a key component linking LAR phosphatase to CDK1. Consistent with these findings, c-Abl localises to adhesion complexes and the Drosophila ortholog of LAR, DLAR, is known to interact with Abl to regulate axon guidance, an actin- and adhesion-dependent process (Wills et al., 1999; Zaidel-Bar et al., 2007). The question remains as to how c-Abl is regulating CDK1 activity and our data indicate this is achieved via Akt as we observed that inhibition of Akt blocked LAR-dependent activation of CDK1 (Fig. 5D). As LAR has been reported to regulate PDGF signalling via c-Abl (Zheng et al., 2011) and Akt is well-established as a signalling component downstream of receptor tyrosine kinases including PDGFR (Andrae et al., 2008), one possible explanation for our findings is that LAR regulates CDK1 and hence cell adhesion via the PDGF pathway. The decreased Akt Thr^{308} phosphorylation seen in LARΔP cells following PDGF stimulation (Fig. 5B) would support this idea and Akt has also been reported to regulate PDGF-dependent phosphorylation of FAK at Tyr^{397} (Higuchi et al., 2013). However, we find no evidence that LAR regulates FAK activity and phosphorylation of CDK1 Thr^{161} in LARΔP cells is not affected by PDGF (Fig. 4B). In addition, we observe that LAR can regulate adhesion complex formation and cell adhesion independent of PDGF stimulation (Figs 1,2). One surprising observation was that whilst inhibition of c-Abl restored phosphorylation of CDK1 Thr^{161} in un-stimulated LARΔP cells, thus identifying c-Abl as a component of the pathway linking LAR to activation of CDK1, stimulation with PDGF resulted in a steady decrease in CDK1 Thr^{161} phosphorylation over time. One possible explanation for this is that in the absence of LAR and c-Abl activity a phosphatase is activated that can dephosphorylate CDK1. Irrespective of this, our data clearly demonstrate a role for c-Abl in regulating LAR-mediated phosphorylation of CDK1 and taken together, our data indicate that whilst PDGF signalling can act to modulate LAR-dependent effects on adhesion, LAR can also regulate CDK1 and cell adhesion independent of PDGF signalling (Fig. 7).

We have observed that LAR positively regulates attachment to fibronectin and, whilst there is no evidence for LAR interacting directly with fibronectin, LAR has been reported to interact with the laminin-nidogen complex (O'Grady et al., 1998). This would suggest that LAR might play a role in regulating both ‘inside-out’ and ‘outside-

in' signalling in a manner analogous to integrins (Hynes, 2002) and it would be interesting to analyse whether different extracellular matrix substrates elicit distinct LAR-dependent signalling events. Interestingly, whilst we have clear evidence that LAR regulates cell adhesion, loss of LAR activity had no effect on cell migration (Fig. 2). This would be consistent with our data showing that LAR does not affect activity of FAK (Fig. 3), a known regulator of cell migration in fibroblasts (Sieg et al., 1999). Given the ability of LAR to regulate both PDGF-dependent mitogenic signalling and adhesion to extracellular matrix it is tempting to speculate that LAR may play a pivotal role in integrating signals required for cell cycle progression. Similarly, LAR might also be important in anoikis, where detachment of a normal, adherent cell is a prelude to programmed cell death (Reddig and Juliano, 2005).

In summary, we have identified a novel mechanism whereby a membrane associated protein tyrosine phosphatase positively regulates adhesion complex formation and cell adhesion via a signalling pathway involving c-Abl, Akt and CDK1.

MATERIALS AND METHODS

Antibodies

Akt (#9272), phospho-Akt Thr^{308} (#9275), cyclin B1 (#4135), paxillin (#2542), phospho-paxillin Tyr^{118} (#2541), phospho-CDK1 Thr^{161} (#9114) and c-Abl (#2862) antibodies were obtained from Cell Signaling Technology (Danvers, MA). CDK1 (#sc-54), phospho-FAK Tyr^{407} (#sc-16664) and PY99 phosphotyrosine (#SC-7020) antibodies were obtained from Santa Cruz Biotechnology (Dallas, TX). FAK (#610087) and phospho-FAK Tyr^{397} (#611722) antibodies were purchased from BD Biosciences (Franklin Lakes, NJ). FLAG M2 antibody (#F3165) was from Sigma-Aldrich (Poole, UK). LAR antibody (#73-193) was purchased from Neuromab (UC Davis, CA). The Alix antibody was a gift from Carl Hendrik Heldin (Karolinska Institute, Sweden) (Lennartsson et al., 2006). Alexa 594-conjugated phalloidin and DAPI were from Life Technologies (Paisley, UK). Goat anti-mouse IgG IRDye-conjugated antibody and goat anti-rabbit IgG HRP-conjugated antibodies were from LI-COR Biosciences (Lincoln, NE).

Cell Culture

All cells were grown in DMEM supplemented with 10 % fetal bovine serum (FBS), 100 U/mL penicillin, 10 mg mL^{-1} streptomycin, and 250 µg mL^{-1} amphotericin B and periodically tested for, and shown to be free of, mycoplasma contamination. WT and LARΔP mouse embryonic fibroblasts (MEFs) were kind gifts from Wiljan Hendriks (Radboud University Medical Centre, Netherlands) (Schaapveld et al., 1997). Cell culture reagents were purchased from Gibco Life Technologies (Paisley, UK). For growth factor stimulation experiments cells were grown to 80 % confluence and starved for 16 hours in serum free DMEM before being treated with 20 ng mL^{-1} recombinant PDGF-BB (Cell Signaling Technology) at 37 ˚C. In some experiments, cells were incubated with 10 µM InSolution Akt Inhibitor VIII (Barnett et al., 2005) (Merck Millipore, Watford UK) to inhibit Akt, 10 µM AG957 (Sigma-Aldrich, Poole UK) to inhibit c-Abl or RO-3306 (Merck Millipore, Watford UK) to inhibit CDK1. The carrier DMSO was added to control cells.

Cell lysis, immunoprecipitation and immunoblotting

Cell lysates were prepared, separated by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted using previously described protocols (Zheng et al., 2011). Immunoblots were visualized using fluorescence detection on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln NE). Densitometric analysis was carried out using ImageJ. Immunoprecipitation of cyclin B1 or CDK1 using Dynabeads protein G (Novex, Life Technologies) was performed as per the manufacturer's protocol.

Cell adhesion and scratch wound assays

Adhesion assays were performed as described previously (Lock and Hotchin, 2009) with the modification that adherent cells were quantitated by addition of 5 mg mL^{-1} of Thiazolyl blue tetrazolium bromide (MTT) (Sigma-Aldrich, Poole UK) for 1 hour at 37 ˚C followed by addition of 100 µL of isopropanol for 15 minutes at room temperature after which absorbance was measured at 570 nm. For the scratch wound assays, cells were plated in 96 well cell culture plates and cultured to confluency. Scratch wounds were made using an automated wound maker (Essen BioScience, Hertfordshire, UK) and images collected at hourly intervals post-wounding using an IncuCyte ZOOM imaging system (Essen BioScience, Hertfordshire, UK).

Immunocytochemistry

WT and LARΔP cells were grown on acid-etched glass coverslips before being cultured overnight in serum-free medium. Where indicated, seeded cells were incubated with either 10 µM CDK1 RO-3306 specific inhibitor for one hour or 10 µM InSolution Akt Inhibitor VIII or in DMSO overnight. Cells were stimulated with 20 ng mL^{-1} PDGF-BB for 14 minutes and then incubated on ice for 5 minutes. Fixed and permeabilised cells were immunostained as previously described (Lock and Hotchin, 2009). Cells were visualized using TIRF Nikon A1R confocal microscope with NIS-Elements Software and images were analysed using *ImageJ* 1.48. To quantitate adhesion complex area, background-subtracted images of a minimum of 4 cells per experiment were thresholded to identify and measure adhesion pixels using ImageJ software using a previously published method (Webb et al, 2004).

RNA interference

Cells were transiently transfected with 80 pmol of SMARTpool ON-TARGETplus siRNA for murine LAR (L-042444-00-0005; Dharmacon, Lafayette, CO) using Lipofectamine RNAiMAX (Life Technologies, Paisley UK) as described elsewhere (Lock and Hotchin, 2009). Non-silencing control (NSC) siRNA was used as a control for each experiment.

cDNA expression

To 'rescue' CDK1 activity, LARΔP cells were transiently transfected with a constitutively active form of CDK1 (CDK1-AF) (Hagting et al., 1998) using Lipofectamine 2000 (ThermoFisher Scientific) according to manufacturer's instructions. In the LAR phosphatase trap experiment 293T cells were transiently transfected using Lipofectamine 2000 with cDNA constructs expressing either wild type c-Abl (WT c-Abl) (Johannessen et al., 2010) or WT LAR, LARC1538S (LAR C/S) or LARD1506A (LAR D/A) (Wang et al., 2007). The LAR constructs were kindly provided by Ruey-Hwa Chen (National Taiwan University, Taipei, Taiwan). The CDK1-AF construct was obtained from Addgene (Cambridge MA).

Phosphoproteomics and bioinformatics

Potential CDK1 substrates were identified using the Group-based Prediction System (GPS; version 2.1.2) (Xue et al., 2008). To minimize false positives, the highest threshold was applied. Protein-protein interaction (PPI) network analyses were performed using Cytoscape (version 3.3.0) (Shannon et al., 2003). GO enrichment analyses were performed using (DAVID; version 6.7) (Huang da et al., 2009).

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Competing Interests

JKH is an Editor for Journal of Cell Science. There are no other competing interests.

Author Contributions

Conceived and designed the experiments: ARS, MGT, CH, JKH, DLC, NAH. Performed the experiments and analysed the data: ARS, TRP, ARC, DLC. Wrote the manuscript: ARS, TRP, MGT, JKH, DLC, NAH.

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Data Availability

The mass spectrometry proteomics data, including MaxQuant output, used in this study have been deposited in the ProteomeXchange Consortium via the PRIDE partner repository (www.ebi.ac.uk/pride/archive dataset identifier PXD002545).

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Figures

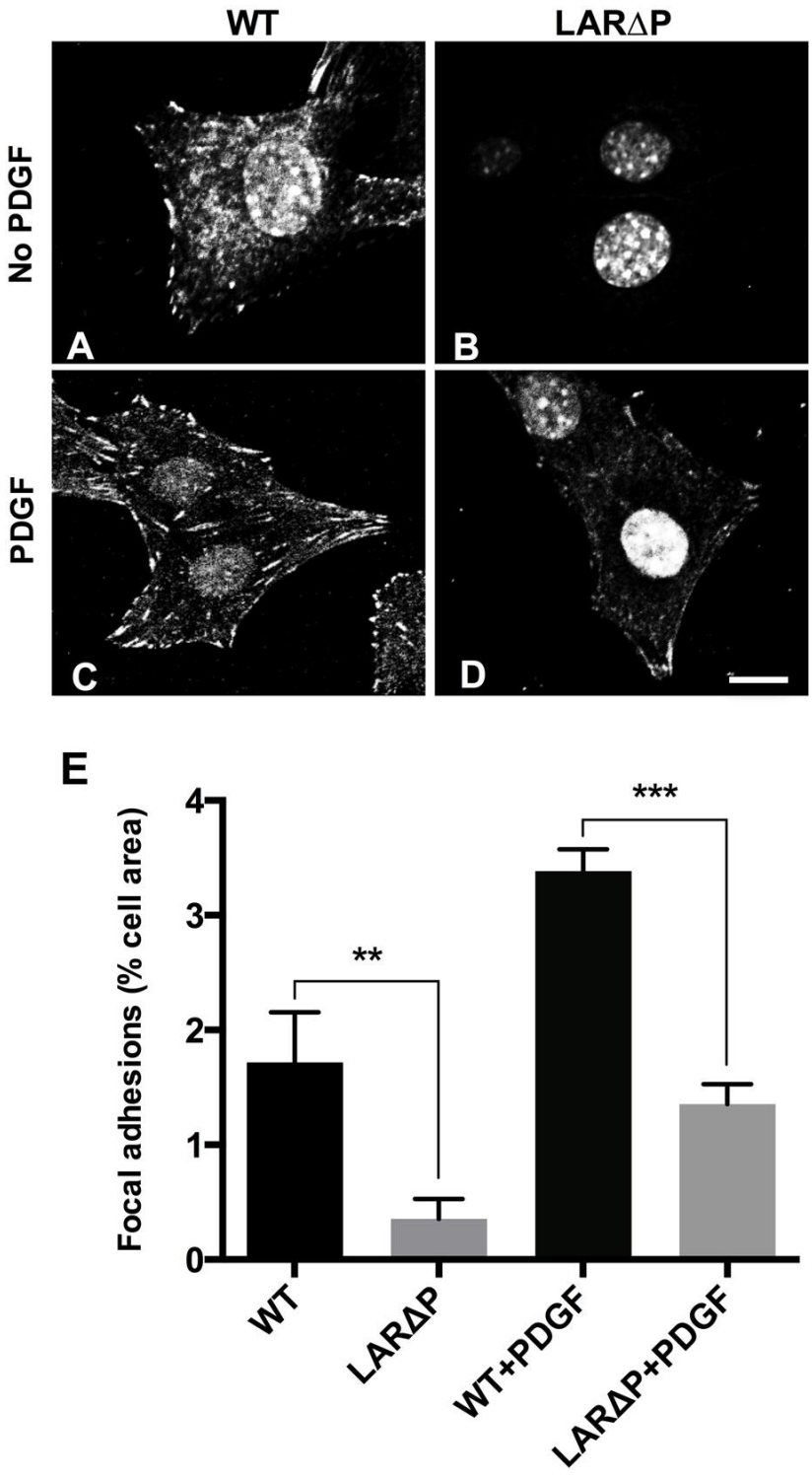

Figure 1: **Loss of LAR phosphatase activity is associated with decreased adhesion complex formation.** (A-D) WT and LARΔP MEFs were serum-starved overnight before being cultured in the presence or absence of 20ng mL^{-1} PDGF BB for 14

minutes. Fixed and permeabilised cells were stained using an antibody against phosphorylated paxillin Tyr^{118} to visualise adhesion complexes. Nuclei were visualised using DAPI. Scale bar represents 20 μm. (E) The cell area occupied by adhesion complexes was calculated using ImageJ and expressed as a percentage of the total cell area. Data presented in E are from 3 separate experiments with 4-5 representative cells analysed per experiment. Statistical significance was calculated by first normalising the data by arcsine transformation, followed by a one-way ANOVA with post hoc Tukey's test (** $p<0.01$; *** $p<0.001$).

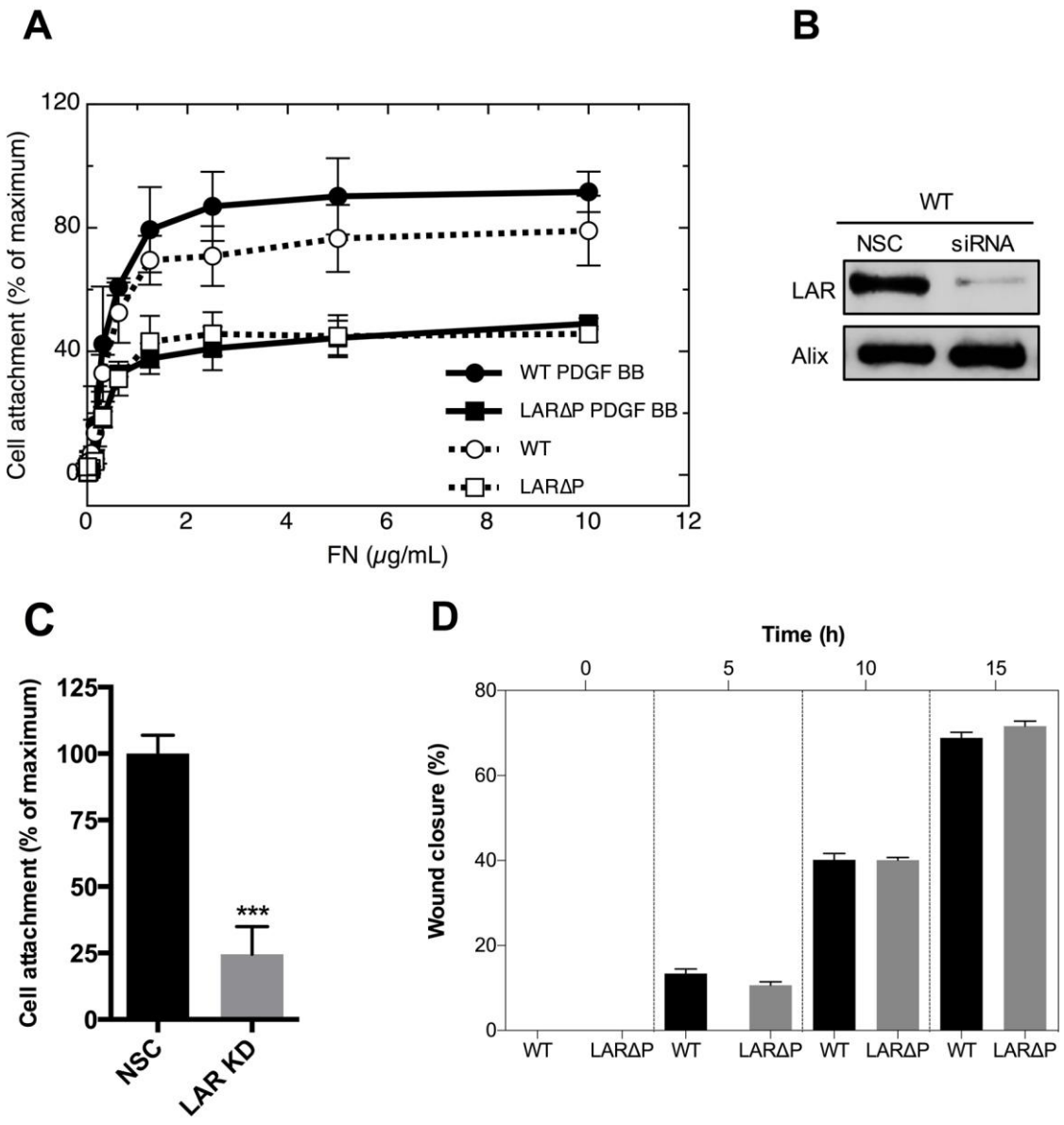

Figure 2: LAR phosphatase is required for optimal cell adhesion to fibronectin. (A) Serum-starved WT and LARΔP MEFs were allowed to attach to fibronectin for 30 minutes in the presence or absence of 20 ng mL^{-1} PDGF BB. Non-adherent cells were discarded and percentage cell attachment to increasing concentrations of fibronectin calculated. Data are the mean and standard error of 3 separate experiments. (B, C) WT MEFs were transiently transfected with siRNA oligos targeted against LAR or with control non-silencing oligos (NSC). 48 hours after transfection cells were serum-starved overnight and either (B) cell lysates were prepared for immunoblotting with antibodies against LAR and Alix (loading control) or (C) cells were allowed to attach

to 10 μg mL^{-1} fibronectin for 30 minutes and percentage attachment calculated as described in A. Statistical significance was calculated by first normalising the data by arcsine transformation, followed by a Student's T test. (D) WT and LARΔP MEFs were wounded using an automated scratch maker and images of 5 separate wounds taken at 0, 5, 10 and 15 hours post wounding for each cell line using the IncuCyte ZOOM imaging system. Data are the mean and standard error of 3 separate experiments.

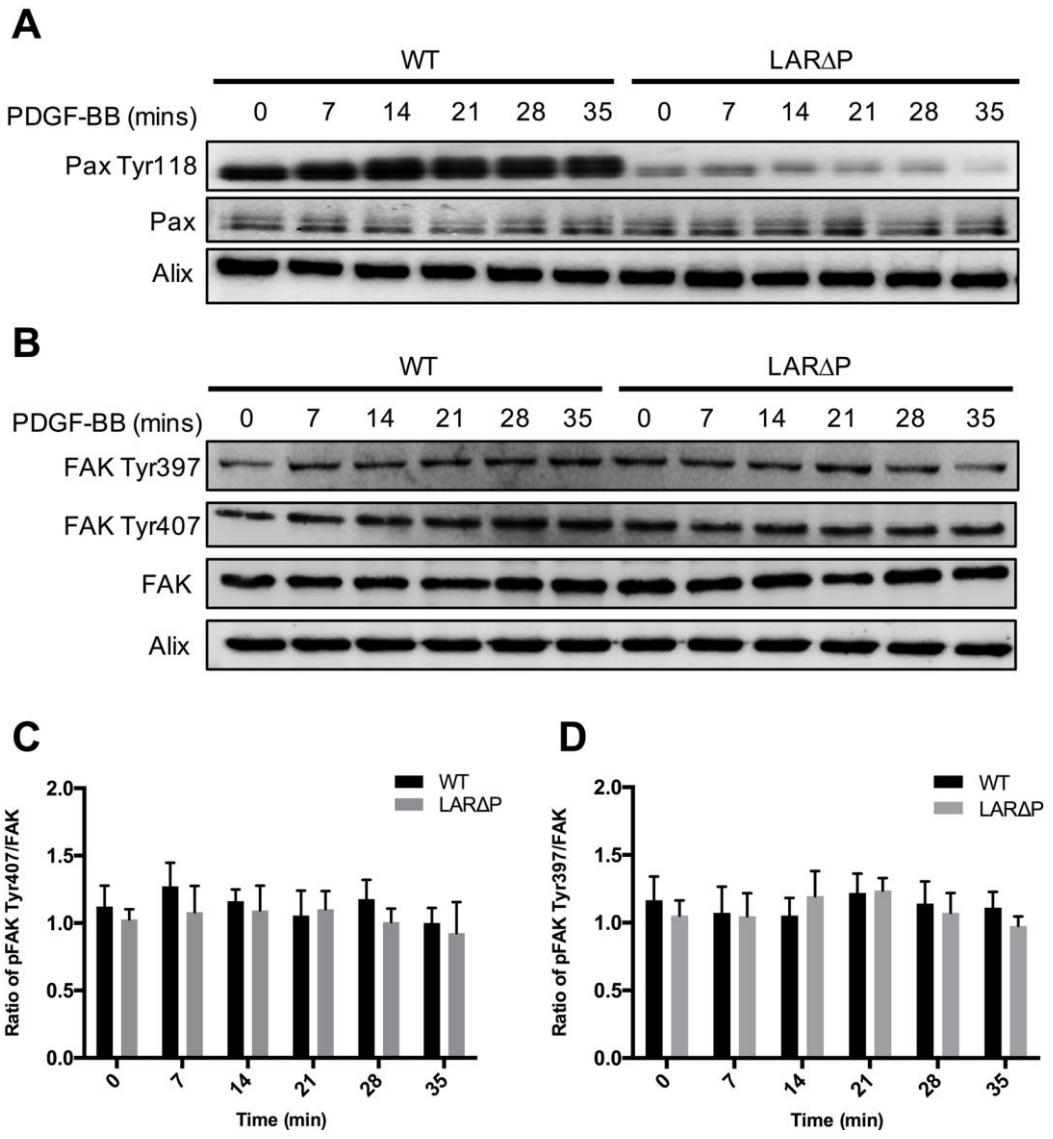

Figure 3: **FAK tyrosine phosphorylation is unaffected by loss of LAR activity**. (A, B) Serum-starved WT and LARΔP MEFs were stimulated with 20 ng mL^{-1} PDGF BB for increasing periods of time before protein lysates were prepared and immunoblotted using antibodies against (A) paxillin (Pax) and phospho-paxillin Tyr^{118} or (B) FAK, phospho-FAK Tyr^{397} and phospho-FAK Tyr^{407}. In both (A) and (B) expression of Alix was used as a loading control and results are representative of three independent experiments. (C, D) The ratio of either phosphorylated FAK Tyr^{407} (C) or phosphorylated FAK Tyr^{397} (D) to total FAK was calculated using densitometric analysis of immunoblots. Data are the mean and standard error from 3 separate experiments.

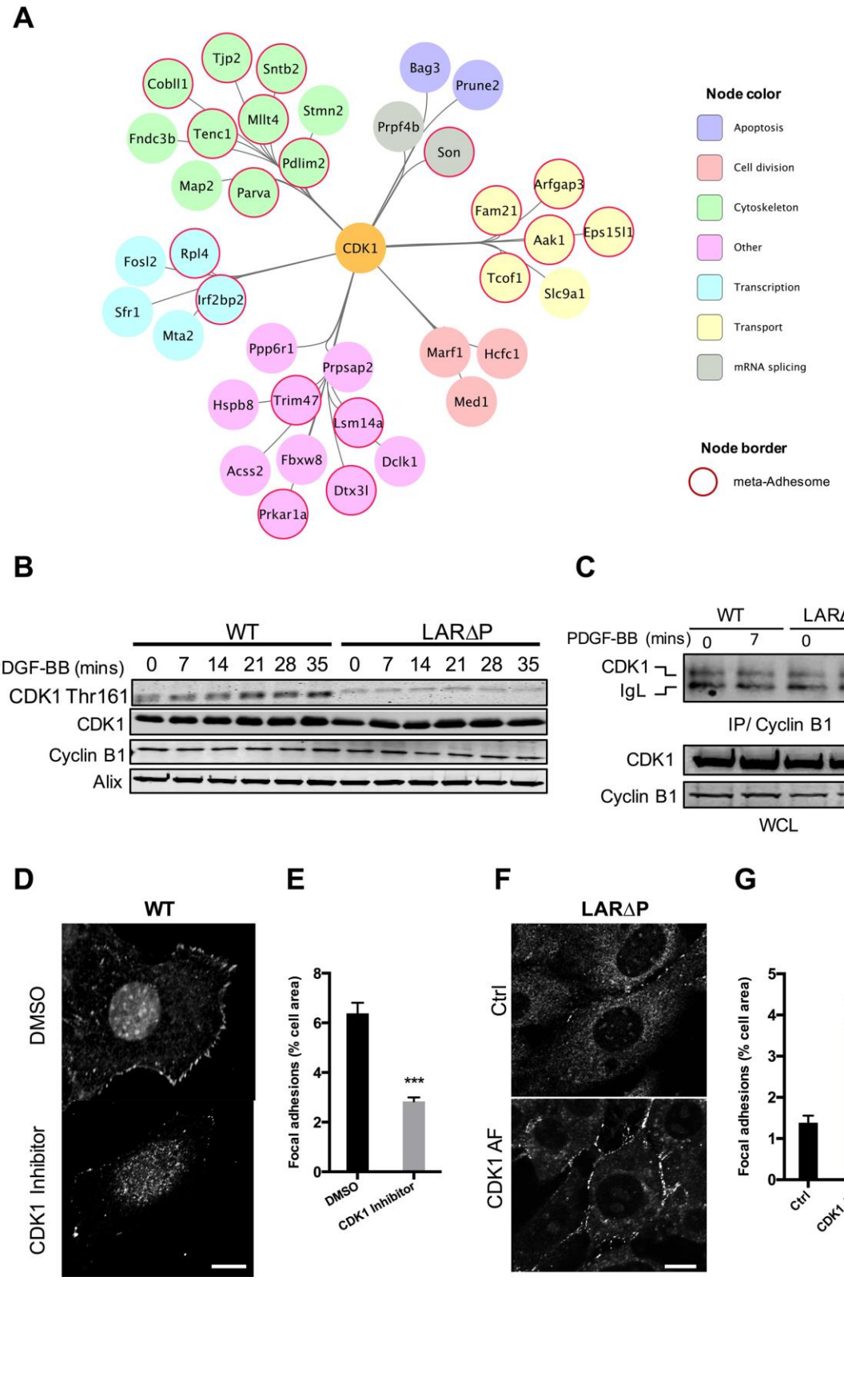

Figure 4: **CDK1 functions downstream of LAR to regulate focal adhesion complex formation.** (A) CDK1 substrates were analysed using the Group-based Prediction System (GPS). Of 270 phosphopeptides, 38 were identified as potential CDK1 substrates. Protein-protein interaction network analyses were performed using Cytoscape. Nodes are clustered according to functional group and nodes identified as being part of the meta-adhesome are highlighted with a red border. (B) Serum-starved WT and LARΔP MEFs were stimulated with 20 ng mL^{-1} PDGF BB for increasing periods of time before protein lysates were prepared and immunoblotted using antibodies against CDK1, CDK1 phospho-Thr^{161}, cyclin B1 and Alix (loading control). Results are representative of three independent experiments. (C) Serum-starved WT and LARΔP MEFs were stimulated with 20 ng mL^{-1} PDGF BB for 7 min, Cyclin B1 immunoprecipitated and immunoblotted with CDK1 or Cyclin B1 (D) WT MEFs were treated with a CDK1 inhibitor (RO-3306) or DMSO (carrier control) for 60 minutes before being stimulated with 20 ng ml^{-1} PDGF-BB for 14 minutes, fixed and stained with a phospho-paxillin (Y^{118}) antibody to visualise focal adhesions. Scale bar represents 20 μm. (E) (G) The cell area occupied by adhesion complexes was calculated using ImageJ and expressed as a percentage of the total cell area. Data are the mean and standard error of 3 independent experiments with a minimum of 4 cells analysed per experiment. Statistical significance was calculated by first normalising the data by arcsine transformation, followed by a one-way ANOVA with post hoc Tukey's test (*** $p<0.001$). (F) LARΔP cells were transfected with constitutively active form of CDK1 (CDK1-AF) before being stimulated with 20 ng mL^{-1} PDGF-BB for 14 minutes, fixed and stained with a phospho-paxillin (Y^{118}) antibody to visualise focal adhesions.

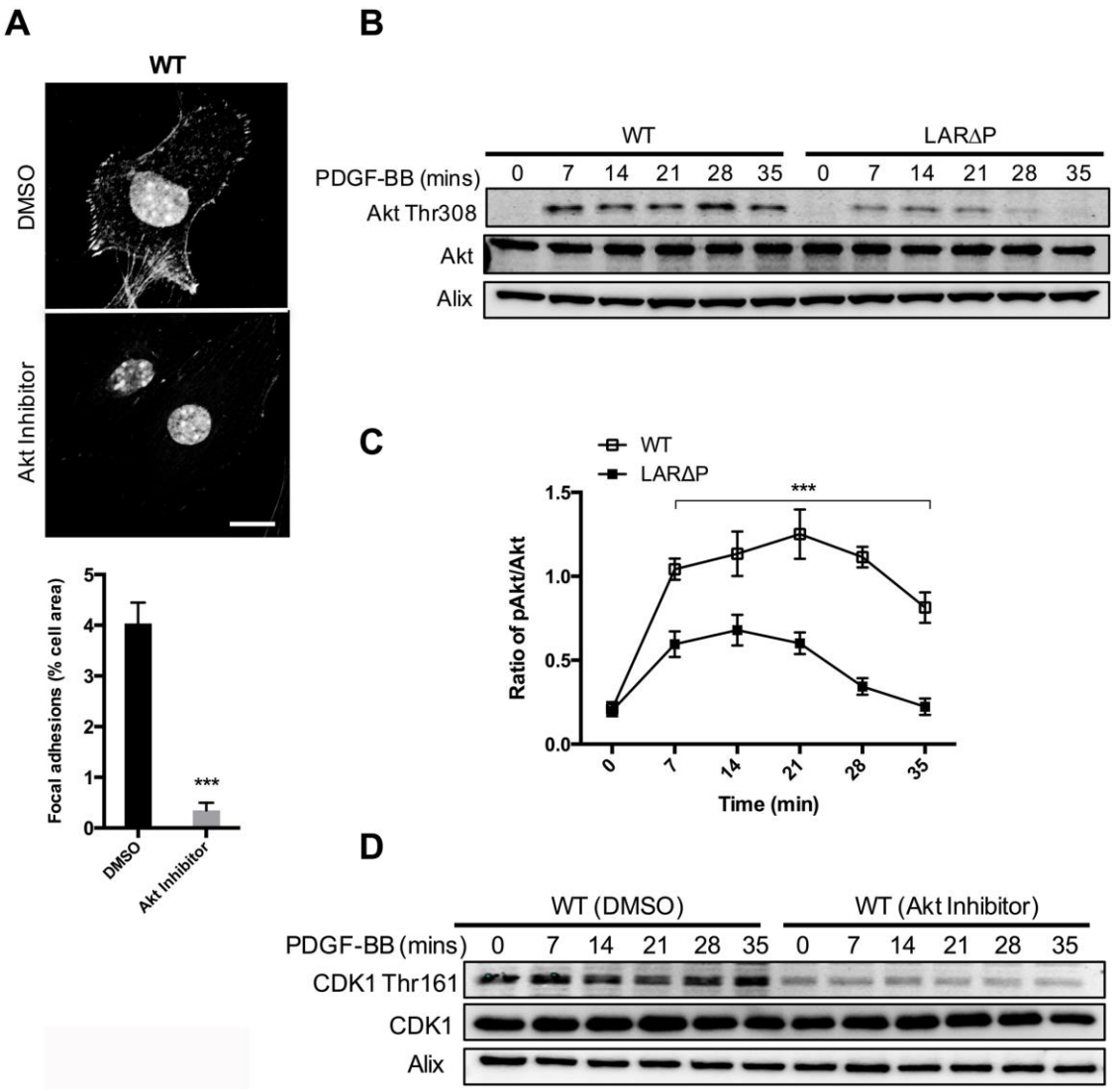

Figure 5: **Akt is required for LAR-mediated regulation of CDK1 and adhesion complex formation**. (A) WT MEFs cultured in normal growth medium were treated with an Akt inhibitor (InSolution Akt Inhibitor VIII) or DMSO (carrier control) overnight before being stimulated with 20 ng mL^{-1} PDGF-BB for 14 minutes, fixed and stained with a phospho-paxillin (Y^{118}) antibody to visualise focal adhesions. The cell area occupied by adhesion complexes was calculated using ImageJ and expressed as a percentage of the total cell area. Data are the mean and standard error of 3 independent experiments with a minimum of 4 representative cells analysed per experiment. Statistical significance was calculated by first normalising the data by arcsine transformation, followed by a two-way ANOVA with Sidak's test (*** p <

0.001). Scale bar represents 20 μm. (B, C) Serum-starved WT and LARΔP MEFs were stimulated with 20 ng mL^{-1} PDGF BB for increasing periods of time before protein lysates were prepared and immunoblotted using antibodies against Akt, phospho-Akt Thr^{308} and Alix (loading control). (C) Densitometric analysis of data from 3 independent experiments using ImageJ. (D) Serum-starved WT MEFs were stimulated with 20 ng mL^{-1} PDGF BB for increasing periods of time in the presence of InSolution Akt Inhibitor VIII (Akt inhibitor) or DMSO (carrier control) before protein lysates were prepared and immunoblotted using antibodies against CDK1, phospho-CDK1 Thr^{161} and Alix (loading control).

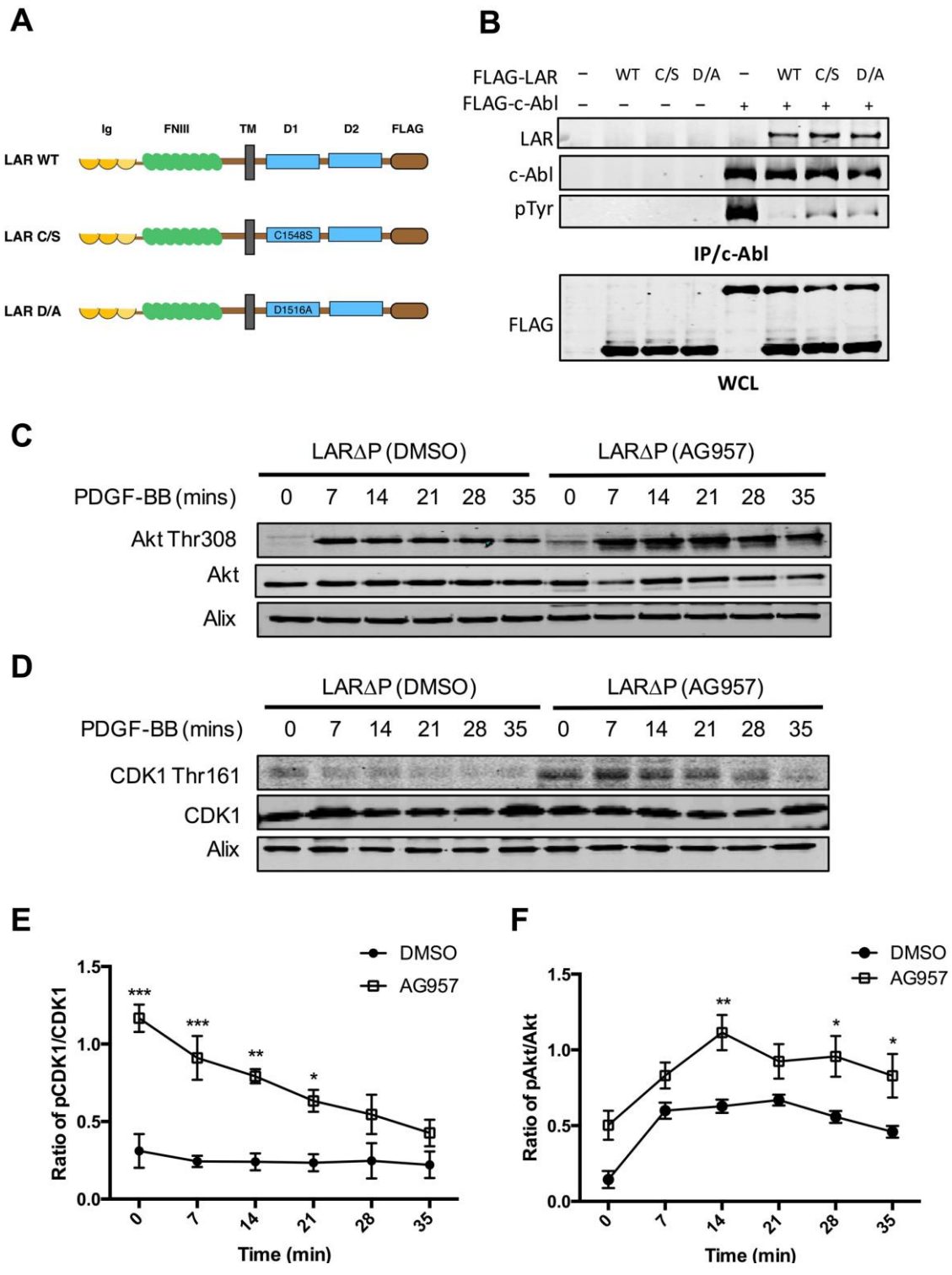

Figure 6: Inhibition of c-Abl restores phosphorylation of CDK Thr^{161} in cells lacking LAR phosphatase activity. (A) Schematic structure of LAR substrate-trapping mutants. Full-length FLAG-tagged wild-type LAR (WT), substrate-trapping

mutants C/S (Cys1548 in D1 domain replaced by Ser1548) and D/A (Asp1516 in D1 domain replaced by Ala1516). Ig, immunoglobulin-like domain; FNIII, fibronectin type III repeat domain; TM, transmembrane; D1, protein tyrosine phosphatase domain 1; D2, protein tyrosine phosphatase domain 2. (B) FLAG-tagged wild-type LAR (WT) and substrate-trapping mutants of LAR along with WT c-Abl were either expressed alone or co-expressed in 293T cells. c-Abl was immunoprecipitated using a c-Abl-specific antibody and immunoprecipitates were immunoblotted with either LAR, c-Abl or total phosphotyrosine (PY99) antibodies. To control for expression levels, whole cell lysates (WCL) were immunoblotted with FLAG antibody. (C, D) Serum-starved LARΔP cells were stimulated with 20 ng mL^{-1} PDGF BB for increasing periods of time in the presence of a c-Abl inhibitor (AG957) or DMSO (carrier control) before protein lysates were prepared and immunoblotted using antibodies against Akt, phospho-Akt Thr^{308} and Alix (loading control) (C) or CDK1, phospho-CDK1 Thr^{161} and Alix (loading control) (D). For both CDK1 (E) and Akt (F) blots from 3 independent experiments were analysed using ImageJ and statistical significance was calculated by first normalising the data by arcsine transformation, followed by a two-way ANOVA with Sidak's test (** $p < 0.01$; * $p < 0.05$).

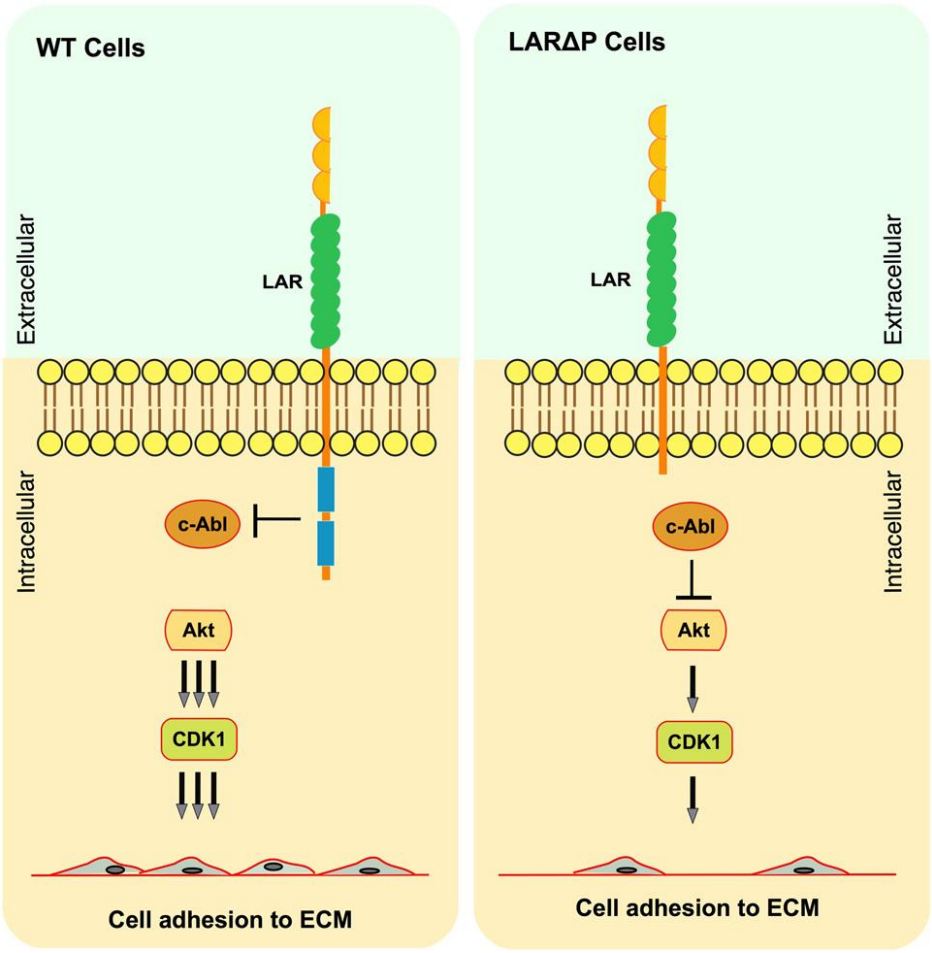

Figure 7: Model showing how LAR phosphatase regulates cell adhesion via CDK1

In wild type MEFs, LAR dephosphorylates and inactivates c-Abl resulting in enhanced Akt and CDK1 signalling, leading to increased cell adhesion to extracellular matrix (ECM). In the absence of LAR phosphatase activity, c-Abl inhibits Akt, resulting in decreased CDK1 activity and decreased cell adhesion to extracellular matrix.