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RESEARCH ARTICLE

LAR protein tyrosine phosphatase regulates focal adhesions through CDK1

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ABSTRACT

Focal adhesions are complex multi-molecular structures that link the actin cytoskeleton to the extracellular matrix through integrin adhesion receptors and play a key role in regulation of many cellular functions. LAR (also known as PTPRF) 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 through c-Abl and Akt family proteins. In summary, we have identified a new role for a receptor protein tyrosine phosphatase in regulating CDK1 activity and hence cell adhesion to the extracellular matrix.

KEY WORDS: CDK1, Cell adhesion, Focal adhesions, LAR phosphatase, PTPRF

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 through 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 through 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, also known as PTK2) 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, although 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 new signalling pathway in which LAR phosphatase signals through CDK1 and c-Abl and Akt family proteins 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 MEFs expressing either WT LAR 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 min prior to fixation

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and staining using an antibody against phosphorylated paxillin (Tyr[118]) (hereafter phospho-paxillin) 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 (Fig. 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 (Fig. 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 20 ng ml^{-1} PDGF-BB for 30 min. 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 10 µg ml^{-1} (Fig. 2A). Treatment with PDGF-BB did not significantly affect attachment of either WT or LARΔP cells (Fig. 2A). To confirm these data, we used RNA interference (RNAi) to knockdown expression of LAR in WT cells (Fig. 2B). Small interfering RNA (siRNA)-mediated reduction of LAR expression in WT cells was accompanied by a significant decrease in cell attachment to 10 µg ml^{-1} fibronectin when compared to WT cells transfected with a non-silencing control (NSC) oligonucleotide (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 MEFs but has no effect on cell migration.

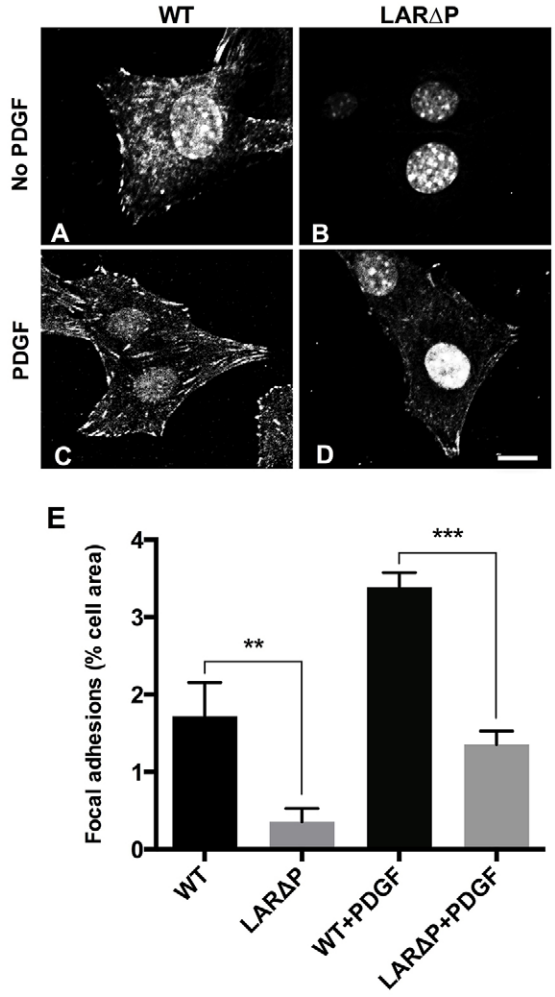

Fig. 1. Loss of LAR phosphatase activity is associated with decreased adhesion complex formation. (A–D) WT and LARΔP MEFs were serum-starved overnight before being cultured in the presence or absence of 20 ng ml^{-1} PDGF-BB for 14 min. Fixed and permeabilised cells were stained using an antibody against phosphorylated paxillin Tyr[118] to visualise adhesion complexes. Nuclei were visualised using DAPI. Scale bar: 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 mean±s.e.m. from three separate experiments with four or five 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).

Regulation of adhesion complexes by LAR is independent of FAK activity

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-BB (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 (Fig. 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; http://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 CDK1 phosphorylation motifs were identified with significantly reduced phosphorylation in LARΔP cells. Of these, 19 (50%) formed part of the recently characterised meta-adhesome (Horton et al., 2015) (Fig. 4A).

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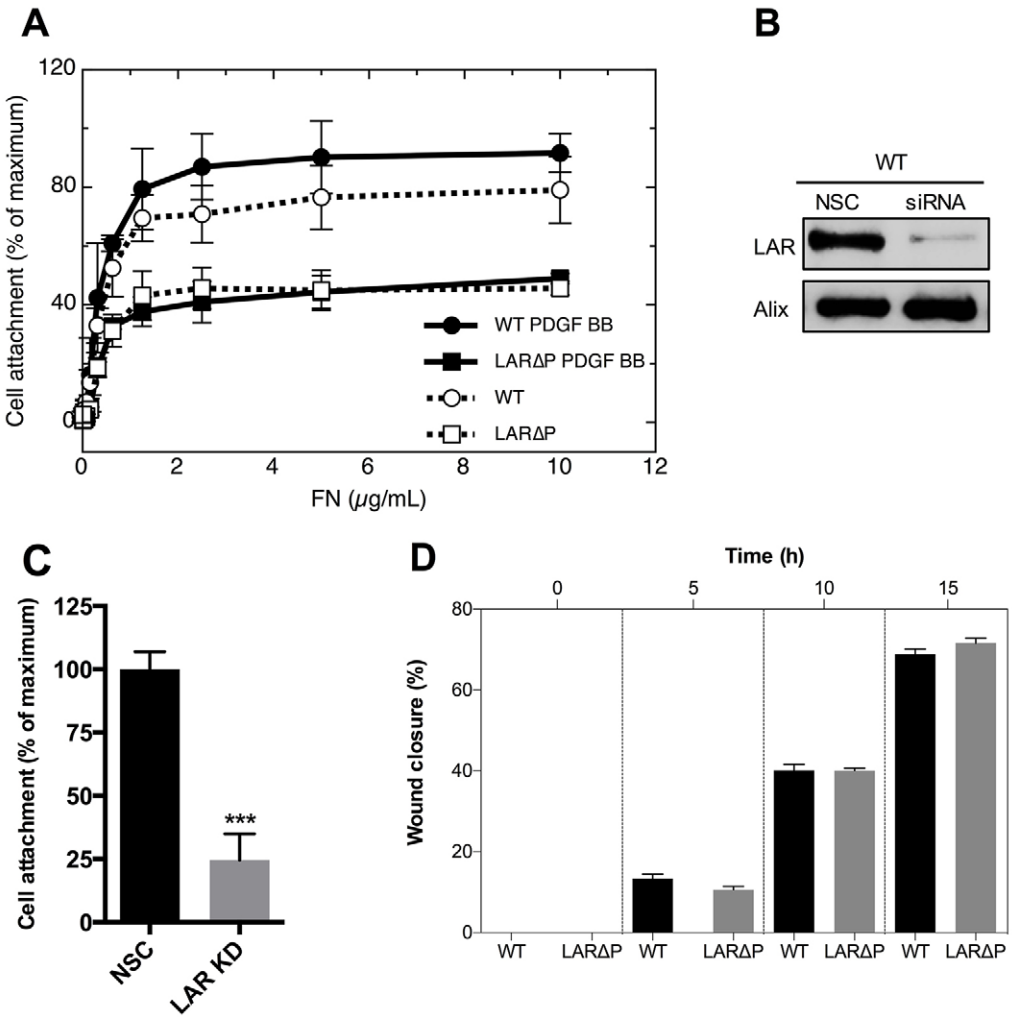

Fig. 2. LAR phosphatase is required for optimal cell adhesion to fibronectin. (A) Serum-starved WT and LARΔP MEFs were allowed to attach to fibronectin for 30 min in the presence or absence of 20 ng ml^{-1} PDGF-BB. Non-adherent cells were discarded and the percentage cell attachment to increasing concentrations of fibronectin calculated. Data are the mean±s.e.m. of three separate experiments. (B,C) WT MEFs were transiently transfected with siRNA oligonucleotides targeted against LAR or with control non-silencing oligonucleotides (NSC). At 48 h 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 min and the percentage attachment was calculated as described in A. Statistical significance was calculated by first normalising the data by arcsine transformation, followed by a Student's *t*-test (***P<0.001). (D) WT and LARΔP MEFs were wounded using an automated scratch maker and images of five separate wounds were taken at 0, 5, 10 and 15 h post wounding for each cell line using the IncuCyte ZOOM imaging system. Data are the mean±s.e.m. of three separate experiments.

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-BB (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 that 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 (Fig. 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 (Fig. 4F,G). These data establish CDK1 as a protein kinase acting downstream of LAR to regulate adhesion complex formation.

LAR signals to CDK1 through Akt family proteins

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 proteins might 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 showing 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 (Fig. 5B,C) and significantly reduced phosphorylation of Thr^{308} in LARΔP cells at all time points (Fig. 5B,C). Treatment of WT cells with the Akt

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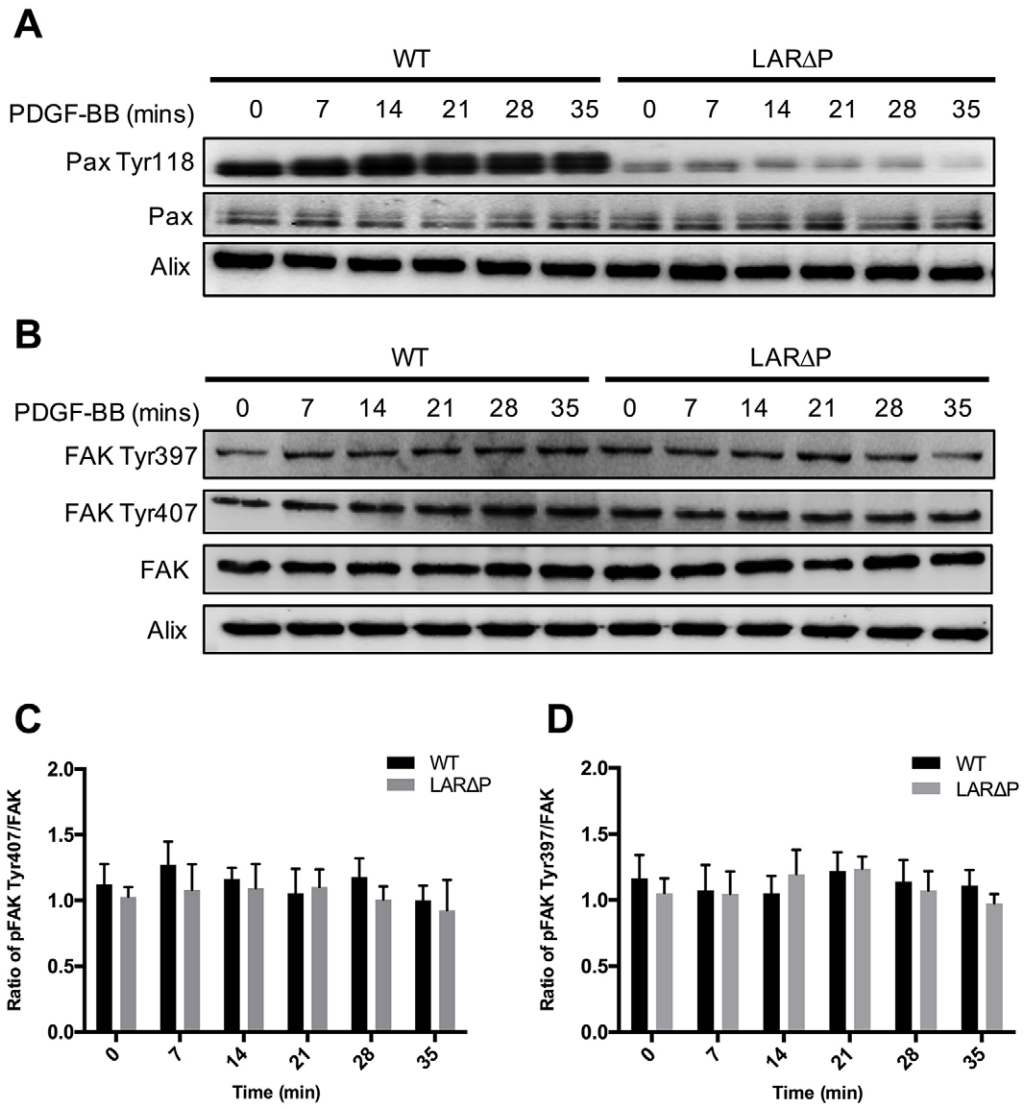

Fig. 3. FAK tyrosine phosphorylation is unaffected by loss of LAR activity. (A,B) Serum-starved WT and LARΔP MEFs were stimulated with 20 ng ml^{-1} PDGF-BB for increasing periods of time before protein lysates were prepared and immunoblotted using antibodies against (A) paxillin (Pax) and phospho-paxillin 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± s.e.m. from three separate experiments.

inhibitor, in the absence or presence of PDGF-BB, 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 new role of LAR in regulation of cell adhesion through 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 protein family in LARΔP cells and Abl has been identified as a potential substrate for the *Drosophila* LAR ortholog (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} (Fig. 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 (Um and Ko, 2013). *Drosophila* LAR localises to synaptic junctions and regulates synaptic growth and axonal guidance through regulation of the actin cytoskeleton through 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 *Drosophila* LAR 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

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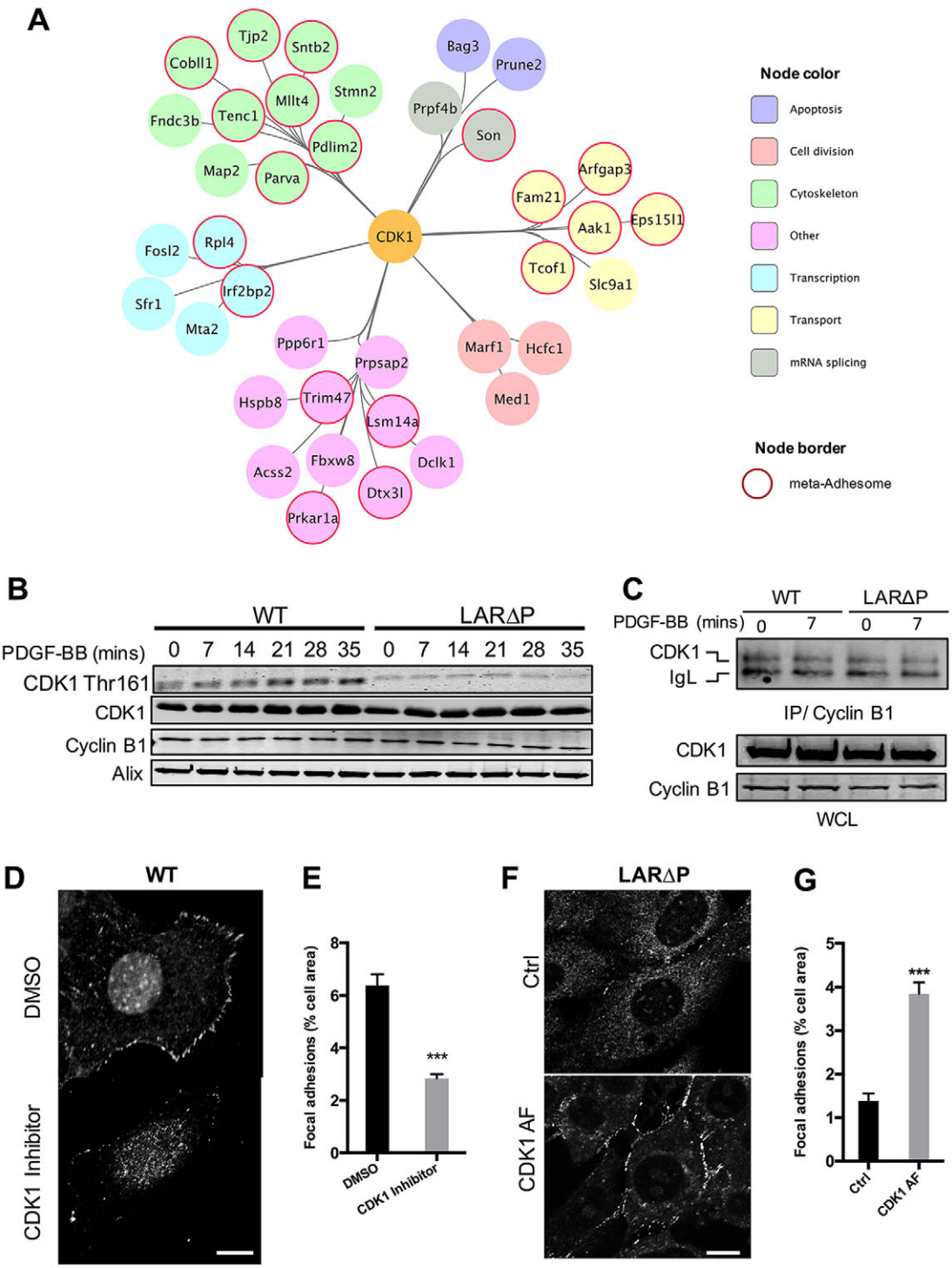

Fig. 4. CDK1 functions downstream of LAR to regulate focal adhesion complex formation. (A) CDK1 substrates were analysed using the Group-based Prediction System (GPS). Of 270 phosphopeptides, 38 were identified as potential CDK1 substrates. Protein–protein interaction network analyses were performed 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 was immunoprecipitated (IP) and precipitates were immunoblotted for CDK1 or cyclin B1. (D–G) (D) WT MEFs were treated with a CDK1 inhibitor (RO-3306) or DMSO (carrier control) for 60 min before being stimulated with 20 ng ml^{-1} PDGF-BB for 14 min, fixed and stained with an anti-phospho-paxillin (Y^{118}) antibody to visualise focal adhesions. (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 min, fixed and stained with a phospho-paxillin (Y^{118}) antibody to visualise focal adhesions. Scale bars: 20 µm. (E,G) The cell area occupied by adhesion complexes in experiments shown in D and F was calculated using ImageJ and expressed as a percentage of the total cell area. Data are the mean±s.e.m. of three independent experiments with a minimum of four 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).

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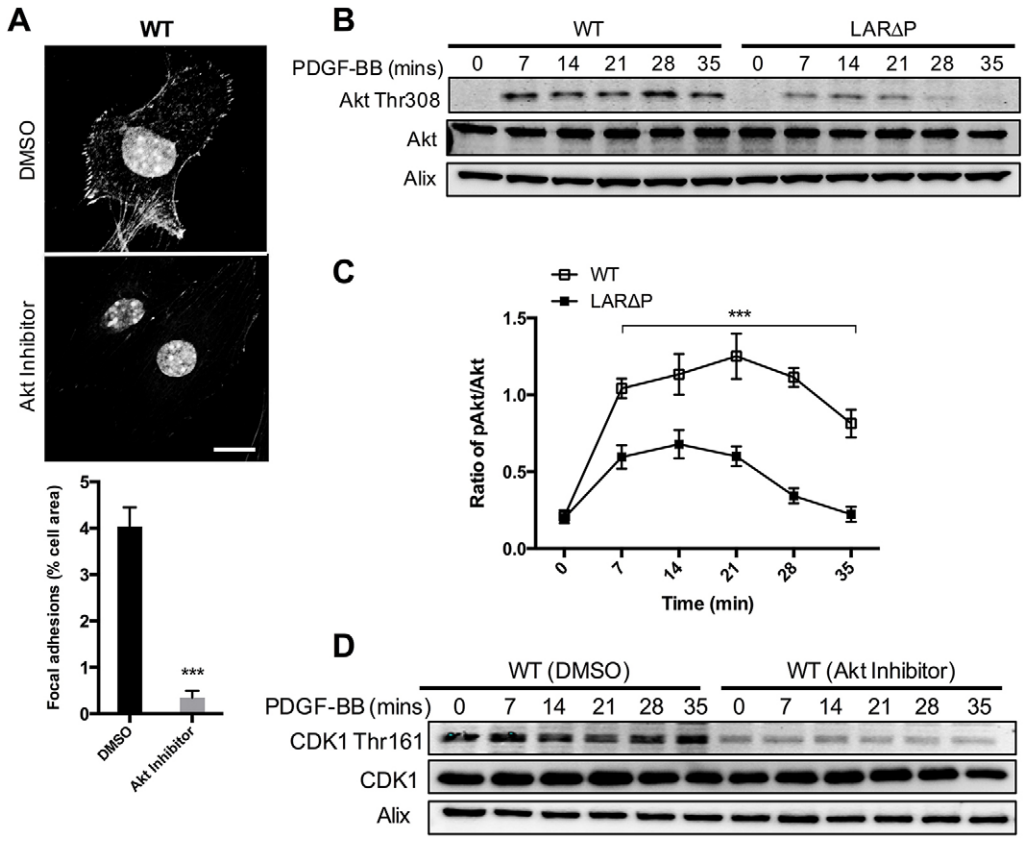

Fig. 5. Akt is required for LAR-mediated regulation of CDK1 and adhesion complex formation. (A) WT MEFs cultured in normal growth medium were treated with an Akt inhibitor (InSolution Akt Inhibitor VIII) or DMSO (carrier control) overnight before being stimulated with 20 ng ml^{-1} PDGF-BB for 14 min, fixed and stained with an anti-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±s.e.m. of three independent experiments with a minimum of four 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: 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 three independent experiments using ImageJ. Results are mean±s.e.m. (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).

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 might regulate 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 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 through 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 through 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 through 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

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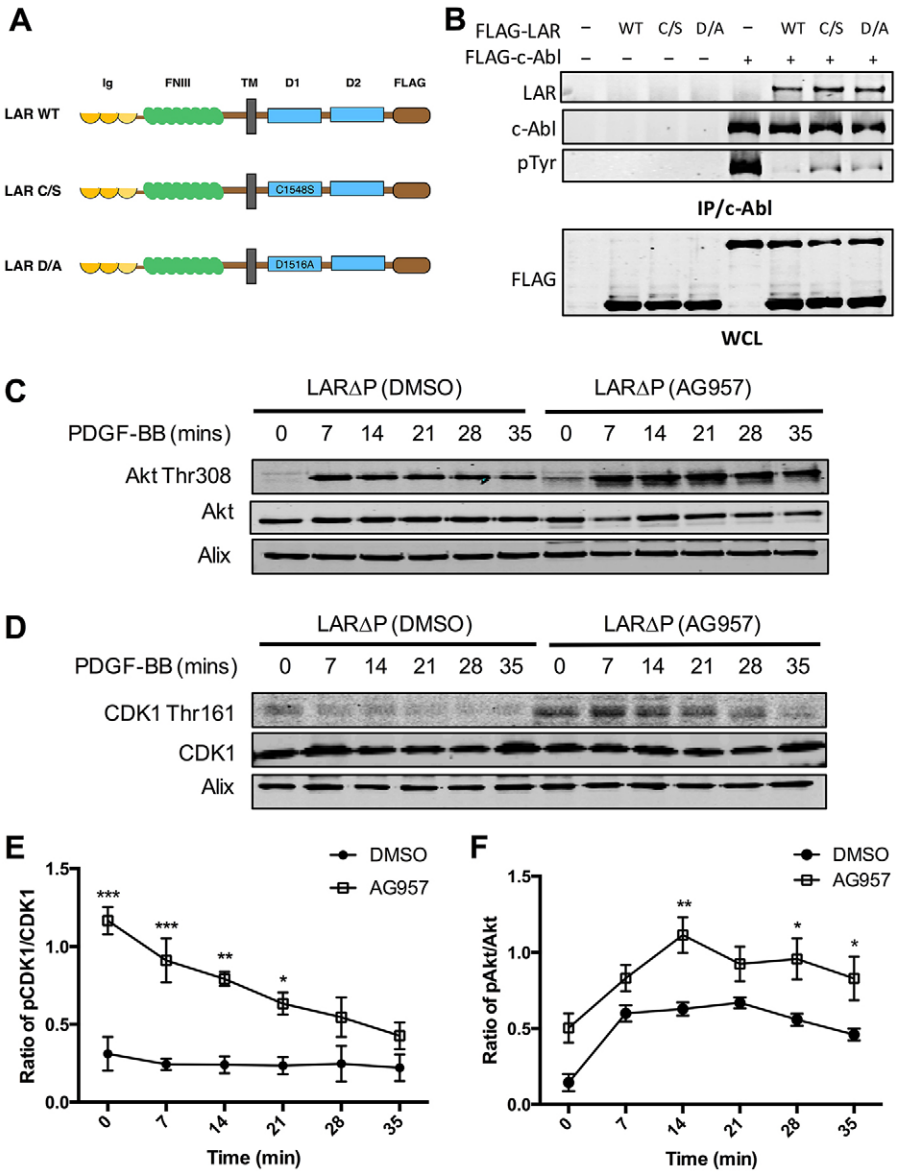

Fig. 6. Inhibition of c-Abl restores phosphorylation of CDK Thr^{161} in cells lacking LAR phosphatase activity. (A) Schematic structure of LAR substrate-trapping mutants. Full-length FLAG-tagged wild-type LAR (WT), and substrate-trapping mutants C/S (Cys1548 in D1 domain replaced by Ser1548) and D/A (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 WT LAR 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 antibodies against LAR, c-Abl or total phosphotyrosine (PY99) antibodies. To control for expression levels, whole cell lysates (WCL) were immunoblotted with anti-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 three 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<0.05, **P<0.01).

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 observed that LAR can regulate adhesion complex formation and cell adhesion independently of PDGF stimulation (Figs 1 and 2). One surprising observation was that although inhibition of c-Abl restored phosphorylation of CDK1 Thr^{161} in unstimulated 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 although PDGF signalling can act to modulate LAR-dependent effects on adhesion, LAR can also regulate CDK1 and cell adhesion independently of PDGF signalling (Fig. 7).

We have observed that LAR positively regulates attachment to fibronectin and, although 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, although 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 did not affect the 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 plays 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 new mechanism whereby a membrane-associated protein tyrosine phosphatase positively

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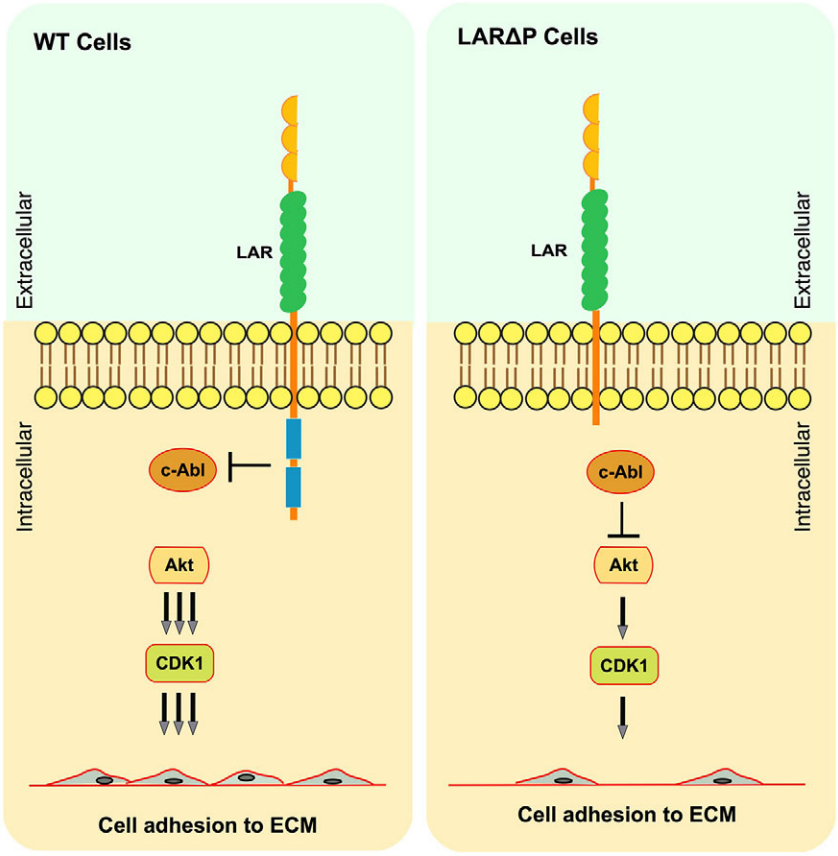

Fig. 7. Model showing how LAR phosphatase regulates cell adhesion through CDK1. In wild-type MEFs, LAR dephosphorylates and inactivates c-Abl resulting in enhanced Akt and CDK1 signalling, leading to increased cell adhesion to extracellular matrix (ECM). In the absence of LAR phosphatase activity, c-Abl inhibits Akt, resulting in decreased CDK1 activity and decreased cell adhesion to extracellular matrix.

regulates adhesion complex formation and cell adhesion through a signalling pathway involving c-Abl, Akt and CDK1.

MATERIALS AND METHODS

Antibodies

For immunoblotting, antibodies were diluted 1:1000 and, for immunocytochemistry, antibodies were diluted 1:100. Antibodies against Akt (#9272), phospho-Akt Thr^{308} (#9275; antibody recognises equivalent epitopes on Akt1, Akt2 and Akt3), cyclin B1 (#4135), paxillin (#2542), phospho-paxillin Tyr^{118} (#2541), phospho-CDK1 Thr^{161} (#9114) and c-Abl (#2862) were obtained from Cell Signaling Technology (Danvers, MA). Antibodies against CDK1 (#sc-54), phospho-FAK Tyr^{407} (#sc-16664) and PY99 phosphotyrosine (#SC-7020) were obtained from Santa Cruz Biotechnology (Dallas, TX). Antibodies against 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). Anti-LAR antibody (#73-193) was purchased from Neuromab (UC Davis, CA). The anti-Alix antibody was a gift from Carl Hendrik Heldin (Karolinska Institute, Sweden) (Lennartsson et al., 2006). Alexa-Fluor-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 Dulbecco's modified Eagle's medium (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 MEFs were kind gifts from Wiljan Hendriks (Radboud University Medical Centre, The 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 h 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 visualised 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 h at 37°C followed by addition of 100 µl of isopropanol for 15 min 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 1 h or 10 µM InSolution Akt Inhibitor VIII or in DMSO overnight. Cells

were stimulated with 20 ng ml^{-1} PDGF-BB for 14 min and then incubated on ice for 5 min. Fixed and permeabilised cells were immunostained as previously described (Lock and Hotchin, 2009). Cells were visualised using TIRF Nikon A1R confocal microscope with NIS-Elements Software and images were analysed using ImageJ 1.48. To quantify the adhesion complex area, background-subtracted images of a minimum of four 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 the manufacturer's instructions. In the LAR phosphatase trap experiment, 293T cells were transiently transfected using Lipofectamine 2000 with cDNA constructs expressing either WT c-Abl (WT c-Abl) (Johannessen et al., 2010) or WT LAR, LAR-C1538S (LAR C/S) or LAR-D1506A (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 minimise false positives, the highest threshold was applied. The 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 et al., 2009).

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Competing interests

J.K.H. is an Editor for Journal of Cell Science. There are no other competing interests.

Author contributions

A.R.S., M.G.T., C.H., J.K.H., D.L.C. and N.A.H. conceived and designed the experiments. A.R.S., T.R.P., A.R.C. and D.L.C. performed the experiments and analysed the data. A.R.S., T.R.P., M.G.T., J.K.H., D.L.C. and N.A.H. wrote the manuscript.

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Data availability

The mass spectrometry proteomics data, including the 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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