Received: 11 June 2019 | Revised: 27 January 2020 | Accepted: 11 February 2020

DOI: 10.1096/fj.201901443R

RESEARCH ARTICLE

THE FASEB JOURNAL

LIM and SH3 protein 1 (LASP-1): A novel link between the slit membrane and actin cytoskeleton dynamics in podocytes

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Funding information
Innovative Medizinische Forschung (IMF), Grant/Award Number: KR110904; Deutsche Forschungsgemeinschaft (DFG), Grant/Award Number: GE 2158/3-1

Abstract

The foot processes of podocytes exhibit a dynamic actin cytoskeleton, which maintains their complex cell structure and antagonizes the elastic forces of the glomerular capillary. Interdigitating secondary foot processes form a highly selective filter for proteins in the kidney, the slit membrane. Knockdown of slit membrane components such as Nephrin or Neph1 and cytoskeletal adaptor proteins such as CD2AP in mice leads to breakdown of the filtration barrier with foot process effacement, proteinuria, and early death of the mice. Less is known about the crosstalk between the slit membrane-associated proteins and cytoskeletal components inside the podocyte foot processes. Our study shows that LASP-1, an actin-binding protein, is highly expressed in podocytes. Electron microscopy studies demonstrate that LASP-1 is found at the slit membrane suggesting a role in anchoring slit membrane components to the actin cytoskeleton. Live cell imaging experiments with transfected podocytes reveal that LASP-1 is either part of a highly dynamic granular complex or a static, actin cytoskeleton-bound protein. We identify CD2AP as a novel LASP-1 binding partner that regulates its association with the actin cytoskeleton. Activation of the renin-angiotensin-aldosterone system, which is crucial for podocyte function, leads to phosphorylation and altered localization of LASP-1. In vivo studies using the Drosophila nephrocyte model indicate that Lasp is necessary for the slit membrane integrity and functional filtration.

KEYWORDS

actin cytoskeleton, angiotensin, CD2AP, nephrocyte, slit membrane

Abbreviations: ABL-1, Abelson tyrosine kinase 1; Actn-4, Actinin 4; ANF, atrial natriuretic factor; ANGII, angiotensin II; AT1R, angiotensin receptor subtype 1; cAMP, cyclic adenosine mono phosphate; CD2AP, CD2-associated protein; cGMP, cyclic guanosine mono phosphate; Dm, Drosophila melanogaster; dsRNA, double-stranded RNA; FSGS, focal segmental glomerulosclerosis; GAL, galactosidase; GFP, green fluorescence protein; LASP-1, LIM and SH3 protein 1; LPP, lipoma preferred partner; MEK1/2, mitogen-activated protein kinase kinase 1/2; PKA, protein kinase A; PKG, protein kinase G; PRD, proline-rich domain; pS146, phospho serine 146; RAAS, renin-angiotensin-aldosterone system; RSK, p90 ribosomal S6-kinase; SH3, src-homology 3; Sns, sticks-and-stones; UAS, upstream activating sequence; VASP, vasodilator-stimulated phosphoprotein; Y2H, yeast two hybrid; ZO-1, zona occludens protein 1.

Carolin Lepa, Annika Möller-Kerutt, Miriam Stölting, Britta George, Joachim Kremerskothen and Hermann Pavenstädt contributed equally to this study.

1 | INTRODUCTION

The glomerular ultrafiltration barrier is built up by three layers; fenestrated endothelium, basement membrane, and podocytes.[1] Podocytes are highly differentiated postmitotic cells with long foot processes that interdigitate among each other and mediate the formation of the slit membrane, a modified adherens junction that bridges the space between the foot processes.[2] To maintain their integrity, podocyte foot processes developed a well-organized cytoskeleton that combines strength and flexibility, counteracts against the elastic forces of the capillary wall and connects the slit membrane complex with the podocyte focal adhesions.[1,3] The linkage of the slit membrane proteins (for example, Nephrin, Neph1, P-cadherin, and Podocin) to actin filaments is mediated by a group of adapter proteins including zona occludens protein 1 (ZO-1), Catenin, and the CD2-associated protein (CD2AP).[2,4] In the kidney, CD2AP is expressed primarily in podocytes at the cytoplasmic site of the slit membrane where it anchors proteins such as Nephrin and Podocin to the actin cytoskeleton.[5] CD2AP-deficiency in podocytes leads to the accumulation of ubiquitinated proteins and $CD2AP^{-/-}$ mice die within 6 weeks of age by massive proteinuria and renal failure.[2,6]

LIM and SH3 protein 1 (LASP-1) was originally identified from a cDNA library of metastatic axillary lymph nodes and was shown to be overexpressed in human breast and ovarian cancer.[7] LASP-1plays an essential role in actin cytoskeleton organization due to its localization within multiple sites of dynamic actin assembly such as focal adhesions, lamellipodia, or membrane ruffles.[7] Beside this, LASP-1 directly binds to F-actin via its two nebulin-like repeats and interacts with other actin-associated proteins such as Palladin, Zyxin, vasodilator-stimulatedphosphoprotein (VASP), and lipoma preferred partner (LPP) via its carboxyterminal src-homology 3 (SH3) domain.[8,9] The LIM domain of LASP-1 with its internal zinc-finger module can mediate direct binding to DNA suggesting a role for LASP-1 in transcriptional regulation.[7,10] Inside the cell, LASP-1 shows an accumulation at the actin cytoskeleton and at stable focal adhesion sites but is also enriched at highly dynamic, vesicle-like microdomains in the cytosol.[7,10] In addition, a shuttling of LASP-1 between the cytosol and nucleus was shown indicating an impact on transcription.[11,12] LASP-1 is phosphorylated by cAMP- and cGMP-dependent protein kinases (PKA and PKG) and by Abelson tyrosine kinase 1 (ABL-1) at several phosphorylation residues within a region located between the nebulin repeats and the SH3 domain.[13] In cancer cells, phosphorylation of LASP-1 regulates its function as well as its localization (for example at focal contacts).[14,15]

In this report, we show that LASP-1 is highly expressed in mammalian podocytes and is partially localized in foot processes close to the slit membranes. Protein-protein interaction studies revealed that LASP-1 binds to CD2AP and that this interaction affects the LASP-1 association with the actin cytoskeleton. Furthermore, activation of the renin-angiotensin-aldosterone system by angiotensin II enhances the LASP-1 phosphorylation and alters its localization indicating a regulatory impact of angiotensin signaling on LASP-1 function in podocytes. Finally, using the Drosophila nephrocyte model, we could show that *in vivo* Lasp is necessary for the slit membrane integrity and filtration.

2 | MATERIALS AND METHODS

2.1 | Reagents

Culture media and cell culture antibiotics were obtained from PAA (Cölbe, Germany) and Invitrogen (Mannheim, Germany). Standard highest-grade reagents were from Sigma-Aldrich (Deisenhofen, Germany).

2.2 | Fly strains

Overexpression and transgenic RNAi studies were performed using the *UAS-Gal4* system. All flies were grown at 25°C or 29°C on standard fly food. RNAi stocks were obtained from the Vienna Drosophila Resource Center: *Control dsRNA* targeting *or83b* (100825), which is an olfactory receptor that is not expressed in nephrocytes; Lasp *dsRNA1* (47127), Lasp *dsRNA2* (47126). S*ns-Gal4* flies were a gift from Tobias Huber (UKE, Hamburg).

2.3 | Antibodies

Antibodies used for staining procedures or Western blot detection are listed in Table S1.

2.4 | Plasmids

LASP-1 constructs including truncation mutants were either subcloned or produced by PCR and cloning into the Yeast Two-Hybrid bait vector pAS2.1 (Clontech), modified pEGFP-C2 (Clontech) or a modified pGEX vector (GE Healthcare Biosciences, Freiburg, Germany). CD2AP constructs were generated by cloning human cDNA fragments into the pACTII vector (Clontech). Cloning details are available from J.K.

2.5 | Cell culture and transfection

Human immortalized podocytes (AB8), podocytes overexpressing AT1R,[16] and immortalized mouse $CD2AP^{+/+}$ and $CD2AP^{-/-}$ podocytes (generous gift from M. Schiffer) were cultivated as previously described.[17] Cells were grown in standard RPMI-1640 medium containing Penicillin/

Streptomycin, 10% FBS, and supplements at the permissive temperature of 37°C on collagen-coated glass coverslips. Transient transfection of podocytes was performed using Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

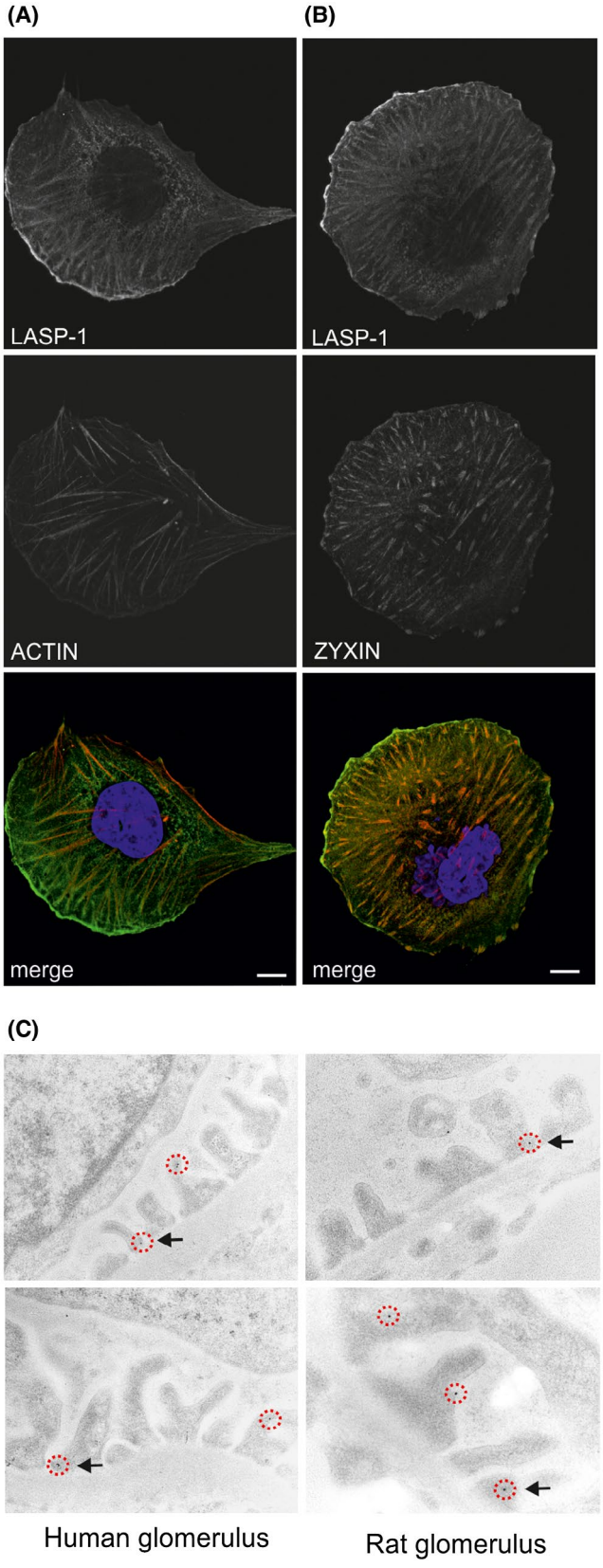


FIGURE 1 LASP-1 is an actin- and slit membrane-associated protein in podocytes. A, Human immortalized podocytes were fixed and stained for actin and LASP-1. The merged picture reveals that LASP-1 localizes predominantly along actin stress fibers and at focal adhesions but also shows a granular distribution in the cytosol. Nuclei (merged picture) were stained with DAPI. Scale bar represents 10 µm. B, Immunofluorescence staining of LASP-1 and its known binding partner Zyxin in cultured podocytes demonstrates a co-localization of both proteins at focal adhesions (merge). Nuclei were stained with DAPI. Scale bar represents 10 µm. C, Human and rat kidney sections were fixed and stained for LASP-1. Immunogold particles (10 nm) are partially found close to the slit membrane of podocyte foot processes (marked by arrowheads and red-dotted circles)

2.6 | Lysate preparation

Cells were either scraped directly into Laemmli sample buffer and boiled for 5 minutes or into lysis buffer (LB, 20 mM Tris-HCl (pH 7.5), 25 mM NaCl, 50 mM NaF, 15 mM $Na_4P_2O_7$, 1% TritonX-100, 1 mM EDTA, *Complete* Protease Inhibitor Mix [Roche]) and lysed by 10 passes through a 26-gauge needle. Lysates were centrifuged at 14, 000 *g* and supernatants were stored at –80°C until further use.

2.7 | Production of recombinant proteins and GST pull down

For the production of GST-LASP-1 fusion proteins, *E. coli* BL21 cells were transformed with pGEX-KG, pGEX-4T1-LASP-1, or pGEX-LASP-1ΔSH3, respectively. After IPTG-induced expression, bacteria were pelleted, resuspended in PBS containing 1% Triton-X, and the *Complete* protease inhibitor mix (Roche) and were sonicated on ice. Bacterial lysates were then centrifuged for 1 hours at 14 000 *g* (4°C) and supernatants were stored at −80°C. For GST pull-down experiments, equivalent amounts of recombinant proteins (GST, GST-LASP-1, GST-LASP-1 ΔLIM, or GST-LASP-1 ΔSH3) were immobilized on glutathione sepharose beads (GE Healthcare, Freiburg, Germany). The loaded beads were washed with LB and then incubated overnight at 4°C with cell extracts. After washing with LB, bound proteins were eluted from the beads into Laemmli sample buffer and were analyzed by Western blotting.

2.8 | Western blotting

Semidry Western blotting was performed using standard techniques. Blot membranes were incubated with primary antibodies diluted in blocking buffer containing 5% skim milk powder in PBS/Tween. After washing, the blot membranes were incubated with secondary antibodies coupled to horseradish peroxidase. Finally, the membranes were washed

and developed using the Lumi Light chemiluminescence detection kit (Roche) and an X-ray film developer.

2.9 | Immunofluorescence staining

Immortalized podocytes were fixed in 4% PFA for 20 minutes at room temperature, then treated with 50 mM NH_4Cl in 1× PBS for 10 minutes, and permeabilized with Saponin (30 µg/mL). Cells were blocked with 10% goat serum, 0.2% TX-100, 0.2% Gelatine (w/v) in 1× PBS. Primary antibody incubation was performed over night at room temperature in a humidity chamber diluted in 2% goat serum, 0.2% TX-100, 0.2% Gelatine (w/v) in 1× PBS. Cells were then incubated with the secondary antibodies (diluted in 2% goat serum, 0.2% TX-100, 0.2% Gelatine (w/v) in 1× PBS) and DAPI for 30 minutes at room temperature. F-actin was stained with Alexa Fluor 594-Phalloidin and nuclei were stained with DAPI. Coverslips were mounted with Mowiol and allowed to dry for 1 hour at 37°C.

Images were obtained using an Observer Z1 microscope with Apotome 2.0, Axiocam MRm (Zeiss), and EC Plan Apochromat 63×/1.40 Oil M27. For detecting green labeling we used the filter set 38 HE eGFP shift free (Zeiss). Filter sets 43 HE Cy3 shift free (Zeiss) was used to detect red labeling. Image files in CZI format (Zeiss Zen 2.3 blue edition software) were further processed by Zen 2.3 blue edition (Zeiss). After that, final images were mounted to complex figures by CorelDRAW Graphics suite 6× software (Adobe).

Garland cell nephrocytes were dissected from L3 larvae, fixed with 4% paraformaldehyde, and stained using a standard protocol.[18] Images of nephrocyte surface sections were obtained using the confocal microscope Leica Sp8.

2.10 | Transmission electron and immunogold microscopy

Garland cell nephrocytes of L3 larvae were dissected and fixed in 2.5% glutaraldehyde in Soerensen buffer for 3 days at 4°C. Samples were treated with osmium tetroxide for 1 hours, dehydrated in an ascending alcohol series, and infiltrated with epon using a series of mixtures of epon and the propylene oxide and pure epon in the last step. After embedding in epon and polymerization at 60°C for 36 hours, ultrathin slices of 60 nm were cut, contrasted with uranyl acetate, and lead citrate for 45 seconds. Images were obtained with a Phillips CM10 transmission electron microscope.

Immunogold electron microscopy was performed on ultrathin frozen sections of human kidney biopsies and dissected rat kidneys. In brief, tissue samples were fixed in 4% formaldehyde and 0.1% distilled glutaraldehyde (Merck, Darmstadt, Germany) in 100 mM phosphate buffer (pH 7.2) for 6 to 12 hours at 4°C. Samples were soaked in sucrose, frozen, and stored in liquid nitrogen until processing for indirect immunogold labeling, as described earlier.[19]

2.11 | Live cell imaging

Live cell imaging with human immortalized podocytes transfected with pEGFP-LASP-1 (wt), pEGFP-LASP-1ΔSH3, or pEGFP-Lasp1ΔLIM was performed 20 hours after transfection using an Observer Z1 microscope with Apotome 2.0, Axiocam MRm (Zeiss), and EC Plan Apochromat 63×/1.40 Oil M27. For detecting of EGFP-tagged fusion proteins, we used the filter set 38 HE eGFP shift free (Zeiss). Images were taken every 10 seconds for a period of 30 minutes. Image files

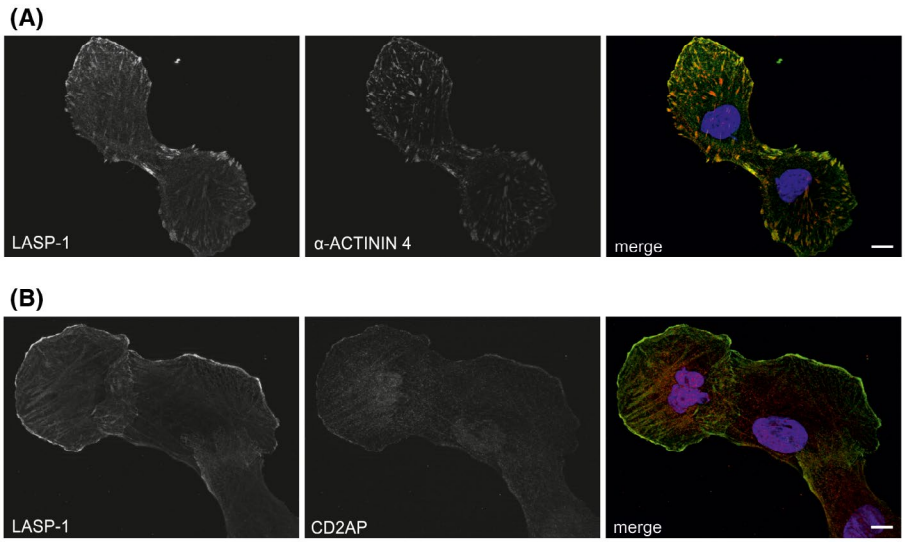


FIGURE 2 LASP-1 co-localizes with α-Actinin 4 and CD2AP in podocytes. Cultured human podocytes were fixed and co-stained for LASP-1, α-Actinin 4, or CD2AP, respectively. Merged pictures reveal a partial co-localization of LASP-1 with α-Actinin 4 (upper panel) and CD2AP (lower panel). Nuclei were stained with DAPI. Scale bars represent 10 µm

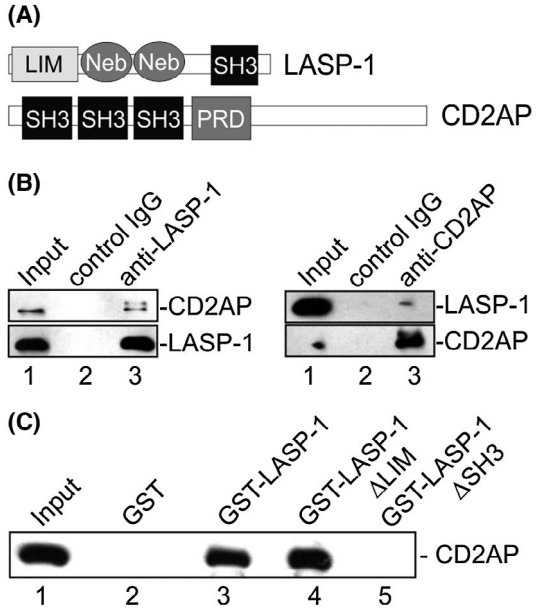


FIGURE 3 LASP-1 interacts with CD2AP. A, Domain structure of LASP-1 and CD2AP. The LASP-1 protein consists of an aminoterminal LIM domain, two internal nebulette-like repeats (Neb) and a carboxyterminal SH3 domain. CD2AP contains three aminoterminal SH3 domains and an internal proline-rich domain (PRD). B, Immunoprecipitation of LASP-1/CD2AP complexes. Control IgG and anti-LASP-1 or anti-CD2AP antibodies were used to precipitate the proteins and their binding partners from human podocyte extracts. Western blotting experiments reveal that CD2AP can be detected in the precipitate using anti-LASP-1 antibodies (left part, lane 3) and that LASP-1 is found in precipitates using anti-CD2AP antibodies (right part, lane 3). No signals were obtained with precipitates using control IgG (lane 2 in both parts). 5% of the lysates for the immunoprecipitation experiments were used as an input to detect the endogenous level of LASP-1 and CD2AP, respectively (lane 1 in both parts). C, GST pull-down experiment demonstrating the interaction between LASP-1 and CD2AP. Equal amounts of GST (lane 2), GST-LASP-1 (lane 3), and the truncation mutants GST-LASP-1ΔLIM (lane 4) as well as GST-LASP-1ΔSH3 (lane 5) were incubated with sepharose glutathione beads and lysates of cultured human podocytes (lane 1). Western blotting analysis of purified proteins demonstrated a binding between endogenous CD2AP and GST-LASP-1 (lane 3) or GST-LASP-1ΔLIM (lane 4). In contrast, GST and GST-LASP-1ΔSH3 did not interact with CD2AP (lanes 2 and 5). 5% of the lysates for the GST pull-down assays were used as an input to detect endogenous CD2AP

in CZI format (Zeiss Zen 2.3 blue edition software) were further processed by Zen 2.3 blue edition (Zeiss). For this, the first 40 pictures were transformed into the AVI file format.

2.12 | LASP-1 phosphorylation assays

PKA-dependent phosphorylation was induced by treatment of AB8 cells with 10 μM forskolin for 5 minutes. Activation of AT1R signaling was performed by incubation of AT1R-overexpressing cells[15] with AngII (100 nM, 15 minutes). Preincubation with specific inhibitors were performed for 2 hours (Losartan, 10 μM; U0126, 20 μM; BID1870, 20 μM) or 4 hours (Rp-8- Br-cAMP, 0.5 mM; Rottlerin, 5-100 μM; GF109203X, 1 μM), respectively.

TABLE 1 Summary of yeast two-hybrid (Y2H) assays about the binding between LASP-1 and CD2AP

Bait	Prey	Interaction
LASP-1	CD2AP	+
LASP-1ΔLIM	CD2AP	+
LASP-1ΔSH3	CD2AP	–
LASP-1	CD2APΔSH3	+
LASP-1	CD2AP SH3	–
LASP-1	CD2AP PRD	+

Note: Y2H experiments showed that the LASP-1 SH3 and the proline-rich domain (PRD) of CD2AP are sufficient and necessary for the direct interaction between the two proteins.

2.13 | GFP accumulation assay

Virgin females of the genotype *UAS::Dcr; sns-Gal4, ubi::ANF-GFP-GFP/CyO* were crossed with *UAS::dsRNA* males at 25°C and transferred to 29°C 48 hours after egg laying. Garland cell nephrocytes of L3 larvae were dissected and stained with wheat germ agglutinin (WGA) coupled to Alexa555 (Invitrogen) and DAPI (Invitrogen). Images of nephrocytes were obtained using a Zeiss LSM700 confocal microscope.[18] For quantification, the mean value of ANF-GFP-GFP fluorescence per nephrocyte area was measured with ImageJ. An unpaired, two-tailed Mann-Whitney U test was applied for statistical analysis. Results are presented as mean fluorescence/μm^2, normalized to control.

2.14 | Image processing and statistical analysis

Image and video exports were done with ZEN (Zeiss, Oberkochen) or ImageJ (National Institutes of Health, Bethesda, MD). Data are expressed as mean ± SE.

3 | RESULTS

3.1 | LASP-1 is highly expressed in podocytes

The actin cytoskeleton and effectors controlling its dynamics and architecture are crucial for podocyte function.[1,3] First evidence that the actin-binding protein LASP-1 is expressed in glomerular podocytes came from earlier proteomic studies.[20]

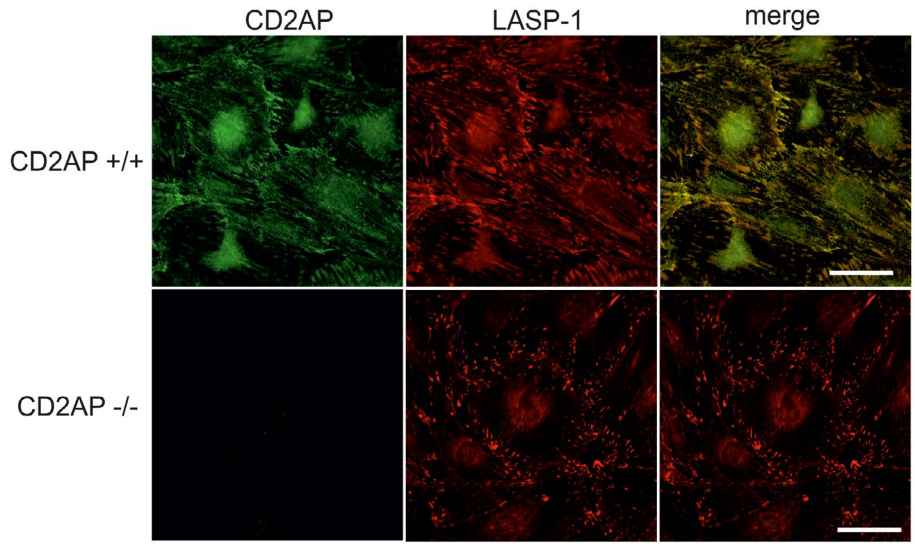


FIGURE 4 CD2AP controls the intracellular localization of LASP-1. LASP-1 localization was determined by immunofluorescence staining in control and CD2AP-deficient ($CD2AP^{-/-}$) immortalized mouse podocytes. In the presence of endogenous CD2AP ($CD2AP^{+/+}$, green), LASP-1 (red) displayed a mainly cytosolic localization with an enrichment at actin stress fibers and focal adhesions (left column). Weak signals can be also detected in the nucleus. In the absence of CD2AP ($CD2AP^{-/-}$), LASP-1 is predominately found at focal adhesion-like structures close to the cell border but is almost absent in the cytosol (right column). Scale bars represent 25 μm

Therefore, we decided to examine the LASP-1 expression in human immortalized podocytes (AB8) as well as in human and rat kidney glomerula. In human podocytes, LASP-1 was strongly expressed and was localized in the cytosol as well as along actin stress fibers (Figure 1A). LASP-1 accumulated at focal adhesions where it displayed a co-localization with its known binding partner Zyxin (Figure 1B). Furthermore, immunogold electron microscopy showed that LASP-1 was present in foot processes of human and rat glomerular podocytes where it partially localizes close to the slit membrane (Figure 1C).

3.2 | LASP-1 co-localizes with the slit membrane-associated proteins α-Actinin 4 and CD2AP

To test a putative co-localization of LASP-1 with the known slit membrane-associated proteins α-Actinin 4 and CD2AP, we performed immunofluorescence studies in cultured human podocytes. In these cells LASP-1 and α-Actinin 4 co-localized along actin stress fibers and at focal adhesions (Figure 2A), whereas LASP-1 and CD2AP co-localization appeared within punctated structures in the cytosol but was also detected at lamellipodia or cell borders (Figure 2B).

3.3 | CD2AP binds to LASP-1 and controls its localization

As CD2AP is a crucial regulator of glomerular filtration and podocyte function,[5,6] we next analyzed whether LASP-1 interacts with CD2AP. For this purpose, we performed immunoprecipitation studies with cytosolic podocyte lysates probed with control IgG, anti-CD2AP, or anti-LASP-1 antibodies, respectively. Whereas control IgG did not precipitate LASP-1 or CD2AP, CD2AP was precipitated with anti-LASP-1 antibodies (Figure 3B, left part) and LASP-1 employing anti-CD2AP antibodies (Figure 3B, right part). Subsequent mapping of the binding domains of LASP-1 and CD2AP (Figure 3A) was performed using in vitro GST pull-down assays (Figure 3C) and a Yeast Two-Hybrid (Y2H) approach (Table 1). Both assays showed that CD2AP interacts with the full length LASP-1 protein and LASP-1 lacking the LIM domain (LASP-1ΔLIM; Table 1) but not to LASP-1 lacking the SH3 domain (LASP-1ΔSH3). An internal proline-rich domain (PRD, amino acids 329-428) of CD2AP was sufficient to bind to the LASP-1 SH3 domain (Table 1). These experiments demonstrated that LASP-1 directly interacts via its SH3 domain with the proline-rich motif of CD2AP.

To test whether LASP-1 localization is controlled via its interaction with CD2AP, we analyzed LASP-1 in immortalized control ($CD2AP^{+/+}$) and CD2AP-deficient ($CD2AP^{-/-}$) mouse podocytes.[17] In control cells, LASP-1 partially co-localized with CD2AP in the cytosol and at actin stress fibers or focal adhesions (Figure 4). In contrast, $CD2AP^{-/-}$ podocytes showed accumulation of LASP-1 at peripheral focal adhesions whereas actin-associated LASP-1 mostly disappeared (Figure 4).

3.4 | LASP-1 dynamics in podocytes

LASP-1 is known as a stable component of focal adhesions and the actin cytoskeleton. In addition, LASP-1-containing

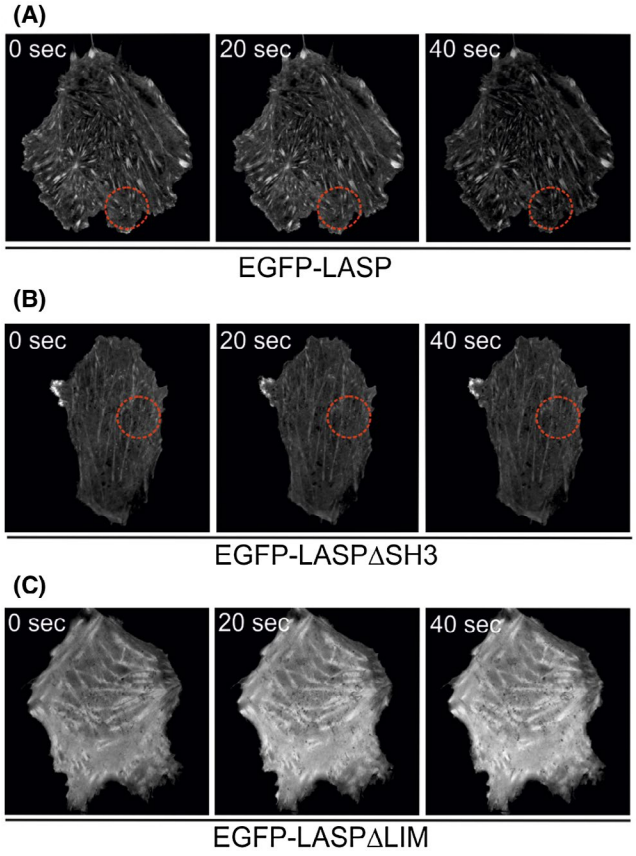


FIGURE 5 The LIM domain regulates intracellular LASP-1 dynamics. A, Dynamics of EGFP-LASP-1 in transfected human podocytes. An immobile fraction of EGFP-LASP-1 is found at actin stress fibers and focal contacts. Red-dotted circles indicate a region with highly motile EGFP-LASP-1 granules. Pictures were taken every 10 seconds. B, Dynamics of EGFP-LASP-1ΔSH3 in transfected human podocytes. Red-dotted circles indicate a region with highly motile EGFP-LASP-1ΔSH3 granules. C, Localization and dynamics of EGFP-LASP-1ΔLIM in transfected human podocytes. EGFP-LASP-1ΔLIM is found at actin stress fibers and diffusely distributed in the cytosol. There are no dynamic granular structures containing EGFP-LASP-1ΔLIM

microdomains display a dynamic intracellular transport and shuttle between the cytosol and the nucleus.[7-12] To analyze the dynamics of LASP-1 in podocytes, we used live cell imaging experiments with transfected human podocytes expressing enhanced green fluorescent protein (EGFP)-LASP-1 fusion protein. A population of EGFP-LASP-1 displayed a static localization along the actin fibres, at focal adhesions or at cell borders (Figure 5A and Supplemental video 1). In addition, a second fraction of EGFP-LASP-1 showed a high intracellular motility with strong bi-directional transport of granulated molecules (Figure 5A, Supplemental video 1).

Next, experiments with recombinant EGFP-LASP-1 truncation mutants (LASP-1ΔLIM, LASP-1ΔSH3) were performed to determine the role of LASP-1 domains for intracellular dynamics of LASP-1 in human podocytes. Unexpectedly, overexpression of the EGFP-LASP-1ΔSH3 truncation mutant did not alter the dynamics compared to full length LASP-1 fused to EGFP (Figure 5B and Supplemental video 2), whereas EGFP-LASP-1ΔLIM displayed a markedly reduced motility and was diffusely distributed in the cytosol or accumulated along the actin stress fibers (Figure 5C and Supplemental video 3).

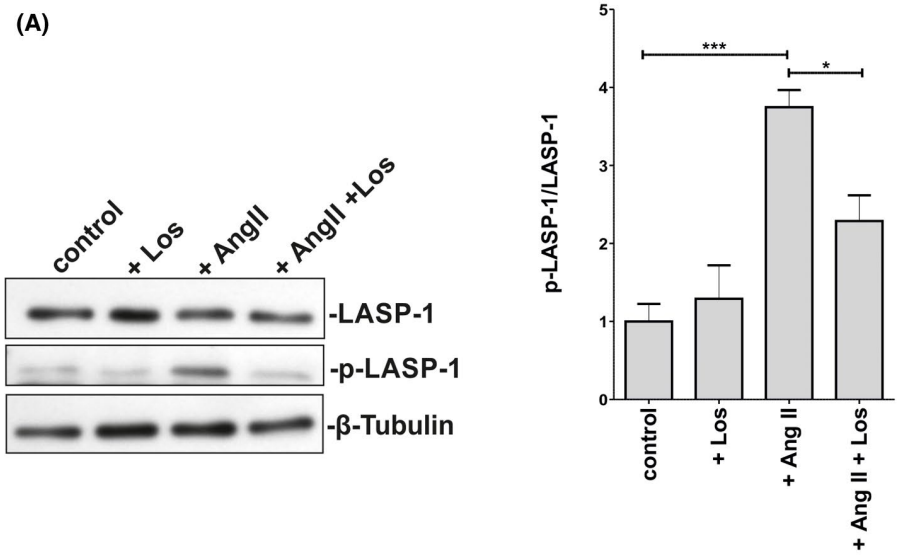
3.5 | Angiotensin regulates LASP-1 phosphorylation in podocytes

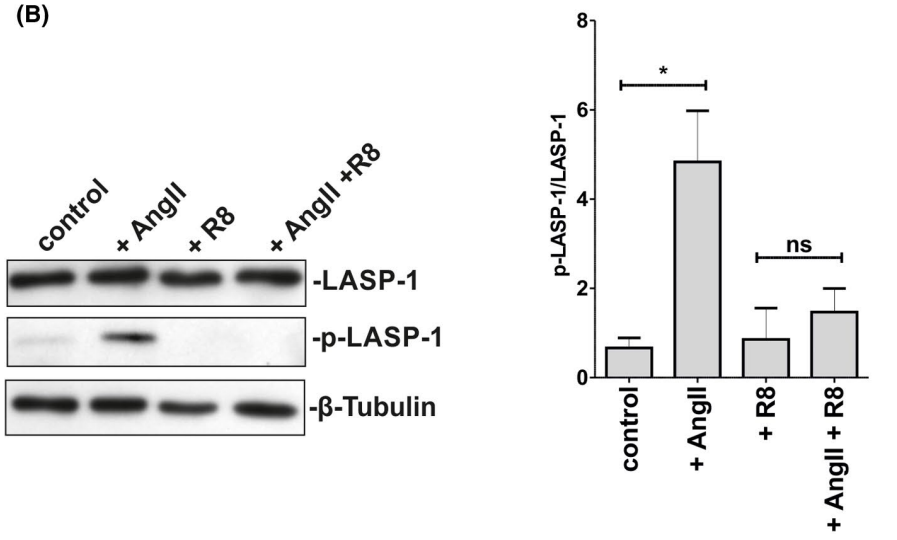
Previous studies demonstrate that cAMP-dependent phosphorylation of human LASP-1 at serine 146 (S146) by protein kinase A (PKA) affects its cellular function.[12,13] However, little is known about the upstream pathway of PKA and extracellular effectors that stimulate LASP-1 phosphorylation. To analyze this posttranslational LASP-1 modification in podocytes, we treated AB8 cells with the PKA activator forskolin and detected phospho-S146 (pS146) within LASP-1 using phospho-specific antibodies**.** Forskolin treatment dramatically increased the amount of pS146 LASP-1 (p-LASP-1) in human podocytes, whereas the overall amount of LASP-1 protein remained unchanged (Supplemental Figure 1A).

We recently demonstrated that the hormone angiotensin II (AngII), a crucial regulator of the renin-angiotensin-aldosterone system (RAAS) that activates the angiotensin receptor subtype 1 (AT1R), mediates the phosphorylation of various cytoskeleton-associated proteins in podocytes via different kinases including PKA, protein kinase C (PKC), mitogen-activated protein kinase kinase 1/2 (MEK 1/2), and p90 ribosomal S6-kinase (RSK).[21] Therefore, we tested a putative AngII-mediated S146 phosphorylation of LASP-1 in human podocytes overexpressing AT1R. Treatment of these cells with AngII significantly increased the level of p-LASP-1, whereas the overall amount of LASP-1 protein was not affected (Figure 6A). Losartan, an AngII antagonist and inhibitor of AT1R signaling, mostly abolished the effect of AngII on LASP-1 phosphorylation (Figure 6A).

Inhibition of PKA activity with Rp-8-Br-cAMPS (R8) prevented AngII-mediated LASP-1 phosphorylation (Figure 6B), confirming that PKA is involved in this signaling pathway. In contrast, inhibition of PKC with GF109203X or Rottlerin, of MEK 1/2 with U0126, and of RSK with BID1870 had no significant effects on AngII-induced LASP-1 phosphorylation (Supplementary Figure S1B-E).

As LASP-1 phosphorylation is known to affect its intracellular localization, we performed immunofluorescence studies with AngII-treated AT1R podocytes. While LASP-1 was detected predominantly along actin stress fibers and


(A)
control
+ Los
+ AngII
+ AngII +Los
-LASP-1
-p-LASP-1
-β-Tubulin
p-LASP-1/LASP-1
***
*
control
+ Los
+ Ang II
+ Ang II + Los


(B)
control
+ AngII
+ R8
+ AngII +R8
-LASP-1
-p-LASP-1
-β-Tubulin
p-LASP-1/LASP-1
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control
+ AngII
+ R8
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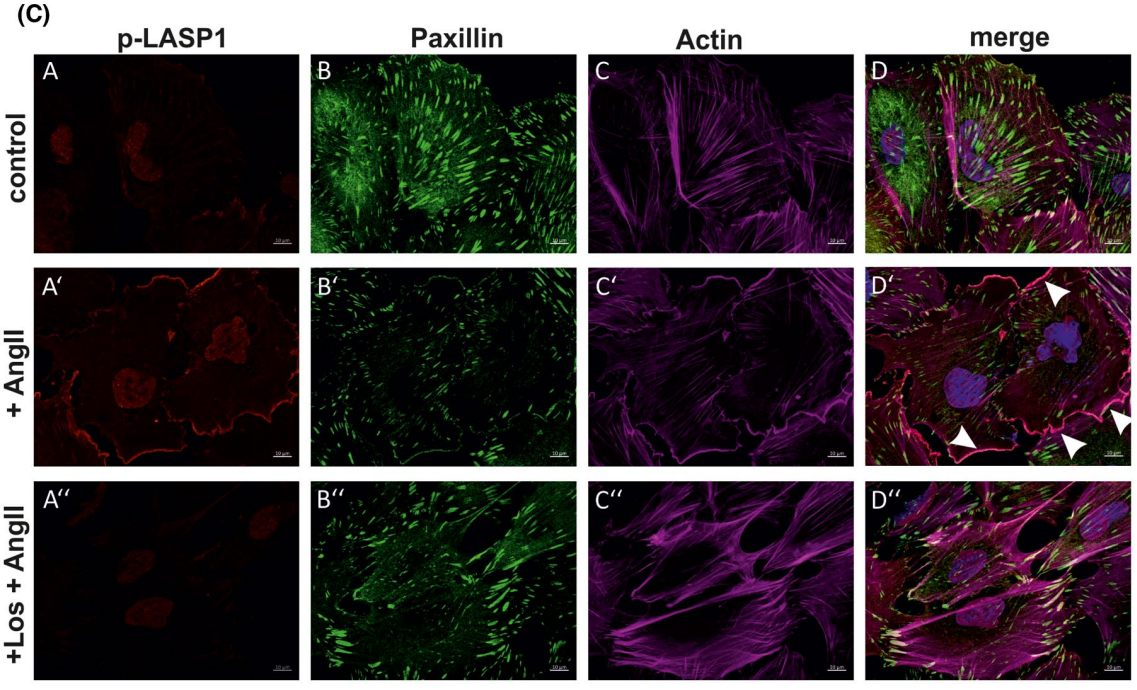
(C)
p-LASP1
Paxillin
Actin
merge
control
+ AngII
+Los + AngII
A
B
C
D
A′
B′
C′
D′
A″
B″
C″
D″

FIGURE 6 AngII stimulation of podocytes results in S146 phosphorylation and altered localization of p-LASP-1. A: Representative Western blot analysis of lysates from AT1R-overexpressing podocytes treated with AngII (100 nM, 15 minutes; left part). Where indicated, podocytes were preincubated with the AT1R blocker Losartan (Los; 10 μM, 2 hours). Quantification of signals from four independent experiments was used to determine the ratio between phosphorylated pS146-LASP-1 (p-LASP-1) and total amount of LASP-1 (right part). β-Tubulin was used as a loading control. B, Representative Western blot from AB8-AT1R podocytes that were treated with AngII (100 nM, 15 minutes; left). Preincubation with the PKA-specific inhibitor Rp-8-Br-cAMP (R8; 500 μM, 4 hours) diminishes AngII induced LASP-1 S146 phosphorylation. Quantification determines the ratio between p-LASP-1 and total amount of LASP-1 from four independent experiments (right part). β-Tubulin was used as a loading control. C, Immunofluorescence staining of endogenous p-LASP-1 (red in A, A′ and A′′), the focal adhesion marker Paxillin (green in B, B′ and B′′), and F-actin (purple in C, C′ and C′′) before (upper panel) and 15 minutes after stimulation of the AB8-AT1R cells with AngII (100 nM, middle panel) indicates an AngII-dependent accumulation of p-LASP-1 at lamellipodia (arrow heads in D′). Losartan (10 μm, lower panel) abolishes the effect of AngII treatment on p-LASP-1 localization. Scale bars represent 20 μm

co-localized with the focal adhesion component Paxillin in non-stimulated podocytes, AngII treatment led to a partial enrichment of LASP-1 at lamellipodia (Supplemental Figure 2). Further experiments with phosphospecific antibodies revealed that p-LASP-1 predominately accumulated at lamellipodia of AngII-treated podocytes where it co-localizes with Paxillin and F-actin. Losartan silenced the effect of AngII on intracellular distribution of p-LASP-1 (Figure 6C). These data indicate that AngII-mediated activation of RAAS affects LASP-1 phosphorylation and localization in podocytes.

3.6 | Lasp is necessary for Drosophila nephrocyte filter function

To analyze the in vivo role of Lasp in filtration processes, we employed the *Drosophila melanogaster* (Dm) nephrocyte model.[18,22,23] Drosophila only expresses one Lasp protein compared to mice which express LASP-1 and the highly related isoform LASP-2.[24] Nephrocytes are similar to mammalian podocytes as they exhibit a slit membrane-like structure composed of orthologs of known slit membrane proteins such as Nephrin (Sticks-and-stones [Sns] in Dm), Neph1 (Kin-of-irre in Dm), and Podocin (Mec2 in Dm).[23] To test whether Lasp is expressed in Dm nephrocytes, we dissected these cells from L3 larvae and performed immunofluorescence analysis with anti-Lasp and anti-Pyd (ortholog of the peripheral membrane protein zona occludens 1 [ZO-1]) antibody. Images revealed Lasp expression mainly in the cytoplasm of nephrocytes, (Figure 7A). To analyze whether *lasp* knockdown impaired nephrocyte function and filtration, we employed an assay in which genetically modified fly larvae ubiquitously express the protein ANF-GFP-GFP that is secreted into the hemolymph and taken up by nephrocytes. The fluorescent molecule can be measured as a surrogate of nephrocyte filter function and functional endocytosis.[18] We then analyzed whether *lasp* knockdown impaired nephrocyte function and filtration. Nephrocytes with knockdown of *lasp* that was achieved by two different dsRNA exhibited significantly reduced uptake of ANF-GFP-GFP (Figure 7B and Supplemental Figure S3). To determine whether Lasp is necessary for intact nephrocyte slit membranes, we crossed flies that contain a *sns::Gal4* transgene with flies encoding a *UAS-dsRNA* for *lasp* which led to a nephrocyte-specific *lasp* knockdown. While intact slit membranes were observed by transmission electron microscopy (TEM) in control nephrocytes, flies from both *lasp* knockdown strains showed a markedly reduced number of slit membranes (Figure 7C).

Using indirect immunohistochemistry, slit membrane proteins can be visualized in a highly regular, fingerprint-like pattern on the surface of nephrocytes by confocal microscopy which represents the intact slit membranes.[18] To analyze the impact of *lasp* on slit membrane architecture, we stained control and *lasp* knockdown nephrocytes for the slit membrane proteins Pyd and Sns and made surface sections of these nephrocytes by confocal microscopy. While control nephrocytes stained for Pyd and Sns exhibited the typical regular fingerprint-like pattern, *lasp* knockdown nephrocytes showed wider gaps between the thin lines that represent the slit membrane (Figure 7D). This is consistent with a reduced number of slit membranes and confirms our TEM data (Figure 7C). As Lasp is known as a regulator of actin dynamics in mammalian cells, we hypothesized that *lasp* knockdown may alter the actin cytoskeleton of nephrocytes. To analyze this, we stained control and *lasp* knockdown nephrocytes with phalloidin to visualize F-actin. While in control nephrocytes F-actin was mainly visualized near the plasma membrane and presented as a thin line, *lasp* knockdown resulted in mislocalization of F-actin which accumulated in a broader area next to the plasma membrane and in the cytoplasm (Figure 7E).

4 | DISCUSSION

The composition of the actin cytoskeleton is crucial for podocyte function and changes in pathways regulating the cytoskeleton and its connection with the slit membrane often lead to podocyte foot process effacement and proteinuria.[2,3,25] Loss of CD2AP, an actin-binding and slit membrane-associated protein, results in focal segmental glomerulosclerosis (FSGS). CD2AP deficient mice die within 6 weeks after birth due to renal failure.[5,6,17]

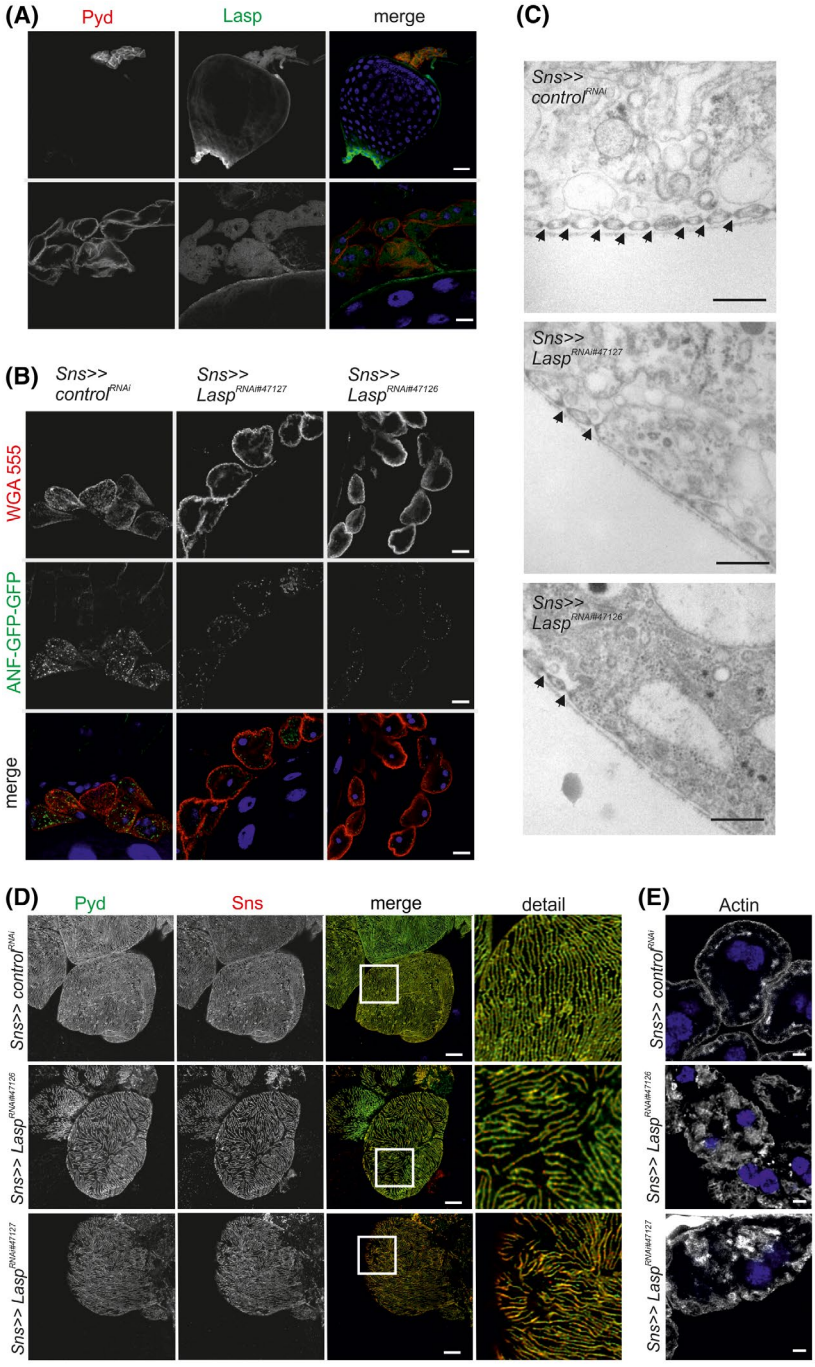

FIGURE 7 Lasp is necessary for Drosophila nephrocyte filter integrity. A, Proventriculi with adjacent garland nephrocytes (lower panel) were prepared from L3 Drosophila larvae and were used for immunofluorescence analysis with antibody specific for Pyd (membrane marker) and Lasp. Nuclei were stained with DAPI. The lower part shows a 10-fold magnification of the nephrocytes. Scale bars: 100 µm (upper panel) and 10 µm (lower panel). B, Uptake of fluorescent ANF-GFP-GFP in dissected control (control RNAi) or *lasp* knockdown nephrocytes from two different lines. *lasp* knockdown in nephrocytes was generated by employing *sns::Gal4* and two different *dsRNA* specific for *lasp*. For visualization of membranes samples were stained with wheat germ agglutinin-(WGA)-Alexa^{555}. Merged images are shown in the lower panel. Scale bars represent 10 µm. C, Transmission electron microscopy of nephrocytes from larvae of control (*sns>>control*^{RNAi}) and two *lasp* knockdown strains (*sns-Gal4->>lasp*$^{RNAi\#47127}$, *sns-Gal4->>lasp*$^{RNAi\#47126}$). The number of slit membranes (arrows) is markedly decreased in nephrocytes with *lasp* knockdown. Scale bars represent 200 nm. D, Surface section of control (*sns>>control*^{RNAi}), and *lasp* knockdown nephrocytes (*sns-Gal4->>lasp*$^{RNAi\#47127}$, *sns-Gal4->>lasp*$^{RNAi\#47126}$) co-stained for Pyd (green) and Sns (red) revealing a fingerprint-like pattern. A detailed view of the marked area of the merged image is shown in the column to the right. Scale bars represent 5 µm. E, Cross section of control (*sns>>control*^{RNAi}), and *lasp* knockdown nephrocytes (*sns-Gal4->>lasp*$^{RNAi\#47127}$, *sns-Gal4->>lasp*$^{RNAi\#47126}$) stained for F-actin. Nuclei are visualized by DAPI (blue). Scale bars represent 2.5 µm

Our data place LASP-1 into the group of actin regulators that are involved in cytoskeleton organization in foot processes and suggest an important role for LASP-1 in podocyte function. We show that LASP-1 is not only highly expressed in podocytes but is also partially localized close to the slit membrane (Figure 1). This is consistent with our finding that in podocytes, LASP-1 and the slit diaphragm-associated protein CD2AP interact (Figure 3). CD2AP is known to link slit membrane proteins like Nephrin to the actin cytoskeleton of podocyte foot processes and CD2AP may also recruit LASP-1 to the slit membrane protein complex. Indeed, we observe a co-localization of LASP-1 and CD2AP in granular structures in the cytosol and at cell borders in cultured podocytes (Figure 2). A similar granular localization of CD2AP was observed before by Welsch et al. who speculate that CD2AP may regulate endosomal sorting or trafficking of multiprotein complexes.[26] In this context, it is worth to mention that LASP-1 is also implicated in trafficking processes via its interaction with the large GTPase Dynamin 2.[11] CD2AP affects the LASP-1 localization as CD2AP-deficient podocytes display an altered LASP-1 localization with an accumulation at peripheral focal contacts and less cytosolic LASP-1 (Figure 4). Taken together, our data suggest that the LASP-1-CD2AP interaction is beneficial for trafficking processes of the protein complex in podocytes and that CD2AP may mediate anchoring of LASP-1 to the actin cytoskeleton close to the slit membrane.

Further evidence for a regulated balance of intracellular shuttling and the cytoskeleton anchoring of LASP-1 is maintained by our live cell imaging experiments (Figure 5). We observe two different LASP-1 fractions that exhibit different intracellular dynamics showing either a high motility in vesicle-like structures or a more static behavior at focal adhesions (Figure 5A). We show that the truncation of the LIM domain, but not of the SH3 domain, exhibits a dramatic effect on the intracellular dynamic of LASP-1 (Figure 5B,C). The exact function of the LASP-1 LIM domain still needs to be investigated in more detail but it is becoming clear that this domain not only enables putative DNA-binding of LASP-1 but is also important for the intracellular motility of the protein.

Our experiments show that the SH3 domain has no crucial role on the intracellular dynamics of LASP-1. Moreover, CD2AP regulates the intracellular localization of LASP-1 probably via the interaction with the LASP-1 SH3 domain. This indicates that CD2AP is predominantly involved in recruiting LASP-1 to specific intracellular subdomains but has no or only a minor impact on LASP-1 dynamics.

We demonstrate that an AngII treatment of podocytes leads to phosphorylation of LASP-1 at serine 146 and a subsequent enrichment at cell borders (Figure 6). This is of special interest as LASP-1S146 phosphorylation was shown to induce LASP-1 translocation to the cytosol in non-podocyte cell types.[11,13] We speculate, that angiotensin-mediated LASP-1 phosphorylation regulates its anchoring at intracellular subdomains in podocytes which is then further stabilized by a slit membrane-associated protein such as CD2AP. The RAAS plays a crucial role in the pathogenesis of glomerular injury and that its inductor AngII acts as a pro-fibrotic cytokine involved in glomerulosclerosis.[27,28] Therefore, AngII induced LASP-1 phosphorylation and altered localization mediated by PKA might be crucial for the glomerular function and sheds light on upstream cues that regulate the role of LASP-1 in podocytes.

To proof the impact of LASP-1 on filtration processes, we used the Drosophila nephrocyte model. This experimental model is an excellent tool for in vivo studies on proteins with a proposed role in glomerular filtration as a number of their encoding genes are conserved between invertebrates and mammalians. Interestingly, knockdown of *Cindr*, the homolog of mammalian CD2AP, impairs nephrocyte slit membranes and filtration.[29] This phenotype is very similar to what we observe in nephrocytes with reduced Lasp expression which suggests a role for LASP-1 in the mammalian kidney filter.

In summary, we demonstrate the functional interaction of LASP-1 and the slit membrane-associated protein CD2AP as well as the AngII-regulated phosphorylation of LASP-1. The functional role of LASP-1 in podocytes is further supported by our findings that LASP is necessary for intact slit membranes, architecture of the actin cytoskeleton, and filtration in Drosophila nephrocytes.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interests.

AUTHOR CONTRIBUTIONS

C. Lepa, A. Möller-Kerutt, M. Stölting, M.-L. Eddy, C. E. Butt, D. Kerjaschki, B. George, B. Vollenbröker, H. Korb-Pap, T. Weide, J. Kremerskothen, H. Pavenstädt designed research. C. Lepa, A. Möller-Kerutt, M.-L. Eddy, M. Stölting, C. Picciotto, J. Kremerskothen, and Jens Möller performed research. C. Lepa, A. Möller-Kerutt, M.-L. Eddy, M. Stölting, B. Vollenbröker, B. George, and J. Kremerskothen analyzed data; C. Lepa, A. Möller-Kerutt, M. Stölting, M.-L. Eddy, B. George, and J. Kremerskothen wrote the paper.

ACKNOWLEDGMENTS

We would like to thank Helga Schachner and Karin Wacker for their excellent technical assistance. We are thankful to Mario Schiffer for providing us with the immortalized CD2AP$^{-/-}$ cells, and Jürgen Klingauf for support with the electron microscopic analyses. This work was supported by a DFG grant to BG (GE 2158/3-1) and an IMF grant to JK.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

How to cite this article: Lepa C, Möller-Kerutt A, Stölting M, et al. LIM and SH3 protein 1 (LASP-1): A novel link between the slit membrane and actin cytoskeleton dynamics in podocytes. *The FASEB Journal*. 2020;34:5453–5464. https://doi.org/10.1096/fj.201901443R