Cardiovascular Research (2013) **100**, 461–471
doi:10.1093/cvr/cvt207

Cysteine-rich protein 2 alters p130Cas localization and inhibits vascular smooth muscle cell migration

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Received 10 March 2013; revised 8 August 2013; accepted 14 August 2013; online publish-ahead-of-print 23 August 2013

Time for primary review: 34 days

Aims Cysteine-rich protein (CRP) 2, a member of the LIM-only CRP family that contains two LIM domains, is expressed in vascular smooth muscle cells (VSMCs) of blood vessels and functions to repress VSMC migration and vascular remodelling. The goal of this study was to define the molecular mechanisms by which CRP2 regulates VSMC migration.

Methods and results Transfection of VSMCs with CRP2-EGFP constructs revealed that CRP2 associated with the actin cytoskeleton. In response to chemoattractant stimulation, Csrp2 (mouse CRP2 gene symbol)-deficient ($Csrp2^{-/-}$) VSMCs exhibited increased lamellipodia formation. Re-introduction of CRP2 abrogated the enhanced lamellipodia formation and migration of $Csrp2^{-/-}$ VSMCs following chemoattractant stimulation. Mammalian 2-hybrid and co-immunoprecipitation assays demonstrated that CRP2 interacts with p130Cas, a scaffold protein important for lamellipodia formation and cell motility. Immunofluorescence staining showed that CRP2 colocalized with phospho-p130Cas at focal adhesions (FAs)/terminal ends of stress fibres in non-migrating cells. Interestingly, in migrating cells phospho-p130Cas localized to the leading edge of lamellipodia and FAs, whereas CRP2 was restricted to FAs and stress fibres. Furthermore, we demonstrated that p130Cas expression and phosphorylation promote neointima formation following arterial injury.

Conclusion These studies demonstrate that CRP2 sequesters p130Cas at FAs, thereby reducing lamellipodia formation and blunting VSMC migration.

Keywords Vascular smooth muscle cells • Migration • Cysteine-rich protein 2 • P130Cas

1. Introduction

Vascular smooth muscle cells (VSMCs) constitute a major component of the medial layer of mature blood vessels. Under normal physiological conditions, VSMCs display a quiescent, differentiated, and contractile phenotype.[1] However, in response to a variety of injurious stimuli, VSMCs switch from a contractile phenotype to a synthetic, migratory, and proliferative phenotype. The migration and proliferation of VSMCs into the intima contribute to arterial thickening and subsequent arteriosclerosis.[1,2] As atherosclerotic lesions progress, the thinning of the fibrous cap that overlies a large, lipid-filled necrotic core leads to plaque rupture and acute thrombus formation.[3] Thus, VSMC accumulation at the fibrous cap can stabilize plaques and prevent plaque rupture. Despite the importance of VSMC migration in the development of occlusive vascular disease, the molecular mechanisms regulating VSMC migration have not been elucidated completely.[4]

Cysteine-rich protein (CRP) 2, expressed in VSMCs,[5,6] is a member of the LIM-only CRP family and contains two LIM domains.[7] The LIM domain, a two zinc finger structural motif, functions as modular protein–protein binding interface.[8] As such, LIM proteins play critical functions in diverse biological processes.[9,10] CRP proteins, including CRP1, CRP2, and CRP3, have been implicated in promoting protein assembly along the actin-based cytoskeleton.[7,11] *In vitro* binding assays show that CRPs interact with two cytoskeletal proteins—the actin crosslinking protein α-actinin and the adhesion plaque protein zyxin.[7] Purified recombinant CRP2 binds directly to F-actin in co-sedimentation and ELISA assays.[12,13] In addition to cytoskeletal roles, CRPs localize to the nucleus.[8] For example, CRP3 interacts with muscle-specific transcription factor MyoD and can promote myogenesis.[14] CRP1 and CRP2 can form complexes with SRF and GATA transcription factors, facilitating the expression of some smooth muscle (SM) marker genes.[15] By gene deletion experiments, we previously demonstrated that the

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absence of CRP2 increases neointima formation following arterial injury.[16] Importantly, the increased intimal thickening in Csrp2 (mouse CRP2 gene symbol)-deficient ($Csrp2^{-/-}$) mice correlated with enhanced VSMC migration into the intima.[16] This migratory role emphasizes a cytoskeleton-associated function of CRP2 in VSMCs.

In response to migratory cues from cytokines and extracellular matrix (ECM) components, the initiation of cell migration involves dynamic and spatially regulated changes in the cytoskeleton and cell adhesion.[17,18] These processes are controlled by interactions involving ECM, integrins, cellular components associated with focal adhesions (FAs), and the actin cytoskeleton.[19] Upon receiving signals to migrate, new adhesions to the ECM are initiated at the leading edge of the cell by recruiting adaptor proteins to the focal complex. Subsequent transmission of signals leads to lamellipodia extension from the leading edge in the direction of migration.[20,21] The small Rho GTPase Rac1 co-ordinates the cellular responses required for cell migration.[20,22] Indeed, the increased cell motility in the absence of CRP2 strongly correlates with enhanced Rac1 activation.[16] One of the signalling pathways leading to Rac1 activation in migrating cells is the assembly of the p130Cas (Crk-associated substrate)-Crk signalling complexes present in nascent adhesive complexes.[23–25] p130Cas is a versatile scaffold molecule containing several structural motifs including a Src homology 3 (SH3) domain, a proline-rich region, a substrate domain with 15 YxxP repeats ($(YxxP)_{15}$), and a Cas-family C-terminal homology domain.[26,27] Through interaction with a variety of partners including tyrosine kinases, p130Cas assembles, and transmits migratory signals.[23,27,28] Interestingly, the LIM proteins zyxin, TRIP6, and Ajuba associate with p130Cas, and might regulate downstream signalling events that control migration.[29–31]

Given the cytoskeleton association of CRP2, we hypothesized that, similar to other LIM protein family members, CRP2 might regulate VSMC motility through interactions with p130Cas-associated signalling complexes. In the current study, we show that in response to chemoattractant stimulation, $Csrp2^{-/-}$ VSMCs exhibited increased lamellipodia formation. Mammalian-2-hybrid and co-immunoprecipitation assays demonstrated that CRP2 interacted with p130Cas. Further, CRP2 colocalized with phospho-p130Cas (p-p130Cas) at FAs in non-migrating cells. Importantly, in migratory cells p-p130Cas was present both at the leading edges of lamellipodia and FAs, whereas CRP2 was only found at FAs. Our results indicate that CRP2 sequesters p130Cas at FAs, thereby reducing lamellipodia formation and inhibiting VSMC migration. Furthermore, we demonstrated that p130Cas expression and phosphorylation promote neointima formation following arterial injury.

2. Methods

An expanded Methods section is provided in the Supplementary material online.

2.1 Cell culture and transient transfection

Primary mouse VSMCs were cultured[16] and transient transfections performed using electroporation.

2.2 Construction of expression plasmids

Expression plasmids containing various lengths of CRP2 and for mammalian two-hybrid assays were constructed and confirmed by DNA sequencing.

2.3 Adhesion, spreading, and migration assays

To assess VSMC adhesion to and spreading on ECM, cells were plated on plates coated with ECM proteins for different periods of time, stained, and cell number counted. To assess migration, $Csrp2^{-/-}$ VSMCs electroporated with various CRP2-EGFP expression plasmids were placed in the upper chamber of transwell plates and migration assays performed.

2.4 Immunofluorescence

Immunofluorescence staining was performed to assess subcellular localization of CRP2, p-p130Cas, and paxillin in VSMCs.

2.5 Mammalian two-hybrid assay

Protein–protein interactions were evaluated using the mammalian two-hybrid assay kit (Stratagene).

2.6 Mouse vascular remodelling model and analysis

Approximately 12-week-old male mice were used in a mouse model of neointima formation after carotid artery cessation of blood flow.[32] The left common carotid artery was dissected and ligated near the bifurcation with a 5-0 suture essentially as described.[32] Pluronic gel (Sigma) was used to deliver siRNA or inhibitor to the carotid artery as described.[33] Ligated and control vessels were harvested and analysed by histological and western blot analysis.

2.7 Co-immunoprecipitation

Co-immunoprecipitation experiments of CRP2 and p-p130cas were performed using specific antibodies and protein G magnetic beads (Millipore), followed by western blot analysis.

2.8 Statistical analysis

Data are presented as means $\pm$ SE. The statistical significance between two groups was determined by Student's *t*-test and statistical significance was accepted at $P < 0.05$.

3. Results

3.1 Effects of CRP2 on cellular adhesion, spreading, and lamellipodia formation

To define the mechanisms by which CRP2 alters migration, we first tested the effects of CRP2 on VSMC adhesion and spreading on ECM. These processes can influence cell migration.[34] $Csrp2^{+/+}$ and $Csrp2^{-/-}$ VSMCs adhered similarly to fibronectin, collagen I, and laminin (*Figure 1A*). In addition, phalloidin staining revealed that $Csrp2^{+/+}$ and $Csrp2^{-/-}$ VSMCs exhibited a similar amount of spreading on fibronectin at 15 and 60 min after plating (*Figure 1Ba–Bd*). Quantitative assessment showed that there were no differences in the spreading rates between $Csrp2^{+/+}$ and $Csrp2^{-/-}$ VSMCs up to 120 min (*Figure 1C*). Given that lamellipodia extension and membrane ruffling are the first steps in initiating cell migration,[21] we then examined lamellipodia formation of migrating VSMCs. Unstimulated $Csrp2^{+/+}$ and $Csrp2^{-/-}$ VSMCs had very little lamellipodia formation (*Figure 1Da, Db, and E*). However, in response to PDGF-BB stimulation, $Csrp2^{+/+}$ VSMCs produced small lamellipodia (*Figure 1Dc*), whereas $Csrp2^{-/-}$ VSMCs had increased membrane ruffles and lamellipodia formation (*Figure 1Dd*). In contrast to 28 ± 2 lamellipodia per 100 $Csrp2^{+/+}$ VSMCs, CRP2 deficiency increased the number of lamellipodia to 85 ± 4 per 100 cells ($P < 0.05$ vs. $Csrp2^{+/+}$; *Figure 1E*). PDGF-BB also significantly increased lamellipodial size in $Csrp2^{-/-}$ VSMCs ($Csrp2^{+/+}$ 588 ± 115 vs. $Csrp2^{-/-}$ 1016 ± 52 μm^2/lamellipodium; $P < 0.05$) (*Figure 1F*). These results suggest that the increased migration of

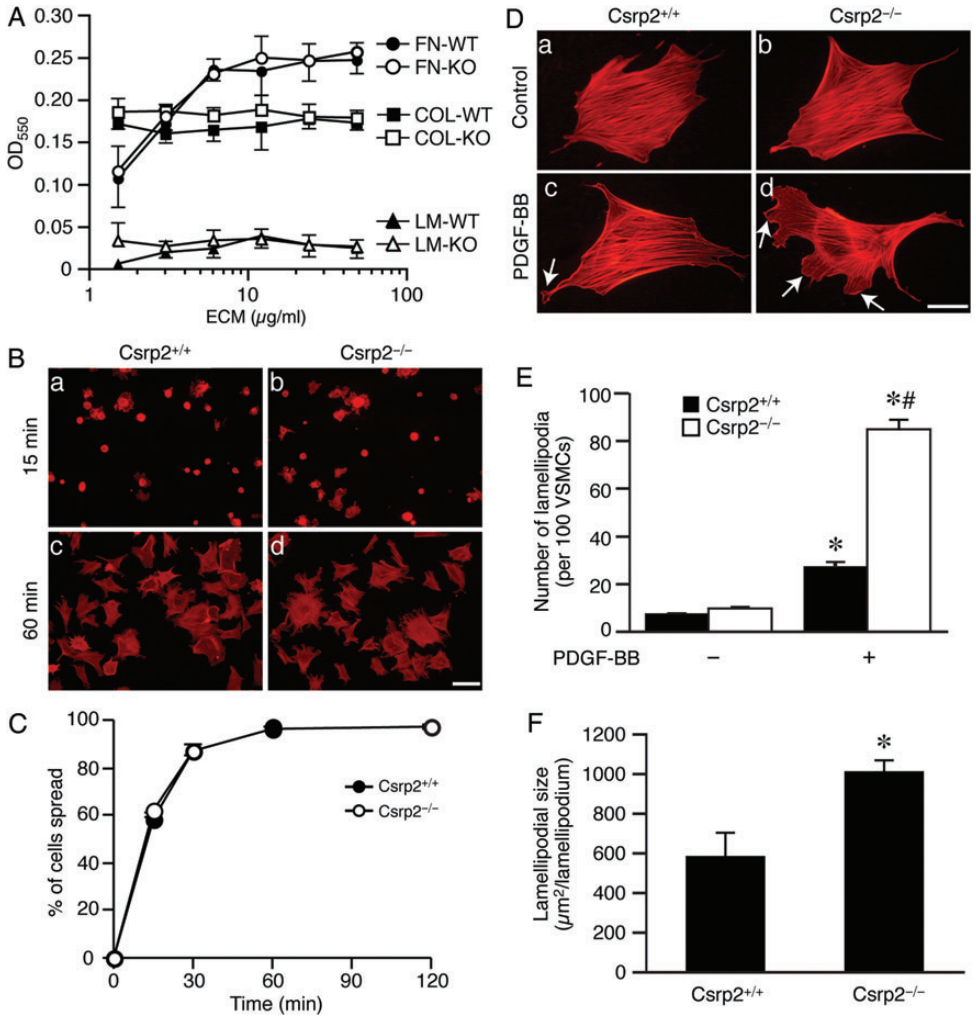

Figure 1 CRP2 on VSMC adhesion, spreading, and lamellipodia formation. (*A*) The adhesion of Csrp2$^{+/+}$ (WT, black) and Csrp2$^{-/-}$ (KO, white) VSMCs to indicated ECM proteins (FN, fibronectin; COL, collagen I; LM, laminin) was determined by measuring OD_{550}. (*B*) Csrp2$^{+/+}$ (a and c) and Csrp2$^{-/-}$ (b and d) VSMCs were plated on fibronectin-coated chamber slides, fixed, and stained with Alexa Fluor 546-Phalloidin to detect F-actin (red) at different time points following plating. Representative images of stained cells at 15 (a and b) and 60 min (c and d) are shown. Scale bar, 100 μm. (*C*) Quantitative analysis of cell spreading at different time points. The percentage of spread cells was calculated as the number of cells spread divided by total (round and spread) cell number and multiplied by 100. (*D*) To assess lamellipodia formation, VSMCs were serum starved for 2 days prior to stimulation. Csrp2$^{+/+}$ (a and c) and Csrp2$^{-/-}$ (b and d) cells were then stimulated without (a and b) or with PDGF-BB (c and d) for 30 min and stained with Alexa Fluor 546-Phalloidin (red). Arrows indicate lamellipodia. Scale bar, 50 μm. (*E*) Lamellipodia were counted in at least 100 cells stimulated with or without PDGF-BB and expressed as the number of lamellipodia per 100 cells. $^{*}P < 0.05$ vs. without PDGF-BB; $^{\#}P < 0.05$ vs. Csrp2$^{+/+}$ cells with PDGF-BB. (*F*) After PDGF-BB stimulation, lamellipodial size was measured using the NIH ImageJ software and expressed as μm^2 per lamellipodium. $^{*}P < 0.05$ vs. Csrp2$^{+/+}$ cells. Values are means ± SE of three experiments and images are representatives from three experiments.

Csrp2$^{-/-}$ VSMCs was due to enhanced lamellipodia formation (in both number and size) rather than altered adhesion to or spreading on ECM.

3.2 CRP2 associates with actin stress fibres in VSMCs

CRP family members, when expressed in adherent fibroblasts, associate with the actin cytoskeleton.[7] To investigate subcellular localization of CRP2 in VSMCs and to define the domains of CRP2 that direct subcellular locations, we generated a series of CRP2 full length and deletion expression constructs with EGFP fused at the C-terminus (see Supplementary material online, *Figure S1A*). When VSMCs were transfected with pEGFP vector green fluorescence was localized to the cytosol and the nucleus (see Supplementary material online, *Figure S1B*) rather than actin stress fibres, which were revealed by red phalloidin staining (see Supplementary material online, *Figure S1C* and *D*). In contrast, full-length CRP2-EGFP (see Supplementary material online, *Figure S1E*) was colocalized with stress fibres (see Supplementary material online, *Figure S1F*) in actin cytoskeleton (see Supplementary material online, *Figure S1G*, yellow). Interestingly, LIM1 domain of CRP2 also colocalized with stress fibres (see Supplementary material online, *Figure S1H–J*). However, LIM2 domain of CRP2 failed to associate with actin stress fibres (see Supplementary material online, *Figure S1K–M*), suggesting LIM1 is critical for CRP2 targeting to stress fibres.

3.3 Restoring CRP2 expression in Csrp2$^{-/-}$ VSMCs inhibits lamellipodia formation and migration following PDGF-BB stimulation

To assess the cytoskeletal function of CRP2 in VSMCs, we restored the expression of CRP2 or its LIM domains in Csrp2$^{-/-}$ cells and examined lamellipodia formation following chemoattractant stimulation. Exogenous expression of EGFP did not affect PDGF-BB-induced lamellipodia formation of Csrp2$^{-/-}$ cells (*Figure 2A–C*). In contrast, the expression of the CRP2-EGFP fusion reduced the formation of lamellipodia after PDGF-BB stimulation (*Figure 2D–F*). The LIM1 domain was sufficient to decrease PDGF-BB-induced lamellipodia formation (*Figure 2G–I*) while expression of the LIM2 domain had no effect (*Figure 2J–L*). These results indicate that localization to actin stress fibres correlated with CRP2's ability to inhibit lamellipodia formation. Next, we wanted to determine whether restoring CRP2 or its LIM domain affected VSMC migration. Csrp2$^{-/-}$ VSMCs were transfected with EGFP, CRP2-EGFP, CRP2-LIM1-EGFP, or CRP2-LIM2-EGFP plasmids, and evaluated in migration assays (*Figure 2M*). In response to chemoattractant stimulation, full-length CRP2 reduced migration to 45 $\pm$ 4% compared with controls (*Figure 2M*), further demonstrating an inhibitory role for CRP2 in cellular migration. CRP2-LIM1 also reduced migration in these cells (*Figure 2M*), suggesting the functional importance of the LIM1 domain. In contrast, CRP2-LIM2 failed to reduce the migration of Csrp2$^{-/-}$ VSMCs (*Figure 2M*), indicating LIM2 domain may be dispensable in CRP2-mediated migration. Together these results demonstrated that the enhanced migration of Csrp2$^{-/-}$ VSMCs was specifically due to loss of CRP2; furthermore, the ability of CRP2 to inhibit lamellipodia formation strongly correlated with its ability to reduce migration.

3.4. CRP2 interacts with scaffold molecule p130Cas

Since CRPs interact with zyxin in blot overlay assays[7] and that zyxin interacts with p130Cas in regulating cell motility,[29,31] we postulated that zyxin might serve as a bridge for CRP2 to associate with the p130Cas complex. To test our hypothesis, we first evaluated the interaction between CRP2 and zyxin using a mammalian two-hybrid system. We cotransfected the reporter pFR-Luc and bait and target plasmids into AD293 cells. Protein–protein interactions were then assessed by luciferase assays 24 h after transfection. Unexpectedly, CRP2 did not interact with zyxin despite high levels of fusion protein expression (*Figure 3A*). Consistent with previous reports, p130Cas interacted with zyxin in these assays (*Figure 3A*).[29,31]

We next tested the possibility that CRP2 might interact with p130Cas directly. Indeed, compared with empty vector or negative TRAF2 controls, CRP2 interacted with p130Cas as revealed by enhanced luciferase activity (*Figure 3B*). Western blot analysis confirmed similar levels of all fusion protein expression (*Figure 3B*). These data demonstrated that CRP2 interacts directly with the scaffold protein p130Cas. To identify domains in CRP2 that mediated its association with p130Cas, we generated plasmids pBD-CRP2-LIM1 and pBD-CRP2-LIM2 containing respective LIM1 or LIM2 domain of CRP2, fused to the GAL4-DNA binding domain. Mammalian two-hybrid assays showed that the LIM1 domain of CRP2 interacted strongly with p130Cas, to the same extent as full-length CRP2 (*Figure 3C*). In contrast, the LIM2 domain failed to interact with p130Cas (*Figure 3C*). The failure of CRP2-LIM2 domain to interact with p130Cas was not due to the lack of fusion protein expression as evidenced by abundant BD-CRP2-LIM2 levels (*Figure 3C*). Together these results indicated that CRP2 interacted with p130Cas via its LIM1 domain.

3.5 Phospho-p130Cas expression in the normal and injured arterial wall

To investigate the potential role of p130Cas in vascular injury and because phosphorylation of p130Cas is critical for its function, we used a mouse model of neointima formation after carotid artery cessation of blood flow.[32] Un-injured arteries exhibited high p-p130Cas levels in the medial SM layers as indicated by strong brown staining (*Figure 3D*), which was substantially diminished 4 days after carotid ligation (*Figure 3E*). Interestingly, p-p130Cas positive cells started to appear again in medial SM layers and in the small neointima 7 days after ligation (*Figure 3F*). Fourteen days after ligation, p-p130Cas levels were further enhanced in the large neointima (*Figure 3G*). Together these results suggest an important role of p-p130Cas in vascular remodelling.

To demonstrate the function of p130Cas in vascular remodelling, we performed p130Cas knockdown experiments. After confirming the knockdown of p130Cas by siRNA (see Supplementary material online, *Figure S2A*), we applied pluronic gel containing control or p130Cas siRNA to the carotid artery 15 min before ligation and the arteries harvested 14 days later. Verhoeff's elastin staining revealed that in comparison with control siRNA, suppression of p130Cas expression in the ligated carotid artery decreased neointima formation (*Figure 3H* and *I*). To further confirm the critical importance of p130Cas and its phosphorylation in vascular injury, we inhibited p130Cas phosphorylation. Calyculin A, which has been shown to inhibit p130Cas phosphorylation and thus may block its function,[35] inhibited p130Cas tyrosine phosphorylation in VSMCs (see Supplementary material online, *Figure S2B*). Indeed, compared with vehicle DMSO inhibition of p130Cas phosphorylation decreased neointima size 14 days after ligation (*Figure 3J* and *K*). We next examined p130Cas phosphorylation levels in the ligated carotid arteries from Csrp2$^{+/+}$ and Csrp2$^{-/-}$ mice at different time points following ligation. Consistent with immunohistochemistry results, p-p130Cas levels were reduced 4 days after ligation but increased thereafter at 7 days (*Figure 3L*). Intriguingly, in comparison with Csrp2$^{+/+}$ arteries p130Cas phosphorylation was enhanced in ligated Csrp2$^{-/-}$ carotid arteries (*Figure 3L*).

3.6 PDGF-BB stimulated Csrp2$^{-/-}$ VSMCs exhibit increased p-p130Cas at the leading edges of lamellipodia

Phosphorylation of p130Cas is important for its ability to act as a scaffolding protein and to control cell migration.[27,36] In addition, p-p130Cas is found at FAs in fibroblasts.[37] We examined the localization of p-p130Cas in non-migrating and migratory VSMCs. As in fibroblasts,[37] p-p130Cas was found at FAs in non-migrating Csrp2$^{+/+}$ (*Figure 4A*) and Csrp2$^{-/-}$ (*Figure 4E*) VSMCs that had been serum starved for 48 h. We also observed non-specific nuclear staining as previously noted.[37] Colocalization of p-p130Cas with FA marker paxillin further verified that p-p130Cas was indeed localized at FAs of Csrp2$^{+/+}$ and Csrp2$^{-/-}$ VSMCs (see Supplementary material online, *Figure S3*). The actin stress fibres of Csrp2$^{+/+}$ and Csrp2$^{-/-}$ VSMCs were revealed by green phalloidin staining (*Figure 4B* and *F*). The localization of p-p130Cas at FAs/terminal ends of actin stress fibres was further confirmed in the merged and magnified images in Csrp2$^{+/+}$ (*Figure 4C* and *D*) and Csrp2$^{-/-}$ (*Figure 4G* and *H*) VSMCs. Following PDGF-BB stimulation, in Csrp2$^{+/+}$ cells p-p130Cas was present in FAs and leading edges

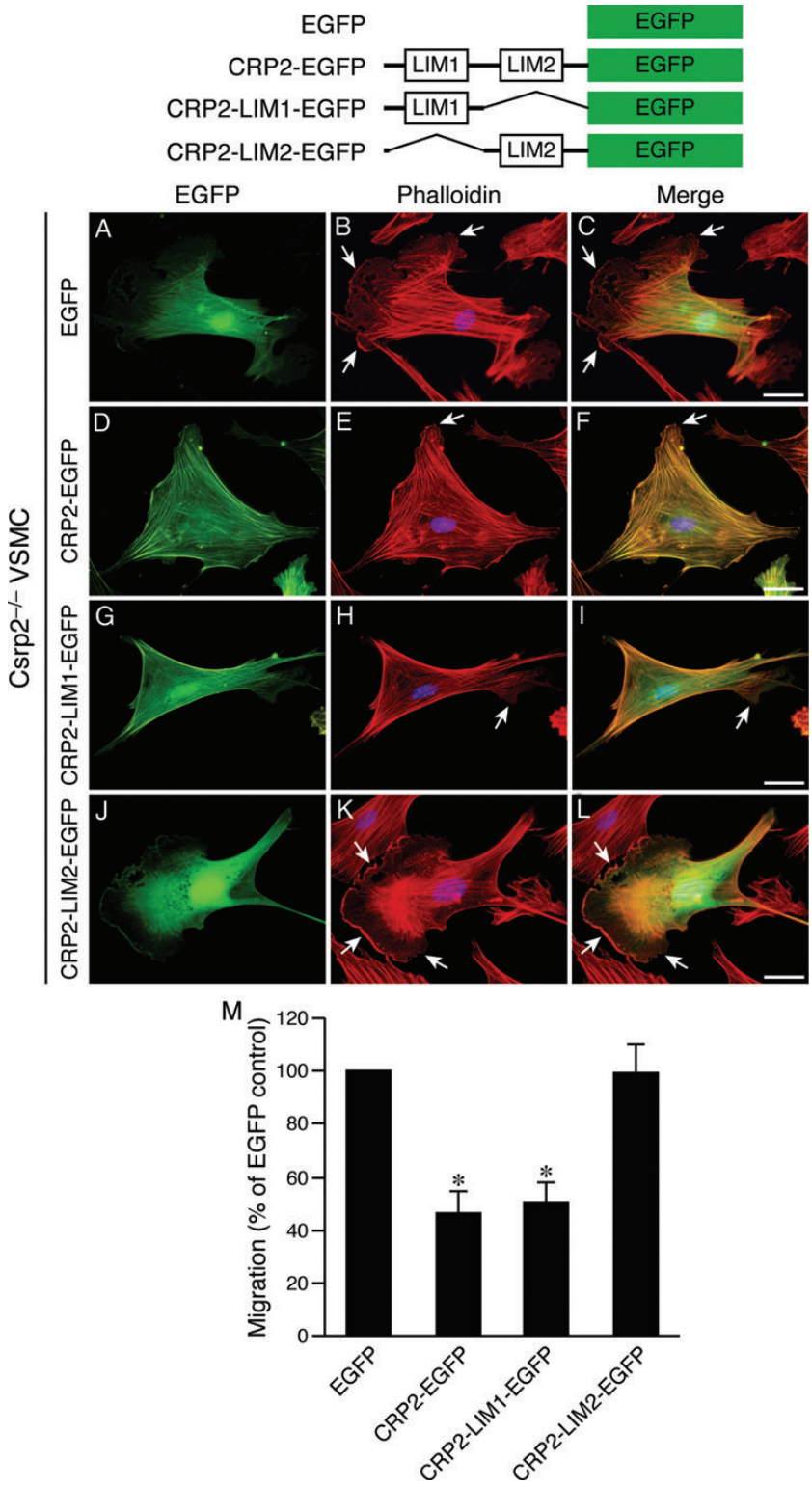

Figure 2 Re-expression of CRP2 in Csrp2$^{-/-}$ VSMCs inhibits PDGF-BB-induced lamellipodia formation and migration. Csrp2$^{-/-}$ VSMCs were transfected with pEGFP vector (*A*–*C*), pCRP2-EGFP (*D*–*F*), pCRP2-LIM1-EGFP (*G*–*I*), or pCRP2-LIM2-EGFP (*J*–*L*) expression plasmids, plated overnight, and serum-starved for 2 days. Cells were then treated with PDGF-BB for 30 min, fixed, and stained with Alexa Fluor 546 phalloidin. The green (GFP; *A*, *D*, *G*, and *J*), red (phalloidin; *B*, *E*, *H*, and *K*), and blue (DAPI) fluorescence were imaged. Colocalization of GFP and actin stress fibres is revealed by yellow colour in merged images (*C*, *F*, *I*, and *L*). Arrows indicate lamellipodia. Images are representatives from three independent experiments. Scale bar, 50 μm. (*M*) Csrp2$^{-/-}$ VSMCs were transfected with various expression plasmids for migration assays. The green cells migrating through the membranes in response to PDGF-BB after 4 h were quantified. The number of migrated green cells was normalized to total number of green cells initially plated on the upper chamber for each construct. Migration is expressed relative to the pEGFP control vector. Values are means ± SE of three experiments. $^{*}P < 0.05$ vs. EGFP.

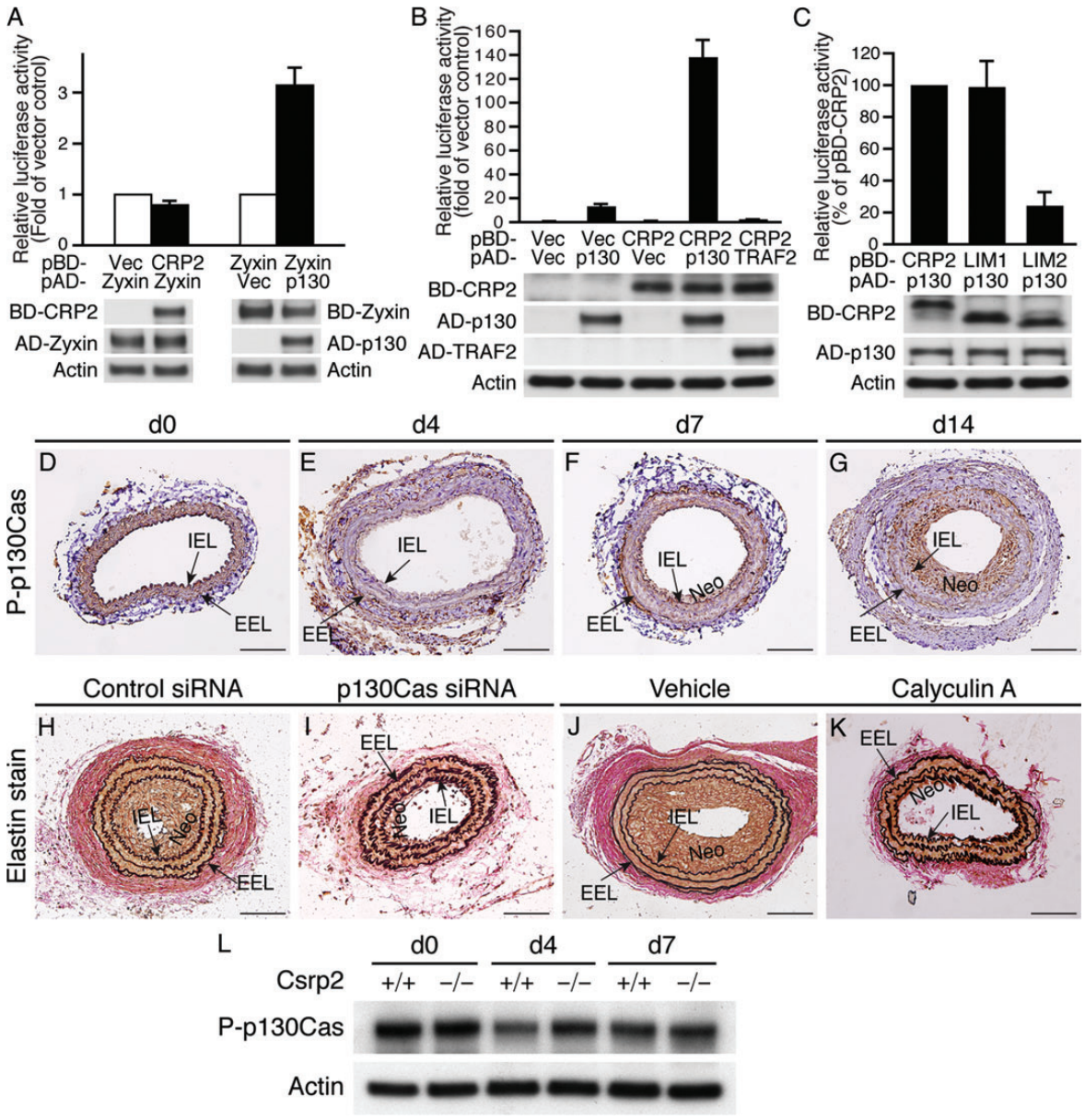

Figure 3 CRP2 interaction with p130Cas and the role of p130Cas in vascular remodelling. (*A*–*C*) CRP2 interacts with p130Cas by mammalian two-hybrid assays. AD293 cells were cotransfected with the mammalian two-hybrid reporter construct pFR-Luc and pBD- and pAD-expression plasmids. After 24 h, cells were harvested for luciferase activity as a measure of protein–protein interaction. (*A*) Upper: luciferase activity of zyxin with respective empty vector was set as 1. Lower: protein expressions were verified by western analyses. The BD-bait fusion proteins were detected by GAL4-BD antibody and the AD-target fusion proteins were detected by NF-κB antibody. (*B*) Upper: luciferase activity is expressed relative to pBD- and pAD-empty expression vectors. pAD-TRAF2 was included as a negative interaction control. Lower: immunoblot analyses reveal expression of the corresponding fusion proteins. (*C*) Interactions of p130Cas with a series of BD-CRP2 fusion plasmids harbouring full length, LIM1 domain, or LIM2 domain. Luciferase activity resulting from pBD-CRP2 and pAD-p130Cas interaction is set as 100%. Western blotting was performed to confirm fusion protein expression. Luciferase values are means ± SE of at least three experiments. For western blotting, equal loading of total proteins was verified by actin expression and a representative of three independent experiments is shown. (*D*–*G*) P-p130Cas expression in the normal and injured arterial wall. Male mice were subjected to a neointima formation model by ligating the left common carotid artery (LCCA). At different days following ligation, the ligated LCCA were harvested for immunohistochemistry to detect p-p130Cas levels. Brown colour indicates positive p-p130Cas staining. Internal elastic lamina (IEL) and external elastic lamina (EEL) are indicated by arrows. The neointima (Neo) in 7 days (*F*) and 14 days (*G*) of ligated LCCA is marked. (*H*–*K*) Pluronic gel containing siRNA (*H*, control; *I*, p130Cas) or p130Cas phosphorylation inhibitor (*J*, vehicle DMSO; *K*, calyculin A) was placed around LCCA 15 min before ligation. Ligated LCCA was harvested 14 days later for Verhoeff's staining to reveal elastin (black). IEL and EEL are indicated by arrows and neointima is indicated by Neo. Scale bar, 100 μm. The representative sections are shown ($n = 3$ for each group). (*L*) The LCCA of $Csrp2^{+/+}$ and $Csrp2^{-/-}$ were ligated and proteins extracted after different days for western blot analysis to detect p-p130Cas levels. At 4 days following ligation, the p-p130Cas levels were higher in $Csrp2^{-/-}$ LCCA than in $Csrp2^{+/+}$ LCCA. Equivalent loading was verified by actin expression.

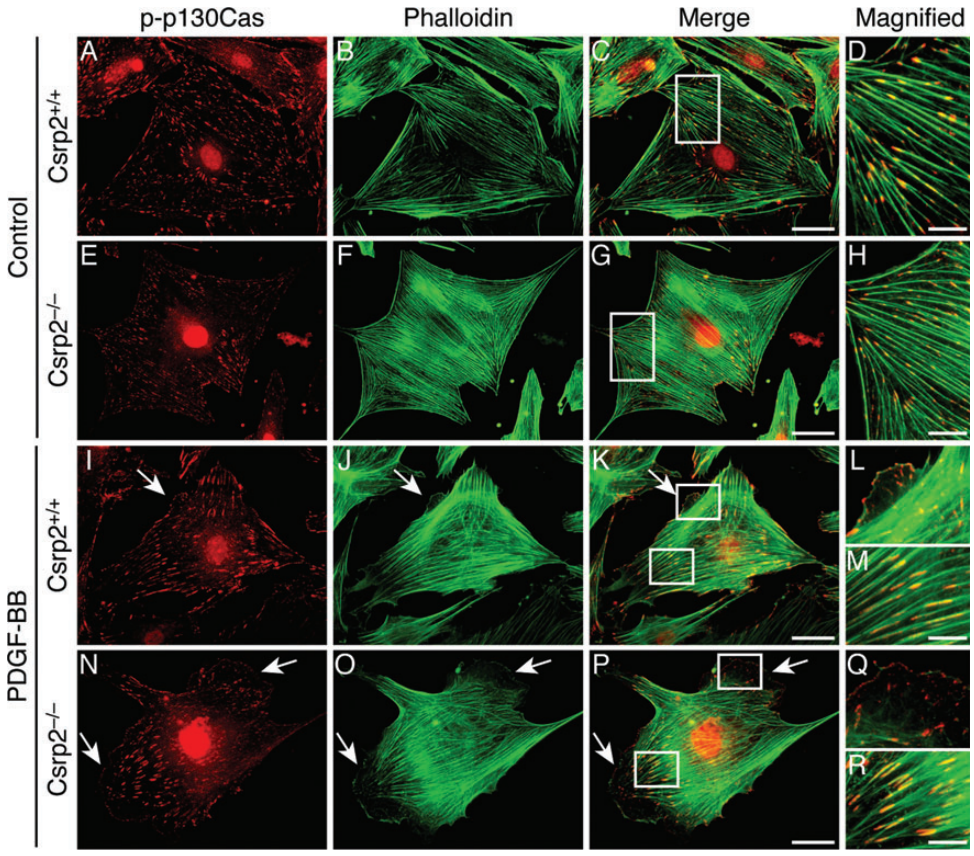

Figure 4 Subcellular localization of p-p130Cas in non-migrating and migratory Csrp2$^{+/+}$ and Csrp2$^{-/-}$ VSMCs. Cells were plated overnight, serum starved for 2 days, and treated without or with PDGF-BB for 30 min, fixed, and co-stained with p-p130Cas (p-p130Cas) antibody (*A*, *E*, *I*, and *N*; red) and Alexa Fluor 488 phalloidin (*B*, *F*, *J*, and *O*; green). In Csrp2$^{+/+}$ (*A*–*D*) and Csrp2$^{-/-}$ (*E*–*H*) control cells without PDGF-BB stimulation, p-p130Cas is localized to FAs (*A* and *E*). (*B* and *F*) Actin stress fibres are shown by green phalloidin staining. (*C* and *G*) Yellow colour in merged images indicates colocalization of p-p130Cas and actin stress fibres. (*D* and *H*) Higher magnification of the boxed areas in *C* and *G*, respectively. Following PDGF-BB stimulation, migrating Csrp2$^{+/+}$ cells (*I*–*M*) exhibit small lamellipodia (arrow). (*I* and *K*) P-p130Cas is present at FAs and leading edge of the small lamellipodia. (*L* and *M*) Higher magnification of the two boxed areas of K for lamellipodia (*L*) and FAs (*M*). (*N*–*R*) Increased p-p130Cas at the leading edge of lamellipodia in Csrp2$^{-/-}$ VSMCs following PDGF-BB stimulation. Migrating Csrp2$^{-/-}$ cells (*N*–*P*) exhibit increased lamellipodia formation (arrows). (*N* and *P*) P-p130Cas is present at FAs and leading edge of the large lamellipodia. (*Q*) Higher magnification of lamellipodia from the upper boxed area in *P* reveals the presence of p-p130Cas in the broad leading edge of lamellipodia. (*R*) Higher magnification of FAs from the lower boxed area in *P*. Scale bar, 50 μm in merged and 15 μm in magnified images.

of the small lamellipodia (*Figure 4I–M*). In comparison with Csrp2$^{+/+}$ cells, enhanced p-p130Cas staining was observed on the leading edges of large lamellipodia in Csrp2$^{-/-}$ cells (*Figure 4N–R*).

3.7 CRP2 colocalizes with p-p130Cas at FAs but not at leading edges of lamellipodia in migratory VSMCs

Given that p-p130Cas was localized to the leading edges of lamellipodia in migratory VSMCs (*Figure 4L* and *Q*) and that CRP2 interacted with p130Cas (*Figure 3*), we next evaluated whether CRP2 colocalized with p-p130Cas at the tips of lamellipodia. To avoid the influence of endogenous CRP2, we transfected pEGFP vector or pCRP2-EGFP plasmid into Csrp2$^{-/-}$ VSMCs, stimulated cells without or with PDGF-BB, and then examined EGFP and p-p130Cas localization. In pEGFP-transfected cells, EGFP green fluorescence was distributed to the cytosol and the nucleus (*Figure 5A* and *E*). P-p130Cas was detectable at FAs without PDGF-BB stimulation (*Figure 5B–D*, red). PDGF-BB increased lamellipodia formation and p-p130Cas was localized at both FAs and lamellipodia (*Figure 5F–G*). Higher magnification further revealed the presence of p-p130Cas at the leading edges of lamellipodia (*Figure 5H*, *arrow*), corroborating the results of *Figure 4Q*. CRP2-EGFP expression (*Figure 5I* and *M*) revealed that CRP2 colocalized with p-p130Cas at FAs/terminal ends of stress fibres in control (*Figure 5J–L*) and PDGF-BB-stimulated cells (*Figure 5N–P*). As expected, CRP2-EGFP expression reduced PDGF-BB-induced lamellipodia formation (*Figure 5N* and *O*). Intriguingly, only p-p130Cas but not CRP2 was present at the leading edges of the small lamellipodia (*Figure 5P*, *arrow*), suggesting CRP2 might sequester p-p130Cas at FAs in migratory VSMCs.

To examine the association of CRP2 and p130Cas in VSMCs, we performed co-immunoprecipitation assays using cell lysates from pEGFP- or pCRP2-EGFP-transfected Csrp2$^{-/-}$ cells. Expression of endogenous p130Cas and exogenous EGFP or CRP2-EGFP was confirmed by western blot analyses (*Figure 5Q*, *left*). Importantly, GFP antibody

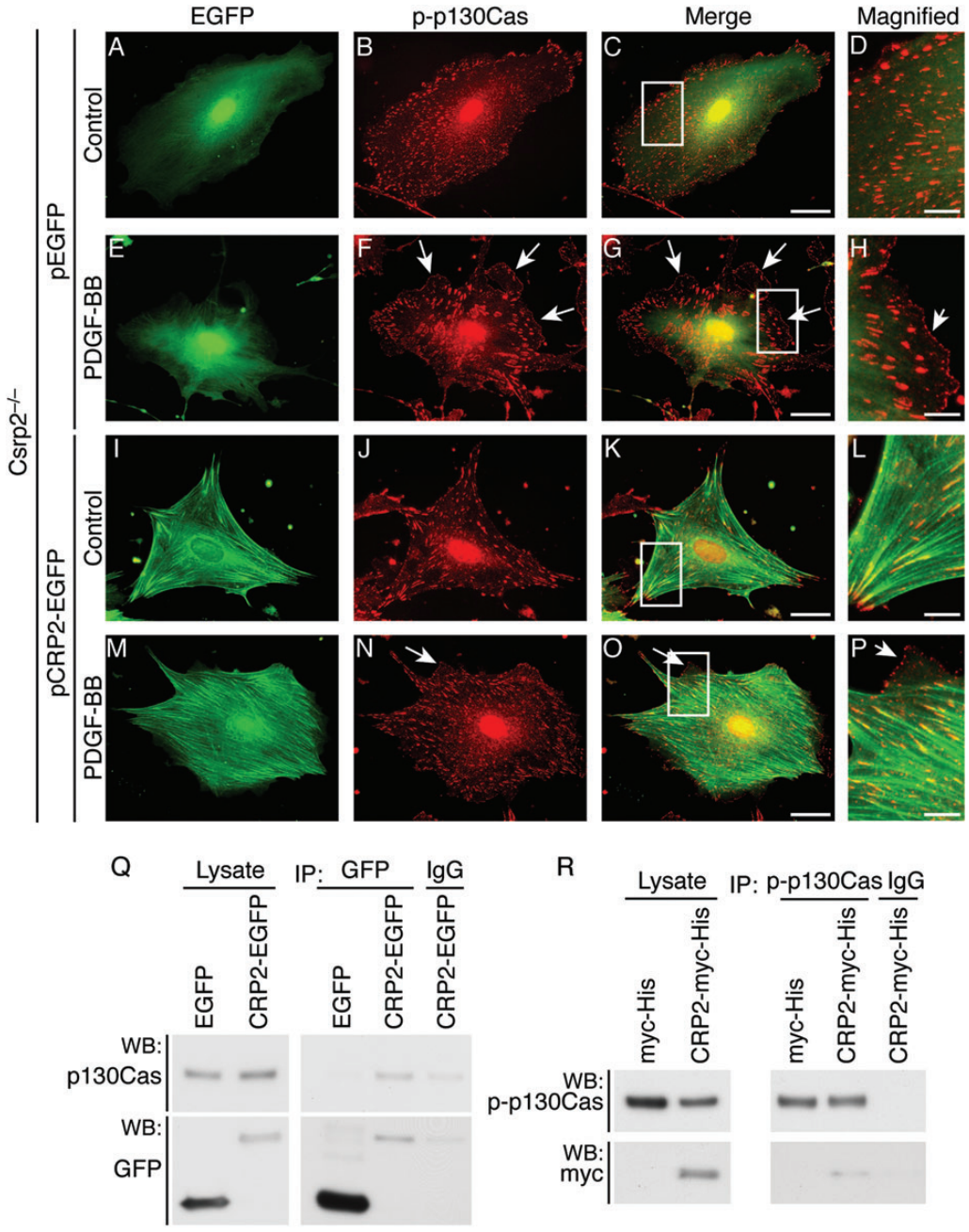

Figure 5 CRP2 colocalizes with p-p130Cas at FAs but not leading edge of lamellipodia in VSMCs. Csrp2$^{-/-}$ VSMCs were transfected with pEGFP (*A*–*H*) or pCRP2-EGFP (*I*–*P*) plasmids, treated without (*A*–*D* and *I*–*L*) or with PDGF-BB (*E*–*H* and *M*–*P*) for 30 min and immunostained with p-p130Cas antibody. Green fluorescence indicates EGFP (*A*, *E*, *I*, and *M*) while red fluorescence indicates p-p130Cas (*B*, *F*, *J*, and *N*). In control pEGFP-transfected cells, EGFP is localized to the cytosol, whereas p-p130Cas is localized to FAs (*A*–*D*). (F–G) PDGF-BB stimulation increased lamellipodia (*arrows*) and p-p130Cas is present at FAs and lamellipodia. Higher magnification of the box in *G* reveals p-p130Cas localizes at the leading edge of lamellipodia (*H*, *arrow*). In control pCRP2-EGFP-transfected cells, EGFP is localized to actin stress fibres (*I*) while p-p130Cas at FAs (*J*). Merged images reveal that CRP2 colocalizes with p-p130Cas at FAs/terminal ends of stress fibres (*K* and *L*, *yellow*). CRP2-EGFP expression reduces PDGF-BB-stimulated lamellipodia formation (*M*–*O*). Higher magnification of the box in (*O*) shows that only p-p130Cas, but not CRP2 is present at the leading edge of lamelliipodia (*P*, *arrow*). Scale bar, 50 μm in merged and 15 μm in magnified images. (*Q*) CRP2 associates with endogenous p130Cas in VSMCs. Csrp2$^{-/-}$ VSMCs were transfected with control pEGFP or pEGFP-CRP2. Cell lysates were harvested 48 h after transfection and immunoprecipitated (IP) with anti-GFP or control normal IgG antibody. Immunoblotting (WB) was subsequently performed with anti-p130Cas and anti-GFP antibodies. A representative of three independent experiments is shown. (*R*) Csrp2$^{-/-}$ VSMCs were transfected with pcDNA3.1/myc-His vector or pcDNA3.1/CRP2-myc-His plasmids and co-immunoprecipitation performed using p-p130Cas or control IgG antibodies. Membranes were then blotted with p-p130Cas or myc antibodies.

co-immunoprecipitated p130Cas from pCRP2-EGFP-transfected but not pEGFP-transfected cell lysate while control IgG immunoprecipitated barely detectable p130Cas and CRP2-EGFP (*Figure 5Q, right*). To demonstrate further the association of CRP2 with phosphorylated p130Cas, we transfected Csrp2$^{-/-}$ VSMCs with another set of expression plasmids—pcDNA3.1/myc-His vector and pcDNA3.1/CRP2-myc-His. Co-immunoprecipitation assays with p-p130Cas antibody (IgG served as a negative control) demonstrated that endogenous p-p130Cas was associated with CRP2 (*Figure 5R*).

3.8 The substrate domain of p130Cas is required for CRP2 interaction

We next sought to identify domains in p130Cas that mediate its interaction with CRP2. The SH3 domain of p130Cas is critical for p130Cas to target to FAs, thus we first deleted the N-terminal SH3 domain (pAD-p130CasΔSH3) (*Figure 6A*) and tested whether this deletion would affect the interaction with CRP2 in mammalian two-hybrid assays. Compared with full-length p130Cas (pAD-p130Cas(2−870)), deletion of the SH3 domain did not affect the interaction between p130Cas and CRP2 (*Figure 6B*). Since the substrate domain of p130Cas has been reported to mediate the association with zyxin family members of TRIP6 and zyxin,[29] we next created a mutant construct with N-terminal deletion that included SH3, proline-rich, and substrate domains (p130CasΔSH3-Pro-SD). This further deletion abrogated luciferase activity (*Figure 6B*), suggesting the substrate domain might mediate the interaction. Indeed, internal deletion of substrate domain (pAD-p130CasΔSD) substantially reduced luciferase activity, indicating disruption of the interaction with CRP2 (*Figure 6B*). Western blotting showed comparable expression levels of various fusion proteins, indicating that the lack of interaction was not due to differences in fusion protein expression (*Figure 6B, lower*). These data demonstrate that the p130Cas substrate domain is required for interaction with CRP2.

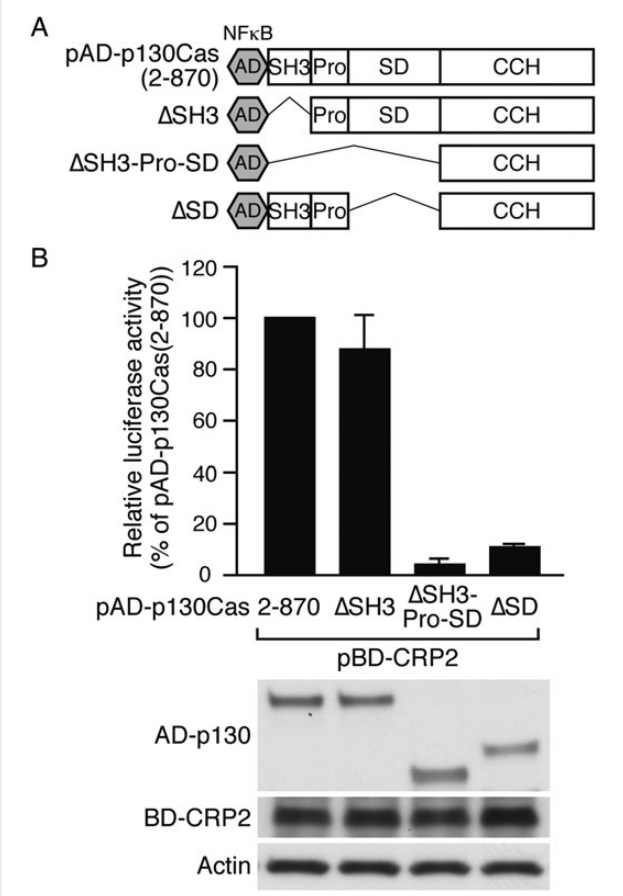

Figure 6 The substrate domain of p130Cas is required for interaction with CRP2. (*A*) pAD-p130Cas deletion constructs with NFκB activation domain (NFκB AD) fused at the N-terminus. SH3, Src homology 3 domain; Pro, proline-rich domain; SD, substrate domain containing 15 repeats of YxxP; CCH, Cas family C-terminal homology domain. (*B*) AD293 cells were cotransfected with the mammalian two-hybrid reporter construct pFR-Luc, pBD-CRP2, and pAD-p130Cas deletion constructs. After 24 h, cells were harvested for luciferase activity as a measure of protein−protein interaction. Upper: luciferase activity of pAD-p130Cas(2−870) with pBD-CRP2 was set as 100%. Values are means ± SE of three experiments. Lower: western analyses were performed with total protein. Blots were incubated with NFκB antibody to detect various AD-p130Cas fusion proteins. BD-CRP2 fusion proteins were detected by GAL4 antibody. Blots were subsequently probed with pan-actin antibody to verify equal loading. A representative of three independent experiments is shown.

4. Discussion

We previously reported that the absence of CRP2 promotes VSMC migration and increases neointima formation following arterial injury,[16] demonstrating an important function of CRP2 in vascular remodelling. In the present study, we show that in response to chemoattractant stimulation, CRP2 reduces lamellipodia formation and subsequent cellular migration. CRP2-reduced migration is dependent upon its actin stress fibre-targeting ability that is mediated by LIM1 domain. Through its LIM1 domain, CRP2 associates directly with the FA scaffold protein p130Cas via the substrate domain of p130Cas. More importantly, the interaction of CRP2 and p-p130Cas at FAs sequesters p130Cas at FAs, thereby reducing lamellipodia formation and VSMC migration. Furthermore, our study reveals a previously unrecognized critical function of p130Cas and its phosphorylation in the response to arterial injury.

Although CRP2 has been reported to form complexes with SRF and GATA transcription factors in the nucleus and facilitate SMC marker gene expressions,[15] we show that CRP2 is predominantly localized to actin stress fibres of VSMCs (*Figures 2* and *5*, see Supplementary material online, *Figure S1*). This is consistent with initial reports implicating the CRP family of proteins in promoting protein assembly along the actin-based cytoskeleton,[7,11] and emphasizes a critical cytoskeletal function of CRP2 in VSMCs. Indeed, CRP2 associated directly with FA scaffold protein p130Cas (*Figures 3* and *5*), which is in agreement with previous findings that several LIM proteins can interact with p130Cas and regulate downstream signalling for cell motility.[29−31]

It is well established that p130Cas serves as a versatile scaffold in signalling networks that modulate numerous cellular processes.[27,28] A critical function of p130Cas in regulating cell migration has been shown in various cell types such as carcinoma, glioma, and endothelial cells.[23,38] In VSMCs, nitric oxide decreases cell motility via increasing PTP-PEST activity and dephosphorylation of its substrate p130Cas[39,40] while angiotensin II causes Src and p130Cas association, phosphorylation of p130Cas and subsequent VSMC migration.[41] Neuropilin-1 mediates PDGF-stimulated VSMC migration and signalling via p130Cas.[42] In migratory VSMCs, our results indicate that CRP2 colocalizes with

p-p130Cas at FAs but not leading edges of lamellipodia (*Figure 5*) and did not alter cellular adhesion and spreading (*Figure 1*). Taken together, these findings support a model where CRP2 might function to sequester p-p130Cas at FAs in migrating VSMCs. These findings are in contrast to that of LIM protein Ajuba.[30] Ajuba-null mouse embryonic fibroblasts (MEFs) have reduced lamellipodia formation and migratory cues-induced Rac1 activation, whereas Csrp2$^{-/-}$ VSMCs have increased lamellipodia formation (*Figures 1* and *2*) and enhanced Rac1 activation.[16] While Ajuba functions to localize p130Cas to nascent focal complexes at the leading edges of migrating MEFs, we found that CRP2 likely plays an opposite role by sequestering p130Cas at FAs (*Figure 5*). Interestingly, while Ajuba interacts with the p130Cas C-terminal homology domain, CRP2 interacts with its substrate domain (*Figure 6*). These findings partially explain the differential functions of Ajuba and CRP2 in altering p130Cas subcellular localization. Furthermore, CRP2 might block the function of substrate domain that is important for downstream Rac1 activation. Supporting this notion, substrate domain of p130Cas has been shown to be functionally important for VSMC migration as expression of a substrate domain-deleted p130Cas decreases motility of VSMCs.[39] Interestingly, zyxin interacts with substrate domain of p130Cas[29] and zyxin-null fibroblasts exhibit enhanced migration.[43] This further supports the model that CRP2 and zyxin attenuate p130Cas migratory signalling by inhibiting substrate domain function of p130Cas. Whether CRP2 and zyxin compete in binding to p130Cas at the same site remain to be investigated. While our results show that LIM1 is essential for CRP2's cytoskeletal functions LIM2 can associate with GATA transcription factors to promote SMC gene transcription,[15] suggesting that LIM1 and LIM2 may play distinct roles.

Consistent with a role of p130Cas and its phosphorylation in vascular remodelling, vascular injury modulated p130Cas phosphorylation levels (*Figure 3D–G*). The high levels of p-p130Cas in medial SM layers of normal arteries might reflect the requirement for p-p130Cas at FAs for the differentiated VSMC phenotype. Interestingly, p-p130Cas levels were down-regulated 4 days after carotid artery ligation. It has been reported that 4 days after vascular injury medial SMCs start to exhibit a migratory phenotype.[44,45] Therefore, the rapid down-regulation of p-p130Cas might indicate that at this time point medial SMCs are changing to a de-differentiated phenotype and disassemble FAs to facilitate migration. Supporting this notion, p-p130Cas levels increased thereafter in the medial layers and neointima at 7 days and were further enhanced in the neointima 14 days after ligation. The critical importance of p130Cas expression and phosphorylation in neointima formation was further demonstrated by that p130Cas knockdown or pharmacological inhibition of p130Cas phosphorylation resulted in reduced neointima size (*Figure 3H–K*). Intriguingly, 4 days after carotid artery ligation p-p130Cas levels were higher in Csrp2$^{-/-}$ than in Csrp2$^{+/+}$ carotids (*Figure 3L*), suggesting that enhanced p130Cas phosphorylation might contribute to increased Csrp2$^{-/-}$ VSMC migration and the resultant larger neointima.[16] It remains to be investigated as how p-p130Cas levels are elevated in the carotid artery from Csrp2$^{-/-}$ mice 4 days after ligation.

In conclusion, we demonstrate that CRP2 associates with actin cytoskeleton of VSMCs through its LIM1 domain, which is required for CRP2's interaction with the FA scaffold protein p130Cas. In response to migratory cues, the interaction of CRP2 with p130Cas attenuated lamellipodia formation, leading to reduced migratory ability of VSMCs. Furthermore, we show for the first time that p130Cas and its phosphorylation promote neointima formation following arterial injury. Given that the migration-related proteins are not VSMC restricted, the preferential expression of CRP2 in arteries might serve as a key regulatory step in VSMC migration and subsequent vascular remodelling. Taken together, LIM1 domain of CRP2 and p130Cas/p-p130Cas might be potential therapeutic targets for vascular disease.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Acknowledgements

This research was conducted under the Graduate Program of Biotechnology in Medicine sponsored by the National Tsing Hua University and the National Health Research Institutes. We thank the Pathology Core Laboratory of the National Health Research Institutes for processing and embedding of arterial samples. We thank Wun-Chu Fan for assistance in histological analysis.

Conflict of interest: none declared.

Funding

This work was supported by the National Science Council (Taiwan) (101-2320-B-400-002-MY3 and 98-2320-B-400-004-MY3 to S.F.Y.); National Health Research Institutes (CS-102-PP-05 to S.F.Y.); and the National Institutes of Health (HL-078869 to M.D.L.).

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