

Interaction of the Human Prostacyclin Receptor with the PDZ adapter protein PDZK1: Role in Endothelial Cell Migration and Angiogenesis

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Running Title: Interaction of PDZK1 with the human Prostacyclin Receptor.

Abbreviations

DDO, double drop-out; FTI, Farnesyl transferase inhibitor; HA, hemagglutinin; hIP, human prostacyclin receptor; HUVEC, human umbilical vein endothelial cell; IP, prostacyclin receptor; PKA, cAMP-dependent protein kinase A; PDZ, Postsynaptic density-95, Discs large, Zonula occludens-1; PDZK1, PDZ domain-containing protein 1; PPAR, peroxisome proliferator-activated receptor; QDO, quadruple drop-out; RBD, Rab11 binding domain; TP, thromboxane receptor; Y2H, yeast-two-hybrid.

Abstract.

Prostacyclin is increasingly implicated in re-endothelialization and angiogenesis but through largely unknown mechanisms. Herein, the HDL scavenger receptor class B, type 1 (SR-B1) adapter protein PDZ domain-containing protein 1 (PDZK1) was identified as an interactant of the human prostacyclin receptor (hIP) involving a Class I PDZ ligand at its carboxyl-terminus and PDZ domains 1, 3 and 4 of PDZK1. While the interaction is constitutive, it may be dynamically regulated following cicaprost-activation of the hIP through a mechanism involving cAMP-dependent protein kinase (PK)A-phosphorylation of PDZK1 at Ser⁵⁰⁵. While PDZK1 did not increase overall levels of the hIP, it increased its functional expression at the cell surface enhancing ligand binding and cicaprost-induced cAMP generation. Consistent with its role in re-endothelialization and angiogenesis, cicaprost-activation of the hIP increased endothelial cell migration and tube formation/*in vitro* angiogenesis, effects completely abrogated by the specific IP antagonist RO1138452. Furthermore, similar to HDL/SR-B1, *siRNA*-targeted disruption of PDZK1 abolished cicaprost-mediated endothelial responses but did not affect VEGF-responses. Considering the essential role played by prostacyclin throughout the cardiovascular system, identification of PDZK1 as a functional interactant of the hIP sheds significant mechanistic insights into the protective roles of these key players, and potentially HDL/SR-B1, within the vascular endothelium.

Introduction.

Eukaryotic proteins are modular by nature. The frequently encountered Postsynaptic density-95, Discs large, Zonula occludens-1 (PDZ) domain mediates protein:protein interactions by binding to the 'PDZ ligand' located most typically, but not exclusively, at the extreme *C-termini* of target proteins (Jemth and Gianni, 2007; Tonikian *et al.*, 2008; Lee and Zheng, 2010). Through the formation of multi-protein complexes, PDZ interactions can participate in the co-ordination of key intra- and inter-cellular signalling systems including intracellular routing or localization of proteins, cell polarity as well as in the regulation of cell:cell interactions (Jemth and Gianni, 2007; Tonikian *et al.*, 2008; Lee and Zheng, 2010). Structurally, the PDZ domain is composed of compact globular modules containing six *anti*-parallel β -strands (β A to β F) and two α -helices (α A and α B) with a highly conserved 'GLGF motif' within its hydrophobic binding pocket that is responsible for the sequence-specific recognition of the PDZ ligand within the target protein(s). Depending on the nature of the three residues at their extreme C-termini, the PDZ ligand of the target protein itself may belong to one of three classes, namely Class I (Ser/Thr-X- Φ -COOH), Class II (Φ -X- Φ -COOH) or Class III (Asp/Glu-X- Φ -COOH), where Φ represents a hydrophobic amino acid and X can be any residue (Jemth and Gianni, 2007; Tonikian *et al.*, 2008; Lee and Zheng, 2010).

The intracellular scaffold or adapter protein PDZ domain-containing protein 1 (PDZK1) is a member of the Na^+ , H^+ exchanger regulatory family (NHERF) and is predominantly expressed in the brush border of the kidney and small intestine, in epithelial and endothelial cells, in macrophages and in the liver (Kocher *et al.*, 1998; Lamprecht and Seidler, 2006; Kocher and Krieger, 2009). PDZK1 contains 4 PDZ domains, facilitating its binding to highly specific interacting partners including various ion transporters (e.g the cystic fibrosis transmembrane conductance regulator (CFTR) and apical organic cation transporters OCTN1 and OCTN2), inducible nitric oxide synthase (iNOS) and certain members of the G-protein coupled receptor (GPCR) superfamily (Kato *et al.*, 2005; Navarro-Lerida *et al.*, 2007; Hu *et al.*, 2009). Most notably, through its interaction with the high density lipoprotein (HDL) high affinity scavenger receptor class B, type 1 (SR-B1), PDZK1 is essential for both reverse cholesterol transport (RCT) and for HDL-mediated vascular re-endothelialization (Zhu *et al.*, 2008; Kocher and Krieger, 2009). More specifically, by binding to its C-terminal PDZ ligand, PDZK1 plays an essential role in maintaining hepatic SR-B1 levels, thereby controlling HDL cholesterol levels, and is now also known to play a key role in HDL/SR-B1-dependent regulation of endothelial cell migration, promoting re-endothelialization and protecting against the development of atherosclerosis (Kocher *et al.*, 2003; Zhu *et al.*, 2008; Fenske *et al.*, 2009).

The prostanoid prostacyclin, or prostaglandin I_2 , plays a central role in haemostasis acting as a potent inhibitor of platelet aggregation and as an endothelium-derived vasodilator (Gryglewski, 2008; Kawabe *et al.*, 2010a). It exerts an important cytoprotective role within the myocardium (Ribeiro *et al.*, 1981) and, within the wider vasculature, promotes angiogenesis and limits restenosis promoting re-endothelialization/vascular repair in response to injury (Kawabe *et al.*, 2010a). The importance of prostacyclin for haemostasis and cardiovascular integrity has been highlighted by the finding that certain COXIBs, selective inhibitors of cyclooxygenase 2, disproportionately depress prostacyclin generation, leaving subjects at increased risk of thrombotic stroke and myocardial infarction (Fitzgerald, 2004). Moreover, prostacyclin receptor ($\text{IP}^{-/-}$) null mice display enhanced tendency toward thrombosis, intima hyperplasia, atherosclerosis and restenosis (Murata *et al.*, 1997; Yuhki *et al.*, 2011). More recently, in an experimental model of endothelial damage, it was established that endothelial progenitor cells (EPCs) from $\text{IP}^{-/-}$ null mice fail to promote re-endothelialization and vessel repair (Kawabe *et al.*, 2010b). Hence, somewhat similar to the HDL/SR-B1-mediated pathway, the prostacyclin receptor (IP) plays a central protective role in promoting re-endothelialization, limiting neointima hyperplasia and vascular remodeling in response to vessel wall injury that frequently accompanies atherosclerosis or surgical procedures such as angioplasty or carotid endarterectomy, for example (Qu *et al.*, 2009; Kawabe *et al.*, 2010b). Somewhat consistent with this, several single nucleotide polymorphisms have been identified within the IP gene that predispose individuals to CV disease including enhanced risk of deep vein thrombosis and intimal hyperplasia (Archart *et al.*, 2008; Patrignani *et al.*, 2008).

While, as stated, prostacyclin mainly signals through the IP, a member of the GPCR superfamily (Gryglewski, 2008; Kawabe *et al.*, 2010a), it can also modulate the peroxisome proliferator-activated receptor (PPAR) δ signalling pathway(s), also with important clinical implications for angiogenesis (Pola *et al.*, 2004; Biscetti *et al.*, 2008; Biscetti and Pola, 2008; He *et al.*, 2008; Biscetti *et al.*, 2009). The IP is primarily coupled to Gs/adenylyl cyclase activation but may regulate other effectors in a cell- and/or species specific manner (Lawler *et al.*, 2001b; Miggin *et al.*, 2002). The IP is somewhat unique among GPCRs in that it undergoes both isoprenylation and palmitoylation within its carboxyl-terminal tail (C-tail) domain, modifications that are critical for its signalling and function and intracellular trafficking (Hayes *et al.*, 1999; Lawler *et al.*, 2001a;

Miggin *et al.*, 2002, 2003; O'Meara and Kinsella, 2004b, a, 2005; Reid *et al.*, 2010). More specifically, the human IP (hIP) undergoes farnesylation at Cys³⁸³ within its carboxy-terminal conserved -C³⁸³SLC³⁸⁶, or 'CaaX', motif (Hayes *et al.*, 1999; Miggin *et al.*, 2002) and palmitoylation at Cys³⁰⁸, Cys³⁰⁹ and Cys³¹¹, residues adjacent to a recently identified Rab11 binding domain (RBD) within the proximal C-tail of the hIP (Miggin *et al.*, 2003; Reid *et al.*, 2010).

While, as stated, recent emerging data further highlights a central role for prostacyclin and the IP as a critical protective agent within the vascular endothelium, the underlying mechanisms whereby they do so remains to be defined. Herein, we report the identification of a novel specific interaction between the hIP and the intracellular SR-B1 adapter protein PDZK1. The aim of this study was to characterise this novel protein:protein interaction and to explore its impact on hIP function, not least within vascular endothelial cells. Data presented herein identify a novel mechanism of agonist- regulated interaction of PDZK1 with the hIP involving direct cAMP-dependent protein kinase (PK)A phosphorylation of PDZK1 in response to agonist-activation of the hIP itself. Moreover, in the context of re-endothelialization, it was established that, similar to SR-B1, the interaction of the hIP with PDZK1 is functionally critical for prostacyclin-mediated endothelial cell migration and angiogenesis in recognised *in vitro* models. Bearing in mind the critical roles of the hIP, PDZK1 and, indeed, SR-B1 within the vascular endothelium (Kocher and Krieger, 2009; Kawabe *et al.*, 2010a), the discovery of the interaction between the hIP and PDZK1 is likely to shed significant new insights into the mechanistic processes involved in endothelial integrity and in protection against CV disease.

Results

Identification of PDZK1 as an interactant of the hIP in yeast and mammalian cells

We recently carried out a yeast-two-hybrid (Y2H) screen of a human kidney cDNA library to identify proteins that specifically interact with the carboxyl (C)-terminal tail domain of the hIP (hIP²⁹⁹⁻³⁸⁶; **Figure 1A**). Through those studies, Rab11a was identified as a direct binding partner of the hIP through an interaction dependent on a 14 residue Rab11 binding domain (RBD) located within its proximal C-tail domain, comprising Val²⁹⁹-Val³⁰⁷ adjacent to the palmitoylated residues at Cys³⁰⁸ – Cys³¹¹ (Wikstrom *et al.*, 2008; Reid *et al.*, 2010). Herein, we report the identification of the intracellular adapter protein PDZK1 as a direct interactant of the hIP. Using the hIP²⁹⁹⁻³⁸⁶ as the initial bait protein, Y2H screening identified several independent clones that encode amino acids 1 – 154 of PDZK1 (PDZK1¹⁻¹⁵⁴), encompassing its entire PDZ domain 1 (PDZ^{D1}) and part of its PDZ^{D2} (Hu *et al.*, 2009; LaLonde and Bretscher, 2009).

To initially investigate specificity, the interaction of PDZK1¹⁻¹⁵⁴ was examined through extended Y2H mating-type studies with various sub-fragments of the C-tail domain derived from either the wild type (hIP^{299-386,WT}) or isoprenylation-defective (hIP^{299-386,SSLC}) forms of the hIP (Wikstrom *et al.*, 2008; Reid *et al.*, 2010). As controls for those studies, the interaction of PDZK1¹⁻¹⁵⁴ with the C-tail domains of the TPα (TPα³¹²⁻³⁴³) and TPβ (TPβ³¹²⁻⁴⁰⁷) isoforms of the human thromboxane (TX) A₂ receptor was examined while comparison of the interaction of the hIP-derived sub-fragments with PDZK1¹⁻¹⁵⁴ relative to that of Rab11a served as additional reference controls (**Figure 1B**). While all of the bait and prey yeast strains mated successfully to form diploids (**Figure 1B**; DDO), PDZK1¹⁻¹⁵⁴ only interacted with sub-fragments containing the C-terminal residues of the hIP domain (hIP²⁹⁹⁻³⁸⁶ or hIP³²⁰⁻³⁸⁶; **Figure 1B**; QDO). Conversely, PDZK1¹⁻¹⁵⁴ did not interact with the internal hIP²⁹⁹⁻³²⁰ sub-fragment, encoding the RBD only, or indeed with any of the controls including TPα³¹²⁻³⁴³, TPβ³¹²⁻⁴⁰⁷, p53 or the vector alone (**Figure 1B**; QDO). Furthermore, the interaction of PDZK1¹⁻¹⁵⁴ with the hIP²⁹⁹⁻³⁸⁶ or hIP³²⁰⁻³⁸⁶ sub-fragments was independent of the presence of an isoprenylation 'CaaX' motif associated with the wild type hIP (-C³⁸³LSC; e.g in hIP^{299-386,WT} and hIP^{320-386,WT}) or an isoprenylation-defective variant (-S³⁸³SLC; e.g in hIP^{299-386,SSLC} and hIP^{320-386,SSLC}) at its C-terminus. In addition, consistent with previous reports, but distinct from that of PDZK1¹⁻¹⁵⁴, Rab11a only interacted with sub-fragments containing an intact RBD, namely the hIP^{299-386,WT/SSLC} and hIP²⁹⁹⁻³²⁰ sub-fragments (Reid *et al.*, 2010), while p53 only interacted with SV-40 large T antigen which acted as an additional Y2H control (**Figure 1B**). Based on these data, PDZK1¹⁻¹⁵⁴ specifically interacts with the C-tail domain of the hIP through an interaction that is not affected by mutation of its CaaX motif (-C³⁸³LSC to -S³⁸³LSC).

Thereafter, the ability of full length PDZK1 (PDZK1¹⁻⁵¹⁹, here-on-in referred to as PDZK1) to interact with haemagglutinin (HA)-tagged forms of either the wild type hIP, hIP^{SSLC} or hIP^{Δ383} expressed in the previously characterised HEK.hIP, HEK.hIP^{SSLC} and HEK.hIP^{Δ383} cell lines (Miggin *et al.*, 2003) was examined through co-immunoprecipitations. PDZK1 was detected in the *anti*-HA immunoprecipitates with both the wild type hIP and isoprenylation defective hIP^{SSLC} and at similar levels in both cases (**Figure 1C**). In contrast, only trace amounts of PDZK1 was co-immunoprecipitated with the hIP^{Δ383} mutant devoid of its extreme four C-terminal residues including the entire CaaX motif (**Figure 1C**). PDZK1 was completely absent from the corresponding *anti*-HA:TPα immunoprecipitates from the control HEK.TPα cell line (**Figure 1C**). Such differences in the co-immunoprecipitation of PDZK1 with the HA-tagged receptors were not due to failure or variations of the immunoprecipitations *per se* or indeed due to differences in the levels of the Flag-tagged PDZK1 present in the cell lysates prior to immunoprecipitation (**Figure 1C**).

To further examine the possible influence of isoprenylation of the hIP on its interaction with PDZK1, the effect of inhibition of farnesylation of the hIP using the selective farnesyl transferase inhibitors (FTIs) R115777 and SCH66336 (O'Meara and Kinsella, 2004b, 2005) was examined. As a control for these studies, the ability of both FTIs to inhibit protein farnesylation was confirmed whereby they both efficiently inhibited farnesylation of the molecular chaperone HDJ-2 as evidenced by the increased accumulation of its non-farnesylated (49 kDa) in addition to its farnesylated (45 kDa) species (**Figure 2**; lower panels) (O'Meara and Kinsella, 2004b). Moreover, neither R115777 nor SCH66336 FTIs affected the co-immunoprecipitation of PDZK1 with the hIP (**Figure 2**).

Collectively, these data identify a novel physical interaction between the hIP and PDZK1 in both yeast and mammalian cells. While the interaction with PDZK1 requires the presence of the four C-terminal residues of the hIP, it is largely independent of the presence of a functional -CaaX motif or of the isoprenylation status of the hIP *per se*.

Characterisation of the 'PDZ ligand' within the hIP

PDZ domains have been described as ‘protein interaction modules’ that recognise and bind the C-terminal residues (also termed the ‘PDZ ligand’) of their target protein(s) and, as stated, depending on their sequence composition can be broadly classified into Classes I-III (Tonikian *et al.*, 2008). Y2H-based approaches were used as a convenient means to characterise the putative PDZ ligand at the C-terminus of the hIP whereby the effect of mutation of residues at the 0 to -3 positions (here-on-in defined as P₀, P₋₁, P₋₂ and P₋₃; **Figure 3**) to corresponding Ser or Ala residues, either alone or in combination, was investigated. As previous, each of the bait and prey strains mated successfully (**Figure 3**; DDO). Specific mutation of residues at the P₋₃ (hIP^{299-386,SSLC}) or P₋₁ (hIP^{299-386,CSAC}, a variant not predicted to be isoprenylated or hIP^{299-386,CSSC}, a variant predicted to be isoprenylated) positions did not affect interaction of the hIP with PDZK1¹⁻¹⁵⁴ (**Figure 3**). Conversely, mutation at the P₀ (hIP^{299-386,CSLS}) abolished the interaction while mutation at the P₋₂ (hIP^{299-386,CALC}) substantially reduced the interaction with PDZK1, suggesting that the C-terminal Cys³⁸⁶, at P₀, and Ser³⁸⁴ at P₋₂ are specifically required for the interaction. Moreover, combined mutation of the P_{0,-1,-2} (hIP^{299-386,CAAAA}), P_{0,-1,-2,-3} (hIP^{299-386,SAAAA}) or deletion of P_{0,-1,-2} (IP^{299-386,C-stop} or hIP^{299-386,S-stop}) generated forms of the hIP that failed to interact with PDZK1¹⁻¹⁵⁴ (**Figure 3**). Failure to detect an interaction was not due to altered/reduced expression of the various hIP²⁹⁹⁻³⁸⁶-derived bait proteins, as confirmed by immunoblotting (**Supplemental Figure 1**). Collectively, these data reveal critical roles for the residues at P₀ and P₋₂, but not at P₋₁ or P₋₃, within the PDZ ligand of the hIP in defining its interaction with PDZK1 and thereby suggests that it can be classified as a Class I type PDZ ligand.

Disruption of the GLGF motif within the PDZ domains of PDZK1

PDZK1 is a multi-PDZ domain adapter or scaffold protein containing four well defined PDZ domains, here-on-in referred to as PDZ^{D1}, PDZ^{D2}, PDZ^{D3} and PDZ^{D4}, in addition to a short regulatory domain at its C-terminus (Hu *et al.*, 2009; LaLonde and Bretscher, 2009). While the initial Y2H screen identified PDZK1¹⁻¹⁵⁴, corresponding to its entire PDZ^{D1} and only part of its PDZ^{D2}, as the region that interacts with the hIP, it was sought to investigate the specificity of the interaction of PDZ^{D1} and to establish whether any of the other PDZ domains, namely PDZ^{D2}, PDZ^{D3} and PDZ^{D4}, may also contribute to the interaction with the hIP. Typically, mutation of the conserved hydrophobic ‘GLGF motif’ within the carboxyl-binding loop/binding pocket of a given PDZ domain will disrupt interaction with its target PDZ ligand(s) and, hence, can be used experimentally to identify/validate specific PDZ protein:protein interactions (Lee and Zheng, 2010).

Herein, mutation of the GLGF motif within PDZ^{D1} completely disrupted interaction of PDZK1¹⁻¹⁵⁴ with each of the hIP²⁹⁹⁻³⁸⁶-derived subfragments examined through Y2H-based studies (**Supplemental Figure 1B**). In agreement with previous data, in mammalian cells full length PDZK1 strongly and specifically co-immunoprecipitated with the wild type hIP, but not with the control TP α , from their respective HEK.hIP and HEK.TP α stable cell lines (**Figure 4A & 4B**). While PDZK1^{PDZD2*}, carrying a mutated GLGF motif within PDZ^{D2}, appeared to co-immunoprecipitate along with the hIP at slightly reduced levels, statistical comparisons established that those levels were not significantly different from those of the wild type PDZK1 ($P = 0.1439$). Conversely, the interactions of PDZK1^{PDZD1*} ($P < 0.001$) and PDZK1^{PDZD4*} ($P < 0.01$) with the hIP were substantially reduced, while the interaction of PDZK1^{PDZD3*} ($P < 0.001$) was almost completely abolished (**Figure 4A**). Moreover, none of the PDZK1 variants carrying the disrupted GLGF motifs within PDZ^{D1}, PDZ^{D2}, PDZ^{D3} or PDZ^{D4} co-immunoprecipitated with TP α , a further endorsement of the specificity of the interaction between PDZK1 and hIP (**Figure 4B**). Furthermore, immunoblot analysis showed that differences in immunoprecipitation of PDZK1 or its GLGF motif variants were not due to differences in their expression levels or in the efficiency of the *anti*-HA immunoprecipitations *per se* (**Figures 4A & 4B**; Lower and Middle panels, respectively). Hence, collectively these data confirm a constitutive physical interaction between the hIP and PDZK1 in both yeast and mammalian cells and establish critical roles for PDZ^{D1}, PDZ^{D3} and PDZ^{D4}, but not PDZ^{D2}, of PDZK1 in that interaction.

Effect of agonist-activation of the hIP on its interaction with PDZK1

The selective IP agonist cicaprost (Kawabe *et al.*, 2010a) was used to investigate the possible influence of agonist-activation of the hIP on its interaction with PDZK1. Consistent with previous data (**Figure 1C**), in the absence of agonist, PDZK1 showed a strong constitutive interaction with the hIP (**Figure 5A**). In response to cicaprost-stimulation, the interaction of PDZK1 with the hIP was dynamically regulated in a time-dependent manner, for example being significantly diminished at 10 min while returning to basal levels at 30 - 60 min and decreasing again at 2 - 4 hr post-agonist stimulation (**Figure 5A**). Differences in levels of PDZK1 associated with the immune-complexes were not due to variations in the overall levels of PDZK1

expression or in the efficiency of the *anti*-HA immunoprecipitations (**Figure 5A**, lower & middle panels, respectively).

PDZK1 has been established to undergo phosphorylation by cAMP-dependent protein kinase (PK) A at Ser⁵⁰⁵ within its C-terminal regulatory region (Nakamura *et al.*, 2005). Furthermore, phosphorylation of PDZK1 by PKA at Ser⁵⁰⁵ critically regulates its interaction with many of its target proteins including that of the HDL scavenger receptor SR-B1 (Nakamura *et al.*, 2005). Hence, to establish whether the observed dynamic interaction of PDZK1 with the hIP may involve agonist-regulated phosphorylation of PDZK1, the effect of cicaprost-stimulation on the phosphorylation of PDZK1 was examined using *anti*-Phospho^{Ser} antibodies (**Figure 5B**). In the absence of agonist, PDZK1 was found to be basally or net hypophosphorylated (**Figure 5B**). In response to cicaprost-stimulation, the level of PDZK1 phosphorylation was dynamically regulated, being substantially increased at 10 - 30 min post agonist-stimulation (net hyperphosphorylated) while returning to the basal, hypo-phosphorylated levels at 60 - 120 min and increasing again, such as 240 min post-stimulation (**Figure 5B**). Confirmation that the enhanced cicaprost-induced phosphorylation of PDZK1 is specifically mediated through activation of the hIP was established whereby the selective IP antagonist RO1138452 (Clark *et al.*, 2004) blocked both basal and cicaprost-induced phosphorylation of PDZK1 (**Figure 5C**).

The hIP is primarily coupled to Gs-mediated activation of adenylyl cyclase leading to increases in cAMP concentrations and, in turn, downstream activation of PKA (Lawler *et al.*, 2001b) while, as stated, PDZK1 has been established to undergo phosphorylation by PKA where Ser⁵⁰⁵ within its C-terminal regulatory region represents the critical phospho-target (Nakamura *et al.*, 2005). Herein, it was established that pre-incubation with the PKA inhibitor H-89 also completely inhibited both basal and cicaprost-induced PDZK1 phosphorylation (**Figure 5D**). Furthermore, it was also established that the PDZK1 variant PDZK1^{S505A} was not phosphorylated under basal conditions, in the absence of agonist, and did not undergo enhanced phosphorylation in response to cicaprost-stimulation (**Figure 5E**). Differences in levels of phosphorylation of PDZK1, either in the absence or presence of cicaprost-stimulation or in the presence of RO1138452 or H-89, or indeed phosphorylation of PDZK1^{S505A} were not due to variations in the overall levels of PDZK1 or PDZK1^{S505A} expression *per se* (**Figure 5B – 5E**, lower panels). Hence, collectively, these data demonstrate that PDZK1 constitutively interacts with the hIP but that the interaction is regulated in a transient and dynamic manner in response to agonist-activation of the hIP. Furthermore, in the absence of agonist, PDZK1 is basally or hypo-phosphorylated but that it undergoes enhanced cicaprost-induced phosphorylation in a transient, dynamic manner through a mechanism involving PKA phosphorylation of PDZK1 at Ser⁵⁰⁵.

Hence, to further investigate the role of Ser⁵⁰⁵ in contributing to the dynamic nature of the interaction of PDZK1 with the hIP, the effect of cicaprost-stimulation on the interaction with both its phosphorylation-defective PDZK1^{S505A} and phospho-mimetic PDZK1^{S505D} variants were examined through co-precipitations. To begin, the interaction of PDZK1^{S505A} and PDZK1^{S505D}, relative to that of the wild type PDZK1, with the hIP was examined. In the absence of agonist, interaction of PDZK1^{S505D} with the hIP was directly comparable to that of PDZK1 while the interaction between PDZK1^{S505A} and the hIP was substantially reduced (**Figure 6A**, upper panel). Furthermore, consistent with the phosphorylation data (**Figure 5B-5E**), pre-incubation with two independent PKA inhibitors, namely H-89 and KT5720 (Davies *et al.*, 2000) substantially reduced with interaction of PDZK1 with the hIP while pre-incubation with the protein kinase C inhibitor Gö6983 had no effect (**Figure 6A**). On the other hand, preincubation with H-89, KT5720 or Gö6983 had no effect on the interaction of either PDZK1^{S505A} or PDZK1^{S505D} with the hIP in the absence of cicaprost (**Supplemental Figure 2**). In response to agonist-activation, the interaction of PDZK1^{S505A} with the hIP was completely lost at 10 min and PDZK1^{S505A} was not found in the *anti*-HA:hIP immune complexes even following sustained (4 hr) cicaprost-stimulation (**Figure 6B**, Upper panel). Conversely, the interaction of PDZK1^{S505D} with the hIP was not regulated in response to agonist-stimulation, remaining largely unaffected by cicaprost regardless of the incubation period (**Figure 6C**, Upper panel). As previous, the observed differences in immunoprecipitation of PDZK1, PDZK1^{S505A} and PDZK1^{S505D} with the hIP were not due to differences in their levels of expression or in the efficiency of the *anti*-HA:hIP immunoprecipitations (**Figure 6B & 6C**; Middle and lower panels).

Hence, collectively, these data confirm a constitutive interaction between the hIP and PDZK1 but that, in response to agonist-activation, the interaction is modulated in a dynamic manner through a mechanism that is dependent, at least in part, on regulated PKA phosphorylation of PDZK1 at Ser⁵⁰⁵ in response to cicaprost-stimulation.

Effect of PDZK1 on the Expression and Signalling of the hIP

While PDZK1 has been shown to affect the level of expression and/or function of certain of its targets, such effects are protein and/or cell-type specific, as exemplified in the case of SR-B1 (Kocher and Krieger, 2009). Hence, herein, the effect of ectopic expression of PDZK1 on hIP expression levels and function was examined by immunoblot and flow cytometry analyses and through assessment of its radioligand binding and agonist-induced cAMP generation (**Figure 7 & Supplemental Figure 3**). While immunoblot analysis suggested that PDZK1 did not affect overall IP expression levels, radioligand binding assays (RLBAs) established that ectopic expression of PDZK1 resulted in a 1.5-fold increase in binding of its selective radioligand [³H]iloprost in crude membrane preparations ($P < 0.001$; **Figure 7A**; 30 °C). Immunofluorescence image analyses also confirmed that the hIP and PDZK1 show some degree of co-localization, particularly at cell membranes, but there was no detectable change in the overall level of hIP expression following ectopic expression of PDZK1 (**Supplemental Figure 3C & data not shown**). To assess whether the increase in [³H]iloprost binding in the presence of PDZK1 is actually due to alterations in the levels of the hIP at the cell surface, RLBAs were also carried out at 4 °C on whole cells, as opposed to at 30 °C, thereby blocking any internalization of the ligand-bound receptor complex (**Figure 7A**). Consistent with the radioligand binding on membrane preparations, over-expression of PDZK1 led to similar increases in [³H]iloprost binding and, hence, suggested that PDZK1 enhances hIP expression at the cell surface ($P < 0.001$; **Figure 7A**; 4 °C). These findings were also corroborated by flow cytometric analysis whereby the relative fluorescence intensity due to *anti*-HA hIP expression was increased approximately 1.4 fold in the presence of over-expressed PDZK1 (**Supplemental Figure 3A & 3B**).

Furthermore, over-expression of PDZK1 resulted in a significant increase in cicaprost-induced cAMP generation in HEK 293 cells (**Figure 7B**). Disruption of the GLGF domains in PDZ^{D1} and PDZ^{D3}, but not PDZ^{D2} or PDZ^{D4}, of PDZK1 completely abrogated these effects, further implicating an involvement of both PDZ^{D1} and PDZ^{D3} in the specific interaction with the hIP (**Figure 7C & 7D & Figure 4B**). More specifically, similar to wild type PDZK1, ectopic expression of PDZK1^{PDZD2*} and PDZK1^{PDZD4*} resulted in significant increases in both [³H]iloprost binding and agonist-induced cAMP generation whereas over-expression of PDZK1^{PDZD1*} and PDZK1^{PDZD3*} had no effect. Furthermore, immunoblotting confirmed equivalent expression of PDZK1 and its variants and, hence, that the observed effects on functional expression of the hIP were not due to variations in PDZK1 expression levels (**Figure 7C & 7D**). Combined, these data show that increased expression of PDZK1 leads to enhanced functional expression of the hIP at the plasma membrane, resulting in its increased radioligand ([³H]iloprost) binding and agonist-induced cAMP generation. Additionally, PDZ domains 1 and 3, but not 2 and 4, were shown to be critical for the ability of PDZK1 to mediate these effects.

Effect of PDZK1 on Cicaprost-induced Endothelial Cell Migration and Angiogenesis.

Both the hIP and PDZK1 are expressed within the vascular endothelium where they are individually functionally important, not least through their aforementioned regulation of endothelial cell migration and/or angiogenesis (Zhu *et al.*, 2008; Kawabe *et al.*, 2010b). Hence, as an initial means of exploring the functional significance of the interaction in a more physiologically relevant setting, the interaction between the hIP and PDZK1 was investigated in primary human umbilical vein endothelial cells (1° HUVECs). PDZK1 was detected in immune-complexes with the hIP from 1° HUVECs (**Supplemental Figure 4A**), where immunoprecipitations were performed using an affinity purified antibody directed to the intracellular loop (IC)₂ domain of the hIP (Turner and Kinsella, 2010). PDZK1 was not present in the precipitate employing the control pre-immune IgG and failure to detect PDZK1 in the pre-immune complex was not due to differences in its expression levels (**Supplemental Figure 4A**).

As stated, the IP has also been implicated in endothelial cell migration (Pola *et al.*, 2004) and angiogenesis (Kawabe *et al.*, 2010b) and studies herein demonstrate a highly specific interaction between the hIP and PDZK1. Therefore, it was sought to investigate prostacyclin-induced endothelial cell migration and angiogenesis and, more specifically, to examine the possible influence of PDZK1 on those effects, where studies involving HDL/SR-B1 and VEGF acted as reference controls. PDZK1, through its interaction with SR-B1, is known to play a critical role in HDL-mediated endothelial cell migratory and angiogenic responses whereas such VEGF-mediated responses are entirely independent of PDZK1 (Zhu *et al.*, 2008) (**Figure 9B**). Stimulation of 1° HUVECs with the selective IP agonist cicaprost led to significant increases in endothelial cell migration, an effect completely abrogated by the IP antagonist RO1138452 (**Figure 8A(i)**). Consistent with numerous studies, VEGF and HDL also promoted endothelial cell migration, which was further augmented by cicaprost co-stimulation (**Figure 8A(ii) and 8A(iii)**; $P = 0.04$ and $P = 0.015$, respectively, by

2-way ANOVA). Collectively, these data reveal a specific role for the hIP in promoting basal-, VEGF- and HDL- mediated migration of 1° HUVECs.

To investigate the role of PDZK1 in migration of 1° HUVECs, *small interfering RNA (siRNA)* was used to disrupt its expression in 1° HUVECs (**Figure 8D**). Consistent with previous reports (Zhu *et al.*, 2008), it was confirmed that targeted knockdown of PDZK1 using *siRNA*_{PDZK1}, but not the scrambled control *siRNA*_{CONTROL}, almost completely abrogated HDL-mediated cell migration in 1° HUVECs (**Figure 8C**) and also resulted in small, but significant, reductions in basal migration (**Figure 8C & Supplemental Figure 4B**). In contrast, the effect of *siRNA*_{PDZK1} on VEGF-mediated migration was not significantly different than its effect on basal migration (**Figure 8C**), confirming that PDZK1 does not influence or participate in VEGF-migration. In contrast to this, targeted disruption PDZK1 by *siRNA*_{PDZK1} completely inhibited cicaprost-induced cell migration in 1° HUVECs while the *siRNA*_{CONTROL} (**Figure 8C**) or *siRNA*_{LAMIN A/C} (**Data not shown**) had no effect.

The influence of the interaction between hIP and PDZK1 on endothelial tube formation, a further essential step in the angiogenic process, was also investigated. In the presence of cicaprost, there was a significant increase in mean tube length (**Figure 8B**), an effect completely abrogated by co-incubation with the IP antagonist RO1138452 (**Figure 8B**). Stimulation with both VEGF and HDL led to significant increases in mean tube lengths and co-stimulation with cicaprost further enhanced both these effects (**Figure 8B**; $P = 0.043$ and $P = 0.02$, respectively, by 2-way ANOVA). Similar to the findings for endothelial cell migration, while targeted disruption of PDZK1 with *siRNA*_{PDZK1} led to small, but significant, reductions in basal tube formation in the absence of stimulation (**Supplemental Figure 4C**), it did not lead to further reductions in VEGF-induced migration (**Figure 8E**). In contrast, and consistent with our migration data (**Figure 8C**), disruption of PDZK1 expression impaired HDL-mediated endothelial tube formation (**Figure 8E**). Furthermore, targeted disruption of PDZK1 by *siRNA*_{PDZK1} also completely inhibited cicaprost-induced endothelial tube formation in 1° HUVECs while the *siRNA*_{CONTROL} (**Figure 8E**) or *siRNA*_{LAMIN A/C} (**Data not shown**) had no effect.

Taken together, these data confirm an important role for the hIP in endothelial cell migration and in promoting angiogenesis through endothelial tube formation *in vitro*. Furthermore, and similar to that previously found to occur for HDL/SR-B1, they establish a critical role for PDKZI in mediating both cicaprost/IP-induced endothelial migration and *in vitro* angiogenesis. Collectively, the data presented herein, identifies a novel physical interaction between the hIP and the intracellular adapter protein PDZK1 that impacts on hIP-mediated endothelial function and sheds critical new insights into knowledge of the interplay between the hIP, but also of PDZK1 and HDL/SR-B1, as key players in the maintenance of endothelial monolayer integrity.

Discussion

In this study, we report the discovery of a novel interaction between the human (h) prostacyclin receptor (IP) with PDZK1, a member of the Na⁺-H⁺ exchanger regulatory factor (NHERF) family of PDZ domain-containing scaffolding proteins (Hu *et al.*, 2009; Kocher and Krieger, 2009). The direct interaction occurs through binding of a Class I type PDZ ligand at the C-terminus of the hIP with the domains PDZ^{D1}, PDZ^{D3} and PDZ^{D4} of PDZK1. The ability of the hIP to bind several PDZ domains is consistent with the binding preferences of certain other proteins to bind PDZK1 and is, for example, identical to CFTR in that they both bind PDZ^{D1}, PDZ^{D3} & PDZ^{D4} but not PDZ^{D2} (Wang *et al.*, 2000). As stated, the hIP is somewhat unusual among GPCRs in that it undergoes isoprenylation within a conserved carboxyl terminal -C³⁸³SLC³⁸⁶, or 'CaaX' motif (Hayes *et al.*, 1999; Miggin *et al.*, 2002) while data herein establish that this sequence also serves as a PDZ ligand. Such an interaction of an isoprenylated protein with PDZ domain proteins described herein for the hIP is not without precedence, being exemplified by the interaction of Gyl3 with PSD95, Veli-2 and SAP97 involving classic PDZ domain:PDZ ligand type interactions (Li *et al.*, 2006). Furthermore, while several lines of evidence herein suggest that the interaction of PDZK1 with the hIP is largely independent of its isoprenylation status, additional follow up studies involving more direct biophysical approaches are required to ascertain whether the farnesylated, fully processed form of the hIP actually interacts with PDZK1.

Agonist-activation plays an important role in regulating the signalling pathways of members of the GPCR superfamily, including the hIP (Lawler *et al.*, 2001b; O'Keefe *et al.*, 2008; Wikstrom *et al.*, 2008; Reid *et al.*, 2010). The ability of PDZK1 to regulate the expression of the high affinity HDL SR-B1 receptor is subject to functional regulation by phosphorylation whereby cAMP-dependent PKA phosphorylation of Ser⁵⁰⁵ within the C-terminal regulatory region of PDZK1 is the critical phosphorylation event (Nakamura *et al.*, 2005). Herein, several lines of evidence suggest that PDZK1 may also undergo PKA phosphorylation at Ser⁵⁰⁵ in response to cicaprost-activation which, in turn, may provide a molecular basis for the observed agonist-regulated dynamic interaction between PDZK1 and the hIP. Based on data presented in **Figure 5 and 6**, a model presented in **Figure 9A** proposes that PDZK1 is recruited into a complex with the hIP in a basal non-phosphorylated or overall net hypo-phosphorylated state at Ser⁵⁰⁵. In response to cicaprost-induced receptor activation, while (i) PDZK1 dissociates from the hIP complex, it also undergoes enhanced (ii) cAMP dependent-PKA phosphorylation at Ser⁵⁰⁵ which, in time, (iii) triggers re-association of PDZK1 with the hIP followed by (iv) dephosphorylation of PDZK1 to basal levels (**Figure 9A**). Evidence for the model is substantiated by the finding that the phosphorylation defective variant PDZK1^{S505A} interacts, *albeit* weaker than PDZK1, with the hIP under basal conditions and dissociates from the complex in response to agonist-activation of the hIP. However, unlike wild type PDZK1, the mutant PDZK1^{S505A} cannot undergo cicaprost-induced PKA phosphorylation and, therefore, does not undergo phosphorylation-induced re-association with the hIP. Furthermore, and in contrast to this, it is proposed that the mutant PDZK1^{S505D} mimics the hyper-phosphorylated state (iii). Hence, while it is possible that PDZK1^{S505D} may undergo dissociation in response to agonist-activation of the hIP, such effects are not observed using the experimental approaches used in the current study, at least. By mimicking the hyper-phosphorylated state, it is proposed that any dissociation of PDZK1^{S505D} would be followed by its rapid recruitment into the complex with the hIP, therefore leading to the observed stabilized or prolonged interaction of PDZK1^{S505D} with the hIP either in the absence or presence of agonist-activation (**Figure 9A**, (iii)). While certain aspects of the agonist-dependent regulation of PDZK1 with the hIP remain to be elucidated including, (a) whether PDZK1 is phosphorylated or not at Ser⁵⁰⁵ when bound to the hIP under basal conditions, (b) the identity of the phosphatase(s) that regulate the dephosphorylation of PDZK1 and (c) the nature of the agonist-induced conformational trigger(s) that promotes the dissociation at the level of the hIP itself, regulation of PDZ interactions by phosphorylation is not confined to PDZK1/NHERF3 (Voltz *et al.*, 2007). Critically data herein now also establish that agonist-activation of the hIP leads to direct PKA phosphorylation of PDZK1 to modulate their interaction and it will be of interest to establish whether hIP-mediated phosphorylation of PDZK1 modulates its interaction with other targets, such as the SR-B1 or CFTR.

In contrast to the quite rapid temporal association and dissociation between the hIP and PDZK1, the hIP has also been established to undergo internalization and subsequent recycling to the plasma membrane in response to agonist-stimulation but such events are much slower, occur maximally at 2 -4 hr post agonist-stimulation (O'Keefe *et al.*, 2008; Wikstrom *et al.*, 2008; Reid *et al.*, 2010). Hence, the temporal association and dissociation of PDZK1 with the hIP is a much more dynamic event, being largely regulated by phosphorylation/dephosphorylation, while the agonist-induced internalization of the hIP is a much slower event, requiring engagement and participation of the intracellular trafficking machinery including members

of the Rab GTPases, as we have previously reported (O'Keeffe *et al.*, 2008; Wikstrom *et al.*, 2008; Reid *et al.*, 2010). Therefore, it is unlikely that there is a relationship between the temporal association between the hIP with PDZK1 and the processes regulating agonist-induced trafficking of the hIP. While PDZK1 has been shown to affect the level of expression and/or function of certain of its targets, such effects are protein and/or cell-type specific (Kocher *et al.*, 2003; Zhu *et al.*, 2008). PDZK1 does not substantially regulate overall expression of the hIP, in the cell type examined at least, but led to increased functional expression of the hIP at the cell surface enhancing ligand binding and cAMP generation. While disruption of the GLGF motif within PDZ^{D1} and PDZ^{D3} abrogated these effects, disruption of PDZ^{D4} did not appear to be functionally important, despite its ability to influence the interaction of PDZK1 with the hIP.

By binding to its C-terminal PDZ ligand, PDZK1 plays an essential role in maintaining hepatic SR-BI levels thereby controlling HDL metabolism/reverse cholesterol transport (RCT) and HDL/SR-BI-dependent endothelial cell migration, tube formation and proliferation, protecting against the development of atherosclerosis (Fenske *et al.*, 2008; Zhu *et al.*, 2008; Fenske *et al.*, 2009). PDZK1^{-/-} mice display marked hypercholesterolaemia due to a 95% decrease in hepatic SR-B1 expression and thereby represent an important preclinical model of CV disease (Kocher and Krieger, 2009). Furthermore, while PDZK1 does not influence SR-B1 levels within the vascular endothelium, PDZK1^{-/-} mice also impaired EC migration/re-endothelialization, contributing to decreased vascular repair and increased atherosclerosis (Zhu *et al.*, 2008). Hence, through its interaction with SR-B1, PDZK1 is critically involved in maintaining endothelial monolayer integrity. In contrast, PDZK1 does not influence endothelial cell migration or angiogenesis by vascular endothelial growth factor (VEGF) and, therefore, it was suggested that it is uniquely required for signalling by HDL/SR-B1 within the endothelium (Zhu *et al.*, 2008).

Categorical evidence of the importance of prostacyclin to vascular repair and angiogenesis was recently demonstrated whereby regenerative endothelial progenitor cells (EPCs) from IP^{-/-} mice failed to promote re-endothelialization highlighting a critical protective role for the IP in vascular remodelling in response to injury similar to that of the HDL/SR-B1-mediated pathway (Qu *et al.*, 2009; Kawabe *et al.*, 2010b). In the current study, as outlined in the model in **Figure 9B**, it was established that PDZK1 also plays a critical role in IP-mediated endothelial cell migration and *in vitro* angiogenesis (**Figure 8**). While a number of studies have suggested that prostacyclin-induced endothelial migration and angiogenesis occurs through its regulation of PPAR δ , rather than through the IP *per se* (Pola *et al.*, 2004; He *et al.*, 2008), those studies used iloprost as agonist which, unlike the highly selective IP agonist cicaprost employed herein, is known to activate both the IP and PPAR δ (Kawabe *et al.*, 2010a). Moreover, in the current study, the effects of cicaprost were blocked by the IP antagonist RO1138452 and by targeted knockdown of PDZK1 using siRNA_{PDZK1}. Taken together, our findings establish a specific and critical role for the hIP in modulating endothelial migratory and angiogenic responses and, similar to the HDL/SR-B1 pathway, that those agonist-induced responses are PDZK1-dependent (**Figure 9B**). Considering the central role of the hIP and SR-B1 within the vasculature, including in re-endothelialization in response to injury, coupled with the finding herein of a direct interaction of PDZK1 with the hIP, it will be of significant interest to investigate the possible interplay between the critical prostacyclin/IP and HDL/SR-B1 pathways, both of which are now known to be critically regulated by PDZK1.

Materials and Methods

Materials Cicaprost was obtained from Schering AG (Berlin, Germany). RO1138452 was obtained from Cayman Chemicals; H-89, KT5720 and GÖ6983 were from Merck Biochemicals. Mouse monoclonal *anti*-hemagglutinin (HA) 101R antibody was from Cambridge Biosciences; mouse polyclonal *anti*-PDZK1, normal rabbit IgG, horseradish peroxidase (HRP)-conjugated goat *anti*-rabbit and goat *anti*-mouse secondary antibodies were from Santa Cruz; rat monoclonal *anti*-HA 3F10-HRP-conjugated antibody was from Roche; *anti*-Myc (9B11) mouse monoclonal antibody was from Cell Signalling Technology; rabbit *anti*-PhosphoSer antibody was from Invitrogen (61-8100); recombinant human vascular endothelial growth factor (VEGF) 165 (293-VE/CF) was from R&D Systems; high density lipoprotein (HDL; purified from human plasma; L1567), mouse monoclonal *anti*-FLAG and *anti*-FLAG-HRP-conjugated M2 antibody were from Sigma-Aldrich; *anti*-HDJ-2 (DNAJ protein) was from Neomarkers; AlexaFluor488 goat *anti*-rabbit, AlexaFluor488 goat *anti*-mouse and AlexaFluor594 goat *anti*-mouse antibodies were from Molecular Probes. Plasmids pCRE-Luc, pRL-TK and pCMVTag2C were from Agilent Technologies. Farnesyl transferase inhibitors R115777 and SCH66336 were obtained from Janssen Pharmaceuticals and Schering Plough, respectively. [³H]iloprost was obtained from Perkin Elmer. All oligonucleotides were synthesised by Sigma Genosys.

Subcloning and site-directed mutagenesis

The plasmids pHM6:hIP^{WT}, pHM6:hIP^{SSLC} and pHM6:hIP^{Δ383}, encoding HA epitope-tagged forms of the wild type human prostacyclin receptor (hIP), isoprenylation defective hIP^{SSLC}, or CaaX-truncated hIP^{Δ383}, respectively, have been previously described (Hayes *et al.*, 1999). pACT2:Rab11a, pGBKT7:hIP²⁹⁹⁻³⁸⁶, pGBKT7:hIP^{299-386,SSLC}, pGBKT7:hIP³²⁰⁻³⁸⁶, pGBKT7:hIP^{320-386,SSLC} and pGBKT7:hIP²⁹⁹⁻³²⁰ have been described (Wikstrom *et al.*, 2008). The plasmids pGBKT7:TPα³¹²⁻³⁴³ and pGBKT7:TPβ³¹²⁻⁴⁰⁷ have also been described (Reid *et al.*). The plasmids pGBKT7:hIP^{299-386,CSLS}, pGBKT7:hIP^{299-386,CSAC}, pGBKT7:hIP^{299-386,CSSC}, pGBKT7:hIP^{299-386,CALC}, pGBKT7:hIP^{299-383,C-STOP}, pGBKT7:hIP^{299-383,S-STOP}, pGBKT7:hIP^{299-386,CAAA} and pGBKT7:hIP^{299-386,SAAA} were generated by subcloning the respective region of the wild type or mutant hIP from the corresponding pHM6-based plasmids into the *EcoRI*-*Bam*HI sites of the yeast bait vector pGBKT7 (Clontech), such that fragments were in-frame with the DNA-binding domain (DBD) of the yeast GAL4 transcriptional activator. The plasmid pACT2:PDZK1^{PDZD1*} was generated by QuikChange™ site-directed mutagenesis (Agilent Technologies) using the PDZK1 library clone identified in the Y2H screen, pACT2:PDZK1, as template and the primer pair shown in **Supplemental Table 1**. The plasmid pOBT7:PDZK1 was obtained from ImaGenes and the plasmid pCMVTag2C:PDZK1 was generated by subcloning the full-length sequence from pOBT7:PDZK1 into the *Bam*HI-*Xho*I sites of the mammalian expression vector pCMVTag2C, such that fragment was in-frame for FLAG epitope-tagged protein expression. The plasmids pCMVTag2C:PDZK1^{PDZD1*}, pCMVTag2C:PDZK1^{PDZD2*}, pCMVTag2C:PDZK1^{PDZD3*}, and pCMVTag2C:PDZK1^{PDZD4*}, where the respective hydrophobic binding pocket (“GLGF”) sequence for each PDZ domain (Doyle *et al.*, 1996) was mutated from N¹⁹YGF²² to K¹⁹YRS²² (Domain 1), S¹⁴⁴YGF¹⁴⁷ to S¹⁴⁴YRE¹⁴⁷ (Domain 2), G²⁵²YGF²⁵⁵ to G²⁵²YRE²⁵⁵ (Domain 3), and G³⁸⁷YGF³⁹⁰ to G³⁸⁷YRE³⁹⁰ (Domain 4), were generated by QuikChange™ site-directed mutagenesis using pCMVTag2C:PDZK1 as template and the primer pairs shown in **Supplemental Table 1**. Mutations were designed to disrupt PDZ ligand-binding at each domain solely through destabilization of the hydrophobic binding pocket of each PDZ domain, and were validated through the use of the online protein domain organization tool, Pfam (Finn *et al.*, 2010). Conversion of Ser⁵⁰⁵ to Ala⁵⁰⁵ or Asp⁵⁰⁵ to generate pCMVTag2C:PDZK1^{S505A} and pCMVTag2C:PDZK1^{S505D} was also achieved by QuikChange™ site-directed mutagenesis using pCMVTag2C:PDZK1 as a template and the primer pairs shown in **Supplemental Table 1**. All mutations were validated by DNA sequence analysis.

Yeast-2-hybrid screening and yeast matings

Yeast-2-hybrid (Y2H) screening of a human kidney cDNA library with the carboxyl-terminal (C-tail) domain, encoding amino acids 299 – 386, of the hIP (hIP²⁹⁹⁻³⁸⁶) as specific bait was carried out as previously described (Wikstrom *et al.*, 2008; Reid *et al.*, 2010). Y2H screening identified several independent clones encoding amino acids 1-154 of PDZK1, expressed in the yeast prey plasmid pACT2:PDZK1, as an interactant of the hIP. pGBKT7 and pGBKT7:p53, encoding the GAL4 DBD alone or as a fusion with p53, were obtained from Clontech. All yeast protocols were standard procedures as previously described (Wikstrom *et al.*, 2008). For analysis of protein expression in *S.c.* AH109 (pGBKT7) bait transformants,

protein was extracted, resolved by SDS-PAGE, and screened by western blot analysis using *anti-Myc* (9B11), with chemiluminescence detection.

Cell Culture and transfections

Human embryonic kidney (HEK) 293 cells were obtained from American Type Culture Collection and grown in minimal essential medium (MEM) containing 10 % foetal bovine serum (FBS). HEK 293 cells were transiently or stably transfected using the calcium phosphate/DNA co-precipitation or EffecteneTM procedures, as previously described (O'Keeffe *et al.*, 2008; Wikstrom *et al.*, 2008). In brief, approximately 48 hr prior to transfection, cells were routinely plated at a density of 2×10^6 cells per 10 cm culture dish in 8 ml media. Thereafter, cells were transiently co-transfected with pcDNA/pCMV-based vector in the presence of pADVA (Gorman *et al.*, 1990) at a ratio of 2.5:1 using either the calcium phosphate/DNA co-precipitation procedure, where a total of 35 μ g DNA was used, or the Effectene® (Qiagen) transfection procedure, where a total of 2 μ g DNA was used. HEK.hIP, HEK.hIP^{SSLC}, HEK.hIP ^{Δ 383} cells stably over-expressing HA-tagged forms of the wild type and mutated hIPs, respectively, have been described (Miggin *et al.*, 2003). Primary (1°) human umbilical vein endothelial cells (HUVECs), obtained from Lonza (IRT9-048-0904D), were routinely cultured in M199 media (Sigma-Aldrich) supplemented with 0.4 % (v/v) Endothelial Cell Growth Supplement/Heparin (ECGS/H; Lonza), 20 % (v/v) FBS and 0.2 % (v/v) L-glutamine. 1° HUVECs were used between passages 2 - 8. All mammalian cells were grown at 37 °C in a humid environment with 5 % CO₂ and confirmed to be mycoplasma free.

Immunoprecipitations.

HEK.hIP, HEK.hIP^{SSLC}, HEK.hIP ^{Δ 383} and HEK.TP α cells were transiently co-transfected with pADVA and either pCMVTag2C:PDZK1 or mutant variants where specified, using Effectene®. To assess the effect of the farnesyl transferase inhibitors (FTIs), R115777 and SCH66336, on the interaction between the hIP and PDZK1, HEK.hIP^{WT} cells, transiently co-transfected with pCMVTag2C:PDZK1 were incubated 24 hr post-transfection with R115777 and SCH66336 at concentrations indicated in the figure legends, or, as a control, with 0.1 % DMSO (vehicle), for 24 hr at 37 °C prior to immunoprecipitation. IP-mediated phosphorylation of PDZK1 was examined in HEK.hIP^{WT} cells, transiently co-transfected with pCMVTag2C:PDZK1, whereby cells were pre-incubated with RO1138452 (10 μ M; 10 min) prior to cicaprost stimulation. To assess the effect of the kinase inhibition, H-89, KT5720 and GÖ6983, on the interaction between the hIP and PDZK1, HEK.hIP^{WT} cells, transiently co-transfected with pCMVTag2C:PDZK1 or mutated variants were incubated 48 hr post-transfection with H-89, KT5720 and GÖ6983 at concentrations indicated in the figure legends, or, as control, with 0.1 % DMSO (vehicle), for 10 min at 37 °C prior to immunoprecipitation or stimulation with cicaprost, as indicated. Primary HUVECs were plated onto 10 cm dishes to achieve > 80 % confluence. In all cases prior to immunoprecipitation, cells were washed in the appropriate serum-free media and either incubated with vehicle or with 1 μ M cicaprost for the times indicated in the figure legends. Thereafter, cells were washed, lysed and clarified as per previously detailed protocols (Wikstrom *et al.*, 2008; Reid *et al.*, 2010). HA-tagged hIP or TP α , were immunoprecipitated using *anti-HA* (101R; 1:300) antibody, FLAG-tagged PDZK1 was immunoprecipitated with *anti-FLAG* M2 (1: 200), endogenously expressed hIP with the affinity purified rabbit polyclonal *anti-hIP* (1: 50) antibody (Reid *et al.*, 2010) or as control, with normal rabbit IgG (Santa Cruz). Thereafter, lysates were incubated for 1 hr with either 50 % slurry of protein G-sepharose (10 μ l) or protein A-sepharose (immunoprecipitation with rabbit *anti-hIP*; 40 μ l), prior to repeated washing with RIP followed by PBS (2-3 times). Immunoprecipitates were resolved by SDS-PAGE and subjected to successive immunoblotting with *anti-Flag* (1:2500), *anti-HA* (3F10; 1:500), *anti-PDZK1* (1:1000), *anti-HDJ-2* (1:4000), and *anti-PhosphoSer* (1:1000) antibodies, as indicated.

Immunofluorescence Microscopy

To examine the localisation of PDZK1 and the hIP, HEK.hIP cells transiently co-transfected with pCMVTag2C:PDZK1 plus pADVA were grown on poly-L-lysine treated coverslips, in 6-well plates for at least 48 hr post-transfection. Thereafter, cells were fixed using 3.7 % paraformaldehyde in PBS pH 7.4 for 15 min at RT prior to washing in PBS. Cells were permeabilized by incubation with 0.2 % Triton X-100 in PBS for 10 min on ice, followed by washing in TBS. Non-specific sites were blocked by incubating cells with 1 % BSA in TBS, pH 7.4 for 1 hr at RT. Cells were incubated with the affinity purified rabbit polyclonal *anti-hIP* antibody (1:250; 1 % BSA in TBS) to label the hIP and *anti-PDZK1* (1:500; 1 % BSA in TBS) to label PDZK1 for 1 hr at RT. The antibody solution was removed and cells were washed with TBS followed by a further incubation with 1 % BSA in TBS-T for 30 min. Cells were then incubated with

AlexaFluor488 goat *anti-rabbit* IgG secondary antibody (1:2000; 1% BSA in TBS-T) to detect the IP receptor and AlexaFluor594 goat *anti-mouse* IgG secondary antibody (1:4000; 1% BSA in TBS-T) to detect PDZK1 for 1 hr at RT. After washing, all slides were counterstained with DAPI (1 µg/ml in H₂O) prior to mounting coverslips in DakoCytomation fluorescence mounting medium. Imaging was carried out using the Zeiss Axioplan 2 microscope and Axioplan Version 4.4 imaging software. Data presented are representative images for at least three independent experiments from which at least 10 fields were viewed at 63X magnification, where the horizontal bar represents 10 µM.

Radioligand Binding Assays

To examine the effect PDZK1 on hIP expression, HEK.hIP cells were transiently co-transfected with pADVA in the presence of pCMVTag2C:PDZK1 or, as controls, with pCMVTag2C. Radioligand binding assays (RLBAs) of the hIP were carried out as previously described (Hayes *et al.*, 1999). For saturation binding studies, RLBAs were carried out on cell membranes in the presence of 4 nM [³H]iloprost (15.3 Ci/mmol) at 30 °C for 1 hr using 50 – 100 µg of protein in 100 µl reactions. Non-specific binding was determined in the presence of 0.2 mM iloprost for saturation-binding studies. Alternatively to analyse cell surface hIP expression, following harvesting and washing in PBS, cells were resuspended in MES/KOH buffer and RLBAs were carried out at 4 °C for 1 hr using whole cell protein in 100 µl reactions. Incubations were terminated by the addition of 4 ml of ice-cold Resuspension Buffer followed by filtration through Whatman GF/C filters, which had been pretreated in 0.33 % polyethyleneimine (PEI) for 1 – 24 hr. The filters were washed three times with Resuspension Buffer (4 ml) and then subjected to liquid scintillation counting in scintillation fluid (5 ml/filter) using a Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer, Waltham, MA, USA).

Flow cytometry

HEK.hIP cells were transiently co-transfected with pCMVTag2C:PDZK1 or, as a control, with pCMVTag2C along with pADVA using Effectene[®]. Seventy-two hr post-transfection, cells were washed twice with PBS, harvested and resuspended at 10⁶ cells/ml in ice-cold PBS/2% BSA. Cell suspensions were incubated with 2.5 µg/ml *anti*-HA (101R) antibody or, as a control, with 2.5 µg/ml normal mouse IgG to quantify background fluorescence. After 1 hr on ice, the cells were centrifuged at 500 g for 5 min and washed twice in ice-cold PBS/2% BSA. Cell suspensions were then incubated with 1.5 µg/ml AlexaFluor488 goat *anti*-mouse antibody for 1 hr on ice, centrifuged, washed twice in ice-cold PBS/2% BSA and fixed with 3.7% paraformaldehyde in PBS. Surface fluorescence was analyzed on a CyAn ADP using Summit 4.3 software (Dako). The isotype-matched normal mouse IgG antibody was used to set the gates for positive staining and positively-stained cells were gated by forward- and side-scatter. Fluorescent intensity was corrected for background fluorescence and results are presented as mean fold increase in fluorescent intensity upon PDZK1 over-expression where levels upon control transfection are expressed as 1.

Measurement of agonist-induced cAMP generation

A gene reporter-based assay was performed to investigate the effect of over-expression of PDZK1 on changes in intracellular cAMP levels in response to stimulation of the hIP with its selective agonist cicaprost, essentially as previously described (Turner and Kinsella, 2010). In brief, the plasmids pHM6:hIP (1.5 µg), pCMVTag2C:PDZK1 (2 µg), pCMVTag2C:PDZK^{PDZD1*} (2 µg), pCMVTag2C:PDZK^{PDZD2*} (2 µg), pCMVTag2C:PDZK^{PDZD3*} (2 µg), pCMVTag2C:PDZK^{PDZD4*} (2 µg) or, as a negative control, pCMVTag2C, were each transiently co-transfected into HEK 293 cells with the luciferase reporter pCRE-Luc (1 µg), pRL-TK (50 ng) and pADVA (0.5 µg) using Effectene[®] reagent as per the manufacturers' instructions (Qiagen). Cells were treated 72 hr post-transfection with 3-isobutyl-1-methylxanthine (IBMX; 100 µM) at 37 °C for 30 min and then stimulated with either vehicle (V; 0.01 % DMSO) or 1 µM cicaprost at 37 °C for 3 hr. Firefly and renilla luciferase activity was assayed 76 hr post-transfection using the Dual Luciferase Assay System[®]. Relative firefly to renilla luciferase activities (arbitrary units) were calculated as a ratio and were expressed in relative luciferase units (RLU).

Disruption of PDZK1 expression by small interfering (si)RNA

For small interfering (si) RNA experiments, 1^o HUVEC cells were plated at ~2.5 x 10⁵ cells /35- mm plate and some 24 hr prior to transfection such that cells reach ~50 % confluence. Thereafter, cells were transfected with 30 nM PDZK1 siRNA (siRNA_{PDZK1}; 5'-GAAGAACAUGAUCUGGUAtt; nucleotides 828 – 846; Dharmacon), 30 nM Lamin A/C siRNA (siRNA_{LaminA/C}; 5'-CUGGACUUCAGAAAGAACAtt;

Qiagen) or 30 nM scrambled negative control *siRNA* (*siRNA*_{CONTROL}; 5'-AATTCTCCGAACGTGTCACGTt-3'; Qiagen) using RNAiFECT transfection reagent (Qiagen), as per manufacturer's instructions. To confirm the efficacy of the *siRNA* to disrupt PDZK1 expression, following transfection 1° HUVECs were harvested after incubation at 0 – 72 hr and subject to SDS-PAGE (10 – 15 µg/lane on 10 % polyacrylamide gels) followed by electroblotting onto PDVF membranes (Roche). Membranes were successively screened using *anti*-PDZK1 and *anti*-Lamin A/C antibodies and then screen using *anti*-HDJ-2 antibody to confirm uniform protein loading. For migration and tube formation assays, 24 hr post-*siRNA* transfection cells were placed in reduced serum growth media (2.5 % FBS) for 16 hr before experiments were performed.

Cell Migration assays

In order to monitor changes in 1° HUVEC migration, cells were plated in 12-well plates such that they were ≥ 90 % confluent 24 hr post-seeding. Thereafter, cells were pre-incubated in reduced serum media (2.5 % FBS) 12-16 hr prior to scoring the cells from top-to-bottom perpendicular to pre-drawn lines (2 parallel lines approx 3-4 mm) on the underside of the well using a 200 µl pipette tip. Loose cells and debris were washed away with serum-free media and replaced with reduced serum media (2.5 % FBS). Cells were pre-incubated with vehicle (0.01% PBS) or 10 µM RO1138452 for 10 min prior to stimulation with either vehicle (0.01% PBS), cicaprost (1 µM), VEGF (50 ng/ml) or HDL (50 µg/ml), alone or in combination. Immediately (0 hr) and after 12 hr incubations, the reduction in scratch paths were visualised and imaged using a Nikon TMS inverted microscope with Matrox Intellicam software (Version 2.07) and analysed with TScratch software (Version 1.0). Migration was expressed as percentage of basal cell migration. All experiments were performed in triplicate and each experiment was repeated at least three times.

In Vitro Tube Formation Assays

Matrigel tube formation assays were performed to assess *in vitro* angiogenesis. Growth factor-reduced Matrigel (BD Biosciences) was placed in 96-well tissue culture plates (50 µl/well) and allowed to gel at 37 °C for at least 30 min. Then 1° HUVECs (4 x 10³ cells/well) were seeded in 100 µl reduced serum media (2.5 % FBS) and pre-incubated with vehicle (0.01% PBS) or 10 µM RO1138452 for 10 mins prior to stimulation with either vehicle (0.01% PBS), cicaprost (1 µM), VEGF (50 ng/ml) or HDL (50 µg/ml), alone or in combination. After 12 hr incubation, cell morphology was visualised and imaged using a Nikon TMS inverted microscope with Matrox Intellicam software (Version 2.07; 4 times at random per field). The length of tube was measured at 40X magnification with WCIF ImageJ software (Version 1.37c) and expressed as percentage of basal tube length. All experiments were performed at least in triplicate and each experiment was repeated at least two times.

Data analyses

Statistical analyses of differences were carried out using 1-way or 2-way ANOVA followed by post-hoc Dunnett's multiple comparison *t* tests, as indicated, throughout employing GraphPad Prism, version 4.00 package. *P*-values of less than or equal to 0.05 were considered to indicate a statistically significant difference. As relevant, single, double and triple symbols signify $p \leq 0.05$, ≤ 0.01 and ≤ 0.001 , respectively, for post-hoc Dunnett's multiple comparison *t*-test analyses.

ACKNOWLEDGEMENTS

This work was supported by Science Foundation of Ireland (Grant SFI:05/IN.1/B19).

We are grateful to Dr Maria Hill and Karol English, B.Sc for carrying out the initial Y2H screen.

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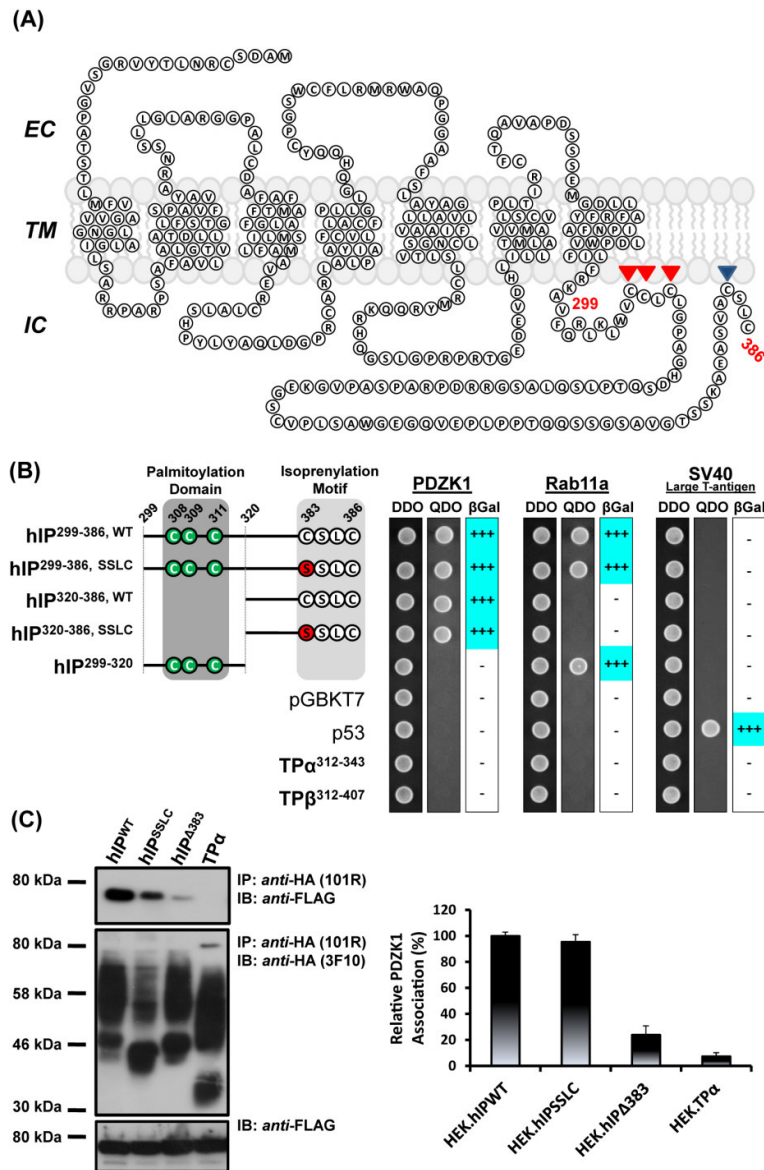


Figure 1. Interaction of PDZK1 with the human Prostacyclin Receptor.

Panel A: Schematic of the human prostacyclin receptor (hIP). The hIP undergoes palmitoylation at Cys³⁰⁸, Cys³⁰⁹ and Cys³¹¹ and isoprenylation/farnesylation at Cys³⁸³ which, together, are proposed to introduce fourth (IC₄) and fifth (IC₅) intracellular loops within the C-tail domain of the hIP. **Panel B:** *S.c* Y187 (pACT2:PDZK1) or, as controls, *S.c* Y187 (pACT2:Rab11a) and *S.c* Y187 (pTD1-1), encoding SV-40 large T-antigen, prey strains were mated with *S.c* AH109 bait strains transformed with recombinant pGBKT7 encoding the listed hIP subfragments and, as controls, pGBKT7.TPα, pGBKT7.TPβ and p53 or with the vector pGBKT7 alone. Diploids were selected on DDO medium, whereas interactants were selected on QDO medium and by their ability to express β-galactosidase (β-Gal). Data: *n* ≥ 3. **Panel C:** HEK.hIP^{WT}, HEK.hIP^{SSLC}, HEK.hIP^{Δ383} or, as controls, HEK.TPα cells, each transiently transfected with pCMVTag2C:PDZK1^{FL}, were subject to immunoprecipitation with anti-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB), as indicated. Uniform expression of Flag-tagged PDZK1 was verified by immunoblotting of whole cell lysates (50 μg/lane) with anti-FLAG antibody (lower panel). The bar charts show mean relative levels of PDZK1-associated with the anti-HA 101R immunoprecipitates (relative protein, % ± SEM, *n* = 3) where levels associated with the anti-HA.hIP immunoprecipitates are expressed as 100%.

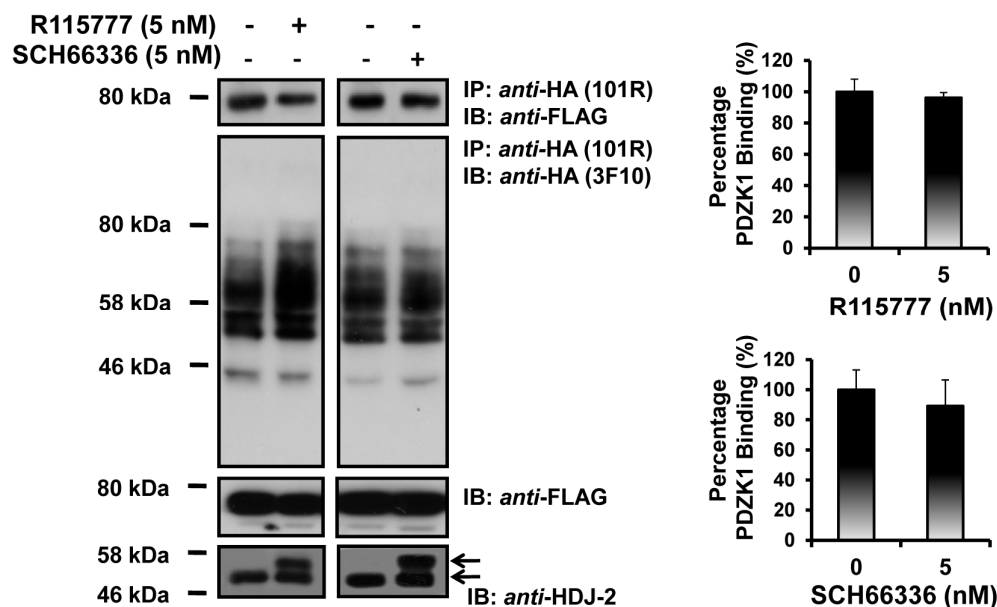


Figure 2. Effect of isoprenylation of the hIP on its interaction with PDZK1.

HEK.hIP cells, transiently transfected with pCMVTag2C:PDZK1^{FL}, were pre-incubated with vehicle, R115777 (5 nM) or SCH66336 (5 nM) for 24 hr prior to immunoprecipitation with *anti*-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB), as indicated. Uniform expression of Flag-tagged PDZK1 was verified by immunoblot analysis of whole cell lysates (50 µg/lane) with *anti*-FLAG antibody (middle panels). The efficacy of R115777 or SCH66336 to inhibit protein farnesylation was validated by immunoblot analysis of whole cell lysates (50 µg/lane) for the farnesylated (~45-46 kDa) and non-farnesylated (49 kDa) species of the molecular chaperone HDJ-2 (*anti*-HDJ-2; lower panels). The bar charts show mean relative levels of PDZK1-associated with the *anti*-HA.hIP immunoprecipitates in the absence or of presence R115777 and SCH66336 (relative protein, % ± SEM, *n* = 3).

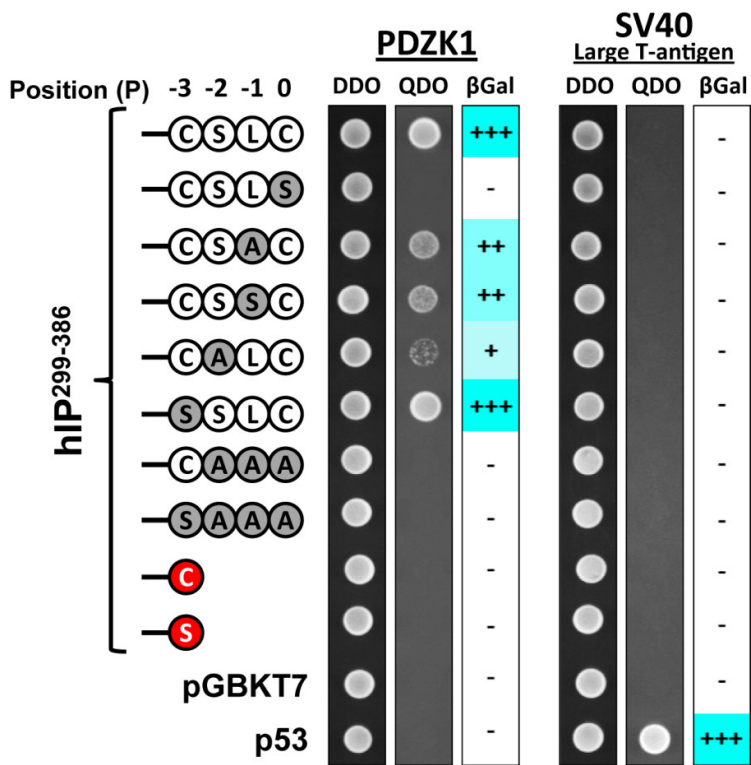


Figure 3. Characterisation of the PDZ ligand within the C-tail domain of the hIP. *S.c* Y187 (pACT2:PDZK1) or, as a control, *S.c* Y187 (pTD1-1) prey strains were mated with *S.c* AH109 bait strains transformed with pGBKT7.hIP²⁹⁹⁻³⁸⁶ subfragment with its wild type (-C³⁸³SLC³⁸⁶, corresponding to the positions (P)₀, P₋₁, P₋₂, and P₋₃ of its PDZ ligand, respectively) carboxyl-terminal residues, or the listed mutated variants, and as controls, p53 or transformed with the vector pGBKT7 alone. Data: n ≥ 3.

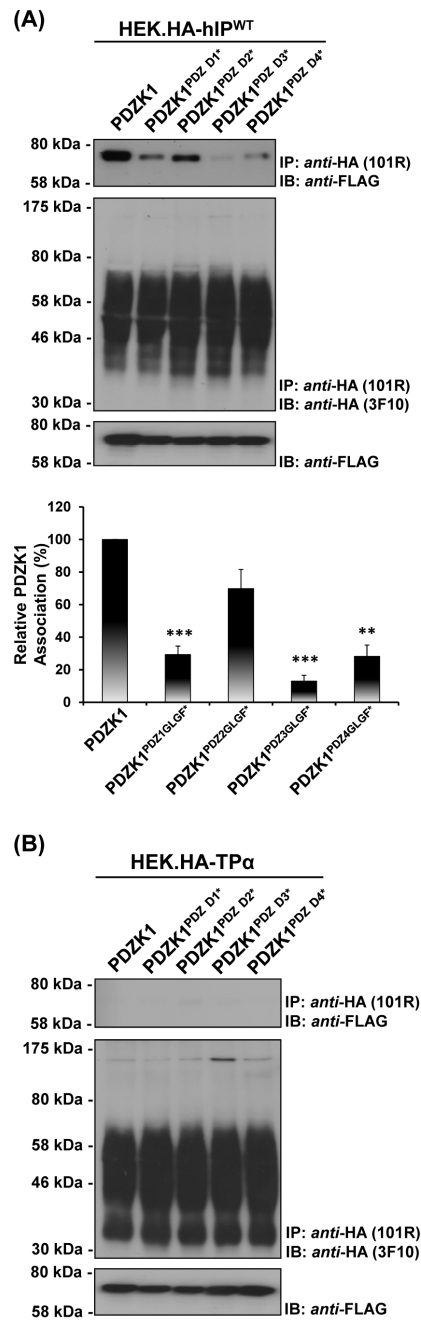
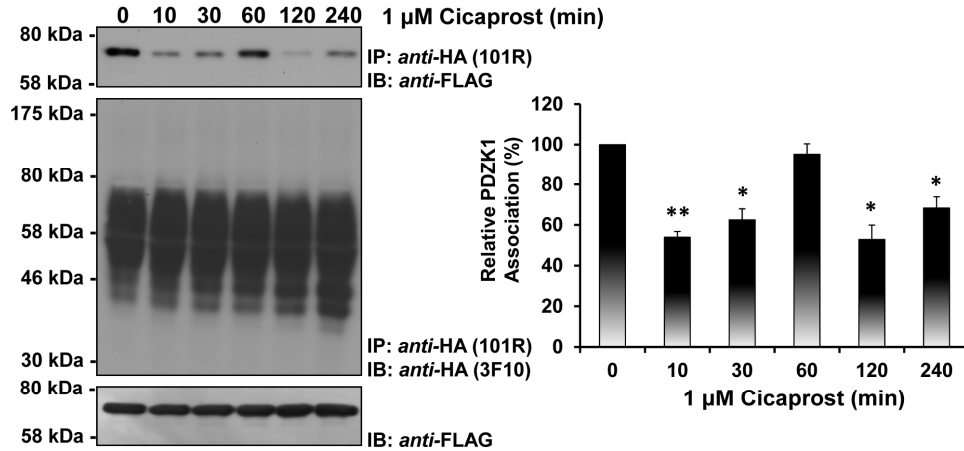
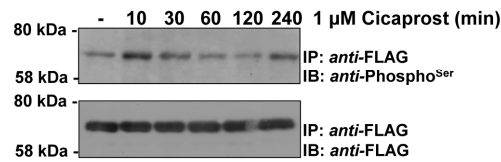
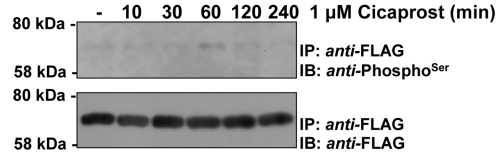
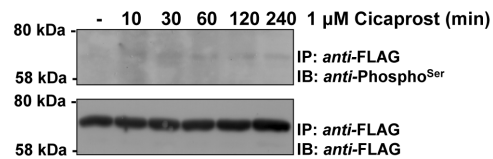


Figure 4. Identification of the PDZ domains involved in the interaction of PDZK1 with the hIP.

HEK.hIP (**Panel A**) or, as controls, HEK.TP α (**Panel B**) cells, each transiently transfected with pCMVTag2C encoding FLAG-tagged PDZK1^{FL}, PDZK1^{PDZD1*}, PDZK1^{PDZD2*}, PDZK1^{PDZD3*}, or PDZK1^{PDZD4*}, were subject to immunoprecipitation with *anti*-HA 101R antibody. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB), as indicated. The bar charts show mean relative levels of the wild type and mutated forms of PDZK1 associated with the *anti*-HA.hIP 101R immunoprecipitates (relative protein, % $\pm$ SEM, $n = 3$) where levels of the wild type PDZK1 are expressed as 100%. The asterisks indicate where PDZK1 mutation resulted in significant reductions in complex associated PDZK1 where ** and *** indicates $p < 0.01$ and $p < 0.001$, respectively, for post-hoc Dunnett's multiple comparison t -test analysis.

(A) PDZK1**(C) PDZK1 + RO1138452****(B) PDZK1****(D) PDZK1 + H-89****(E) PDZK1^{S505A}****Figure 5. Effect of Agonist-Activation of the hIP on the Interaction and Phosphorylation of PDZK1.**

Panel A: HEK.hIP cells, transiently transfected with pCMVTag2C:PDZK1, were stimulated with cicaprost (1 μ M; 0 – 240 min). HA-tagged hIPs were immunoprecipitated with *anti*-HA 101R antibody; immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB), as indicated. The bar charts show mean relative levels of the PDZK1 associated with the *anti*-HA.hIP 101R immunoprecipitates as a function of cicaprost-stimulation (relative protein, % $\pm$ SEM, n = 3) where levels in the absence of agonist are expressed as 100%. The asterisks indicate that cicaprost stimulation resulted in significant reductions in levels of PDZK1 associated with the hIP immune-complexes where * and ** indicates p < 0.05 and p < 0.01, respectively, for post-hoc Dunnett's multiple comparison t -test analysis. **Panels B – E:** HEK.hIP cells, transiently transfected with either pCMVTag2C:PDZK1 (**Panels B - D**) or pCMVTag2C:PDZK^{S505A} (**Panel E**) were pre-incubated with vehicle (**Panels B & E**), RO1138452 (10 μ M; 10 min; **Panel C**) or H-89 (10 μ M; 10 min; **Panel D**) prior to stimulation with cicaprost (1 μ M; 0 – 240 min). Cells were then subject to immunoprecipitation with *anti*-FLAG antibody to immunoprecipitate PDZK1. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB), as indicated. The bar chart below Panel B shows mean relative levels of phosphorylated PDZK1 in the *anti*-FLAG immunoprecipitates as a function of cicaprost-stimulation (relative protein, % $\pm$ SEM, n = 3) where levels in the absence of agonist are expressed as 100%. The asterisks indicate that cicaprost stimulation resulted in significant changes in levels of PDZK1 phosphorylation, where * and ** indicates p < 0.05 and p < 0.01, respectively, for post-hoc Dunnett's multiple comparison t -test analysis.

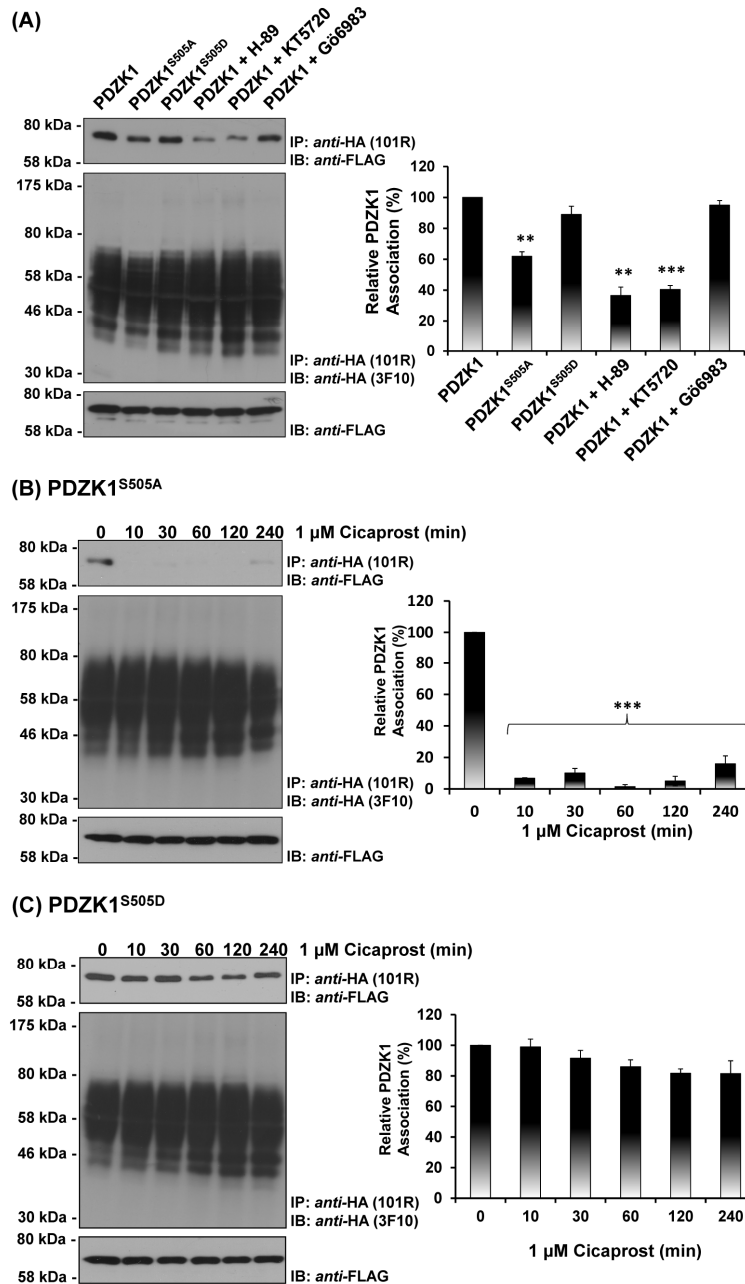


Figure 6. Effect of Agonist-activation of the hIP on its Interaction with PDZK1, PDZK1^{S505A} and PDZK1^{S505D}.

Panel A: HEK.hIP cells, transiently transfected with pCMVTag2C encoding PDZK1, PDZK1^{S505A} or PDZK1^{S505D}, were incubated with either vehicle (0.01 % DMSO; 10 min), H-89 (10 μM; 10 min), KT5720 (5 μM; 10 min) or GÖ6983 (1 μM; 10 min), as indicated. Alternatively, HEK.hIP cells, transiently transfected with pCMVTag2C encoding PDZK1^{S505A} (**Panel B**) or PDZK1^{S505D} (**Panel C**), were stimulated with cicaprost (1 μM; 0 – 240 min). HA-tagged hIPs were immunoprecipitated with anti-HA 101R antibody; immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB), as indicated. The bar charts show mean relative levels of the PDZK1, PDZK1^{S505A} and PDZK1^{S505D}-associated with the anti-HA.hIP 101R immunoprecipitates as a function of cicaprost-stimulation (relative protein, % ± SEM, $n = 3$) where levels in the absence of agonist are expressed as 100%. The asterisks indicate that inhibition of PKA (**Panel A**) or cicaprost stimulation (**Panels B & C**) resulted in significant reductions in PDZK1 levels in anti-HA immunoprecipitates, where ** and *** indicates $p = 0.01$ and $p = 0.001$, respectively, for post-hoc Dunnett's multiple comparison t -test analysis.

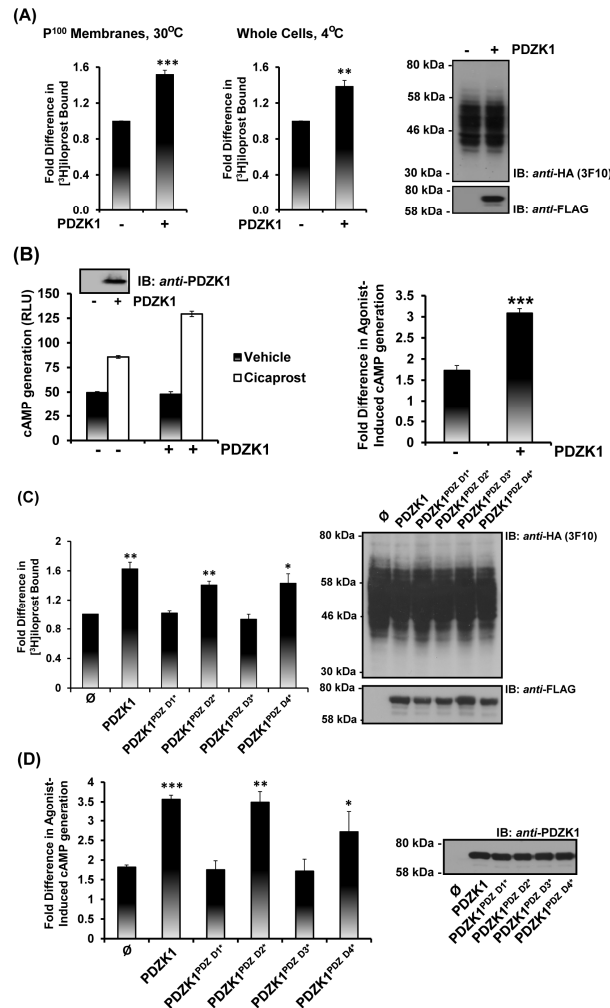


Figure 7. Effect of PDZK1 on the Expression and Signalling of the hIP.

Panels A & C: HEK.hIP cells were transiently transfected with pCMVTag2C encoding either PDZK1 (**Panels A**) or PDZK1^{PDZ D1*}, PDZK1^{PDZ D2*}, PDZK1^{PDZ D3*} and PDZK1^{PDZ D4*} or, as controls, pCMVTag2C vector ($\emptyset$) alone (**Panel C**). Radioligand binding analysis was performed 72 hr post-transfection in the presence of 4 nM [³H]iloprost for 60 min using either crude membrane (P₁₀₀) fractions (30 °C; **Panels A & C**) or whole cells (4 °C; **Panel A**). Data are presented as fold increases in [³H]iloprost bound as a function of PDZK1 expression where levels in the presence of wild type PDZK1 are expressed as 1. **Panels B & D:** HEK 293 cells were transiently co-transfected with pHM6:hIP, pADVA, pCRE-LUC and pRL-TK in the presence of pCMVTag2C encoding PDZK1^{FL} (**Panel B**) or PDZK1^{FL}, PDZK1^{PDZ D1*}, PDZK1^{PDZ D2*}, PDZK1^{PDZ D3*} and PDZK1^{PDZ D4*} or, as controls, pCMVTag2C vector ($\emptyset$) alone (**Panel D**). Cells were incubated either with vehicle or cicaprost (1 μ M; 3 hr) prior to determination of cAMP generation (RLU $\pm$ SEM; $n = 3$), where data are represented as levels of agonist-induced-cAMP generation (**Panel B**, left bar charts) and as fold inductions in agonist-induced cAMP accumulation (**Panel B**, right bar charts and **Panel D**). Expression of the HA-tagged hIP and Flag-tagged PDZK1 proteins were verified by immunoblot analysis of the respective whole cell lysates (50 μ g/lane), as indicated. The asterisks indicate where ectopic expression of PDZK1 resulted in significant fold increases in [³H]iloprost bound (**Panels A & C**) or agonist-induced cAMP accumulation (**Panels B & D**) where *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively, for post-hoc Dunnett's multiple comparison t -test analysis. Levels of [³H]iloprost binding in HEK.hIP cells were 1.1 ± 0.04 pmol/mg of cell protein ($n = 4$). Basal levels cAMP generation in HEK.hIP cells was 0.70 ± 0.04 pmol/mg of cell protein ($n = 4$) and was not affected by ectopic expression of PDZK1 or its mutated variants.

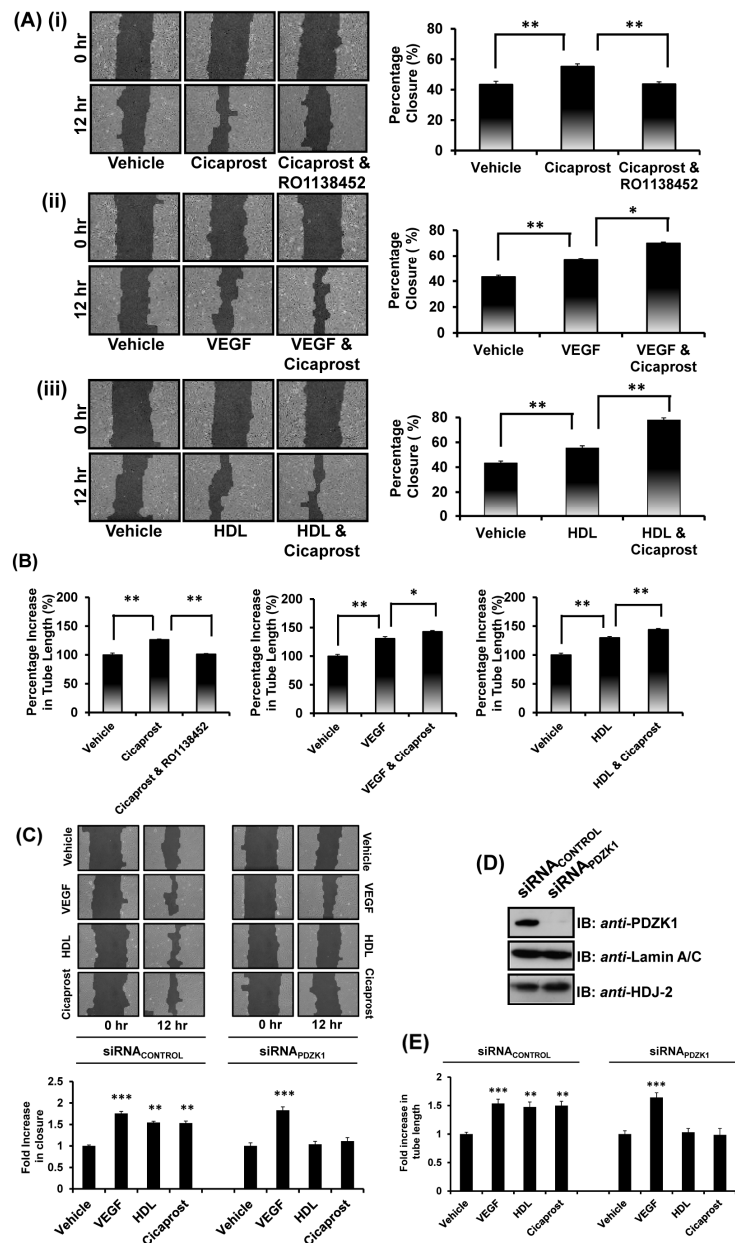


Figure 8. Effect of PDZK1 on hIP agonist-induced migration and endothelial tube formation in 1° HUVECs.

Panel A & B: Migration after scratch wounds in 1° HUVEC monolayers (**Panel A**) or tube formation of HUVECs seeded on Matrigel™ (**Panel B**) in the presence of cicaprost (1 μM), VEGF (50 ng/ml), or HDL (50 μg/ml), alone or in combination and in the absence or presence of RO1138452 (10 μM; 10 min pre-incubation) were analysed at 12 hr. Bar charts represent mean percentage closure (% ± SEM; *n* = 3) at 12 hr (**Panel A**) or mean percentage of basal tube length at 12 hr (% ± SEM; *n* = 3; **Panel B**). **Panel C - E:** 1° HUVECs were transfected with siRNA_{PDZK1} or siRNA_{CONTROL} where immunoblot analysis confirmed specific disruption of PDZK1 expression (**Panel D**). Migration (**Panel C**) and tube formation (**Panel E**) was analysed in the presence of vehicle, VEGF, HDL or cicaprost, as indicated, at 0 hr and 12 hr. Bar charts represent mean fold increases (± S.E.M.; *n* = 3) in either wound closure or tube length in the presence of vehicle, VEGF, HDL or cicaprost, as indicated, at 12 hr. The asterisks indicate either significant agonist-induced increases in migration (**Panel A**), significant agonist-induced increases in tube length (**Panel B**), significant fold increases in agonist-induced migration in comparison to vehicle treated cells (**Panel C**), significant fold increases in agonist-induced tube length in comparison to vehicle treated cells (**Panel E**) where *, ** and *** indicates *p* < 0.05, *p* < 0.01 and *p* < 0.001, respectively, for post-hoc Dunnett's multiple comparison *t*-test analysis.

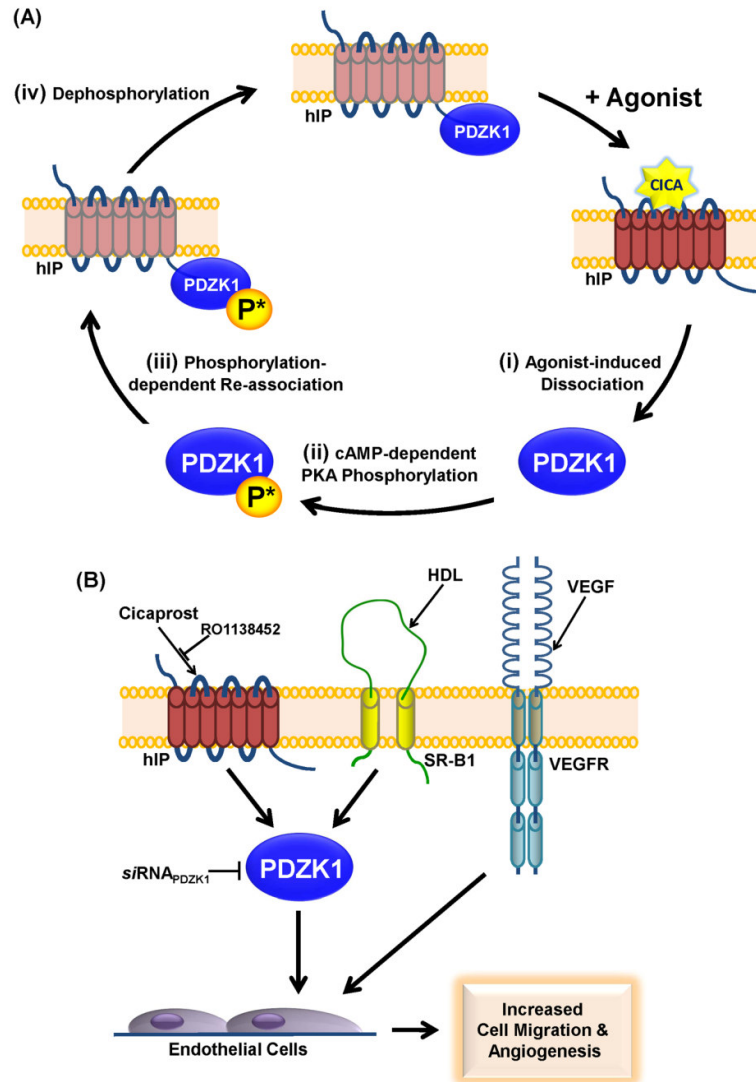
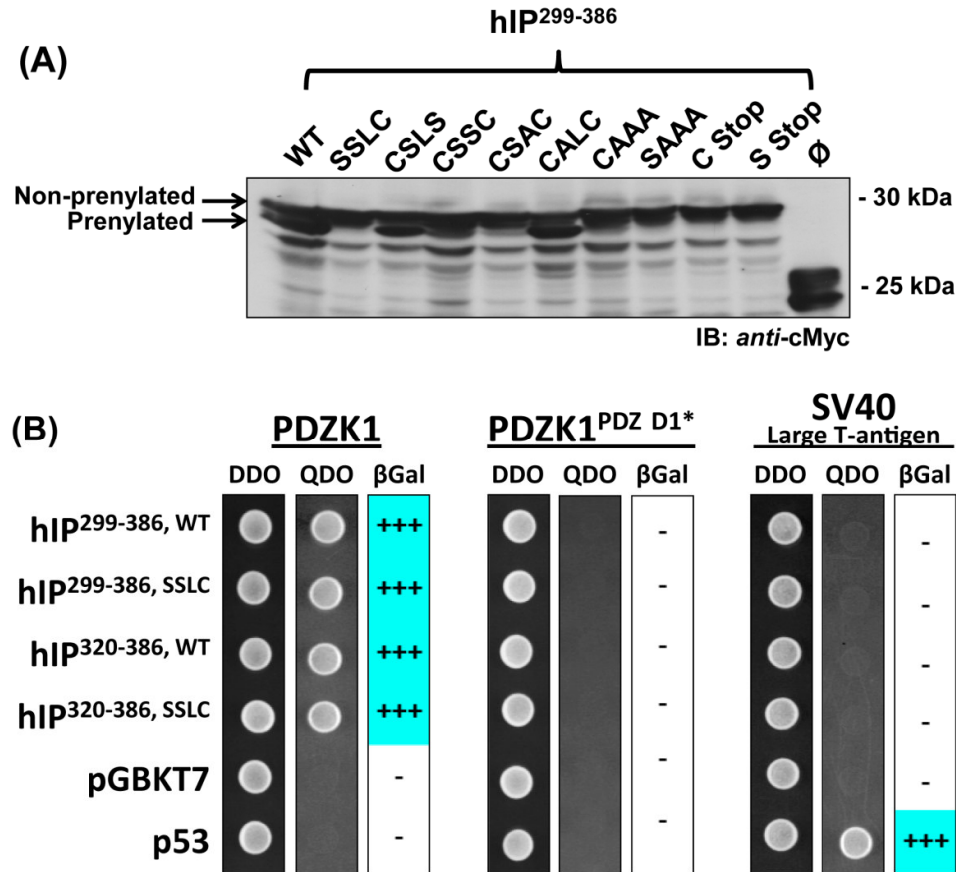


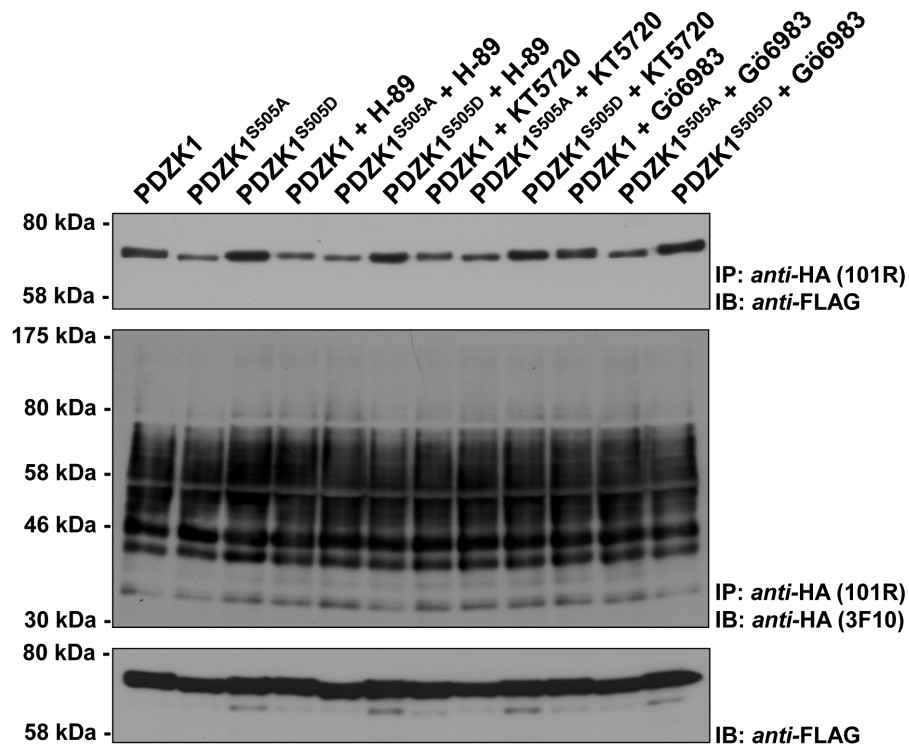
Figure 9. Proposed model of the interaction of PDZK1 with the hIP and its implications for endothelial cell migration and *in vitro* angiogenesis.

Panel A: In the absence of agonist, PDZK1 is constitutively associated in a complex with the hIP where PDZK1 is either not phosphorylated or basally/ hypo-phosphorylated. Upon cicaprost-stimulation, (i) the hIP undergoes an agonist-induced conformational activation leading to dissociation of PDZK1. (ii) Released PDZK1 is then subject to enhanced hIP induced cAMP-dependent PKA phosphorylation at Ser⁵⁰⁵ and (iii) this enhanced or nett hyper-phosphorylated PDZK1 triggers its re-association with the hIP. The re-association of PDZK1 and hIP is coincident with regulated (iv) dephosphorylation of PDZK1 and its return to basal or hypo-phosphorylated levels. Consistent with this model, the phospho-defective PDZK1^{S505A} is found in a constitutive complex with the hIP and undergoes agonist-induced dissociation but cannot undergo phosphorylation-induced re-association in response to receptor activation. In contrast, the phospho-mimetic PDZK1^{S505D} is hypothesised to mimic the ‘hyper-phosphorylated’ protein state (state iii) whereby any transient agonist-induced dissociation in the interaction of this mutant with the hIP is immediately recovered due to it mimicking the ‘hyper-phosphorylated’ state. **Panel B:** Agonist-activation of the hIP, SR-B1 and VEGFR leads to enhanced endothelial cell migration and tube formation/*in vitro* angiogenesis. Consistent with a previous study (Zhu *et al.*, 2008), HDL/SR-B1-, but not VEGF/VEGFR-, mediated endothelial cell migration is dependent on its interaction with PDZK1. Herein, it was established that, similar to that of HDL/SR-B1, cicaprost-activation of the RO1138452-sensitive hIP promotes endothelial cell migration and tube formation and that these effects are dependent on PDZK1. siRNA-disruption of PDZK1 inhibits both cicaprost- and HDL-, but not VEGF-, induced endothelial cell responses.

Supplemental Data

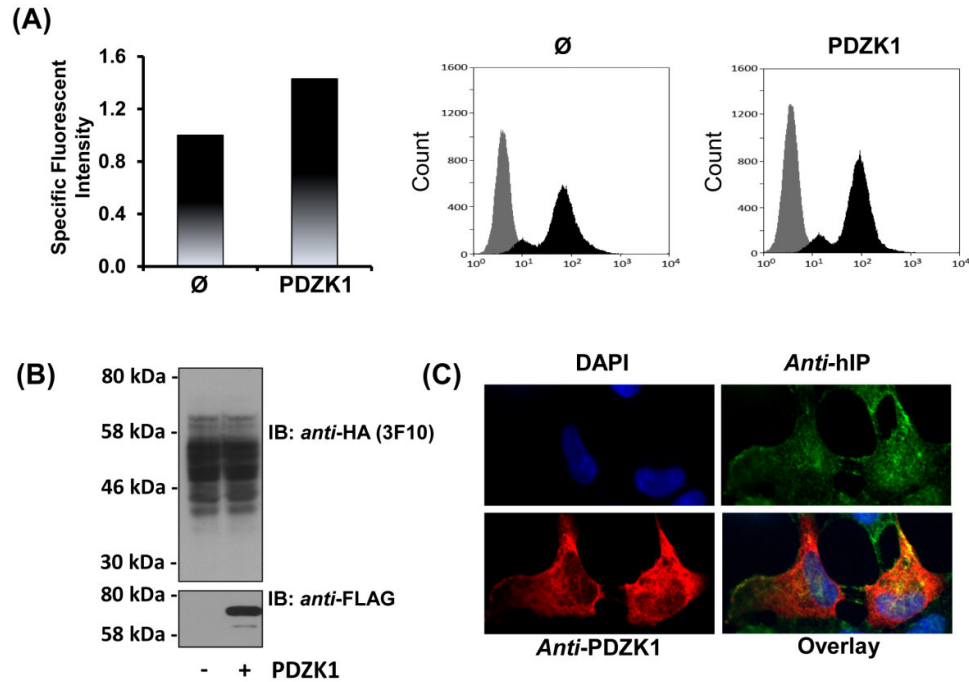


Supplemental Figure 1. Western Blots Analysis of hIP Bait Proteins Expressed in *S.c* AH109.
Panel A: Immunoblot analysis of protein extracted from the *S.c* AH109 bait strains transformed with recombinant pGBKT7 encoding the hIP²⁹⁹⁻³⁸⁶ subfragment with its wild type (-C³⁸³SLC³⁸⁶) or the listed mutated variant sequences at its carboxyl terminus and, as a control, with the vector pGBKT7 (∅) alone. Proteins were resolved by SDS-PAGE and immunoblotted with *anti-Myc* (9B11) antibody. The arrows to the left of the panel indicate the non-isoprenylated and isoprenylated forms of the hIP. **Panel B:** *S.c* Y187 (pACT2:PDZK1), *S.c* Y187 (pACT2:PDZK1^{PDZ D1*}) and, as controls, *S.c* Y187 (pTD1-1) prey strains were mated with *S.c* AH109 bait strains transformed with recombinant pGBKT7 encoding the listed hIP subfragments and, as controls, p53 or with the vector pGBKT7 alone. Diploids were selected on DDO medium, whereas interactants were selected on QDO medium and by their ability to express β-Gal. Data: n ≥ 3.



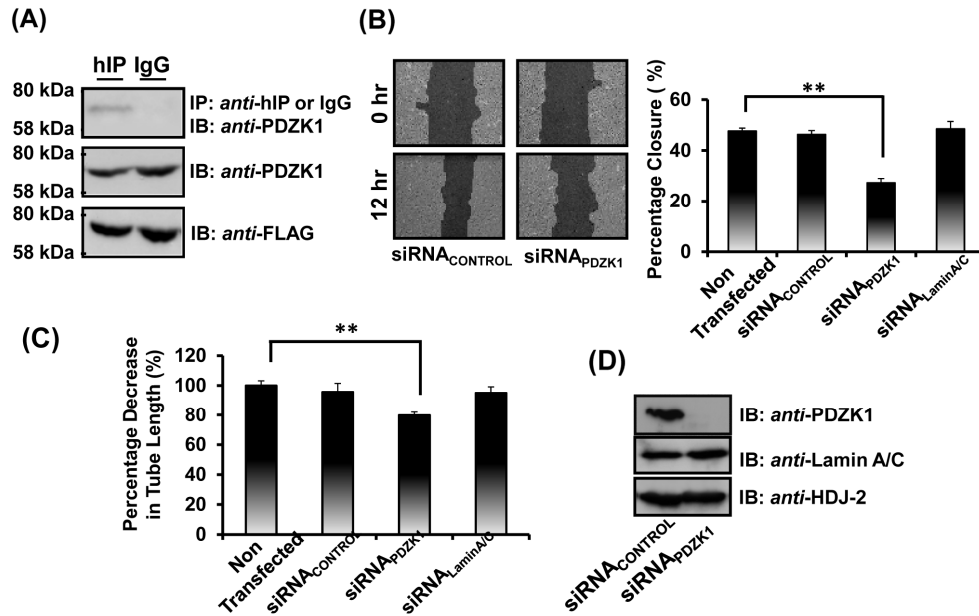
Supplemental Figure 2. Effect of Kinase Inhibition on the Interaction of PDZK1 with the hIP.

HEK.hIP cells, transiently transfected with pCMVTag2C encoding PDZK1^{FL}, PDZK1^{S505A} or PDZK1^{S505D} were pre-incubated with vehicle (0.01 % DMSO), H-89 (10 μ M; 10 min), KT5720 (5 μ M; 10 min) or with Gö6983 (1 μ M; 10 min), as indicated. HA-tagged hIPs were immunoprecipitated with *anti*-HA 101R antibody; immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB), as indicated. Uniform expression of the Flag-tagged PDZK1 proteins was verified by immunoblot analysis of whole cell lysates (50 μ g/lane) with *anti*-FLAG antibody (lower panels). Data: $n \geq 3$.



Supplemental Figure 3. Effect of PDZK1 on the expression of and co-localization with the hIP.

Panels A & B: Flow cytometric and immunoblot analysis of the effect of PDZK1 on hIP expression: HEK.hIP cells were transiently transfected with pCMVTag2C:PDZK1 or, as control, pCMVTag2C vector ($\emptyset$) alone. Cell surface HA-tagged hIP expression was examined by flow cytometry (**Panel A**), using *anti*-HA 101R antibody and AlexaFluor 488 goat *anti*-mouse IgG secondary antibody. Fluorescent intensity was corrected for background fluorescence using a control isotype IgG. Bar charts represent fold increases in fluorescent intensity upon PDZK1 over-expression where levels upon control transfection ($\emptyset$) are expressed as 1. Representative flow cytometry histograms show the specific cell surface expression of HA-tagged hIP (black) relative to background (grey), where the horizontal axis shows fluorescent intensity and the vertical axis represents cell count. Expression of the HA-tagged hIP and Flag-tagged PDZK1 proteins were verified by immunoblot analysis of the respective whole cell lysates (50 μ g/lane) using *anti*-HA 3F10 and *anti*-FLAG (upper and middle panels, respectively; **Panel B**). Data: $n \geq 3$. **Panel C:** HEK.hIP cells were immunolabelled with *anti*-hIP and *anti*-PDZK1 antibodies under permeabilizing conditions followed by detection with *anti*-Rabbit AlexaFluor488 (*anti*-hIP) and *anti*-Mouse AlexaFluor594 (*anti*-PDZK1) conjugated secondary antibodies, respectively, or both (overlay) and then counterstained with DAPI. Data: $n \geq 3$.



Supplemental Figure 4. Effect of Disruption of PDZK1 Expression on Endothelial Cell Migration and Tube Formation under Basal Conditions.

Panel A: 1^o HUVECs, transiently transfected with pCMVTag2C:PDZK1^{FL}, were subject to immunoprecipitation with *anti*-hIP antibody or, as a control, with the pre-immune IgG. Immunoprecipitates (IP) were resolved by SDS-PAGE and immunoblotted (IB), as indicated. Expression levels of PDZK1 were verified by immunoblotting of whole cell lysates with *anti*-PDZK1 (middle panel) and *anti*-FLAG (lower panel) antisera. Data: $n \geq 3$. **Panel B & C:** 1^o HUVECs were transfected with siRNA_{PDZK1}, siRNA_{LaminA/C} or siRNA_{CONTROL}. Some 36 hr later, transfected or, as a control, non-transfected cells were either scratched and wounds analysed immediately (0 hr) or after 12 hr (**Panel B**), seeded on MatrigelTM and tube formation evaluated after 12 hr (**Panel C**) or immunoblot analysis (**Panel C**) assessed PDZK1 (Upper panel) and Lamin A/C (Middle Panel) abundance 36 hr post-transfection. Analysis of HDJ-2 expression (Lower panel) was used as a loading control. Bar charts represent mean percentage closure at 12 hr (% $\pm$ SEM; $n = 3$; **Panel B**) or percentage decrease in basal tube length at 12 hr (% $\pm$ SEM; $n = 3$; **Panel C**). The asterisks indicate significant siRNA_{PDZK1}-mediated decreases in migration (**Panel B**) or tube formation (**Panel C**), where ** indicates $p < 0.01$ for post-hoc Dunnett's multiple comparison t -test analysis.

SUPPLEMENTAL TABLE 1. PRIMERS USED FOR SITE-DIRECTED MUTAGENESIS OF THE ‘GLGF MOTIFS’ WITHIN PDZ DOMAIN (PDZD) 1 TO PDZD4 OF PDZK1 & SERINE⁵⁰⁵ MUTANTS.

WILD TYPE ‘GLGF MOTIF’	MUTATED ‘GLGF MOTIF’	RECOMBINANT PLASMID GENERATED	OLIGONUCLEOTIDE PRIMER*
<u>PDZD1:</u> N ¹⁹ YGF ²²	<u>K</u> ¹⁹ <u>YRS</u> ²²	pACT2: PDZK1 ^{PDZD1*} / pCMVTag2C:PDZK1 ^{PDZD1*}	5'GGCAAGCAAGAAGGGCAAAAGTAT <i>GGCTTCTTCCTGCGAATTGAGAAGG</i> -3'
<u>PDZD2:</u> S ¹⁴⁴ YGF ¹⁴⁷	S ¹⁴⁴ <u>YRE</u> ¹⁴	pCMVTag2C:PDZK1 ^{PDZD2*}	5'CTGGAAGGAAGGAGGCAGCTAT <i>CGC</i> <i>GAGTCTCTGAAAAGTGTCCAA</i> -3'
<u>PDZD3:</u> G ²⁵² YGF ²⁵⁵	G ²⁵² <u>YRE</u> ²⁵⁵	pCMVTag2C:PDZK1 ^{PDZD3*}	5'GAAGAAAGGAAGCAATGGCTAT <i>CGT</i> <i>GAGTATCTGAGGGCAGGCTCAGAA</i> -3'
<u>PDZD4:</u> G ³⁸⁷ YGF ³⁹⁰	G ³⁸⁷ <u>YRE</u> ³⁹⁰	pCMVTag2C:PDZK1 ^{PDZD4*}	5'GGCTAAAGGTGAAAATGGCTAT <i>CGC</i> <i>GAGCACTTAAATGCGATTCGGGGTC</i> -3'

RECOMBINANT PLASMID GENERATED	OLIGONUCLEOTIDE PRIMER*
pCMVTag2C:PDZK1 ^{S505A}	5'-GCAAAAGAACGGGCCCAC <i>GCT</i> TACAGCCTCACATTCTTC-3'
pCMVTag2C:PDZK1 ^{S505D}	5'-GCAAAAGAACGGGCCCAC <i>GAT</i> TACAGCCTCACATTCTTC-3'

* Sequences given correspond to those of the sense primer only where the identity of the mutator codon(s) is in boldface italics and antisense primer sequences are inferred.