The Guidance Receptor Plexin D1 Moonlights as an Endothelial Mechanosensor

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Shear stress imparted by blood flow on arteries is critical for vascular development and homeostasis but can also be an instigator of atherosclerosis[1]. Endothelial cells (ECs) lining the vasculature use molecular mechanosensors to directly detect shear stress profiles that will ultimately lead to atheroprotective or atherogenic responses[2]. Plexins are key cell-surface receptors for the Semaphorin family of cell-guidance signalling proteins and can regulate cellular patterning by modulating the cytoskeleton and focal adhesion structures[3-5]. However, a role for Plexins in mechanotransduction has not been examined. Here, we demonstrate a hitherto unrecognised role of Plexin D1 (PlxnD1) in mechanosensation and mechanically-induced disease pathogenesis. PlxnD1 is required for the EC response to shear stress *in vitro* and *in vivo* and regulates the site-specific distribution of atherosclerotic lesions. PlxnD1 is a direct force sensor in ECs and forms a mechano-complex with Neuropilin-1 (NRP1) and VEGFR2 that is necessary and sufficient for conferring mechanosensitivity upstream of the junctional complex and integrins. PlxnD1 achieves its binary functions as either a ligand or force receptor by populating two distinct molecular conformations. Our results establish a novel mechanosensor in ECs that regulates cardiovascular pathophysiology and provide a mechanism by which a single receptor can exhibit a binary biochemical nature.

ECs are constantly exposed to the haemodynamic forces of blood flow, including the frictional force of fluid shear stress that, depending on the vessel geometry, can be protective or pathogenic. While disturbed or atheroprone flow patterns found in curvatures and bifurcations are associated with upregulation of pro-inflammatory genes and deposition of atherosclerotic lesions, uniform or atheroprotective shear stress induces cytoskeleton remodelling and alignment of ECs in the direction of flow [1,6]. The critical importance of shear stress in cardiovascular development and function has fuelled intense investigation into the identification of endothelial mechanosensors, as they are the first responders to changes in the mechanical environment [2].

Plexins are cellular receptors that play a range of important roles in axon guidance, tumour progression and immune cell regulation[7]. To date, Plexins are known to work primarily by binding to semaphorin ligands, cell-bound or free in solution, along with other co-receptors, resulting in intracellular signalling events that lead to large scale changes in the cytoskeleton and cell adhesion [3,4]. Here, we show that the guidance receptor PlxnD1 moonlights as a novel mechanosensor in ECs, regulating vascular function and the site-specific distribution of atherosclerosis.

To determine the role of PlxnD1 under flow conditions, we transfected bovine aortic ECs (BAECs) with either Scrambled (Scr) or PlxnD1 siRNAs (Extended Fig. 1a), and subjected them to shear stress. Knockdown of PlxnD1 attenuated shear stress-induced activation of key signalling mediators Akt, ERK1/2 and eNOS (Extended Fig. 2a). PlxnD1-dependent mechanotransduction is independent of its ligand Sema3E, as incubation with a Sema3E function blocking antibody did not affect the flow-induced activation of signalling cascades (Extended Fig. 3). Next, we examined the role of PlxnD1 in the hallmark response to atheroprotective shear stress by examining alignment in the direction of flow. EC alignment with flow direction is

highly correlated with atheroresistant regions of arteries and plays an important role in the activation of anti-inflammatory pathways. PlxnD1-depleted ECs showed a striking failure to align in response to shear stress and displayed fewer and more disorganised actin stress fibres (Extended Fig.2b). Quantification of alignment by measuring the orientation angle and the elongation factor indicate that PlxnD1 is required for EC alignment with flow. We also examined levels of Kruppel-like factors KLF2 and KLF4, key anti-inflammatory transcription factors which are known to be upregulated by atheroprotective shear stress.[8,9] Congruently, we found that knockdown of PlxnD1 attenuated flow-induced upregulation of both these genes compared to control cells. (Fig. 1a). We then asked if PlxnD1 could mediate the endothelial response to disturbed shear stress. We subjected ECs to atheroprone flow for 24h and examined mRNA levels of pro-inflammatory genes Monocyte Chemoattractant Protein-1 (MCP-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1)[10]. We noted that knockdown of PlxnD1 in ECs with siRNA significantly reduced the upregulation of both genes in response to atheroprone shear stress (Fig. 1b). Combined, these data demonstrate that PlxnD1 is a critical mediator of key shear stress responses in ECs.

To explore the biological relevance of our findings, we used a transgenic mouse model to enable endothelial-specific inducible deletion of PlxnD1 ($PlxnD1_{iECKO}$) (Extended Fig.1b, 7b). Confocal imaging of the endothelial actin filaments and staining for the junctional marker β-catenin revealed reduced EC elongation and intensity of actin stress fibres in the absence of PlxnD1 (Fig.1c), consistent with *in vitro* observations (Extended Fig.2b).

Given the decrease in inflammatory gene expression in response to atheroprone shear stress *in vitro* observed with loss of PlxnD1 (Fig.1b), we assessed the role of endothelial PlxnD1 in a pathophysiogical setting. Atherosclerotic lesions are known

to occur in regions of the vasculature with low/disturbed blood flow, flow reversal and other complex spatial/temporal flow patterns[1]. Systemic risk factors, such as hypercholesterolaemia, interact with local biomechanical factors to initiate and advance atherosclerotic plaque deposition. To assess whether endothelial deletion of PlxnD1 affected atherosclerosis *in vivo*, we crossed PlxnD1$^{fl/fl}$ and PlxnD1^{iECKO} into the hypercholesterolaemic Apolipoprotein E deficient (ApoE$^{-/-}$) background[11] and subjected them to a high-fat diet for 10 weeks. Although animal body weights and lipid levels were unaffected by loss of PlxnD1 (Extended Fig. 4a), quantification revealed a significant decrease in plaque burden for both the whole aorta and the arch in PlxnD1^{iECKO}/ApoE$^{-/-}$ animals (Fig. 1d, e). To explore these differences further, we examined expression of inflammatory markers in the inner curvature of the aortic arch. Immunostaining and qPCR analysis showed reduced levels of MCP-1 and VCAM-1 in PlxnD1^{iECKO}/ApoE$^{-/-}$ compared to PlxnD1$^{fl/fl}$/ApoE$^{-/-}$ mice (Fig. 1f, Extended Fig. 4b). Given the atheroprotective role of laminar shear stress and the reduced alignment with loss of PlxnD1, we examined the effects in the atheroprotected descending aorta. After high-fat feeding for an extended period of 20 weeks, we observed increased plaque burden in the descending aortas of PlxnD1^{iECKO}/ApoE$^{-/-}$ (Extended Fig. 5); these plaques also appeared to correlate with intercostal branch points that have flow disturbances. Together, these results show that endothelial PlxnD1 is required for the endothelial response to fluid shear stress and the site-specific distribution of atherosclerosis.

The requirement for PlxnD1 in flow-mediated responses *in vitro* and *in vivo*, prompted us to ask if this is because PlxnD1 is simply a player in mechanochemical signalling cascades, or functions as a mechanoreceptor, capable of detecting mechanical force. We applied tensional forces, using a magnetic system[12], to paramagnetic beads coated with an antibody that recognises the extracellular domain of PlxnD1 and examined force responses using 4 different readouts. First,

force on PlxnD1 induced activation of the same signalling cascades (ERK1/2, Akt, VEGFR2) (Fig. 2a), as those induced by shear stress (Extended Fig. 2a)[13]. Second, we observed robust transient increase in intracellular calcium levels in ECs when force was applied on PlxnD1 (Fig. 2b), similar to the response observed for other recently discovered mechanosensors[14,15]. Third, we examined cytoskeletal responses[12]: ECs responded to application of force on PlxnD1 by exhibiting a robust increase in both vinculin-positive focal adhesions (Fig. 2c) and ligated integrin β1 staining (Extended Fig. 6a). Notably, the mechanotransduction response was not restricted to the vicinity of the magnetic bead under tension, but was a global cell-wide phenomenon. Fourth, we examined phosphorylation of vinculin at Y822, a site known to be phosphorylated when force is applied on E-cadherin[16], and observed a significant increase in its activation following force on PlxnD1 (Fig. 2d). These mechanoresponses were specific to PlxnD1 because ECs incubated with beads coated with another transmembrane receptor CD44 or Poly-L-lysine did not respond to force. These data clearly demonstrate that PlxnD1 is a direct force sensor that can elicit robust and global mechanical signalling in ECs.

Cell-cell and cell-matrix adhesions represent two highly mechanically active sites within ECs. These include the junctional mechanosensory complex comprising PECAM-1, VEGFR2 and VE-cadherin and integrins at the cytoskeleton-extracellular matrix interface[13,17] (Fig.3a). We interrogated the relationship between PlxnD1 and these mechanical 'hotspots' in ECs. *En face* confocal imaging revealed robust and similar expression of PlxnD1 in ECs in both arch and descending aorta and co-localisation with PECAM-1 at cell-cell junctions (Extended Fig. 7a). Staining was specific as it was not observed in PlxnD1$_{iECKO}$ aortas (Extended Fig. 7b). Sema3E was also observed in *en face* sections with its expression being lower in the arch (Extended Fig. 7c). Co-immunoprecipitation experiments showed flow-induced association of PlxnD1 with components of the junctional mechanosensory complex

(PECAM-1, VEGFR2, VE-cadherin and PI3K/p85) (Extended Fig. 7d). To explore if PlxnD1 is just another component of the junctional complex or whether it operates upstream, we used immunoprecipitation to interrogate complex formation both at the junctional mechanosensory complex and integrin-matrix adhesions. We found that responses at the junctional complex, such as shear stress-induced phosphorylation of VEGFR2 and association of the p85 subunit of PI3K and VE-cadherin with VEGFR2[13], were all abrogated by knockdown of PlxnD1 (Fig. 3b). In agreement, both inhibition of VEGFR2 receptor kinase (Fig. 3d) and deletion of PECAM-1 abrogated force-induced signalling, suggesting that junctional mechanosensory components are necessary intermediates for the PlxnD1 force response (Extended Fig. 8a). Similarly, flow-induced complex formation at integrin-matrix adhesions (as assayed by association of Shc with integrin $\alpha_v\beta_3$[18,19]) was also strongly reduced with loss of PlxnD1 (Fig. 3c). Recent work has highlighted a role for Piezo1 and G_q/G_{11}-mediated mechanosignalling, although there are conflicting reports as to whether these pathways are linked[20,21] or independent of each other[22,23]. Force application on PlxnD1 showed that loss of G_q/G_{11} abolished the PlxnD1 force response, while knockdown of Piezo1 had no effect (Extended Fig. 1e,f; Extended Fig., 8b,c).

To investigate molecular mechanisms even further, we examined the role of the PlxnD1 co-receptor neuropilin-1 (NRP1). NRP1 is a cell surface transmembrane protein that acts as a Sema3 and VEGF co-receptor for PlxnD1 and VEGFR2, respectively[24] and its presence in neurons switches the Sema3E signal from repulsion to attraction[25]. We found a requirement for NRP1 in the PlxnD1 force response, as both knockdown (Extended Fig. 1d) and inhibition of NRP1 abolished force-induced phosphorylation of vinculin (Fig. 3d). We also observed that shear stress induced the formation of a complex between PlxnD1, VEGFR2 and NRP1 (Fig. 3e, f) and this complex was dependent on NRP1 (Fig. 3g). Taken together,

these data show that PlxnD1 associates with NRP1 and VEGFR2 in response to flow and operates upstream of both the junctional complex and integrins.

To test if PlxnD1 (and its molecular partners) is sufficient to confer mechanosensitivity in a heterologous cell line, we transfected Cos7 cells with plasmid constructs expressing PlxnD1, NRP1 and/or VEGFR2 and applied shear stress (Fig. 3h). These cells do not express any of the components of the junctional complex (i.e. PECAM-1 or VE-cadherin) and, thus, constitute an ideal system to monitor mechanical responses specifically due to PlxnD1. Cos7 cells expressing all three proteins (VEGFR2, NRP1 and PlxnD1) showed activation of early signalling responses, including phosphorylation of VEGFR2, association of VEGFR2 with Src tyrosine kinase, and PlxnD1/VEGFR2/NRP1 complex formation in response to shear stress. Importantly, none of these responses occurred in the absence of PlxnD1, thus providing further evidence that PlxnD1 is a specific and direct force sensor (Fig. 3i). Overall, these data provide evidence for a necessary and sufficient role of PlxnD1 in the shear stress-induced response. To demonstrate that PlxnD1 operates as a specific force sensor even further, we applied force on other elements of the complex. As shown in Extended Fig. 9, application of force on either NRP1 or VEGFR2 failed to elicit downstream responses. Taken together, these data unambiguously show that PlxnD1 is a specific and direct mechanosensor.

The mechanical response of PlxnD1 is in stark contrast to the ligand response, as force on PlxnD1 increases focal adhesions, whereas Sema3E treatment reduces focal adhesions and leads to collapse of the actin cytoskeleton [4] (Extended Fig. 6b). Structure-function studies on Semaphorins, Plexins and their cognate complexes, have established that the ligand-binding response requires a dimeric semaphorin to engage the N-terminal sema domains of two plexin receptors[26]. Recent crystal structures and negative stain electron microscopy (EM) analyses of entire, ten domain, class A plexin (PlxnA) ectodomains revealed a distinctive ring-like

conformation that is suitable for coupling extracellular semaphorin-based dimerization through to the transmembrane and cytoplasmic regions to transduce the ligand-binding response [7,27]. However, the negative stain EM studies also revealed that the PlxnA ectodomain is capable of flexion, with distinctive minor populations of more open conformations. We carried out negative stain EM analysis of the PlxnD1 ectodomain and found evidence that it can flex to a more open conformation although the dominant state is ring-like (Fig. 4a, 4b and Extended Data Fig. 10a). We speculated that the ability to have flexion and switch between these two conformation states might provide an explanation for the binary nature of PlxnD1 function (Fig. 4c). To examine this, we generated a double mutant of PlxnD1, Y517C A1135C, designed to promote formation of an intramolecular disulphide bond between domain 1 and domain 9 of the PlxnD1 ectodomain (Fig. 4d). Based on structural analysis, we predicted that the introduction of this disulphide bridge would lock the receptor ectodomain into the ring-like conformation, still allowing triggering of the ligand-binding reponse by Sema3E, but preventing switching to the “open” and putative mechanosensory conformation. Purification of the protein and subsequent quantitative assay using a thiol-reactive fluorescent dye, as well as negative stain EM, demonstrated that the protein did indeed contain the desired covalent disulphide linkage (Extended Fig. 10b, 10c).

PlxnD1-depleted ECs were infected with adenovirus expressing either WT or mutant PlxnD1 and were assayed for their ability to respond to the ligand Sema3E or mechanical force. Treatment with Sema3E resulted in a decrease in focal adhesions in both WT and mutant PlxnD1 expressing cells (Fig. 4e), showing that the PlxnD1 ectodomain, when locked into a ring-like conformation, maintains its ability to bind Sema3E and signal to cause the disassembly of the cytoskeleton. We then tested if trapping the PlxnD1 in the semaphorin binding ring-like conformation was permissive of its mechanosensory function. We found that cells expressing the mutant PlxnD1

did not respond to mechanical force, as assayed by activation of early signalling responses (pVEGFR2, pAkt and pERK1/2 in Fig. 4h), cytoskeleton signalling (pVinculin in Extended Fig. 11) and focal adhesion maturation (Fig. 4f). To further determine the requirement for PlxnD1 flexion in mechanotransduction, we examined the effects of mutant PlxnD1 in shear stress signalling. In contrast to ECs expressing WT PlxnD1, ECs expressing mutant PlxnD1 were unable to activate Akt, ERK1/2 or eNOS in response to shear stress (Fig. 4i). Additionally, reconstitution of mutant PlxnD1 in Cos7 cells blocked early shear stress responses, including phosphorylation of VEGFR2, association of VEGFR2 with Src tyrosine kinase and shear stress-induced VEGFR2 and NRP1 complex formation (Fig. 4g). Collectively, these results demonstrate that trapping PlxnD1 in its ring-like conformation maintains its ligand-dependent signalling function but compromises its ability to sense and respond to mechanical force.

Our work identifies the semaphorin-binding receptor PlxnD1 as a novel force detector in ECs. One of the best-characterised mechanosensors to date is the junctional mechanosensory complex, with PECAM-1 being the molecule that can sense and respond to mechanical force [13,28] [12,29]. Given the proven crucial role of shear stress in cardiac and vascular development [30,31], it was always hard to reconcile the lack of developmental defects in the PECAM-1 knockout. We now identify a novel mechanosensor in ECs that operates upstream of the junctional complex. We show that onset of shear stress induces formation of a mechano-complex of PlxnD1/NRP1/VEGFR2; this complex requires the presence of NRP1 as well as flexion in the PlxnD1 ectodomain. Endothelial PlxnD1 regulates signals at junctions and integrins and downstream cellular responses to shear stress that ultimately regulate the site-specific distribution of atherosclerosis. The developmental cardiovascular defects observed in global [32], as well as EC-specific[33], PlxnD1 knockouts are in agreement with a requirement for this mechanosensor during

development, and as our data now demonstrate also in the adult. Despite the critical importance of mechanosensation in biology, knowledge of how mechanoreceptors detect physical force is extremely limited. Our data identify a novel mechanosensor in ECs and provide a new framework for understanding how ligand-dependent and mechanical signals can be channelled through a single receptor.

Methods

Experimental Animals – All animal experiments were approved and authorized by both the University of Oxford Local Animals Ethics and Welfare Committee and by the Home Office, UK. Project licences used in this work were 30/3080 and P0C27F69A. PlxnD1$^{fl/fl}$ animals were obtained from J. Epstein (University of Pennsylvania). To obtain endothelial specific deletion of PlxnD1, these mice were crossed with mice expressing an inducible Cre recombinase under the *Cadherin-5 Cre* driver, obtained from Ralf Adams (Max Planck Institute, Muenster). Three consecutive intra-peritoneal injections of tamoxifen (2mg each) in adult animals (6-8 weeks of age) resulted in the deletion of endothelial PlxnD1 to generate PlxnD1 inducible endothelial cell knockout PlxnD1^{iECKO} mice. For atherosclerosis studies, the PlxnD1$^{fl/fl}$-Cdh5Cre animals were crossed into the hypercholesterolemic Apolipoprotein E deficient (ApoE$^{-/-}$) background. All mice used in this study were maintained on a C57BL/6J background. For *en face* immunofluorescent analysis and qPCR of aortas, tissue was harvested 2 weeks after the last tamoxifen injection. For atherosclerosis studies, high fat diet was commenced 1 week after the last tamoxifen injection.

All animals were housed in individually ventilated cages at 22°C, 56 % relative humidity, light/dark cycle of 12h/12h and fed on a standard chow diet (B&K Ltd., UK). For high fat diet experiments carried out on animals in the hypercholesterolemic ApoE$^{-/-}$ background, the animals were fed Western RD (P) VP 25kGy diet containing 20% fat, 0.15% cholesterol (829108, SDS, UK) for 10 weeks or 20 weeks. Water and food were available *ad lib* at all times.

Genotyping – Genotyping was determined by PCR analysis of DNA in ear notches, collected for identifying the animals, using the Phire Tissue Kit (F140-WH, Thermo Scientific).

***En face* preparations** - Animals were induced a terminal general anaesthesia with isoflurane, followed by exsanguination and perfusion fixation with 4% paraformaldehyde. The entire length of aorta was dissected out and the surrounding connective tissue and adventitial fat were removed. The aorta was fixed in 4% paraformaldehyde and stored at 4°C in PBS until staining. Atheroprone areas from the inner curvature of the aortic arch were isolated and atheroprotective areas from the thoracic aorta were dissected and processed for immunofluorescent studies.

Oil red O staining for atherosclerosis study- Fixed aortas were rinsed in absolute propylene glycol and stained with Oil-red-O (01516, Sigma Aldrich). After washing in 85% propylene glycol solution and distilled water, the aortas were opened

longitudinally to the iliac bifurcation and a coverslip was placed to flatten down the aorta with endothelial surface facing upwards. Images were acquired using Olympus SZX7 fitted with a 1x lens and image processing was performed using Image-Pro (Media Cybernetics). The plaque area was quantified as a percentage of the area of both the total aorta and the aortic arch.

Lipid Profile Analysis – Blood was sampled by cardiac puncture under terminal general anaesthesia in plasma collection tubes. Plasma samples were shipped to MRC Harwell where they were analysed for total cholesterol, triglycerides, high density lipoprotein and low density lipoprotein levels on an automated AU680 Clinical Chemistry Analyser.

Cell culture, shear stress and transfections – Bovine aortic endothelial cells (BAECs), PECAM knockout (-/-) and PECAM reconstituted (+/+) cells were cultured as previously described[13]. Mouse lung endothelial cells were isolated from $PlxnD1^{fl/fl}$ mice and maintained in EGM2 growth medium (Lonza), supplemented with 10% fetal bovine serum (FBS). Cos7 cells were maintained in DMEM with 10% FBS. All cell types were maintained at 37°C in 5% CO_2 in a humidified incubator. Cells were subjected to shear stress either using a parallel plate chamber[13] or a cone and plate viscometer as previously described[10]. For Sema3E blocking antibody experiments, cells were treated with the antibody for 1 hour prior to and during shear stress. siRNA reverse transfections for PlxnD1 were performed using the Lipofectamine RNAimax Reagent (Invitrogen). siRNAs used in this study were from Dharmacon and as follows:-

Mouse ECs – Acell Mouse PlxnD1 SMARTpool consisting of GUAUCGACCACAGAUCAUG, CGUGGACCUUGAAUGGUUU, CUAUUAUAAACAGAUCCAA and CCAACAAGCUUCUGUACGC;
Acell Mouse Piezo1 SMARTpool consisting of CUAUCAGACACCAUUUAUC, GCCUCAUCCUCUAUAAUGU, UCAUCAUCUCUAAGAAUAU, CUGUUACGCUUCAAUGCUC;
Acell Mouse GNA11 SMARTpool consisting of CCAUUUUCUAAGUUAUUGA, CUUUUGAGCACCAGUAUGU, CUGUGACCCUUGUAUAUUA, CUGUCAGAUUUCUUUACUU ;
Acell Mouse GNAQ SMARTpool consisting of UUGUCAAGUUGUACGAAUU, CCAGGAUCAUAAGUGUUAA, GUAUAGUGCAAUUAUGAAU, CGAUCAUACUAGGAGGGAU
Acell NRP1 SMARTpool consisting of GCAGGAUUUUCCAUACGUU, CUUGAAUGCACUUAUAUUG, UGGUUAUCCUCAUUCUUAU, UCCUGGAAUUUGAAAGCUU

Bovine ECs – PlxnD1 custom duplex consisting of GGGAAAACAUCGAGGCCAAUU and UUGGCCUCGAUGUUUUCCCUU

Transfections of plasmids expressing NRP1, VEGFR2 and PlxnD1 in Cos7 cells were performed with Lipofectamine-2000 (Invitrogen) as per manufacturer's instructions.

RNA Extraction and Q-PCR – Total RNA extraction was performed from cells or from tissue using the RNeasy Plus mini kit (Qiagen), with an additional genomic DNA wipeout step. Reverse transcription was performed using the Superscript III cDNA synthesis kit. Quantitative real-time PCR was performed in triplicate with SYBR green and CFX96TM real-time system. Thermocycling conditions were 95°C for 3 minutes, followed by 40 cycles of 95°C for 15s, 60°C for 45s. Gene expression was

normalised to the constitutively expressed housekeeping gene 18s rRNA, and relative expression was calculated and plotted employing the ΔΔCt method. Primer sequences used were as follows:-

KLF2 – 5'-CTAAAGGCGCATCTGCGTA-3'
5'-TAGTGGCGGGTAAGCTCGT-3'
KLF4 – 5'-CGACTAACCGTTGGCGTGA-3'
5'-GAGGTCGTTGAACTCCTCGG-3'
MCP-1 – 5'-CATCCACGTGTTGGCTCA-3',
5'GATCATCTTGCTGGTGAATGAGT-3'
VCAM-1 – 5'-GCTATGAGGATGGAAGACTCTGG-3',
5'-ACTTGTGCAGCCACCTGAGATC-3'
18s rRNA – 5'-AGGAATTGACGGAAGGGCACCA-3
5'-GTGCAGCCCCGGACATCTAAG-3′

Immunofluorescence- Tissue and cell permeabilization were performed by incubation with 0.5% TritonX-100 overnight and 0.2% TritonX-100 respectively and blocked with 10% normal goat serum/1% BSA. Inner curvatures of aortic arch were incubated with primary antibodies (CD106 (VCAM), 553330; BD Biosciences and MCP-1, ab7202; Abcam) and descending aorta segments were incubated with primary antibodies (PlxnD1 (PA5-21605; ThermoFisherScientific) and PECAM-1 (553369; BD Biosciences) before incubation with Alexa Fluor 488– and Alexa Fluor 568–conjugated secondary antibodies (1:100; Invitrogen). Cells subjected to flow or tissues were incubated at 4°C overnight in beta catenin (610153; BD Biosciences) followed by 1 hr incubation of Alexa Fluor 488-conjugated phallodin (Invitrogen) at RT and DAPI (Invitrogen). Tissues were mounted *en face* with Prolong Gold Antifade mountant (Invitrogen) for confocal imaging using Olympus FluoView3000.

Image analysis- For *in vitro* flow experiment quantification, cell alignment in the direction of flow was determined by measurement of the angle between the flow direction and the long axis of the cell as determined visually[34]. Cell elongation was estimated as the ratio of cell length to cell width in both *in vitro* and *in vivo* study[35]. Measurement of the fluorescence intensity of VCAM, MCP-1 and phallodin was performed using ImageJ software (Analyze→Set Measurements→Mean gray value→ Measure). Quantification of the colocalization was performed using coloc2 plugin on ImageJ.

Co-immunoprecipitation and Western blotting – Cells were harvested in lysis buffer as described in [19] and supplemented with protease and phosphatase inhibitor cocktail tablets. Lysates were pre-cleared with 10 µl protein A/G plus sepharose beads (Santa Cruz Biotechnology) for 1 hr at 4°C. The pre-cleared lysates were then incubated with 20 µl of protein A/G plus sepharose beads, which had previously been coupled to the appropriate primary antibody for 2 hours at 4°C on an orbital shaker. The beads were washed three times with the lysis buffer supplemented with protease and phosphatase inhibitors. The immunoprecipitation complexes were eluted from the beads by boiling in 2X SDS buffer for 5 minutes.

For all western blotting analyses, protein lysates/co-immunoprecipitation complexes were resolved on a 4-12% gradient gel with the appropriate primary antibodies and IRDye-conjugated anti-mouse, anti-goat or anti-rabbit secondary antibodies, as appropriate. Images were acquired on a LICOR Odyssey infra-red scanner. Densitometric quantification of bands was performed using the ImageStudio software (LICOR Biosciences).

Inhibitors, Antibodies and other Reagents– The antibodies used for western blotting included phospho(p)-ERK1/2$_{T\text{-}202;Y\text{-}204}$, total(t)-ERK1/2, pAkt$_{S473}$, tAkt, p-

eNOS$_{S1177}$, pVEGFR2$_{Y1175}$, tVEGFR2 (all antibodies from Cell Signaling Technology), t-eNOS (BD Biosciences), p-Vinculin$_{Y822}$ (AbCam), t-Vinculin (Sigma Aldrich), PI3K/P85 (Upstate), integrin $\alpha_v\beta_3$ (clone LM609, Merck), Shc (Abcam),VE-cadherin (Santa Cruz), PlxnD1 (Thermo Fisher Scientific and Abcam), Piezo1 (Abcam), $G_{q/11}$ (Santa Cruz Biotechnology), Src (Upstate)

The inhibitors used in the study included VEGFR2 tyrosine kinase inhibitor SU1498 (Sigma Aldrich). Recombinant Semaphorin 3E was purchased from R&D Systems (Bio-techne, Minneapolis, MN, USA) and used at 10 μM. NRP1 blocking antibody was purchased from R&D Systems and Sema3E blocking antibody was from Thermo Fisher Scientific USA.

Bead pulling/Magnetic tweezer system – Tosyl activated paramagnetic beads (4.5 μm) were washed with PBS and coated with an antibody to the extracellular domain of PlxnD1 (Santa Cruz) or CD44 (clone 5D2-27 from Developmental Studies Hybridoma Bank, USA) Beads were quenched in 0.2M Tris, pH 7.4 prior to use, to eliminate any remaining tosyl groups. ECs were incubated with the beads (and inhibitor or blocking antibody, if appropriate) prior to force application for 5-30 minutes at 37°C. For immunofluorescence, ECs grown on fibronectin-coated coverslips were fixed for 20 minutes in PBS containing 2% formaldehyde, permeabilized with 0.2% Triton X-100 and blocked with 10% goat serum for 1 hour at room temperature. Antibody incubations for vinculin and HUTS4 were performed as previously described[12]. Focal adhesion number was quantified as previously described[36]. Ligated β1 integrin staining was quantified by determining the mean fluorescence intensity on ImageJ software. For examining phosphorylation of vinculin, cells were lysed as described above and lysates immunobloted with a primary antibody to phospho-vinculin (Abcam).

Calcium Imaging - BAECs were cultured in 33mm glass bottom dishes to form a sub-confluent monolayer. After the cells had fully attached and spread, 4 μM of Fluo-8 AM calcium binding dye (Abcam) was added to the media. Cells were incubated for 30 minutes with the dye. Beads conjugated with either PlxnD1 or Poly L-Lysine were added to the cells and incubated for another 30 minutes. To assess the calcium influx as a result of mechanical stimulation, cells with Fluo-8 and magnetic beads were subjected to 1 nN force applied with magnetic tweezers. Time-lapse video of epifluorescent calcium imaging was acquired with Nikon Ti-e microscope (60x objective) during 10s pre stimulation, 20s stimulation and 30s post stimulation. Acquired image sequences were analysed by measuring mean fluorescence intensity (mean pixel value) for each cell at each frame. Mean peak amplitude for each phase (pre-stimulation and during stimulation) was calculated and normalised against pre-stimulation fluorescence intensity for each cell.

Sema3E Challenge – BAECs in which endogenous PlxnD1 was knocked down with siRNA were infected with either WT or mutant PlxnD1-expressing adenoviruses. Cells were serum-starved and treated with recombinant Sema3E before processing for immunofluorescence with phalloidin, DAPI and anti-vinculin antibody. Images were taken on a Zeiss LSM 880 Airy Scan Confocal microscope and analysed using ImageJ (Schneider et al., 2012) using an in-house macro to measure cell area, focal adhesion number and focal adhesion area. Statistical analyses were performed using GraphPad Prism 7 (La Jolla, CA, USA). Comparisons between groups were assessed by 2-way ANOVA with a Tukey multiple comparisons post hoc test. Difference were considered significant when $P < 0.05$.

Site-directed mutagenesis - In order to lock the ectodomain of PlxnD1 in the ring-like conformation we designed a double mutant introducing two single point

mutations Y517C and A1135C in the sema domain (domain 1) and IPT5 domain (domain 9), respectively. Site-directed mutagenesis of full length PlxnD1, and of PlxnD1 ectodomain, was carried out by multiple-step overlap-extension PCR, and the resulting PCR products were cloned into a pHLSec vector[37].

Protein production Constructs encoding the ectodomain (residues 47-1271) of mouse PlxnD1 or double mutant PlxnD1 Y517C, A1135C were cloned into pHLsec vector in frame with a C-terminal hexahistidine (His6) tag. Protein was produced by transient transfection in HEK293T cells at 37°C. Five days post-transfection, the conditioned medium was collected and buffer exchanged using a QuixStand diafiltration system (GE Healthcare). The double mutant of PlxnD1 was secreted at a similar level to the wild-type protein. Proteins were purified by immobilized metal-affinity chromatography using a HisTrap FF column (GE Healthcare) followed by size-exclusion chromatography using a Superdex 200 Increase 10/300 column (GE Healthcare).

Alexa Fluor labelling of PlxnD1 Y517C, A1135C for validation of disulphide bond formation - PlxnD1 Y517C, A1135C at a concentration of 10 µM in PBS was labelled with a 20-fold molar excess of a thiol-reactive fluorescent dye, Alexa Fluor 488 C5 maleimide (ThermoFisher), and the reaction was allowed to proceed for 24 h at 6°C in the dark. Unreacted dye was removed from the labelled protein using a Sephadex G-25 (GE Healthcare). The degree of labelling (*n*) was determined using the following formula (Eq.1):

$$n = \frac{A_{488}\, M_w}{\varepsilon c} \qquad \text{(Eq.1)}$$

where A_{488} is the absorbance at 488 nm, M_w is the molecular weight of protein, ε is the molar extinction coefficient of the dye and c is the protein concentration in mg/ml. Hen egg ovalbumin (GE Healthcare) was used as a positive control.

Negative stain electron microscopy (EM) - A drop of 2.5 µl freshly gel-filtrated PlxnD1 ectodomain at a concentration of 1-5 µ g/ml in 10 mM HEPES, pH 7.5 and 150 mM sodium chloride was adsorbed to the newly glow-discharged carbon-coated copper grid, washed with two drops of 50 µl deionized water, and stained with two drops of 50 µl 0.75% uranyl formate. The excess stain on the grids was removed with filter paper before air-drying. Samples were imaged at room temperature using an FEI Tecnai T12 electron microscope equipped with a LaB6 filament operating at an acceleration voltage of 120 kV and a dose of 15 electrons per square Å. Images were taken using a 4k × 4k FEI Eagle TM CCD camera at a magnification of 57,000 × with under-focus values ranging from 1.0 to 1.5 µm and a pixel size of 2.16 Å. The particle images were normalized, re-scaled, filtered before being subjected to reference-free classification in EMAN2 [38]

. The PlxnD1 structural models were generated manually using The PyMOL Molecular Graphics System (Schrödinger, LLC).

Cloning and Adenoviral Generation – WT and Mutant PlxnD1 were cloned into the pENTR/TOPO entry vector of the Gateway System® (Invitrogen) using the KOD Hot Start High Fidelity polymerase. Following confirmation of successful cloning by Sanger sequencing, the constructs were sub-cloned into the pAd/CMV/V5-Dest destination vector by LR Clonase II reaction. All steps were performed as per manufacturer's instructions. The destination vector was linearized by PacI digestion and transfected into HEK-293A cells for adenoviral generation and subsequent amplification, as per manufacturer's instructions. In experiments where adenoviral

over-expression was employed, endogenous levels of PlxnD1 were knocked down with siRNA pool to minimise any background signals.

Statistics – Data are presented as means ± SEM. All experiments were performed at least three times independently. Statistical significance was tested by employing either analysis of variance test or unpaired Student's t-tests, as indicated in Results. Data were tested for normality using the Shapiro-Wilk test and equality of variance using the Levene test. Where necessary data were log transformed before being analyzed for statistical significance. All image analysis was performed by operators who were blinded to the treatments administered.

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Author Contributions

V.M. performed/was involved in majority of the experiments and analysis. K.L.P. performed *en face* staining and imaging of all aortas, staining and imaging for *in vitro* alignment, most qPCR experiments and quantification of the data. D.R. designed and validated the ring-locked PlxnD1 mutant. K.N. performed activation of signalling mediators in response to shear stress and initial magnetic force application experiments. A.K. performed semaphorin challenge experiments and analysed the data. D.L. performed the calcium imaging experiments. Y.K. provided structural analysis of the PlxnD1 ectodomain. D.K. and M.A. carried out the negative stain EM analysis. J.H. performed the initial PlxnD1 siRNA experiments. Y.F. provided the design of the cone-and-plate. A.H. led and supervised the calcium imaging experiments. Y.F. provided the design of the cone and plate system. J.S.R. co-supervised and interpreted data, conceived and developed the idea of the binary conformations of PlxD1 and performed cloning of the PlxnD1 WT and mutant constructs into adenovirus. E.Y.J. led and supervised the structural biology based components of the study. E.T. initiated the project, generated research funds and ideas, directed and coordinated the project. V.M., J.S.R., E.Y.J. and E.T. designed experiments, interpreted data and wrote the manuscript, with inputs from all authors.

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Figure Legends

Figure 1 – PlxnD1 mediates the endothelial cell response to fluid shear stress and regulates the site-specific distribution of atherosclerosis (a.b) Mouse ECs were transfected with either Scr or PlxnD1 siRNA and exposed to either atheroprotective or atheroprone flow for 24 hours, using a cone and plate viscometer. Q-PCR was performed for KLF2 and KLF4 on samples subjected to atheroprotective flow, and inflammatory markers MCP-1 and VCAM-1 on samples subjected to atheroprone flow; n=4 biological replicates **(c)** The descending thoracic aorta was isolated and prepared *en face* from $PlxnD1^{fl/fl}$ and $PlxnD1^{iECKO}$ mice and stained for β-catenin, phalloidin and DAPI to visualise the cell junctions, actin stress fibres and nuclei. Quantification of alignment was performed using ImageJ; 3-5 images (each image has n≤100 cells) taken from three regions along the length of descending aorta collected from 5 animals of each genotype (for exact *n*, please refer to source data). **(d)** Representative *en face* preparations of whole aortas showing atherosclerosis in $PlxnD1^{fl/fl}$; $ApoE^{-/-}$ and $PlxnD1^{iECKO}$; $ApoE^{-/-}$ mice after 10 weeks of high fat diet feeding, visualised by Oil Red O staining. **(e)** Quantification of lesion area in whole aortas and aortic arches from $PlxnD1^{fl/fl}$; $ApoE^{-/-}$ and $PlxnD1^{iECKO}$; $ApoE^{-/-}$ mice; n=8 **(f)** Aortic arches from $PlxnD1^{fl/fl}$; $ApoE^{-/-}$ and $PlxnD1^{iECKO}$, $ApoE^{-/-}$ mice were isolated and Q-PCR was performed for inflammatory markers VCAM-1 and MCP-1; n=5. The data represent mean±SEM. P-values were obtained by performing two-tailed Student's *t* test using Graphpad Prism. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; scale bar represents 20 μm

Figure 2 – PlxnD1 is a mechanosensor that mediates the EC response to force (a) Mouse ECs were incubated with anti-PlxnD1 or C44 (negative control) antibody-coated beads and subjected to force (10pN) for the indicated time periods. Phosphorylation of VEGFR2, Akt and ERK1/2 was determined by western blotting and quantified using Image Studio Lite Ver 5.2, n=3 biological repeats; *p<0.05 relative to no force condition, #p<0.05 relative to the respective force application time point with PlxnD1. **(b)** BAECs were loaded with Fluo-8AM dye and then incubated with beads coated with an antibody to the extracellular domain of PlxnD1 or Poly L-lysine (negative control). The beads were then subjected to force (1nN). Calcium responses were measured by calculating the fluorescent intensity of individual cells

before (10 seconds), during (20 seconds), and after (30 seconds) stimulation. Representative images are shown along with quantification. n=18 cells for PlxnD1 and n=19 cells for control over 3 independent biological replicates. ***p < 0.001 relative to unstimulated controls, scale bar represents 10 μm. Representative trace for calcium influx response over time has also been shown. The arrow marks the start of the stimulation. **(c)** BAECs were incubated with anti-PlxnD1-coated beads and subjected to force (10pN) for 30min. ECs were fixed and stained with an anti-vinculin antibody to mark focal adhesions. Focal adhesion number was quantified using ImageJ software. Values were normalised to the "no force" condition. Location of the beads are highlighted in yellow circles (n=50 cells for each condition from 3 independent biological replicates;****p<0.0001; scale bar represents 10 μm **(d)** Mouse ECs were incubated with anti-PlxnD1 or C44 coated beads and subjected to 10pN force for the indicated time periods. Phosphorylation of vinculin was determined by western blotting and quantified using Image Studio Lite Ver 5.2, n=3 biological repeats; *p<0.05 relative to no force condition, #p<0.05 relative to the respective force application time point with PlxnD1. The data represent mean±SEM. P-values were obtained by performing two-tailed Student's *t* test using Graphpad Prism.

Figure 3 - PlxnD1, NRP1 and VEGFR2 mechano-complex functions upstream of known mechanosensory hotspots and is sufficient for responsiveness to shear stress (a) Schematic showing signalling at the junctional complex and integrins. **(b)** Mouse ECs were transfected with Scr or PlxnD1 siRNA and exposed to shear stress for 2min (12 dynes/cm$_2$ using a parallel plate system) or left as static controls before immunoprecipitating VEGFR2 and examining its phosphorylation and association with the p85 subunit of PI3K and VE-Cadherin, n=3. Normal rabbit IgG was used as a negative control and showed no reactivity with any of the antibodies examined (data not shown). **(c)** Mouse ECs were transfected with Scr or PlxnD1 siRNA and exposed to shear stress for 30min (12 dynes/cm$_2$ using a parallel plate system) or left as static controls before immunoprecipitating integrin $\alpha v\beta_3$ and examining its association with Shc, n=3. Normal mouse IgG was used as a negative control and showed no reactivity with any of the antibodies examined (data not shown). **(d)** ECs were incubated with a VEGFR2 tyrosine kinase inhibitor SU1498, transfected with an siRNA to NRP1 or treated with a NRP1 blocking antibody, then incubated with anti-PlxnD1-coated beads and subjected to force for 5min before examining phosphorylation of vinculin. (n=3 biological repeats; $^*p < 0.05$) The data represent mean±SEM. P-values were obtained by performing two-tailed Student's *t* test using Graphpad Prism. **(e)** Mouse ECs were exposed to shear stress for the indicated times (12 dynes/cm$_2$ using a parallel plate system) or left as static controls before immunoprecipitating VEGFR2 and examining its phosphorylation and association with PlxnD1, NRP1 and Src; n=3. **(f)** Mouse ECs were exposed to shear stress for the indicated times (12 dynes/cm$_2$ using a parallel plate system) or left as static controls before immunoprecipitating NRP1 and examining its association with PlxnD1 and VEGFR2; n=3. **(g)** Mouse ECs transfected with either Scr or NRP1 siRNA were exposed to shear stress for the indicated times (12 dynes/cm$_2$ using a parallel plate system) or left as static controls before immunoprecipitating VEGFR2 and examining its association with PlxnD1; n=3. **(h)** Schematic showing reconstitution of PlxnD1, VEGFR2 and NRP1 in Cos7 cells confers shear stress sensitivity to these cells. **(i)** Cos7 cells were left untransfected or transfected with NRP1 and VEGFR2, with or without PlxnD1. Cells were then subjected to shear stress for 2min (12 dynes/cm$_2$ using a parallel plate system) and VEGFR2 was immunoprecipitated. Shear stress sensitivity was assessed by examining phospho-VEGFR2 levels, complex formation between VEGFR2 and Src as well as complex formation of PlxnD1, VEGFR2 and NRP1, n=3. Normal rabbit IgG was used as a negative control and showed no

reactivity with any of the antibodies examined (data not shown).

Figure 4 - PlxnD1 flexion is required for mechanotransduction.
(a) Schematic domain organisation of mouse PlxnD1 spanning amino acids 1-1925. SS, signal sequence; TM, transmembrane region; c, cytoplasmic region. **(b)** Representative negative stain class averages of the PlxnD1 ectodomain and corresponding structural models showing the ring-like and open conformations, scale bar represents 10nm. The 2D class averages were obtained from single experiment by classifying 1357 particles into 10 classes. **(c)** Model of opening the ring-like ectodomain which confers PlxnD1 mechanosensory functions. **(d)** Design of PlxnD1 mutant with an intramolecular disulphide bond to lock the ring-like structure. Zoom-in view shows the disulphide bond between the sema domain (domain 1) and IPT5 domain (domain 9). **(e)** ECs in which endogenous PlxnD1 was knocked down were infected with adenoviruses expressing WT or Mutant PlxnD1, treated with Sema3E for 30min and immunostained with anti-vinculin antibodies. Focal adhesion number was quantified using ImageJ; n=30 cells over 4 biological replicates. **(f)** ECs in which endogenous PlxnD1 was knocked down were infected with adenoviruses expressing WT or Mutant PlxnD1 and incubated with anti-PlxnD1 paramagnetic beads followed by force application (10pN; 30min). Cells were fixed and immunostained with anti-vinculin antibodies. Focal adhesion number was quantified using ImageJ; n=30 cells over 3 biological replicates ****p<0.0001; scale bar represents 10 μm. **(g)** Cos7 cells were transfected with plasmids expressing either WT or mutant PlxnD1, NRP1 and VEGFR2. Cells were then subjected to shear stress for 2 minutes (12 dynes/cm_2 using a parallel plate system) and VEGFR2 was immunoprecipitated. Shear stress sensitivity was assessed by examining phospho-VEGFR2 levels, complex formation between VEGFR2 and Src and complex of PlxnD1, VEGFR2 and NRP1, n=3. Normal rabbit IgG was used as a negative control and showed no reactivity with any of the antibodies examined (data not shown). **(h)** ECs in which endogenous PlxnD1 was knocked down were infected with adenoviruses expressing WT or Mutant PlxnD1 and incubated with anti-PlxnD1 paramagnetic beads followed by force application (10pN for the indicated times). Phosphorylation of Akt, ERK 1/2 and VEGFR2 was determined by western blotting and quantified using Image Studio Lite Ver 5.2, n=3 biological repeats; *p<0.05 relative to “no force” condition; #p<0.05 relative to the respective WT force time point. **(i)** ECs in which endogenous PlxnD1 was knocked down were infected with adenoviruses expressing WT or Mutant PlxnD1 and subjected to fluid shear stress for the indicated times (12 dynes/cm_2 using a parallel plate system). Phosphorylation of Akt, ERK 1/2 and eNOS was determined by western blotting and quantified using Image Studio Lite Ver 5.2, n=3 biological repeats; *p<0.05 relative to static condition; #p<0.05 relative to the respective WT shear time point. All data are presented as mean±SEM. P-values were obtained by performing two-tailed Student's *t* test using Graphpad Prism.