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Review Article

The DCBLD receptor family: emerging signaling roles in development, homeostasis and disease

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The discoidin, CUB, and LCCL domain-containing (DCBLD) receptor family are composed of the type-I transmembrane proteins DCBLD1 and DCBLD2 (also ESDN and CLCP1). These proteins are highly conserved across vertebrates and possess similar domain structure to that of neuropilins, which act as critical co-receptors in developmental processes. Although DCBLD1 remains largely uncharacterized, the functional and mechanistic roles of DCBLD2 are emerging. This review provides a comprehensive discussion of this presumed receptor family, ranging from structural and signaling aspects to their associations with cancer, physiology, and development.

Introduction: cloning and early work

The discoidin, CUB, and LCCL domain-containing (DCBLD) receptor family consists of two paralogous type-I transmembrane proteins, DCBLD1 and DCBLD2, which are conserved across vertebrates. DCBLD2 was first characterized in two separate cloning experiments almost 20 years ago [1,2], however, the function of the DCBLD family is not yet fully delineated. To date, DCBLD2, also endothelial and smooth muscle cell-derived neuropilin-like protein (ESDN) and CUB, LCCL-homology, and coagulation factor V/VIII-homology domains protein 1 (CLCP1), is the better-studied family member and, necessarily, will be the primary focus of the functional and mechanistic discussion herein. This review synthesizes the current understanding of the DCBLD protein family and offers interpretations of the complexities concerning the functional biology and mechanistic signaling these proteins have been observed to modulate.

Kobuke et al. [1] were the first to describe cloning of the novel cDNA *ESDN* (*DCBLD2*) from human, mouse, and rat coronary arterial endothelial and smooth muscle cells (CAECs and CASMCs) using a signal sequence trap method in yeast. They were interested in characterizing novel proteins that entered the secretory pathway of CAECs and CASMCs and identified ESDN, among other novel transmembrane and secreted proteins. Further analysis in cultured vascular smooth muscle cells (VSMCs) revealed a robust up-regulation of *ESDN* mRNA in response to stimulation with the homodimeric BB isoform of the platelet-derived growth factor (PDGF-BB) and, to a lesser degree, with fetal calf serum [1], which suggested that this protein may play a role in cell growth and proliferative processes.

Shortly thereafter, Koshikawa et al. [2] cloned a cDNA they named *CLCP1* (*DCBLD2*) from highly metastatic lung cancer cells. They first identified up-regulation of the gene at the RNA level in a highly metastatic lung cancer cell line (LNM35) relative to a low-metastatic clone (N15) of the LNM35 parental line. Additionally, they identified increased CLCP1 expression in several clinical lung cancer cases, with particularly strong expression in lymph node metastases in comparison with normal tissue [2]. This group went on to look at possible extracellular cues important in the metastatic abilities of LNM35 cells using a phage display assay and identified a binding peptide with homology to a portion of the semaphorin domain of SEMA4B [3]. The authors hypothesized that the extracellular domains of CLCP1 and SEMA4B could interact and that together they might modulate tumor

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progression [3]. They demonstrated an interaction between CLCP1 and both SEMA4B-Fc and full-length SEMA4B by co-immunoprecipitation and observed an increase in CLCP1 ubiquitylation and a decrease in fully modified CLCP1 levels induced by SEMA4B-Fc co-expression [3].

This early work set the foundation for investigations into the functional and mechanistic roles of DCBLD2 [4–9]. The observed interaction with SEMA4B, which serves as a local repulsive cue in axon guidance and vascular development, presented the possibility that DCBLD2 might be involved in neuronal pathfinding and angiogenic processes, as well as tumorigenesis and the progression of certain cancers.

Structural features and conservation

DCBLD1 and DCBLD2 amino acid sequences are highly conserved across vertebrates (Figures 1A,B and 2A,B) [10–12]. Presumed DCBLD homologs in a handful of invertebrates have been curated in the Uniprot (uniprot.org) and NCBI (ncbi.nlm.nih.gov) protein databases [12,13], although the length and domain structure of these proteins deviate substantially from vertebrate sequences (Supplementary Table S1). Many of the invertebrate sequences do not possess the extracellular domains or the transmembrane region common to canonical vertebrate sequences, suggesting that DCBLD1/2 vertebrate function may not be conserved in these species.

Full-length human sequences of DCBLD1 and DCBLD2 span 715 and 775 amino acids, respectively. Each family member possesses a signal sequence, followed by CUB, LCCL, and Coagulation Factor V/VIII type-C (also Discoidin) domains (Figures 1C and 2C) [12,14]. A single-pass transmembrane region precedes the intracellular C-terminal scaffolding domain. Although the intracellular region does not possess any currently described modular domains, it contains several phosphorylation, acetylation, and ubiquitylation sites (Figure 3) as well as SH2 domain-binding motifs [4,6]. DCBLD domain structure closely resembles that of neuropilins, transmembrane proteins that possess two CUB and discoidin domains and act as co-receptors for class 3 semaphorins and growth factors in axon guidance and angiogenesis [15,16].

Conservation of DCBLD1 and DCBLD2 domain structure in canonical and isoformic protein products is shown across representative vertebrates in Figures 1C and 2C. The human DCBLD2 signal peptide is composed of 66 amino acids and is the longest signal sequence in the human proteome [17]. Resch et al. characterized distinct domains within the DCBLD2 signal peptide that exhibit different functionality: a N-terminal domain, a transition region, and a C-terminal domain. The C-domain acts as a fully functional signal peptide, possessing the characteristic sequence of positively charged amino acids followed by a hydrophobic stretch and a polar C-terminus, and alone is a sufficient target to the secretory pathway [17]. Interestingly, the N-domain, in the absence of its C-terminal region, targets proteins to the mitochondria. It remains unknown whether the N-domain is biologically cleaved, thereby freeing it for alternative functions, or if in some cases it shuttles DCBLD2 to the mitochondria. The N-domain is also required for glycosylation, suggesting that it plays a role in DCBLD2 maturation [17]. Although non-mammalian vertebrates possess shorter signal sequences with lengths closer to the average signal peptide length (22 amino acids in humans [17]), mammalian DCBLD2 signal sequences are remarkably long and highly conserved (Figure 2A,C). This would suggest that the mammalian signal peptide may have a unique functionality that has not yet been fully characterized.

Following the signal peptide are the CUB and the LCCL domains, which are conserved in canonical vertebrate sequences (Figures 1C and 2C). CUB domains (~110 AAs) are represented in many developmentally regulated extracellular and plasma membrane-associated proteins, including components of the compliment cascade, adhesive molecules, proteases, and certain growth factors [18,19]. Although the functional role of the CUB domain is not yet fully delineated, it is thought to facilitate protein–protein or protein–carbohydrate interactions. For example, in the C and D isoforms of PDGF, the CUB domain serves to sterically inhibit PDGF-C and -D binding partners from interacting with the growth factor domain before the CUB domain is cleaved by a regulatory protease [5]. The DCBLD1/2–CUB domain could similarly prevent binding of extracellular interactors prior to cleavage of the extracellular domain. The two CUB domains of neuropilins are involved in Semaphorin binding [20], suggesting that DCBLD2–CUB domain might be involved in the DCBLD2/SEMA4B interaction. The LCCL domain (~100 AAs) is, similarly, poorly characterized, although this module is thought to be involved in structural integrity and immune function, specifically in binding lipid A of the endotoxin lipopolysaccharide [21–23].

The third conserved domain located N-terminal to the transmembrane region is the Coagulation Factor V/VIII type C, or Discoidin, domain. This ~150 AA module is found within membrane and extracellular proteins, including discoidin domain receptor tyrosine kinase (DDR) family members, which are involved in focal adhesion dynamics, proliferation, and extracellular matrix degradation [24]. Although the function of this

Biochemical Journal (2019) **476** 931–950
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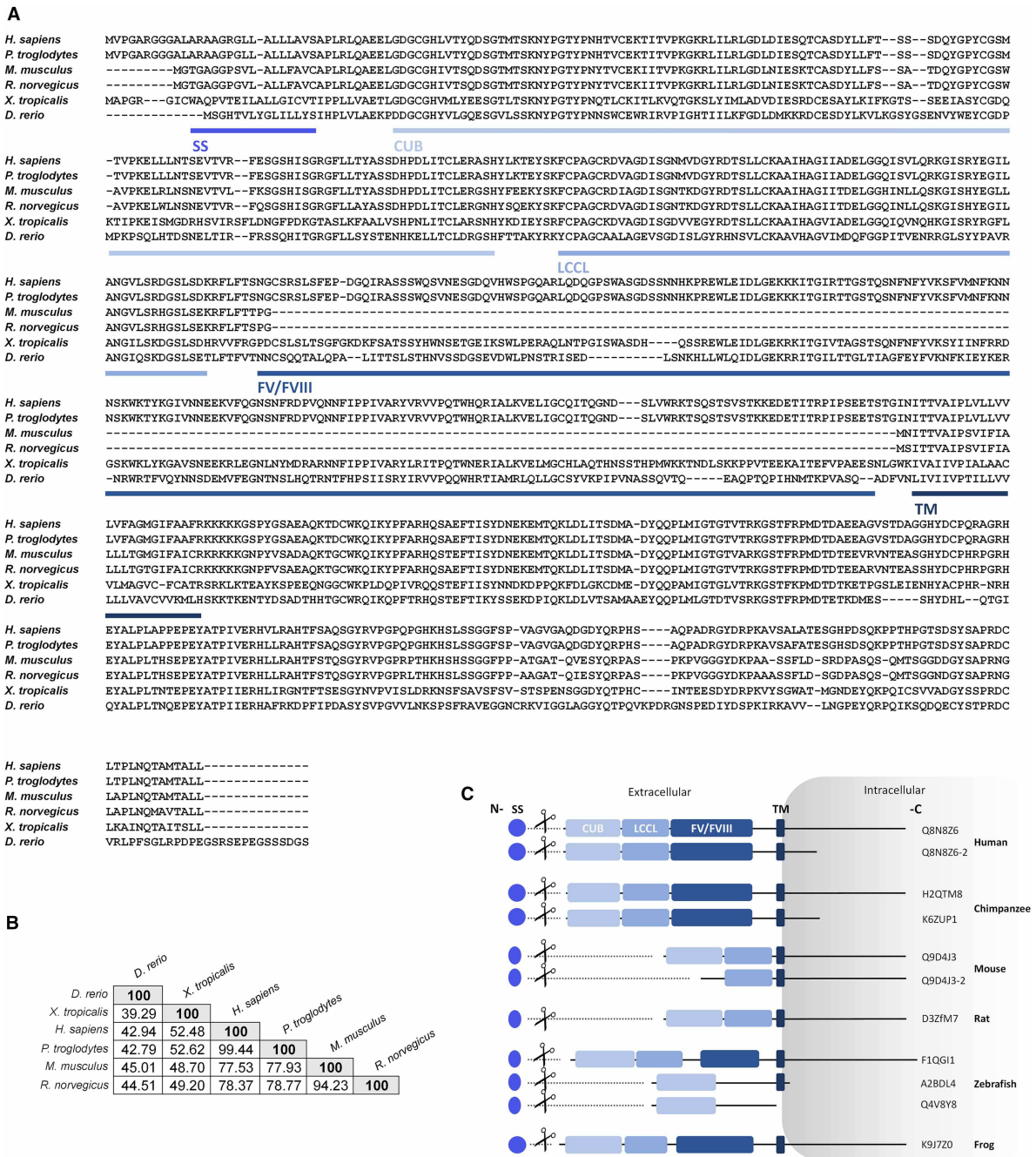

Figure 1. Multiple sequence alignments, conservation, and domain structure of the DCBLD1 protein in vertebrate species.

(**A**) Alignments and (**B**) percent conservation of canonical DCBLD1 sequences across representative vertebrates (*H. sapiens, P. troglodytes, M. musculus, R. norvegicus, X. tropicalis, D. rerio*) were constructed using ClustalOmega [10,11]. Regions of the alignment that are part of conserved domains are indicated. (**C**) Domain structure of all DCBLD1 isoforms of representative vertebrates in the UniprotKB database (uniprot.org) [12]. SS = signal sequence, TM = transmembrane.

domain is similarly unknown, it shares homology with regions of Factors V and VIII that are required for anionic phospholipid binding [25,26]. The Discoidin domain is, interestingly, not conserved in rodent DCBLD1 (Figure 1C), suggesting that it either is not essential to the conserved function of this gene or other rodent proteins (e.g. DCBLD2) may otherwise functionally compensate. The discoidin domains of neuropilins

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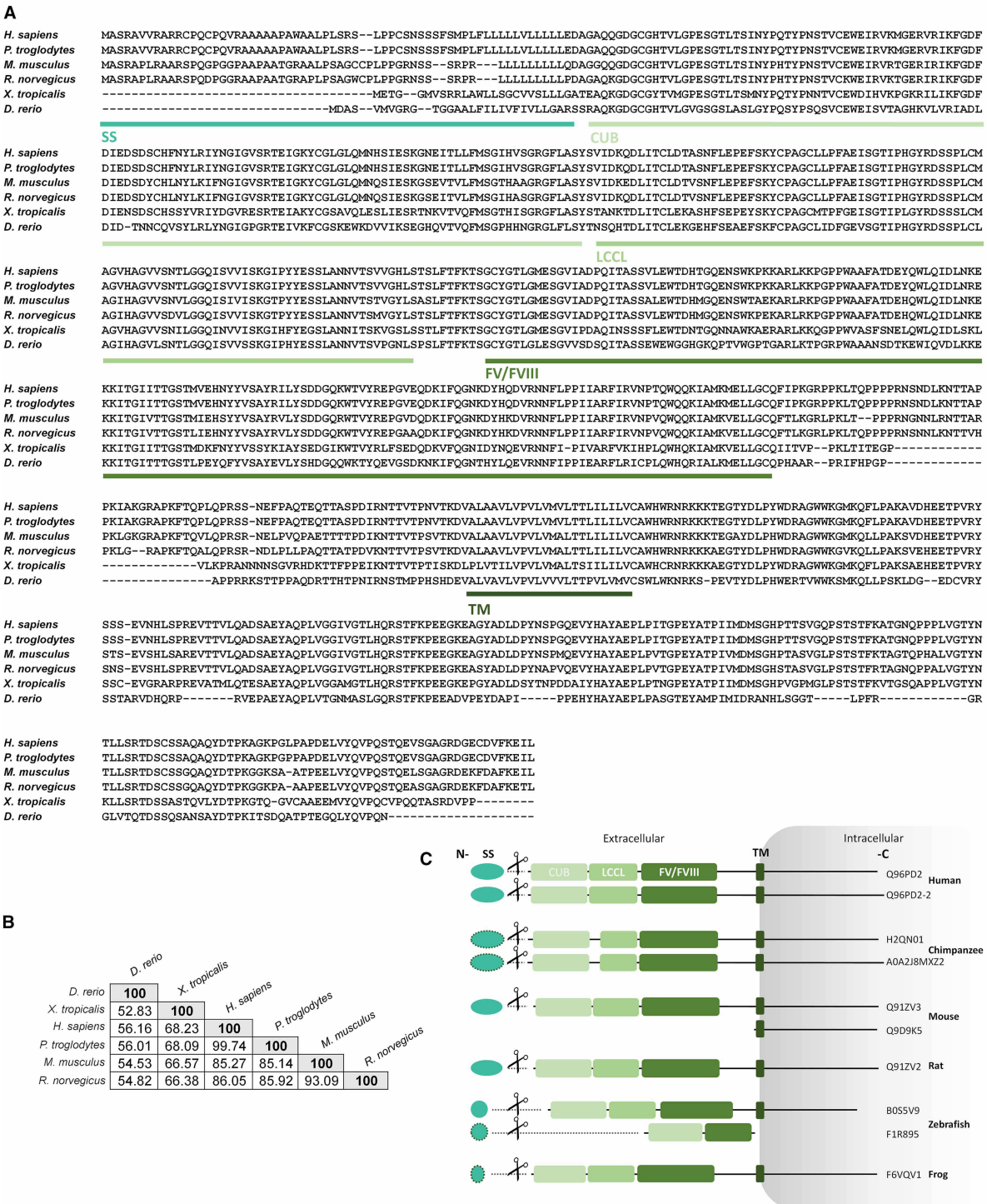

Figure 2. Multiple sequence alignments, conservation, and domain structure of the DCBLD2 protein in vertebrate species.

(**A**) Alignments and (**B**) percent conservation of canonical DCBLD2 sequences across representative vertebrates (*H. sapiens, P. troglodytes, M. musculus, R. norvegicus, X. tropicalis, D. rerio*) were constructed using ClustalOmega [10,11]. Regions of the alignment that are part of conserved domains are indicated. (**C**) Domain structure of all DCBLD2 isoforms of representative vertebrates in the UniprotKB database (uniprot.org) [12]. Domain size is proportional to relative amino acid lengths, as annotated by UniprotKB. Signal sequences (SS) are separated from the mature protein with a dotted line. SSs that were not annotated in UniprotKB were predicted using SignalP 4.1 [14] (dotted outlines). SS = signal sequence, TM = transmembrane.

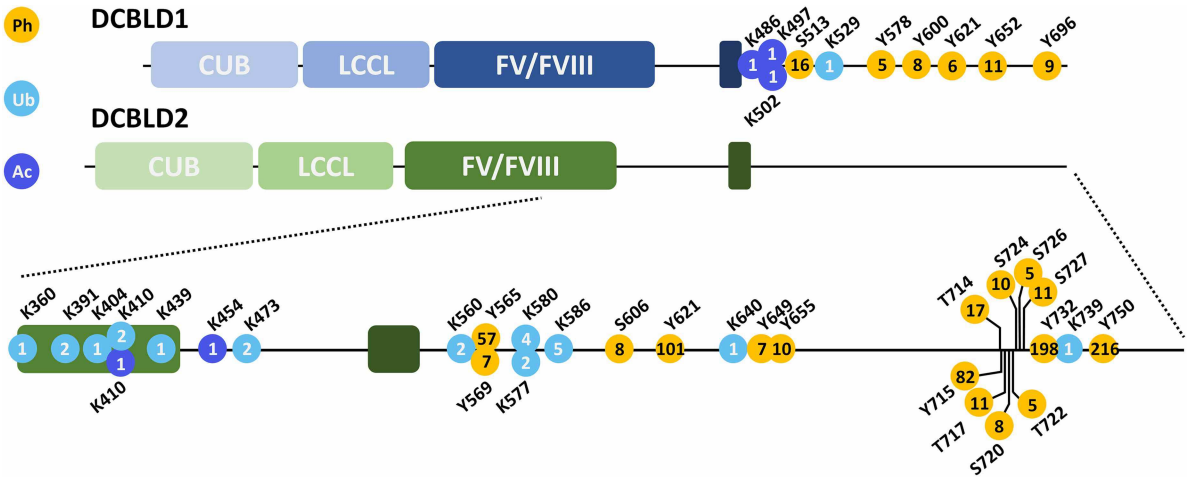

Figure 3. Experimentally observed post-translational modifications (PTMs) of DCBLD1 and DCBLD2.
Sites of PTMs are indicated by the amino acid position along each protein sequence, and numbers indicate the number of high and low-throughput experiments, conducted either by Cell Signal Technology or curated from the literature, obtained from the PhosphoSitePlus [45] database on August 22, 2018. Observed PTMs include phosphorylation (Ph, yellow), ubiquitylation (Ub, light blue), and acetylation (Ac, dark blue). Due to the high abundance of observed phosphorylation sites on DCBLD1 and DCBLD2, the number of experimentally identified phosphorylation sites are only included if > 5, although all ubiquitylation and acetylation identifications are included.

have been shown to bind growth factors of the VEGF family and other heparin-binding proteins [20,27], suggesting that this domain may similarly interface with secreted factors, either in direct contribution to growth factor receptor signaling or by an alternative mechanism.

Several splice variants exist for both DCBLD1 and DCBLD2 (uniprot.org; Figures 1C and 2C) [12], some of which could have interesting functional implications. In mice, there is a DCBLD1 isoform with no CUB domain, which could act to prevent important CUB-mediated interactions, as well as a DCBLD2 isoform with no extracellular region (Figures 1C and 2C). Interestingly, zebrafish Dcbld1 splice variants include Dcbld1–CUB and Dcbld1–CUB–TM species, and a Dcbld2 splice variant possessing only the CUB and Discoidin domains (Figures 1C and 2C). The signal peptide remains intact in these species; therefore, these variants are presumed secretory pathway targets. These isoforms suggest an important mechanism that links extracellular CUB-mediated interactions to intracellular signal transduction. This could either be achieved by removing the potential for CUB-mediated interactions, as in the mouse variant, or by removing the potential for intracellular signal transduction stimulated through CUB domain interactions, as in zebrafish variants. The DCBLD1 CUB domain could act as a paracrine signal, and the zebrafish isoforms could promote signaling through an unknown Dcbld1/2–CUB receptor.

In addition, the zebrafish Dcbld1–CUB–TM isoform has an abbreviated intracellular domain that terminates after the first intracellular tyrosine, similar to human and chimpanzee isoforms with stop codons that falls directly before the first CRK/CRKL–SH2 binding motif (pYXXP) in the canonical sequence (Figure 1C). These variants would prevent scaffolding of intracellular binding partners important in DCBLD1/2 signaling, and thus are predicted to act as dominant-negatives to interfere with DCBLD signaling and reduce the binding potential of intracellular interactors. Secreted forms may serve as either dominant negative species that bind ligands in nonfunctional complexes, or conceivably as paracrine factors capable of forward or 'reverse' signaling when considering semaphorins such as SEMA4B.

Expression and localization

Expression profile

Currently available RNA-seq data generated by the Human Protein Atlas project (www.proteinatlas.org) [28] demonstrate that *DCBLD2* exhibits generally higher expression levels than *DCBLD1* (Figure 4). Of the represented tissues, *DCBLD2* transcripts were concentrated most in reproductive and muscle tissue, as well as the

Biochemical Journal (2019) **476** 931–950
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Figure 4. Expression levels of DCBLD family members.

DCBLD1 and DCBLD2 expression levels across (**A**) tissues and (**B**) cell types. Tile plots display *DCBLD1* and *DCBLD2* RNA transcripts per million (TPM) detected from human tissues and cell types by RNA sequencing conducted as part of the Human Protein Atlas project (www.proteinatlas.org) [28].

parathyroid gland (Figure 4A). *DCBLD1* transcripts were present at relatively lower concentrations, with highest transcripts-per-million (TPM) found in the parathyroid gland, placenta, and gallbladder. Of the queried cell types, *DCBLD2* was represented at similar or higher levels than *DCBLD1* and was most highly expressed in glioma (U-87 MG) and myoblast (LHCN-M2) cell lines (Figure 4B). *DCBLD2* tissue- and cell-specific expression analyses have also been published by several investigative groups (Table 1) [1,2,7,8,29,30]. These studies similarly demonstrate high *DCBLD2* expression in reproductive and muscle tissues, as well as muscle-derived cell types.

DCBLD2 is expressed highly in proliferating cells in culture and is up-regulated *in vivo* following vascular injury [9], suggesting its expression is tightly regulated in developmental and repair processes. *DCBLD2* expression in a variety of human fetal tissues has been described [2], although there is little data describing developmental expression patterns of DCBLD family members. Modulated *DCBLD1* and *DCBLD2* expression levels are associated with several types of cancer (Table 2) [2,3,5,31–43]. Currently available *DCBLD1/2* RNA-seq data generated by the Cancer Genome Atlas (TCGA) Research Network (http://cancergenome.nih.gov) is included in Supplementary Figure S1A,B, which compares expression of DCBLD family members in tumors to that in normal tissue [44]. Given the proliferative phenotypes and links to cancer, the developmental role of DCBLD proteins will likely become important subjects of study and, therefore, necessitates further investigation into developmental expression patterning.

Subcellular localization

The DCBLD family are a class of type-I transmembrane proteins that are thought to localize to the plasma membrane, however, there is a lack of reproducible, robust experimental evidence clearly demonstrating their subcellular localization. Koshikawa et al. [2] described plasma membrane localization of transiently expressed DCBLD2 in A549 cells via immunofluorescence, and the same research group later replicated these findings following proteasome inhibition with MG-132 [3]. Similarly, immunohistochemistry of DCBLD2 in clinical myxofibrosarcoma specimens revealed plasma membrane localization [34]. Kobuke et al. [1] also examined DCBLD2 localization by immunofluorescence in COS7 cells transiently transfected with DCBLD2-FLAG and

Table 1. Expression of DCBLD2 protein and mRNA in tissue and cell types
Data were compiled from low-throughput studies reported in primary literature.

Highest expression	Other tissues/Cell types	Species	Age	Organism	References	Tissue/ Cell
Heart	brain, lung, spleen, stomach, small intestine, colon, kidney, testis	mRNA	-	Rat	[1]	Tissue
Skeletal muscle, heart, testis	pancreas, kidney, liver, placenta, brain, colon, small intestine, ovary, prostate	mRNA	Adult	Human	[2]	Tissue
Heart	lung, brain, kidney, adrenal gland	mRNA	Fetal	Human	[2]	Tissue
Heart, lung, aorta	brain, spleen, stomach, kidney, skeletal muscle, liver	protein	-	Mouse	[8]	Tissue
-	liver, muscle	protein	18–20 wk	Mouse	[7]	Tissue
Megakaryocytes	erythrocytes, HUVECs	mRNA	-	Human	[29]	Cell
CASMCs	hCAECs, HeLa	mRNA	-	Rat, human	[1]	Cell
DRG neurons	-	mRNA	e15.5	Mouse	[30]	Cell

observed strong fluorescence at the cell membrane using both anti-FLAG as well as antibodies raised against peptides within the CUB and FV/FVIII extracellular domains of DCBLD2.

In HUVECs, Nie et al. observed a strong signal of endogenous DCBLD2 at the plasma membrane, the endoplasmic reticulum, and the perinuclear space; however, there was also significant staining throughout the cytoplasm. It is possible that this staining indicates sequestration or trafficking in vesicles. Interestingly, the localization at the plasma membrane appeared to be non-uniform [8], suggesting that DCBLD2 could be integrated into lipid micro-domains and possibly involved in polarizing processes. Li et al. [7] observed fluorescence of endogenous DCBLD2 in VSMCs that was primarily localized to vesicles. They detected a small degree of membrane localization, apparent in concentrated patches, although the published images suggest less of a polarization effect than that observed by Nie et al.

Post-translational modifications and processing

The human DCBLD2 protein has a predicted molecular mass of ∼80 kDa based on its amino acid sequence, although it regularly displays an effective molecular mass of ∼130 kDa via SDS–PAGE [3,4,6]. A significant portion of this reduced electrophoretic mobility was shown to be the result of glycosylation [3]. Commonly, two protein bands of different molecular mass (110 and 130 kDa) are observed with antibodies to a C-terminal epitope tag [1,3]. A third species of ∼100 kDa, although less frequently reported, is prominently observed when cells expressing DCBLD2 are treated with the proteasome inhibitor MG-132 [3], suggesting that this variant is more susceptible to degradation.

Intracellular phosphorylation will likely emerge as an important regulator of DCBLD1/2 biological function. With no enzymatic activity, these transmembrane proteins are hypothesized to act as scaffolds for the formation of signaling hubs at the plasma membrane. Large-scale mass spectrometric studies curated at PhosphoSitePlus (phosphositeplus.org) [45] have revealed high spectral counts of tyrosine phosphorylation, as well as the identification of ubiquitylation and acetylation sites (Figure 3). The most well-characterized DCBLD2 phosphorylation site is Tyr750. This site has been identified as a direct substrate of the receptor tyrosine kinase (RTK) EGFR using an *in vitro* kinase assay [5] and has been shown to be phosphorylated downstream of EGF and hyperactive EGFR [5,32]. Activity of Abl and the SFK Fyn has also been shown to induce DCBLD2 tyrosine phosphorylation [4,6], although direct phosphorylation of DCBLD2 by these cytoplasmic kinases has yet to be demonstrated.

In addition to functional regulation via glycosyl and phosphoryl modification, DCBLD2 has recently been identified as a novel substrate of the serine protease RHBDL2 [46]. Catalyzing proteolysis of extracellular domains of transmembrane proteins localized at the plasma membrane, RHBDL2 is a key positive regulator of EGF signaling [47] and its activity is implicated in wound healing and cancers of the airway and digestive tract [48–50]. Johnson et al. [46] demonstrated that the ∼80 kDa RHBDL2-cleaved DCBLD2 product was detectable

Biochemical Journal (2019) **476** 931–950
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Table 2. Associations of DCBLD family members with cancer
Data were compiled from primary literature reports.

Cancer type	Family member	Cell type studied	Phenotype/Association	Molecular insights	References
Lung adenocarcinoma	DCBLD1	Clinical specimens	Associated with EGFR mutations	N/A	[36,42]
Myxofibrosarcoma	DCBLD2	Clinical specimens	Associated with invasive properties	N/A	[34]
Lung cancer	DCBLD2	Clinical specimens, A549	Promotes cell motility	May interact with SEMA4B	[2,3]
Neuroendocrine	DCBLD2	Clinical specimens	Involved in invasion, progression, metastasis	N/A	[33]
Glioma, head-and-neck cancer	DCBLD2	U87, SNB19, PCI-158	Required for EGFR-driven tumorigenesis; Associated with poor prognosis	EGFR-mediated pTyr750 facilitates TRAF6-mediated Akt activation	[5]
Glioma	DCBLD1	Clinical specimens	High expression	N/A	[43]
Gastric cancer	DCBLD2	Clinical specimens, SNU-016, SNU-601, SNU-620, SNU-638	Inhibits colony formation and invasion	High promotor methylation	[35]
Colorectal cancer	DCBLD2	Clinical specimens, HT29, RKO	Reduced in distant metastases; Associated with good prognosis	PPARγ and TNF-α signaling regulate NT5E and DCBLD2 levels	[40]
Melanoma	DCBLD2	Clinical specimens, HeLa	High levels associated with decreased migration	Expression repressed by AP2-alpha	[37–39]
Cervical cancer	DCBLD2	A431	N/A	pTyr target downstream of EGF-signaling	[32]
Pancreatic cancer	DCBLD2	Clinical specimens	Associated with poor survival, vascular invasion, and an aggressive squamous subtype	Part of a 5-gene signature with ADM, ASPM, E2F7, and KRT6A	[41]

in the growth medium and not in the cell lysate, when both proteins were ectopically expressed in HEK293-ET cells. Using mass spectrometry and site-directed mutagenesis, the authors identified the cleavage site as Ala531, which falls directly N-terminal to the DCBLD2 transmembrane domain (Figure 5B). They demonstrated that transiently transfected DCBLD2 was cleaved by endogenous RHBDL2, but not by ADAM metalloproteases, in HeLa cells and HEK cells, respectively. In addition, they identified DCBLD2 as a substrate of the ER membrane protease RHBDL4 [50,51] when co-expressed in HEK cells [46], which may play a role in degradation of misfolded DCBLD2 at the endoplasmic reticulum (Figure 5C). The identification of DCBLD2 as an RHBDL2 substrate presents interesting functional implications; DCBLD2 ectodomain shedding could modulate cell–cell contact or the released product could possess altered bioactivity, functioning as an autocrine or paracrine signal.

Alteration of DCBLD2 gene function

DCBLD2 has been shown to have both inhibitory and activating effects on cell proliferation and migration, supporting the mounting evidence that the function of DCBLD2 is highly specific to its cellular environment [1,5,7–9,29,52].

Phenotypes at the cellular level

In a mouse model of graft arteriosclerosis, the DCBLD2 protein co-localizes with proliferative cells [9]. Sadeghi et al. [9] demonstrated that siRNA-mediated knockdown (KD) of DCBLD2 increased proliferation of vascular smooth muscle cells (VSMCs), while overexpression had a reciprocal effect. Robust evidence of the inhibitory

Biochemical Journal (2019) **476** 931–950
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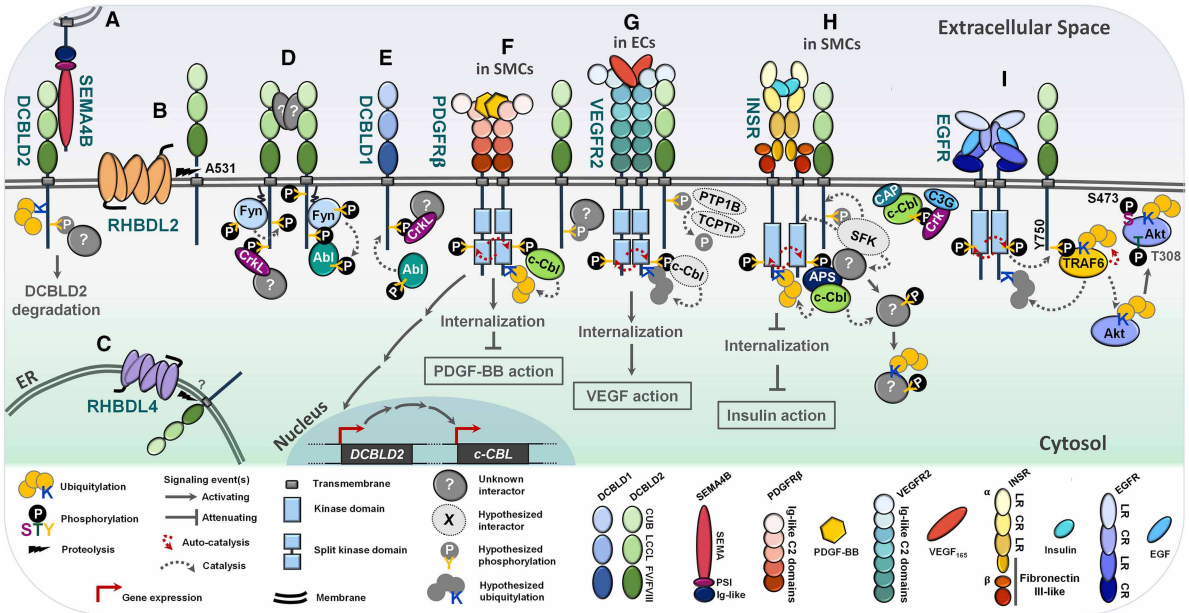

Figure 5. Molecular interactions and signaling of DCBLD family members.

(**A**) SEMA4B increases ubiquitylation of DCBLD2, leading to degradation of plasma membrane-localized DCBLD2 [3]. This likely occurs through an interaction between the SEMA4B and DCBLD2 extracellular domains, leading to DCBLD2 intracellular phosphorylation and the recruitment of E3 ubiquitin ligases. (**B**) RHBDL2 activity releases the DCBLD2 extracellular region, either allowing this cleaved protein to act as a paracrine signal or to prevent DCBLD2 signaling through a ligand-binding mechanism [46]. (**C**) DCBLD2 is a substrate of the endoplasmic reticulum (ER) localized protease RHBDL4 [46], likely as a result of misfolded DCBLD2. (**D**) Extracellular ligand binding clusters DCBLD2 molecules, leading to activation of constitutively-bound Fyn and, subsequently, cytosolic Abl [4,6]. Both kinases are stabilized via their SH2 domain at DCBLD2 Fyn-/Abl-mediated pTyr sites. CRK/CRKL are recruited to DCBLD2 pYXXP motifs, bringing with them unknown CRK/CRKL-SH3 interactors [4,6]. This mechanism could conceivably be activated by SEMA4B (A). (**E**) Abl-mediated DCBLD1 tyrosine phosphorylation leads to the recruitment of CRK/CRKL to DCBLD1 pYXXP sites [6]. Stimuli for Abl activation in this context are unknown. (**F**) PDGF-BB binding to PDGFRβ leads to up-regulation of DCBLD2 expression and, subsequently, of the PDGFRβ negative regulator c-Cbl [1,52]. DCBLD2 could recruit other negative regulators to or titrate positive regulators away from PDGFRβ. G) DCBLD2 promotes VEGFR2 internalization and downstream signaling through direct binding [8]. DCBLD2 could scaffold positive regulators of VEGFR2 signaling or titrate away VEGFR2 phosphatases. H) DCBLD2 attenuates insulin signaling through a direct interaction with INSR [7]. DCBLD2 alters the ratio of bound INSR negative regulators c-Cbl and NEDD4, likely through the complexation of regulatory proteins [7]. For example, the recruitment of cytoplasmic tyrosine kinases would increase phosphorylation of members within the INSR complex, increasing the recruitment rate of negative regulators. (**I**) Active EGFR phosphorylates DCBLD2 at Tyr750, which resides within the preferred binding motif of TRAF6 (PXEXXpY) [5]. Membrane-recruited TRAF6 is autoubiquitylated and activated, leading to TRAF6-mediated Akt ubiquitylation and translocation to the membrane, where it is activated via phosphorylation of Thr308 and Ser473 [5].

effect of DCBLD2 on PDGF-BB-driven proliferation of VSMCs was shown by Guo et al. [52] who observed a ~7-fold increase in PDGF-BB-induced VSMC proliferation in the presence of *DCBLD2* siRNA, as well as a more subtle increase in PDGF-BB-induced VSMC migration. Given the robust up-regulation of DCBLD2 expression following PDGF-BB treatment observed in early work by Kobuke et al. [1], and the apparent inhibitory effect of DCBLD2 on PDGF-induced proliferation, up-regulation of DCBLD2 could be an important negative feedback loop that decreases the rate of PDGF-driven proliferation and migration at later time-points.

In addition to PDGF-driven processes, DCBLD2 has been found to modulate phenotypic effects of other mitogens and growth factors, including insulin, vascular endothelial growth factor (VEGF), and epidermal growth factor (EGF) [5,7,8]. Li et al. [7] observed increased insulin-induced proliferation and migration in mouse-derived DCBLD2 knock-out (*Dcbld2*$^{-/-}$) VSMCs. In contrast, overexpression of DCBLD2 promoted VEGF-driven proliferation and migration in human umbilical vein endothelial cells (HUVECs), and

Biochemical Journal (2019) **476** 931–950
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proliferation was inhibited in cells transfected with *DCBLD2* siRNA [8]. Although no investigations into the phenotypic effects of other RTK ligands have been made in normal cell lines, DCBLD2 is required for EGF- and hyperactive EGFR-driven cell growth, proliferation, and migration in a variety of cancer cell lines, including those derived from glioma, head-and-neck cancer (HNC), lung cancer, and melanoma [5].

Organismal level knockout and knockdown

Nie et al. [8] generated global and endothelial cell (EC)-specific *Dcbld2*$^{-/-}$ mice by homologous recombination resulting in deletion of the *Dcbld2* promotor region and exon 1. These mice exhibit impaired neovascularization in adult mice following both injury and exogenous VEGF administration and, more subtly, impaired developmental angiogenesis. The authors observed less severe phenotypes in EC-specific knockout mice than in the global knockout, which was likely due to compensation by non-EC DCBLD2 or other proteins [8]. Although DCBLD2 has been shown to exhibit an inhibitory effect on PDGF-BB-induced proliferation in smooth muscle cells [9,52], cellular cross-talk could be an important factor in the phenotypic effects of DCBLD2 during vascular development and regeneration. The fact that these mice are viable suggests that DCBLD1 could be compensating for the loss of DCBLD2 gene function, which could also explain the lack of severity in phenotypes. In addition to studying the effects of gene alteration in mice, Nie et al. investigated the developmental role of Dcbld2 in zebrafish vasculogenesis using morpholino (MO)-induced *dcbld2* KD. They demonstrated that *dcbld2* KD resulted in decreased anastomosis of intersegmental vessels (ISVs), a process in which VEGF-A signaling plays a central role [8].

Li et al. went on to explore the effects of DCBLD2 on insulin-related signaling in the global knockouts, and reported improved insulin sensitivity in *Dcbld2*$^{-/-}$ mice relative to wild type (WT) mice. *Dcbld2*$^{-/-}$ mice exhibited significantly lower blood glucose levels in the course of both glucose and insulin tolerance testing regardless of whether they were fed a normal or high-fat diet, despite no differences in secreted insulin levels [7]. The enhanced glucose metabolism in *Dcbld2*$^{-/-}$ mice coupled with the proliferative effects observed in VSMCs derived from *Dcbld2*$^{-/-}$ mice suggests that DCBLD2 negatively affects insulin signaling proximal to the insulin receptor (INSR).

O'Connor et al. [29] identified Dcbld2 as a novel platelet membrane protein in a functional genomics screen in zebrafish and demonstrated increased thrombus surface area in *dcbld2* KD fish, suggesting that Dcbld2 plays an inhibitory role in thrombus formation.

Vascular repair and angiogenesis are concerted processes that require heterocellular cross-talk between endothelial and smooth muscle cells, and similar mechanisms function in neuronal developmental, as well as tumorigenic and metastatic processes. The dynamic phenotypic effects observed in the regulation of DCBLD2 expression are likely due to differential environment-specific modifications and/or expression of interacting partners. DCBLD2 is likely differentially phosphorylated or otherwise post-translationally modified downstream of various mitogens and growth factors, resulting in the association/dissociation of different intracellular signaling molecules that ultimately affect RTK signaling in distinct ways.

The negative regulatory effects of DCBLD2 on glucose uptake and thrombus formation present DCBLD2 as a potential therapeutic target in individuals in which these processes are compromised. Impaired angiogenesis in *Dcbld2*$^{-/-}$ mice and *dcbld2* KD zebrafish indicates that there may be a broader developmental role of DCBLD2 that remains as yet unexplored. For example, signaling mechanisms that govern angiogenesis are similarly central to proper nervous system development [53,54]. It will be important to utilize these available models to query the developmental role of DCBLD2, as this will not only continue to unravel the complexities of and complications associated with developmental processes but will also inform potential targets to combat cancers in which progression relies on overexpression or suppression of this gene.

Role in cancer and disease

As has been discussed of the roles of DCBLD family members in normal cellular processes, their roles in cancers likewise appear highly environment-specific. The observed activating and inhibitory roles of DCBLD1 and DCBLD2 in tumorigenesis and progression are summarized in Table 2 [2,3,5,31–43]. DCBLD family members have also been associated with various physical abnormalities. Increased DCBLD2 levels are linked to enlarged Heschyl's gyrus [55] and nasal polyposis in asthmatics [56,57]. Increased DCBLD1 levels are associated with COPD and emphysema [58].

DCBLD2 expression is suppressed by AP-2α in invading cells of melanomic origin

Regulation of DCBLD2 expression by members of the AP-2 family of transcription factors (TFs) has been characterized in cervical and breast adenocarcinoma, breast ductal carcinoma, and melanoma [37–39]. AP-2 TFs are known to be involved in progression of certain cancers and have been shown to play both inhibitory [59,60] and oncogenic [61] roles. Orso et al. [38] demonstrated that knockdown of AP-2α and AP-2γ is associated with increased cell proliferation at early stages of tumor growth, and reduced migration and invasion in breast (MDA-MB-435 and MDA-MB-231) and cervical (HeLa) adenocarcinoma lines. In AP-2α-deficient HeLa cells, they observed a significant increase in *DCBLD2* mRNA levels, and this effect was reversed by AP-2α overexpression [38]. The observed ∼5-fold increase in migration of DCBLD2-deficient HeLa cells [38] indicates that DCBLD2 could either inhibit cell migration or act in promotion of cell adhesive properties. They later demonstrated that AP-2α binds directly to the *DCBLD2* promotor and acts as a transcriptional repressor of DCBLD2 expression [37].

Like DCBLD2, the AP-2 family is known to play important roles in embryogenic processes, including modulation of cell proliferation, differentiation, and apoptosis. This mechanism of DCBLD2 repression in invasive malignant cells could be similarly involved in dynamic modulation of cell migration during development.

DCBLD2 expression is associated with good prognosis in colorectal cancer

Pagnotta et al. [40] found that high levels of DCBLD2 expression coupled with low NT5E expression in colorectal cancer (CRC) were a robust predictor of patient survival, with 100% of the patients in the study surviving after 5 years. Up-regulation of NT5E is known to be important in tumor invasion and metastasis [62]. Pagnotta et al. [40] identified NT5E as a novel target gene of the TNFα/NFκB inflammatory cascade in HEK293T cells. PPARγ, a nuclear receptor that represses cell growth and promotes differentiation in epithelial cells [63,64] and is a known tumor suppressor via negative regulation of NFκB [65,66], was found to also negatively regulate NT5E expression [40]. The authors treated stable cell lines derived from both colon carcinoma (RKO cells), which possess low levels of PPARγ, and colorectal adenocarcinoma (HT29 cells), which possess high levels of PPARγ, with TNFα and found an increase in NT5E expression and a reciprocal decrease in DCBLD2 expression in RKO cells, but not in HT29 cells [40]. When these same cells lines were treated with the PPARγ agonist, DCBLD2 protein levels were dramatically increased in HT29 cells, but not in RKO cells [40]. These findings suggest that PPARγ activity either prevents degradation of DCBLD2 or directly interferes with TNFα signaling.

Together with the observed AP-2α-induced transcriptional repression of *DCBLD2* as well as the observed increase in *DCBLD2* mRNA levels downstream of PDGF signaling, the dynamic modulation of DCBLD2 levels by TNFα/NFκB and alternatively by PPARγ further underlines the importance of high regulation of DCBLD2 expression, and likely of DCBLD2 protein stability, in distinct signaling mechanisms governing cellular functions important both in developmental and in oncogenic and metastatic processes.

DCBLD1 and DCBLD2 associations with EGFR-driven tumorigenesis and progression

Although high levels of DCBLD2 can have inhibitory effects on tumorigenesis and progression in some malignant cell types, DCBLD family members are also associated with oncogenic properties in other cancers, including glioma, lung adenocarcinoma, myxofibrosarcoma, neuroendocrine cancer, and head and neck cancer (Table 2).

DCBLD2 was identified as a phosphotyrosine target downstream of EGF-induced activation of EGFR in a cervical cancer cell line (A431) [32]. DCBLD2 co-expression has also been shown to be important for hyperactive EGFR-driven tumorigenesis in gliomas and HNCs through a DCBLD2 pTyr750-dependent mechanism of Akt activation [5]. Analysis of clinical specimens of gliomas and HNCs revealed that high levels of pTyr1172 EGFR and pTyr750 DCBLD2 were severely correlated with decreased patient survival [5].

Signaling

The signaling roles of DCBLD1 and DCBLD2 in normal and diseased states remain largely undefined, although a handful of publications provide insight into the potential biological mechanisms involving this protein family (Figure 5) [3–8,46,52].

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Interface with receptor tyrosine kinases (RTKs)

The majority of publications investigating molecular interactors involving DCBLD family members and their potential avenues of mechanistic signaling focus on the interplay between DCBLD2 and the receptor tyrosine kinases EGFR, VEGFR, PDGFR, and INSR. DCBLD2 co-expression has been shown to have both positive and negative effects on signaling downstream of growth factors and hormones [5,7,8,52], however, the specific mechanisms by which DCBLD2 differentially affects RTK-mediated signaling are poorly understood.

Intracellular signaling following RTK activation involves differential activation of a subset of generally conserved pathways, although the mechanisms of activation and regulation can be highly variable across RTK families [67–69]. This may contribute to some of the observed differences in DCBLD2-mediated RTK modulation. In general, a ligand binds to the extracellular domain of a RTK, inducing dimerization and autophosphorylation within the receptor's intracellular domain, leading to the activation of downstream signaling via complex formation surrounding intracellular phosphorylation sites of RTKs and/or their substrates. RTK tyrosine phosphorylation can recruit and activate ubiquitin ligases, leading to internalization of the receptor, from which RTKs are either recycled to the plasma membrane or degraded. Determinants in receptor fate include protein interactors, such as co-receptors, which can regulate the level and type of receptor ubiquitylation.

Drawing from the first description of DCBLD2, which reported its up-regulation during vascular remodeling and in response to PDGF-BB treatment in VSMCs [1], Guo et al. [52] investigated the potential role of DCBLD2 in modulating signaling of PDGFRß, the primary VSMC receptor for PDGF-BB. Following *DCBLD2* siRNA-mediated knockdown, they observed a subtle increase in PDGF-BB-induced activation of Erk1/2, Src and, more robustly, PDGFRß [52].

Guo et al. [52] went on to show that DCBLD2 can alter the ratio of receptor bound-to-unbound PDGF-BB, demonstrating that DCBLD2 KD increased the total amount of PDGF-BB bound to VSMCs without affecting PDGFRß levels. While it remains unclear whether this effect is through a direct interaction between DCBLD2 and PDGFRß or indirectly via secondary messengers, these data suggest that DCBLD2 interacts with PDGFRß or other PDGF-BB receptors to reduce the total amount of bound ligand. The observed increase in bound PDGF-BB is most likely attributed to increased surface levels of PDGF-BB receptors in the absence of DCBLD2. This particular hypothesis has been tested [52], however, the published results were inconclusive. The mechanism of the observed DCBLD2-mediated decrease in bound PDGF-BB is not likely achieved by effecting a conformation of PDGFRß that inhibits ligand binding regions from interacting with PDGF-BB, as the receptor-ligand dissociation constant remained unaltered in WT and DCBLD2 KD VSMCs [52]. It remains possible that full-length DCBLD2 is involved in intercellular interactions with PDGF-BB receptors or, given the recent report that RHBDL2 mediates DCBLD2 ectodomain shedding [46], the released ectodomain could compete for PDGF-BB receptor binding sites. RHBDL2 is up-regulated during wound healing and angiogenesis and it is known to play important roles in the migratory and proliferative properties of keratinocytes and ECs [49,70].

In addition to investigating the effects of DCBLD2 on ligand binding, Guo et al. demonstrated a subtle decrease in PDGFRß ubiquitylation in the presence of *DCBLD2* siRNA and investigated whether DCBLD2 KD had an effect on the expression of the E3 ubiquitin ligase c-Cbl, an important negative regulator of PDGFRß [71]. They demonstrated that both c-Cbl protein and mRNA were decreased by levels similar to DCBLD2 following siRNA-mediated *DCBLD2* KD [52]. Although the implications of this relationship require further investigation, it is possible that DCBLD2 promotes the expression of c-Cbl, thereby indirectly modulating PDGFRß degradation and decreasing PDGFRß surface levels (Figure 5F).

This group went on to create a *Dcbld2*$^{-/-}$ mouse, described above, and investigated the effect of *Dcbld2* knockout (KO) on the signaling of other RTKs. Nie et al. investigated the effect of *Dcbld2* KO on VEGF signaling, given the similarity in domain structure of DCBLD2 to that of neuropilins as well as the known role of Nrp1 as a co-receptor of $VEGF_{165}$ with VEGFR2 [72]. In murine lung endothelial cells (MLECs) derived from WT and *Dcbld2*$^{-/-}$ mice, they found that *Dcbld2* KO prevented VEGF-induced phosphorylation of eNOS, indicating that Akt signaling was impaired in the absence of DCBLD2 [8]. Further investigation into signaling downstream of VEGF revealed a dramatic reduction in VEGFR-induced Erk1/2 and Akt phosphorylation, with a more subtle effect on p38 activity [8]. This inconsistency with the observed effect on signaling downstream of PDGFRß further supports the context-dependent nature of the effect of DCBLD2 on RTK signaling.

In HUVECs, Nie et al. [8] observed a modest *DCBLD2* siRNA-mediated decrease in VEGF-induced VEGFR2 autophosphorylation of Tyr1054/1059 in the kinase domain, Tyr1175, which regulates Akt and Erk1/

2 signaling via SHB and PLCγ binding, respectively, and Tyr1214, which has been implicated in CDC42/p38 activation [73,74]. This suggests that DCBLD2 could either promote VEGFR2 dimerization by interacting with the receptor or its ligand, or prevent the complexation of VEGFR2 with its negative regulators, such as phosphatases [75] and VE-cadherin [76,77]. They tested this latter hypothesis in MLECs and found a subtle increase in levels of VEGFR2-bound PTP1B, TCPTP, VE-cadherin in *Dcbld2*$^{-/-}$ cells [8]. In addition, they were able to marginally recover MAPK, Akt, and VEGFR2 phosphorylation by knocking down expression of PTP1B and TCPTP in *Dcbld2*$^{-/-}$ cells, but not to the level of activity observed in WT cells [8].

Internalization is a key step in VEGFR2 signaling post-ligand binding and is facilitated by EphrinB2 [78] and potentially additional interacting partners. DCBLD2 could play a role in the recruitment of positive-regulatory signaling molecules to the membrane that reduces binding of VEGFR2 to regulators that prevent receptor internalization, such as titrating away VEGFR2 phosphatases, or DCBLD2 could directly interact with VEGFR2 to promote its internalization (Figure 5G). Nie et al. [8] observed co-immunoprecipitation of the two transmembrane proteins, although it was not determined whether this was through a direct interaction or as part of a larger signaling complex. VEGFR2 internalization is also negatively regulated by VEGFR1, which can titrate VEGF away from VEGFR2 [79,80]. It would be interesting to investigate whether, like PDGFRß, DCBLD2 could affect VEGF binding to its receptors, and whether DCBLD2 co-expression or knockdown could affect the ratio of VEGF bound to VEGFR1 and VEGFR2. Interestingly, Nie et al. [8] did not find any evidence of reduced c-Cbl expression in *Dcbld2*$^{-/-}$ MLECs, which suggests that the observed effect of DCBLD2 on c-Cbl expression in smooth muscle cells is indirect and is not a factor in ECs. Alternatively, knocking out DCBLD2 could allow for the development of compensatory mechanisms that would be observed in the transient knockdown of gene expression.

c-Cbl is a known negative regulator of VEGFR2 signaling, promoting degradation of the receptor [81,82]. However, VEGFR2 requires internalization for proper signaling [76,78], and the presence of Nrp-1 as a co-receptor promotes sequestration in specific vesicles, altering the signaling output and preventing VEGFR2 degradation [83]. In environments rich in VEGFR2 and DCBLD2, DCBLD2 could similarly promote VEGFR2 signaling for a specific output.

In addition to the alteration of VEGFR2 signaling in *Dcbld2*$^{-/-}$derived ECs, Li et al. [7] investigated the effect of *Dcbld2* KO on insulin receptor (INSR) signaling. In liver and muscle tissue excised from *Dcbld2*$^{-/-}$ mice, insulin-induced Erk1/2 and Akt activity was increased in *Dcbld2*$^{-/-}$ over WT mice [7]. Li et al. [7] reported a subtle increase in INSR phosphorylation in *Dcbld2*$^{-/-}$ liver, muscle and VSMCs. More robustly, the authors observed a marked increase in INSR ubiquitylation in *Dcbld2*$^{-/-}$ liver and muscle tissue in a manner that was independent of insulin stimulation [7], suggesting that DCBLD2 constitutively inhibits INSR ubiquitylation. DCBLD2 could alter the association of adaptors and regulatory proteins that modulate INSR phosphorylation and activity, however, these would likely be constitutively bound if they were to affect INSR ubiquitylation in unstimulated cells. Given the importance of ubiquitylation in INSR activation and downstream signaling [84], Li et al. [7] focused on the potential for a DCBLD2-mediated interaction between INSR and the adaptor proteins Grb10 and APS, which bring the INSR-regulatory E3 ubiquitin ligases Nedd4 and c-Cbl to the membrane.

In liver and muscle tissue excised from *Dcbld2*$^{-/-}$ mice, Li et al. [7] observed a subtle increase and reciprocal decrease in INSR-associated APS/c-Cbl and Grb10/Nedd4, respectively. More definitive findings were in the combined effect of *Dcbld2* KO and Grb10 or APS knockdown in VSMCs. A dramatic increase in insulin-induced INSR activity in WT VSMCs in the absence of Grb10 was observed [7], which is consistent with the known negative regulatory effect of Grb10 on INSR activity [85]. Strikingly, this increase in INSR phosphorylation was abolished in *Dcbld2*$^{-/-}$ VSMCs treated with *GRB10* siRNA [7]. This suggests that the effect of DCBLD2 on insulin receptor signaling is complex and is not simply explained by the promotion of the Grb10/INSR complex and inhibition of the APS/INSR complex. Both the Grb10/Nedd4 complex and DCBLD2 are presumed negative regulators of insulin signaling, and yet DCBLD2 was required for INSR activation in the absence of Grb10 [7]. *APS* siRNA-mediated KD did reduce insulin-induced INSR phosphorylation, although this appeared to be independent of DCBLD2 expression [7]. Grb10 is known to attenuate INSR signaling by binding to multiple phosphotyrosine residues that serve as docking points for downstream effectors of insulin signaling via the Grb10 SH2 and BPS domains [85]. Additionally, Grb10 prevents substrates from engaging with the binding pocket of the INSR kinase domain by acting as a pseudo-substrate INSR inhibitor [85]. DCBLD2 could act as a scaffold for cytoplasmic tyrosine kinases of INSR, increasing the rate of INSR tyrosine phosphorylation, signal propagation, and INSR down-regulation.

Biochemical Journal (2019) **476** 931–950
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Although Grb10 and, to a lesser degree, APS, KD in *Dcbld2* null cells have potent and distinct effects on INSR activity, these INSR/adaptor interactions likely do not explain the change in the observed insulin-independent INSR ubiquitylation state in *Dcbld2*$^{-/-}$ cells [7]. Recruitment of these SH2 domain-containing adaptors and their E3 ubiquitin ligase cargo requires insulin-induced tyrosine phosphorylation of INSR [86,87]. Membrane translocation of Grb10 or APS could also be mediated by DCBLD2 tyrosine phosphorylation, which would bring the E3 ligase cargo in close proximity to INSR, given the reported insulin-independent complexation of INSR and DCBLD2 [7]. Conceivably, the loss of DCBLD2 expression could free available adaptor/E3 ligase complexes or other INSR positive regulators that normally interact with DCBLD2. In this manner, DCBLD2 could modulate insulin signaling by titrating away certain positive regulators of INSR. For example, c-Cbl is a known negative regulator of INSR through ubiquitin-mediated receptor internalization and degradation [88,89], however, c-Cbl also directly participates in and promotes glucose uptake in parallel to the canonical IRS/PI3K/Akt pathway [87,90]. c-Cbl is recruited to the INSR complex via APS where it is tyrosine-phosphorylated [87]. c-Cbl can then migrate with the associated CAP to lipid rafts, leading to the recruitment of Crk/C3G to c-Cbl phosphotyrosine residues, activation of TC10, and translocation of the GLUT4 complex to the membrane for glucose transport [87,90]. The recruitment of cytoplasmic kinases (e.g. SFKs) to the DCBLD2 intracellular scaffold downstream of insulin/INSR binding could promote phosphorylation and c-Cbl-mediated ubiquitylation of downstream effectors, thereby altering the pool of c-Cbl involved in positive vs. negative regulation of glucose uptake (Figure 5H).

The signaling interface between DCBLD2 and EGFR, characterized in glioma and HNC cell lines, has produced robust biochemical evidence. However, the particular signaling pathway explored may not be a conserved mechanism, as it requires a mammalian-specific TRAF6 binding motif (PXEXXY; Figure 2A). Feng et al. demonstrated that EGFR, activated through EGF-binding or mutation-induced hyperactivity, phosphorylates DCBLD2 Tyr750, near its C-terminus, recruiting the E3 ubiquitin ligase TRAF6 [5,6]. Upon binding to the DCBLD2 PXEXXpY motif containing the EGFR substrate tyrosine, the TRAF6 ubiquitin ligase is activated, inducing Akt ubiquitylation and ultimately resulting in Akt activation via ubiquitin-mediated translocation of Akt to the plasma membrane [91]. Interestingly, they demonstrated that DCBLD2 pTyr750 is not induced by HGF or PDGF-A in variety of cell types (343T, SNB19, PCI-15B, A375), further supporting the evidence of DCBLD2 context-dependent phosphorylation and signaling. Whether additional DCBLD2 tyrosine residues are phosphorylated in these cell lines downstream of EGF, HGF, and PDGF-A, as well as directly by the EGFR, remains to be determined.

Signaling via SFKs and Abl

In addition to RTK-dependent signaling, there is evidence to suggest that DCBLD1 and DCBLD2 might participate in RTK-independent signaling. DCBLD proteins are known to act as scaffolds for the Src homology 2 (SH2) domain of the ubiquitously-expressed adaptor protein CRKL which, along with its homolog CRK, plays central roles in cytoskeletal and focal adhesion dynamics, among many other fundamental cellular processes [92]. The CRK/CRKL-SH2 domain binds phosphorylated tyrosine residues in YXXP motifs, which is the preferred substrate motif of several receptor and non-receptor tyrosine kinases, including Abl and SFKs [93]. The interaction between DCBLD proteins and CRKL requires SFK- and/or Abl-induced phosphorylation of intracellular tyrosine residues within YXXP motifs [4,6], although it remains formally possible that other kinases are also involved. Whether this interaction is dependent or independent of RTK signaling remains unexplored.

The DCBLD2/CRKL-SH2 interaction was first described by Aten et al. and emerged from a proteomics screen that aimed to identify SFK-induced binding partners of the CRKL-SH2 domain in HEK293 cells. They demonstrated that SFKs and Fyn, specifically, could induce DCBLD2 to bind the CRKL-SH2 domain, and that this interaction required phosphorylation of at least one of the seven intracellular YXXP motifs within the DCBLD2 sequence [4]. In addition, they identified the Fyn-SH2 domain as novel pYXXP-dependent DCBLD2 interactor [4], suggesting a possible mechanism by which Fyn could be stabilized in its active conformation upon binding to DCBLD2 pTyr residues. In support of an RTK-independent mechanism of DCBLD2 signaling, they demonstrated that DCBLD2 tyrosine phosphorylation could be induced by adding a DCBLD2 ectodomain-specific antibody [4], thereby clustering the transmembrane proteins. Possessing no intracellular kinase domain, DCBLD2 phosphorylation upon receptor clustering would require a constitutively-bound kinase or other regulatory protein normally in an inactive state that, upon clustering, could autophosphorylate/autoactivate and either directly phosphorylate/modify DCBLD2 and/or lead to the recruitment of other tyrosine kinases to the membrane. While this evidence of a potential RTK-independent pathway is

interesting and likely important, an extracellular ligand that can induce DCBLD2 dimerization has yet to be discovered.

Schmoker et al. described a similar and yet differentially regulated interaction between DCBLD1 and CRKL. Although Fyn kinase activity was sufficient to induce a subtle DCBLD1/CRKL-SH2 interaction, the effect of Fyn on DCBLD2/CRKL-SH2 binding was more robust [6]. Furthermore, the effect of Fyn co-expression on induction of the DCBLD1/CRKL-SH2 interaction was much weaker than the induction by H_2O_2 treatment [6], which increases cellular levels of tyrosine phosphorylation by endogenous kinases. This suggested that another kinase was involved in mediating this interaction and led to the identification of Abl as non-receptor tyrosine kinase that could mediate the interaction between DCBLD1/2 and the CRKL-SH2 domain [6]. Abl was shown to be more important for the DCBLD1/CRKL-SH2 interaction while DCBLD2 was equally induced to bind the CRKL-SH2 domain by Fyn and Abl (Figure 5D,E) [6]. These findings were further supported by the mapping and quantification of Fyn- and Abl-induced tyrosine phosphorylation sites using mass spectrometry, which demonstrated that Abl was the primary kinase of DCBLD1, while Fyn and Abl were found to phosphorylate both common and distinct YXXP and non-YXXP DCBLD2 tyrosine residues [6]. As was demonstrated for the Fyn-SH2 domain by Aten et al. [4], the Abl-SH2 domain was found to bind to DCBLD2 in a pYXXP-dependent manner using site-directed mutagenesis (Figure 5D) [6].

As both Fyn and Abl can be activated downstream of RTKs, phosphorylation of DCBLD1 and DCBLD2 by these non-RTKs is likely also a factor in RTK signaling. Although the interface of DCBLD2 with RTKs is likely important in developmental and tumorigenic processes, it will be interesting to explore the implications of non-RTK-related DCBLD2 signaling defined by the binding of an as of yet unknown ligand. The increased ubiquitylation state of DCBLD2 observed with co-expression of SEMA4B coupled with the loss of the highest molecular mass variant of DCBLD2 [3] suggests that SEMA4B could be a ligand of DCBLD2. This highest molecular mass variant was shown to be the product of glycosylation and could represent the fraction of fully matured DCBLD2 that is localized at the plasma membrane, which is degraded in the presence of SEMA4B (Figure 5A) [3]. SEMA4B-mediated clustering of DCBLD2 could activate bound SFKs, leading to phosphorylation of DCBLD2 tyrosine residues and the recruitment of CRK/CRKL (Figure 5D), as well as negative regulatory molecules including ubiquitin ligases. As the induction of signaling related to DCBLD1 phosphorylation has yet to be explored, it will be important to determine whether DCBLD1 is similarly activated by RTKs or whether DCBLD1 phosphorylation can be achieved by DCBLD1 clustering.

Future perspectives and outlook

The body of investigations into the DCBLD receptor family discussed here reveal that cellular context is highly influential in determining the outcomes of DCBLD2 expression and signaling, at both the cellular and organismal levels. Temporal regulation of DCBLD2 action is achieved through modulating expression and stability of the scaffolding receptor, although the precise function of DCBLD2, and whether DCBLD1 expression is similarly regulated, remains unknown. The dynamic cross-talk between ECs, SMCs, pericytes, and fibroblasts governed by receptor/ligand interactions during angiogenesis and neovascularization facilitates the migration and proliferation of these cell types at precise time-points. Similar interactions are essential to proper neuronal migration and pathfinding, as well as cancer cell invasion and metastasis [31,94–96]. The implications of DCBLD2 in blood vessel [1,8,9], and in tumorigenesis and metastasis [2,3,5,32–34,41], suggest that its functions, likely involving receptor/ligand interactions, are similarly conserved throughout these processes.

Known DCBLD2 molecular interactors are closely related to those of neuropilins in their angiogenic roles. Neuropilins (Nrps) bind Class III semaphorins and $VEGF_{165}$ in tandem with and separately from VEGFR2 [97]. Nrp1 acts as a co-receptor for VEGFR2 and enhances the $VEGF_{165}$/VEGFR2 interaction [98,99], while the SEMA3A/Nrp1 interaction prevents VEGF form binding the Nrp1/VEGFR2 complex [100,101]. SEMA4B binds to DCBLD2 and induces its ubiquitylation and, presumably, its degradation, suggesting that SEMA4B acts to attenuate DCBLD2 action [3]. SEMA4B could act as a repulsive cue by inducing DCBLD2 degradation in migrating cells, thereby affecting focal adhesions or cytoskeletal dynamics. SEMA4B is known to potently inhibit Akt-driven metastasis of non-small cell lung cancers [102], indicating a potential link to the role of DCBLD2 downstream of EGFR in aberrant Akt signaling. DCBLD2 also modulates VEGF-induced VEGFR2 activation, although it is not yet known whether this occurs through a co-receptor mechanism or otherwise.

During angiogenic migration, endothelial tip cells extend lamellopodia toward a VEGF gradient to stimulate VEGFR2 signaling and secrete PDGF-BB to recruit PDGFRß-expressing pericytes and SMCs. DCBLD2 promotes growth factor-mediated signaling, proliferation, and growth in ECs through VEGF/VEGFR2, while

Biochemical Journal (2019) **476** 931–950
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attenuating the same processes in SMCs through PDGF-BB/PDGFRß [8,52]. However, a population of RTKs are expressed within a given cell type, therefore, the action of DCBLD2 on the signaling of a specific RTK within a specific cell type may not be a sufficient representation of DCBLD2 function. This is further complicated by our evolving understanding of the promiscuity of growth factor/RTK interactions, such as PDGF/VEGFR and VEGF/PDGFR interactions [103–105]. Although DCBLD2 was found to decrease PDGFRß signaling in SMCs [52], the effect on the signaling of PDGFRß or of other RTKs in ECs was not considered. DCBLD2 could play a role in determining the fate of cells within a population migrating toward a permissive signal; for example, DCBLD2 could promote VEGFR2 signaling in cells in close proximity to concentrated VEGF while attenuating signaling through other RTKs within the same cell type.

The binding of different ligands to RTKs is known to modulate the amount of time a signal is propagated and, therefore, the phenotypic outcome of the receptor-ligand interaction. Co-receptors also influence these interactions by altering receptor conformation, post-translational modifications and protein–protein interactions, and therefore downstream signaling events. DCBLD2 could act as a co-receptor for certain RTKs, to alter the phenotypic outcome of the signal. Internalization of VEGFR, PDGFRß, EGFR, NGF, FGF, and TGFß receptors is known to promote their signaling from endosomal compartments [76,106–111], while INSR is thought to signal predominately at the cell surface, although some evidence of INSR endosomal signaling has been reported [110]. Although the evidence of cellular environment and molecular context surrounding DCBLD2/RTK interactions are variable across studies, the unifying features, either implied or explicitly demonstrated, include DCBLD2-mediated regulation of (i) expression/association of RTK-regulators including phosphatases and ubiquitin ligases, and (ii) surface levels of RTKs, which can either promote or attenuate downstream signaling. Our current understanding of the DCBLD2/RTK interface implies that DCBLD2 could affect receptor fate post-internalization, potentiating RTK membrane recycling, degradation, or vesicular sequestration.

Although there remains much to uncover surrounding the characterization of DCBLD1/2 signaling and the resulting functional implications, the preliminary investigations discussed here demonstrate the importance of this understudied receptor family in modulating the phenotypic outcomes of several well-studied systems. Future studies will need to focus on distinguishing ligand-induced DCBLD2 signaling from its interface with RTK signaling, and to determine whether these mechanisms are conserved in DCBLD1 functionality. Given the conservation of these protein sequences among vertebrates, and their apparent roles in fundamental biological processes, DCBLD1 and DCBLD2 will likely emerge as important subjects of study in understanding vertebrate development, and in the improvement of therapeutic agents to combat progression of certain cancers.

Abbreviations

ADAM, a disintegrin and metalloprotease; CAEC, coronary artery endotheial cell; CASMC, coronary artery smooth muscle cell; CLCP1, CUB, LCCL-homology, and coagulation factor V/VIII-homology domains 1; CRK(L), CT10 regulator of kinase(-like); CUB, complement C1r/C1s, Uegf, and the Bmp1; DCBLD1/2, discoidin, CUB, and LCCL domain-containing 1/2; EC, endothelial cell; EGF, epithelial growth factor; EGFR, epithelial growth factor receptor; ER, endoplasmic reticulum; ESDN, endothelial smooth muscle cell-derived neuropilin-like; HEK, human embryonic kidney; HGF, hepatocyte growth factor; HUVEC, human umbilical vein endothelial cell; IGF, insulin-like growth factor; IGFR, insulin-like growth factor receptor; IR, insulin receptor; ISV, intersegmental vessel; LCCL, Limulus clotting factor C, Coch-5b2, and Lgl1; MO, morpholino; NFκB, nuclear factor kappa B; NT5E, 5′-nucleosidase ecto; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PPARγ, peroxisome proliferator-activated receptor gamma; RGC, retinal ganglion cell; RHBDL2/4, Rhomboid-related protein 2/4; RTK, Receptor tyrosine kinase; SEMA4B, Semaphorin 4B; SFK, Src family kinase; SH2, Src Homology 2; TM, transmembrane; TNFα, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; VSMC, vascular smooth muscle cell; WT, wild type.

Author Contribution

A.M.S. wrote the manuscript. B.A.B. edited the manuscript. B.A.B. and A.M.E. provided important conceptual insights.

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Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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