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## Integrin signaling in vascular function

**Nikolay L. Malinin, Elzbieta Pluskota, and Tatiana V. Byzova**

Department of Molecular Cardiology, Taussig Cancer Center, Lerner Research Institute, Cleveland Clinic

### Structured summary

**Purpose of review**—In the current review, we summarize recent progress on vasculature-specific function and regulation of integrins and integrin-associated proteins, including advances in our understanding of inside-out integrin activation. The studies on regulation of integrin activation received new impulse in 2009 with the identification of Kindlin protein family members as crucial mediators of integrin inside-out signaling. In the current review, we outline the recent findings on the role of Kindlins in the vascular system, as well as new studies that have begun shaping the mechanistic model of Kindlins' function.

**Recent findings**—Several tissue-specific knockout models for integrins and genes associated with the integrin functions have been recently presented, including smooth muscle-specific ILK and endothelial-specific FAK and Talin-1 ablation. In the heterozygous animal knockout model, Kindlin-2 has been demonstrated as a crucial modulator of angiogenesis and vascular permeability. As a number of papers have advanced our understanding of Kindlin function, they are reviewed and discussed in further detail. New findings include an additional lipid binding site within the Kindlin molecule and preferential binding of non-phosphorylated form of  $\beta$ -integrins.

**Summary**—The role of integrins in angiogenesis has been demonstrated to include, in addition to cell adhesion and mechanotransduction, specific signaling functions. The importance of integrin inside-out pathway in vascular physiology has been unequivocally proven, and endothelial permeability is directly regulated by this process. Inhibition of Kindlin-dependent steps in the inside-out pathway as an approach to block platelet aggregation should be paralog-specific, as it may have adverse effects on vascular permeability.

### Keywords

integrins; endothelial permeability; inside-out; adhesion; Kindlin

### Introduction

Integrins are crucial in vasculogenesis, angiogenesis, and vascular integrity as major mediators of vascular cell adhesion and migration through the extracellular matrix, as well as signaling co-receptors of the receptor tyrosine kinases, including VEGFR – the “master switch” of angiogenesis [1]. While studies determining unique versus redundant roles of a particular integrin pair are currently under way, utilization of tissue specific knockouts of integrin subunits has allowed an accurate assessment of integrin functions in endothelial and

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Author of correspondence: Tatiana V. Byzova, PhD, FAHA, Staff/Professor, Department of Molecular Cardiology, JJ Jacobs Center for Thrombosis and Vascular Biology, Taussig Cancer Center, Lerner Research Institute, CLEVELAND CLINIC, 9500 Euclid Ave., NB50, Cleveland OH 44195, Fax: 216/445-8204, Office: 216/445-4312, Lab: 216/445-8237, <http://www.lerner.ccf.org/moleccard/byzova/>, byzovat@ccf.org.

#### Conflicts of interest

Authors declare no conflict of interest.

smooth muscle cells, as reviewed here. An important feature of integrins is the regulation of their affinity towards the extracellular matrix (ECM) ligands. In endothelial cells, integrin activation is induced by growth factors, including VEGF [1], and mechanical stress [2, 3]. Activation is achieved by binding two FERMT domain containing proteins, Talin and Kindlin, to NxxY motifs within the  $\beta$  integrin cytoplasmic domain. The role of Talin in integrin activation has been reported [4], however, the significance of Kindlin is demonstrated more recently [5–8] and will be further discussed in this review. Kindlin paralogs Kindlin-1, -2, and -3 have a high degree of sequence identity among them, yet exhibit different patterns of expression [9]. The presence of at least one Kindlin paralog is required to achieve integrin activation. Once integrins are engaged by ECM, they function as docking modules for a plethora of intracellular signaling proteins, altogether known as “adhesome” [10]. Integrin Linked Kinase (ILK) and Focal Adhesion Kinase (FAK) are required for the integrin link with the cytoskeleton and signal transduction. As global knockouts of ILK and FAK are lethal [11, 12], recently reported inducible and tissue specific knockouts of ILK and FAK have demonstrated the unique roles of these genes in smooth muscle and endothelial cells, respectively [13, 14].

## Tissue-specific functions of integrins

We summarize here the recent findings in gene knockout (KO) studies addressing integrin function. Previously, knockout of  $\beta 1$ -integrin has been reported as peri-implantation lethal [15], and endothelial-specific gene ablation resulted in severe vascular development defects [16, 17]. Two new reports addressed the mechanism underlying the impaired vascular development in  $\beta 1$  integrin KO using blastocyst-derived embryonic cells [18] and endothelial-specific knockout [19]\*\*. The first study shows CD31+ endothelial precursors from wild type, but not  $\beta 1$  KO, embryos spontaneously undergo clustering and sprouting in vitro. In striking difference to wild type, the basal membrane of  $\beta 1^{-/-}$  embryonic bodies (EB) was absent, as a result of diminished expression of ECM proteins [18]. Re-introduction of  $\beta 1$  expression under the CD31 promoter rescued all of the defects of tubulogenesis observed in EB, demonstrating a cell-autonomous effect of  $\beta 1$  ablation in the endothelial cells [18]. The other study addressed the  $\beta 1$  integrin's role in vasculogenesis using VE-cadherin (VE-Cad) driven Cre expression [19]\*\*. Unlike Tie-2-driven  $\beta 1$  integrin ablation [16], which is lethal at E10, the VE-Cad-driven knockout resulted in lethality around E17, allowing studies of embryos [19]\*\*. Similar to Tie-2-driven KO, the VE-Cad KO of the  $\beta 1$  integrin resulted in vascular rupture and hemorrhage. The remarkable feature of these VE-Cad KO embryos was the defective lumen formation. The lumen was formed in the aorta, but not in mid-size vessels, which is likely due to the timing of the  $\beta 1$  excision. In agreement with the other report, the effect is endothelial cell-autonomous. An unexpected consequence of the  $\beta 1$  ablation was the loss of Par3 expression, but not other proteins of the polarity complex. Therefore, vacuoles over-accumulated in the endothelial cells' cytoplasm due to the lack of coordinated vacuoles movement and fusion. Over-expression of Par3 partially rescued the phenotype, thereby solidifying the role of  $\beta 1$  integrin-Par3 axis in vascular lumen formation [19]\*\*. Alpha subunits of integrins have important roles in vascular functions, including lower vertebrates [20]. Using Tie-2-driven ablation of gene expression, Richard Hynes's group has shown  $\alpha 5$  and  $\alpha v$  integrin subunits have overlapping functions in developmental angiogenesis [21]\*\*. In the double knockout, development appeared normal until E11.5, then exhibited impaired vascular remodeling, resulting in death at midgestation. Surprisingly, a low percentage of double KO mice survived, indicating functional compensation by other alpha subunits. As Tie-2-driven ablation of the  $\beta 1$  integrin results in death at earlier stages than in  $\alpha 5/\alpha v$  double KO, some other alpha subunits function in a complex with  $\beta 1$  at the earlier stages [21]\*\*. Another interesting study utilized endothelial-specific ablation of  $\alpha 3$  and demonstrated a suppressive effect of this integrin on tumor angiogenesis [22].

Together, these elegant *in vivo* experiments with tissue specific deletions demonstrate not only the importance of individual integrin subunits, but also high plasticity and adaptability of cell adhesion machinery. The multiple levels of functional redundancy underscore the key role of integrins in vascular biology.

### Tissue-specific role of integrin modulators in vivo

Talin-1 appears to be the only isoform expressed by endothelial cells [23, 24]. Since global deletion of Talin-1 results in early embryonic arrest [25], inducible and endothelial specific Talin-1 KO are of interest [23]. Talin-1 ablation at E8.5 and endothelium specific ablation resulted in vascular defects and embryo death. Vasculature was reported to be dilated and poorly branching as a result of the endothelial cells' inability to spread [23]. A recent study [26] using embryoid bodies from Talin-1 null embryos suggests that Talin-1 is needed for the maintenance of the  $\beta 1$  integrin expression, but not its activation. Most surprisingly, either expression of the  $\beta 1$  integrin or proteasome inhibition largely rescued defective cell adhesion in Talin-1 null cells. Thus, Talin-1 binding to the integrin  $\beta 1$  appears to merely prevent  $\beta 1$  degradation by proteasome in K48 ubiquitination-dependent manner. In this connection, identification of the SHARPIN protein as a novel negative modulator of integrin activation is particularly intriguing [27], as SHARPIN has been previously identified within linear ubiquitin chain assembly complex LUBAC [28]. SHARPIN binds to a conservative peri-membrane GFFRK motif of at least three alpha subunits, presumably "locking" integrins in inactive conformation [27].

Similar to Talin, ILK and FAK are essential for integrin dependent adhesion and signaling. Both global and endothelial specific knockouts were reported for the FAK gene [12, 29]. Hodivala-Dilke and colleagues, using inducible endothelial-specific FAK ablation, have now reported a dramatic decrease in tumor neo-vascularization. As expected, the effect was not confined to tumor angiogenesis, but was also present in other *in vivo* settings [14]. ILK knockout models have been reported previously [11, 30, 31]. In the new report, ablation of ILK expression governed by SM22 smooth muscle specific promoter resulted in aneurismal dilation of the aorta, lethal at postnatal day P1 [13]. The effects are cell autonomous and correlated with decreased expression of genes associated with an SMC contractile phenotype [13].

Two new *in vivo* studies are focused on "the master switch" of integrin-dependent functions, Rap1 small G protein. Rap1b KO and VEGFR2 inhibition have an additive inhibitory effect on angiogenesis *in vivo*, indicating non-overlapping functions and emphasizing that integrin activation is required for the VEGFR2 function, thus providing a positive feedforward loop [32]. In the other study, Zhang *et al.* demonstrated that  $\alpha I I b \beta 3$ -dependent outside-in signaling activates Rap-1b, which is crucial for platelet spreading and clot retraction [33]. Thus, activation of Rap-1 plays a dual role in platelet function: agonist-induced Rap-1 leads to integrin activation and platelet granule secretion, while integrin-mediated Rap-1 activation is required for clot retraction and platelet spreading [33].

### Kindlin-2 mechanistic and structural studies

The Kindlin proteins have been implicated in integrin activation [4, 9]. The Kindlin family consists of three members: Kindlin-1 (FERMT1), Kindlin-2 (FERMT2), and Kindlin-3 (FERMT3), all of which are structurally very similar. However, it appears that Kindlins cannot compensate one another, as deficiencies of each Kindlin show distinct phenotypes [9]. Since Kindlins were demonstrated to be crucial for integrin activation [5–8], the focus of follow-up studies branched into two major directions – the role of Kindlin paralogs in different physiological settings, and the mechanistic studies of Kindlin function and regulation. Kindlins, similar to the Talin head region, are largely composed of FERM

domains. In Kindlins, however, these FERM domains are further intersected with the pleckstrin homology (PH) domain [4, 9]. Structurally, Kindlins' integrin binding site resembles a phosphotyrosine binding domain (PTB) [34]. As the integrin is phosphorylated in the Kindlin binding motif NxxY [35], Bledzka *et al.* have now demonstrated that phosphorylation of this motif in the  $\beta 3$  cytoplasmic tail disrupts its interaction with Kindlin-2 [36]\*\*. These studies suggest that NxxY motifs might act as a dual switch in a control of integrin activation.

To propose the mechanistic model of the Kindlin function, the crystal structure of the Kindlin protein in a complex with the  $\beta$ -integrin cytoplasm tail is essential, however, it is not yet reported. Crystallization of the PH domain of Kindlin-2 has been announced, although the structural model is not finalized [37]. Kindlin-2 PH domain is reported to bind phosphatidylinositol phosphate with preference for PIP3, and overexpression of PH-deficient Kindlin-2 reduced integrin activation in podocytes [37, 38]. Apparently, the PH domain is not the only lipid binding region within the Kindlin-2 protein, as the F0 sub-domain of the Kindlin-2 FERM domain has been demonstrated to interact with PIP2 [39]\*. Kindlin-2 F0 is unlikely to contribute to the protein microdomain targeting, as the depletion of PIP2 from focal adhesions impeded recruitment of Talin-1 and Vinculin, but not Kindlin-2 [40].

## Kindlin-2 in heart and vasculature

Kindlin-2 is the most widely expressed Kindlin family member, and multiple lines of evidence point to its crucial role in integrin activation in vascular cells. *In vitro*, the Kindlin-2 knockdown in endothelial cells led to a defect in adhesion, spreading, and migration on the  $\beta 3$  integrin ligands [41]. Studies by Dowling and colleagues show the essential role of Kindlin-2 in heart development [42]. The depletion of Kindlin-2 led to severe myocardial defects resulting from disrupted intercalated disc formation [42]. Early embryonic lethality of the Kindlin-2 KO mice did not permit the detailed analysis of the role of this molecule in endothelium or pathological vascularization. However, analysis of embryonic bodies generated from the Kindlin-2 deficient embryonic stem cells revealed impaired adhesion, spreading, and integrin activation [43]. Unlike homozygous mice, Kindlin-2 heterozygous mice are viable and, when challenged, showed significantly reduced angiogenesis in growing tumors and Matrigel implants [44]\*\*. The RM1 prostate tumors grown in the Kindlin-2<sup>+/-</sup> mice were smaller and had reduced vessel density and vascular area. New blood vessels formed in these mice were immature, had thinner basement membrane, and severely reduced pericyte content – all leading to vascular leakage [44]\*\*. Likewise, Kindlin-2 knockdown in zebrafish resulted in multiple abnormalities in developmental angiogenesis, including thin, shortened intersegmental vessels and failure to form a continuous dorsal longitudinal anastomotic vessel. Aortic endothelial cells (ECs) isolated from the Kindlin-2<sup>+/-</sup> mice showed diminished  $\beta 3$  integrin activation and, consequently, all of the  $\beta 3$  integrin-dependent cellular responses were impaired, while the  $\beta 1$  integrin function appeared to be preserved [44]\*\*. Since Kindlin-2 is known to interact with and activate both  $\beta 1$  and  $\beta 3$  integrins, this differential integrin specificity in ECs is intriguing. Interestingly, the baseline permeability of preexisting blood vessels was enhanced by 70% in Kindlin-2<sup>+/-</sup> mice, while VEGF, a potent inducer of vascular permeability, robustly increased permeability in the WT vasculature; the Kindlin-2<sup>+/-</sup> blood vessels remained unresponsive [44]\*\*. Remarkably, VEGF and PLGF did not induce intracellular signaling in Kindlin-2<sup>+/-</sup> ECs and VEGF did not rescue aberrant angiogenesis observed in zebrafish with Kindlin-2 knockdown. Another interesting question is the mechanism by which Kindlin-2 regulates vascular permeability. It has been demonstrated that Kindlin-2 co-localizes with E-cadherin in keratinocyte cell-cell junctions and is present in adherence junctions in the colon and heart [42, 45]. However, the functions of Kindlin-2

at these sites or the mechanism underlying its recruitment are not clear and remain an exciting field for investigation. Kindlin-3, which is robustly expressed in hematopoietic cells, is a key molecule in the control of hemostasis and inflammation. Bialkowska *et al.* demonstrated that in addition to Kindlin-2, Kindlin-3 is also present in ECs [46]\*. While Kindlin-2 is needed for  $\alpha V\beta 3$ -mediated adhesion to vitronectin, Kindlin-3 is required for  $\alpha 5\beta 1$ -dependent adhesion to fibronectin, suggesting that the two Kindlins do not have overlapping functions in ECs. Additionally, although both Kindlins co-localize with the  $\beta 1$  and  $\beta 3$  integrins, they do not co-localize with each other, as Kindlin-2 was detected in the focal adhesions, where Kindlin-3 was absent [46]\*. What segregates Kindlin-2 and Kindlin-3 and the molecular mechanism responsible for functional differences between these two proteins in the same cell remains to be elucidated.

On the mechanistic side, new cases of Leukocyte Adhesion Disorder type III (LADIII) help to shape the structure-functional studies on Kindlin-3, as the G308R point mutation has been reported as specifically affecting cell migration, but not cell adhesion, signifying the finely tuned regulation of several inter-related processes by Kindlin proteins [47]\*. Recent studies in leukocytes shed light on the signaling events preceding the final steps of integrin activation. The authors identified two types of protein complexes: one containing RAPL/Mst1, and the second one containing RIAM/Mst1/Kindlin-3 [48]. These results laid a foundation for future work on protein-protein interaction studies for Kindlin paralogs. (figure 1)

## Conclusions and future directions

Most recent studies further demonstrated the importance of integrins and their signaling in vascular biology. Deletions of integrin subunits and key regulators of integrin function result in distinctive vascular phenotypes. However, a substantial functional redundancy remains between several integrin subunits. Talin-1's essential role for vasculogenesis is clearly demonstrated using *in vivo* knockout approach. However, we may witness a revision of Talin function from the integrin activation to a link between the integrin and cytoskeleton required for normal levels of integrin expression. Kindlin paralogs were shown to serve as essential regulators of integrin-dependent functions in various cell types. It became clear that the expression levels of Kindlin-2 are critical for the endothelial cell functions as shown in the heterozygous animal model. The authors expect a series of reports on conditional knockouts of Kindlin paralogs expanding our knowledge on the proteins' function. From the mechanistic perspectives, the next breakthrough in integrin signaling is expected to result from structural studies. As Kindlin studies progress further, the big picture of the molecular events leading to integrin activation remains under constant revision. As Kindlins are found in the nucleus and at cell-cell junctions, identification integrin-independent functions of these proteins remain an intriguing possibility.

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## Abbreviations

<b>VEGFR</b>	vascular endothelial growth factor receptor
<b>ILK</b>	Integrin Linked Kinase



<b>FAK</b>	Focal Adhesion Kinase
<b>PIP2</b>	phosphatidylinositol-(4,5)-biphosphate

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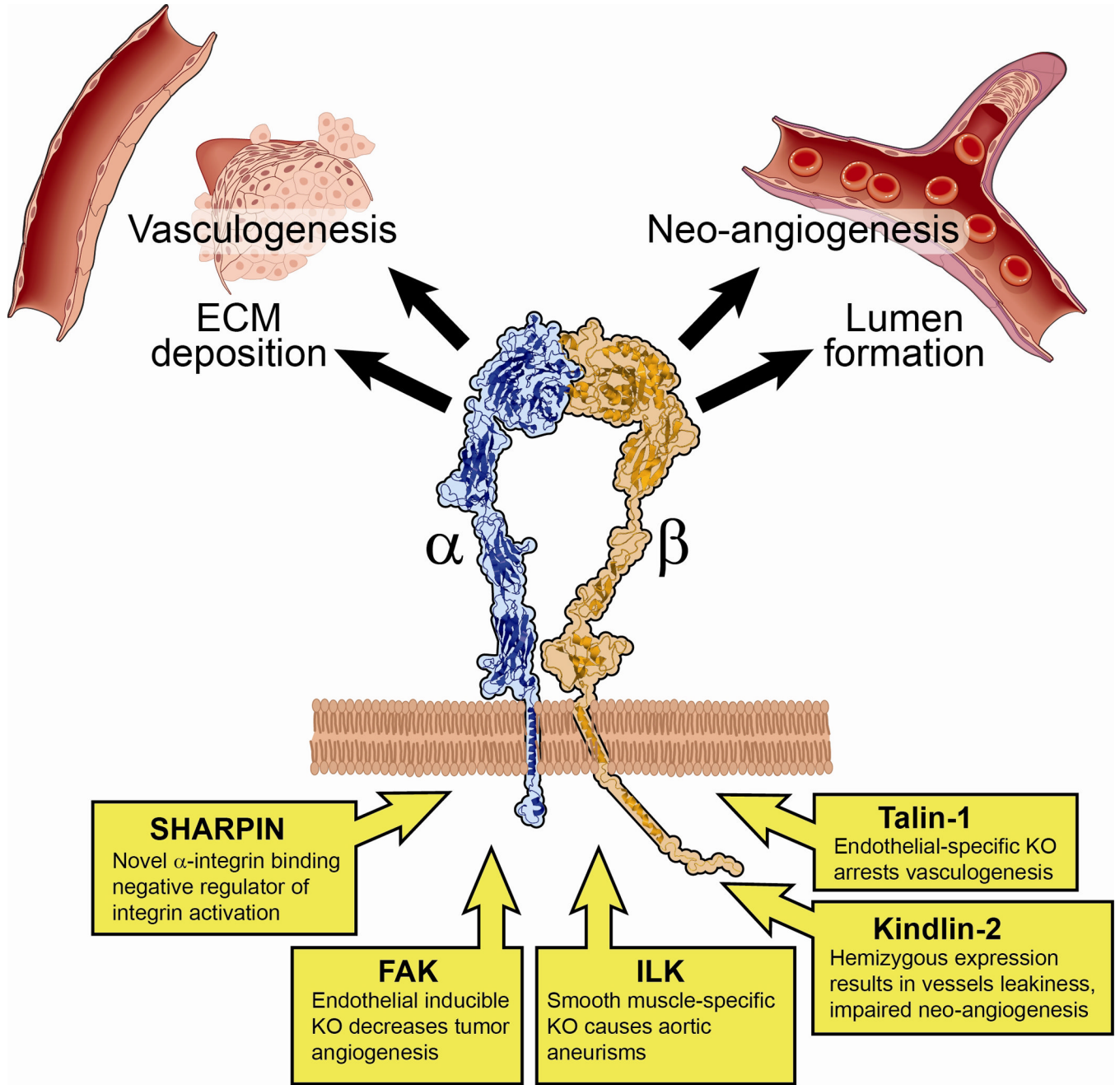
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### Key points

- ◆ Plethora of integrins heterodimers is crucial for both development and homeostasis of the circulatory system. There is a considerable degree of functional redundancy between integrin subunits on endothelial cells.
- ◆ Integrins as signaling entities are crucial for ECM deposition and vessel lumen formation.
- ◆ Endothelial expression of Talin-1 is essential for vasculogenesis.
- ◆ Kindlins are implicated in vasculogenesis and vascular permeability and postnatal angiogenesis.
- ◆ Conditional/inducible knockouts of Kindlin genes are required to fully address their roles in vascular biology.



**Figure 1. Integrin and integrin-binding proteins in vasculature biology**

New reports identify the unique role of integrins and integrin-binding proteins in the vasculature. Ablation or decreased expression of integrin modulators Talin-1 and Kindlin-2, respectively, disable vasculature development and regulation of permeability. Focal Adhesion Kinase or Integrin Linked Kinase gene ablation result in the unique phenotypes as indicated. Integrin  $\beta 1$  gene ablation has a profound effect on vasculogenesis through disabled cell signaling for secretion of the extracellular matrix proteins and lumen formation, as well as through impaired cell adhesion and spreading. Novel  $\alpha$ -integrin binding modulator of integrin activity, SHARPIN, is shown as well.

Source: original