Brag2 differentially regulates #1- and #3-integrin-dependent adhesion in endothelial cells and is involved in developmental and pathological angiogenesis

Citation: Manavski, Yosif et al. "Brag2 Differentially Regulates ß1- and ß3-Integrin-Dependent Adhesion in Endothelial Cells and Is Involved in Developmental and Pathological Angiogenesis." Basic Research in Cardiology, vol. 109, February 2014, pp. 1-19.

As Published: http://dx.doi.org/10.1007/s00395-014-0404-2

Publisher: Springer-Verlag

Persistent URL: http://hdl.handle.net/1721.1/104868

Version: Author's final manuscript: final author's manuscript post peer review, without publisher's formatting or copy editing

Basic Res Cardiol (2014) 109:404
DOI 10.1007/s00395-014-0404-2

ORIGINAL CONTRIBUTION

Brag2 differentially regulates β1- and β3-integrin-dependent adhesion in endothelial cells and is involved in developmental and pathological angiogenesis

Yosif Manavski · Guillaume Carmona · Katrin Bennewitz · Zhongshu Tang · Fan Zhang · Atsuko Sakurai · Andreas M. Zeiher · J. Silvio Gutkind · Xuri Li · Jens Kroll · Stefanie Dimmeler · Emmanouil Chavakis

Received: 22 August 2013 / Revised: 19 January 2014 / Accepted: 28 January 2014 / Published online: 13 February 2014

Abstract β1-Integrins are essential for angiogenesis. The mechanisms regulating integrin function in endothelial cells (EC) and their contribution to angiogenesis remain elusive. Brag2 is a guanine nucleotide exchange factor for the small Arf-GTPases Arf5 and Arf6. The role of Brag2 in EC and angiogenesis and the underlying molecular mechanisms remain unclear. siRNA-mediated Brag2-silencing reduced EC angiogenic sprouting and migration. Brag2-siRNA transfection differentially affected α5β1- and αVβ3-integrin function: specifically, Brag2-silencing increased focal/fibrillar adhesions and adhesion on β1-integrin ligands (fibronectin and collagen), while reducing the adhesion on the αVβ3-integrin ligand, vitronectin. Consistent with these results, Brag2-silencing enhanced surface expression of α5β1-integrin, while reducing surface expression of αVβ3-integrin. Mechanistically, Brag2-mediated αVβ3-integrin-recycling and β1-integrin endocytosis and specifically of the active/matrix-bound α5β1-integrin present in fibrillar/focal adhesions (FA), suggesting that Brag2 contributes to the disassembly of FA via β1-integrin endocytosis. Arf5 and Arf6 are promoting downstream of Brag2 angiogenic sprouting, β1-integrin endocytosis and the regulation of FA. In vivo silencing of the Brag2-orthologues in zebrafish embryos using morpholinos perturbed vascular development. Furthermore, in vivo intravitreal injection of plasmids containing Brag2-shRNA reduced pathological ischemia-induced retinal and choroidal neovascularization. These data reveal that Brag2 is essential for developmental and pathological angiogenesis by promoting EC sprouting through regulation of

Y. Manavski and G. Carmona contributed equally.

Electronic supplementary material The online version of this article (doi:10.1007/s00395-014-0404-2) contains supplementary material, which is available to authorized users.

Y. Manavski · G. Carmona · S. Dimmeler · E. Chavakis
Institute of Cardiovascular Regeneration, Goethe University Frankfurt, Frankfurt, Germany

Present Address:
G. Carmona
David H Koch Institute for Integrative Cancer Research at Massachusetts Institute of Technology, Cambridge, MA 02139-4307, USA

K. Bennewitz · J. Kroll
Department of Vascular Biology and Tumor Angiogenesis, Center for Biomedicine and Medical Technology Mannheim (CBTM), Medical Faculty Mannheim, Heidelberg University, Mannheim, Germany

Z. Tang · X. Li
State Key Laboratory of Ophthalmology, Zhongshan Ophthalmic Center, Sun Yat-Sen University, Guangzhou 510060, People's Republic of China

F. Zhang
NEI, National Institutes of Health, Bethesda, MD, USA

A. Sakurai · J. S. Gutkind
Oral and Pharyngeal Cancer Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA

A. M. Zeiher · E. Chavakis (✉)
Department of Internal Medicine III, Cardiology, Goethe University of Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany
e-mail: chavakis@em.uni-frankfurt.de

J. Kroll
Division of Vascular Oncology and Metastasis, German Cancer Research Center (DKFZ-ZMBH Alliance), Heidelberg, Germany

adhesion by mediating β1-integrin internalization and link for the first time the process of β1-integrin endocytosis with angiogenesis.

Keywords Angiogenesis · Brag2 · Endocytosis · Integrins · Migration

Introduction

Angiogenesis, the formation of new capillaries from pre-existing ones by endothelial cell (EC) sprouting, is involved in many physiologic and pathologic conditions, including embryonic development, wound healing, rheumatoid arthritis, tumor growth and metastasis and proliferative retinopathy [5]. Beyond endothelial cells also proangiogenic cells circulating in blood contribute to angiogenesis by paracrine effects [28, 32].

Integrins are heterodimeric transmembrane proteins consisting of non-covalent bound α- and β-subunits mediating cell adhesion to extracellular matrix (ECM) proteins and bidirectional signaling [24]. Beyond angiogenic growth factors, there is evidence that integrins are implicated in angiogenesis [23]. Specifically, endothelial-restricted deficiency of β1-integrins and of the α5-integrin subunit of the β1-integrins impairs in vivo angiogenesis [4, 31, 49, 50, 52]. However, the effects of increased integrin-dependent EC adhesion on angiogenesis are still elusive.

Integrins are regulated by distinct mechanisms: (a) the level of expression, (b) regulation of integrin affinity and integrin valency (avidity) and (c) integrin trafficking, which affects integrin localization in the cells and the assembly/disassembly of focal adhesions. Previous studies from our group provided insight in the regulation of integrin affinity in EC and their role in angiogenesis [6]. However, less is known about the mechanisms of integrin trafficking in EC and specifically their contribution to angiogenesis. Cell migration requires a tight spatiotemporal regulation of focal adhesion assembly and disassembly. Integrin trafficking (endocytosis/recycling) seems to be essential for cell migration by contributing to the turnover of focal and fibrillar adhesions during migration [8–10, 17, 53].

Arf6 is a small GTPase cycling between an inactive GDP- and active GTP-bound form involved in the regulation of actin cytoskeleton and receptor trafficking [14, 19, 35]. Several stimuli, such as integrin binding to fibronectin, as well as stimulation with growth factors, cytokines and guidance molecules may induce the activation of Arf6 in EC [18, 20, 27, 44]. Arf6 can be activated by several guanine exchange nucleotide factors (GEFs) [7]. Brag2 (GEP100/*IQSEC1*) is a GEF activating the small Arf-GTPases Arf4, Arf5 and Arf6 [33, 48] and is expressed in EC [20, 45]. Brag2 was found to promote the invasive activity of breast cancer cells [34] and myoblast fusion [11, 37]. Furthermore, Brag2 was shown to be involved in trafficking processes such as the endocytosis of synaptic AMPA-receptors, E-Cadherin recycling, and phagocytosis of monocytes [21, 46, 47]. Silencing of Brag2 in HeLa cells increased the surface expression of β1-integrins [15] and a later study indicated that Brag2 might contribute to β1-integrin endocytosis in HeLa cells [33]. However, the role of Brag2 in the regulation of integrins in EC was not studied thus far. In EC VEGF stimulation induces the association of VEGFR2 with Brag2 and induces the activation of Arf6 [20]. Interestingly, Semaphorin 3E-induced over-activation of the Brag2/Arf6 pathway results in collapse of EC and angiogenesis inhibition [45]. On the other side, it was demonstrated that silencing of Brag2 reduces endothelial tube formation in vitro and angiogenesis in a model of Angioreactor-induced angiogenesis [20]. Nevertheless, the role of Brag2 for in vivo physiological developmental and pathological angiogenesis and the underlying molecular mechanisms remain elusive.

In our present work, we provide direct evidence that Brag2 plays a key role in the regulation of integrin-dependent angiogenic functions such as sprouting, migration and adhesion of EC in vitro. Specifically, we show that Brag2 differentially affects β1- and αVβ3-integrin function and promotes angiogenic sprouting by contributing to the endocytosis of β1-integrins, thereby Arf5- and Arf6-dependently regulating focal/fibrillar adhesions and reducing adhesion in EC. In accordance with these results, silencing of Brag2 impaired developmental angiogenesis and vascular patterning in zebrafish. Moreover, in vivo knockdown of Brag2 significantly reduced pathological angiogenesis in mice. Taking together, our work for the first time links a mechanism of integrin endocytosis with the process of angiogenesis.

Materials and methods

Cells

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Basel, Switzerland) and cultured in endothelial basal medium (EBM) supplemented with 1 μg/mL hydrocortisone, 12 μg/mL bovine brain extract, 50 μg/mL gentamicin, 50 ng/mL amphotericin-B, 10 ng/mL epidermal growth factor, and 10 % fetal calf serum until the third passage. Cos-7 cells were cultured in DMEM containing 10 % fetal calf serum.

RNAi transfections

To silence Brag2, α5-integrin, Arf5, Arf6 we performed transfection of small-interfering RNA (siRNA) duplex

using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. Brag2 siRNAs were synthesized by Eurogentec and Sigma-Aldrich. The Brag2 target sequences were 5′-AGAACUCGGUGACGUACAG-3′ (Brag2 siRNA-1), and 5′-GGAUGCAGUUCUCCUUUGA-3′ (Brag2 siRNA-2). A nonrelated scrambled siRNA was used as a control. The target sequence targeting α5-integrin is 5′-AATCCTTAATGGCTCAGACAT-3′ (Qiagen). For silencing human Arf5 and Arf6, we used ON-TARGETplus SMARTPool siRNAs and the respective controls from Dharmacon.

Plasmid constructs and transfection

COS-7 cells (1.5×10^5 cells per 3.5 cm-well) were grown to 60–70 % confluence and then transfected with 4 μg plasmids using Superfect transfection reagent (QIAGEN) according to the manufacturer's protocol. The Brag2a-GFP (GEP100-GFP) plasmid was kindly provided by Dr. J.E. Casanova (Department of Cell Biology, University of Virginia Health System, Charlottesville, VA, USA). The Brag2a-ΔSec-GFP, Brag2a-ΔPH-GFP plasmids were kindly provided by Dr. M. Vaughan (Pulmonary-Critical Care Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, USA) and by Dr. A. Someya (Juntendo University, School of Medicine, Tokyo). The Arf5-GFP and Arf6-GFP were purchased by Addgene. Site-directed mutagenesis was performed to generate the rapid cycling Arf5-T161A-GFP and Arf6-T1571A-GFP mutants with the QuikChange II XL site-directed mutagenesis kit (Stratagene/Agilent, Waldbronn, Germany).

Spheroid-based angiogenic sprouting assay

Endothelial-cell spheroids were generated as described previously [6]. In brief, HUVEC were suspended in culture medium containing 0.2 % (w/v) carboxymethylcellulose (Sigma-Aldrich) 24 h after siRNA transfection and seeded in round-bottomed 96-well plates (Greiner) to form spheroids. The following day, spheroids were embedded into rat collagen I (BD Biosciences) containing gels. Angiogenic sprouting was stimulated where indicated by adding human vascular endothelial growth factor (VEGF, 50 ng/mL; PeproTech, Hamburg, Germany) containing medium after gel polymerization. In the experiments with the neutralizing integrin antibodies endothelial-cell spheroid embedded in the collagen gels were incubated with control antibody (BD Biosciences), anti-αVβ3-integrin antibody (Clone LM609) or anti-β1-integrin antibody (clone: mAb13, BD Biosciences) in the presence of VEGF. After 24 h, images were acquired using an Axiocam MR digital camera with an Axiovert 100 M inverted microscope using as objective a Plan-NEOFLUAR (at 10×/0.30) and were processed using AxioVision Rel 4.5 digital imaging software (all from Carl Zeiss, Jena, Germany). In vitro capillary sprouting was quantified by measuring the cumulative sprout length of each spheroid using a computer-assisted microscope [AxioVision 4.5 software (Carl Zeiss)] and the mean cumulative sprout length of 10 spheroids/condition was calculated.

Cell–matrix adhesion

Ninety-six-well plates were coated overnight with 1 μg/mL soluble recombinant human collagen I (Millipore) or 2.5 μg/mL human fibronectin (Sigma), 2.5 μg/mL human vitronectin (Millipore), or 2.5 μg/mL bovine serum albumin (BSA) in PBS at 4 °C and then blocked for 1 h at room temperature with 3 % (w/v) heat-inactivated (2 h, 56 °C) bovine serum albumin (BSA; Sigma-Aldrich). HUVEC were stained (48 h after the siRNA transfection) with 2′7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF, AM; Molecular Probes) and after detachment with trypsin were resuspended in EBM containing 0.05 % BSA. Then, 50,000 cells/well were seeded and allowed to attach for 60 min at 37 °C. After removal of non-adhering cells by washing with warm EBM, adherent cells were quantified in triplicates with a fluorescence plate reader (Synergy HT; Bio-Tek Instruments, Bad Friedrichshall, Germany). Data are presented as mean specific adhesion % of input ± SEM.

Zebrafish lines, morpholinos and vessel quantification

Embryos of AB wt and the *tg(fli1:EGFP)* line were raised and staged as described [30]. Embryos were kept in E3 solution at 28.5 °C with or without 0.003 % 1-phenyl-2-thiourea (Sigma) to suppress pigmentation and staged according to somite number or hours post-fertilization [16]. Morpholinos were diluted in 0.1 M KCl and 1 nL of morpholino dilution was injected through the chorion of one-cell or two-cell stage embryos. To attenuate possible off-target effects, a p53 Mo was co-injected 1.5-fold to the other morpholinos used. The following splice-blocking morpholinos (Gene Tools) were used:

IQSEC1a-MO-A: CTCCACCTGTGAAAACAAGGATGAA (targeting intron 4/exon 5 boundary); 2.5 ng per embryo

IQSEC1a-MO-B: GCTGACTGAATGGAGAAACAATAAC (targeting intron 7/exon 8 boundary); 2 ng per embryo

IQSEC1b-MO-A: CTGGCTAAACAAAGAAACACACCAC (targeting intron4/exon 5 boundary); 2 ng per embryo

IQSEC1b-MO-B: AACCCCTTTCAAAACGCAAGCACAA (targeting intron5/exon6 boundary); 3 ng per embryo
control morpholino (control Mo): CCTCTTACCTCAG TTACAATTTATA

Quantification of vessel defects in *tg(fli1:EGFP)* embryos of intersomitic vessels (ISV) and the dorsal longitudinal anastomotic vessel (DLAV) was performed as recently described [16]. Parachordal vessel/parachordal lymphangioblasts (PL) were quantified as recently described [16]. In brief, embryos were divided in three groups according to the presence (2), partial (1) or complete absence (0) of the parachordal lymphangioblasts. For the quantification of CCV defects following criteria were used: 2—broad, all cells have intercellular contacts to each other and are homogenously regular distributed, not branched; 1—thin vessel, branched, not all cells have contacts to each other and as consequence there exist gaps between cells; 0—very thin or interrupted vessel, shortened vessel with brittle structure.

In the experiments of the Supplemental Figure S6 following conditions were compared in order to exclude off-target effects of double injections of morpholinos: (1) Control MO + p53 MO; (2) IQSEC1a-MO-B + p53 MO; (3) IQSEC1a-MO-B + Control MO; (4) IQSEC1b-MO-A + Control MO and (5) IQSEC1b-MO-B + Control MO.

Retinopathy of prematurity (ROP) model and model of laser-induced choroidal neovascularization

All animal experiments were approved by the Animal Care and Use Committee at the NEI/National Institutes of Health (NIH) and were performed according to the NIH guidelines and regulations. Briefly, 7-day-old (p7) C57BL/6J mice were exposed to 75 % oxygen in a chamber for 5 days with their nursing mothers. At p12, the mice were returned to room air. Brag2 shRNA vector (TRCN0000340707, Open Biosystems) or the control vector PLKO.1 (Sigma) was delivered to the eyes of anesthetized neonatal mice by intravitreal injections with the in vivo-jetPEI™ reagent (Polyplus Transfection) at p12. The eyes with severe vitreous hemorrhage or disturbed development after injection were excluded from assessment. Mice were euthanized and the eyes harvested at P17. Whole-mount retinae were stained with Isolectin GS-IB4 Alexa Fluor® 488 (Invitrogen). Image analysis was performed using a Carl Zeiss Imager Z1 and the image-processing software: AxioVision (Carl Zeiss), Photoshop CS (Adobe). Quantification of neovascularization was performed as previously described [13].

In the model of laser-induced choroidal neovascularization female mice (age 6 weeks) were used for experiments. Four laser spots were introduced (75 μm spot size, 75 ms, 90 mW power; OcuLight Infrared Laser System 810 nm, Iridex) in the area surrounding the optic disk in the eye. Mouse Brag2 shRNA (2 μg/eye, Open Biosystems, catalogue number TRCN0000340707) or PLKO1 empty plasmid was injected intravitreally 2 days before laser treatment together with the transfection reagent in vivo-jetPEI™ (Polyplus Transfection, New York, NY) according to the manufacturer's instructions. The CNV area was analyzed at 1 week after laser treatment using isolectin B4 (IB4, Invitrogen) staining. For eye tissue (choroid and retina) isolation, the anterior segment and the vitreous of the eyes were removed. The retina was dissected from the RPE-choroid eye cup. The dissected tissues were fixed for morphological analysis.

Statistical analysis

Continuous variables are expressed as mean ± SEM or where indicated mean ± SD. Comparisons between groups were analyzed by *t* test (2-sided) or analysis of variance (ANOVA) (post hoc test: Newman–Keuls) for experiments with more than 2 subgroups (GraphPad Prism version 5.0). In the zebrafish experiments the Kruskal–Wallis test was used. P values <0.05 were considered statistically significant.

Results

Role of Brag2 in the VEGF-induced activation of Arf6

Consistent with previous studies [20, 45] HUVEC and human microvascular endothelial cells express Brag2a and Brag2b protein as assessed by Western blot (data not shown). Moreover, we confirmed that VEGF stimulation rapidly increases the activity of the small GTPase Arf6 in EC (supplemental Figures S1A and S1B) as previously reported [18, 20, 27]. To address the role of Brag2 as guanine nucleotide exchange factor (GEF) in the VEGF-induced activation of Arf6, we employed siRNA targeting Brag2. Silencing of Brag2 with siRNAs effectively reduced the expression of Brag2 mRNA and protein (supplemental Figures S1C and S1D) while not significantly affecting the expression of other Arf6 GEFs such as Cytohesin-1, Cytohesin-2 and Cytohesin-3 (data not shown). Furthermore, silencing of Brag2 reduced basal and VEGF-induced activation of Arf6 (supplemental Figures S1A and S1B). Thus, Brag2 is required for Arf6 activation under basal conditions and upon VEGF stimulation. Likewise, Semaphorin 3E also activates Arf6 in a Brag2-dependent manner; however, the Semaphorin 3E-induced Arf6-activation is delayed and much more sustained ([44] and data not shown).

Role of Brag2 for angiogenic sprouting, tube formation and EC migration

We next explored the contribution of endogenous Brag2 in angiogenesis in vitro. Silencing of Brag2 with siRNA significantly reduced the capability of 3-dimensional EC spheroids of angiogenic sprouting in the presence or absence of VEGF (Fig. 1a, b) or bFGF (data not shown) in comparison to scrambled siRNA-transfected HUVEC. As a control for the specificity of this approach, a second Brag2-siRNA was generated and gave similar results (Fig. 1a, and supplemental Figures S1C and S1D). Moreover, transient overexpression of Brag2a-GFP rescued the inhibition of angiogenic sprouting exerted by a siRNA targeting the 3′UTR region of Brag2 (data not shown). In accordance with these data, silencing of Brag2 significantly impaired tube/network-forming activity of HUVEC in the matrigel assay (Fig. 1c, d). Furthermore, Brag2 silencing significantly impaired the VEGF-induced 3-dimensional invasion and the 2-dimensional migration of HUVEC on fibronectin in a scratch wound assay (Fig. 2a, b), while not significantly affecting HUVEC proliferation (Supplemental Figure S1E). Taken together, Brag2 is essential for in vitro angiogenesis and EC migration.

Brag2 differentially regulates β1- and αVβ3-integrins

The regulation of integrin-mediated cell adhesion on matrix proteins is essential for cell migration [42]. Therefore, we studied the role of Brag2 in the β1-integrin-

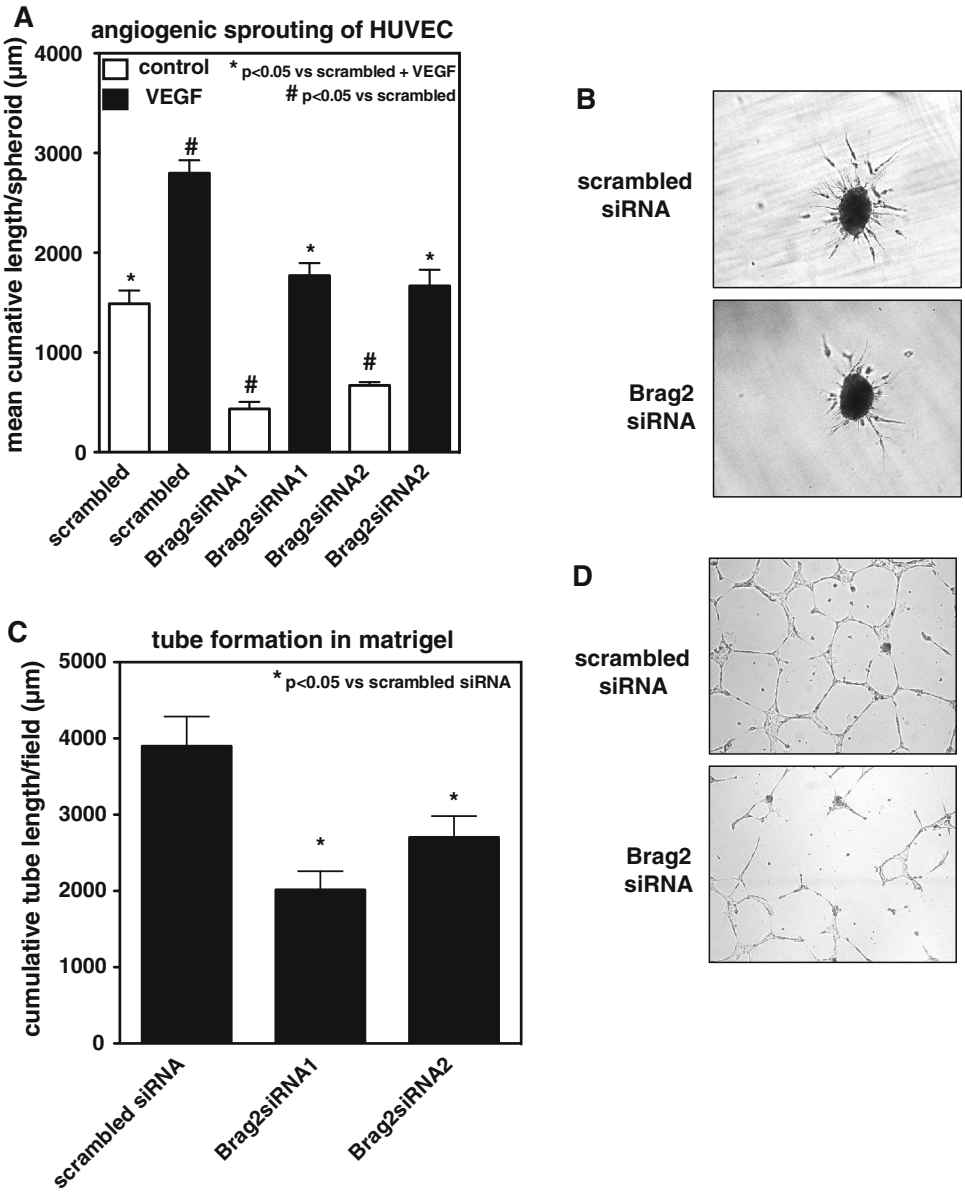


Fig. 1 Silencing of Brag2 with siRNA blocks in vitro angiogenesis. **a** HUVEC were transfected with siRNAs targeted against Brag2 or scrambled siRNA. Two different sequences were used as indicated by Brag2siRNA1 and Brag2siRNA2. Three-dimensional in vitro angiogenic sprouting in a spheroidal culture system with collagen-embedded spheroids in the presence or absence (control) of VEGF 50 ng/mL. The mean cumulative length of sprouts per spheroid was assessed after 24 h ($^{\#}P < 0.05$ vs. scrambled siRNA, $^{*}P < 0.05$ vs. scrambled + VEGF, $n = 4$). **b** Representative figures of spheroidal sprouting assay performed with HUVEC transfected as indicated with Brag2-siRNA or scrambled siRNA in the presence of VEGF 50 ng/mL. **c** HUVECs were seeded on Matrigel Basement Membrane Matrix 48 h after transfection with scrambled siRNA, Brag2siRNA1 or Brag2siRNA2. The length of capillary-like structures/networks was measured in 5 different high-power fields by light microscopy after 24 h ($^{*}P < 0.05$ vs. scrambled siRNA, $n = 3$–6). **d** representative micrographs of the tube-forming activity of HUVEC after transfection with scrambled siRNA or Brag2 siRNA

dependent EC adhesion on ECM proteins such as fibronectin and collagen. Interestingly, silencing of Brag2 significantly enhanced the steady-state β1-integrin-dependent HUVEC adhesion on fibronectin and collagen and significantly increased spreading (cell area) on fibronectin in comparison to scrambled siRNA-transfected HUVEC (Fig. 2c–e). Remarkably, the effect on adhesion seems to be specific for the β1-integrins since silencing of Brag2

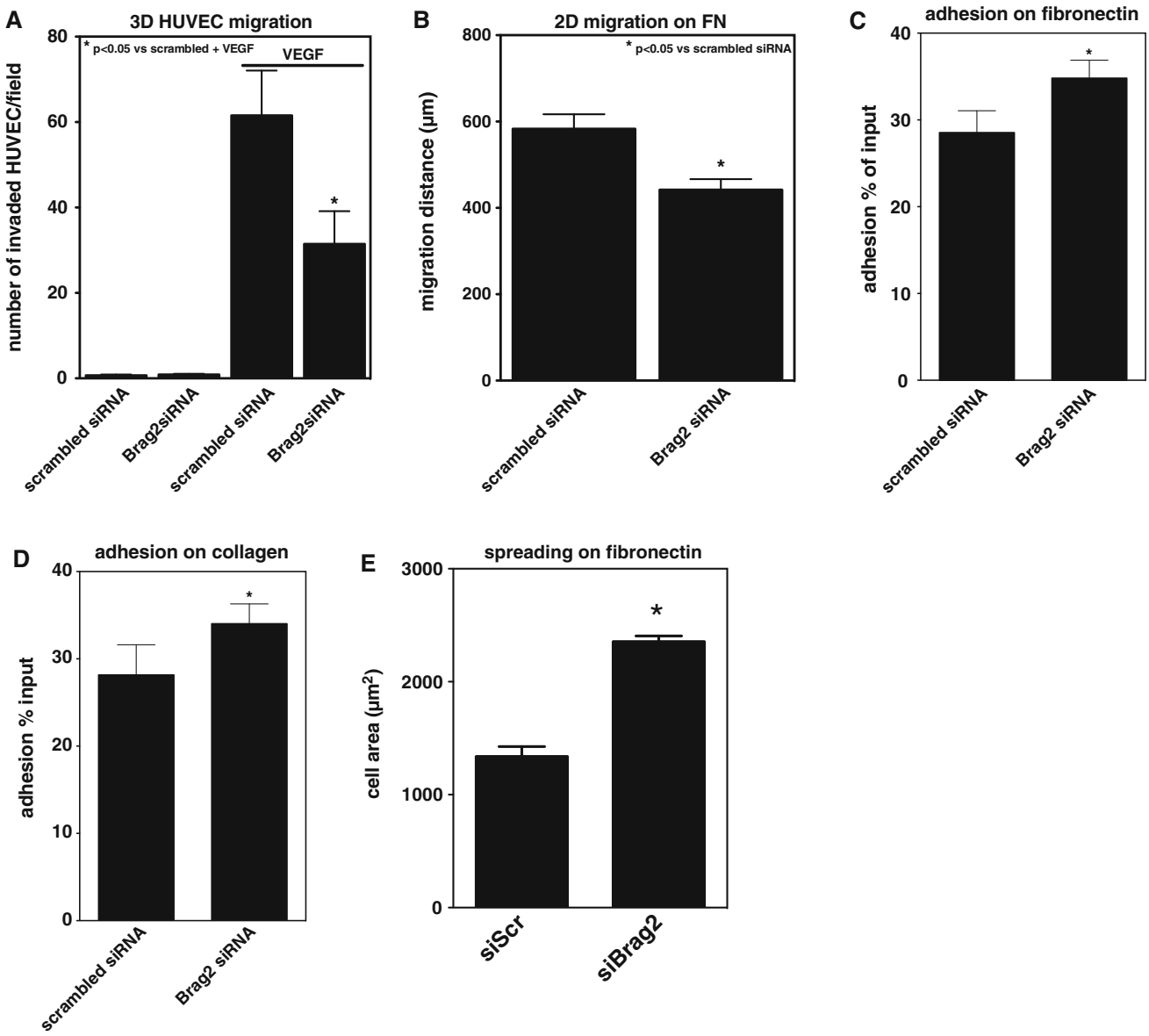
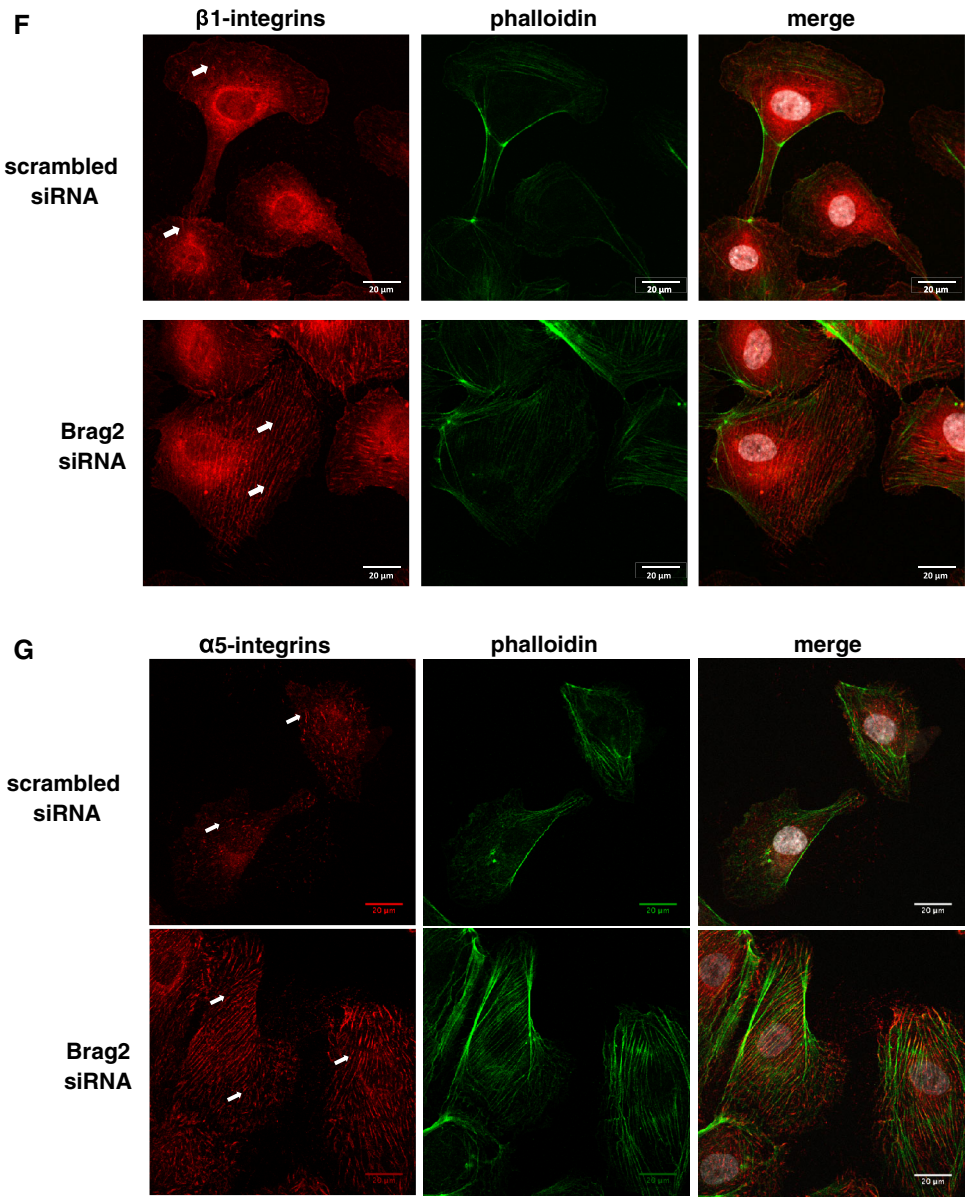


Fig. 2 Role of Brag2 for EC migration and β1-integrin-dependent adhesion and spreading. **a** 3-Dimensional migration (invasion) assay on Matrigel Basement Membrane Matrix with HUVEC transfected with siRNAs targeted against Brag2 or scrambled siRNA. After 48 h, cells were seeded in the upper chamber of modified Boyden chambers. HUVEC migration was assessed using VEGF (50 ng/mL) as chemoattractant (in the lower chamber). Data are presented as mean migrated cells/field ± SEM (*$P < 0.05$ vs. VEGF + scrambled siRNA, $n = 8$). **b** 2-Dimensional migration assay (wound healing/scratch wound assay) with HUVEC transfected with siRNAs targeted against Brag2 or scrambled siRNA. Data are presented as mean migration distance ± SEM (*$P < 0.05$ vs. scrambled siRNA, $n = 4$). **c**, **d** Adhesion assay with EC transfected with siRNAs targeted against Brag2 or scrambled siRNA on fibronectin (**c**) or collagen (**d**). After 48 h, cells were allowed to adhere for 1 h on coated wells ($n = 5$). Data are presented as mean percentage of adhering cells % input ± SEM (*$P < 0.05$ vs. scrambled siRNA). **e** Spreading assay of HUVEC on fibronectin 48 h after transfection with scrambled or Brag2 siRNA. Data are presented as mean cell area ± SEM (*$P < 0.05$ vs. scrambled siRNA, $n = 3$). **f**, **g** Immunofluorescence staining for β1-integrin (**f**) or active α5-integrin subunit (clone SNAKA51) (**g**) in HUVEC adhering on fibronectin, 48 h after transfection with scrambled siRNA or Brag2 siRNA. Microphotographs demonstrating focal and fibrillar adhesions were acquired at the basal surface of the cells, where focal/fibrillar adhesions are localized. Red fluorescence indicates the active α5-integrin or β1-integrin subunit, green fluorescence indicates phalloidin staining for F-actin and white color indicates nuclei. The *white arrows* indicate focal and fibrillar adhesions

Fig. 2 continued



significantly reduced HUVEC adhesion on the β3-integrin ligand, vitronectin (supplemental Figure S1F). In accordance with these results, silencing of Brag2 increased β1-integrin- and active α5-integrin-containing focal and fibrillar adhesions and enhanced the formation of actin stress fibers in HUVEC adhering on fibronectin, suggesting that Brag2 is a negative regulator of β1-integrin-containing focal/fibrillar adhesions (Fig. 2f, g). Quantification of the focal/fibrillar adhesion area by performing immunofluorescent staining with an antibody recognizing activated/matrix-bound α5β1-integrins localized in fibrillar and partly in focal adhesions (clone SNAKA51) [12] confirmed these results (Fig. 2g and Supplemental Figure S1G). These data raised the hypothesis that Brag2 may contribute to the disassembly of β1-integrin-containing focal/fibrillar adhesions in EC.

Silencing of Brag2 increases α5β1-integrin- and reduces αVβ3-integrin surface expression on EC

To understand the differential function of Brag2 in EC adhesion, we determined the role of Brag2 for the cell surface expression of β1- and αVβ3-integrins. Indeed, silencing of Brag2 by siRNA increased the cell surface abundance of the α5β1-integrin on HUVEC as assessed by FACS (Fig. 3a), while not affecting the total α5-integrin and β1-integrin protein expression as assessed by Westernblot (Supplemental Figures S2A and S2B). In addition, silencing of Brag2 also slightly increased the expression of α3β1- and α6β1-integrin on the EC surface (data not shown). In contrast, knockdown of Brag2 significantly reduced the surface expression of the αVβ3-integrin

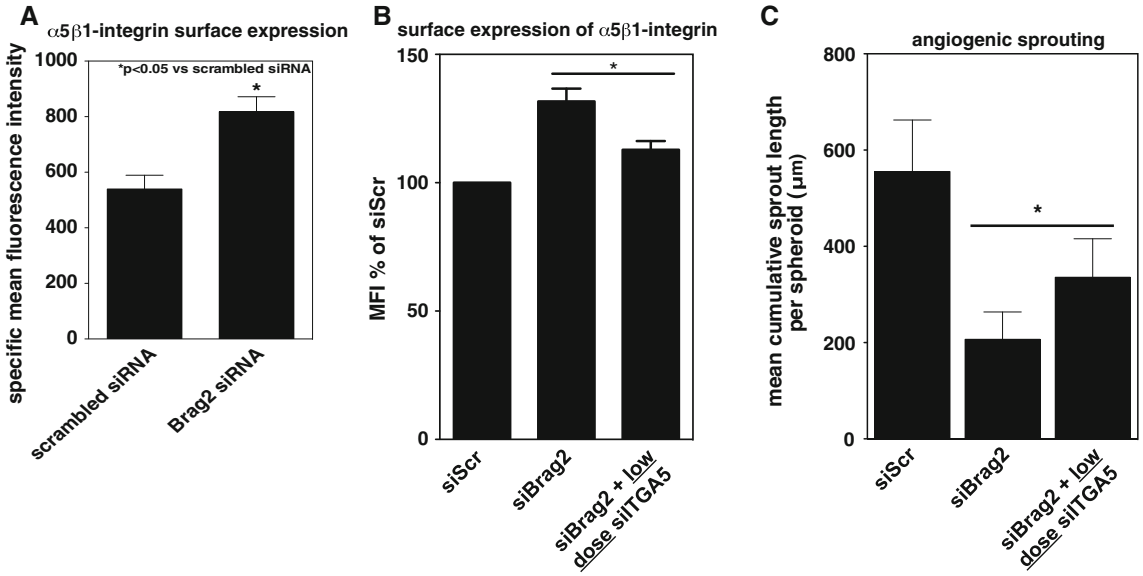


Fig. 3 Role of Brag2-mediated regulation of β1-integrin surface expression for angiogenic sprouting. **a** Steady-state surface expression of the α5β1-integrin on EC as assessed by FACS, 48 h after transfection with scrambled siRNA or Brag2 siRNA. Data are presented as specific mean fluorescence intensity ± SEM (*$P < 0.05$ vs. scrambled siRNA, $n = 5$). **b** HUVEC were transfected with scrambled siRNA (siScr), Brag2siRNA (siBrag2) or with the combination of Brag2siRNA and low dose (6.5 nM) of siRNA targeting α5-integrin (low-dose siITGA5). After 48 h, the steady-state surface expression of the α5β1-integrin on EC was assessed by FACS. Data are presented as specific mean fluorescence intensity % of siScr ± SEM (*$P < 0.05$ vs. siScr, $n = 8$). **c** Spheroidal sprouting assays using HUVEC transfected with scrambled siRNA (siScr), Brag2 siRNA (siBrag2) or Brag2 siRNA and low-dose ITGA5 siRNA (siBrag2 + low-dose siITGA5) were performed. The mean cumulative length of sprouts per spheroid was assessed after 24 h. Data are presented as mean cumulative length of sprouts per spheroid ± SEM (*$P < 0.05$, siBrag2 vs. siBrag2 + low-dose siITGA5, $n = 7$)

(Supplemental Figures S2C and S2D), which is in accordance with the reduction of EC adhesion to the αVβ3-integrin ligand, vitronectin (supplemental Figure S1F). Thus, silencing of Brag2 differentially affects β1- and β3-integrin expression on the EC surface and adhesion on fibronectin and vitronectin.

Brag2 promotes angiogenesis by regulating of α5β1-integrin expression on the EC surface

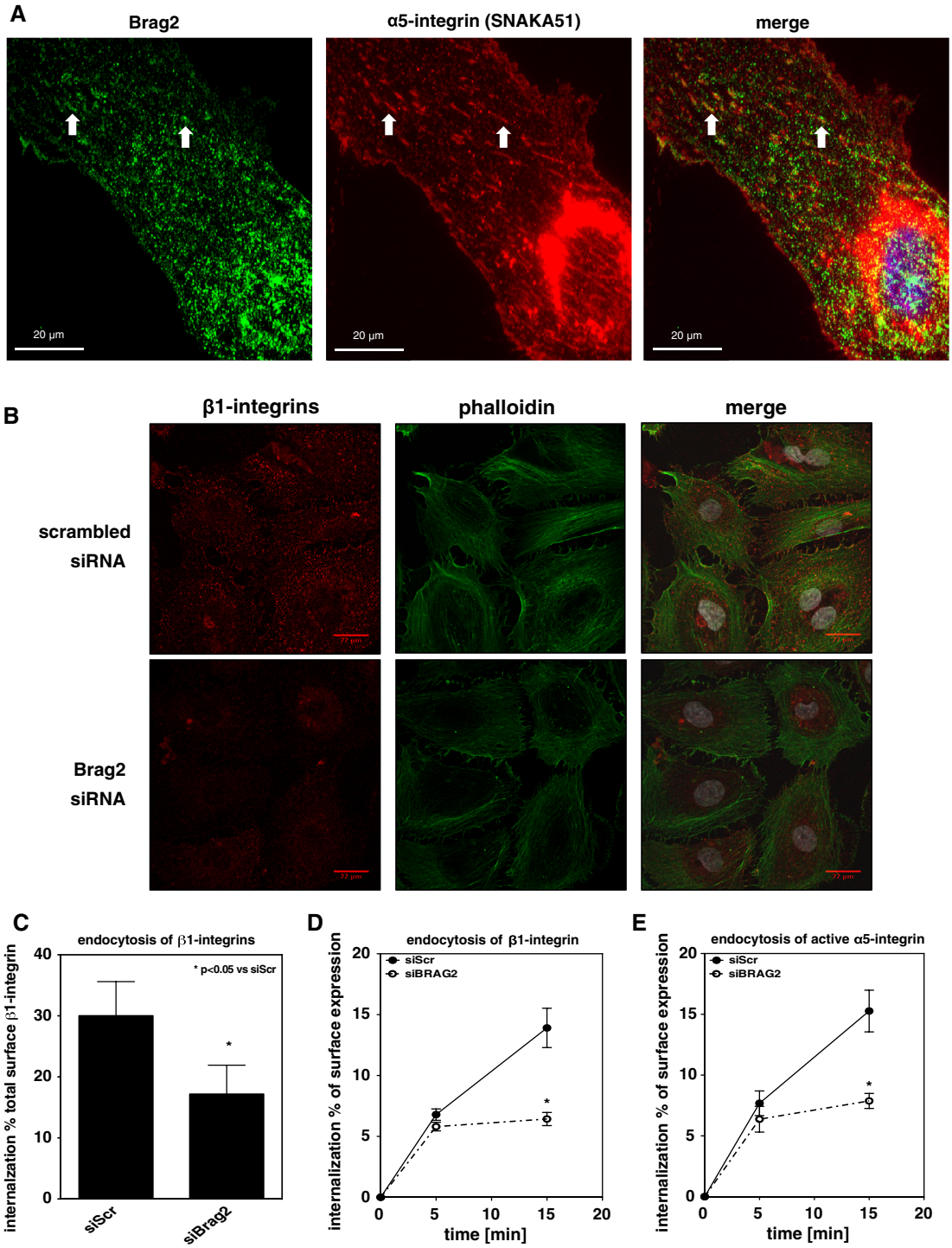
β1-integrin inhibition with neutralizing antibodies significantly blocked angiogenic sprouting of HUVEC, while αVβ3-integrin inhibition did not (Supplemental Figure S1H). In line with these results, genetic evidence supports that β1-integrins are essential for in vivo angiogenesis [3, 4, 41, 49, 52]. Since silencing of Brag2 enhanced β1-integrin-dependent adhesion and α5β1-integrin surface expression, we investigated the underlying mechanisms affecting β1-integrin function in EC. Specifically, we addressed the role of the enhanced surface expression of α5β1-integrins for the inhibitory effect of Brag2 silencing on angiogenic sprouting. For this purpose, we performed silencing of α5-integrin subunit of the α5β1-integrin using a low dose of siRNA in Brag2 siRNA-transfected HUVEC, in order to achieve a slight reduction of the α5-integrin on the cell surface reaching the levels observed in scrambled siRNA-transfected EC as assessed by FACS (Fig. 3b). Strikingly, a reduction of the α5β1-integrin surface abundance in the Brag2-siRNA-transfected HUVEC partly rescued the inhibitory effect of Brag2 silencing on angiogenic sprouting (Fig. 3b, c). Hence, Brag2 partly mediates

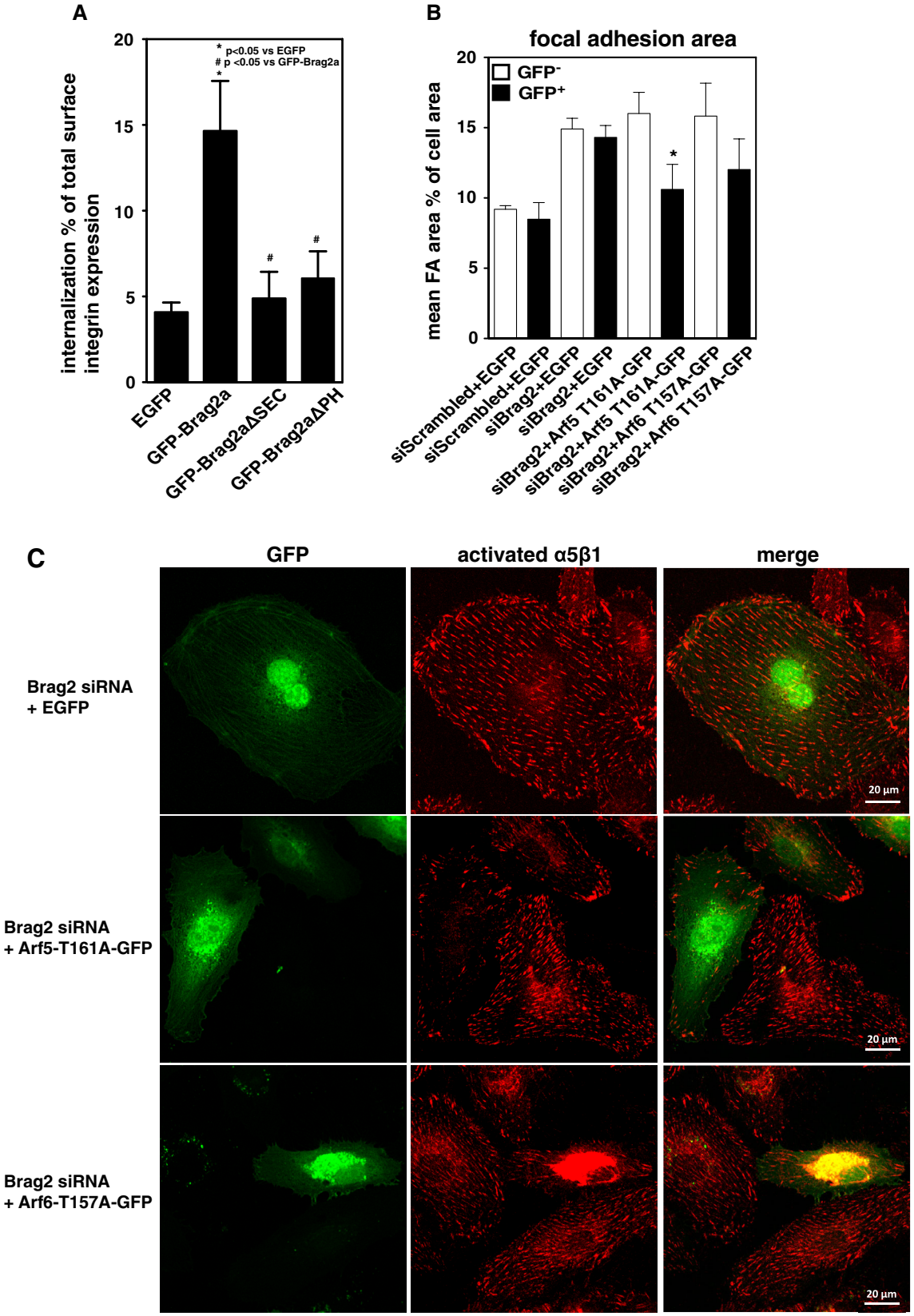
Fig. 4 Brag2 mediates β1-integrin internalization. **a** Immunofluorescent staining for Brag2 (green fluorescence) and activated/matrix-bound α5β1-integrin (clone SNAKA51) present in focal and fibrillar adhesions. The *blue color* indicates nuclear staining (DAPI). The *white arrow* indicates Brag2 co-localized or in the vicinity of focal/fibrillar focal adhesions. TIRF microscopy with a ×100 objective was performed, in order to assess the co-localization in the basal level of HUVEC. **b** Pulse-chased endocytosis assay based on immunofluorescence staining for β1-integrin subunit in HUVEC adhering on fibronectin, 48 h after transfection with scrambled siRNA or Brag2 siRNA. HUVEC were allowed to internalize β1-integrin for 10 min as described in the "Materials and methods". *Red fluorescence* indicates the internalized β1-integrin subunit, green fluorescence indicates phalloidin staining for F-actin and white color indicates nuclei. **c** Pulse-chased endocytosis assay of the β1-integrin subunit in HUVEC as determined with FACS as described in the "Materials and methods" section. Data are presented as mean internalized β1-integrin % of total surface β1-integrin ± SEM (*$P < 0.05$ vs. scrambled siRNA, $n = 6$). **d**, **e** Biochemical biotinylation-based internalization assay of β1-integrin (**d**) and of activated α5-integrin subunit (**e**) were performed for the indicated time-points (0, 5 and 15 min) as described in the "Materials and methods" section. Data are presented as mean internalized integrin % of total surface integrin ± SEM (*$P < 0.05$ vs. scrambled siRNA at 15 min of endocytosis, $n = 3$)

angiogenic sprouting by regulating the surface expression of the α5β1-integrin on the EC surface. Remarkably, silencing of the α5-integrin subunit employing a high dose of α5-integrin-siRNA leading to a profound reduction of the α5-integrin on the EC surface significantly blocked in vitro angiogenic sprouting (Supplemental Figure S2E) suggesting that a tight regulation of β1-integrin surface expression is essential for efficient angiogenic sprouting.

Brag2 promotes endocytosis of β1-integrins and recycling of αVβ3-integrins in EC

As assessed by TIRF microscopy, Brag2 partly co-localized with focal/fibrillar adhesions containing activated α5β1-integrins indicating a direct role of Brag2 in the regulation of focal/fibrillar adhesions (Fig. 4a). Mechanisms of endocytosis were shown to contribute to the disassembly of focal




A
internalization % of total surface integrin expression
* p<0.05 vs EGFP
# p <0.05 vs GFP-Brag2a
EGFP
GFP-Brag2a
GFP-Brag2aΔSEC
GFP-Brag2aΔPH
B
focal adhesion area
mean FA area % of cell area
GFP-
GFP+
siScrambled+EGFP
siBrag2+EGFP
siBrag2+Arf5 T161A-GFP
siBrag2+Arf6 T157A-GFP
C
GFP
activated α5β1
merge
Brag2 siRNA + EGFP
Brag2 siRNA + Arf5-T161A-GFP
Brag2 siRNA + Arf6-T157A-GFP
20 µm

◀**Fig. 5** Role of Arf GTPases for Brag2-mediated β1-integrin internalization, angiogenic sprouting and regulation of focal/fibrillar adhesions. **a** Pulse-chased endocytosis assay of the β1-integrin subunit in COS7 cells as determined with FACS. Briefly, Cos7 cells were transfected with wild-type Brag2a-GFP, Brag2a-ΔSec-GFP, Brag2a-ΔPH-GFP or EGFP empty vector. After 24 h, a pulse-chased endocytosis assay of the β1-integrin subunit was performed for 10 min and the MFI of internalized β1-integrin was determined in GFP^+ COS7 cells by FACS. Data are presented as mean internalized β1-integrin % of total surface β1-integrin ± SEM (*$P < 0.05$ vs. EGFP; #$P < 0.05$ vs. GFP-Brag2a, $n = 7$). **b** HUVEC were transfected with scrambled or Brag2 siRNA. After 24 h, cells were transiently transfected where indicated with EGFP empty vector, rapid cycling Arf5-T161A-GFP mutant, or a rapid cycling Arf6-T157A-GFP mutant. After 24 h, immunofluorescent staining was performed with an antibody recognizing activated/matrix-bound α5β1-integrin (clone SNAKA51) present in focal and fibrillar adhesions. The focal adhesion area in GFP^+ and GFP^- cells of each condition was quantified by confocal microscopy. Data are presented as focal adhesion area % total cell area ± SEM (*$P < 0.05$ vs. siBrag2 + EGFP-GFP^+ and siBrag2 + Arf5-T161A-EGFP-GFP^-; $n = 3$–4). **c** Representative images for Fig. 5b. Microphotographs demonstrating focal and fibrillar adhesions were acquired at the basal surface of the cells, where focal/fibrillar adhesions are localized. *Red fluorescence* indicates the active/matrix-bound α5-integrin, green fluorescence indicates GFP^+ cells (for each condition). **d** Overexpression in HUVEC of wild-type myc-Brag2 or pcDNA3.1 empty vector in combination with siRNAs targeted against Arf5, Arf6 or scrambled control. Angiogenic sprouting of endothelial-cell spheroids was determined. Data are presented as mean % pcDNA3.1 + scrambled siRNA ± SEM (*$P < 0.05$ vs. myc-Brag2 + scrambled siRNA; $n = 6$–7)

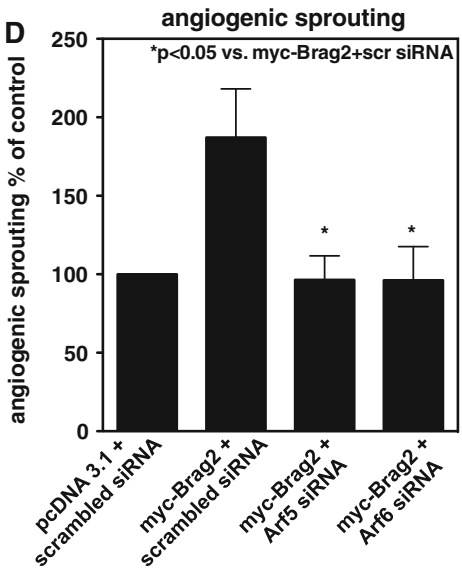


Fig. 5 continued

adhesions during cell migration [8–10, 17]. Therefore, we hypothesized that Brag2 may mediate endocytosis/internalization of β1-integrins in EC. Silencing of Brag2 with siRNA significantly reduced the internalization of the β1-integrin as assessed by a pulse-chased immunofluorescence-based endocytosis assay (Fig. 4b). To quantify the internalization of β1-integrins, we additionally employed a FACS-based endocytosis assay (Fig. 4c). Indeed, silencing of Brag2 with siRNA significantly reduced β1-integrin internalization (Fig. 4c). Since binding of antibodies to surface receptors and cell detachment by trypsin may affect internalization rates of surface receptors, we also employed a biochemical internalization assay based on biotinylation of surface proteins [43, 53]. Indeed, silencing of Brag2 significantly reduced the internalization of the β1-integrin subunit in comparison to scrambled siRNA-transfected HUVEC (Fig. 4d). Since silencing of Brag2 increased focal/fibrillar adhesions in HUVEC adhering to the α5β1-integrin ligand fibronectin (Fig. 2f, g) and since focal and fibrillar adhesions contain activated (matrix-bound) integrins [2], we also specifically explored the role of Brag2 for the internalization of activated/matrix-bound α5β1-integrin by using an antibody, which recognizes activated α5β1-integrins localized in fibrillar and partly in focal adhesions (clone SNAKA51) [12]. Silencing of Brag2 significantly reduced the internalization of activated α5β1-integrins (Fig. 4e). Interestingly, silencing of Brag2 reduced the internalization of activated α5β1-integrins even in the presence of the recycling inhibitor primaquine, suggesting that Brag2 is mediating internalization without affecting recycling of activated α5β1-integrin (data not shown). In summary, our data indicate that Brag2 may promote EC migration and angiogenesis by mediating β1-integrin endocytosis thereby contributing to the turnover of focal/fibrillar adhesions and regulating β1-integrin-dependent EC adhesion. Interestingly, silencing of Brag2 did not significantly affect endocytosis of αVβ3-integrins (data not shown), but significantly reduced their recycling (Supplemental Figure S2F). Taking together, Brag2 mediates endocytosis of β1-integrins and recycling of αVβ3-integrins.

Brag2 promotes endocytosis of β1-integrins in EC and angiogenic sprouting in an Arf-GTPase-dependent manner

To gain insight in the molecular mechanisms involved in Brag2-mediated β1-integrin endocytosis, we studied the domains of Brag2 required in this process. Therefore, we overexpressed following constructs in COS7 cells: WT-Brag2a-GFP, Brag2a-ΔSec-GFP lacking the catalytic domain Sec7, which is essential for the activation of GTPases of the Arf family, Brag2a-ΔPH-GFP lacking the pleckstrin homology domain, which is required for the activation of the enzymatic activity of Brag2 by binding PI(4,5)P2 on membranes [26, 45], or EGFP control vector and assessed β1-integrin endocytosis in a pulse-chased endocytosis assay by FACS in GFP^+ cells. Overexpression of WT-Brag2a-GFP increased the internalization of β1-integrin in comparison to EGFP-transfected COS7 cells (Fig. 5a). Overexpression of Brag2a-ΔSec7-GFP or

Brag2a-ΔPH-GFP did not promote the endocytosis of β1-integrins (Fig. 5a), indicating that Brag2 is mediating endocytosis of β1-integrins by activation of Arf-GTPases. Consistent with these results, overexpression of the rapid cycling mutant Arf5-T161A-GFP significantly reduced the increased SNAKA51-positive focal/fibrillar adhesions observed upon silencing of Brag2 (Fig. 5b, c). Moreover, also the rapid cycling mutant Arf6-T157A-GFP reduced, even if not significantly, focal/fibrillar adhesions induced by Brag2-silencing (Fig. 5b, c). To explore the role of Arf5 and Arf6 as possible downstream effectors of Brag2 in angiogenesis, we overexpressed myc-Brag2a in combination with scrambled siRNA, Arf5 siRNA or Arf6 siRNA in HUVEC and assessed angiogenic sprouting. Whereas overexpression of Brag2a enhanced angiogenic sprouting in scrambled siRNA-transfected cells, silencing of Arf5 or Arf6 blocked Brag2a-induced sprouting (Fig. 5d and Supplemental Figures S2G, S2H and S2I). Taken together, Arf5 and Arf6 are acting downstream of Brag2 in EC thereby promoting β1-integrin endocytosis, regulating focal adhesions and promoting angiogenic sprouting.

Role of Brag2 for developmental angiogenesis in zebrafish

Having demonstrated that Brag2 regulates in vitro angiogenesis, EC migration and matrix adhesion, we next explored whether Brag2 contributes to endothelial sprouting and vessel formation in vivo. For this purpose, we studied the effect of Brag2/*IQSEC1* silencing during embryonic development of zebrafish in the transgenic *tg(fli1:EGFP)* zebrafish line expressing EGFP in vessels under control of the fli-1 promoter. We identified 2 orthologue *IQSEC1* genes (Brag2/GEP100) in zebrafish using the ENSEMBL database: ENSDARG00000016551 (*IQSEC1a*) and ENSDARG00000073822 (*IQSEC1b*). RT-PCR of FACS-sorted EGFP-positive EC from the developing *tg(fli1:EGFP)* zebrafish at 24hpf [51] revealed that *IQSEC1a* and *IQSEC1b* mRNA are present in EC in zebrafish yielding a band of 367 and 403 bp, respectively (Supplemental Figure S3). To determine the role of these genes in developmental angiogenesis, we designed specific splice-blocking morpholinos targeting these genes. Knockdown of *IQSEC1a* or *IQSEC1b* or both was achieved by injection of splice-blocking morpholinos resulting in an aberrant splicing of the *IQSEC1a* and *IQSEC1b* mRNA, respectively (Supplemental Figure S4A and S4B).

Silencing of *IQSEC1a* using the splice-blocking *IQSEC1a*-MO-A morpholino led to vascular defects in 48hpf old *tg(fli1:EGFP)* zebrafish embryos. Specifically, silencing of *IQSEC1a* significantly induced defects of the dorsal longitudinal anastomotic vessel (DLAV) and intersomitic vessels (ISV) (Fig. 6a, c, d) and significantly reduced the formation of the parachordal lymphangioblasts (PL), the precursor to the lymphatic system at 48hpf and 72hpf (Fig. 6e, Supplemental figure S6C and S6D). For a control of the specificity of the *IQSEC1a*-MO-A morpholino, we designed a second splice-blocking morpholino (*IQSEC1a*-MO-B) silencing *IQSEC1a* expression, which led to similar results in the vasculature in *tg(fli1:EGFP)* embryos (Fig. 6a, c–e and Supplemental Figure S6A, S6B, S6C and S6D). Silencing of *IQSEC1b* with two distinct splice-blocking morpholinos (*IQSEC1b*-MO-A and *IQSEC1b*-MO-B) led to a milder vascular phenotype. Specifically, silencing of *IQSEC1b* only significantly impaired formation of the parachordal vessel/parachordal lymphangioblast (PL) at 48hpf and 72hpf (Fig. 6e, Supplemental Figures S6C and S6D) but did not significantly affect the formation of ISV and DLAV (Fig. 6a, Supplemental figures S6A and S6B). Strikingly, silencing of both *IQSEC1a* and *IQSEC1b* led to a more severe vascular phenotype strongly affecting vascular patterning in *tg(fli1:EGFP)* zebrafish embryos (Fig. 6b). Indeed, silencing of *IQSEC1a* and *IQSEC1b* genes induced more severe defects in the ISV and in the DLAV (Fig. 6b–d) and led to a highly penetrant absence of the PL (Fig. 6e). In addition, silencing of both genes, *IQSEC1a* and *IQSEC1b,* led to defects in the formation of the common cardinal vein (CCV) in *tg(fli1:EGFP)* zebrafish embryos at 48hpf (Fig. 6f, g). Remarkably, silencing of both genes, *IQSEC1a* and *IQSEC1b* also led to vascular edema in the tail and to formation of hydrocephalus (Supplemental Figure S5A and Figure S5B). Taken together, the Brag2 orthologue genes *IQSEC1a* and *IQSEC1b* play an essential synergistic role in the developmental angiogenesis of the zebrafish and in vessel stability and affect vascular patterning.

Inhibition of Brag2 in vivo reduces pathological angiogenesis in mice

Next, we attempted to address whether Brag2 is also involved in pathological angiogenesis. The ROP model mimics the ischemia-induced angiogenesis observed in retinopathy of prematurity. Intravitreal injection of Brag2 shRNA reduced the in vivo expression of murine Brag2 by 73.5 % as assessed by qPCR (Supplemental Figure S7). Intravitreal injection of Brag2 shRNA significantly inhibited retinal neovascularization in comparison to empty vector (Fig. 7a, b). In addition, laser-induced choroidal neovascularization is a disease model of the wet form of age-related macular degeneration. In this model, a laser is used to disrupt the Bruch's membrane leading to the invasion of the choroidal vasculature into the subretinal pigment epithelium. Injection of Brag2 shRNA led to a significant reduction of choroidal neovascularization in comparison to mice treated with the control vector (Fig. 7c,

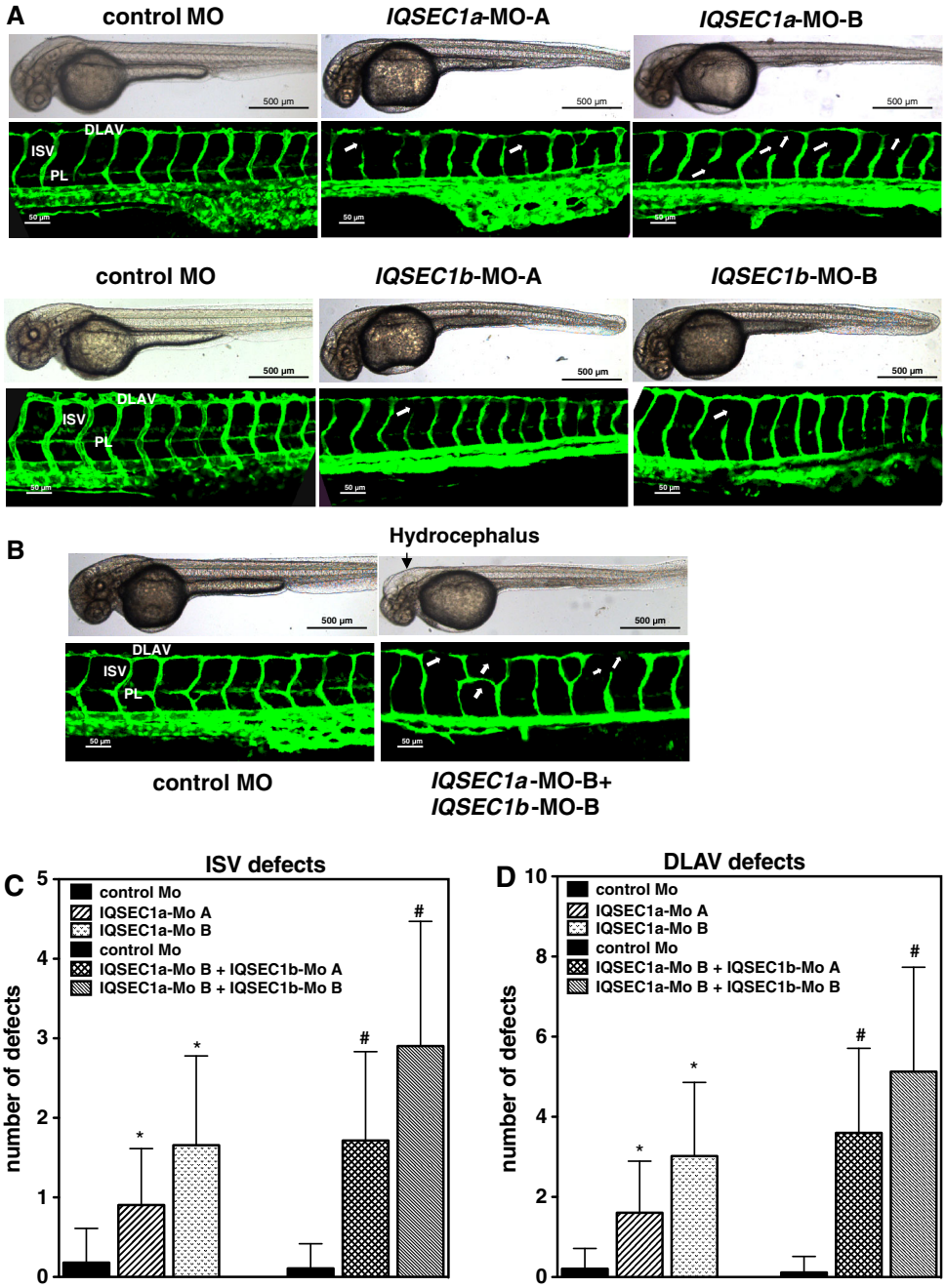


Fig. 6 Brag2 (*IQSEC1a and IQSEC1b*) regulates developmental angiogenesis in *tg(fli1:EGFP)* zebrafish embryos. **a** Representative bright field and confocal fluorescence images of vessels in the posterior part of *tg(fli1:EGFP)* zebrafish embryos after injection of indicated splice-blocking morpholinos or control morpholino at 48hpf. *Arrows* indicate vessel defects. **b** Representative bright field and confocal fluorescence images of vessels in the posterior part of *tg(fli1:EGFP)* zebrafish embryos after injection of *IQSEC1a*–MO-B and *IQSEC1b*–MO-B morpholinos or control morpholino at 48hpf. *Arrows* indicate vessel defects. **c**, **d** Quantification of defects in ISVs (**c**) and DLAVs (**d**) in IQSEC1a, or IQSEC1a + IQSEC1b and control morphants. The data are presented as the number of defects per zebrafish ± SD (*$P < 0.05$ vs. respective control Mo, $n = 73$–111; $^{\#}P < 0.05$ vs. respective control Mo, $n = 93$–112). **e** Quantification of parachordal lymphangioblasts (PL) for IQSEC1a, IQSEC1b, IQSEC1a + IQSEC1b and control morphants at 48hpf and 72hpf. The data are presented as the number of PL ± SD (*$P < 0.05$ vs. respective control morpholino, 48 hpf, $^{\#}P < 0.05$ vs. respective control morpholino, 72hpf; $n = 63$–135). **f** Representative confocal fluorescence images of the common cardinal vein (CCV) in control morphants (*left*) and in IQSEC1a-MO-B + IQSEC1b-MO-A morphants (*right*) at 48hpf. **g** Quantification of CCV defects in IQSEC1a + IQSEC1b morphants and control morphants at 48hpf. The data are presented as mean ± SD (*$P < 0.05$ vs. control morpholino, 48hpf; $n = 35$–43)

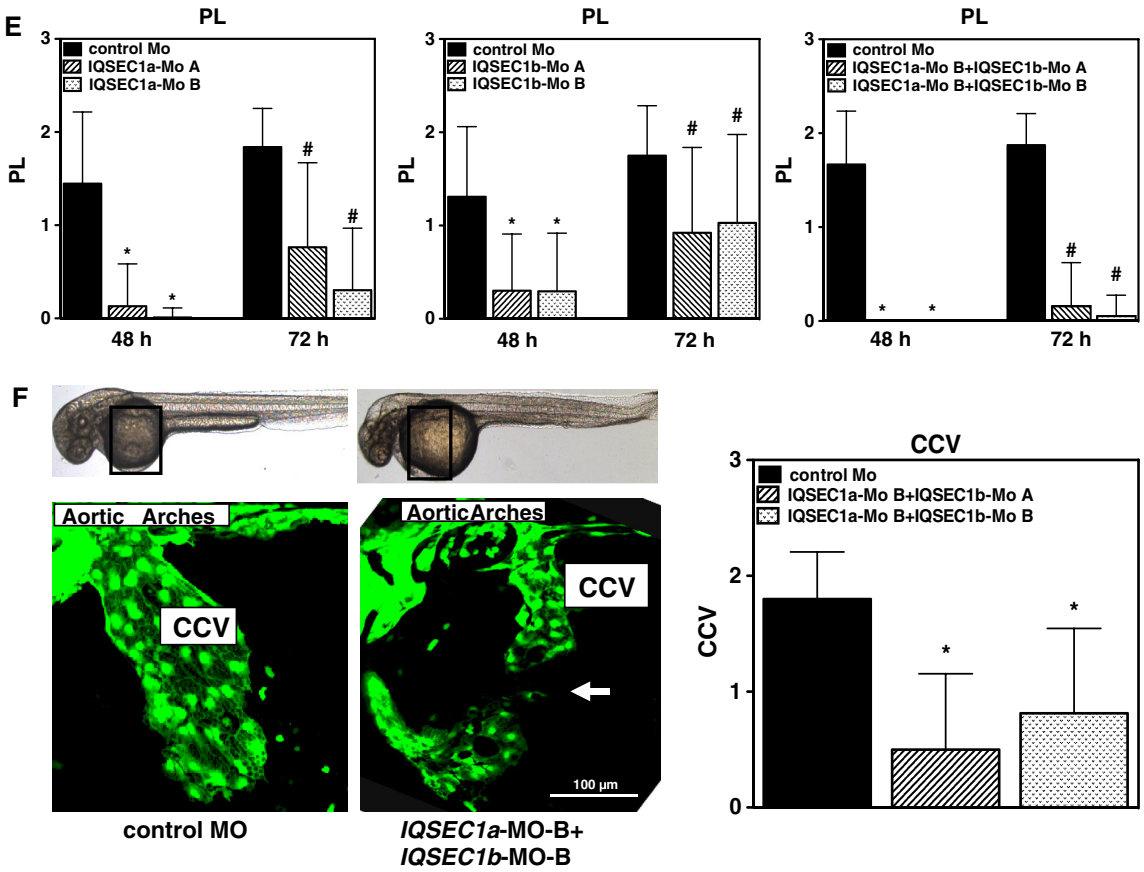


Fig. 6 continued

d). Taking together, Brag2 is also involved in pathological angiogenesis.

Discussion

The present study underscores the relevance of Brag2 for regulation of integrin function in EC and in angiogenesis. Specifically, our findings revealed that: (1) endothelial Brag2 mediates migration and in vitro angiogenic sprouting in an Arf-GTPase-dependent manner partly by regulating the surface expression of α5β1-integrins; (2) Brag2 differentially regulates α5β1- and αVβ3-integrins in EC. Specifically, Brag2 promotes the endocytosis of active/matrix-bound α5β1-integrins, thereby regulating α5β1-integrin abundance on the cell surface, contributing to the turnover of focal adhesions, and reducing β1-integrin-dependent adhesion. In contrast, Brag2 promotes the recycling of αVβ3-integrins, thereby increasing αVβ3-integrin cell surface abundance and promoting EC adhesion on the αVβ3-integrin ligand vitronectin; (3) Brag2 genes (*IQSEC1a* and *IQSEC1b*) are essential for developmental angiogenesis in zebrafish, thereby contributing to vascular patterning and finally; (4) inhibition of Brag2 in vivo reduces pathological retinal angiogenesis and choroidal neovascularization. Thus, the present study provides insights into the mechanisms of integrin endocytosis in EC and their contribution to the process of angiogenesis and unravels a novel function of Brag2 as key mediator of physiological and pathological neovascularization. Moreover, inhibition of Brag2 seems to be a promising therapeutic target for treatment of pathological angiogenesis.

Adhesion to fibronectin and VEGF was shown to stimulate activation of the Arf6 GTPase [18, 20, 27]. Our present work shows that Brag2 is required for basal and VEGF-induced Arf6 activity in EC. These data are consistent with the data of Hashimoto et al. [20] demonstrating that VEGF stimulation leads to the association of VEGFR2 to Brag2 thereby mediating Arf6 activation. Interestingly, a pharmacological inhibitor of the Arf6-GEFs cytohesins 1–3, SecinH3, reduced VEGF-induced activation of Arf6 in EC [27], suggesting that not only Brag2, but also Arf6-GEFs of the cytohesin family may contribute to the VEGF-induced stimulation of Arf6 activity.

Remarkably, silencing of Brag2 blocked angiogenic sprouting and tube-forming activity of EC. In line with these results, knock down of the orthologue genes of Brag2 (*IQSEC1a* and/or *IQSEC1b*) in zebrafish perturbed

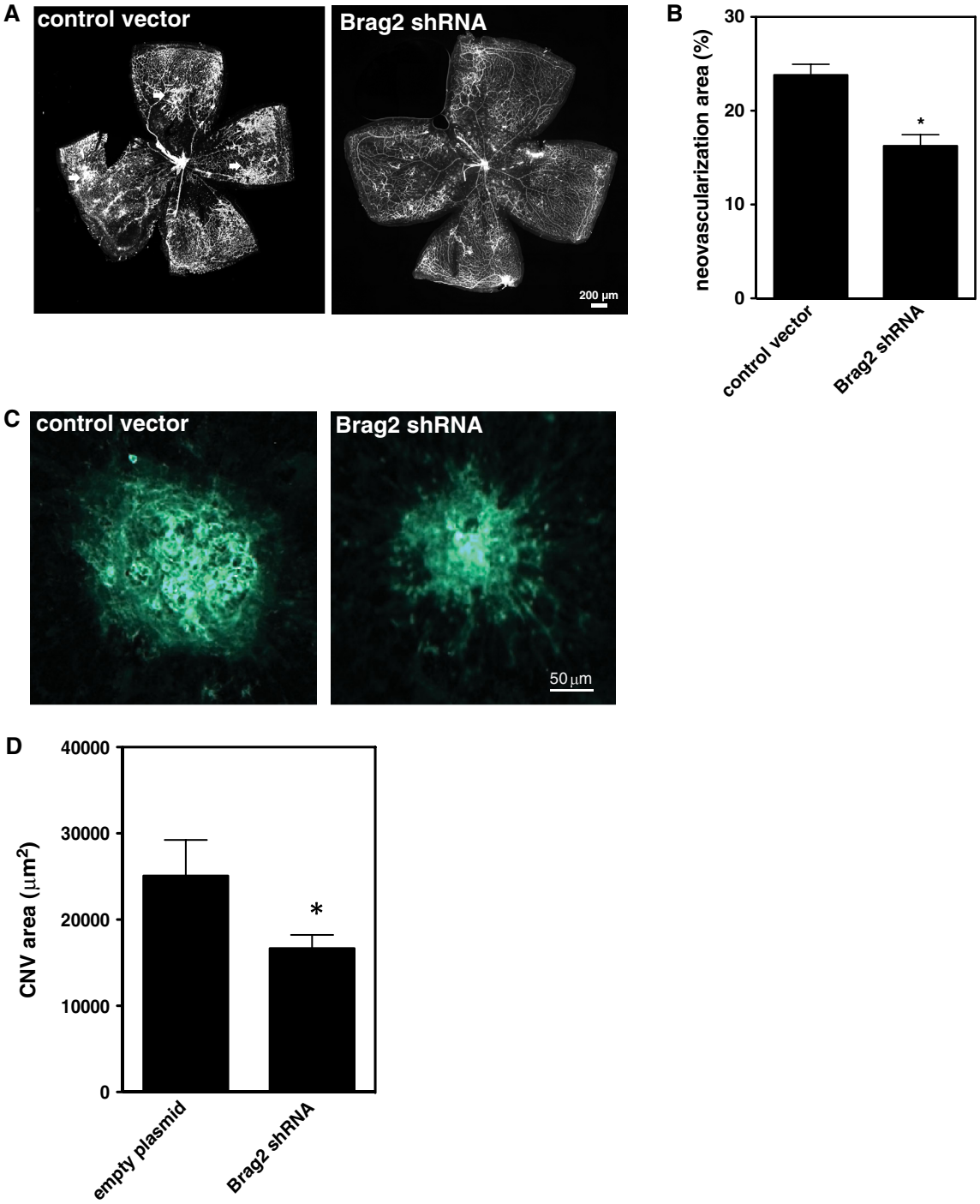


Fig. 7 Silencing of Brag2 suppresses pathological angiogenesis in the ROP model and choroidal neovascularization**. a** Representative images of whole-mount retinae stained with IB4 after intravitreal injection of vector containing Brag2 shRNA or control empty vector in the ROP model. *White arrows* indicate neovascular tufts. **b** Quantification of the neovascularization area after intravitreal injection of a Brag2 shRNA or control plasmid in the ROP model. Data are presented as mean neovascularization area % total area ± SEM (*$P < 0.05$ vs. empty plasmid, $n = 8$). **c** Representative images of choroidal neovascularizations stained with IB4 after intravitreal injection of a plasmid containing Brag2 shRNA or an control plasmid (PLKO.1) in the choroidal neovascularization model. **d** Quantification of the neovascularization area after intravitreal injection of a plasmid containing Brag2 shRNA or control plasmid in the choroidal neovascularization model. Data are presented as neovascularization area (μm^2) ± SEM (*$P < 0.05$ vs. empty plasmid, $n = 16$–20)

developmental angiogenesis and vascular patterning. Interestingly, silencing of *IQSEC1a* and *IQSEC1b* also impaired the formation of the parachordal vessel/parachordal lymphangioblasts, which is considered as a precursor of the lymphatic system [55] and additionally led to vascular edema and hydrocephalus. Beyond developmental angiogenesis, in vivo silencing of Brag2 significantly reduced pathological angiogenesis in the disease model of retinopathy of prematurity and in the model of choroidal neovascularization. Consistent with our results, also in a model of Angioreactor-induced angiogenesis, silencing of Brag2 inhibited neovascularization [20]. Furthermore, the pharmacological inhibition of Arf6-GEFs of the cytohesin family with SecinH3 reduced angiogenesis [27]. Intriguingly, not only inhibition but also an over-activation of the Brag2/Arf6 pathway, as achieved by stimulation with Semaphorin 3E, blocks angiogenesis [44, 45]. In conclusion, a stringent and balanced activation of Arf-GTPases by Brag2 is mandatory for efficient angiogenesis.

A relevant question is how Brag2 promotes angiogenesis. EC migration is essential for angiogenesis. We found that Brag2 promotes 2- and 3-dimensional EC migration, while not affecting proliferation. Cell migration requires the dynamic establishment of new integrin-mediated focal adhesions by binding of integrins to ECM proteins in the migrating front, concomitant with the disassembly/turnover of mature focal/fibrillar adhesions allowing cell retraction and forward movement [8, 9]. Integrin trafficking is involved in the assembly/disassembly of focal/fibrillar adhesions. Indeed, focal/fibrillar adhesion disassembly includes the internalization/endocytosis of integrins [8–10, 17, 53]. Strikingly, we provide here direct evidence that Brag2 partly mediates angiogenesis by reducing α5β1-integrin surface expression. Specifically, we show that Brag2 promotes β1-integrin endocytosis in EC, thereby reducing focal/fibrillar adhesions and EC adhesion to β1-integrin ligands. In addition, silencing of Brag2 also slightly increased α3β1- and α6β1-integrin on the EC surface (data not shown). In line with our results, Dunphy et al. [15] demonstrated that silencing of Brag2 in HeLa cells increased surface expression of β1-integrins and cell spreading. Moreover, another study suggested that Brag2 may promote β1-integrin endocytosis [33]. Remarkably in our present work, we demonstrated that Brag2 is involved in the internalization/endocytosis of activated (matrix-bound) α5β1-integrins, as assessed by an activation-dependent α5β1-integrin antibody recognizing an active α5β1-integrin conformation present in fibrillar and partly in focal adhesions [12], supporting the idea that Brag2 contributes to the disassembly of focal/fibrillar adhesions by mediating endocytosis of active/matrix-bound α5β1-integrins. Interestingly, silencing of Brag2 also reduced the recycling of αVβ3-integrins to the cell surface and consequently decreased the cell surface expression of αVβ3-integrins and adhesion to vitronectin. However, in our hands inhibition of αVβ3-integrins (in contrast to β1-integrin inhibition) did not affect angiogenic sprouting. In summary, our data indicate that Brag2 promotes EC migration and angiogenesis by mediating β1-integrin endocytosis thereby contributing to the turnover of focal/fibrillar adhesions and regulating α5β1-integrin-dependent EC adhesion.

An important question is how Brag2 mediates integrin endocytosis. In this regard, we found that the integrin endocytosis-promoting activity of Brag2 is dependent on the activation of Arf-GTPases. However, the effector proteins downstream of Arf-GTPases signaling mediating integrin internalization in EC are unclear. Arf6 was shown to contribute via PIP5K and recruitment of AP2 and clathrin to receptor endocytosis [22, 29, 39]. Moreover, a recent study demonstrated that Brag2 could bind clathrin and AP2 itself and that Arf5, an Arf-GTPase also activated by Brag2, may contribute to β1-integrin endocytosis [33]. In line with these results, we found here that Arf5 and Arf6 mediate downstream of Brag2 angiogenic sprouting and the regulation of focal/fibrillar adhesions containing activated α5β1-integrins in EC. Interestingly, it was shown that Arf6 may also mediate recycling of β1-integrins to the cell surface [40]. Additional studies are mandatory in order to define the downstream mechanisms promoting Brag2-dependent integrin endocytosis.

An additional mechanism by which Brag2 may affect angiogenesis includes the regulation of vascular permeability. Indeed, silencing of Brag2 increases in vitro permeability of EC monolayers ([20] and our unpublished data), while reducing the surface abundance of VE-Cadherin [20]. Moreover, silencing of Brag2 (*IQSEC1a* and *IQSEC1b*) in zebrafish led to hydrocephalus and vascular edema, probably as a consequence of vascular hyperpermeability. Nevertheless, the permeability regulating activity of Brag2 does not explain the observed inhibition of EC migration and angiogenic sprouting induced by the knockdown of Brag2 in our study. Indeed, VE-Cadherin-induced cell–cell interactions were shown to block EC migration and angiogenic sprouting [1]. So, one would expect that, since silencing of Brag2 reduces VE-Cadherin-mediated cell–cell adhesion (adherens junctions) and increases vascular permeability ([20] and our unpublished data), would rather lead to a promigratory and proangiogenic EC phenotype. However, as we clearly demonstrated in our present work silencing of Brag2 blocked angiogenic sprouting, EC migration and in vivo angiogenesis. Furthermore, specifically Arf6 was shown to modify VEGFR2 function in endothelial cells [25]. However, silencing of Brag2 did not reduce VEGF-induced VEGFR2 phosphorylation and Akt activation (data not shown), suggesting that the inhibitory effect of Brag2 knockdown on angiogenic sprouting cannot be ascribed to an inhibition of VEGFR2-induced signaling.

Surprisingly, Brag2 exerted opposing effects on β1- and β3-integrins in EC. A potential explanation for these opposing effects is that the trafficking of α5β1- and αVβ3-integrins are inversed coupled to each other [54]. However, additional studies will be required to clarify the underlying molecular mechanisms in EC. Based on the fact that β1-integrins are essential for angiogenesis [4, 49, 52], we focused in the present study on the effects of Brag2 on β1-integrins in EC.

Taken together, our data demonstrate that Brag2 is a key mediator of developmental and pathological angiogenesis by promoting β1-integrin endocytosis thereby regulating β1-integrin-containing focal/fibrillar adhesions, and consequently EC migration and sprouting. Thus, our present work provides evidence that mechanisms involved in integrin endocytosis are essential in angiogenesis and that increased EC adhesion as a consequence of reduced integrin endocytosis may negatively affect angiogenesis. In line with our results, Palecek et al. [38] demonstrated that high cell-substratum adhesiveness impairs cell motility, and that maximum cell speed occurs at intermediate adhesiveness. Since silencing of Brag2 did not completely abolish β1-integrin internalization in EC, additional pathways may also contribute to this process as reported elsewhere [36, 53]. Finally, Brag2 inhibition seems to be a promising target for the development of new antiangiogenic approaches to treat patients with pathological angiogenesis such as in proliferative retinopathies and in cancer.

Acknowledgments We thank Peggy Schuster and Carmen Janczyk for excellent technical assistance. We thank Dr. M. Vaughan (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, U.S.A.) and Dr. A. Someya (Juntendo University, School of Medicine, Tokyo, Japan) for providing us Brag2 plasmids and Dr. J.E. Casanova (Department of Cell Biology, University of Virginia Health System, Charlottesville, VA, USA) for providing us Brag2 plasmids and the Brag2 antibody. Moreover, we thank Dr. Martin J. Humphries (Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Manchester, UK) for providing us the SNAKA51 antibody. In addition, we thank Dr. J.C. Norman (Beatson Institute for Cancer Research, Glasgow, Scotland, UK) for providing us the protocol for the biotinylation-based integrin internalization assay. This work was supported by DFG (Deutsche Forschungsgemeinschaft, Transregional Collaborative Research Centre SFB/TR23, Project A2 to E.C. and S.D., Project Z5 to J.K.) and by the Else Kröner-Fresenius-Stiftung (2013_A2 to E.C.). E.C. and S.D. are members of the Excellence Cluster Cardiopulmonary System (DFG; Exc147-1), the German Centre for Cardiovascular Research (BMBF) and the LOEWE Center for Gene and Cell Therapy (Hessen). Moreover, J.S.G., A.S., and F.Z. are supported by the Intramural Research Program of the NIH.

Conflict of interest The authors declare no competing financial interests.

References

1. Abraham S, Yeo M, Montero-Balaguer M, Paterson H, Dejana E, Marshall CJ, Mavria G (2009) VE-Cadherin-mediated cell–cell interaction suppresses sprouting via signaling to MLC2 phosphorylation. Curr Biol 19:668–674. doi:10.1016/j.cub.2009.02.057
2. Askari JA, Tynan CJ, Webb SE, Martin-Fernandez ML, Ballestrem C, Humphries MJ (2010) Focal adhesions are sites of integrin extension. J Cell Biol 188:891–903. doi:10.1083/jcb.200907174
3. Bader BL, Rayburn H, Crowley D, Hynes RO (1998) Extensive vasculogenesis, angiogenesis, and organogenesis precede lethality in mice lacking all alpha v integrins. Cell 95:507–519. doi:10.1016/S0092-8674(00)81618-9
4. Carlson TR, Hu H, Braren R, Kim YH, Wang RA (2008) Cell-autonomous requirement for beta1 integrin in endothelial cell adhesion, migration and survival during angiogenesis in mice. Development 135:2193–2202. doi:10.1242/dev.016378
5. Carmeliet P, Jain RK (2000) Angiogenesis in cancer and other diseases. Nature 407:249–257. doi:10.1038/35025220
6. Carmona G, Gottig S, Orlandi A, Scheele J, Bauerle T, Jugold M, Kiessling F, Henschler R, Zeiher AM, Dimmeler S, Chavakis E (2009) Role of the small GTPase Rap1 for integrin activity regulation in endothelial cells and angiogenesis. Blood 113:488–497. doi:10.1182/blood-2008-02-138438
7. Casanova JE (2007) Regulation of Arf activation: the Sec7 family of guanine nucleotide exchange factors. Traffic 8:1476–1485. doi:10.1111/j.1600-0854.2007.00634.x
8. Caswell P, Norman J (2008) Endocytic transport of integrins during cell migration and invasion. Trends Cell Biol 18:257–263. doi:10.1016/j.tcb.2008.03.004
9. Caswell PT, Vadrevu S, Norman JC (2009) Integrins: masters and slaves of endocytic transport. Nat Rev Mol Cell Biol 10:843–853. doi:10.1038/nrm2799
10. Chao WT, Ashcroft F, Daquinag AC, Vadakkan T, Wei Z, Zhang P, Dickinson ME, Kunz J (2010) Type I phosphatidylinositol phosphate kinase beta regulates focal adhesion disassembly by promoting beta1 integrin endocytosis. Mol Cell Biol 30:4463–4479. doi:10.1128/MCB.01207-09
11. Chen EH, Pryce BA, Tzeng JA, Gonzalez GA, Olson EN (2003) Control of myoblast fusion by a guanine nucleotide exchange factor, loner, and its effector ARF6. Cell 114:751–762. doi:10.1016/S0092-8674(03)00720-7
12. Clark K, Pankov R, Travis MA, Askari JA, Mould AP, Craig SE, Newham P, Yamada KM, Humphries MJ (2005) A specific alpha5beta1-integrin conformation promotes directional integrin translocation and fibronectin matrix formation. J Cell Sci 118:291–300. doi:10.1242/jcs.01623
13. Connor KM, Krah NM, Dennison RJ, Aderman CM, Chen J, Guerin KI, Sapieha P, Stahl A, Willett KL, Smith LE (2009) Quantification of oxygen-induced retinopathy in the mouse: a model of vessel loss, vessel regrowth and pathological angiogenesis. Nat Protoc 4:1565–1573. doi:10.1038/nprot.2009.187
14. D'Souza-Schorey C, Chavrier P (2006) ARF proteins: roles in membrane traffic and beyond. Nat Rev Mol Cell Biol 7:347–358. doi:10.1038/nrm1910
15. Dunphy JL, Moravec R, Ly K, Lasell TK, Melancon P, Casanova JE (2006) The Arf6 GEF GEP100/BRAG2 regulates cell adhesion by controlling endocytosis of beta1 integrins. Curr Biol 16:315–320. doi:10.1016/j.cub.2005.12.032

16. Epting D, Wendik B, Bennewitz K, Dietz CT, Driever W, Kroll J (2010) The Rac1 regulator ELMO1 controls vascular morphogenesis in zebrafish. Circ Res 107:45–55. doi:10.1161/CIRCRESAHA.109.213983
17. Ezratty EJ, Bertaux C, Marcantonio EE, Gundersen GG (2009) Clathrin mediates integrin endocytosis for focal adhesion disassembly in migrating cells. J Cell Biol 187:733–747. doi:10.1083/jcb.200904054
18. Gambardella L, Hemberger M, Hughes B, Zudaire E, Andrews S, Vermeren S (2010) PI3K signaling through the dual GTPase-activating protein ARAP3 is essential for developmental angiogenesis. Sci Signal 3:ra76 doi:10.1126/scisignal.2001026
19. Grant BD, Donaldson JG (2009) Pathways and mechanisms of endocytic recycling. Nat Rev Mol Cell Biol 10:597–608. doi:10.1038/nrm2755
20. Hashimoto A, Hashimoto S, Ando R, Noda K, Ogawa E, Kotani H, Hirose M, Menju T, Morishige M, Manabe T, Toda Y, Ishida S, Sabe H (2011) GEP100-Arf6-AMAP1-cortactin pathway frequently used in cancer invasion is activated by VEGFR2 to promote angiogenesis. PLoS ONE 6:e23359. doi:10.1371/journal.pone.0023359
21. Hiroi T, Someya A, Thompson W, Moss J, Vaughan M (2006) GEP100/BRAG2: activator of ADP-ribosylation factor 6 for regulation of cell adhesion and actin cytoskeleton via E-cadherin and alpha-catenin. Proc Natl Acad Sci USA 103:10672–10677. doi:10.1073/pnas.0604091103
22. Honda A, Nogami M, Yokozeki T, Yamazaki M, Nakamura H, Watanabe H, Kawamoto K, Nakayama K, Morris AJ, Frohman MA, Kanaho Y (1999) Phosphatidylinositol 4-phosphate 5-kinase alpha is a downstream effector of the small G protein ARF6 in membrane ruffle formation. Cell 99:521–532. doi:10.1016/S0092-8674(00)81540-8
23. Hynes RO (2007) Cell-matrix adhesion in vascular development. J Thromb Haemost 5(Suppl 1):32–40. doi:10.1111/j.1538-7836.2007.02569.x
24. Hynes RO (2002) Integrins: bidirectional, allosteric signaling machines. Cell 110:673–687. doi:10.1016/S0092-8674(02)00971-6
25. Ikeda S, Ushio-Fukai M, Zuo L, Tojo T, Dikalov S, Patrushev NA, Alexander RW (2005) Novel role of ARF6 in vascular endothelial growth factor-induced signaling and angiogenesis. Circ Res 96:467–475. doi:10.1161/01.RES.0000158286.51045.16
26. Jian X, Gruschus JM, Sztul E, Randazzo PA (2012) The pleckstrin homology (PH) domain of the Arf exchange factor Brag2 is an allosteric binding site. J Biol Chem 287:24273–24283. doi:10.1074/jbc.M112.368084
27. Jones CA, Nishiya N, London NR, Zhu W, Sorensen LK, Chan AC, Lim CJ, Chen H, Zhang Q, Schultz PG, Hayallah AM, Thomas KR, Famulok M, Zhang K, Ginsberg MH, Li DY (2009) Slit2-Robo4 signalling promotes vascular stability by blocking Arf6 activity. Nat Cell Biol 11:1325–1331. doi:10.1038/ncb1976
28. Kanzler I, Tuchscheerer N, Steffens G, Simsekyilmaz S, Konschalla S, Kroh A, Simons D, Asare Y, Schober A, Bucala R, Weber C, Bernhagen J, Liehn EA (2013) Differential roles of angiogenic chemokines in endothelial progenitor cell-induced angiogenesis. Basic Res Cardiol 108:310. doi:10.1007/s00395-012-0310-4
29. Krauss M, Kinuta M, Wenk MR, De Camilli P, Takei K, Haucke V (2003) ARF6 stimulates clathrin/AP-2 recruitment to synaptic membranes by activating phosphatidylinositol phosphate kinase type Igamma. J Cell Biol 162:113–124. doi:10.1083/jcb.200301006
30. Lawson ND, Weinstein BM (2002) In vivo imaging of embryonic vascular development using transgenic zebrafish. Dev Biol 248:307–318. doi:10.1006/dbio2002.0711
31. Li L, Welser-Alves J, van der Flier A, Boroujerdi A, Hynes RO, Milner R (2012) An angiogenic role for the alpha5beta1 integrin in promoting endothelial cell proliferation during cerebral hypoxia. Exp Neurol 237:46–54. doi:10.1016/j.expneurol.2012.06.005
32. Lorenzen JM, Dietrich B, Fiedler J, Jazbutyte V, Fleissner F, Karpinski N, Weidemann F, Wanner C, Asan E, Caprio M, Ertl G, Bauersachs J, Thum T (2013) Pathologic endothelial response and impaired function of circulating angiogenic cells in patients with Fabry disease. Basic Res Cardiol 108:311. doi:10.1007/s00395-012-0311-3
33. Moravec R, Conger KK, D'Souza R, Allison AB, Casanova JE (2012) BRAG2/GEP100/IQSec1 interacts with clathrin and regulates alpha5beta1 integrin endocytosis through activation of ADP ribosylation factor 5 (Arf5). J Biol Chem 287:31138–31147. doi:10.1074/jbc.M112.383117
34. Morishige M, Hashimoto S, Ogawa E, Toda Y, Kotani H, Hirose M, Wei S, Hashimoto A, Yamada A, Yano H, Mazaki Y, Kodama H, Nio Y, Manabe T, Wada H, Kobayashi H, Sabe H (2008) GEP100 links epidermal growth factor receptor signalling to Arf6 activation to induce breast cancer invasion. Nat Cell Biol 10:85–92. doi:10.1038/ncb1672
35. Myers KR, Casanova JE (2008) Regulation of actin cytoskeleton dynamics by Arf-family GTPases. Trends Cell Biol 18:184–192. doi:10.1016/j.tcb.2008.02.002
36. Nishimura T, Kaibuchi K (2007) Numb controls integrin endocytosis for directional cell migration with aPKC and PAR-3. Dev Cell 13:15–28. doi:10.1016/j.devcel.2007.05.003
37. Pajcini KV, Pomerantz JH, Alkan O, Doyonnas R, Blau HM (2008) Myoblasts and macrophages share molecular components that contribute to cell–cell fusion. J Cell Biol 180:1005–1019. doi:10.1083/jcb.200707191
38. Palecek SP, Loftus JC, Ginsberg MH, Lauffenburger DA, Horwitz AF (1997) Integrin-ligand binding properties govern cell migration speed through cell-substratum adhesiveness. Nature 385:537–540. doi:10.1038/385537a0
39. Poupart ME, Fessart D, Cotton M, Laporte SA, Claing A (2007) ARF6 regulates angiotensin II type 1 receptor endocytosis by controlling the recruitment of AP-2 and clathrin. Cell Signal 19:2370–2378. doi:10.1016/j.cellsig.2007.07.015
40. Powelka AM, Sun J, Li J, Gao M, Shaw LM, Sonnenberg A, Hsu VW (2004) Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. Traffic 5:20–36 [pii]: 150
41. Reynolds LE, Wyder L, Lively JC, Taverna D, Robinson SD, Huang X, Sheppard D, Hynes RO, Hodivala-Dilke KM (2002) Enhanced pathological angiogenesis in mice lacking beta3 integrin or beta3 and beta5 integrins. Nat Med 8:27–34. doi:10.1038/nm0102-27
42. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR (2003) Cell migration: integrating signals from front to back. Science 302:1704–1709. doi:10.1126/science.1092053
43. Roberts M, Barry S, Woods A, van der Sluijs P, Norman J (2001) PDGF-regulated rab4-dependent recycling of alphavbeta3 integrin from early endosomes is necessary for cell adhesion and spreading. Curr Biol 11:1392–1402 [pii]: S0960-9822(01)00442-0
44. Sakurai A, Gavard J, Annas-Linhares Y, Basile JR, Amornphimoltham P, Palmby TR, Yagi H, Zhang F, Randazzo PA, Li X, Weigert R, Gutkind JS (2010) Semaphorin 3E initiates antiangiogenic signaling through plexin D1 by regulating Arf6 and R-Ras. Mol Cell Biol 30:3086–3098. doi:10.1128/MCB.01652-09
45. Sakurai A, Jian X, Lee CJ, Manavski Y, Chavakis E, Donaldson J, Randazzo PA, Gutkind JS (2011) Phosphatidylinositol-4-

phosphate 5-kinase and GEP100/Brag2 protein mediate antiangiogenic signaling by semaphorin 3E-plexin-D1 through Arf6 protein. J Biol Chem 286:34335–34345. doi:10.1074/jbc.M111.259499

46. Scholz R, Berberich S, Rathgeber L, Kolleker A, Kohr G, Kornau HC (2010) AMPA receptor signaling through BRAG2 and Arf6 critical for long-term synaptic depression. Neuron 66:768–780. doi:10.1016/j.neuron.2010.05.003
47. Someya A, Moss J, Nagaoka I (2010) The guanine nucleotide exchange protein for ADP-ribosylation factor 6, ARF-GEP100/BRAG2, regulates phagocytosis of monocytic phagocytes in an ARF6-dependent process. J Biol Chem 285:30698–30707. doi:10.1074/jbc.M110.107458
48. Someya A, Sata M, Takeda K, Pacheco-Rodriguez G, Ferrans VJ, Moss J, Vaughan M (2001) ARF-GEP(100), a guanine nucleotide-exchange protein for ADP-ribosylation factor 6. Proc Natl Acad Sci USA 98:2413–2418. doi:10.1073/pnas.05163479898/5/2413
49. Stenzel D, Franco CA, Estrach S, Mettouchi A, Sauvaget D, Rosewell I, Schertel A, Armer H, Domogatskaya A, Rodin S, Tryggvason K, Collinson L, Sorokin L, Gerhardt H (2011) Endothelial basement membrane limits tip cell formation by inducing Dll4/Notch signalling in vivo. EMBO Rep 12:1135–1143. doi:10.1038/embor.2011.194
50. Stenzel D, Lundkvist A, Sauvaget D, Busse M, Graupera M, van der Flier A, Wijelath ES, Murray J, Sobel M, Costell M, Takahashi S, Fassler R, Yamaguchi Y, Gutmann DH, Hynes RO, Gerhardt H (2011) Integrin-dependent and -independent functions of astrocytic fibronectin in retinal angiogenesis. Development 138:4451–4463. doi:10.1242/dev.071381
51. Stoll SJ, Bartsch S, Augustin HG, Kroll J (2011) The transcription factor HOXC9 regulates endothelial cell quiescence and vascular morphogenesis in zebrafish via inhibition of interleukin 8. Circ Res 108:1367–1377. doi:10.1161/CIRCRESAHA.111.244095
52. Tanjore H, Zeisberg EM, Gerami-Naini B, Kalluri R (2008) Beta1 integrin expression on endothelial cells is required for angiogenesis but not for vasculogenesis. Dev Dyn 237:75–82. doi:10.1002/dvdy.21385
53. Valdembri D, Caswell PT, Anderson KI, Schwarz JP, Konig I, Astanina E, Caccavari F, Norman JC, Humphries MJ, Bussolino F, Serini G (2009) Neuropilin-1/GIPC1 signaling regulates alpha5beta1 integrin traffic and function in endothelial cells. PLoS Biol 7:e25. doi:10.1371/journal.pbio.1000025
54. White DP, Caswell PT, Norman JC (2007) alpha v beta3 and alpha5beta1 integrin recycling pathways dictate downstream Rho kinase signaling to regulate persistent cell migration. J Cell Biol 177:515–525. doi:10.1083/jcb.200609004
55. Yaniv K, Isogai S, Castranova D, Dye L, Hitomi J, Weinstein BM (2006) Live imaging of lymphatic development in the zebrafish. Nat Med 12:711–716. doi:10.1038/nm1427