The Bipartite Rac1 Guanine Nucleotide Exchange Factor Engulfment and Cell Motility 1/Deducator of Cytokinesis 180 (Elmo1/Dock180) Protects Endothelial Cells from Apoptosis in Blood Vessel Development*

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Engulfment and cell motility 1/deducator of cytokinesis 180 (Elmo1/Dock180) is a bipartite guanine nucleotide exchange factor for the monomeric GTPase Ras-related C3 botulinum toxin substrate 1 (Rac1). Elmo1/Dock180 regulates Rac1 activity in a specific spatiotemporal manner in endothelial cells (ECs) during zebrafish development and acts downstream of the Ntrn-1/Unc5-homolog B (Unc5B) signaling cascade. However, mechanistic details on the pathways by which Elmo1/Dock180 regulates endothelial function and vascular development remained elusive. In this study, we aimed to analyze the vascular function of Elmo1 and Dock180 in human ECs and during vascular development in zebrafish embryos. In vitro overexpression of Elmo1 and Dock180 in ECs reduced caspase-3/7 activity and annexin V-positive cell number upon induction of apoptosis. This protective effect of Elmo1 and Dock180 is mediated by activation of Rac1/p21-activated kinase (PAK) and AKT/protein kinase B (AKT) signaling. In zebrafish, Elmo1 and Dock180 overexpression reduced the total apoptotic cell and apoptotic EC number and promoted the formation of blood vessels during embryogenesis. In conclusion, Elmo1 and Dock180 protect ECs from apoptosis by the activation of the Rac1/PAK/AKT signaling cascade in vitro and in vivo. Thus, Elmo1 and Dock180 facilitate blood vessel formation by stabilization of the endothelium during angiogenesis.

Angiogenesis is a pivotal process, e.g. during embryonic development, and includes various tightly regulated steps. Stimulation of pre-existing blood vessels with angiogenic growth factors promotes degradation of the basal membrane, detachment of mural cells, selection of tip and stalk cells, and proliferation and migration of endothelial cells (ECs).2 Eventually new blood vessels are built by anastomosis and lumen formation (1, 2). Finally, vessel stabilization and pruning are required to form a mature and hierarchically structured vasculature (1, 2). Pruning or vessel regression is an active, tightly controlled process due to changes in hemodynamics and includes endothelial cell-cell contact reorganization, cell retraction and induction of apoptosis (3–7). The decision whether a newly formed vessel remains or undergoes pruning is made by several factors, such as survival factors, establishment and stabilization of cell-cell contacts, either of ECs or pericytes, deposition of basal membrane, and initiation of blood flow (1, 8, 9). Particularly, ECs are dependent on survival and stabilization factors like VEGF, delta-like ligand 4 (DII4), fibroblast growth factor (FGF), or angiopoietin-1 (Ang-1) to prevent vessel regression (6, 10–15).

The monomeric GTPase Rac1 is a member of the Ras homologue (Rho) family of small G proteins and regulates critical

2 The abbreviations used are: EC, endothelial cell; Elmo1, engulfment and cell motility 1; Dock180, deducator of cytokinesis 180; Rac1, ras-related C3 botulinum toxin substrate 1; Unc5B, Unc5-homologue B; PAK, p21 activated kinase; AKT, AKT/protein kinase B; DII4, delta-like ligand 4; FGF, fibroblast growth factor; Ang-1, angiopoietin-1; Rho, ras homologue; E, embryonic day; GEF, guanine nucleotide exchange factor; DH, Dbl homology; PH, pleckstrin homology; P13K, phosphoinositide 3-kinase; HUVEC, human umbilical vein endothelial cell; BAEC, bovine aortic endothelial cell; ECGM, endothelial cell growth medium; ECBM, endothelial cell basal medium; GTP·S, guanosine 5′-O-(γ-thio) triphosphate; PTU, 1-phenyl-2-thiourea; hpf, hours postfertilization; ISV, intersomitic vessel; Erk-1/2, extracellular signal-regulated kinases 1/2, ROS, reactive oxygen species.

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actions during angiogenesis (16, 17). It is involved in actin cytoskeleton reorganization, lamellipodia formation, and thus migration (18, 19). Furthermore, Rac1 regulates cell cycle progression, EC morphology, capillary survival, and pruning by cell retraction (3, 17). Endothelial loss of Rac1 in mice is embryonic lethal at embryonic day (E) 9.5 due to malformation of major vessels, such as branchial arch arteries and cardiac defects (19). This highlights the important function of this monomeric GTPase in angiogenesis.

Rac1, like other small G proteins, is regulated by guanine nucleotide exchange factors (GEFs). These GEFs mediate the exchange from GDP to GTP and hence activate the monomeric GTPases (20). Usually a multitude of GEFs is available for one small G protein and facilitate cell and context specific actions of the protein (16). An unusual GEF for Rac1 is the protein complex Elmo1/Dock180. Whereas canonical GEFs interact with GTPases of the Rho family via their Dbl homology-pleckstrin homology (DH-PH) domains, Dock180 does not possess such a domain. It interacts with Rac1 by its Docker domain (20). The binding of Dock180 to Elmo1 stabilizes the interaction with nucleotide-free Rac1 and additionally localizes the active protein complex at the plasma membrane (21–23).

Elmo1 and Dock180 have previously been described to activate Rac1, which results in migration of neurons (24–26), glioma cells (27), border cells in Drosophila melanogaster (28), and distal tip cells in Caenorhabditis elegans (29). Furthermore, Elmo1 and Dock180 mediate apoptotic cell clearance in mice and zebrafish, and the uptake of Gram-negative bacteria by endothelial cells (30). Dock180 is important for EC survival and anti-apoptotic action of Netrin-1. In this study, we have identified a novel, cell intrinsic survival function of Elmo1/Dock180 in angiogenesis by maintaining cell survival during embryonic blood vessel development.

EXPERIMENTAL PROCEDURES

Cell Lines, Zebrafish Lines, Antibodies, Inhibitors, and Reagents—Zebrafish embryos of the tg(fli1:EGFP) line (36) were raised and staged as described before (37). Human umbilical vein endothelial cells (HUVECs) were isolated as described previously (38). HUVECs and bovine aortic endothelial cells (BAECs) (39) were cultivated in endothelial cell growth medium (ECGM, PromoCell) containing supplements, 10% FCS (Biochrom), and antibiotics. The following antibodies were used: goat anti-actin (I-19) (Santa Cruz Biotechnology), rabbit anti-Akt (Cell Signaling), mouse anti-pAkt (S473) (587F11) (Cell Signaling), rabbit anti-Dock180 (H-70) (Santa Cruz Biotechnology), goat anti-Elmo1 (Everest Biotech), mouse anti-Rac1 (Transduction Laboratories), mouse anti-ERK1 (E-4) (Santa Cruz Biotechnology), rabbit anti-ERK1 (K-23) (Santa Cruz Biotechnology), mouse anti-human [pSer139]Histone H2AX (9F3) (Enzo Life Science), Cy3-conjugated secondary antibodies (Dianova), and HRP-conjugated antibodies (DAKO). In this study the following inhibitors were used: PI3K inhibitor LY 294002 (Biotrend), PAK inhibitor IPA3 (Tocris Bioscience). VEGF was purchased from R&D Systems, staurosporine from Sigma-Aldrich and Nethrin-1 from Enzo Life Sciences.

Transfection and Transduction of HUVECs—Transfection of HUVECs with siRNAs targeting Elmo1 or Dock180 was performed as described before (35). For adeno viral transduction of HUVECs, full-length cDNA of human Elmo1 or Dock180 was cloned into the adeno viral shuttle vector according to the Vira Power Adenovirus Expression System protocol (Invitrogen). Adenovirus particles of dominant-negative (RacN17) and constitutive-active (RacV12) Rac1 were kindly provided by Dr. Mauro Cozzolino (Santa Lucia Foundation, Rome, Italy). Efficient transduction was confirmed by Western blot analysis or immunofluorescence staining of HUVECs.

In-gel Sprouting Assay—HUVECs were transfected or adenovirally infected 48 h before the assay. The assay was performed as previously described (39). The spheroids were stimulated upon embedding in collagen with 25 ng/ml VEGF for 24 h. The cumulative sprouting length was set in relation to control and is displayed in percent.

Caspase-3/7 and [pSer139]Histone H2AX Assay—To measure caspase 3 and 7 activity in HUVECs the Caspase-Glo® 3/7 Assay System (Promega) was used according to the manufacturer’s protocol. For activity measurement upon serum starvation, HUVECs were seeded 48 h after transduction in a 96-well plate (1 × 10^4 cells/well) and cultivated in ECGM for 22 h, followed by stimulation with 2.5 μM staurosporine in ECGM for 2 h. For activity measurement upon serum starvation, HUVECs were seeded in a 96-well plate (5 × 10^3 cells/well) ~32 h after transduction and cultivated in ECGM overnight. Then cells were starved in endothelial basal medium (ECBM, PromoCell, 0% FCS) for 24 h. For the inhibition of PI3K using LY 294002, medium was changed after 8 h of serum starvation and replaced by fresh ECMB complemented with 10 μM LY (or DMSO in an appropriate concentration in control wells), followed by further 16 h of incubation. For graphical presentation, the caspase-3/7 activity was set in relation to control and is displayed in percent. Detection of [pSer139]Histone H2AX activity was performed after Dock180 siRNA transfection, followed by 4 h of serum starvation in ECBM and subsequent Western blot analysis.

FACS Analysis of Annexin V-positive Cell Fractions—To analyze HUVECs undergoing apoptosis by FACS, cells were stained with APC-conjugated annexin V (BD Pharmingen). To this end, HUVECs were seeded 48 h after adenoviral transduction in 6-well plates (3 × 10^5 cells/well) and cultivated in ECGM

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for 20 h, followed by stimulation with 2.5 μM staurosporine in ECGM for 4 h. For starvation experiments, 1.5 × 10^5 HUVECs were seeded per 6 well and cultivated in ECGM for 24 h. Subsequently, medium was changed to ECBM (0% FCS), and cells were starved for 24 h. At the end of the incubation time, cells were harvested, washed twice with PBS, and resuspended in 1× Binding Buffer (Bender MedSystems). Cell number was adjusted to 1 × 10^6 cells/ml and incubated with annexin V-APC and 7-AAD (Beckman-Coulter). FACS analysis was performed by the Mannheim Cell Sorting Core Facility of the Medical Faculty Mannheim using a BD FACSCanto II. For quantification, the annexin V-positive and 7-AAD-negative cell fraction was considered as the apoptotic cell fraction. The apoptotic cell fraction was set in relation to control (apoptotic cell fraction of pAd-GFP cells) and is displayed in percent.

**Western Blot Analysis**—Western blot analysis was performed as previously described (40) using serum-starved HUVECs (overnight, ECBM, 0% FCS). HUVECs were adenovirally infected 48 h prior to the assay. For inhibition of PI3K by LY 294002 or IPA3, ECBM was changed 30 min (LY 294002) or 1 h (IPA3) before lysis and replaced by ECBM complemented with inhibitor (1 μM LY 294002 or 20 μM IPA3, respectively). Control cells were incubated with ECBM complemented with solvent in appropriate concentrations. For analysis of AKT activity upon Netrin-1 stimulation, BAECs were seeded in a 6-well plate (2.5 × 10^5 cells/well). Approximately 32 h later, cells were starved (ECBM, 2.5% FCS) for 16 h. Cells were treated with 100 ng/ml Netrin-1 for 5 min. Three independent experiments were performed, and a representative Western blot for each experiment is shown.

**Rac1 Pull-down Assay**—Analysis of Rac1 activity was performed using the Rac1 binding domain of PAK1 fused to GST (GST-PBD) as described before (41). Briefly, HUVECs were seeded in a 10-cm dish (2 × 10^6 cells/dish) and adenovirally transduced. 32 h after transduction, cells were starved overnight (ECBM, 0% FCS) followed by lysis, Rac1 pull-down, and Western blot analysis. Incubation of control lysate with 100 μM GTPyS (30 min, 37 °C) served as a positive control for Rac1 activation. Three independent experiments were performed, and a representative Western blot is shown.

**Injection of mRNA in Zebrafish Embryos and Staurosporine Treatment**—Synthesis and injection of zebrafish Elmo1 and Dock180 mRNA were performed using the SP6 mMessage mMachine Kit (Ambion) as previously described (35). For injections, 300 pg of mOrange, Elmo1, or Dock180 mRNA was used. For staurosporine treatment, dechorinated zebrafish embryos were incubated in eggwater containing 0.2% 1-phenyl-2-thiourea (PTU) (Sigma-Aldrich) and 3 μM staurosporine starting at 24 h postfertilization (hpf) (42). Embryos were fixed for TUNEL staining at 30 hpf.

**TUNEL Staining of Zebrafish Embryos**—For whole-mount TUNEL staining of zebrafish embryos the ApoTag® Red In Situ Apoptosis Detection Kit (Chemicon) was used. For this purpose, 30 hpf zebrafish embryos were fixed for 1 h in 4% PFA/PBS, washed in PBST (1× PBS/0.1% Tween-20), followed by dehydration in increasing concentrations of methanol, and stored in 100% methanol overnight at ~20 °C. Embryos were rehydrated in increasing concentrations of PBST and treated with Proteinase K (10 μg/ml, Roche) for 10 min. Embryos were washed in PBST and postfixed in 4% PFA/PBS for 20 min. After further washing with PBST, embryos were incubated in equilibration buffer for 1 h, followed by overnight incubation with working strength TdT enzyme at 37 °C. The reaction was stopped by incubation in working strength stop/wash buffer for 3 h at 37 °C. After washing in PBST, embryos were incubated in rhodamine antibody solution overnight at 4 °C followed by washing with PBST overnight at 4 °C. The embryos were further fixed in 4% PFA/PBS for 30 min, followed by washing in PBST. For quantification of apoptotic cells, embryos were embedded in 1% low melting agarose (Promega) and analyzed by confocal microscopy.

**Imaging of Zebrafish Embryos**—For confocal microscopy, the fixed embryos were embedded in 1% low melting point agarose (Promega) and analyzed using a DM6000 B confocal microscope with Leica TCS SP5 DS scanner (Leica Microsystems).

**Quantification and Statistics**—For quantification of apoptotic cells in zebrafish embryos, confocal images were further processed using ImageJ/Fiji. To exclude counting of nonspecific stained cells in the yolk sac, trunk tissue was selected using the polygon selection tool, and the selected area was measured. Note: the selected area for counting of total apoptotic cell number and apoptotic ECs remained the same in the respective embryo. Total apoptotic cell number was determined by the analyze particles tool of ImageJ/Fiji. Apoptotic ECs were counted manually. Counted apoptotic ECs were marked with the point tool to prevent double counting. The number of total apoptotic or apoptotic ECs per μm² was calculated. For a clearer graphical presentation, values are set in relation to control group (mOrange RNA) and displayed as total apoptotic cells or apoptotic ECs in percent (n = 15–17 at 30 hpf of at least three independent experiments). For quantification of mean intersomitic vessel (ISV) length in 30 hpf zebrafish embryos, length of ISVs was measured using the segmented line tool of ImageJ/Fiji, and the mean number of ISV length per fish was calculated. Results are displayed as mean ISV length/fish in μm (n = 12–14 of three independent experiments). Quantification of Western blot signals was performed using Gel-Pro Analyzer 6.0 software (Media Cybernetics). Sample signals were set in relation to their respective loading controls and are displayed in percent. All results in this publication are expressed as means ± S.E. Comparisons between groups were analyzed by Student’s t-test for *in vitro* assays and Mann-Whitney-*U*-Test for *in vivo* assays (SPSS 21). p values <0.05 were considered statistically significant.

**RESULTS**

**Elmo1 and Dock180 Protect ECs from Apoptosis**—The function of Elmo1 in zebrafish vascular development acting downstream of the Netrin-1/Unc5B signaling cascade has recently been described (35). However, in contrast to the *in vivo* data, Netrin-1 stimulation of cultured ECs did not induce sprouting in an in-gel sprouting assay (35). Since Netrin-1’s identified downstream effector, the small GTPase Rac1 (24, 35), is well known to mediate migration of ECs (18, 19, 43), this raised the question of the precise function of Elmo1 and Dock180 in the endothelium. Since Rac1 has already been described to act
downstream of the VEGFR2 signaling cascade (18, 44), we
aimed to further identify the importance of the Rac1 activator
Elmo1/Dock180 on EC function downstream of VEGFR2. To
this end, we silenced Elmo1 and Dock180 expression in ECs by
siRNA transfection (35) and performed an in-gel sprouting
assay upon VEGF stimulation (Fig. 1, A–C). The stimulation
with VEGF led to an increase of the relative cumulative sprout-
ing length of control cells up to 300%. However, the knock-
down of Elmo1 or Dock180 did not alter the VEGF-increased
sprouting. We further analyzed the sprouting response in an
Elmo1 gain-of-function experiment. To this end, we generated
an adenovirus overexpressing Elmo1. The functionality of the
virus was confirmed by elevated Elmo1 protein level after
adenoviral transduction in ECs (Fig. 1, D) as well as by an
enhanced activity of the Rac1 downstream target extracellular-
signal regulated kinases 1/2 (Erk1/2) (44) (Fig. 1, E). Interest-
ingly, the overexpression of Elmo1 in ECs significantly
increased the basal cumulative sprouting length. Yet, increased
Elmo1 expression had no additional effect on the VEGF-in-
duced sprouting response (Fig. 1, F).

FIGURE 1. Elmo1 and Dock180 do not regulate VEGF-dependent sprouting angiogenesis in ECs. A, representative Western blot analyses shows strong
reduction of the Elmo1 and Dock180 protein levels in HUVECs 48 h after siRNA
transfection. Actin served as loading control. B, Elmo1 knock-down does not alter
VEGF-dependent sprouting angiogenesis in an in-gel sprouting assay. Upon Elmo1 siRNA transfection, spheroids were embedded in collagen and stimulated
with VEGF for 24 h. The graph displays the cumulative sprouting length set in relation to unstimulated control cells (control siRNA) in %. The images show
representative spheroids of each group. Scale bar indicates 100 μm. n = 3. C, Dock180 knock-down does not alter VEGF-dependent sprouting angiogenesis in
an in-gel sprouting assay. Upon Dock180 siRNA transfection, spheroids were embedded in collagen and stimulated with VEGF for 24 h. The graph displays the
cumulative sprouting length set in relation to unstimulated control cells (control siRNA) in %. The images show representative spheroids of each group. Scale
bar indicates 100 μm. n = 3. D, adenoviral infection of HUVECs with pAd-Elmo1 strongly enhances Elmo1 protein level. Western blot analysis (upper panel)
shows the overexpression of Elmo1 48 h after infection. Immunofluorescence staining of HUVECs (lower panel) shows enhanced protein levels of Elmo1 72 h
after adenoviral transduction. Elmo1 is labeled in red, DAPI visualizes nuclei. Scale bar indicates 50 μm. E, Elmo1 and Dock180 overexpression increases the
activity of the Rac1 downstream effector Erk1/2 (pErk1/2) in HUVECs. HUVECs were serum starved 16 h before cell lysis. Total Erk1/2 (tErk1/2) served as loading
control. One representative Western blot for n = 3 experiments is shown. For quantification, values were set in relation to unstimulated control and displayed
in % ± S.E. F, adenoviral overexpression of Elmo1 increases basal sprouting, but does not enhance VEGF-induced sprouting. Spheroids were stimulated with
VEGF for 24 h after Elmo1 (pAd-Elmo1) transduction. The images show representative spheroids of each group. Scale bar indicates 100 μm. n = 4. *, p < 0.05;
#, p < 0.05 versus pAd-GFP; ns, not significant. VEGF concentration: 25 ng/ml. Error bars indicate S.E.
showed no involvement of its activator Elmo1/Dock180 in the VEGF-mediated endothelial sprouting process in vitro (Fig. 1). This finding suggests an additional and new function for Elmo1/Dock180, rather than regulating VEGF-induced cell migration in the endothelium. Recently, an anti-apoptotic and pro-angiogenic function for Netrin-1 and its receptor Unc5B has been identified in the endothelium (45). Since Elmo1 regulates vascular development downstream of Netrin-1 in the zebrafish (35), we addressed the question if Elmo1 and its complex partner Dock180 maintain cell survival in the endothelium. To this end, ECs were transduced with an adenovirus to overexpress Elmo1 and/or Dock180 (Figs. 1D and 2A). To determine apoptosis, the activity of caspases-3/7 was assessed. Induction of apoptosis by treatment of ECs with staurosporine significantly enhanced caspase-3/7 activity in control cells up to 400% (Fig. 2B). However, a significant reduction in caspase-3/7 activity upon staurosporine incubation was detected in Elmo1, Dock180, and Elmo1/Dock180-overexpressing cells compared with control cells. Surprisingly, combined overexpression of Elmo1 and Dock180 did not result in a further reduction of caspase-3/7 activity as compared with single transduction using Elmo1 or Dock180 alone. This suggested that the overexpression of one of the complex partners alone already induced a maximal pro-
protective effect which is likely due to the fact that Elmo1 prevents degradation of its complex partner (46). Alternatively, the overexpression of the catalytic active Dock180 alone might be sufficient for the maximum activation of Rac1 by binding to endogenously expressed Elmo1 and vice versa. A reduction in the number of apoptotic cells was also observed when HUVECs were harvested after staurosporine treatment and stained with annexin V for FACS analysis, a protein which binds phosphatidylserine and therefore marks apoptotic cells (47) (Fig. 2C). The EC fraction positive for annexin V in the staurosporine-treated control group was elevated up to 400%. The overexpression of Elmo1, Dock180, and Elmo1/Dock180 significantly reduced the apoptotic cell number upon staurosporine treatment (Fig. 2C). To further confirm these findings, apoptosis was induced by serum starvation, which more accurately simulates physiological conditions. To this end, ECs were serum starved for 24 h followed by the determination of caspase-3/7 activity. Consistently, enhanced protein levels of Elmo1, Dock180, and Elmo1/Dock180 were able to reduce starvation-induced increase of caspase-3/7 activity (Fig. 2D). In contrast, siRNA-mediated Dock180 loss-of-function experiments increased apoptosis as monitored by increased [pSer139]Histone H2AX phosphorylation (Fig. 2E). This further strengthens the data shown in Fig. 2, B and C, uncovering a new, cell intrinsic pro-survival function for Elmo1/Dock180 in ECs.

Elmo1 and Dock180 Maintain Survival of ECs via the Rac1/AKT Signaling Cascade—To identify the downstream signaling cascade which mediates the endothelial protective function of Elmo1/Dock180 we interfered with known mediators of the activity of one of the key survival factors in ECs, AKT (48). Thus, serum-starved ECs were additionally treated with the PI3K inhibitor LY 294002 (49) to blunt the PI3K-dependent AKT signaling. Although caspase-3/7 activity in Elmo1 or/and Dock180-overexpressing ECs is strongly reduced (Fig. 3A, black bars), the inhibition of PI3K activity in Elmo1 or/and Dock180 expressing cells led to a significant increase in caspase activity (Fig. 3A, gray bars). To further verify the activation of AKT in Elmo1 or/and Dock180 expressing ECs, AKT phosphorylation was determined by Western blot analyses. Overexpression of Elmo1 and/or Dock180 led to an enhanced activation of AKT and, concordantly, inhibition of PI3K activity by LY 294002 strongly attenuated this effect (Fig. 3B). This highlights the dependence of the protective role of Elmo1 and Dock180 in ECs on PI3K and AKT function. We further aimed to address the question, if the activation of AKT by Elmo1 and Dock180 is mediated by Rac1 signaling, as AKT has recently been described acting downstream of Rac1 (48, 50). To this end, Rac1 activity was determined by Rac1 pull-down assays in ECs which showed a strong increase in Rac1 activation when Elmo1 and Dock180 protein levels were enhanced (Fig. 4A). Consequently, to demonstrate Rac1-dependent AKT activation, Western blot analyses for phosphorylated AKT in ECs, which overexpress Elmo1 and/or Dock180 were performed. Yet, to block Rac1 signaling, a dominant-negative form of Rac1 (RacN17) (51) was additionally overexpressed. As already shown before (Fig. 3B), the overexpression of Elmo1, Dock180, and Elmo1/Dock180 alone increased AKT activation. Inhibition of endogenous Rac1 activity by dominant-negative RacN17 expression strongly reduced this effect (Fig. 4B). Since Rac1 is known to mediate PI3K/AKT signaling via activation of its downstream effector PAK1 (48, 52, 53), AKT activity in ECs was further determined in presence of the PAK1 inhibitor IPA3 (Fig. 4C). Inhibition of PAK1 led to a strong reduction of Elmo1 and/or Dock180-mediated AKT phosphorylation, which demonstrates the activation of AKT via PAK1. Thus, Elmo1/Dock180 protects ECs from apoptosis by the activation of the Rac1/AKT signaling cascade in vitro.

Recent data suggested a pro-survival function of Netrin-1 via its receptor Unc5B by decreasing caspase activity in ECs (45). To link the protective action of Netrin-1 to the survival function of Elmo1/Dock180 we performed Western blot analyses to analyze AKT activation upon Netrin-1 stimulation in ECs (Fig. 4D). Netrin-1 stimulation of ECs resulted in enhanced AKT activation. Thus, Netrin-1 activates the same protective machinery, which now has been shown for Elmo1/Dock180 (Fig. 3B).

Elmo1 and Dock180 Maintain EC Survival in the Zebrafish Embryo—To study the protective function of Elmo1/Dock180 in a physiological context, the vascular development in tgf(fli1: EGFP) zebrafish embryos was analyzed. This transgenic line expresses endothelial-specific EGFP and therefore permits the observation of blood vessel formation. For the enhancement of Elmo1 and Dock180 protein expression in zebrafish, the
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![Figure 4](image-url)

**FIGURE 4. Elmo1 and Dock180 increase AKT activity via the Rac1 signaling cascade.** A, Elmo1 and Dock180 overexpression induces Rac1 activity (Rac1-GTP) in cultured ECs. Prior to Rac1 pull-down, ECs were serum-starved for 16 h. Incubation of EC lysate with the non-hydrolyzable GTP-analog GY5 served as a positive control for Rac1 activation. Total Rac1 served as loading control. B, Elmo1- and Dock180-mediated induction of AKT activity (pAKT) is reduced in presence of a dominant-negative form of Rac1 (RacN17). Prior to cell lysis, adenovirally infected ECs were starved for 16 h. RacN17 and a constitutively active form of Rac1 (RacV12) alone served as negative and positive controls for Rac1 activation. Total AKT (tAKT) served as loading control. C, inhibition of the Rac1 effector PAK1 by IPA3 reduces the Elmo1- and Dock180-mediated activation of AKT (pAKT). Adenovirally transduced ECs were serum starved for 16 h and incubated with 20 μM IPA3 for 1 h prior to cell lysis. Total AKT (tAKT) served as loading control. D, Netrin-1 stimulation enhances AKT activity (pAKT) in ECs. Prior to lysis, cells were starved overnight (2.5% FCS) and treated with 100 ng/ml Netrin-1 for 5 min. Total AKT (tAKT) served as loading control. A representative Western blot for n = 3 experiments in each figure is shown. For quantification, values were set in relation to unstimulated control and displayed in % ± S.E.

respective mRNAs were injected into 1-cell stage embryos (Fig. 5A). To induce apoptosis, zebrafish embryos were incubated with staurosporine starting at 24 hpf for 6 h. At 30 hpf, embryos were fixed, and TUNEL staining was performed to visualize total apoptotic and endothelial apoptotic cells in the zebrafish trunk. In control zebrafish embryos, which were injected with mOrange mRNA, only few cells undergo apoptosis without staurosporine treatment (Fig. 5B). However, stimulation with staurosporine results in a strong increase in total apoptotic as well as endothelial apoptotic cell number (Fig. 5, B–D) up to 500%. Apoptotic ECs are localized in all trunk vessels, such as in the dorsal aorta, in the posterior cardinal vein, in intersomitic vessels (ISVs), and in the dorsal longitudinal anastomotic vessel. Importantly, overexpression of Elmo1 or Dock180 in zebrafish embryos strongly reduced the number of apoptotic cells in toto and most important, of apoptotic ECs (Fig. 5, B–D) to a similar extent. In addition to the apoptotic cell phenotype, vascular alterations were identified in the zebrafish embryos. Staurosporine treatment reduced the mean ISV length in control embryos (Fig. 5, B and E). This reduction in ISV length was significantly attenuated when Elmo1 and Dock180 were overexpressed.

Therefore, the in vivo data are in good agreement with the data from cultured ECs (Figs. 2–4) and demonstrate that Elmo1/Dock180 exerts a protective vascular function in a living organism. Taken together, our results demonstrate a so far unknown, cell intrinsic function of the Rac1 guanine nucleotide exchange factor Elmo1/Dock180 in maintaining EC survival in vitro and in vivo (Fig. 6).

**DISCUSSION**

In this study we have identified a novel protective function for the Rac1 activator Elmo1/Dock180 in the endothelium, which is mediated by PAK1, PI3K, and AKT (Fig. 6). First, overexpression of Elmo1 and Dock180 in ECs reduces apoptosis upon staurosporine treatment or serum starvation in a PI3K-, PAK1-, and AKT-dependent manner. Second, in zebrafish embryos, Elmo1 and Dock180 overexpression reduced the number of apoptotic ECs after apoptosis induction and rescued vascular malformation. Thus, Elmo1/Dock180 apparently act as survival factor during early vascular development.

Rac1 regulates apoptosis in several cell types (17, 54). Yet, controversial results have so far been obtained whether Rac1 has a pro- or anti-apoptotic function (17, 54–59). Monomeric GTPases mediate different biological functions due to their cell type, spatial, temporary, and context-dependent regulation by GEFs (16). As has thus also been shown for Rac1 (16), it seems very likely that Rac1 pro- or anti-apoptotic function is mediated by the presence of specific activators or inhibitors. The Rac1 GEF Dock180 mediates survival in glioblastomas, epiblasts, and cardiomyocytes (60–62). So far little is known about apoptosis regulation in the vasculature by Rac1 and the involved Rac1 GEFs (63). Nevertheless, the expression of Elmo1/Dock180 is spatially and temporarily regulated in the zebrafish vasculature during embryonic development. In early stages of vessel formation in zebrafish embryos, Elmo1 is highly expressed in the dorsal aorta, in the posterior cardinal vein and in the ISVs. However, at later stages vascular expression of Elmo1 diminishes, which suggested a specific and transient function in early processes of vascular development (35). During angiogenesis, newly formed blood vessels require stabilizing and survival factors, otherwise they are prone to apoptosis and vessel regression (3–7, 9). Elmo1 expression in the zebrafish vasculature (35), its protective function in cultured ECs and its pro-survival function in zebrafish embryos suggest that Elmo1 and its complex partner Dock180 stabilize newly formed blood vessels. In accordance, the enhanced EC apoptosis induced by staurospo-
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Activation of Rac1 is also known to be required for endothelial migration in response to VEGF. This activation is mediated by the VEGF receptor type 2 and the GEF Vav2 (65). Elmo1/Dock180 is apparently not contributing to VEGF-induced Rac1 activation as gain-of-function and loss-of-function for Elmo1 and Dock180 in ECs did not alter the VEGF-dependent sprouting response. These findings therefore highlight the dependence of Rac1’s different functions in ECs attributed to the spatially and temporally resolved activation of the GTPase by specific GEFs during vascular development.

Although Rac1 has already been described to act downstream of the PI3K/AKT signaling cascade in the endothelium, these data are rather linked to the activation of afore-mentioned VEGFR2-dependent migratory pathways (66). This study provided clear evidence that the Elmo1/Dock180-induced Rac1 activity regulates EC survival via a PAK1- and PI3K-
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FIGURE 6. Elmo1 and Dock180 maintain endothelial cell survival through the Rac1/PI3K/AKT signaling cascade. The role of Elmo1/Dock180 as downstream effector of Netrin-1/Unc5B signaling has been demonstrated in Ref. 35. Elmo1 and Dock180 activate the small G-protein Rac1. Activated Rac1 triggers the survival signal via the activation of PAK, PI3K, and eventually AKT.

dependent activation of AKT (Fig. 6). Thus, the protective effect of Elmo1/Dock180 overexpression was significantly attenuated when PAK1 and PI3K activity was inhibited by IPA3 and LY 294002. In accordance, Elmo1/Dock180 overexpression enhanced AKT activation in a Rac1-, PAK1-, and PI3K-dependent manner. This finding classifies the survival factors PI3K and AKT downstream of Rac1 and PAK1 in ECs and is consistent with recently published studies (50, 67, 68). However, inhibition of PI3K did not result in a complete blockage of the Elmo1/Dock180 protective function in the caspase-3/7 assay. Since Rac1 is known to mediate survival by the regulation of reactive oxygen species (ROS), direct interaction with pro-survival protein Bcl-2, phosphorylation of Bad or transcriptional up-regulation of eNOS (69–72), an additional PI3K/AKT-independent protective role cannot be completely excluded in ECs and will be addressed in subsequent experiments.

Elmo1 has recently been described to mediate angiogenesis downstream of Netrin-1/Unc5B. Although it did not induce angiogenic sprouting in in-gel sprouting assays in vitro, Netrin-1 acted as a guidance factor regulating the pro-angiogenic function of Elmo1 in zebrafish embryos (35). Yet, the exact biochemical mechanisms underlying this pathway remained unclear (35). Some data in the literature suggest an anti-angiogenic function of Netrin-1 and its endothelial receptor Unc5B (73, 74), which is in conflict with recently published data showing a pro-angiogenic and pro-survival function of this signaling cascade (35, 45, 75). However, this controversy can be explained by the dependence receptor function of the Netrin receptors. They induce apoptosis if the ligand is not bound, but mediate survival and other processes if the ligand is present (45, 76). Thus Netrin-1 was shown to mediate survival in ECs (45). Nevertheless the exact signaling cascade has not been elucidated so far. In this study, Western blot analysis of AKT activation in ECs upon Netrin-1 stimulation further supports this pro-survival effect of the Netrin-1/Unc5B signaling cascade. In addition to the identification of Elmo1 as a downstream effector of Netrin-1 (35), the data obtained in this study now explain how Elmo1/Dock180 regulate EC survival and therefore present a pathway by which the Netrin-1/Unc5B/Elmo1/Dock180/Rac1 cascade acts as a pro-survival and pro-angiogenic factor in vascular development (Fig. 6).

In conclusion, this study identified Elmo1/Dock180 as a novel protective factor in ECs. This survival function is mediated by the activation of Rac1 and its downstream signaling cascade. Thus Elmo1/Dock180 acts as a Rac1 GEF that regulates EC survival downstream of Netrin-1.

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