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The Ras Activator RasGRP3 Mediates Diabetes-Induced Embryonic Defects and Affects Endothelial Cell Migration

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Rationale: Fetuses that develop in diabetic mothers have a higher incidence of birth defects that include cardiovascular defects, but the signaling pathways that mediate these developmental effects are poorly understood. It is reasonable to hypothesize that diabetic maternal effects are mediated by 1 or more pathways activated downstream of aberrant glucose metabolism, because poorly controlled maternal glucose levels correlate with the frequency and severity of the defects.

Objective: We investigated whether RasGRP3 (Ras guanyl-releasing protein 3), a Ras activator expressed in developing blood vessels, mediates diabetes-induced vascular developmental defects. RasGRP3 is activated by diacylglycerol, and diacylglycerol is overproduced by aberrant glucose metabolism in diabetic individuals. We also investigated the effects of overactivation and loss of function for RasGRP3 in primary endothelial cells and developing vessels.

Methods and Results: Analysis of mouse embryos from diabetic mothers showed that diabetes-induced developmental defects were dramatically attenuated in embryos that lacked *Rasgrp3* function. Endothelial cells that expressed activated RasGRP3 had elevated Ras-ERK signaling and perturbed migration, whereas endothelial cells that lacked *Rasgrp3* function had attenuated Ras-ERK signaling and did not migrate in response to endothelin-1. Developing blood vessels exhibited endothelin-stimulated vessel dysmorphogenesis that required *Rasgrp3* function.

Conclusions: These findings provide the first evidence that RasGRP3 contributes to developmental defects found in embryos that develop in a diabetic environment. The results also elucidate RasGRP3-mediated signaling in endothelial cells and identify endothelin-1 as an upstream input and Ras/MEK/ERK as a downstream effector pathway. RasGRP3 may be a novel therapeutic target for the fetal complications of diabetes. (*Circ Res.* 2011;108:1199-1208.)

Key Words: blood vessel disruption ■ diabetic embryopathy ■ Ras activator ■ endothelial migration ■ actin cytoskeleton ■ endothelin-1

Receptor-mediated signaling is required for endothelial cell proliferation and migration, processes that are critical to blood vessel formation and function.¹ Signaling downstream of receptor engagement in endothelial cells leads to the activation of several pathways, including phospholipase C- γ activation, to produce diacylglycerol (DAG). DAG in turn activates downstream targets to affect endothelial cell behaviors. Signals that are upregulated in diabetes, such as vascular endothelial growth factor-A (VEGF-A) and endothelin-1 (ET1), generate DAG. DAG is also produced by aberrant glucose metabolism, and DAG levels are elevated in diabetic animals and patients.^{2,3} Diabetic individuals have compromised angiogenesis and blood vessel function, and

fetuses of diabetic mothers have an increased incidence of birth defects, including vascular defects.^{3,4} It is assumed that these vascular defects are mediated, at least in part, by elevated DAG levels, because mouse embryos recovered from diabetic mothers had elevated DAG levels and increased developmental defects³; however, it is not fully understood how elevated DAG leads to vessel dysfunction.

The effects of DAG on cell signaling are mimicked by phorbol esters, tumor promoters that also affect endothelial proliferation, cellular morphology, apoptosis, and barrier function.⁵⁻⁹ DAG and phorbol esters are potent activators of the protein kinase C (PKC) family of proteins. Numerous mammalian PKC isoforms fall into several subfamilies, and DAG/

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Non-standard Abbreviations and Acronyms

DAG	diacylglycerol
ET1	endothelin-1
GFP	green fluorescent protein
HUVEC	human umbilical vein endothelial cell
JNK	C-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
RasGRP3	Ras guanyl-releasing protein 3
STZ	streptozotocin
VEGF-A	vascular endothelial growth factor-A
WT	wild type

phorbol esters activate PKC signaling by binding to C1 domains found in the classic (α , β , γ) and novel (δ , ϵ , η , θ) subgroups of PKCs.¹⁰ Recently, several non-PKC protein families were identified that contain C1 domains and respond to DAG and phorbol esters, which suggests that some DAG/phorbol ester-mediated responses require these proteins.¹¹

Ras guanyl-releasing proteins (RasGRPs) are non-PKC DAG/phorbol ester receptors that function as guanine nucleotide exchange factors and activate the Ras family of GTPases.^{12,13} Ras proteins are localized to membranes, and their activation is controlled in part by proximity to guanine nucleotide exchange factors, such that recruitment of guanine nucleotide exchange factors to membranes can activate Ras. Both DAG and phorbol esters recruit RasGRPs to membranes via the C1 domain. Active Ras in turn activates several effector pathways such as MEK/ERK, p38 mitogen-activated protein kinase (p38 MAPK), and JNK. Ras proteins are activated by phorbol esters in cultured endothelial cells,¹⁴ and PKCs also contribute to Ras and ERK activation in response to phorbol esters in endothelial cells; however, the potential role of non-PKC DAG/phorbol ester receptors in this response has not been investigated.

The RasGRP family of non-PKC phorbol ester receptors has 4 family members. The RasGRPs have limited sites of expression in vivo, and genetic deletion experiments reveal a nonredundant function for the RasGRPs in hematopoietic and endothelial cells. RasGRP1 transduces signals downstream of the T-cell receptor for T-cell maturation, and both RasGRP1 and RasGRP3 affect B-cell function in complex ways.^{15–17} RasGRP2 recently was reported to have effects on vascular development in *Xenopus*.¹⁸ We identified RasGRP3 in a murine-based gene-trap screen as a locus expressed in endothelial cells of developing vessels and required for mediation of the endothelial cell effects of DAG/phorbol esters.¹⁹ The expression profile and DAG/phorbol ester interactions suggested that RasGRP3 might mediate the effects of excess DAG on developing vessels in diabetes.

RasGRP3 activates Ras, Rap, and R-ras in vitro and in mouse embryo fibroblasts.²⁰ In addition to requiring DAG/phorbol ester activity for membrane localization,²¹ RasGRP3 is phosphorylated by PKC at Thr133, and this phosphoryla-

tion is required for RasGRP3 activity in B cells.^{22,23} RasGRP3 also binds dynein light chain, a component of a microtubule minus-end-directed motor, but the significance of this binding in vivo is not clear.²⁴ We showed that RasGRP3 is expressed in angiogenic vessels. Although a genetic loss of function generated by the gene trap did not affect development, embryonic vessels exposed to phorbol ester exhibited dysmorphogenesis that required *Rasgrp3* function, thus identifying RasGRP3 as a novel endothelial phorbol ester receptor.¹⁹ Here, we further characterize the role of RasGRP3-mediated Ras signaling in embryos, endothelial cells, and developing vessels. We find that loss of *Rasgrp3* function significantly reduces diabetes-induced birth defects in vivo, including embryonic vascular defects. We show that Ras is a target of RasGRP3 in endothelial cells and that RasGRP3 is required for ET1-mediated effects on vessel morphogenesis. Our data are consistent with a model in which RasGRP3 signaling in embryonic endothelial cells transduces signals downstream of DAG that affect endothelial cell behaviors and lead to vessel dysmorphogenesis. Thus, excess DAG in diabetes likely overactivates RasGRP3, and this contributes to the perturbed development of fetuses in diabetic environments.

Methods

Diabetic Mice and Embryo Analysis

Female mice (C57Bl/6J purchased from The Jackson Laboratory, Bar Harbor, ME, or *Rasgrp3^{gt/gt}* (a loss-of-function null mutation¹⁹ backcrossed to N8 on the C57Bl/6J background) at 6 to 8 weeks of age were made diabetic according to the protocol in the Animal Models of Diabetic Complications Consortium. Alternatively, *Ins2^{Akita}*/+ mice on the C57Bl/6J background (The Jackson Laboratory, #003548) were bred to obtain *Ins2^{Akita};Rasgrp3^{gt/gt}* mice. Blood glucose was monitored weekly, and mice with blood glucose levels >250 mg/dL were considered diabetic. Mice were mated to genotype-matched males, and embryos were harvested at embryonic day 9.5 (E9.5), fixed, and whole-mount stained for platelet endothelial cell adhesion molecule as described previously.²⁵ Stained embryos were imaged and scored for defects as described in the Online Data Supplement (available at <http://circres.ahajournals.org>).

Whole-embryo culture was performed on embryos dissected at E7.5 or E8.5, in roller bottles as described previously.²⁶ Some embryos were treated with PMA (phorbol 12-myristate 13-acetate) 50 nmol/L and some with glucose 20 mmol/L during the culture period. After 24 hours, embryos were removed and photographed.

Endothelial Cells

Human umbilical vein endothelial cells (HUVECs) were purchased from Clonetics (San Diego, CA), cultured according to the manufacturer's protocol, and used between passages 2 and 8. Transfections were performed with an Amaxa Nucleofector (Lonza, Basel, Switzerland) according to directions. For staining, cells were fixed, permeabilized, blocked, and incubated with Alexa Fluor 555-phalloidin (Molecular Probes, Carlsbad, CA). Migration assays were performed 48 hours after transfection as described in the supplemental Methods. Average velocity and distance to origin of cells were calculated with trajectory measures as diagrammed in Figure 3J.

Mouse endothelial cells (wild type and *Rasgrp3^{gt/gt}*) were generated and expanded as described previously,^{27,28} with minor modifications. Proliferation assays and migration assays were as described in the Online Data Supplement.

Embryonic Stem Cell Differentiation and Analysis

Wild-type (WT; $+/+$) and RasGRP3-deficient (*Rasgrp3^{gt/gt}*) embryonic stem (ES) cells were maintained and differentiated for 8 days as described previously.²⁹ For inhibitor studies, day 7 ES cell cultures

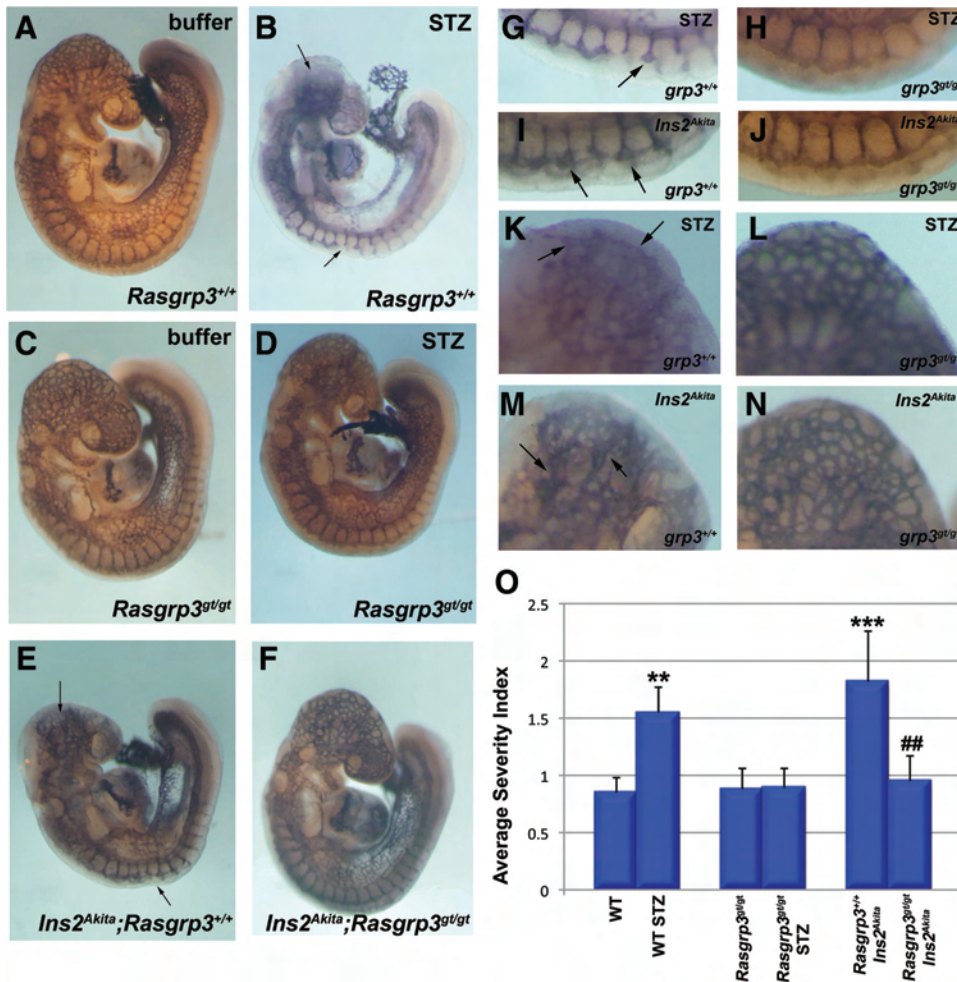


Figure 1. Loss of RasGRP3 attenuates diabetes-induced developmental defects. WT (A, B, G, K) and *Rasgrp3^{gt/gt}* (C, D, H, L) E9.5 embryos were isolated from mothers made diabetic with STZ (B, D, G, H, K, L) or from *Ins2^{Akita}* (E, I, M) or *Ins2^{Akita};Rasgrp3^{gt/gt}* (F, J, N) diabetic mothers and stained for platelet endothelial cell adhesion molecule. A and B, WT embryos from nondiabetic WT mothers (A) had few defects, whereas WT embryos from diabetic mothers (B) had defects in the somitic vasculature (arrow), head plexus (arrow), and somites. C and D, *Rasgrp3^{gt/gt}* embryos from *Rasgrp3^{gt/gt}* nondiabetic mothers (C) had few defects, and *Rasgrp3^{gt/gt}* embryos from *Rasgrp3^{gt/gt}* diabetic mothers (D) were also relatively normal. E and F, Embryos from *Ins2^{Akita};Rasgrp3^{+/+}* mothers (E) had somitic vessel and head plexus defects (arrows), whereas embryos from *Ins2^{Akita};Rasgrp3^{gt/gt}* mothers (F) had few defects. G–J, Close-up of somitic regions of embryos of indicated genotypes; K–N, close-up of head plexus regions of embryos of indicated genotypes. Arrows point to defects in vascular pattern. O, The developmental severity index was calculated on groups of embryos as

described in Methods. Mother's genotype and diabetic status: Lane 1, WT nondiabetic (n=46); lane 2, WT STZ diabetic (n=51); lane 3, *Rasgrp3^{gt/gt}* nondiabetic (n=25); lane 4, *Rasgrp3^{gt/gt}* STZ diabetic (n=27); lane 5, *Ins2^{Akita};Rasgrp3^{+/+}* diabetic (n=17); and lane 6, *Ins2^{Akita};Rasgrp3^{gt/gt}* diabetic (n=19). ** $P \leq 0.001$ for WT versus WT-STZ diabetic; *** $P \leq 0.00001$ for WT versus *Ins2^{Akita}*; ## $P \leq 0.0001$ for *Ins2^{Akita};Rasgrp3^{+/+}* versus *Ins2^{Akita};Rasgrp3^{gt/gt}*.

were pretreated with the appropriate inhibitor for 2 hours before addition of PMA 100 nmol/L for 24 hours. For ET1 stimulation, ET1 100 nmol/L in fresh medium was added daily between days 5 and 8. Antibody staining of ES cell cultures was as described previously.^{25,29} Quantitative image analysis of platelet endothelial cell adhesion molecule–stained ES cell cultures was performed as described previously.²⁵

Ras and ERK Activation Assays

HUVECs (50% to 80% transfected by green fluorescent protein labeling after transfection) or mouse endothelial cells were grown to near confluence for 48 hours, serum-starved overnight, then processed as described in the supplemental Methods for Ras-GTP immunoprecipitation or total Ras, phosphorylated ERK, or total ERK Western blots.

Results

Loss of RasGRP3 Protects Embryos From Diabetes-Induced Birth Defects

Because RasGRP3 is expressed in developing vessels and somites of midgestation mouse embryos,¹⁹ we reasoned that embryos exposed to elevated DAG signaling would be susceptible to effects of RasGRP3-mediated signaling in vivo. Therefore, we examined the effects of elevated DAG/phorbol ester,

glucose, and the diabetic environment on these stages of mouse development. We first mimicked elevated DAG signaling by incubating mouse embryos harvested at E7.5 for 24 hours in phorbol ester under whole-embryo culture conditions (Online Figure I). WT embryos exposed to PMA were severely affected (Online Figure I, A–D), with loss of anterior-posterior landmarks and yolk sacs with large cavities. In contrast, *Rasgrp3^{gt/gt}* mutant embryos incubated under the same conditions were surprisingly intact, and anterior-posterior landmarks were evident, along with intact yolk sacs (Online Figure I, E–H). We next incubated embryos in glucose 20 mmol/L for 24 hours to mimic the diabetic environment (Online Figure II). Glucose exposure between E8.5 and E9.5 led to vascular defects in WT embryos, and likely nonvascular defects such as perturbed somitogenesis and axis defects as well. In contrast, *Rasgrp3^{gt/gt}* mutant embryos were relatively refractory to the teratogenic effects of elevated glucose.

To more precisely define the effects of a diabetic environment on developing embryos, we induced diabetes in female mice, then set up matings and examined embryos. On an outbred background, WT embryos often showed quite severe defects, including aberrant somite formation and vessel patterning, rela-

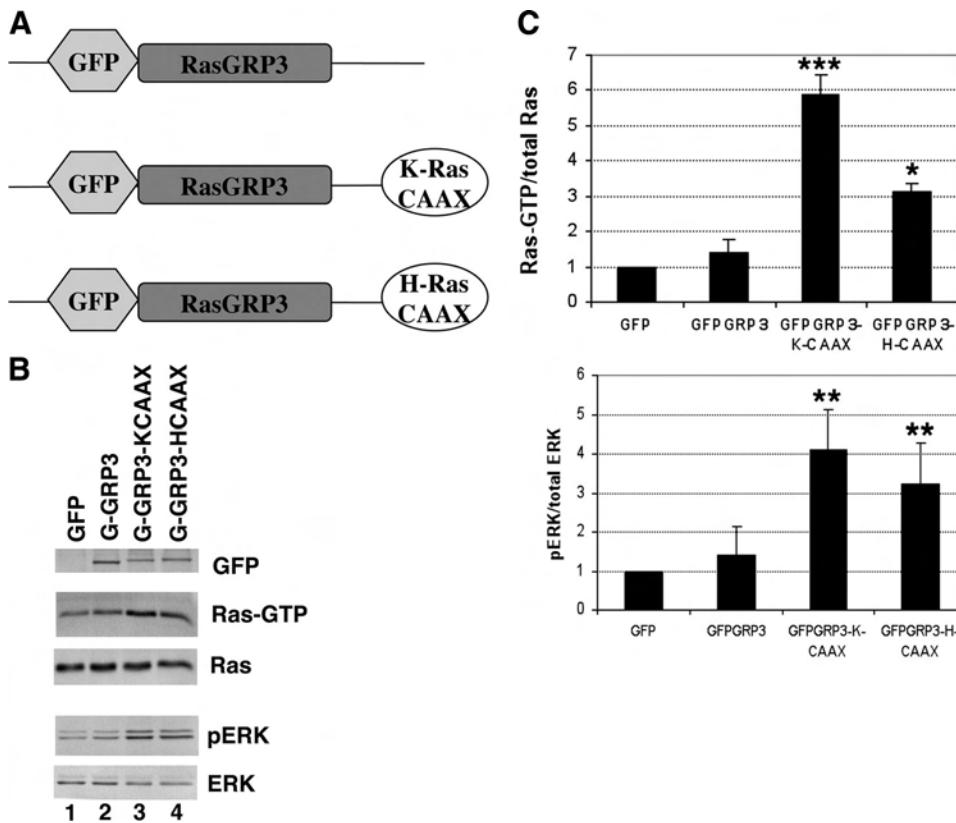


Figure 2. Overexpression of activated RasGRP3 activates Ras and ERK in endothelial cells. **A**, HUVECs were transiently transfected with the constructs shown. **B**, Immunoblotting for green fluorescent protein (GFP) linked to Ras-GRP3 constructs (**Top**) shows relative expression of the introduced genes; Ras-GTP panel is a Ras blot of Ras-RBD (Raf binding domain) immunoprecipitation to show activated Ras; Ras panel is 10% loading of input to show total Ras; pERK panel is blot hybridized with phosphorylated ERK (pERK; activated); ERK is total ERK blot. Lane 1, HUVECs transfected with GFP control; lane 2, HUVECs transfected with GFP-RasGRP3; lane 3, HUVECs transfected with GFP-RasGRP3-KCAAX; lane 4, HUVECs transfected with GFP-RasGRP3-HCAAX. **C**, Relative increase in reactivity for active Ras/total Ras (**Top**) or active ERK/total ERK (**Bottom**) relative to GFP control. * $P \leq 0.01$, ** $P \leq 0.002$, and *** $P \leq 0.0001$ relative to the relevant control.

tive to *Rasgrp3^{gt/gt}* mutant embryos (Online Figure III). To make more rigorous comparisons, we next examined embryos on the C57Bl6/J inbred background. We induced diabetes chemically via streptozotocin (STZ), and we also used mice carrying the *Ins2^{Akita}* mutation that induces diabetes genetically. We monitored the average maternal blood glucose at the time of euthanasia via tail bleed and found the following values: WT STZ, 381 mg/dL; *Rasgrp3^{gt/gt}* STZ, 448 mg/dL; *Ins2^{Akita}; Rasgrp3^{gt/gt}*, 387 mg/dL. Embryos were harvested from diabetic mothers at E9.5 and whole-mount stained for platelet endothelial cell adhesion molecule to visualize vessel development and patterning, and the severity of defects in head plexus vessels, intersomitic vessels, and trunk/somite was scored as described in Methods (Figure 1). WT embryos from mothers with diabetes that was induced either chemically or genetically had a significantly elevated developmental severity index compared with controls (Figures 1A, 1B, 1E, and 1O; Online Figure IV). Closer examination of the somite region showed defects in somites and in the pattern of intersomitic vessels (Figures 1G through 1J), and similar defects were seen in the vascular plexus of the head (Figures 1K through 1N). In contrast, embryos similarly developing in diabetic mothers but genetically deficient for *Rasgrp3* appeared relatively unaffected, and their developmental severity index was significantly lower than WT counterparts and close to control levels (Figures 1C through 1O; Online Figure IV). Analysis of yolk sacs from these embryos showed a low level of vascular defects that did not significantly correlate with genotype, diabetic condition, or the severity of the corresponding embryo (Online Figure V and data not shown). Thus, loss of *Rasgrp3* function had a protective effect on diabetes-induced developmental defects, including vascular defects, which sug-

gests that DAG-mediated activation of RasGRP3 mediates diabetes-induced birth defects.

Ras Is a Target of RasGRP3 in Endothelial Cells

To explore how RasGRP3 signaling contributes to diabetes-induced developmental defects, we first analyzed signaling in HUVECs that upregulated RasGRP3 activity, because the developmental defects are predicted to result from overactivation of RasGRP3 by DAG (Figure 2). WT RasGRP3 linked to a green fluorescent protein reporter and constructs in which a K-Ras CAAX or H-Ras CAAX sequence was linked to the protein were transiently expressed in HUVECs (Figure 2A). The CAAX sequences localize RasGRP3 to membranes,²⁰ which mimics DAG-promoted activation to place the activator in physical proximity to Ras. Thus, these constructs are predicted to be “constitutively active” in terms of their action on Ras and provide gain-of-function activity independent of any overexpression effects. Overexpression of WT RasGRP3 did not significantly increase Ras activation over baseline, but both of the CAAX-tagged RasGRP3 proteins stimulated significant Ras activation (Figures 2B and 2C). ERK activation is downstream of Ras activation, and in HUVECs, both CAAX-tagged RasGRP3 proteins also significantly induced ERK activation (Figures 2B and 2C). These data indicate that Ras is a target of RasGRP3 in endothelial cells.

To examine the cellular responses of endothelial cells to RasGRP3-mediated signaling, we examined the effects of RasGRP3 overactivation on the cytoskeleton, because in other cell types, Ras activation affects the cytoskeleton.^{30,31} HUVECs that expressed the constructs described above were stained for microtubules (α -tubulin, data not shown) and actin

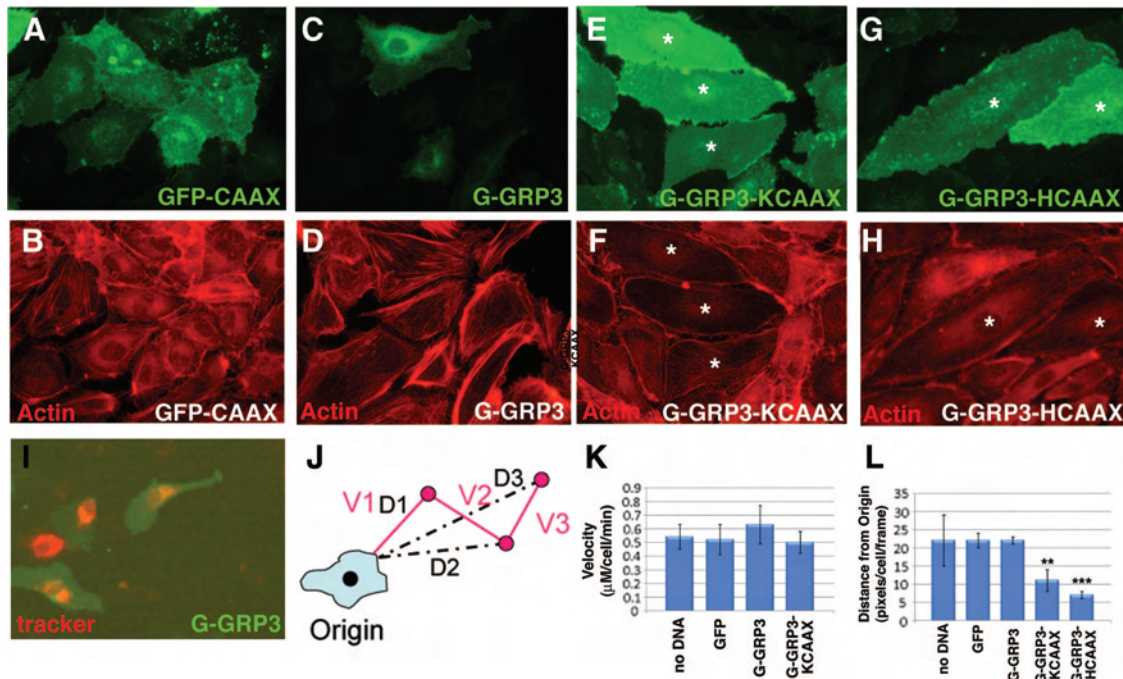


Figure 3. Overexpression of active RasGRP3 perturbs the actin cytoskeleton and migration of endothelial cells. A–H, HUVECs transfected with the indicated constructs were incubated for 48 hours, then fixed and imaged for green fluorescent protein (GFP, green; A–D) or stained with phalloidin (red; B–H). A and B, Control GFP-CAAX; C and D, GFP-RasGRP3; E and F, GFP-RasGRP3-KCAAX; G and H, GFP-RasGRP3-HCAAX. In E–H, * indicates transfected cells with perturbed phalloidin staining. I and J, HUVECs expressing GFP-RasGRP3 (green) and labeled with cell tracker (red). I, HUVECs expressing GFP-RasGRP3 (green) and labeled with cell tracker (red). J, Diagram showing how the average velocity (V) and distance from origin (D) were calculated from cell trajectories. K, Average velocity. L, Average distance from origin. ** $P \leq 0.005$ and *** $P \leq 0.0001$ relative to control.

(phalloidin; Figures 3A through 3H). The microtubule staining pattern was not affected by overexpression of RasGRP3 (data not shown); however, the actin cytoskeleton was dramatically altered in endothelial cells that overexpressed membrane-localized RasGRP3 (Figures 3E through 3H). RasGRP3-CAAX-expressing cells appeared larger and flatter, and they had very few actin stress fibers. Cortical actin was also diminished in endothelial cells that overexpressed RasGRP3-CAAX. Because actin stress fibers are implicated in proper migration, we investigated the migratory behavior of endothelial cells that overexpressed RasGRP3 (Figures 3I through 3L). Endothelial cells that overexpressed RasGRP3 were identified by green fluorescent protein expression, and all cells were labeled with cell tracker (Figure 3I). Cells were imaged, and the average velocity and distance migrated from origin were calculated as described in Methods (Figure 3J). None of the RasGRP3 proteins affected the overall velocity of the cells, but the CAAX-tagged RasGRP3 proteins both significantly inhibited the distance migrated from the origin (Figures 3K and 3L). These results show that endothelial cells that overexpress membrane-localized (and thus activated) RasGRP3 have a perturbed actin cytoskeleton and an attenuated ability to migrate in a forward direction.

To investigate the effects of loss of function for *Rasgrp3* on signaling pathway(s), endothelial cells were isolated and immortalized from WT and *Rasgrp3^{gt/gt}* mice. The cells were expanded clonally, and the endothelial identity of the cells was verified by expression of endothelial markers. The cell lines were more than 95% positive for platelet endothelial cell

adhesion molecule, vascular endothelial cadherin, and intercellular adhesion molecule-2 (Online Figure VI, A–D, and data not shown). RT-PCR analysis showed loss of RasGRP3 expression in *Rasgrp3^{gt/gt}* endothelial cells, and growth curves showed that WT and *Rasgrp3^{gt/gt}* endothelial cells grew at similar rates (Online Figure VI, E–F).

We investigated whether RasGRP3 was necessary for Ras and ERK activation downstream of DAG/phorbol ester stimulation. WT and *Rasgrp3^{gt/gt}* endothelial cells incubated with PMA were evaluated for changes in the levels of Ras and ERK activation. WT endothelial cells stimulated with PMA had elevated levels of active Ras and active ERK. In contrast, *Rasgrp3^{gt/gt}* endothelial cells stimulated with PMA showed no detectable Ras activation, and ERK activation was attenuated (Figure 4A; Online Figure X, A). These results reveal a requirement for RasGRP3 in Ras/ERK signaling downstream of DAG/phorbol ester stimulation in endothelial cells.

We next examined the signaling requirements downstream of RasGRP3 activation in endothelial cells of developing vessels using a mouse ES cell–differentiation model that supports the formation of primitive blood vessels in vitro via a programmed differentiation.^{29,32,33} We previously showed that ES cell–derived blood vessels respond to phorbol ester stimulation with a dramatic vessel dysmorphogenesis that is dependent on *Rasgrp3* function¹⁹ (Online Figure VII, A–D). We therefore investigated whether signaling downstream of activated RasGRP3 in developing vessels used the Ras effector pathways MEK/ERK, p38 MAPK, or JNK. The vessel dysmorphogenesis seen on PMA stimulation was

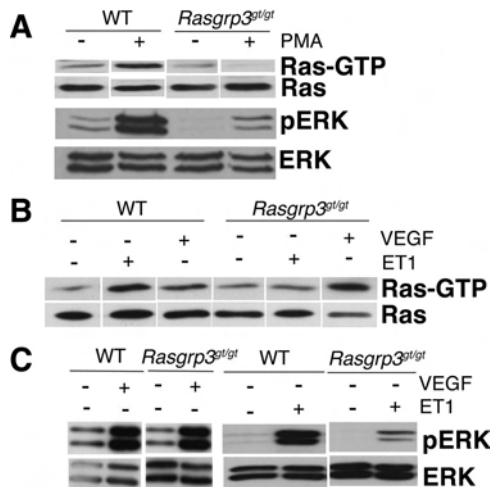


Figure 4. RasGRP3 is required for Ras-ERK activation downstream of phorbol ester and ET1 but not VEGF-A. **A**, WT and *Rasgrp3^{gt/gt}* endothelial cells were treated with PMA and processed for Ras and ERK activation. Compared with WT endothelial cells, *Rasgrp3^{gt/gt}* endothelial cells did not show increased activated Ras (Ras-GTP) and had an attenuated activation of phosphorylated ERK (pERK). **B**, WT and *Rasgrp3^{gt/gt}* endothelial cells were treated with ET1 or VEGF-A and processed for Ras activation. WT endothelial cells had increased levels of activated Ras (Ras-GTP) in response to both VEGF and ET1, whereas *Rasgrp3^{gt/gt}* endothelial cells had increased activated Ras (Ras-GTP) in response to VEGF-A but an attenuated response to ET1. **C**, WT and *Rasgrp3^{gt/gt}* endothelial cells were stimulated with ET1 or VEGF-A and processed for ERK activation. WT endothelial cells had increased levels of pERK in response to both VEGF and ET1, whereas *Rasgrp3^{gt/gt}* endothelial cells had increased pERK in response to VEGF-A but attenuated in response to ET1. Experiments are representative of at least 3 replicates.

significantly attenuated in the presence of the MEK inhibitor U0126 (Online Figure VII, E–F, I). In contrast, the p38 MAPK inhibitor SB203580 had no effect on PMA-induced vessel dysmorphogenesis (Online Figure VII, G–I), and the JNK inhibitor SP600125 showed a similar lack of effect (data not shown). Thus, RasGRP3-dependent DAG/phorbol ester signaling in developing vessels requires MEK but not p38 MAPK or JNK downstream of Ras GTPases.

Activation of RasGRP3 in B cells requires, in addition to membrane localization, phosphorylation by PKC. To test PKC function in endothelial cells, we exposed ES-derived vessels to PKC inhibitors concomitant with exposure to phorbol ester (Online Figure VIII). A general inhibitor of most PKC isoforms, BIM (bisindolylmaleimide), targets the kinase activity of PKC and not C1 domain interactions; thus, BIM inhibits PKC activation without affecting C1-domain-mediated RasGRP3 activation. BIM completely blocked phorbol ester–induced vessel dysmorphogenesis (Online Figure VIII, C–D, I). Likewise, an inhibitor of the conventional PKC isoforms α and β , Gö6976, completely blocked PMA-induced vessel dysmorphogenesis (Online Figure VIII, E–F, I). A third PKC inhibitor, rottlerin, predominantly affects PKC- δ , and it partially blocked PMA-induced vessel dysmorphogenesis (Online Figure VIII, G–I). These results show a requirement for PKC activity in phorbol ester–induced vessel dysmorphogenesis and suggest that endothelial RasGRP3 requires activation by PKC phosphorylation.

RasGRP3 Is Required for ET1-Mediated Ras and ERK Signaling in Endothelial Cells

Although phorbol ester mimics endogenous DAG production and signaling downstream of physiological inputs, it does not identify the physiological signals that normally activate RasGRP3-dependent signaling in endothelial cells. To identify physiologically relevant signals upstream of RasGRP3 in endothelial cells, we investigated signaling mediated by VEGF-A and ET1, 2 angiogenic factors that use DAG-Ras-ERK downstream signaling. WT and *Rasgrp3^{gt/gt}* endothelial cells were stimulated with VEGF or ET1, and levels of active Ras and ERK were analyzed (Figures 4B and 4C; Online Figure X, B). As predicted, WT endothelial cells had increased levels of active Ras and ERK on treatment with either VEGF or ET1. In contrast, although *Rasgrp3^{gt/gt}* endothelial cells had elevated levels of active Ras and ERK with VEGF treatment, they showed no increase in active Ras and attenuated active ERK with ET1 stimulation. Thus, RasGRP3 is required for Ras and ERK activation downstream of ET1 stimulation in endothelial cells. These results identify ET1 as a physiological signal for RasGRP3-mediated signaling in endothelial cells.

RasGRP3 Is Required for ET1-Induced Stimulation of Endothelial Cell Proliferation and Migration

Activation of Ras-ERK signaling by ET1 stimulates endothelial cell proliferation and migration.³⁴ To determine whether ET1-induced endothelial cell proliferation and migration were RasGRP3-dependent, WT and *Rasgrp3^{gt/gt}* endothelial cells were treated with ET1 and evaluated. WT endothelial cells exhibited more than a 2-fold increase in mitotic index over baseline with ET1 treatment, whereas *Rasgrp3^{gt/gt}* endothelial cells did not exhibit a significant change in their mitotic index with ET1 treatment (Figures 5A through 5E). In a Boyden chamber migration assay, VEGF induced migration of both WT and *Rasgrp3^{gt/gt}* endothelial cells significantly and to a similar degree; however, ET1 only stimulated migration of WT endothelial cells and did not significantly induce migration of *Rasgrp3^{gt/gt}* endothelial cells (Figure 5F). These results indicate that ET1-induced proliferation and migration require *Rasgrp3* function, whereas VEGF-induced migration occurs independent of RasGRP3.

RasGRP3 Mediates Vessel Dysmorphogenesis Induced by ET1

Because both ET1-induced endothelial cell proliferation and migration are RasGRP3-dependent, we investigated whether ES cell–derived blood vessels had RasGRP3-dependent dysmorphogenesis induced by ET1 (Figure 6). ET1 treatment of WT vessels led to loss of the fine vascular network and significantly increased vascular area (Figures 6A, 6B, and 6E). This response was *Rasgrp3*-dependent, because *Rasgrp3^{gt/gt}* vessels retained the fine vascular network and did not exhibit increased vascular area with ET1 treatment (Figures 6C through 6E). ET1-mediated vessel dysmorphogenesis was also dependent on PKC activity, because PKC inhibition blocked ET1 effects on vessels (Online Figure IX). Thus, developing vessels exhibited vessel dysmorphogenesis in response to ET1, and that response required RasGRP3 and PKC function. Taken together, these data suggest

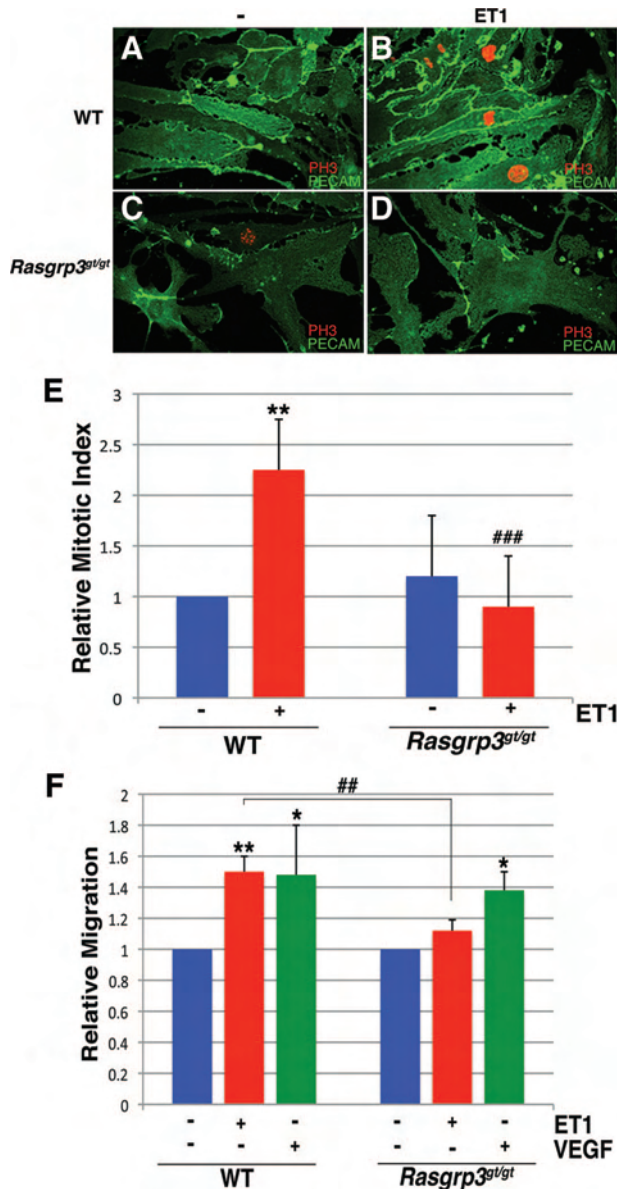


Figure 5. RasGRP3 mediates ET1-induced endothelial cell proliferation and migration. Control (A, C) or ET1-treated (B, D) WT (A, B) or *Rasgrp3^{gt/gt}* (C, D) endothelial cells were stained for platelet endothelial cell adhesion molecule (PECAM; green) and PH3 (red). E, Representative areas were imaged and mitotic cells counted. The relative mitotic index was increased significantly in WT endothelial cells treated with ET1 but not in *Rasgrp3^{gt/gt}* endothelial cells treated with ET1. ** $P \leq 0.001$ relative to WT untreated; ### $P \leq 0.001$ relative to ET1-treated WT endothelial cells. F, Transwell migration assays. WT endothelial cells had significantly increased migration toward both ET1 and VEGF-A compared with control, whereas *Rasgrp3^{gt/gt}* endothelial cells had significantly increased migration toward VEGF-A but not toward ET1. * $P \leq 0.01$ relative to untreated; ** $P \leq 0.0001$ relative to untreated; ### $P \leq 0.0001$, *Rasgrp3^{gt/gt}* ET1-treated versus WT ET1-treated.

that RasGRP3 is required for ET1-induced endothelial cell angiogenic responses.

Discussion

RasGRP3 is an activator of Ras family GTPases that is expressed in angiogenic vessels and is required for the DAG/phorbol ester-mediated responses of these vessels. Here, we

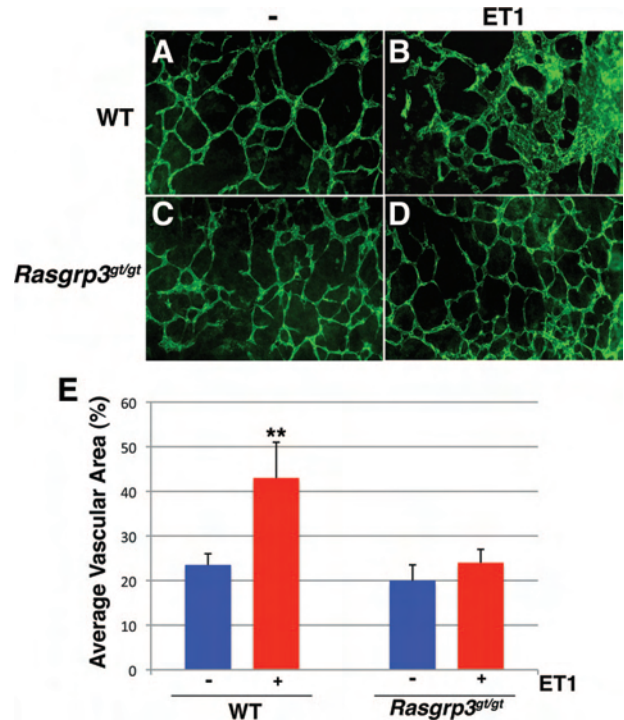


Figure 6. RasGRP3 mediates ET1-induced vessel dysmorphogenesis. ES cell cultures that were WT (A, B) or *Rasgrp3^{gt/gt}* (C, D) were untreated (A, C) or treated with ET1 (B, D), then fixed and stained for platelet endothelial cell adhesion molecule (green) on day 8. E, Representative images were quantified for vascular area. WT vessels treated with ET1 had significantly increased vascular area, whereas ET1-treated *Rasgrp3^{gt/gt}* vessels did not have increased vascular area. ** $P \leq 0.01$ relative to control.

posited that RasGRP3 is overactivated in embryos that develop in a diabetic environment and are susceptible to birth defects, and we showed that loss of RasGRP3 significantly attenuated the detrimental effects of a diabetic environment on embryonic vascular development. We also defined the molecular and cellular processes perturbed by DAG/phorbol ester-induced activation of RasGRP3 activation in endothelial cells of developing vessels. We defined ET1 as an upstream input and Ras as a target of RasGRP3 in endothelial cells, and we showed that manipulation of RasGRP3 perturbs endothelial migration. These data led to a model of RasGRP3-mediated signaling in endothelial cells that includes a molecular mechanism, cellular phenotype, and effects on the developing embryo in a diabetic environment (Figure 7).

We activated RasGRP3 in developing vessels using phorbol ester as a DAG mimic, and RasGRP3-dependent vessel dysmorphogenesis required MEK/ERK signaling. The present data indicate that Ras is a primary target of RasGRP3 in endothelial cells, because overexpression of RasGRP3 linked to either K-Ras CAAX or H-Ras CAAX domains significantly activated both Ras and ERK, whereas loss-of-function analysis showed that RasGRP3 was required for ET1- or DAG/phorbol ester-mediated Ras and MEK/ERK activation. The present data do not rule out that RasGRP3 activates other Ras GTPases in endothelial cells, but they suggest that signaling through Ras to MEK/ERK is critical for the vascular response to DAG. RasGRP3 is normally activated by

ing somites and developing vessels, 2 embryonic organs that express RasGRP3.¹⁹ The vascular defects seen in WT embryos from diabetic mothers were consistent with the endothelial migration defects observed in primary endothelial cells, because the intersomitic vessels were sometimes blunted, with expanded migratory fronts. Interestingly, although yolk sac vessels showed some pattern defects, these did not correlate significantly with embryo genotype, diabetic environment, or overall embryo defects, in contrast to another group who described significant diabetes-induced yolk sac vasculopathy.^{4,47} This indicates that the observed diabetes-induced embryonic defects were not secondary to yolk sac defects that compromised overall embryonic health.

Was the protection afforded by loss of RasGRP3 from the maternal compartment, the embryonic compartment, or both? Several lines of evidence support a critical role for embryonic *Rasgrp3* function in mediating the effects of diabetes. First, another study showed that DAG levels and activated PKC are elevated in embryos from diabetic mothers,³ which indicates that upstream requirements for RasGRP3 activation are in place in the embryo. In the present study, mothers that lacked RasGRP3 became diabetic, and their average blood glucose at the time of euthanasia was higher than WT controls. Whole-embryo culture with medium supplemented with PMA showed that embryos that lacked *Rasgrp3* were significantly protected from the severe PMA-induced perturbations seen in WT embryos. Finally, embryos exposed to elevated glucose had increased defects that were RasGRP3-dependent, which shows that the RasGRP3 status of the embryo is critical to its response to maternal glucose. Taken together, these findings indicate that lack of *Rasgrp3* function in the embryo is critical for protection from developmental defects, and that potential maternal effects of *Rasgrp3* loss do not impact the elevated glucose levels that lead to elevated DAG and diabetes.

The finding that RasGRP3 mediates the effects of a diabetic environment on embryonic development, including vascular development, suggests that RasGRP3 may be a new and useful therapeutic target for prevention of diabetes-associated birth defects. The lack of embryonic defects with *Rasgrp3* loss of function under normal conditions (the present study and Roberts et al¹⁹) indicates that blockade of RasGRP3 is not detrimental to overall development. The focus of the present study was analysis of gain of function and loss of function for RasGRP3 in developing vessels and endothelial cells, and we have shown that RasGRP3-mediated signaling uses Ras/ERK as a target and mediates ET1 effects on angiogenesis. It will be interesting to determine whether RasGRP3-mediated signaling is important in adult vascular pathologies.

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Disclosures

None.

References

- Olsson A-K, Dimberg A, Kreuger J, Claesson-Welsh L. VEGF receptor signaling: in control of vascular function. *Nat Rev Mol Cell Biol*. 2006; 7:359–371.
- Evcimen ND, King GL. The role of protein kinase C activation and the vascular complications of diabetes. *Pharmacol Res*. 2007;55:499–510.
- Hiramatsu Y, Sekiguchi N, Hayashi M, Isshiki K, Yokota T, King GL, Loeken MR. Diacylglycerol production and protein kinase C activity are increased in a mouse model of diabetic embryopathy. *Diabetes*. 2002;51: 2804–2810.
- Pinter E, Mahooti S, Wang Y, Imhof BA, Madri JA. Hyperglycemia-induced vasculopathy in the murine vitelline vasculature: correlation with PECAM-1/CD31 tyrosine phosphorylation state. *Am J Pathol*. 1999;154: 1367–1379.
- Lynch J, Ferro T, Blumenstock F, Brockenauer A, Malik AB. Increased endothelial albumin permeability mediated by protein kinase C activation. *J Clin Invest*. 1990;85:1991–1998.
- Mason J, Yarwood H, Sugars K, Haskard D. Human umbilical vein and dermal microvascular endothelial cells show heterogeneity in response to PKC activation. *Am J Physiol Cell Physiol*. 1997;273:1233–1240.
- Takahashi T, Shibuya M. The overexpression of PKCdelta is involved in vascular endothelial growth factor-resistant apoptosis in cultured primary sinusoidal endothelial cells. *Biochem Biophys Res Commun*. 2001;280: 415–420.
- Huang Q, Yuan Y. Interaction of PKC and NOS in signal transduction of microvascular hyperpermeability. *Am J Physiol*. 1997;273: H2442–H2451.
- Bogatcheva N, Verin AD, Wang P, Birukova A, Birukov K, Mirzopoyazova T, Adyshev D, Chiang E, Crow M, Garcia JG. Phorbol esters increase MLC phosphorylation and actin remodeling in bovine lung endothelium without increased contraction. *Am J Physiol Lung Cell Mol Physiol*. 2003;285:L415–L426.
- Newton A. Diacylglycerol's affair with protein kinase C turns 25. *Trends Pharmacol Sci*. 2004;25:175–177.
- Brose N, Rosenmund C. Move over protein kinase C, you've got company: alternative cellular effectors of diacylglycerol and phorbol esters. *J Cell Sci*. 2002;115:4399–4411.
- Mitin N, Rossman K, Der CJ. Signaling interplay in Ras superfamily function. *Curr Biol*. 2005;15:R563–R574.
- Lambert QT, Reuther GW. Activation of Ras proteins by Ras guanine nucleotide releasing protein family members. *Methods Enzymol*. 2006; 407:82–98.
- Verin AD, Liu F, Bogatcheva N, Borbiev T, Hershenson MB, Wang P, Garcia JG. Role of ras-dependent ERK activation in phorbol ester-induced endothelial cell barrier dysfunction. *Am J Physiol Lung Cell Mol Physiol*. 2000;279:L360–L370.
- Dower NA, Stang SL, Bottorff DA, Ebinu JO, Dickie P, Ostergaard HL, Stone JC. RasGRP is essential for mouse thymocyte differentiation and TCR signaling. *Nat Immunol*. 2000;1:317–321.
- Oh-hora M, Johmura S, Hashimoto A, Hikida M, Kurosaki T. Requirement for Ras guanine nucleotide releasing protein 3 in coupling phospholipase C-gamma2 to Ras in B cell receptor signaling. *J Exp Med*. 2003;198:1841–1851.
- Coughlin JJ, Stang SL, Dower NA, Stone JC. RasGRP1 and RasGRP3 regulate B cell proliferation by facilitating B cell receptor-Ras signaling. *J Immunol*. 2005;175:7179–7184.
- Suzuki K, Takahashi T, Haramoto Y, Onuma Y, Nagamine K, Okabayashi K, Hashizume K, Iwanaka T, Asashima M. XRASGRP2 is essential for blood vessel formation during *Xenopus* development. *Int J Dev Biol*. 2010;54:609–615.
- Roberts DM, Anderson AL, Hidaka M, Swetenburg RL, Patterson C, Stanford WL, Bautch VL. A vascular gene trap screen defines RasGRP3 as an angiogenesis-regulated gene required for the endothelial response to phorbol esters. *Mol Cell Biol*. 2004;24:10515–10528.
- Yamashita S, Mochizuki N, Ohba Y, Tobiume M, Okada Y, Sawa H, Nagashima K, Matsuda M. CalDAG-GEFIII activation of Ras, R-ras, and Rap1. *J Biol Chem*. 2000;275:25488–25493.

21. Lorenzo PS, Kung JW, Bottorff DA, Garfield SH, Stone JC, Blumberg PM. Phorbol esters modulate the Ras exchange factor RasGRP3. *Cancer Res.* 2001;61:943–949.
22. Aiba Y, Oh-hora M, Kiyonaka S, Kimura Y, Hijikata A, Mori Y, Kurosaki T. Activation of RasGRP3 by phosphorylation of Thr-133 is required for B cell receptor-mediated Ras activation. *PNAS.* 2004;101:16612–16617.
23. Zheng Y, Liu H, Coughlin JJ, Zheng J, Li L, Stone JC. Phosphorylation of RasGRP3 on threonine 133 provides a mechanistic link between PKC and Ras signaling systems in B cells. *Blood.* 2005;105:3648–3654.
24. Okamura SM, Oki-Idouchi CE, Lorenzo PS. The exchange factor and diacylglycerol receptor RasGRP3 interacts with dynein light chain 1 through Its C-terminal domain. *J Biol Chem.* 2006;281:36132–36139.
25. Kearney JB, Ambler CA, Monaco KA, Johnson N, Rapoport RG, Bautch VL. Vascular endothelial growth factor receptor Flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division. *Blood.* 2002;99:2397–2407.
26. Nagy A, Gertsenstein M, Vintersten K, Behringer R. *Manipulating the Mouse Embryo*. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Press; 2003.
27. Balconi G, Spagnuolo R, Dejana E. Development of endothelial cell lines from embryonic stem cells: a tool for studying genetically manipulated endothelial cells in vitro. *Arterioscler Thromb Vasc Biol.* 2000;20:1443–1451.
28. Zayad M, Yuan W, Leisner T, Chalothorn D, McFadden A, Schaller M, Hartnett M, Faber J, Parise L. CIB1 regulates endothelial cells and ischemia-induced pathological and adaptive angiogenesis. *Circ Res.* 2007;101:1185–1193.
29. Kearney JB, Bautch VL. In vitro differentiation of mouse ES cells: hematopoietic and vascular development. *Methods Enzymol.* 2003;365:83–98.
30. Scuto A, Zhang H, Zhao H, Rivera M, Yeatman TJ, Jove R, Torres-Roca JF. RbAp48 regulates cytoskeletal organization and morphology by increasing K-Ras activity and signaling through mitogen-activated protein kinase. *Cancer Res.* 2007;67:10317–10324.
31. Shinohara M, Shang W-H, Kubodera M, Harada S, Mitsushita J, Kato M, Miyazaki H, Sumimoto H, Kamata T. Nox1 redox signaling mediates oncogenic Ras-induced disruption of stress fibers and focal adhesions by down-regulating Rho. *J Biol Chem.* 2007;282:17640–17648.
32. Keller G. Embryonic stem cell differentiation: emergence of a new era in biology and medicine. *Genes Dev.* 2005;19:1129–1155.
33. Jakobsson L, Kreuger J, Claesson-Welsh L. Building blood vessels: stem cell models in vascular biology. *J Cell Biol.* 2007;177:751–755.
34. Ribatti D, Conconi M, Nussdorfer G. Nonclassic endogenous novel regulators of angiogenesis. *Pharmacol Rev.* 2007;59:185–205.
35. Pawlak G, Helfman DM. Cytoskeletal changes in cell transformation and tumorigenesis. *Curr Opin Genet Dev.* 2001;11:41–47.
36. Pollock CB, Shirasawa S, Sasazuki T, Kolch W, Dhillon AS. Oncogenic K-RAS is required to maintain changes in cytoskeletal organization, adhesion, and motility in colon cancer cells. *Cancer Res.* 2005;65:1244–1250.
37. Shields JM, Pruitt K, McFall A, Shaub A, Der CJ. Understanding Ras: “it ain’t over ‘til it’s over.” *Trends Cell Biol.* 2000;10:147–154.
38. Castro Barros J, Marshall CJ. Activation of either ERK1/2 or ERK5 MAP kinase pathways can lead to disruption of the actin cytoskeleton. *J Cell Sci.* 2005;118:1663–1671.
39. Pellegrin S, Mellow H. Actin stress fibers. *J Cell Sci.* 2007;120:34910–33499.
40. Ridley AJ, Schwartz MA, Burridge K, Firtel R, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR. Cell migration: integrating signals from front to back. *Science.* 2003;302:1704–1709.
41. Lo C-M, Buxton DB, Chua GCH, Dembo M, Adelstein RS, Wang Y-L. Nonmuscle myosin IIB is involved in the guidance of fibroblast migration. *Mol Biol Cell.* 2004;15:982–989.
42. Rid R, Schiefermeier N, Grigoriev I, Small J, Kaverina I. The last but not the least: the origin and significance of trailing adhesions in fibroblastic cells. *Cell Motil Cytoskeleton.* 2005;61:161–171.
43. Kalani M. The importance of endothelin-1 for microvascular dysfunction in diabetes. *Vasc Health Risk Manag.* 2008;4:1061–1068.
44. Cruz A, Parnot C, Ribatti D, Corvol P, Gasc J-M. Endothelin-1, a regulator of angiogenesis in the chick chorioallantoic membrane. *J Vasc Res.* 2001;38:536–545.
45. Salani D, Tarabozetti G, Rosano L, Di Castro V, Borsotti P, Giavazzi R, Bagnato A. Endothelin-1 induces an angiogenic phenotype in cultured endothelial cells and stimulates neovascularization *in vivo*. *Am J Pathol.* 2000;157:1703–1711.
46. Yoshioka M, Kayo T, Ikeda T, Koizumi A. A novel locus, MODY4, distal to D7MIT189 on chromosome 7 determines early-onset NIDDM in nonobese C57Bl/6 (AKITA) mutant mice. *Diabetes.* 1997;46:887–894.
47. Pinter E, Haigh J, Nagy A, Madri JA. Hyperglycemia-induced vasculopathy in the murine conceptus is mediated via reductions of VEGF-A expression and VEGF receptor activation. *Am J Pathol.* 2001;158:1199–1206.

Novelty and Significance

What Is Known?

- Diabetes leads to elevation of diacylglycerol and endothelin and blood vessel dysfunction.
- Maternal diabetes leads to birth defects.
- Diacylglycerol activates RasGRP3 (Ras guanyl releasing protein 3), a mediator of cell signaling.

What New Information Does This Article Contribute?

- The Ras activator RasGRP3 mediates birth defects associated with diabetes.
- RasGRP3 affects the migration of endothelial cells that form blood vessels.
- RasGRP3 is required for major vessel responses to diacylglycerol and endothelin.

This study focused on the Ras activator, RasGRP3, in blood vessel disruptions induced by diabetes. We show that a lack of RasGRP3 protects mouse embryos from diabetes-induced birth

defects in blood vessels and other tissues. Endothelial cells that expressed activated RasGRP3 had elevated levels of active Ras and ERK. Endothelial cells that lacked RasGRP3 did not activate Ras or ERK in response to a surrogate of diacylglycerol, PMA (phorbol 12-myristate 13-acetate) or endothelin-1. Endothelin-mediated endothelial cell proliferation, migration, and blood vessel formation were attenuated in vessels that lacked RasGRP3. Thus, RasGRP3 is necessary and sufficient for the activation of Ras signaling in endothelial cells and in developing blood vessels in response to signals that are elevated in diabetes. Moreover, RasGRP3 is required for the full spectrum of vascular and nonvascular birth defects induced by diabetes. Our work for the first time demonstrates a critical role for RasGRP3 in the complications of diabetes and reveals a link between RasGRP3 and endothelin-mediated signaling. It also defines Ras as a RasGRP3 target in endothelial cells. This work advances our understanding of pathways that mediate diabetic complications and is significant in the suggestion that RasGRP3 may be a novel therapeutic target for fetal complications induced by diabetes.

1. DETAILED METHODS

Diabetic mice

Female mice (C57Bl6/J purchased from Jackson Laboratories or *Rasgrp3^{gt/gt}* mice backcrossed to N8 on the C57Bl6/J background) at 6-8 weeks of age were made diabetic following the protocol in “Animal Models of Diabetic Complications Consortium”. Mice were fasted and injected IP with streptozotocin (STZ) (stored at -20°C and dissolved to a final concentration of 7.5 mg/mL in 0.1M Na-Citrate buffer (pH 4.5) immediately prior to injection), at a dose of 50 mg STZ/kg of body weight, daily for 5 days. Controls were injected with citrate buffer alone. Alternatively, *Ins2^{Akita}/+* mice on the C57Bl6/J background (Jackson Laboratories) were bred to obtain *Ins2^{Akita}/+*; *Rasgrp3^{gt/gt}* mice. Blood glucose was monitored weekly using a OneTouch Ultra device and a drop of blood from a tail nick. Mice were considered diabetic with blood glucose levels over 250mg/dL. Most mice were diabetic 2-3 weeks after the last injection and remained diabetic long-term. Mice were mated to genotype-matched males.

Embryo and yolk sac analysis

Embryos were harvested at E9.5, fixed in fresh 4% PFA (paraformaldehyde) overnight at 4°C, and whole mount stained for PECAM as described¹. Briefly, embryos were washed 15 min in PBS, dehydrated through a methanol:PBS series, blocked in 5% H₂O₂/methanol 4 hr at RT, then stored overnight in 100% methanol at -20°C. They were rehydrated through a methanol:PBS series, blocked 2 x 1 hr in PBT (0.2%BSA, 0.1% Triton X-100 in PBS), incubated overnight at 4°C in 1:200 rat anti-mouse CD-31 (PECAM) primary antibody (BD Biosciences) in PBT, rinsed 5 x 1hr in PBT and incubated overnight at 4°C in 1:200 goat anti-rat HRP-conjugated secondary antibody (Kirkegaard & Perry, cat. #474-1612) in PBT. Embryos were rinsed 5 x 1hr in PBT, then developed for 20 min at RT in DAB solution (Vector Laboratories, cat. #SK-4100). After developing, embryos were rinsed 2 x 5 min in PBT, 2 x 5 min in PBS, and fixed overnight at 4°C in 2% PFA/0.1% glutaraldehyde in PBS. Embryos were rinsed 3 x 5 min in PBS, then stored in PBS at 4°C. Stained embryos were imaged with an Olympus SZH10 stereo microscope outfitted with a with a Olympus DP71 camera. Embryos were scored for developmental defects on a scale of 0-3, and defects included aberrant intersomitic vessels, lack of neural tube closure, perturbed somite patterning, lack of remodeling and excessive vessel coverage in the head plexus, gaps or excessive vessels dorsal to the somites, and overall body-axis deformities. Embryos with no defects were scored 0; embryos with a single minor defect were scored 1; embryos with several defects were scored 2; and embryos with the most comprehensive and severe defects were scored 3.

Yolk sacs were removed from the embryos after the rehydration step, and stained separately for PECAM as described² with minor modifications. Briefly, yolk sacs were blocked in 5% goat serum, 1% BSA in PBST (PBS with 0.1% Triton 100X) overnight at 4°C, and then incubated in primary antibody (PECAM CD31, BD Pharmingen #557355, rat anti-mouse) at 1:200 in PBST overnight at 4°C. Yolk sacs were washed in SBT (PBS with 0.1% Triton 100X and 2.5% goat serum), 4 times over at least 4 hr. Yolk sacs were then incubated in secondary antibody (Alexa Fluor 488, Molecular Probes #A-11006, goat

anti-rat) overnight at 4°C and washed in SBT 4 times over at least 5 hr, and finally washed in PBS. Yolk sacs were then mounted flat on glass slides with Vectashield Hardest and allowed to harden, then imaged using an Olympus IX 50 Epifluorescence microscope.

Whole embryo culture

Whole embryo culture was performed as described, with minor modifications³. Embryos were dissected into M2 media from maternal decidua with their yolk sacs intact. Dissections were performed at E7.5 (for PMA experiments) or E8.5 (for glucose experiments), and embryos were cultured for 24 hr in 25 mL Nunc screwcap tubes on rollers at 37°C. Culture media contained 50% rat serum, 50% Tyrode's salt solution, and 0.1X penicillin/streptomycin and was sterile filtered using a 0.2 μ M filter. A minimum of 0.75 mL of media was used per embryo. During culture, the media was treated at 12-hr intervals with a gas mixture containing 20% O₂ and 5% CO₂ and the tubes were sealed with vacuum grease. Some embryos were treated with 50nM PMA (phorbol 12-myristate 13-acetate) and some had 20 mM D-glucose added to the media during the culture period. After 24 hr, embryos were removed from the media and photographed.

Endothelial cells

HUVEC

RasGRP3 was amplified by PCR cloning from with appropriate restriction sites at the ends, cloned into pBSISK+/CAAX vector (kindly provided by Dr. Channing Der), and verified by sequence analysis. This version was shuttled into pEGFP-C1 (Clontech, Genbank #U55763) so that eGFP was linked to the N-terminus of RasGRP3-CAAX, and expression was from the CMV promoter. GFP-RasGRP3 was generated by excision of the CAAX domain using Hind III sites.

HUVEC (purchased from Clonetics) were cultured in EGM-2 medium (Lonza) on gelatin-coated dishes and used between passages 2-8. 1×10^6 cells in HUVEC optimized buffer (Amaxa) were transfected with 5 μ g DNA using a nucleofector (Amaxa, #VPB-1492) and HUVEC optimized kit, according to manufacturer's directions. Fresh medium was added 2 hr post-transfection.

For staining, transfected HUVEC were plated in 4-chamber slides, and 48 hr post-transfection starved for 6 hr in HUVEC medium with 0.1% FBS and no growth factors. Cells were fixed in 3.7% formaldehyde for 12 min, permeabilized in PBS/0.1% Triton for 5 min, blocked in PBS/1% BSA for 20 min, and incubated with phalloidin-Alexa 555 (Molecular Probes) diluted 1:50 in blocking buffer for 20 min. Fixation and staining was at RT.

Migration assays were done with HUVEC at 48 hr post-transfection. Cells in slide flasks were labeled with Cell tracker Red (Molecular Probes), and imaged in green and red channels over a 5 hr period with a Nikon TE300 Inverted Microscope (Melville NY) with a Perkin Elmer spinning disc confocal head (Shelton CT) and outfitted with a heated stage. Images were collected at 1 min intervals using Metamorph software (version 6.3r5; Universal Imaging Corp, Downingtown PA) and a Hamamatsu Orca CCD camera

(McHenry IL). Average velocity and distance to origin of cells was calculated using trajectory measures as diagrammed in Fig. 3J.

Mouse endothelial cells

Mouse endothelial cells (wildtype and *Rasgrp3^{gt/gt}*) were generated and expanded as described^{4,5}, with minor modifications. Lungs of P6 wildtype (WT) and *Rasgrp3^{gt/gt}* mice were minced and digested with collagenase (Type 1, #CLS-1, Worthington, Lakewood NJ), diluted to 2 mg/ml in PBS containing Ca⁺⁺ and Mg⁺⁺, for 1 hr at 37°C, and, then incubated with magnetic beads conjugated to anti-mouse PECAM antibody for 20 min at RT. Bead-selected cells were incubated with PyMT virus (2.5 x 10⁷ cfu/ml, cells a gift of Dr. William Sessa) and 8 mg/ml polybrene diluted in complete EBM-2 medium (Bullet kit, Lonza CA) for 8 hr/day for 3 days; cells were allowed to recover in complete EBM-2 medium overnight between virus incubations. Following incubation, cells were treated with 500 mg/ml Geneticin (G418, CalBiochem) for 14 days. Drug-resistant clones were isolated using cloning rings and expanded. Cells were maintained in complete EBM-2 media and incubated at 37°C in 5% CO₂. Growth curves were generated by plating 5 x 10³ cells/well, and trypsinized cells were counted daily using a hemocytometer.

Cells were washed in 1X PBS, fixed with 1:1 cold methanol-acetone or 4% paraformaldehyde (PFA) for 10 min, washed twice in 1X PBS, then blocked with staining media (3% FBS, 0.1% sodium azide in 1X PBS) for 1 hr at 37°C. Cells were incubated in appropriate primary antibodies at 1:1000 in staining media overnight at 4°C. Primary antibodies used were rat anti-mouse PECAM (MEC13.3, #553370, BD Biosciences), rat anti-mouse ICAM-2 (CD102, #553326, BD Biosciences), rat anti-mouse VE-cadherin (CD144, #550548, BD Biosciences), and rabbit anti-phospho-histone H3 (Ser10, #06-570, Millipore). Cultures were washed 2 X in 1X PBS, incubated in AlexaFluor488 goat anti-rat IgG (1:1000) or Alexa Fluor 594 donkey anti-rabbit IgG (#A21206, Invitrogen) diluted in staining media for 2 hr at RT, washed 2 X in 1X PBS and imaged using an inverted epifluorescence microscope (IX-50; Olympus) outfitted with a camera (DP71; Olympus), or a confocal microscope (LSM 5 Pascal, Carl Zeiss, Inc.) using PASCAL Release version 4.2 SP1 acquisition software (Carl Zeiss, Inc.).

Endothelial cells were harvested and RNA isolated using Trizol (Invitrogen). RNA was purified using the RNAeasy kit (Qiagen), and cDNA was generated as described⁶. Equivalent amounts of cDNA were amplified by RT-PCR using RasGRP3 primers (forward 5'-AGAGAACCACTGCCTCGTAC-3'; reverse 5'-GTGTTGCCGCTT TCCCGAGC-3') and *lacZ* primers (forward 5'-TTGAAAATGGTCTGCTGCTG-3'; reverse 5'-TTGGCTTCATCCACCACATA-3') as described⁷. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers (forward 5'-AGCCCATCACCATCTTCC-3'; reverse 5'-GCCATCCACAGTCTTCTGG-3') were used as a control.

For mitotic index determination, cells were plated at 2.5 x 10⁴ cells in chamber slides, serum-starved in 1% FBS overnight, then stimulated with 100nM ET1 in 1% FBS for 24 hr. Cells were fixed with 1:1 methanol: acetone and stained for PECAM and PH3 as described. Ten images from each sample were taken at 10X magnification, and the mitotic index was calculated by counting the number of PH3-positive cells divided by the PECAM-positive cells. For migration assays, Boyden chambers were used as described

⁸, with minor modifications. Transwell chambers fitted with 8- μ m pore size filter membranes (Corning Incorporated, Corning NY) were immersed in medium. Cell suspensions were prepared in 1% FBS, and 2×10^4 cells in 100 μ l were plated in the upper chamber. Cells were incubated overnight at 37°C, then incubated with 600 μ l of 1% FBS containing 2.5 nM VEGF-A, or 100 nM ET1, placed in the bottom of each transwell chamber for 6 hr at 37°C. Cells on the upper surface of the filter were removed, and cells on the bottom surface of the filter were fixed and stained with Hema 3 stain (#122-911, Fisher, Pittsburgh PA). Migration was quantified by counting the total number of cells in 10 fields per chamber at 20X magnification using an Olympus IX-50 epifluorescence microscope attached to a camera.

ES cell differentiation

Wild type (WT, ^{+/+}) and RasGRP3 deficient (*Rasgrp3^{gt/gt}*, where *gt* represents a gene trap insertion in the RasGRP3 locus that results in a loss-of-function mutation ⁷) ES cells were maintained and differentiated in vitro as previously described ⁹. For inhibitor studies, wild type ES cell cultures were differentiated to day 7, and then pre-treated with the appropriate inhibitor for 2 hr prior to PMA (phorbol 12-myristate 13-acetate) or ET1 addition. Cultures were stimulated with 100 nM PMA for 24 hr, and then fixed with 1:1 methanol/acetone and stored in PBS at 4°C. Unless otherwise stated, inhibitors remained on the ES cell cultures for the duration of PMA treatment. Dose curves ranging from 1 μ M to 20 μ M were performed, and the most effective concentration that maintained wild-type vessels was used. Inhibitor concentrations used were: FTI-2153 (farnesyl transferase inhibitor) at 10 μ M, U0126 (MEK inhibitor) at 10 μ M, SB203580 (p38 MAPK inhibitor) at 5 μ M, SP600125 (JNK inhibitor) at 10 μ M, Bisindolymaleimide I (BIM; general PKC inhibitor) at 10 μ M, Gö6976 (PKC α and PKC β inhibitor) at 6 μ M, and rottlerin (PKC δ inhibitor) at 10 μ M. Inhibitors were purchased from Calbiochem (San Diego, CA). For ET1 studies, ES cultures were treated with fresh medium containing 100 nM ET1 daily on days 5-8 or fresh medium containing 10 μ M ET1 on day 7-8, then fixed with 1:1 methanol:acetone and stored in PBS at 4°C.

Antibody staining

Antibody staining of ES cell cultures was as previously described ^{1, 9}. Briefly, fixed ES cell cultures were blocked in staining media (3% fetal bovine serum and 1% sodium azide in 1X PBS) for 1 hr at 37°C. Cultures were then incubated with rat anti-mouse PECAM (Mec 13.3; BD Pharmingen, San Diego, CA) at 1:1000 in staining media for 1 hr at 37°C and subsequently washed 3 times with staining media. The cultures were incubated with goat anti-rat Alexa 488 (Molecular Probes, Eugene, OR) at 1:200 for 1 hr at 37°C, rinsed in staining medium 3 times, then stored in 1X PBS at 4°C. Staining was visualized using either an Olympus IX-50 inverted microscope and epifluorescence, or a Zeiss 510 confocal microscope.

Quantitative image analysis

Quantitative image analysis of PECAM-stained day 8 ES cell cultures was performed as previously described ¹. Briefly, 10X images of non-overlapping areas with complete cell coverage were acquired. In general, the imaged area of each well was more than 60% of the total area. Images were analyzed using Metamorph software to determine the percent area of PECAM staining normalized to controls. On average, three

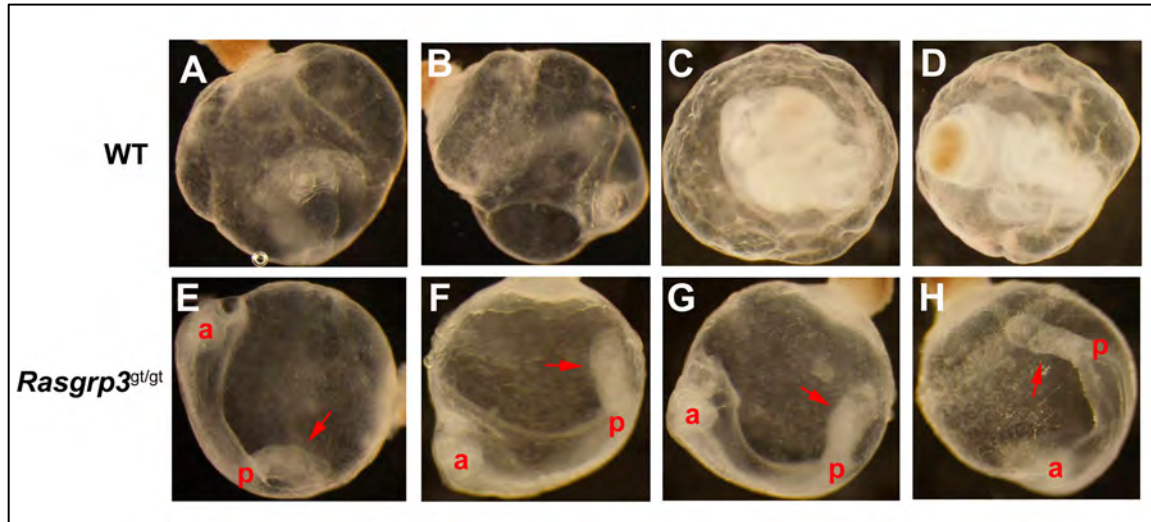
to four wells of each condition were used for analysis. All values were compared by the two-tailed Student's T test, and p values <0.05 were considered significant.

Ras and ERK activation assays

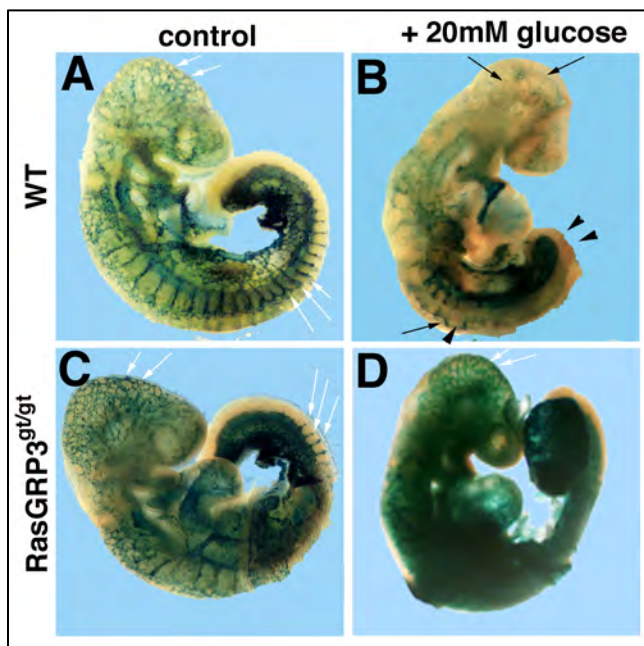
Ras and ERK activation assays were performed as described, with minor modifications^{10, 11}. HUVEC were grown to near confluency for 48 hr after transfection (50-80% transfected by GFP labeling), serum starved as described overnight, then put in lysis buffer (50 mM Tris (pH 7.4), 0.15 M NaCl, 1% Triton, 10% glycerol, 1 mM EDTA, 10mM MgCl₂) with protease inhibitors. Mouse endothelial cells were grown to 60% confluence, serum-starved in 0.1% FBS/EBM-2 media overnight, then left untreated or treated with 100 nM PMA, 5 nM VEGF-A, or 100 nM ET1 diluted in 0.1% FBS/EBM-2 medium for 30 min (PMA) or 5 min (VEGF-A, ET1) prior to lysis. Ras activation assays were performed using the Ras Activation Assay Kit (Upstate, Lake Placid, NY) per manufacturer's protocol, and 10% of the lysate was used for total Ras determination. The remaining lysate was incubated with 10 µl of agarose beads conjugated to the Ras binding domain of Raf (RBD) for 1 hr at 4°C, briefly centrifuged to pellet the beads, then washed with lysis buffer. Samples were suspended in 50 µl of 2X sample buffer and boiled for 5 min before loading on a 15% acrylamide gel for electrophoresis. Proteins were transferred to a PVDF membrane, blocked in 5% milk in 1X TBST (Tris-Buffered Saline with 0.1% Tween), and incubated with mouse monoclonal anti-Ras antibody (Clone Ras10, Upstate) at 1:1000 in blocking solution overnight at 4°C. After washing 3 times for 15 min, the membrane was incubated for 2 hr at RT with a goat anti-mouse secondary antibody conjugated to HRP (Upstate) at 1:5000 in blocking solution. After washing the membrane was developed using ECL Plus according to the manufacturer's instructions.

For phospho-ERK blots, most conditions were the same as for Ras assays, except cells were lysed in RIPA buffer and loaded on a 10% acrylamide gel. Primary antibody incubation was with rabbit anti-mouse pERK (Cell Signaling) at 1:1000 overnight at 4°C, and total ERK was determined by incubation with mouse anti-mouse ERK1/2 (Abcam) at 1:1000 overnight at 4°C. Secondary antibody incubation was with goat anti-rabbit HRP or goat anti-mouse HRP (Promega) at 1:5000 for 2 hr at RT. Blots were quantified using the integrated density function of ImageJ (Version 1.44a, NIH, USA) or a phosphoimager (Storm 840, Molecular Dynamics, Sunnyvale CA). Ras-GTP was first normalized to total Ras, then normalized to untransfected WT HUVEC. pERK intensities were divided by total ERK, then normalized to untransfected WT HUVEC.

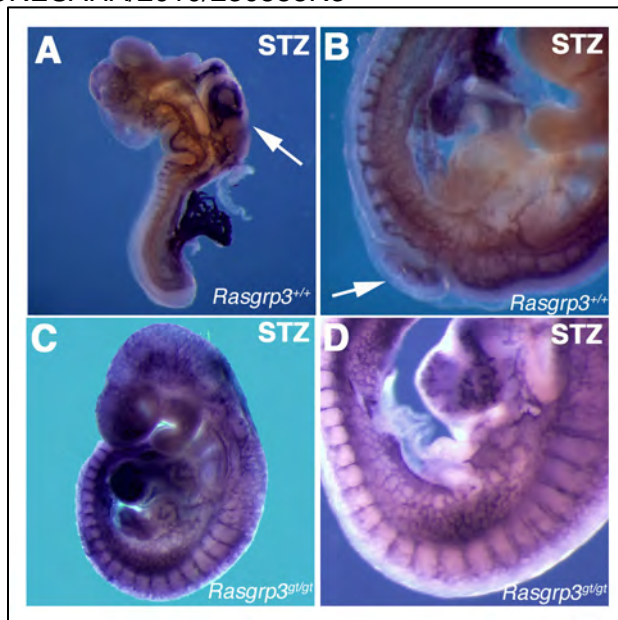
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS



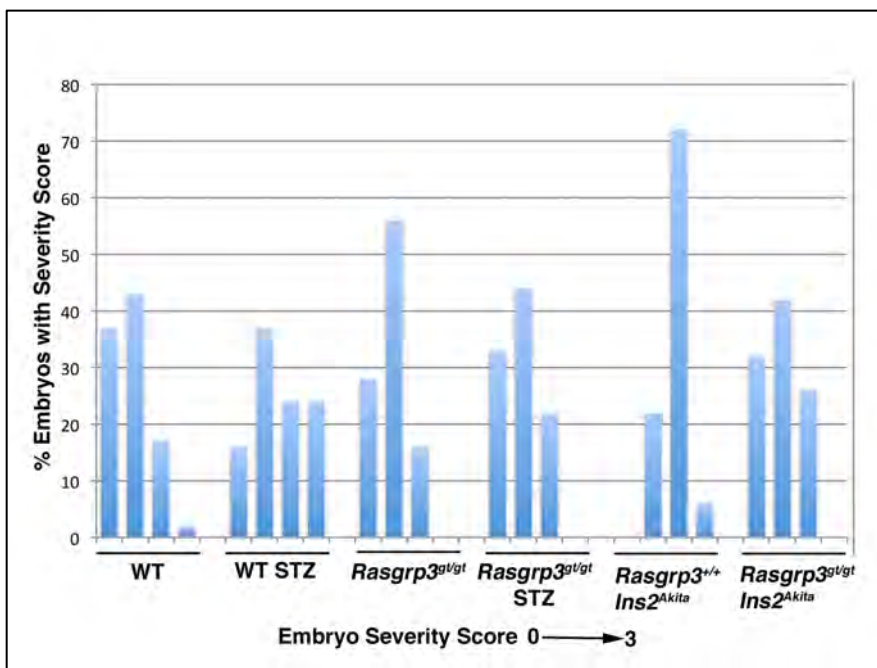
Supplemental Figure I. Loss of *Rasgrp3* protects from PMA-induced developmental perturbations. Mouse embryos that were WT (A-D) or mutant for *Rasgrp3* (E-H) were dissected at E7.5 with yolk sac intact, and incubated for 24 hr in the presence of 50 nM PMA. WT embryos uniformly exhibited severe defects of the yolk sac and embryo proper, while *Rasgrp3*^{gt/gt} embryos were relatively protected from PMA effects, with discernable anterior (a) and posterior (p) and allantois (arrows).



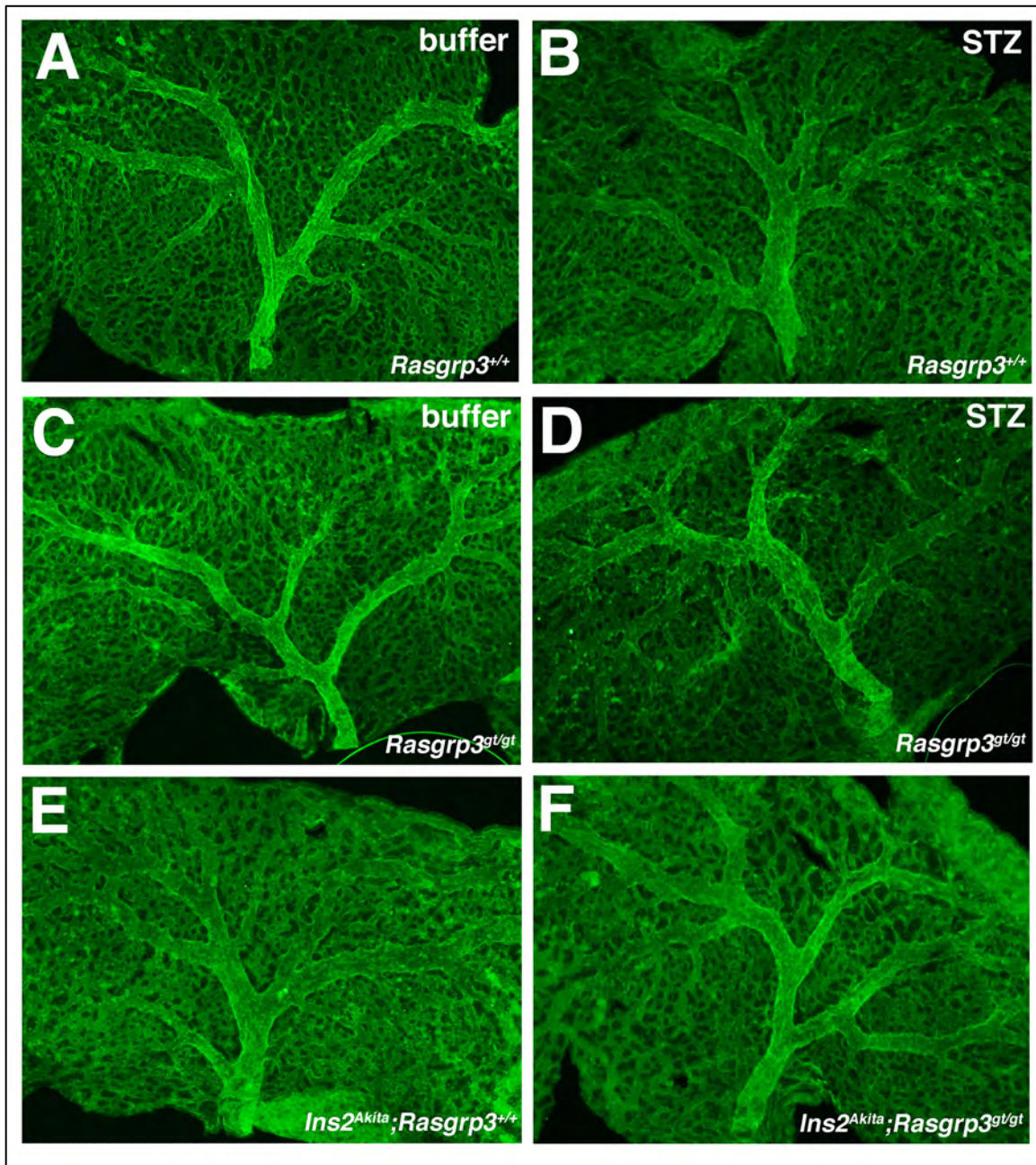
Supplemental Figure II. Loss of *Rasgrp3* protects from glucose-induced defects. WT (A,B) or *Rasgrp3*^{gt/gt} (C, D) embryos were dissected from diabetic mothers at E8.5 and cultured for 24 hr in control media (A, C) or media with 20 mM glucose (B, D), then processed for β -galactosidase activity. White arrows, normal blood vessels in head or inter-somitic areas; black arrows, abnormal head or intersomitic vessels; black arrowheads, abnormal somitogenesis or axis formation.



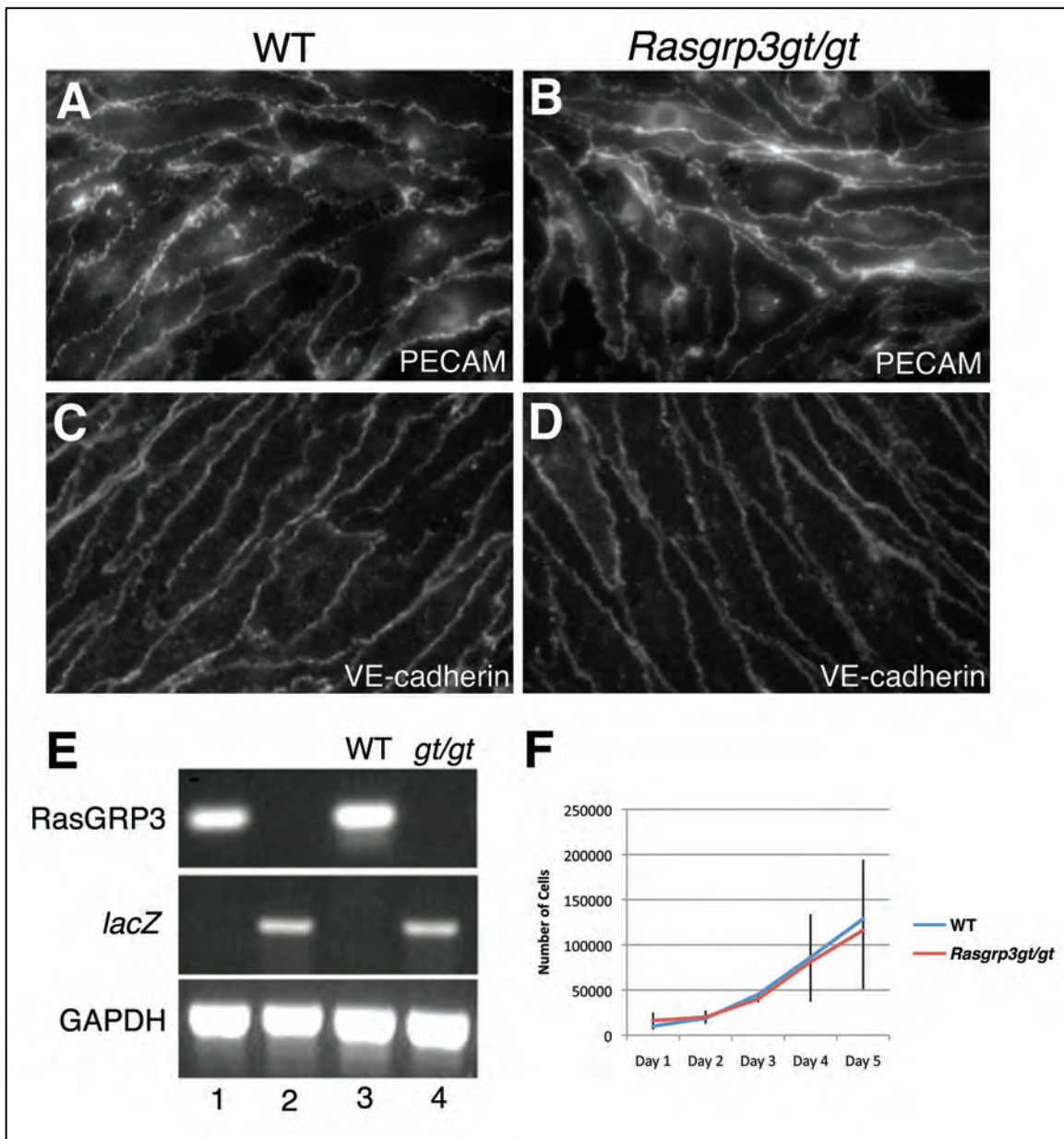
Supplemental Figure III. Loss of *Rasgrp3* protects from diabetes-induced defects. WT (A,B) or *Rasgrp3^{gt/gt}* (C, D) embryos were dissected from diabetic mothers at E9.5 and stained in whole mount for PECAM. (A, B) arrows point to severe defects seen in two different WT embryos; (C, D) two different *Rasgrp3^{gt/gt}* embryos have normal development.



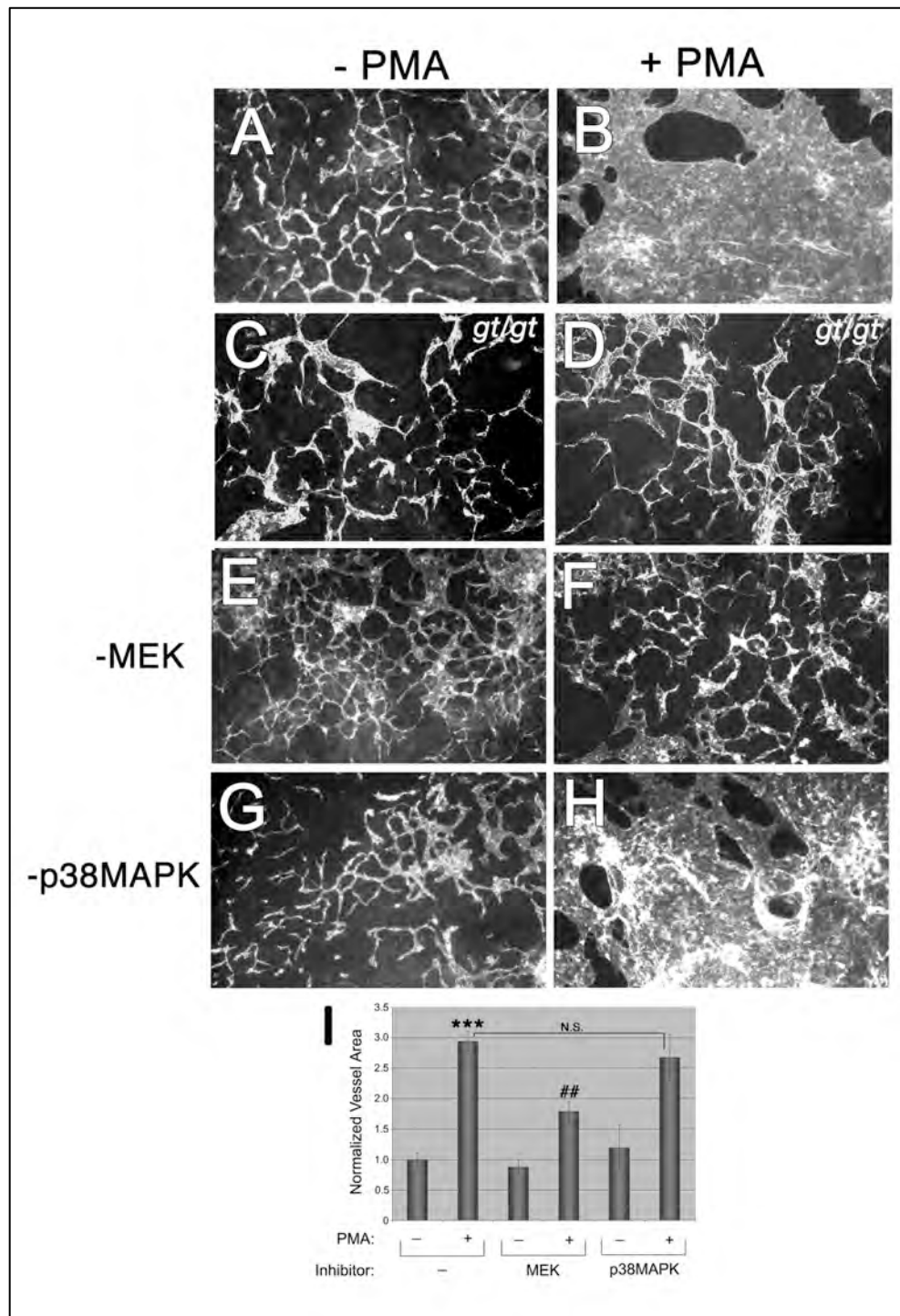
Supplemental Figure IV. Distribution of Embryo Severity Index values relative to genotype/condition. For each group, embryos were scored on a scale from 0 (no defects) to 3 (severe defects) as described. The proportion in each category is indicated. WT, mothers were normo-glycemic; STZ, mothers were treated with streptozotocin and were diabetic.



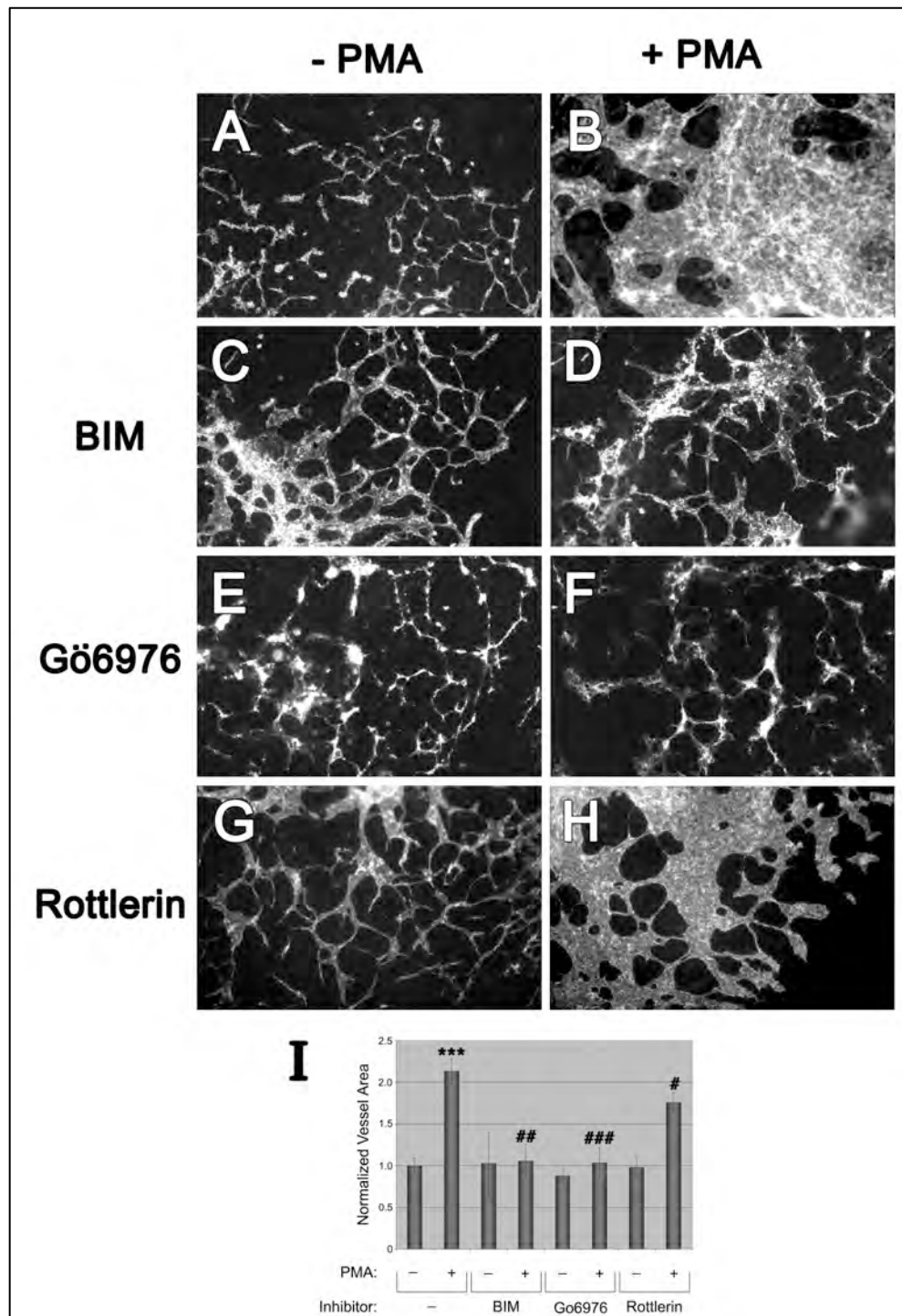
Supplemental Figure V. Lack of yolk sac vascular defects associated with genotype/condition. (A-F) Yolk sacs of E9.5 embryos were processed for PECAM (green) immunofluorescence. No significant trends were found that correlated with genotype, condition, or severity of embryo defects.



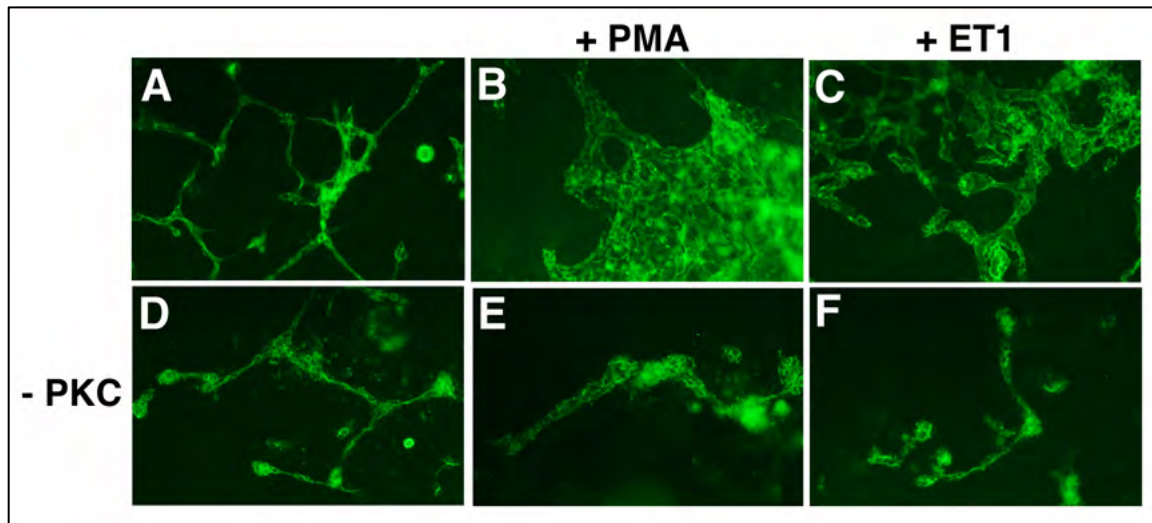
Supplemental Figure VI. Characterization of *Rasgrp3^{gt/gt}* endothelial cells. WT (A, C) and *Rasgrp3^{gt/gt}* (B, D) endothelial cells were isolated as described, and stained for PECAM (A, B) or VE-cadherin (C, D). (E) RT-PCR from RNA isolated from: lane 1, WT dy 8 ES cell cultures; lane 2, *Rasgrp3^{gt/gt}* dy 8 ES cell cultures; lane 3, WT endothelial cells; and lane 4, *Rasgrp3^{gt/gt}* endothelial cells. Appropriate primers amplified a RasGRP3 band in WT embryos and endothelial cells, while *lacZ* primers amplified the reporter inserted in the gene trap (gt) allele in *Rasgrp3^{gt/gt}* embryos and endothelial cells. (F) A growth curve showed similar kinetics for both WT and *Rasgrp3^{gt/gt}* endothelial cells.



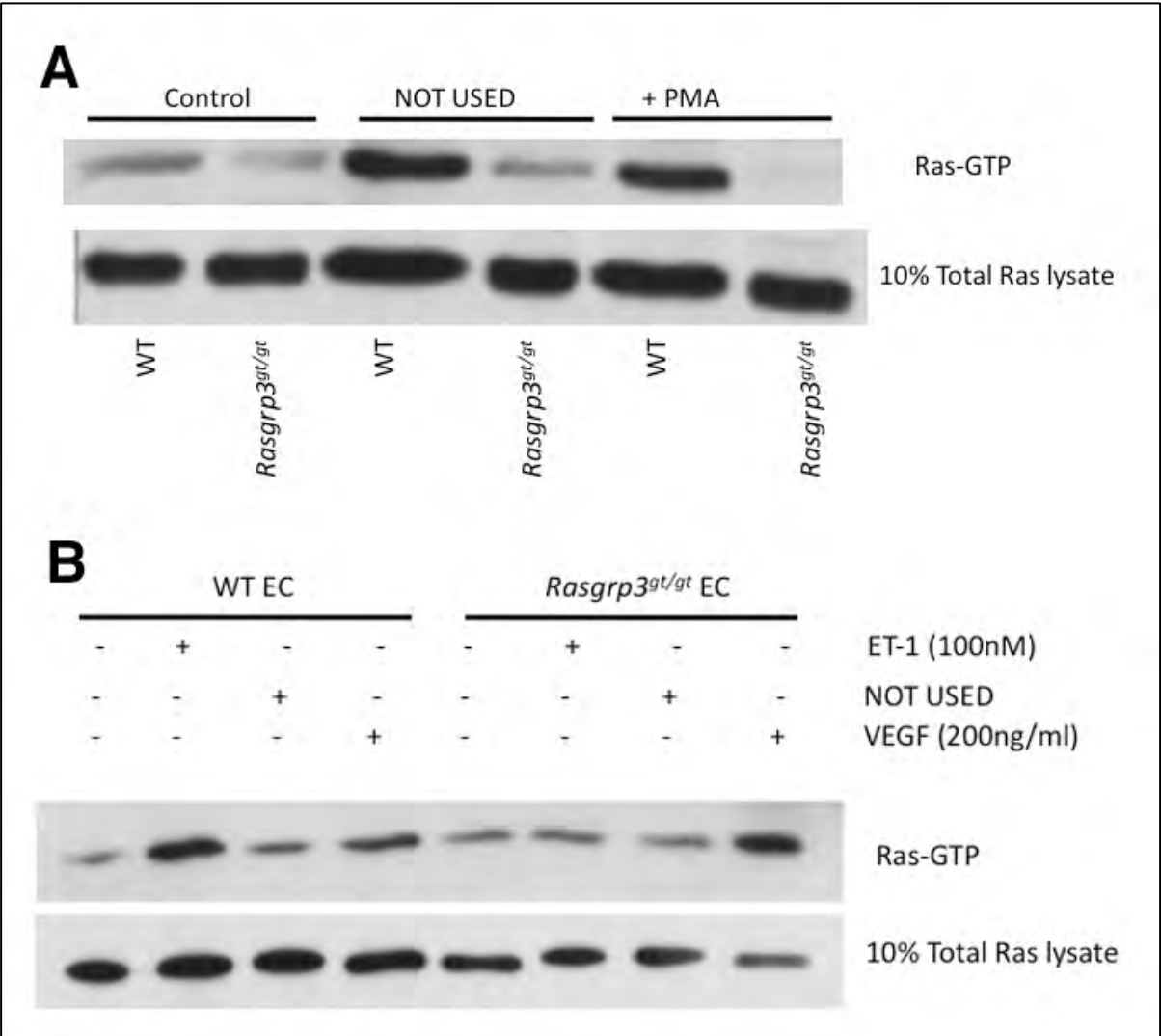
Supplemental Figure VII. RasGRP3-dependent PMA-induced vessel dysmorphogenesis is blocked by MEK inhibition. Day 8 ES cell cultures stained for PECAM. (A, C, E, G) control cultures; (B, D, F, H) cultures incubated with PMA (100 nM) for 24 hr. (A,B) WT vessels with no inhibitor; (C,D) *Rasgrp3^{gt/gt}* vessels with no inhibitor; (E,F) Vessels co-incubated with the MEK inhibitor U0126; (G,H) Vessels co-incubated with the p38 MAPK inhibitor SB203580; (I), Vessel area was quantified. ***, $p \leq 0.00001$ for -PMA (lane 1) vs. +PMA (lane 2); ##, $p \leq 0.0001$ for +PMA (lane 2) vs. +PMA,+MEK inhibitor (lane 4); N.S., $p = 0.24$ for +PMA (lane 2) vs. +PMA,+p38 MAPK inhibitor (lane 6).



Supplemental Figure VIII. RasGRP3-dependent PMA-induced vessel dysmorphogenesis is blocked by PKC inhibition. Day 8 ES cell cultures stained for PECAM. (A, C, E, G) control cultures; (B, D, F, H) cultures incubated with PMA (100 nM) for 24 hr. (A,B) Vessels with no inhibitor; (C,D) Vessels co-incubated with the general PKC inhibitor BIM; (E,F) Vessels co-incubated with the conventional PKC inhibitor Gö6976; (G, H) Vessels co-incubated with the PKC inhibitor rottlerin; (I), Vessel area was quantified. ***, $p \leq 0.00001$ for -PMA (lane 1) vs. +PMA (lane 2); ##, $p \leq 0.001$ for +PMA (lane 2) vs. +PMA, +BIM inhibitor (lane 4); ###, $p \leq 0.0001$ for +PMA (lane 2) vs. +PMA, +Gö6976 inhibitor (lane 6); #, $p \leq 0.01$ for +PMA (lane 2) vs. +PMA, +rottlerin (lane 8).



Supplemental Figure IX. RasGRP3-dependent Endothelin-1 mediated vessel dysmorphogenesis is blocked by PKC inhibition. Day 8 ES cell cultures stained for PECAM. (A-C) control cultures and (D-F) cultures incubated with the general PKC inhibitor BIM and co-incubated with (B, E) PMA, (C, F) ET1. Note the vessel dysmorphogenesis seen in panels B and C is abrogated in panels E and F, with PKC inhibition.



Supplemental Figure X. Original blots for Figure A-B. (A) The original blots used to set up Figure 4A. (B) Original blots used to set up Figure 4B.

SUPPLEMENTAL REFERENCES

1. Kearney JB, Ambler CA, Monaco KA, Johnson N, Rapoport RG, Bautch VL. Vascular endothelial growth factor receptor Flt-1 negatively regulates developmental blood vessel formation by modulating endothelial cell division. *Blood*. 2002;99:2397-2407.
2. Lucitti J, Jones E, Huang C, Chen J, Fraser S, Dickinson M. Vascular remodeling of the mouse yolk sac requires hemodynamic force. *Development*. 2007;134:3317-3326.
3. Nagy A, Gertsenstein M, Vintersten K, Behringer R. *Manipulating the Mouse Embryo*. 3rd ed. Cold Spring Harbor, NY: Cold Spring Harbor Press; 2003.
4. Balconi G, Spagnuolo R, Dejana E. Development of endothelial cell lines from embryonic stem cells: a tool for studying genetically manipulated endothelial cells in vitro. *Arterioscler Thromb Vasc Biol*. 2000;20:1443-1451.
5. Zayad M, Yuan W, Leisner T, Chalothorn D, McFadden A, Schaller M, Hartnett M, Faber J, Parise L. CIB1 regulates endothelial cells and ischemia-induced pathological and adaptive angiogenesis. *Circ Res*. 2007;101:1185-1193.
6. Kappas NC, Zeng G, Chappell J, Kearney JB, Hazarika S, Kallianos K, Patterson C, Annex B, Bautch VL. The VEGF receptor Flt-1 spatially modulates Flk-1 signaling and blood vessel branching. *J Cell Biol*. 2008;181:847-858.
7. Roberts DM, Anderson AL, Hidaka M, Swetenburg RL, Patterson C, Stanford WL, Bautch VL. A Vascular Gene Trap Screen Defines RasGRP3 as an Angiogenesis-Regulated Gene Required for the Endothelial Response to Phorbol Esters. *Mol. Cell. Biol*. 2004;24:10515-10528.
8. Yokoyama U, Patel H, Lai N, Aroonsakool N, Roth D, Insel P. The cyclic AMP effector EPAC integrates pro- and anti-fibrotic signals. *Proc Natl Acad Sci USA*. 2008;105:6386-6391.
9. Kearney JB, Bautch VL. In vitro differentiation of mouse ES cells: hematopoietic and vascular development. *Methods Enzymol*. 2003;365:83-98.
10. Shaul Y, Seger R. The detection of MAPK signaling. *Curr Protocols Mol Biol*. 2006.
11. Taylor S, Resnick R, Shalloway D. Nonradioactive determination of Ras-GTP levels using activated Ras interaction assay. *Methods Enzymol*. 2001;333:333-342.