THE RAS ACTIVATOR RASGRP3 MEDIATES DIABETES-INDUCED EMBRYOPATHY AND ET1-INDUCED VESSEL MORPHOGENESIS

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ABSTRACT

Paramjeet Kaur Randhawa: THE RAS ACTIVATOR RASGRP3 MEDIATES DIABETES-INDUCED EMBRYOPATHY AND ET1-INDUCED VESSEL MORPHOGENESIS
(Under the direction of Victoria L. Bautch)

Blood vessel formation is critical throughout the life of vertebrate organisms, and vessel dysfunction jeopardizes both fetal development and adult survival. Diseases such as diabetes disrupt vessel regulation and contribute to numerous vascular pathologies. Therefore, understanding blood vessel regulation in pathological conditions is essential to devising preventative treatments. The work presented in this dissertation aims to understand how perturbations in the Ras activator, Ras guanyl-releasing protein 3 (RasGRP3), mediate aberrant vessel morphogenesis. We have identified RasGRP3 as a downstream component in an endothelin-1 (ET1) signaling pathway. ET1 signaling is a primary mediator of vascular pathology in diabetes. I hypothesized that RasGRP3 mediates diabetes-induced embryopathy and changes in endothelial cell behavior and vessel morphology. Work by Joanna Fried and Jessica Heinz demonstrated that RasGRP3 is required for embryogenesis in diabetic conditions. In collaboration with Dr. Svetlana Rylova, I determined that activated RasGRP3 promotes Ras-ERK signaling, which can be induced by phorbol ester

or endothelin-1 (ET1). Dr. Rylova demonstrated that overexpression of activated RasGRP3 is sufficient to induce Ras-ERK activation and perturb directional migration. In collaboration with Stephanie Kiser, I showed that RasGRP3 is required for ET1-induced Ras activation, proliferation and migration in endothelial cells. Using an embryonic stem cell model to simulate pathological angiogenesis, we also identified a role for RasGRP3 in ET1-induced vessel dysmorphogenesis. By identifying the molecular requirements that mediate RasGRP3-dependent effects, we can understand how changes in cellular or morphological processes induce vascular pathology. The data herein advance our understanding of RasGRP3-vessel pathology as they may pertain to diabetic conditions.

For my family and my husband

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter I

Chapter II

Chapter III

LIST OF ABBREVIATIONS

CIB1	Calcium and Integrin-binding Protein-1
DAB	3,3'-diaminobenzidine
DAG	Diacylglycerol
DLC1	Dynein light-chain binding domain 1
DMEM-L	Dulbecco's Modified Eagle's Medium, Low Glucose
DNA	Deoxyribonucleic Acid
E7.5	Embryonic Day 7.5
E9.5	Embryonic Day 9.5
ES	Embryonic Stem Cell
ECE	Endothelin-Converting Enzyme
ECM	Extracellular Matrix
EDTA	Ethylenediaminetetraacetic Acid
eGFP	Enhanced Green Fluorescence Protein
EGM-2	Endothelial Growth Media-2
ET1	Endothelin, isoform 1
ET2	Endothelin, isoform 2
ET3	Endothelin, isoform 3
ET_AR	Endothelin Receptor, Type A
ET_BR	Endothelin Receptor, Type B
ERK	Extracellular Regulated Kinase
ES	Embryonic Stem

FGF	Fibroblast Growth Factor
FAK	Focal Adhesion Kinase
FITC	Fluorescein Isothiocyanate
$G\alpha_q$,	$G\alpha_{q/11}$ subunit
gt	Gene-trap
HRP	Horseradish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
I	Inhibitor
ICAM-2	Intercellular Adhesion Molecule 2
IP_3	Inositol Trisphosphate
JNK	c-Jun N-terminal Kinase
MAPK	Mitogen-Activated Protein Kinase
MEK	Mitogen-Activated Protein Kinase Kinase
p-	Phosphorylated
p21	Tumor Protein 21
PAK1	P21/Cdc42/Rac1-activated kinase-1
PBS	Phosphate-buffered Saline
PBT	0.2% Bovine Serum Albumin, 0.1% Triton X-100 in Phosphate-buffered Saline
PECAM-1	Platelet Endothelial Cell Adhesion Molecule-1
PFA	Paraformaldehyde
PI3K	Phosphatidylinositol 3-Kinase
PIP2	Phosphatidylinositol 4,5-biphosphate
PH3	Phospho-histone H3

PLC	Phospholipase-C
PLGF	Placental Growth Factor
PKC	Protein Kinase C
PMA	Phorbol-12-Myristate-13-Acetate (phorbol ester)
PDGF	Platelet-derived Growth Factor
PlGF	Placental Growth Factor
PyMT	Polyoma middle-T
Raf	Raf kinase
RasGAPs	Ras GTPase activating proteins
RasGEFs	Ras guanine nucleotide exchange factors
RasGRFs	Ras-specific guanine nucleotide-releasing factors
RasGRP3	Ras guanyl-releasing protein 3
RasGRPs	Ras guanyl-releasing proteins
REM	Ras-exchanger-motif domain
RT	Room Temperature
RT-PCR	Real-Time Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate
siRNA	Short Interfering Ribonucleic Acid
SOS	Son-of-Sevenless
STZ	Streptozotocin
TGF-β	Transforming Growth Factor β
TRITC	Tetramethyl Rhodamine Isothiocyanate
VEGF	Vascular Endothelial Growth Factor-A
VEGFR-1	Vascular Endothelial Growth Factor Receptor-1

VEGFR-2	Vascular Endothelial Growth Factor Receptor-2
VSMC	Vascular Smooth Muscle Cells
WT	Wildtype

LIST OF SYMBOLS

α	Alpha
β	Beta
δ	Delta
ε	Epsilon
ι	Iota
γ	Gamma
λ	Lambda
η	Eta
θ	Theta
ζ	Zeta

CHAPTER I

GENERAL INTRODUCTION

A functional vasculature is essential for the development and survival of vertebrate organisms. Blood vessels form ordered, branched structures throughout the body to provide nutrients and remove waste from developing organs. Leonardo da Vinci compared this branched network to a tree; he visualized the developing vasculature as sprouting from a seed (the heart) and forming a trunk (aorta, arteries) and roots (capillaries) [1].

Signals regulating the formation and function of this highly ordered 'tree' are critical throughout the life of the organism, and in fact, vessel dysfunction jeopardizes both fetal development and adult survival. In the adult, vessel dysfunction is associated with many cardiovascular diseases such as stroke, myocardial infarction, and peripheral vascular disease. Because vessels deliver a blood supply to all areas of the body, perturbations in blood vessel formation can be detrimental to various organs and contribute to pathology in diseases such as cancer and diabetes. Therefore, understanding blood vessel regulation in pathological conditions is essential to elucidate therapeutic targets.

The work presented in this dissertation aims to understand how perturbations in a Ras activator mediate aberrant vessel morphogenesis. We have identified the Ras activator, Ras guanyl-releasing protein 3 (RasGRP3), as a downstream component in an endothelin-1 (ET1) signaling pathway. ET1 signaling contributes to vascular pathology in diabetes (reviews: [2-5]). We have also demonstrated that RasGRP3 is required to mediate diabetes-induced embryonic defects. By identifying the molecular requirements that mediate RasGRP3-dependent effects, we can understand how changes in cellular or morphological

processes induce vascular pathology. The data herein advance our understanding of RasGRP3-vessel pathology as they may pertain to diabetic conditions.

A. Blood vessel formation. Hypoxic tissues secrete growth factors, including Fibroblast-derived Growth Factor (FGF), angiopoetins, Platelet-derived Growth Factor (PDGF), Transforming Growth Factor-beta (TGF-β), and Vascular Endothelial Growth Factor-A (VEGF), to stimulate blood vessel formation ([6, 7] reviews: [1, 8]). Blood vessel formation occurs by two sequential processes called vasculogenesis and angiogenesis. Vasculogenesis is the process by which angioblasts, or endothelial progenitor cells derived from mesodermal tissue, differentiate into endothelial cells and initiate *de novo* formation of a primitive vessel. This primitive vessel undergoes angiogenesis, a process that induces growth and maturation of the primitive vessel to form a lumenized, branched vascular network ([6, 7], reviews: [1, 8]). The final stages of angiogenesis establish blood flow and induce vessel remodeling to further refine the vasculature [6, 7]. Blood vessels are stabilized by the recruitment of pericytes and vascular smooth muscle cells, which encapsulate the developing blood vessels [9]. Together, blood vessel formation and stabilization establish a functional network to provide nutrients and oxygen throughout the developing organism.

Blood vessel formation also has important roles in the adult: physiological angiogenesis occurs in wound healing or pregnancy, while pathological

angiogenesis occurs in conditions such as arthritis, diabetic retinopathy or tumorigenesis [10]. Inappropriate blood vessel formation or disruption in vessel stability compromises vessel function and contributes to pathology in several diseases such as diabetes [1, 11]. Diabetes is characterized by excess blood glucose, which induces upregulation of many molecules, including diacylglycerol (DAG), VEGF and endothelin-1 (ET1). These molecules impact blood vessel formation and function in both developing embryos and adults.

How angiogenic molecules are differentially regulated in physiological and pathological processes is not well understood but currently under investigation. Seaman et al. [12] broached this subject by analyzing the vascular transcriptome of normal and malignant tissues. Their analysis revealed that some genes are selectively overexpressed in tumor angiogenesis. Many of these genes encode cell surface markers, extracellular matrix (collagen), and placental growth factor (PlGF) [12]. PlGF is a VEGF homolog that has been previously implicated in pathological angiogenesis [13]. Carmeliet et al. [13] showed that deletion of PlGF does not disrupt normal blood vessel formation in the developing embryo. However PlGF is required for several pathological properties mediated by VEGF, including angiogenesis in ischemia, inflammation and cancer [13].

We have identified another protein, RasGRP3, that mediates aberrant blood vessel formation but is not required for physiological angiogenesis. Our research uses a developmental model of blood vessel formation to simulate pathological angiogenesis and address the role of RasGRP3, a Ras activator, in this process. We have demonstrated that genetically disrupting *Rasgrp3* preserves normal

blood vessel formation but inhibit vascular dysmorphogenesis induced by phorbol ester, a DAG mimic [14]. Based on these results and data indicating that angiogenic vessels express *Rasgrp3* in a VEGF-regulated manner [14], we hypothesize that RasGRP3 plays a role in pathological processes. To this end, we address the role of RasGRP3 in pathology induced by excess DAG in diabetic conditions and test the requirement for RasGRP3 in VEGF or ET1 signaling in endothelial cells.

Below, I will briefly discuss VEGF and ET1 signaling pathways and their purported roles in vascular pathology.

B. Vascular Endothelial Growth Factor-A signaling pathways. Work by several groups was seminal to the discovery of a potent angiogenic factor that was diffusible and specifically acted on endothelial cells to promote blood vessel growth or changes in vessel permeability (review: [15]). Senger et al. [16] first demonstrated that conditioned media from tumor cells contained a tumor vascular permeability factor (VPF) that increased vascular leakage. Later work showed that this molecule was identical to a diffusible mitogen named vascular endothelial growth factor (VEGF) by Ferrara and Henzel [17] (review: [15]). Their research has spurred many years of investigation into the role of VEGF in physiological and pathological blood vessel formation and function (review: [15]).

The VEGF family is comprised of several family members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and PlGF. All members regulate growth, migration, survival

and permeability to promote blood vessel formation, although VEGF-C and VEGF-D are specifically involved in lymphangiogenesis ([18], review: [19]). VEGF-A (referred to as VEGF) has been well characterized as a critical molecule is required for blood vessel formation and deletion of VEGF or its receptors causes dramatic vascular defects that result in embryonic lethality [20-22].

VEGF receptors 1 and 2 (VEGFR-1, VEGFR-2) are common VEGF receptors in endothelial cells that mediate vascular patterning. VEGFR-1 is secreted into the extracellular matrix or maintained in a membrane-bound form to regulate vessel patterning by acting as a 'decoy' receptor to sequester VEGF from VEGFR-2 [23-28]. Deletion of *Vegfr-1* acts as a *Vegf* gain-of-function perturbation, which induces vascular overgrowth and compromises the survival of the developing embryo [23]. VEGFR-2 activity is essential for normal angiogenesis and regulates endothelial cell proliferation (via ERK signaling), migration (via p38 MAPK signaling), and survival (via AKT signaling) (Figure 1.1) (review: [29]). Deletion of *Vegfr-2* causes embryonic lethality by perturbing blood island formation and subsequent blood vessel formation [29-34]. A similar phenotype occurs when one or both *Vegf* alleles are deleted [29-34]. These results indicate that there are critical requirements for VEGF concentration or presentation in endothelial cells. While VEGF is required at physiological levels for proper angiogenesis, excess VEGF contributes to vascular pathology by inducing aberrant angiogenesis or vessel dysfunction such as increased vessel permeability in diabetes [35].

Misexpression and misregulation of VEGF induces vessel dysfunction in pathologies including cardiovascular disease and proliferative diabetic retinopathy ([13, 36-41], review: [1]). In diabetes, excess glucose disrupts vessel function and creates hypoxic tissues to increase VEGF production. Elevated VEGF induces endothelial cell activation by promoting several pathways, including ERK [29, 42]. To initiate ERK signaling, VEGFR-2 induces phospholipase C (PLC) activation to generate membrane-bound diacylglycerol (DAG) and cytosolic inositol trisphosphate (IP_3), which stimulates calcium release from the endoplasmic reticulum. A generic version of this pathway is illustrated in Figure 1.2. DAG acts as a second messenger to relay signals from activated receptors to intracellular proteins by recruiting protein kinase C (PKC) and non-PKC proteins with C1 domains such as RasGRP3, to the plasma membrane for activation. PKC phosphorylates Ras activators such as RasGRP3 to mediate Ras GTPase (Ras) activation, which acts through the intermediary proteins Raf kinase (Raf) and mitogen-activated protein kinase kinase (MEK) to stimulate ERK activation [14, 43, 44]. PKC can also bypass Ras to activate Raf and induce ERK activation [45]. The role of RasGRP3 in DAG-mediated signals has been characterized in B-cells using phorbol ester, a DAG mimic [46-52]. Our work extends this investigation to endothelial cells. We also identify an upstream input for the RasGRP3 signaling cascade in endothelial cells that is relevant *in vivo*. Determining when RasGRP3 pathways are active in DAG-PKC signaling cascades has been challenging because DAG-PKC signaling cascades are not limited to VEGF signaling. DAG-PKC signaling cascades are also activated by

many other growth factor or G-protein-coupled receptors, including those downstream of ET1 ([53], reviews: [54-57]).

C. Endothelin-1 signaling pathways. In 1985, Hickey et al. observed that conditioned media from bovine aortic endothelial cells displayed vasoactive effects on isolated rings of the porcine coronary artery. They attributed the changes in vessel tone to the presence of an endothelium-derived contractile factor in the conditioned media [58]. Subsequently, another group purified this contractile factor and named it "endothelin" ([59], reviews: [60, 61]). There are 3 different endothelin (ET) isoforms, ET1, ET2, and ET3 ([62], reviews: [60, 61, 63]). ET is generated through a series of cleavage events. First, a 212-amino acid precursor called pre-proET is cleaved by a furin-like endopeptidase into "big ET", a 38-amino acid protein. "Big ET" is then cleaved by endothelin-converting enzyme (ECE) to form active ET, a 21-amino acid protein ([62], reviews: [60, 61, 63]). I am interested in ET1 because ET1 is the predominant form produced in endothelial cells and the most potent vasoconstrictor in the vasculature ([59], reviews: [60, 61, 63]).

ET signaling is mediated by two G-protein coupled receptors, type A and type B (ET_AR, ET_BR). ET_AR is predominantly expressed in vascular smooth muscle cells and binds ET1 and ET2 with the same affinity but has a much lower affinity for ET3 ([64], review: [57]). ET_BR is primarily expressed in endothelial cells and binds all ET isoforms with equal affinity. Both receptors bind a family of heterotrimeric

G proteins to transmit signals intracellularly and promote activation of the Ras superfamily of small GTPases [65, 66], which I will discuss later. Heterotrimeric G proteins are comprised of α, β, and γ subunits ([67]). Gα subunits consist of several subtypes $G\alpha_{12/13}$, $G\alpha_{q/11}$ ($G\alpha_q$), $G\alpha_i$, and $G\alpha_s$, some of which are activated by ET receptors ([67, 68], review: [69]). ET_AR stimulates the Gα proteins $G\alpha_s$ and $G\alpha_q$, and ET_BR induces $G\alpha_q$ and $G\alpha_i$ activation ([70], review: [69]). The consequences of G protein signaling downstream of ET receptor activation in various cell types, physiologically and pathologically, are not well understood (review: [69]). However, typical $G\alpha_s$ signaling promotes cyclic AMP pathways, which are blocked by $G\alpha_i$ signaling [70]. In endothelial cells, ET1-ET_BR signaling activates PLC through $G\alpha_q$ and Gβ/Gγ to generate DAG, which promotes Ras and ERK activation (Figure 1.2) ([71, 72], review: [69]). $G\alpha_i$ signaling also promotes RasGTPase activity by stimulating a Ras activator called son-of-sevenless [73].

Similar to VEGF-VEGFR-2 signaling, misregulation of ET1-ET_BR signaling can promote neoangiogenesis [41, 74] or endothelial activation by stimulating ERK, p38 MAPK, and AKT pathways to mediate changes in endothelial cell proliferation, migration and survival (Figure 1.3) [75-77]. ET1 also regulates vascular tone by inducing vasodilation in endothelial cells and vasoconstriction in vascular smooth muscle cells (Figure 1.3) ([78, 79], reviews: [80, 81]). ET1 and VEGF display synergistic relationships by exhibiting reciprocal transcriptional upregulation [76, 82] and cooperatively promoting angiogenesis [77]. However, ET1 and VEGF have distinct functions in the vasculature: VEGF has been well

documented as a promoter of angiogenesis while ET1 is better characterized as a regulator of vascular homeostasis [83].

In the developing embryo, homozygous deletions of endothelin-converting enzyme (ECE), ET1 or ET_AR cause craniofacial and cardiac abnormalities, contributing to respiratory failure and postnatal death ([84, 85], reviews: [4, 60]). Deletion of ET_BR also causes lethal colonic aganglionosis, resulting in juvenile death ([85-87], reviews: [4, 60]). These phenotypes illustrate roles for ET1 outside the vasculature. Unlike VEGF, lack of ET1 does not prevent physiological angiogenesis ([79], reviews: [88]). In this way, ET1 is similar to RasGRP3 and PlGF as discussed previously ([13, 14], reviews: [88]). Although elevated ET1 levels have been documented in tumorigenesis (reviews: [88]), the role of ET1 in pathological angiogenesis is not well understood. Previous work has shown that ET1 acts cooperatively with VEGF to induce angiogenesis in a matrigel plug assay in mice [77]. ET1 also induces changes in vascular homeostasis by increasing blood pressure and kidney dysfunction ([89-91], review: [55]). In studies of hypertensive-diabetic rats, ET1-induced hypertension required Ras GTPase signaling [89-91]. These data suggest that Ras signaling is important in ET1-mediated vascular dysfunction.

D. Ras GTPases. The Ras superfamily of small GTPases has over 20 members and regulates many cellular processes, such as cell proliferation, migration, differentiation and apoptosis ([44, 92], reviews: [93-95]). One particular Ras

GTPase subfamily is comprised of several members: R-Ras, K-Ras, H-Ras and N-Ras. With the exception of R-Ras, Ras members recognize distinct effector proteins to modulate downstream signals, promoting ERK activation and inducing proliferation and migration ([92, 96-98], reviews: [93-95]).

K-Ras, H-Ras and N-Ras are upregulated in many cancers ([99], reviews: [100-104]). Studies that assess K-Ras and H-Ras in tumor models demonstrate that Ras is required to promote VEGF transcription and translation to mediate tumor growth and neoangiogenesis ([105, 106], review: [104]). Murine embryos lacking *H-ras* and/or *N-ras* develop normally. However, genetic deletion of *K-ras* disrupts embryogenesis and causes death between embryonic day 12 and birth [107]. To explore the effects of Ras signaling in *K-ras*-specific compartments, Potenza et al. [107] expressed *H-ras* under the control of the *K-ras* promoter. Their elegant experiments demonstrate that while *H-ras* expression rescues embryogenesis, adult mice experience dilated cardiomyopathy associated with increased hypertension. Transgenic mice expressing constitutively activated H-Ras also display hypertension. Interestingly, recent work by Chamorro-Jorganes et al. [108] indicates that although *H-ras*-deficient mice appear normal, they are hypotensive, which occurs as a consequence of increased vasodilation or impaired vasoconstriction. Collectively, these studies indicate that K-Ras and H-Ras pathways mediate angiogenesis and also play important roles in vascular homeostasis [89-91, 105, 107, 108].

Ras activation is mediated by conformational changes between active (GTP-bound) or inactive (GDP-bound) states. Direct interaction with Ras guanine

nucleotide exchange factors (RasGEFs) catalyzes the exchange of GDP for GTP to activate Ras. Active Ras then promotes cell signaling until GTP hydrolysis returns Ras to a GDP-bound state, a process that is enhanced by Ras GTPase activating proteins (RasGAPs) ([109-112], reviews: [94, 113, 114]). Mechanisms that regulate Ras activation are of key interest to understanding how Ras signaling mediates pathologies, such as aberrant angiogenesis or increased hypertension. Several RasGEFs mediate Ras activation including son-of-sevenless (SOS), Ras-specific guanine nucleotide-releasing factors (RasGRFs) and Ras-specific guanine guanyl-releasing proteins (RasGRPs). Growth factor receptor signaling induces SOS activity via complex formation between SOS, two adaptor proteins (Shc and Grb2), and c-Src, a non-receptor family tyrosine kinase ([115], reviews: [114, 116, 117]). RasGRFs are constitutively localized to plasma membranes and regulated by phosphorylation and calcium ([110, 115, 118], review: [114]). RasGRPs are typically regulated by DAG and PKC to mediate Ras activation. Because our lab studies a member of the RasGRP family, RasGRP3, I will discuss this RasGEF family further in the next section.

In addition to these RasGEF families, Ras is also activated by PKC. PKC can stimulate Ras activity through sphingosine kinase or specific RasGRPs, including RasGRP3 [44, 115, 119]. PKC proteins are divided into several functional classes: conventional, novel, and atypical PKCs ([47, 120], reviews: [119, 121, 122]). The conventional PKCs (PKC-α, PKC-β, PKC-γ) are sensitive to calcium, require phospholipids and are activated by DAG. The novel PKCs (PKC-δ, PKC-ε, PKC-θ, PKC-η) are calcium-insensitive, but also require phospholipids and DAG for

activation. Finally the atypical PKCs (PKC- ι/λ, PKC-ζ) do not require calcium or DAG for activation ([47, 120], reviews: [119, 121, 122]). Studies have shown that PKC-δ, PKC-θ, and PKC-ε activate RasGRP3 in a Ras-ERK pathway [47, 48, 51, 123]. PKC-β has also been shown to activate RasGRP3 *in vitro* [47, 48, 51, 123].

E. RasGRPs. The RasGRP family consists of 4 members, RasGRP1-4 ([14, 50, 124] reviews: [125-127]). This family activates Ras to promote signaling pathways including ERK, AKT, p38 MAPK, and JNK to regulate proliferation, migration, survival and differentiation ([50-52, 112, 124, 128-133], reviews: [93-95]).

RasGRPs display an expression pattern that indicates an important role for these proteins in several systems including the hematopoietic system [14, 50, 52, 131-135]. RasGRP1 is expressed in brain, kidney, mast cells, T-cells and B-cells, while RasGRP4 expression is primarily restricted to mast cells [132, 133]. Loss of RasGRP1 causes defects in T-cell development and also autoimmunity, while loss of RasGRP4 causes defects in mast cell differentiation and expression ([132, 133, 136] review: [137]). RasGRP2 is expressed in brain, kidney, platelets, and in blood vessels [14, 138]. Mice lacking RasGRP2 experience impaired platelet aggregation [139]. RasGRP3 is expressed in brain, kidney, endocrine tissue, B-cells, endothelial cells [14, 47, 140] and also vascular smooth muscle cells (PK Randhawa, unpublished data). Interestingly, RasGRP3 expression in endothelial and vascular smooth muscle cells is consistent with cell types that are responsive to ET1 signaling. Loss of RasGRP3 function does not lead to

dramatic defects in the vasculature or normal murine development in general [14]. These mice have a mild B-cell defect, but are otherwise viable and fertile [14, 50]. Compensatory activity by other RasGEFs, such as RasGRP2, may account for the lack of vascular defects.

RasGRPs are characterized by several domains: EF hands, a C1 domain, and a catalytic region, which is comprised of a CDC25 box and a Ras exchange motif (REM box). The catalytic region is required for GTP exchange to activate Ras GTPases. These domains in RasGRP3 are illustrated in Figure 1.4. RasGRP3 also has a recently identified dynein light-chain binding domain (DLC1), which is hypothesized to regulate localization of inactive RasGRP3 [141] (Figure 1.4). EF hands bind calcium, but their role in RasGRP3 signaling has not been elucidated. The C1 domain is homologous to the C1 domain on PKC and is bound by DAG to recruit RasGRP3 to plasma membranes. Once RasGRP3 is localized to the plasma membrane, RasGRP3 is activated by PKC, which phosphorylates RasGRP3 at threonine-133 located in the catalytic region [48, 50]. RasGRP3 activity mediates H-Ras, R-Ras, and Rap1 activation. Rap1 is another GTPase distantly related to Ras that promotes cell adhesion and also modulates migration [142-145].

Until recently, there was relatively little investigation to directly assess the role of RasGRP3 in pathology. Genetic screens have linked elevated RasGRP3 expression to systemic lupus erythematosus, an autoimmune disease, and young-onset hypertension, which suggests a connection to elevated ET1 signaling [146-148]. Work by Yang et al. [112] identified a role for RasGRP3 in prostate

cancer. They demonstrated that RasGRP3 is highly expressed in prostate cancer cells and mediates cell proliferation, migration, Ras, ERK, and AKT activation [112]. They also showed that RasGRP3 is required for these cells to maintain their metastatic phenotype [112]. Together, these studies suggest that RasGRP3 may be instrumental in the progression of several diseases, some of which we hypothesize to have vascular specific effects. Since endothelial cells express RasGRP3, I hypothesize that RasGRP3 regulates ET1-mediated changes in Ras signaling, proliferation and migration in endothelial cells. I also identify a role for RasGRP3 in ET1-induced vessel dysmorphogenesis using an embryonic stem cell model of blood vessel formation.

Roberts et al. have previously shown that *Rasgrp3* is a VEGF-responsive gene in endothelial cells and that genetic disruption of *Rasgrp3* using a gene trap method inhibits phorbol ester-induced vessel dysmorphogenesis [14]. Phorbol ester is an unmetabolizable DAG mimic that recruits PKC and RasGRP3 to the plasma membrane. These data suggest that RasGRP3 is a primary mediator of phorbol ester-induced vessel dysfunction. Phorbol ester stimulation can be used to hypothetically model *in vivo* events downstream of DAG signaling. *In vivo*, DAG signaling is upregulated in diseases such as diabetes [149]. Therefore, phorbol ester can be utilized as a tool to further investigate RasGRP3 function *in vitro*, especially by establishing a requirement for RasGRP3 in phorbol ester-mediated events. In this way, we may understand how RasGRP3 may be important pathologically in conditions of excess DAG, such as in diabetes.

F. Overview of Diabetes Mellitus. Diabetes is characterized by three criteria: excess blood glucose (hyperglycemia), excess free fatty acid release (breakdown products of fat storage), and disrupted insulin signaling (reviews: [150, 151]). The hormone insulin, which is produced by beta cells in the pancreas, modulates blood glucose and free fatty acid levels. There are two main types of diabetes: type 1 and type 2. In type 1 diabetes, genetic abnormalities cause the autoimmune-mediated destruction of the insulin-producing pancreatic beta cells, resulting in overall insulin deficiency in the body [152]. Type 2 diabetes is the more common form of diabetes and is characterized by insulin resistance, which can progress to a decrease in insulin production [152]. A third type, called gestational diabetes, is a temporary diabetic condition that afflicts some women during later stages of pregnancy [152]. Diabetes occurs when there is a lack of insulin production and/or when cells develop insensitivity to insulin, which further promotes hyperglycemic conditions.

Impact on the developing embryo. Poor control of maternal hyperglycemia has consequences for the developing embryo ([149, 153-158], reviews: [159-162]). Embryos developing in a hyperglycemic environment are 3-5 times more likely to suffer from embryonic defects, including defects in the vasculature, neural tube closure, or organ development ([157, 163-165], reviews: [159-162]). These embryos are also prone to cardiac defects ([155], review: [162, 166]) and body axis patterning defects such as caudal regression ([167], review: [162]). There is also evidence of facial malformations in studies of embryos from diabetic mice [163]. In terms of the vasculature, patterning is disrupted, indicative of impaired angiogenesis, which

correlates to reduced endothelial cell proliferation, reduced VEGF signaling, and increased apoptosis [168].

Diabetes-induced embryopathy is also mediated by DAG-PKC signaling [149]. DAG is elevated by multiple mechanisms as a consequence of PLC activation and also glucose metabolism, which induces the *de novo* synthesis of DAG from dihydroxyacetone phosphate and glycerol-3-phosphate ([169], review: [170]). As I previously mentioned, DAG-PKC signaling cascades mediate Ras-ERK signaling [47, 48, 51, 123]. Since PKC also regulates RasGRP3-dependent Ras-ERK signaling pathway [47, 48, 51, 123], we hypothesize that RasGRP3 mediates diabetes-induced embryopathy. Our findings demonstrate that RasGRP3 is instrumental in mediating diabetes-induced embryopathy in mice.

Impact on adults. Diabetes decreases life expectancy in adults by an average of 5-10 years, which correlates to a 5.4% increase in mortality rate [171]. Vascular disease accounts for a large majority of these deaths (review: [172]). Blood vessels undergo many morphological changes such as vessel regression, which creates hypoxic tissues, or angiogenesis, which re-establishes routes for nutrient delivery. Vessel dysfunction is characterized by changes in hypertension, inflammation or thrombosis (reviews: [150, 151]). Pathogenesis is not limited to blood vessels but also occurs in many organs including the heart, nerves, eyes and kidney (reviews: [173, 174]).

Because diabetes is a multi-factorial disease, it is challenging to understand the biochemical and cellular links between elevated blood glucose levels and

endothelial dysfunction. However, many studies have established that glucose metabolism can promote endothelial cell activation by elevating many molecules including DAG, PKC, ET1, and VEGF (reviews: [159, 160, 175-176]). Given the current evidence, we hypothesize that RasGRP3 plays a role in mediating pathways consistent with upregulated DAG, PKC VEGF and ET1 in diabetes. The work presented in this dissertation seeks to elucidate the role of RasGRP3 in endothelial cells and to understand how RasGRP3 mediates endothelial dysfunction in a way that may be consistent with those associated with diabetic complications. Our research demonstrates how perturbations of RasGRP3 impact phorbol ester- and ET1-mediated Ras-ERK signaling and ET1-induced effects on cell behavior and vessel morphology.

Figure 1.1 VEGF-VEGFR2 Signaling Pathways. Through VEGF-R2, VEGF signaling pathways regulate blood vessel formation and function. VEGF induces pathways that mediate proliferation (ERK signaling), migration (p38 MAPK signaling), permeability (AKT signaling), and survival (AKT signaling). VEGF-induced Ras activation also mediates many of these processes.

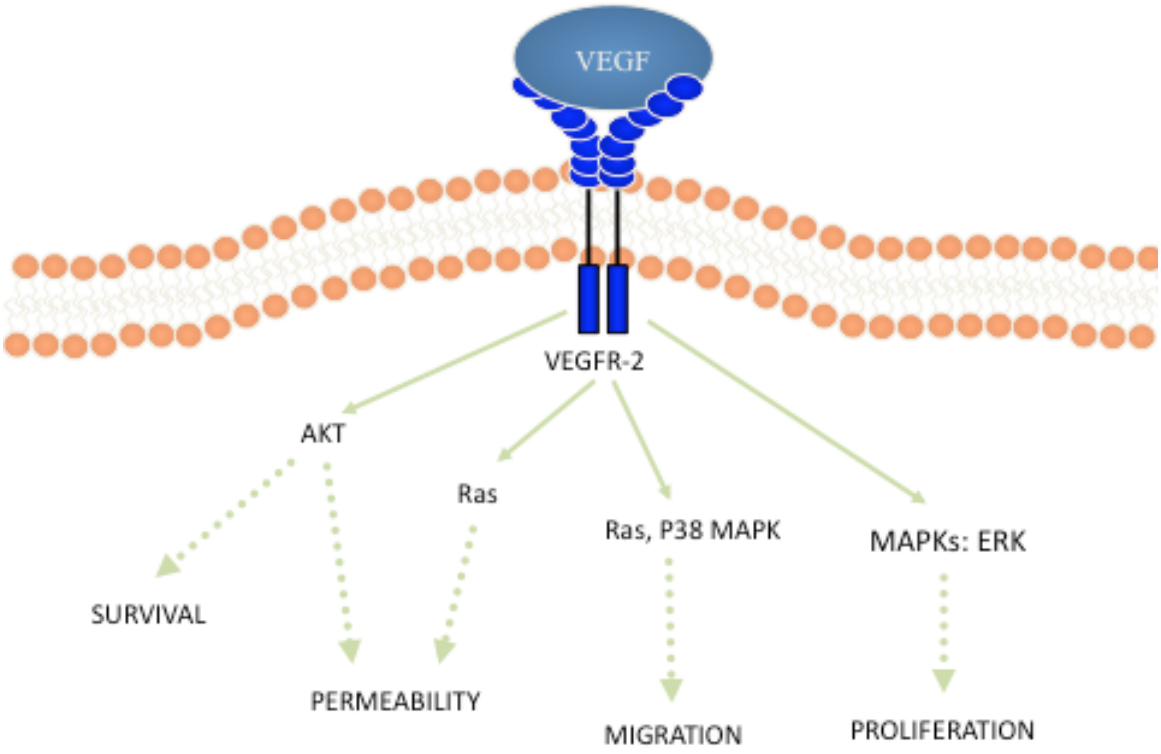

VEGF
VEGFR-2
AKT
Ras
Ras, P38 MAPK
MAPKs: ERK
SURVIVAL
PERMEABILITY
MIGRATION
PROLIFERATION

Figure 1.2. Generic DAG-PKC signaling pathways promote Ras and ERK activation. Ligand-receptor engagement induces activation of PLC, which cleaves PIP_2 to generate cytosolic IP_3 and membrane-bound DAG. DAG binds the C1 domain of PKC, or other non-PKC proteins such as RasGRP3. PKC initiates ERK signaling cascades by activating other Ras activators, such as RasGRP3, or by directly inducing Raf activity. Raf activates MEK, which phosphorylates ERK. DAG-PKC signaling cascades promote endothelial cell activation and vessel dysfunction by inducing changes in endothelial cell behaviors such as proliferation and migration. In this dissertation, we test VEGF and ET1 as two ligands that promote DAG-PKC signaling cascades. We also use phorbol ester (phorbol 12-myristate 13-acetate, PMA) to mimic DAG and pharmacologically activate these signaling cascades.

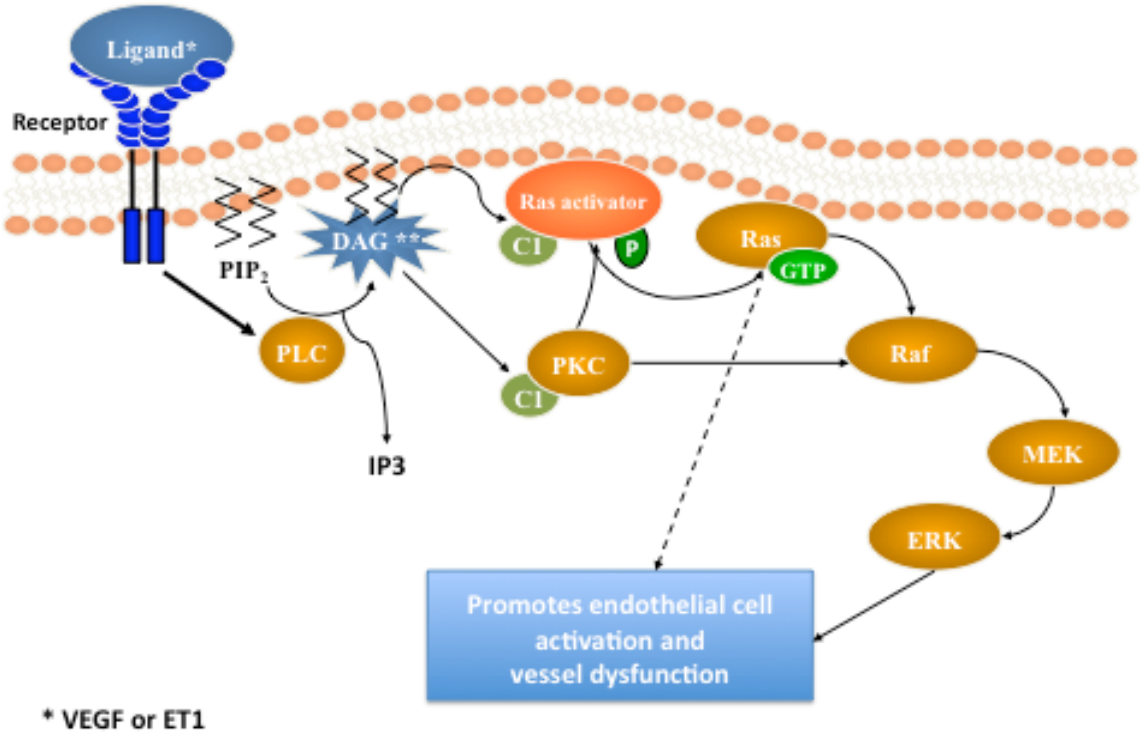

Ligand*
Receptor
PIP2
DAG **
PLC
IP3
Ras activator
Cl
P
Ras
GTP
PKC
Cl
Raf
MEK
ERK
Promotes endothelial cell activation and vessel dysfunction
* VEGF or ET1
** PMA, DAG mimic

Figure 1.3. ET1-ET_BR Signaling Pathways. Through ET_BR, ET1 signaling pathways regulate blood vessel formation and function. ET1 induces pathways that mediate proliferation (ERK signaling), migration (p38 MAPK signaling), and survival (AKT signaling). ET1 signaling also induces vasodilation (NO generation) in endothelial cells and vasoconstriction (AKT signaling) in vascular smooth muscle cells. ET1-induced Ras activation also mediates many of these processes. ET_BR is coupled to heterotrimeric G proteins, $G\alpha_q$ or $G\alpha_i$, and $G\beta$ and $G\gamma$. $G\alpha_q$ and $G\beta/G\gamma$ subunits activate PLC to generate DAG and mediate PKC, Ras, and ERK signals. $G\beta/G\gamma$ subunits stimulate signals to promote AKT pathways and NO generation.

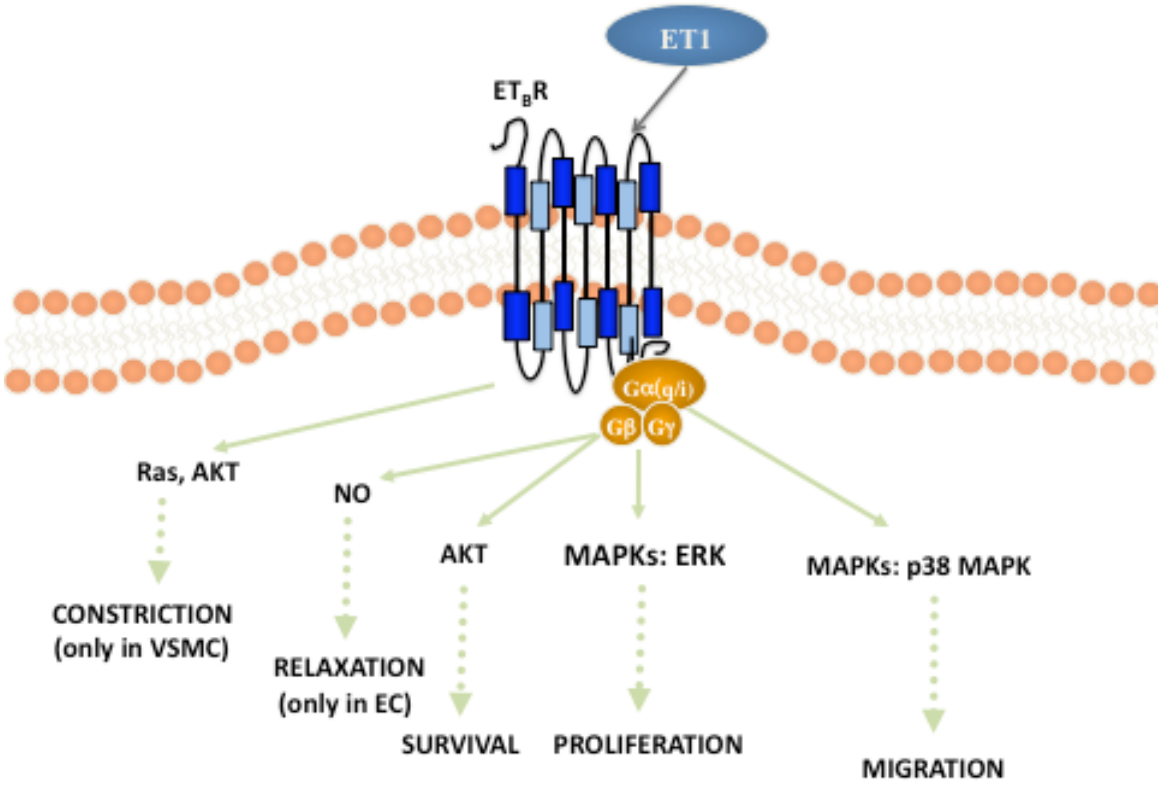
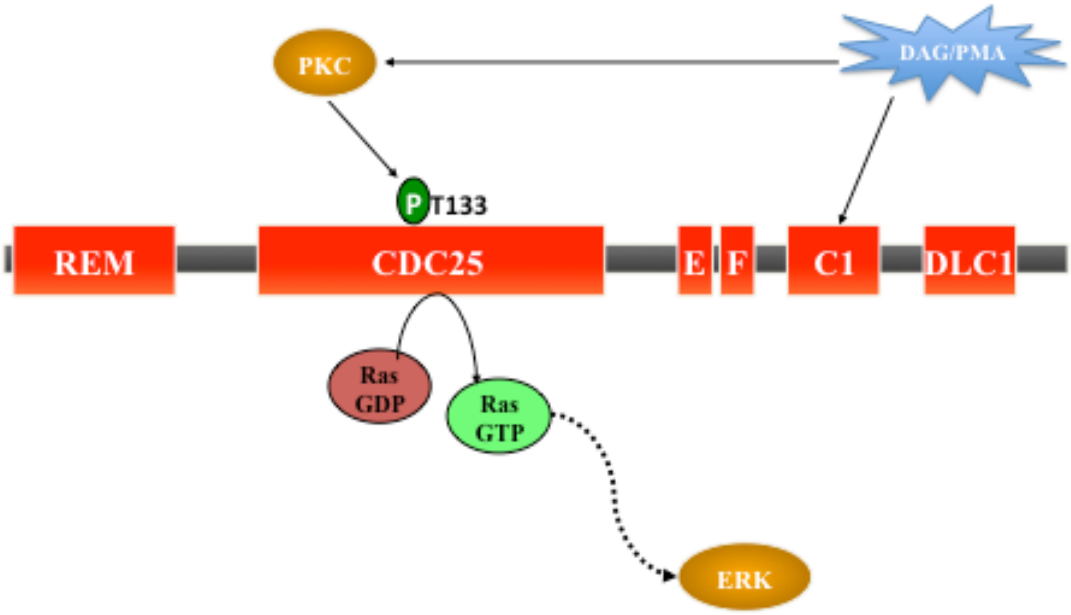

ET1
ET$_B$R
Gα(q/i)
Gβ
Gγ
Ras, AKT
NO
AKT
MAPKs: ERK
MAPKs: p38 MAPK
CONSTRICTION
(only in VSMC)
RELAXATION
(only in EC)
SURVIVAL
PROLIFERATION
MIGRATION

Figure 1.4. RasGRP3: Structure and Function. RasGRP3 consists of several domains. Starting from the C-terminus, RasGRP3 contains a DLC1 binding domain, the function of which *in vivo* is not clear but is hypothesized to regulate RasGRP3 localization. The C1 domain regulates RasGRP3 recruitment to the plasma membrane when bound by DAG or PMA, a DAG mimic. DAG/PMA also bind the C1 domain of PKC, which phosphorylates RasGRP3 at position Threonine 133 (T133) for activation. The function of the EF calcium binding hands in RasGRP3 signaling pathways is not known. The catalytic region is comprised of the Ras-exchanger-motif (REM) and CDC25 domains. The binding of the CDC25 domain to Ras-GDP is stabilized by the REM domain. As a Ras guanyl nucleotide exchange factor, RasGRP3 exchanges GDP for GTP to generate Ras-GTP. In its active form, Ras-GTP stimulates signals to promote ERK activation.


PKC
DAG/PMA
P
T133
REM
CDC25
E
F
C1
DLC1
Ras
GDP
Ras
GTP
ERK

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CHAPTER II

The Ras activator RasGRP3 mediates diabetes-induced embryonic defects and affects endothelial cell migration

This chapter is a manuscript submitted to the journal Circulation Research *(*Randhawa et al., in revision*). My focus is studying the effects of Rasgrp3 loss-of-function in endothelial cells. I characterized endothelial cells isolated from wildtype and Rasgrp3$^{gt/gt}$ mice (Supplemental Figure 2.6). Dr. Svetlana Rylova conducted the experiments using endothelial cells that over-express activated RasGRP3 (Figures 2.2, 2.3). Dr. Rylova and I performed the Ras and ERK activation assays to assess the requirement of RasGRP3 in phorbol ester signaling (Figure 2.4 A). I generated the data demonstrating that RasGRP3 is required for Ras-ERK activation induced by ET1 and not VEGF (Figures 2.4 B, C). I also showed that RasGRP3 is required for ET1-induced endothelial cell proliferation (Figure 2.5). In collaboration with Stephanie Kiser, we generated Figures 2.5 and 2.6. Joanna Fried performed whole embryo culture experiments and characterized embryos on the outbred background (Supplemental Figures 2.1, 2.2, 2.3). Under my mentorship, Jessica Heinz characterized diabetes-induced embryopathy in the inbred background using STZ-injected WT or*

Rasgrp3$^{gt/gt}$ mice and Ins2^{Akita}/+ or Ins2^{Akita}/+;Rasgrp3$^{gt/gt}$ mice (Figure 2.1). Former graduate students, Dave Roberts and Will Dunworth, generated Supplemental Figures 2.7 and 2.8.

A. Abstract

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 one or more pathways activated downstream of aberrant glucose metabolism, since poorly controlled maternal glucose levels correlate with the frequency and severity of the defects.

Objective: We asked whether RasGRP3, a Ras activator expressed in developing blood vessels, mediates diabetes-induced vascular developmental defects.

RasGRP3 is activated by diacylglycerol (DAG), and DAG is over-produced by aberrant glucose metabolism in diabetic individuals. We also investigated the effects of over-activation 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 lacking *Rasgrp3* function. Endothelial cells that expressed activated RasGRP3 had elevated Ras-ERK signaling and perturbed migration, while endothelial cells lacking *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 developing 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.

B. Introduction

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 PLCγ activation to produce diacylglycerol (DAG). DAG in turn activates downstream targets to affect endothelial cell behaviors. Signals that are up-regulated in diabetes, such as 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, since mouse embryos recovered from diabetic mothers had elevated DAG levels and increased developmental defects [3]. 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 PKC family of proteins. Numerous mammalian PKC isoforms fall into several sub-families, and DAG/phorbol esters activate PKC signaling by binding to C1 domains found in the classic (PKCα, β, γ) and novel (PKCδ, ε, η, θ) sub-groups of PKCs [10]. Recently several non-PKC protein families were identified

that contain C1 domains and respond to DAG and phorbol esters, suggesting that some DAG/phorbol ester-mediated responses require these proteins [11].

RasGRPs are non-PKC DAG/phorbol ester receptors that function as guanine nucleotide exchange factors (GEFs) 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 GEFs, such that recruitment of GEFs 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 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 non-redundant 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 was recently 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 mediating 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 phosphorylation is required for RasGRP3 activity in B cells [22, 23]. RasGRP3 also binds dynein light chain, a component of a microtubule plus-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, 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 over-activates RasGRP3, and this contributes to the perturbed development of fetuses in diabetic environments.

C. Materials and Methods

Diabetic mice and embryo analysis

Female mice (C57Bl/6J purchased from Jackson Laboratories, or *Rasgrp3*$^{gt/gt}$ (a loss-of-function null mutation [19]) backcrossed to N8 on the C57Bl/6J background) at 6-8 weeks of age were made diabetic following the protocol in "Animal Models of Diabetic Complications Consortium". Alternatively, *Ins2*^{Akita}/+ mice on the C57Bl6/J background (Jackson Laboratories, #003548) were bred to obtain *Ins2*^{Akita}/+; *Rasgrp3*$^{gt/gt}$ mice. Blood glucose was monitored weekly, and mice with blood glucose levels over 250 mg/dL were considered diabetic. Mice were mated to genotype-matched males, embryos were harvested at E9.5, fixed, and whole mount stained for PECAM as described [25]. Stained embryos were imaged and scored for defects as described (Supplemental Methods).

Whole embryo culture was done on embryos dissected at E7.5 or E8.5, in roller bottles as described [26]. Some embryos were treated with 50nM PMA (phorbol 12-myristate 13-acetate) and some with 20 mM glucose during the culture period. After 24 hr, embryos were removed and photographed.

Endothelial cells

HUVEC were purchased from Clonetics, cultured according to manufacturer's protocol, and used between passages 2-8. Transfections were done using an Amaxa nucleofector according to directions. For staining, cells were fixed,

permeabilized, blocked, and incubated with phalloidin-Alexa 555 (Molecular Probes). Migration assays were carried out 48 hr post-transfection as described (Supplemental Methods). Average velocity and distance to origin of cells was calculated using trajectory measures as diagrammed in Figure 2.3 J.

Mouse endothelial cells (wildtype and *Rasgrp3*$^{gt/gt}$) were generated and expanded as described [27, 28], with minor modifications. Proliferation assays and migration assays were as described (Supplemental Methods).

ES cell differentiation and analysis

Wild type (WT, $^{+/+}$) and RasGRP3 deficient (*Rasgrp3*$^{gt/gt}$) ES cells were maintained and differentiated for 8 days as previously described [29]. For inhibitor studies, day 7 ES cell cultures were pre-treated with the appropriate inhibitor for 2 hr prior to addition of 100 nM PMA for 24 hr. For ET1 stimulation, 100 nM ET1 in fresh medium was added daily between days 5-8. Antibody staining of ES cell cultures was as previously described [25, 29]. Quantitative image analysis of PECAM-stained ES cell cultures was performed as previously described [25].

Ras and ERK activation assays

HUVEC (50-80% transfected by GFP labeling after transfection) or mouse endothelial cells were grown to near confluency for 48 hr, serum starved

overnight, then processed as described (Appendix A) for Ras-GTP immunoprecipitation, or total Ras, pERK, or total ERK Western blots.

D. Results

Loss of Rasgrp3 protects embryos from diabetes-induced birth defects

Because RasGRP3 is expressed in developing vessels and somites of mid-gestation mouse embryos [19], we reasoned that embryos exposed to elevated DAG signaling would be susceptible to effects of RasGRP3-mediated signaling in vivo. Thus 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 hr. in phorbol ester under whole embryo culture conditions (Supplemental Figure 2.1). WT embryos exposed to PMA were severely affected (Supplemental Figures 2.1, 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 (Supplemental Figures 2.1 E-H). We next incubated embryos in 20 mM glucose for 24 hr to mimic the diabetic environment (Supplemental Figure 2.2). Glucose exposure between E8.5 and E9.5 led to both vascular and non-vascular defects in WT embryos, while *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, relative to *Rasgrp3*$^{gt/gt}$ mutant embryos (Supplemental Figure 2.3). To make more rigorous comparisons, we next examined embryos on the C57Bl6/J inbred background. We induced diabetes chemically via STZ, and we also utilized mice carrying the *Ins2*^{Akita} mutation that induces diabetes genetically. We monitored the average maternal blood glucose at sacrifice 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, whole-mount stained for PECAM-1 to visualize vessel development and patterning, and the severity of defects in head plexus vessels, intersomitic vessels, and trunk/somite scored as described in the Methods (Figure 2.1). WT embryos from diabetic mothers induced either chemically or genetically had a significantly elevated developmental severity index compared to controls (Figures 2.1 A-B, E, O; Supplemental Figure 2.4). Closer examination of the somite region showed defects in somites and in the pattern of intersomitic vessels (Figures 2.1 G-J), and similar defects were seen in the vascular plexus of the head (Figures 2.1 K-N). 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 2.1, C-N, O; Supplemental Figure 2.4).

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 (Supplemental Figure 2.5 and data not shown). Thus loss of *Rasgrp3* function has a protective effect on diabetes-induced developmental defects, including vascular defects, suggesting 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 HUVEC that up-regulated RasGRP3 activity, because the developmental defects are predicted to result from over-activation of RasGRP3 by DAG (Figure 2.2). Wild-type RasGRP3 linked to a GFP reporter, and constructs in which a K-Ras CAAX or H-Ras CAAX sequence was linked to the protein, were transiently expressed in HUVEC (Figure 2.2 A). 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 over-expression effects. Over-expression of WT RasGRP3 did not significantly increase Ras activation over baseline, but both of the CAAX tagged RasGRP3 proteins stimulated significant Ras activation (Figures 2.2 B, C). ERK activation is downstream of Ras activation, and in HUVEC both CAAX-tagged RasGRP3

proteins also significantly induced ERK activation (Figures 2.2 B, C). 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 over-activation on the cytoskeleton, since in other cell types Ras activation affects the cytoskeleton [30, 31]. HUVEC expressing the constructs described above were stained for microtubules (α-tubulin, data not shown) and actin (phalloidin) (Figures 2.3 A-H). The microtubule staining pattern was not affected by over-expression of RasGRP3 (data not shown); however, the actin cytoskeleton was dramatically altered in endothelial cells over-expressing membrane-localized RasGRP3 (Figures 2.3 E-H). RasGRP3-CAAX expressing cells appeared larger and flatter, and they had very few actin stress fibers. Cortical actin was also diminished in endothelial cells over-expressing RasGRP3-CAAX. Since actin stress fibers are implicated in proper migration, we investigated the migratory behavior of endothelial cells over-expressing RasGRP3 (Figures 2.3 I-L). Endothelial cells over-expressing RasGRP3 were identified by GFP expression, and all cells were labeled with cell-tracker (Figure 2.3 I). Cells were imaged, and the average velocity and distance migrated from origin was calculated as described in methods (Figure 2.3 J). 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 2.3 K-L). These results show that endothelial cells that over-express 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 verified by expression of endothelial markers. The cell lines were greater than 95% positive for PECAM-1, VE-cadherin, and ICAM-2 (Supplemental Figures 2.6 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 cell grew at similar rates (Supplemental Figures 2.6 E-F).

We asked 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 2.4 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, utilizing a mouse embryonic

stem (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* function [19] (Supplemental Figures 2.7 A-D). We therefore asked whether signaling downstream of activated RasGRP3 in developing vessels utilized the Ras effector pathways MEK/ERK, p38 MAPK or JNK. The vessel dysmorphogenesis seen upon PMA stimulation was significantly attenuated in the presence of the MEK inhibitor U0126 (Supplemental Figures 2.7 E-F, I). In contrast, the p38 MAP kinase inhibitor SB203580 had no effect on PMA-induced vessel dysmorphogenesis **(**Supplemental Figures 2.7 G-H, 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 (Supplemental Figure 2.8). A general inhibitor of most PKC isoforms, BIM (bisindolymaleimide), 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 (Supplemental Figures 2.8 C-D, I). Likewise, an inhibitor of the conventional PKC isoforms α and β, Gö6976, completely

blocked PMA-induced vessel dysmporphogenesis (Supplemental Figures 2.8 E-F, I). A third PKC inhibitor, rottlerin, predominantly affects PKCδ, and it partially blocked PMA-induced vessel dysmorphogenesis (Supplemental Figures 2.8 G-H, 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 signal(s) 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, two angiogenic factors that utilize 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 analyzed (Figures 2.4 B-C). As predicted, WT endothelial cells had increased levels of active Ras and ERK upon treatment with either VEGF or ET1. In contrast, while *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 over a 2-fold increase in mitotic index over baseline with ET1 treatment, while *Rasgrp3*$^{gt/gt}$ endothelial cells did not exhibit a significant change in their mitotic index with ET1 treatment (Figures 2.5 A-E). 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 2.5 F). 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

Since both ET1-induced endothelial cell proliferation and migration are RasGRP3-dependent, we asked whether ES cell-derived blood vessels had RasGRP3-dependent dysmorphogenesis induced by ET1 (Figure 2.6). ET1 treatment of WT vessels led to loss of the fine vascular network and significantly increased vascular area (Figures 2.6 A-B, E). This response was *Rasgrp3*-dependent, as *Rasgrp3*$^{gt/gt}$ vessels retained the fine vascular network and did not exhibit increased vascular area under with ET1 treatment (Figures 2.6 C-D, E). Thus, developing vessels exhibit vessel dysmorphogenesis in response to ET1, and that response requires RasGRP3. Taken together, these data suggest that RasGRP3 is required for ET1-induced endothelial cell angiogenic responses.

E. Discussion

RasGRP3 is an activator of Ras family GTPases that is expressed in angiogenic vessels and required for the DAG/phorbol ester-mediated responses of these vessels. Here we posit that RasGRP3 is over-activated in embryos developing in a diabetic environment and susceptible to birth defects, and we show that loss of RasGRP3 significantly attenuates the detrimental effects of a diabetic environment on embryonic vascular development. We also define the molecular and cellular processes perturbed by DAG/phorbol ester-induced activation of RasGRP3 activation in endothelial cells of developing vessels. We define ET1 as an upstream input and Ras as a target of RasGRP3 in endothelial cells, and we show that manipulation of RasGRP3 perturbs endothelial migration.

These data lead 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 2.7).

We activated RasGRP3 in developing vessels using phorbol ester as a DAG mimic, and RasGRP3-dependent vessel dysmorphogenesis required MEK/ERK signaling. Our data indicates that Ras is a primary target of RasGRP3 in endothelial cells, since over-expression of RasGRP3 linked to either K-Ras CAAX or H-Ras CAAX domains significantly activated both Ras and ERK, while loss-of-function analysis showed that RasGRP3 was required for ET1- or DAG/phorbol ester-mediated Ras and MEK/ERK activation. Our 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 both localization to membranes via DAG binding to its C1 domain and by PKC phosphorylation [12]. Over-expression of RasGRP3 alone was not sufficient to activate endothelial Ras, but over-expression of membrane localized forms was sufficient for activation, suggesting that endogenous PKC levels are not rate-limiting for RasGRP3 activation in endothelial cells. Our studies also reveal a requirement for classical/novel PKC isoform activity in the response of developing vessels to DAG/phorbol esters. While it is possible that the PKC requirement is downstream of the genetic requirement for RasGRP3, we favor the hypothesis that PKC phosphorylation of RasGRP3 is required for its activity in endothelial cells, as has been shown in B cells [22, 23].

Elevated RasGRP3 activity affects specific cellular responses of endothelial cells. Endothelial cells that over-express activated RasGRP3 are flattened relative to controls, a phenotype also observed in neural cells expressing activated RasGRP3 [20]. Consistent with this phenotype, the actin cytoskeleton is perturbed in these cells, with loss of stress fibers and reduction of the cortical actin ring. The perturbation of the actin cytoskeleton in endothelial cells that over-express RasGRP3 is similar to actin perturbations seen in tumor cells that express activated Ras [35-37]. In general, Ras activation reduces actin stress fiber formation, primarily through effects on RhoA activity, but also via MEK signaling to ERK1/2 and ERK5 [30, 31, 38]. Actin stress fibers link to focal adhesions on the ventral side of cells, and this linkage promotes acto-myosin contractility [39, 40]. In terms of migration, stress fibers are thought to act as rudders that keep cells migrating directionally [41, 42]. Consistent with this idea, we find that endothelial cells over-expressing activated RasGRP3 have normal velocity but significantly reduced migration relative to the origin over time. This finding suggests that the cells can move, but their ability to link one movement to the next in a coordinated fashion is impaired. Thus, over-expression of activated RasGRP3 mimics Ras over-activation and prevents orderly forward migration of endothelial cells.

Complementary loss-of-function analysis showed that *Rasgrp3* function is required for DAG/phorbol ester mediated Ras activation, as is predicted to occur in diabetic environments with elevated DAG levels. Interestingly, RasGRP3 is involved in Ras/ERK signaling downstream of ET1 in endothelial cells. ET1 is elevated in diabetic milieus [43] and leads to DAG production, so perhaps two

sources of DAG – DAG produced via elevated glucose and DAG produced via elevated ET1 signaling, contribute to RasGRP3-mediated vascular pathologies in diabetic animals. In contrast, a second signal that is elevated in diabetic environments, VEGF-A, does not appear to require RasGRP3 for Ras-mediated signaling, although RasGRP3 expression is up-regulated by VEGF-A [19]. Several studies reported that ET1 angiogenic effects required VEGF-A [44, 45]. In light of our work, it is possible that this VEGF-A requirement reflects a need for VEGF-A-stimulated RasGRP3 expression. Thus, the requirement for RasGRP3 in ET1-mediated signaling may link the activities of the two pathways in diabetes.

Because diabetes is accompanied by elevated DAG levels and leads to developmental defects, including vascular defects, we hypothesized that in a diabetic fetal environment elevated maternal glucose crosses the placenta, where it is metabolized to DAG. The excess DAG ectopically activates embryonic RasGRP3, and downstream activation of Ras signaling contributes to the increased incidence of developmental problems. This hypothesis predicts that embryos lacking *Rasgrp3* are less susceptible to diabetes-induced birth defects, and in fact embryos lacking *Rasgrp3* were significantly protected from developmental defects produced in a diabetic fetal environment. This was true whether the mothers were diabetic from STZ destruction of pancreatic β cells or via the $Ins2^{Akita}$ mutation, which inactivates the insulin II gene and thus leads to diabetes [46]. The embryonic defects we documented were similar to the defects reported by others [3, 4]. Interestingly, embryos that lacked *Rasgp3* function and developed in diabetic mothers had significantly fewer defects in developing

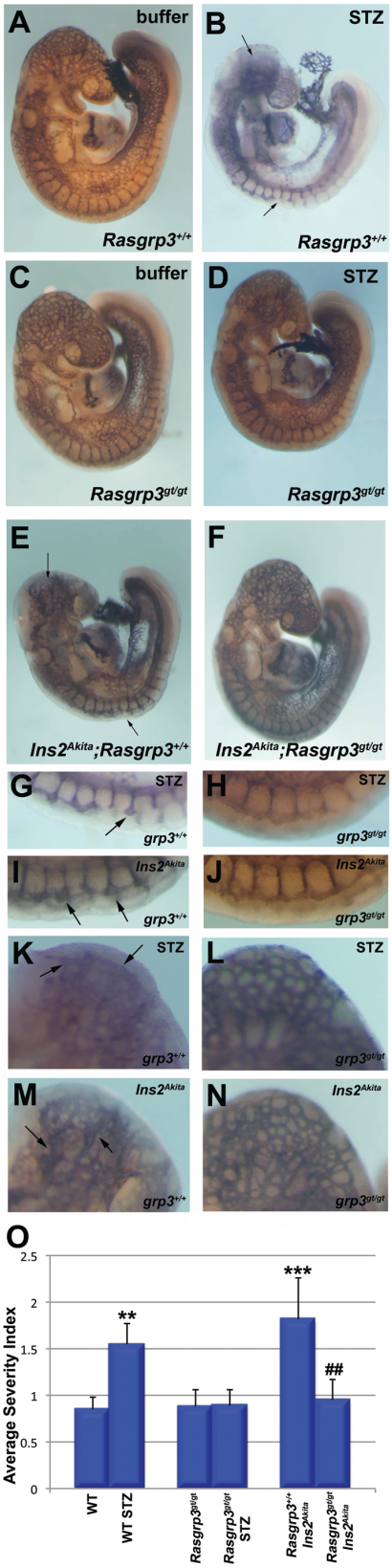
somites and developing vessels, two embryonic organs that express RasGRP3 [19]. The vascular defects seen in WT embryos from diabetic mothers were consistent with the endothelial migration defects observed in primary endothelial cells, since the intersomitic vessels were sometimes blunted, with expanded migratory fronts. Interestingly, while yolk sac vessels showed some pattern defects, these did not significantly correlate 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], indicating that upstream requirements for RasGRP3 activation are in place in the embryo. In our work, mothers lacking RasGRP3 became diabetic, and their average blood glucose at sacrifice was higher than WT controls. Whole embryo culture using medium supplemented with PMA showed that embryos lacking *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, showing 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 (this study and [19]) indicates that blockade of RasGRP3 is not detrimental to overall development. The focus of this study was analysis of gain- and loss-of-function for RasGRP3 in developing vessels and endothelial cells, and we show 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.

Figure 2.1. 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 isolated from *Ins2*^{Akita} (E, I, M) or *Ins2*^{Akita}; *Rasgrp3*$^{gt/gt}$ (F,J, N) diabetic mothers and stained for PECAM. (A) WT embryos from non-diabetic WT mothers have few defects, while (B) WT embryos from diabetic mothers have defects in the somitic vasculature (arrow), head plexus (arrow), and somites; (C) *Rasgrp3*$^{gt/gt}$ embryos from *Rasgrp3*$^{gt/gt}$ non-diabetic mothers have few defects, and (D) *Rasgrp3*$^{gt/gt}$ embryos from *Rasgrp3*$^{gt/gt}$ diabetic mothers are also relatively normal. (E) Embryos from *Ins2*^{Akita}; *Rasgrp3*$^{+/+}$ mothers have somitic vessel and head plexus defects (arrows), whereas (F) embryos from *Ins2*^{Akita}; *Rasgrp3*$^{gt/gt}$ mothers have 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 non-diabetic (n = 46); Lane 2, WT STZ diabetic (n = 51); Lane 3, *Rasgrp3*$^{gt/gt}$ non-diabetic (n = 25); Lane 4, *Rasgrp3*$^{gt/gt}$ STZ diabetic (n = 27); Lane 5, *Ins2*^{Akita}; *Rasgrp3*$^{+/+}$ diabetic (n = 17); Lane 6, *Ins2*^{Akita}; *Rasgrp3*$^{gt/gt}$ diabetic (n = 19). **, p≤0.001 for WT vs. WT-STZ diabetic; ***, p≤0.00001 for WT vs. *Ins2*^{Akita}; ##, p≤0.0001 for *Ins2*^{Akita}; *Rasgrp3*$^{+/+}$ vs. *Ins2*^{Akita}; *Rasgrp3*$^{gt/gt}$.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Rasgrp3+/+ Ins2Akita
Rasgrp3gt/gt Ins2Akita

Figure 2.2. Over-expression of activated RasGRP3 activates Ras and ERK in endothelial cells. (A) HUVEC were transiently transfected with the constructs shown. (B) IB for GFP linked to RasGRP3 constructs (top panel) 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 p-ERK (activated); ERK is total ERK blot. Lane 1, HUVEC transfected with GFP control; Lane 2, HUVEC transfected with GFP-RasGRP3; Lane 3; HUVEC transfected with GFP-RasGRP3-KCAAX; Lane 4; HUVEC transfected with GFP-RasGRP3-HCAAX. (C) Relative increase in reactivity for active Ras/total Ras (top panel) or active ERK/total ERK (bottom panel) relative to GFP control. *, $p \leq 0.01$; **, $p \leq 0.002$; ***, $p \leq 0.0001$, relative to the relevant control.

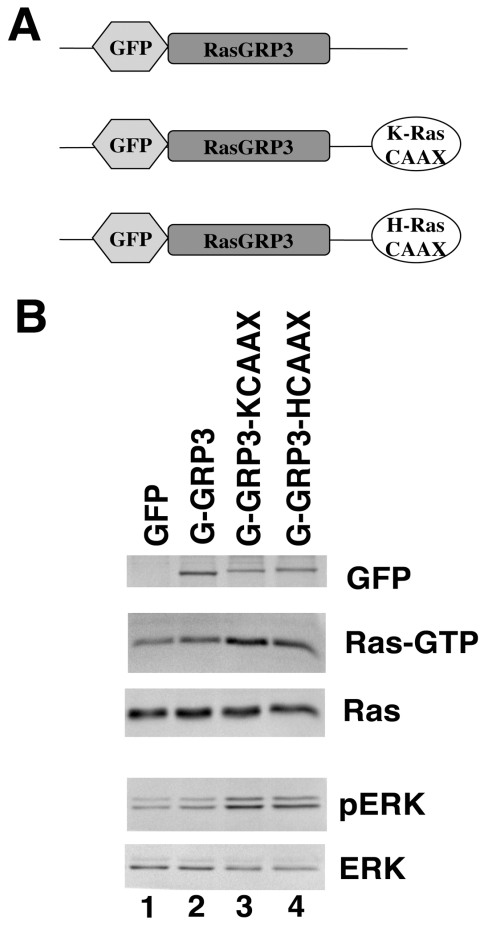
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Figure 2.3. Over-expression of active RasGRP3 perturbs the actin cytoskeleton and migration of endothelial cells. (A-H), HUVEC transfected with the indicated constructs were incubated for 48 hr, then fixed and imaged for GFP (green) (A-D) or stained with phalloidin (red) (B-H). (A-B) control GFP-CAAX; (C-D) GFP-RasGRP3; (E-F) GFP-RasGRP3-KCAAX; (G-H) GFP-RasGRP3-HCAAX. Astericks in panels E-H indicate transfected cells with perturbed phalloidin staining. (I-J), HUVEC were labeled with cell-tracker and live imaged. (I) HUVEC expressing GFP-RasGRP3 (green) and labeled with celltracker (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. **, $p \leq 0.005$; ***, $p \leq 0.0001$, relative to control.

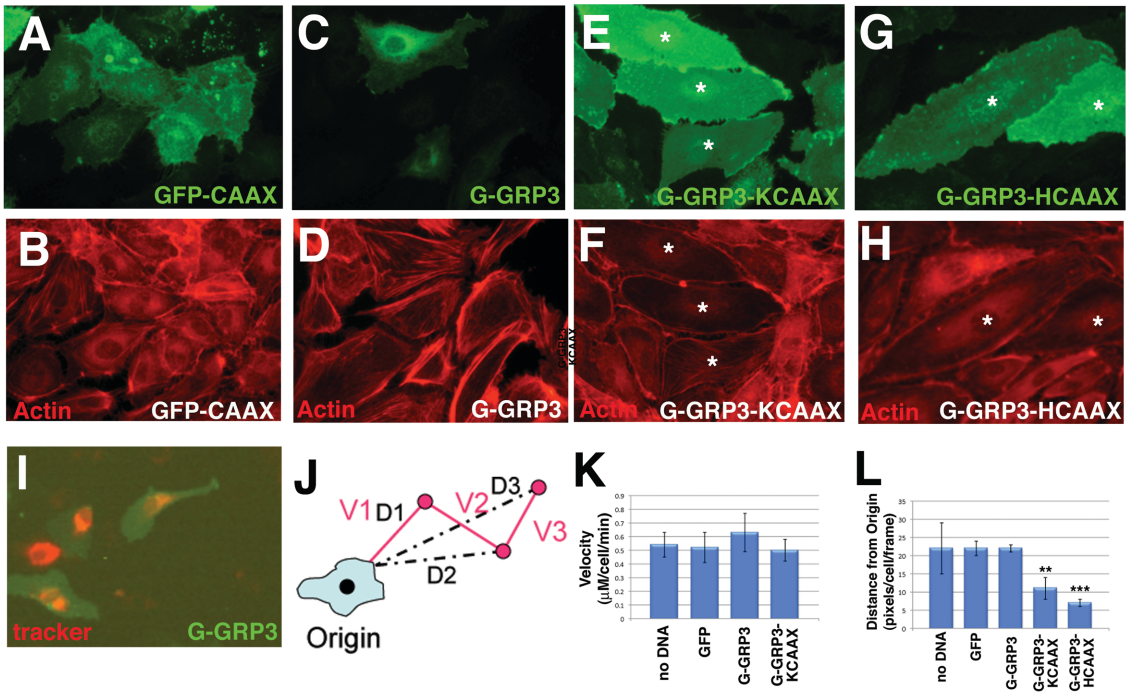
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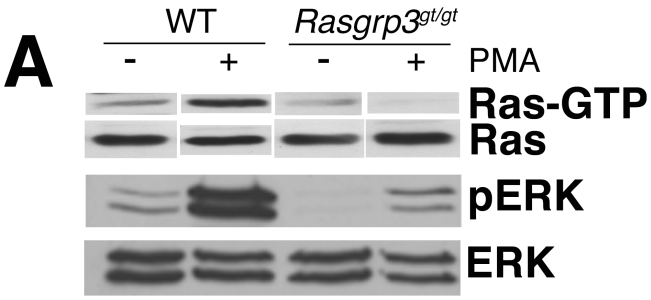
Figure 2.4. RasGRP3 is required for Ras-ERK activation downstream of phorbol ester and ET1 but not VEGF-A. (A) WT and *Rasgrp3*$^{gt/gt}$ endothelial cells were treated with PMA and processed for Ras and ERK activation. Compared to WT endothelial cells, *Rasgrp3*$^{gt/gt}$ endothelial cells did not show increased activated Ras (Ras-GTP) and had an attenuated activation of 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 not in response to ET1. Experiments are representative of at least 3 replicates.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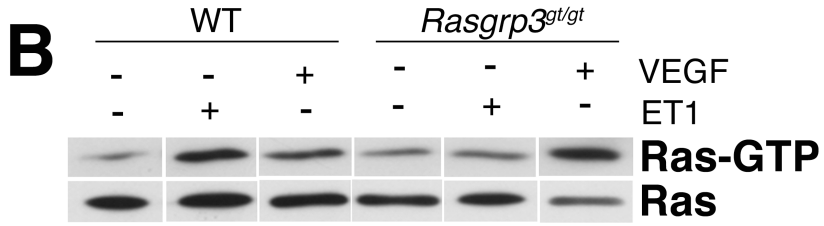
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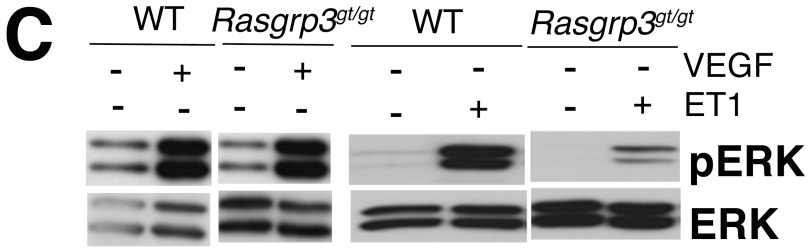
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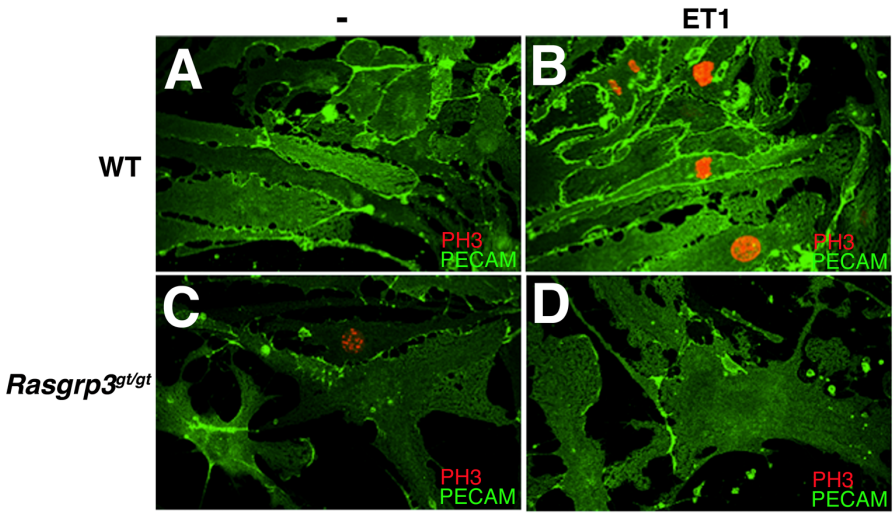
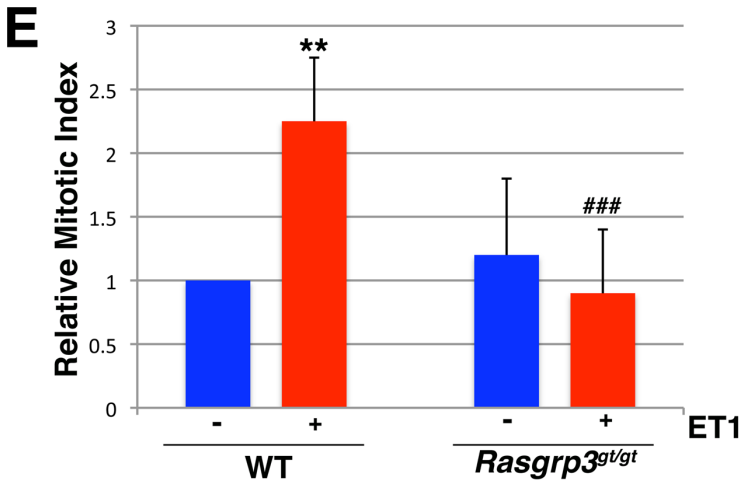
pERK
ERK

Figure 2.5. 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 PECAM (green) and PH3 (red). **(E)** Representative areas were imaged and mitotic cells counted. The relative mitotic index was significantly increased in WT endothelial cells treated with ET1, but was not significantly increased 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 towards both ET1 and VEGF-A compared to control, whereas *Rasgrp3*$^{gt/gt}$ endothelial cells had significantly increased migration towards VEGF-A but not towards ET1. *, $p \leq 0.01$ relative to untreated; **, $p \leq 0.0001$ relative to untreated; ##, $p \leq 0.0001$, *Rasgrp3*$^{gt/gt}$ ET-1 treated vs. WT ET1-treated.


-
ET1
A
B
C
D
WT
Rasgrp3gt/gt
PH3
PECAM


E
Relative Mitotic Index
3
2.5
2
1.5
1
0.5
0
**
###
-
+
-
+
ET1
WT
Rasgrp3gt/gt

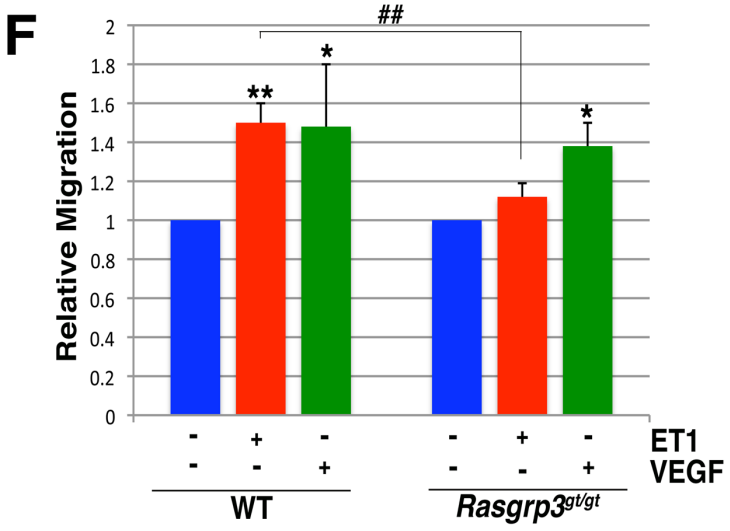
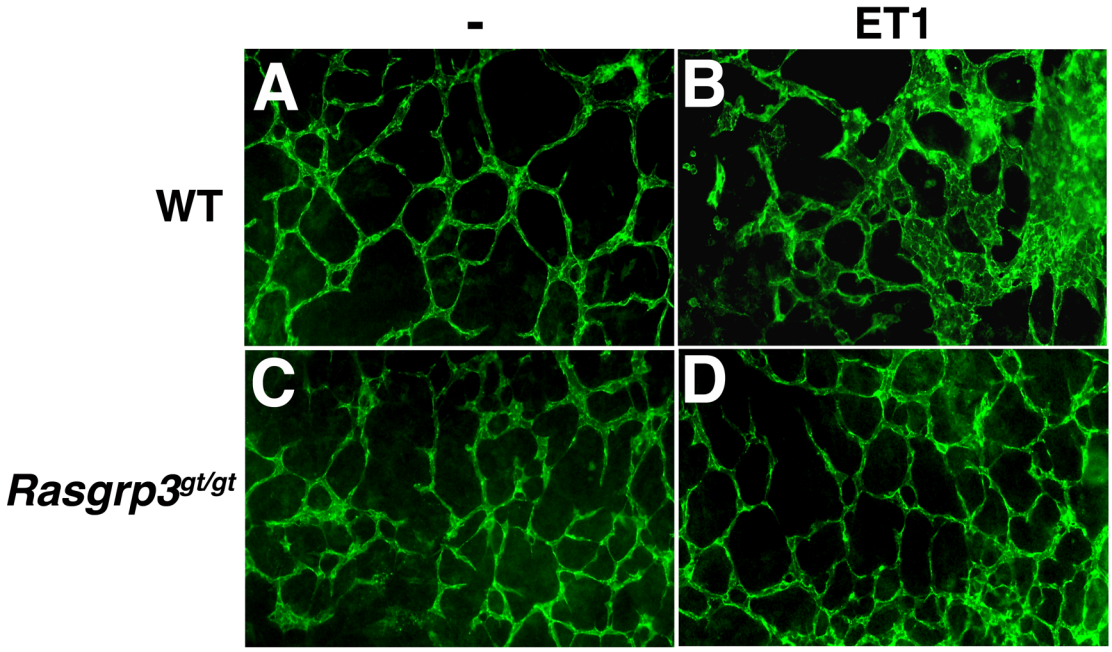

F
Relative Migration
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
##
*
**
*
-
+
-
-
-
+
ET1
VEGF
WT
Rasgrp3gt/gt

Figure 2.6. 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 PECAM (green) on day 8. **(E)** Representative images were quantified for vascular area. WT vessels treated with ET1 had significantly increased vascular area, whereas ET-1 treated *Rasgrp3*$^{gt/gt}$ vessels did not have increased vascular area. **, $p \leq 0.01$ relative to control.


-
ET1
A
B
WT
C
D
Rasgrp3gt/gt

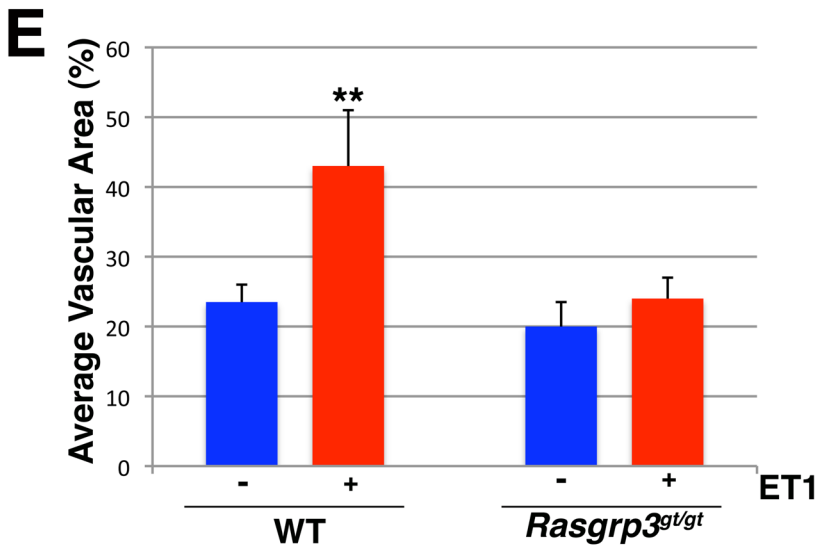
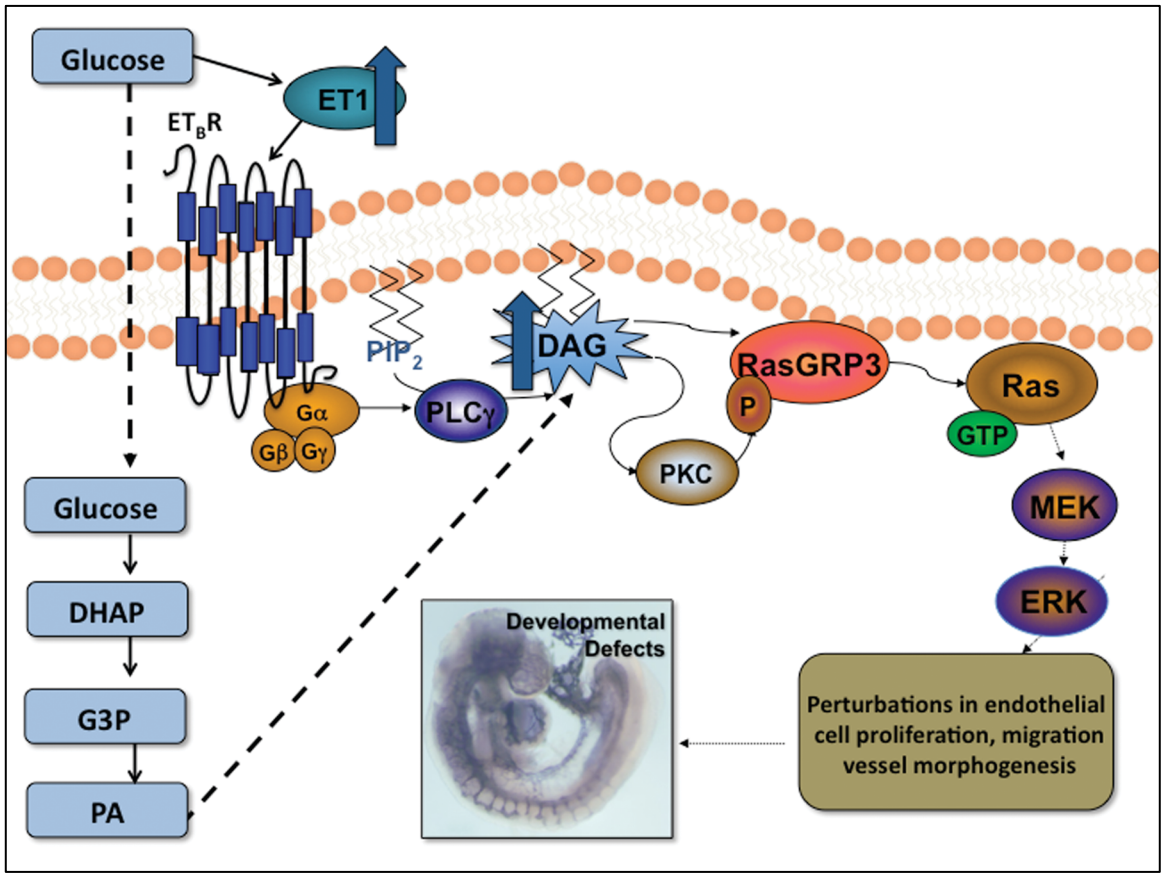
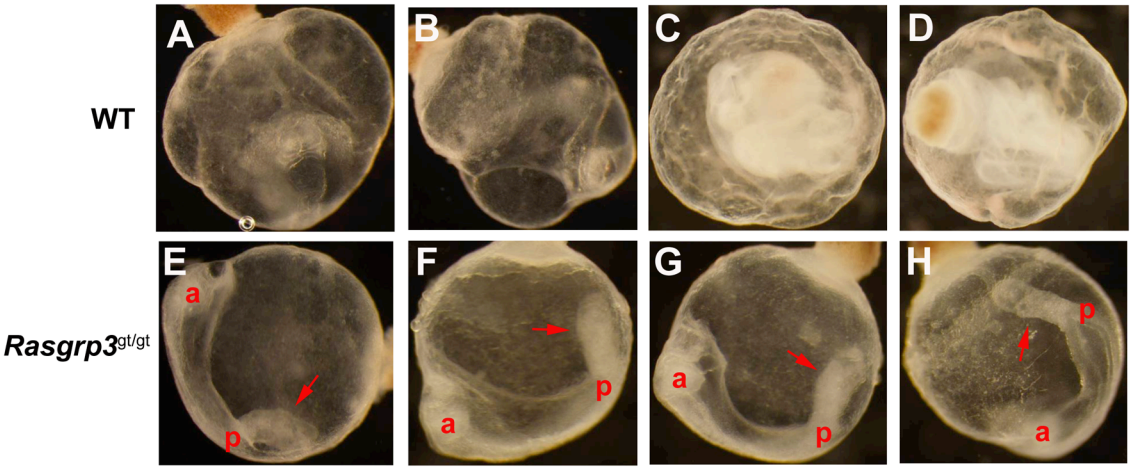

E
Average Vascular Area (%)
60
50
40
30
20
10
0
**
-
+
-
+
ET1
WT
Rasgrp3gt/gt

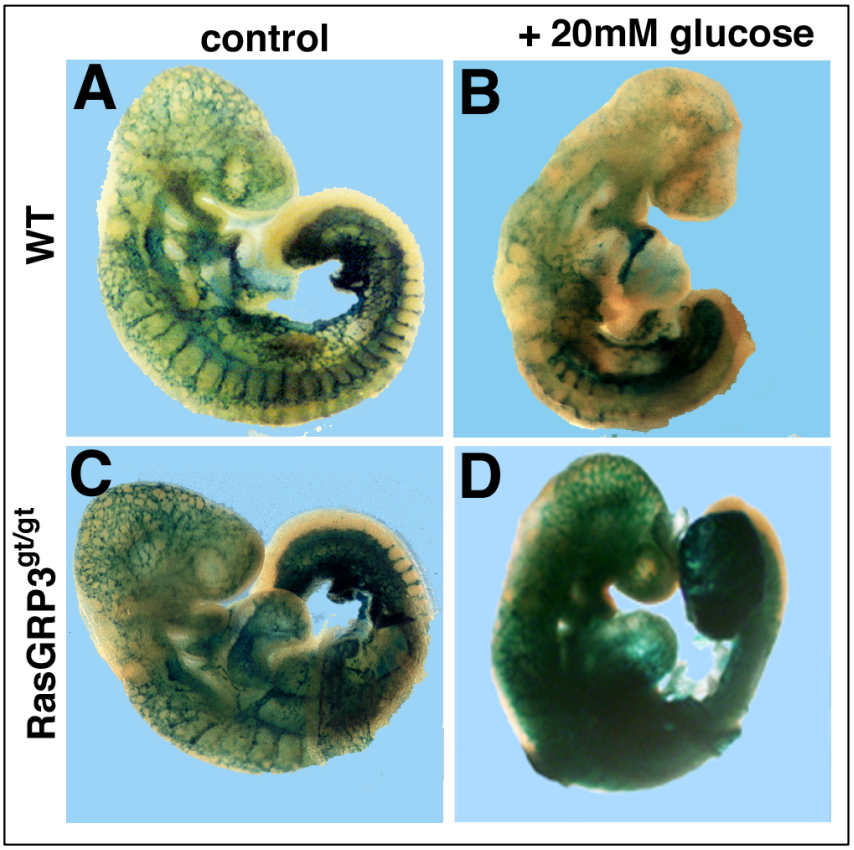
Figure 2.7. Model of activated RasGRP3 signaling and function in endothelial cells. In this model, maternal diabetic conditions induce elevated DAG production in embryos via aberrant glucose metabolism and via ET1 signaling; endothelial RasGRP3 is over-activated by DAG and PKC phosphorylation to activate Ras (Ras-GTP). Ras signaling through MEK/ERK affects the endothelial actin cytoskeleton and perturbs migration, leading to developmental defects.


Glucose
ET1
ET$_B$R
PIP$_2$
DAG
RasGRP3
P
Ras
GTP
Gα
Gβ
Gγ
PLCγ
PKC
MEK
ERK
Glucose
DHAP
G3P
PA
Developmental Defects
Perturbations in endothelial cell proliferation, migration vessel morphogenesis

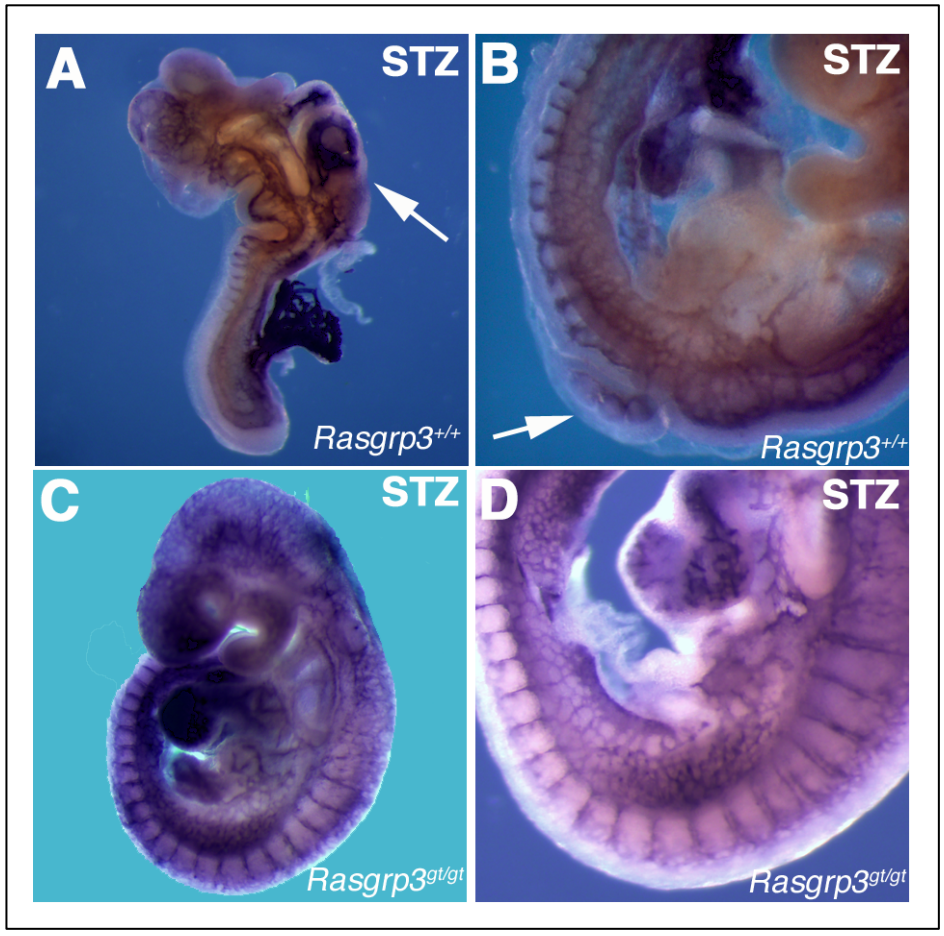
Supplemental Figure 2.1. Loss of *Rasgrp3* protects from PMA-induced developmental perturbations. Mouse embryos that were WT (A-D) or mutant for *Rasgrp3* (E-H) were dissected at E7.5 with yolk sac intact, and incubated for 24 hr in the presence of 50 nM PMA. WT embryos uniformly exhibited severe defects 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).


WT
Rasgrp3gt/gt
A
B
C
D
E
F
G
H
a
p
a
p
a
p
p
a

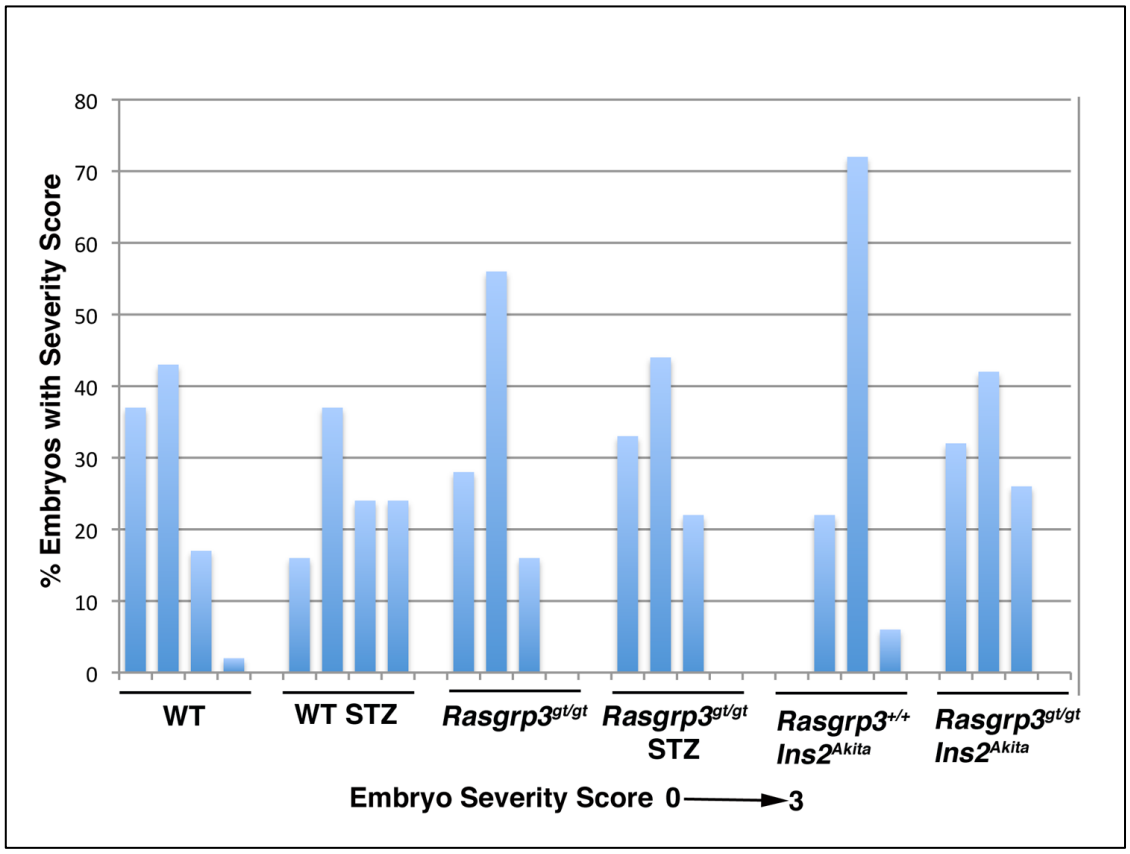
Supplemental Figure 2.2. Loss of *Rasgrp3* protects from diabetes-induced defects. WT (A,B) or *Rasgrp3*$^{gt/gt}$ (C, D) embryos were dissected from diabetic mothers at E8.5 and cultured for 24 hr in control media (A, C) or media with 20 mM glucose (B, D), then processed for β-galactosidase activity.


control
+ 20mM glucose
A
B
C
D
WT
RasGRP3gt/gt

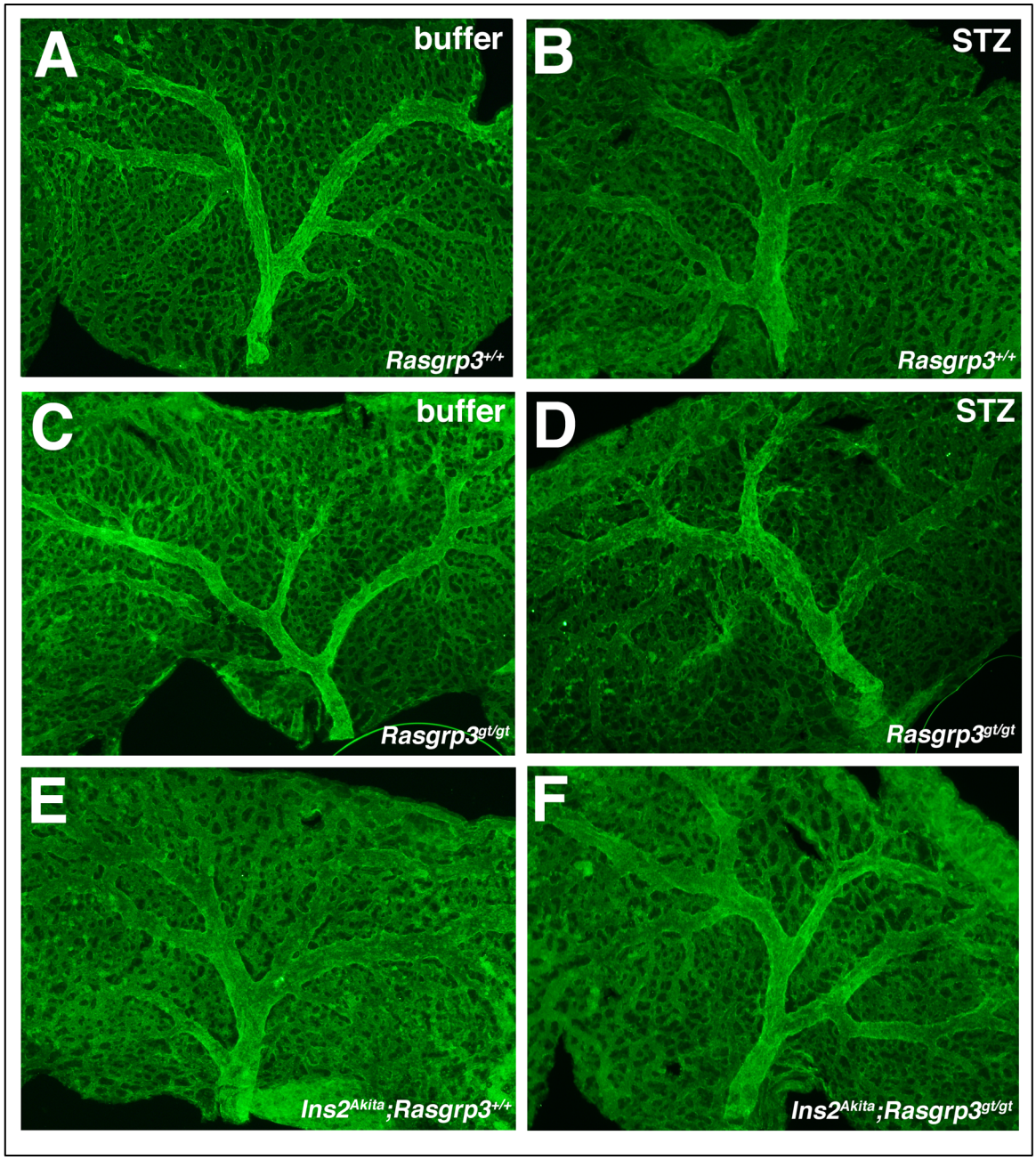
Supplemental Figure 2.3. Loss of *Rasgrp3* protects from diabetes-induced defects. WT (A,B) or *Rasgrp3*$^{gt/gt}$ (C, D) embryos were dissected from diabetic mothers at E9.5 and stained in whole mount for PECAM-1. (A, B) arrows point to severe defects seen in two different WT embryos; (C, D) two different *Rasgrp3*$^{gt/gt}$ embryos have normal development.


A
STZ
Rasgrp3+/+
B
STZ
Rasgrp3+/+
C
STZ
Rasgrp3gt/gt
D
STZ
Rasgrp3gt/gt

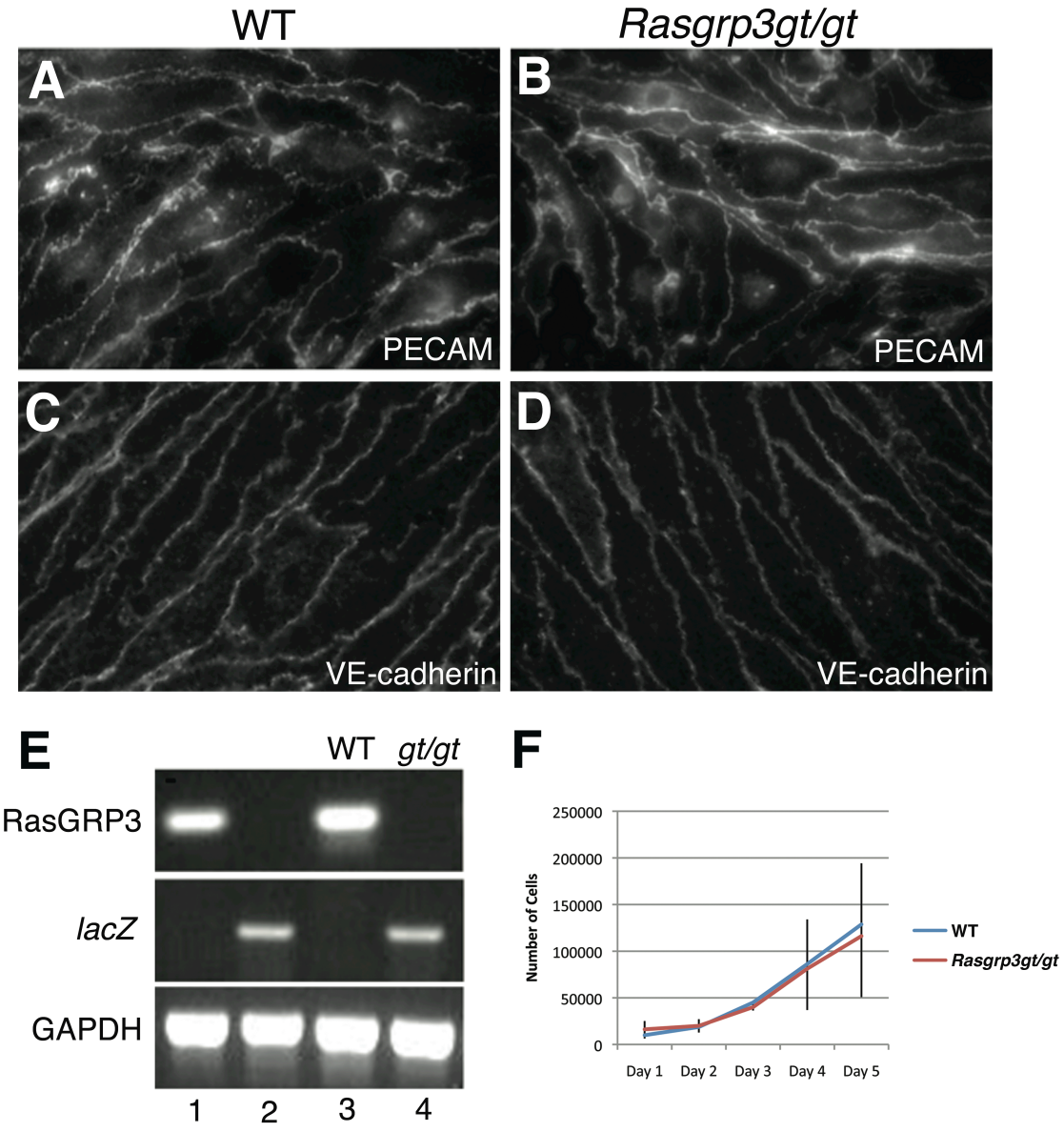
Supplemental Figure 2.4. Distribution of Embryo Severity Index values relative to genotype/condition. For each group, embryos were scored on a scale from 0 (no defects) to 3 (severe defects) as described. The proportion in each category is indicated. WT, mothers were normo-glycemic; STZ, mothers were treated with streptozotocin and were diabetic.


% Embryos with Severity Score
80
70
60
50
40
30
20
10
0
WT
WT STZ
Rasgrp3gt/gt
Rasgrp3gt/gt STZ
Rasgrp3+/+ Ins2Akita
Rasgrp3gt/gt Ins2Akita
Embryo Severity Score 0 → 3

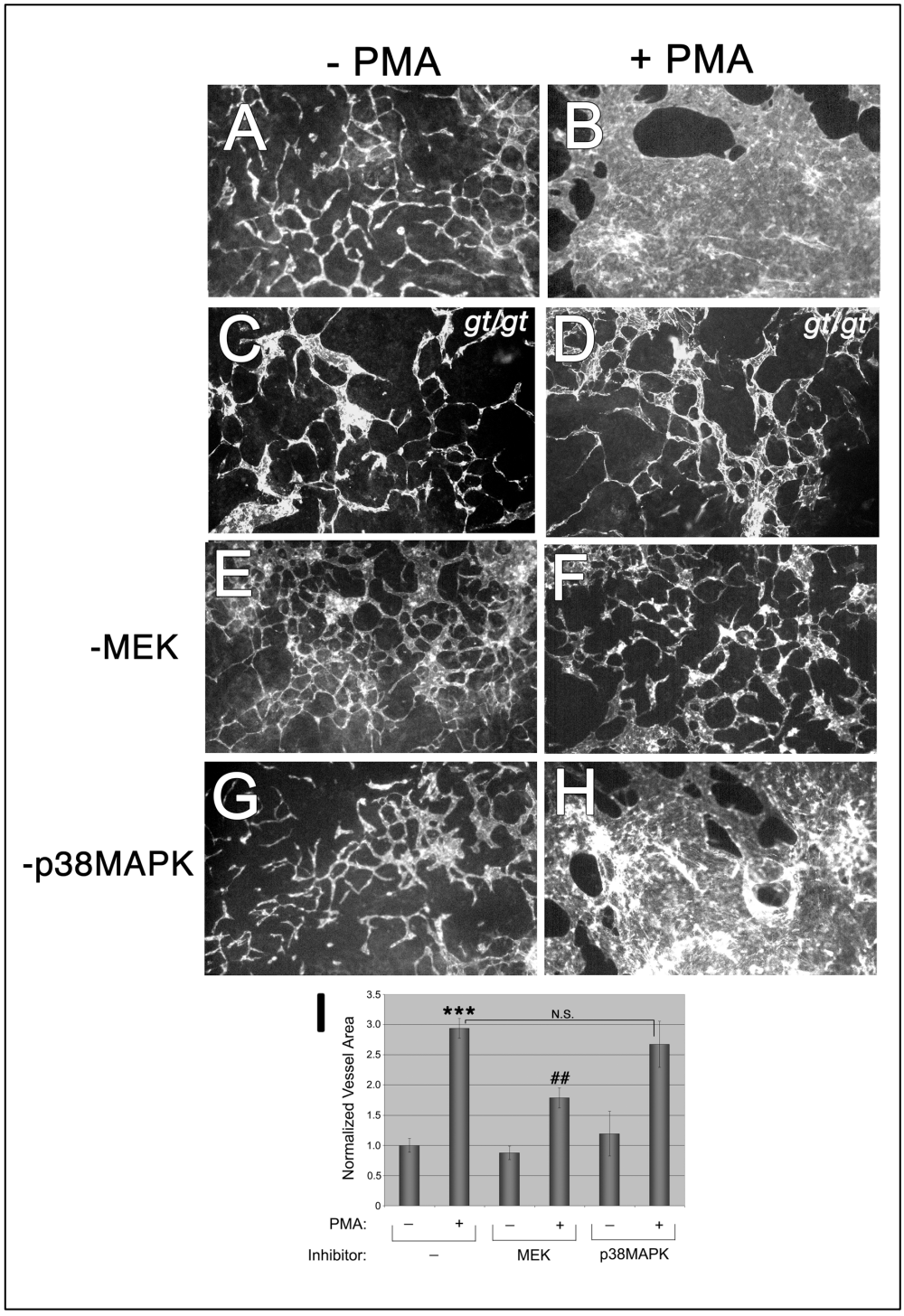
Supplemental Figure 2.5. Lack of yolk sac vascular defects associated with genotype/condition. (A-F) Yolk sacs of E9.5 embryos were processed for PECAM (green) immunofluorescence. No significant trends were found that correlated with genotype, condition, or severity of embryo defects.


A
buffer
Rasgrp3+/+
B
STZ
Rasgrp3+/+
C
buffer
Rasgrp3gt/gt
D
STZ
Rasgrp3gt/gt
E
Ins2Akita;Rasgrp3+/+
F
Ins2Akita;Rasgrp3gt/gt

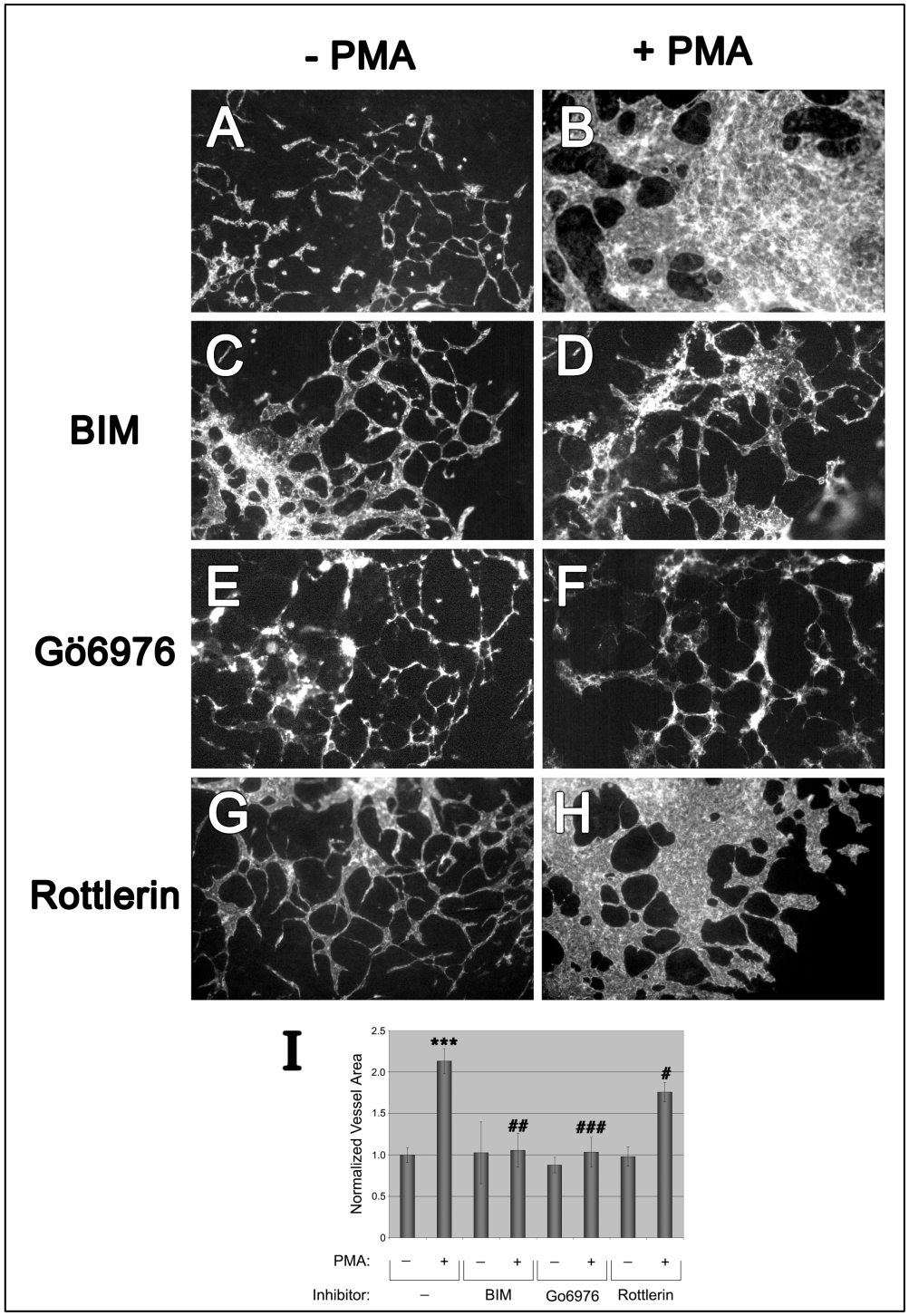
Supplemental 2.6. Characterization of WT and *Rasgrp3$^{gt/gt}$* endothelial cells. WT (**A, C)** and *Rasgrp3$^{gt/gt}$* **(B, D)** endothelial cells were isolated as described, and stained for PECAM-1 **(A, B)** or VE-cadherin **(C, D)**. (E) RT-PCR from RNA isolated from: lane 1, WT day 8 ES cell cultures; lane 2, *Rasgrp3$^{gt/gt}$* day 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.


WT
Rasgrp3gt/gt
A
PECAM
B
PECAM
C
VE-cadherin
D
VE-cadherin
E
WT
gt/gt
RasGRP3
lacZ
GAPDH
1
2
3
4
F
250000
200000
150000
100000
50000
0
Number of Cells
Day 1
Day 2
Day 3
Day 4
Day 5
WT
Rasgrp3gt/gt

Supplemental Figure 2.7. RasGRP3-dependent PMA-induced vessel dysmorphogenesis is blocked by MEK inhibition. Day 8 ES cell cultures stained for PECAM-1. (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≤ 0.00001 for -PMA (lane 1) vs. +PMA (lane 2); ##, p≤ 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).


- PMA
+ PMA
A
B
C
gt/gt
D
gt/gt
E
F
-MEK
G
H
-p38MAPK
I
Normalized Vessel Area
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
***
N.S.
##
PMA:
–
+
–
+
–
+
Inhibitor:
–
MEK
p38MAPK

Supplemental Figure 2.8. RasGRP3-dependent PMA-induced vessel dysmorphogenesis is blocked by PKC inhibition. Day 8 ES cell cultures stained for PECAM-1. (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≤ 0.00001 for -PMA (lane 1) vs. +PMA (lane 2); ##, p≤ 0.001 for +PMA (lane 2) vs. +PMA, +BIM inhibitor (lane 4); ###, p≤0.0001 for +PMA (lane 2) vs. +PMA, +Gö6976 inhibitor (lane 6); #, p≤0.01 for +PMA (lane 2) vs. +PMA, +rottlerin (lane 8).


- PMA
+ PMA
A
B
C
D
E
F
G
H
BIM
Gö6976
Rottlerin
I
Normalized Vessel Area
2.5
2.0
1.5
1.0
0.5
0
***
##
###
#
PMA:
– + – + – + – +
Inhibitor:
–
BIM
Go6976
Rottlerin

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CHAPTER III

GENERAL DISCUSSION

The delivery of nutrients via the vascular system is tightly regulated by insulin signaling. Disruption of regulatory signals significantly elevates blood glucose levels and eventually leads to diabetes. Metabolism of excess blood glucose induces DAG production, and elevated DAG promotes signaling of pathways such as VEGF and ET1 in endothelial cells. Increased levels of DAG, VEGF and/or ET1 perturb endothelial behavior and vessel function. In my work, I sought to understand how the Ras activator, RasGRP3, acts to mediate endothelial cell behaviors, such as proliferation and migration, and vessel dysmorphogenesis. Because RasGRP3 is activated by DAG, RasGRP3-dependent regulation of these phenotypes may significantly contribute to diabetes-induced embryopathy. For the first time, I have also identified ET1-mediated signaling as an upstream input for the RasGRP3 pathway. However, further investigation is required to determine the role of RasGRP3-mediated ET1 signaling in diabetes-induced pathology. Because diabetes causes vessel dysfunction, elucidating the pathophysiological role of RasGRP3 in endothelial cells will be informative to develop strategies that will protect the embryo from maternal hyperglycemia.

Diabetes-induced embryonic defects occur most frequently in the heart and the central nervous system ([1], review: [2-4]). The mechanisms by which maternal hyperglycemia causes embryopathy are not well understood. In Chapter II, work by Joanna Fried and Jessica Heinz assessed embryos from *wildtype* and *Rasgrp3*^{gtgt} diabetic and non-diabetic mice. Their results demonstrate that loss of RasGRP3 confers protection by rescuing diabetes-induced embryopathy, which

suggests that RasGRP3 is a strong mediator of this phenotype. For the purpose of this dissertation, we focused on the role of RasGRP3 in mediating vascular defects, but intriguingly, loss of RasGRP3 also rescues defects in body-axis patterning and neural tube closure. RasGRP3 is expressed in developing somites and in glial cells of the adult brain in mice [5, 6]. Over-activation of RasGRP3 in developing somites may explain diabetes-induced patterning and closure defects. These results warrant further investigation to determine how RasGRP3 mediates diabetes-induced embryopathy, and whether RasGRP3 has currently unidentified roles in other systems regulating body segmentation (skeletal development) or brain patterning (neural network formation).

To understand the contribution of RasGRP3 to diabetes-mediated effects, we must identify cellular signaling pathways that are mediated by RasGRP3. Other groups have provided evidence that perturbations in RasGRP3 signaling significantly impact Ras-ERK signaling [7-13], which we have confirmed in endothelial cells using gain- and loss-of function studies. Work by Dr. Svetlana Rylova illustrates that over-expression of activated RasGRP3 alone in endothelial cells is sufficient to induce Ras and ERK activation. Additionally, I have shown that loss of RasGRP3 effectively inhibits PMA- or ET1-induced Ras and ERK activation. PMA and ET1 promote activity of both PKC and RasGRP3, which are both capable of Ras activation [6-9, 11, 13-15]. Interestingly, our results indicate that PMA- and ET1-induced Ras activation is entirely through RasGRP3. Surprisingly, RasGRP3 does not affect VEGF-induced Ras and ERK activation, suggesting that VEGF either acts independently of RasGRP3 or has redundant

mechanisms to compensate for the loss of RasGRP3. My data imply that RasGRP3 is not globally necessary to mediate Ras-ERK signaling but is only activated by specific stimuli, such as ET1 signaling. The results presented here confirm that RasGRP3 is required for Ras-ERK signaling promoted by either excess DAG, which was simulated with phorbol ester, or by ET1.

The requirement for RasGRP3 in ET1-induced Ras activation provides some perspective on other studies that assess the effects of ET1 signaling in diabetes. Other groups have demonstrated that blockade of Ras signaling alleviates ET1-mediated hypertension in diabetic rats [7-9]. My results suggest that these effects are mediated by RasGRP3, and thus RasGRP3 may be a suitable target for Ras blockade in ET1-induced hypertension. ET1-induced hypertension is primarily regulated through ET_AR, which is highly expressed in vascular smooth muscle cells (VSMC) [10-12]. RT-PCR experiments also show that RasGRP3 is expressed in VSMC (data not shown). In Chapter II, we have demonstrated that RasGRP3 is required for several aspects of ET1 signaling; therefore, it would be interesting to determine whether VSMC-derived RasGRP3 also mediates ET1-induced hypertension *in vivo*. These experiments could be conducted by crossing *Rasgrp3*$^{gt/gt}$ mice with *ET1*$^{+/-}$ mice, which display elevated blood pressure [11, 12]. Increased hypertension in *ET1*$^{+/-}$ mice may be caused by differences in activity between nonendothelial- and endothelial-derived ET1, as mice heterozygous for an endothelial-specific ET1 deletion display normal blood pressure [11]. If RasGRP3 does regulate ET1-induced hypertension, I would expect that *Rasgrp3*$^{gt/gt}$*/ET1*$^{+/-}$ offspring to be protected from elevated blood pressure. To

take this a step further, similar studies could be conducted in hypertensive-diabetic mice. I would still expect that loss of RasGRP3 would confer protection against diabetes-induced hypertension. These experiments would identify a new role for RasGRP3 in ET1-induced hypertension, especially in diabetic backgrounds.

Recent work by Yang et al. [13] determined that RasGRP3 activation increased cell proliferation and migration in prostate cancer cells, which we have also confirmed in endothelial cells. For the first time, I show that loss of RasGRP3 prevents ET1-induced increases in endothelial cell proliferation. These results are consistent with inhibition of Ras-ERK signaling or ET1 signaling in general ([14-17], review: [18]). Work by Dr. Rylova, Stephanie Kiser and myself illustrates that RasGRP3 is required for ET1-mediated migration in endothelial cells. Dr. Rylova shows that over-expression of RasGRP3 causes a reduction in actin stress fibers and inhibits directional migration, while other data confirms that RasGRP3 is required in ET1-induced migration. Changes in the cytoskeleton and cell adhesion are important to facilitate migration. The mechanisms by which RasGRP3 mediates these processes to promote migration are not clear. At first glance, the data seem counter-intuitive: our results demonstrate that RasGRP3 positively mediates ET1-induced migration, but over-expression of RasGRP3 inhibits migration. I hypothesize that these differences are due to a dosage-specific requirement for RasGRP3 to mediate such cellular responses.

Differences in migration can be attributed to specific levels of RasGRP3: activated RasGRP3 signaling normally promotes migration, but excessive

RasGRP3 signaling inhibits migration. This is a common phenotype of Rac GTPase activation [19, 20]. Rac is known to promote migration by initiating signals that down-regulate Rho GTPase to destabilize cell adhesions and stress fibers [20]. However, over-expression of Rac and complete loss of Rho activation completely inhibits migration. This occurs because cells require some level of structure (cytoskeleton) and adhesion to create traction and promote migration. Interestingly, we have evidence that a Rac inhibitor partially rescues PMA-induced vessel dysmorphogenesis, which suggests that Rac may be downstream of RasGRP3 (data not shown). In the future, it would be of interest to determine whether RasGRP3-dependent changes on cytoskeleton remodeling and migration require Rac. As discussed in Chapter II, over-expression of RasGRP3 in endothelial cells inhibits directional migration. To assess the role of Rac in RasGRP3-induced perturbations in migration, I would treat RasGRP3-over-expressing cells with a Rac inhibitor. I expect that these cells would display a dosage-dependent response to the Rac inhibitor. An optimal concentration would promote partial recovery of the actin cytoskeleton and increased migration. These results would implicate a role for Rac downstream of RasGRP3 and help elucidate whether the loss of directional migration is due to RasGRP3-induced Rac over-activation.

There are many other aspects of RasGRP3 signaling that warrant further exploration in order to understand how RasGRP3 functions *in vivo*. In addition to H-Ras, RasGRP3 can also activate R-Ras and Rap, which add dimension and complexity to RasGRP3 signaling [6, 21, 22]. Interactions of multiple signaling

cascades could mediate time-sensitive changes and negative feedback regulation. R-Ras is known to activate phosphatidylinositol 3-kinase (PI3K) to induce an AKT pathway that inhibits proliferation and directional migration [21, 23]. Yang et al. [13] have also provided evidence to support a role for RasGRP3 in AKT signaling and apoptosis in prostate cancer cells. This group showed that siRNA-mediated downregulation of RasGRP3 inhibits Ras, AKT, ERK, proliferation, migration and apoptosis [13]. Similar to H-Ras, Rap can also activate ERK to promote proliferation, but has varying effects on migration. Rap is thought to inhibit migration by promoting cell adhesion, but other studies have shown that Rap also positively mediates migration ([24-28], reviews: [29, 30]). Ultimately, RasGRP3 potentially acts as a signaling hub to integrate positive and negative signals that modulate endothelial cell behaviors. By identifying other key pathways mediated by RasGRP3, we can better understand how RasGRP3 mediates endothelial dysfunction and vessel dysmorphogenesis.

ET1 promotes tumor growth and neoangiogenesis ([39-44], review: [31]). In Chapter II, I show that RasGRP3 is required to mediate ET1-induced vessel dysmorphogenesis in a murine embryonic stem cell model. This dysmorphogenesis is representative of misregulated angiogenic behavior, which prompts the question: Is there a role for RasGRP3 in ET1-induced angiogenesis *in vivo*? Using *wildtype* and *Rasgrp3*$^{gt/gt}$ mice implanted with a matrigel plug, we can test the requirement for RasGRP3 in ET1-induced neoangiogenesis. I expect that only angiogenic vessels derived from *wildtype* and not *Rasgrp3*$^{gt/gt}$ mice would ingress into the ET1-containing matrigel plug. These data would

confirm whether RasGRP3 mediates ET1-induced angiogenesis, further contributing to evidence that RasGRP3 is important in pathologic angiogenesis. In a model derived from this work, ET1-mediated DAG production requires RasGRP3 signaling to promote changes in proliferation or migration, which can induce aberrant vessel growth and other diabetes-induced embryonic vascular defects (Figure 3.1). Our model also predicts that RasGRP3 is required for phorbol ester pathways to mediate similar changes in endothelial cell behavior or vessel morphology (Figure 3.1).

RasGRP3 has a pathophysiological role that has not been well explored. However, there is a growing literature correlating RasGRP3 to various pathologies, including young-onset hypertension, lupus erythematosus and cancer [13, 32]. Previous work by Roberts et al. demonstrated that RasGRP3 expression is upregulated in mammary tumors [5], and our current work identifies a role for RasGRP3 in diabetes-induced embryopathy. RasGRP3 is a curious protein, because it can mediate pathology even though it is not required for normal development. Interestingly, RasGRP3 is not the only protein in the vasculature that behaves this way. In fact, Zayed et al. [33], identify a similar role for calcium and integrin-binding protein-1 (CIB1), a 22 kDa regulatory protein that mediates P21/Cdc42/Rac1-activated kinase-1 (PAK1)-induced ERK signaling. Similar to RasGRP3, CIB1 is not required for physiological angiogenesis as mice lacking CIB1 are viable and appear to develop normally [33, 34]. Zayed et al. [33] demonstrate that loss of CIB1 attenuates VEGF or FGF induced PAK1 and ERK activation in endothelial cells and that CIB1 is required for pathological

angiogenesis induced by ischemia or tumorigenesis. Proteins such as CIB1 or RasGRP3 are attractive targets to treat aberrant vessel formation, while leaving physiological signaling pathways for vessel patterning intact. Ultimately, understanding the cellular outputs of RasGRP3 signaling will help us understand the role of RasGRP3 in disease.

A. Conclusions

We have identified a novel signaling axis that functions in conditions of excess DAG such as diabetes. Previously, the role of RasGRP3 in diabetes was not known, but with this work, we have established that RasGRP3 directly mediates diabetes-induced embryopathy, and we have provided a possible mechanism for this action. We show that perturbations in RasGRP3 alone (gain-of-function or loss-of-function) are sufficient to affect Ras-ERK signaling in endothelial cells, and that RasGRP3 is required for PMA- or ET1-induced Ras-ERK signaling. For the first time, we have identified ET1 as an upstream input for RasGRP3-mediated signaling. We have established that RasGRP3 is required for ET1-induced proliferation and migration in endothelial cells and ET1-induced aberrant vessel growth in embryonic stem cell-derived vessels (Figure 3.2). These novel contributions to the field of vascular biology are important for understanding how RasGRP3-mediated signaling affects vessel dysfunction, especially in diabetic conditions.

Figure 3.1. Hypothetical model for RasGRP3-dependent vessel dysmorphogenesis induced by PMA or ET1-mediated DAG signaling. ET1 signaling promotes DAG generation, which is mimicked by phorbol ester (PMA). DAG/phorbol ester-mediated signals activate RasGRP3. Intact RasGRP3 signaling can promote endothelial proliferation and migration, which leads to vascular dysmorphogenesis and embryopathy in diabetes. Disruption of RasGRP3 signaling pathways inhibits ET1-induced endothelial proliferation and migration and vessel morphogenesis or excess DAG signaling in general, which confers protection in diabetic backgrounds. (*) Preliminary experiments suggest that RasGRP3 is required for PMA-induced migration, but this has not been confirmed.

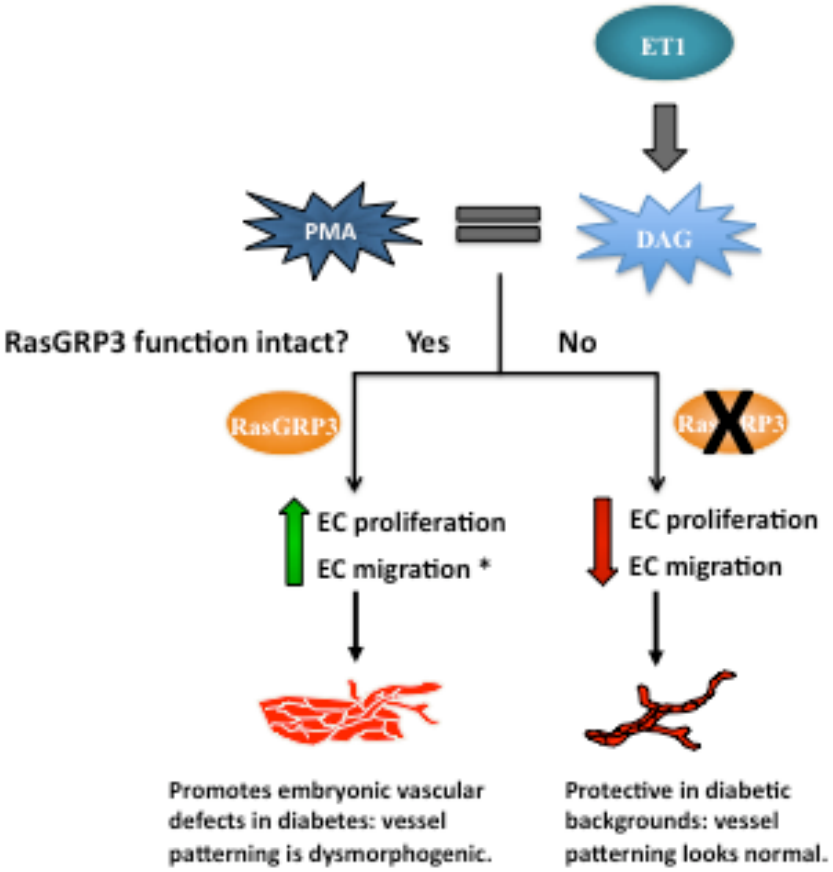
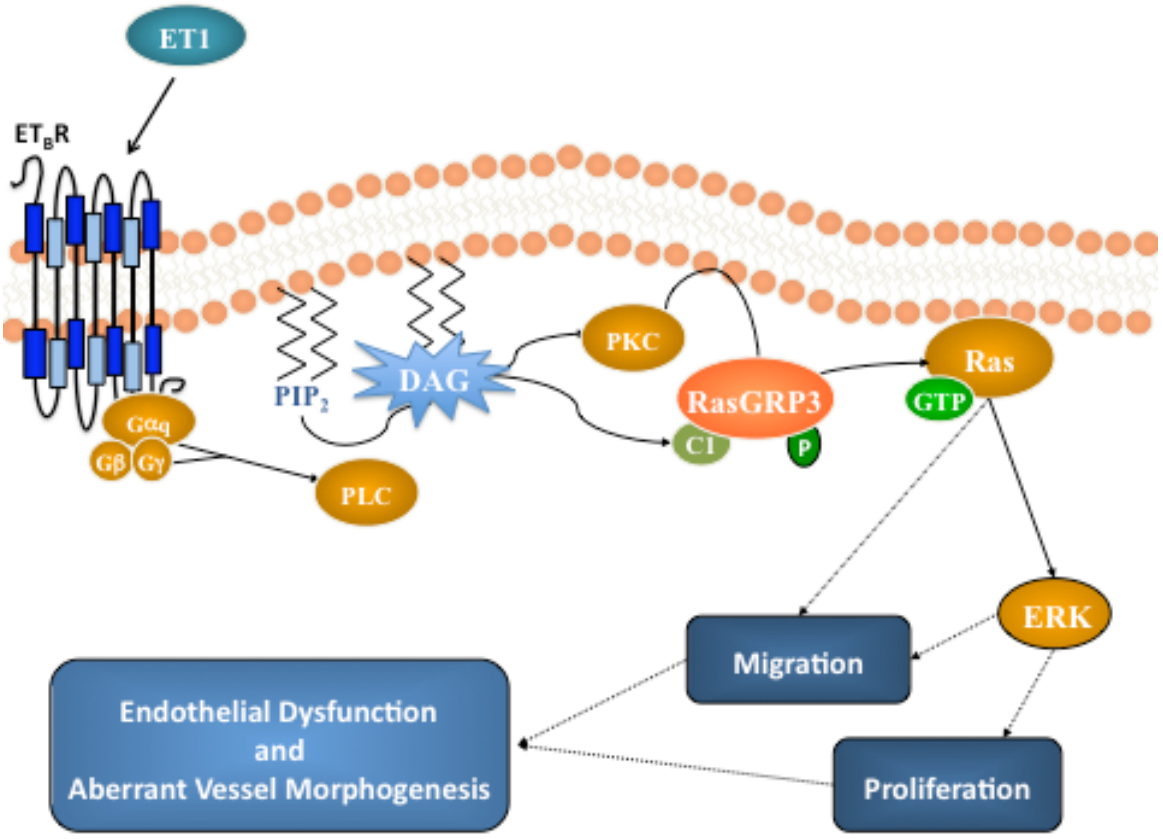

ET1
PMA
DAG
RasGRP3 function intact?
Yes
No
RasGRP3
RasXRP3
EC proliferation
EC migration *
EC proliferation
EC migration
Promotes embryonic vascular defects in diabetes: vessel patterning is dysmorphogenic.
Protective in diabetic backgrounds: vessel patterning looks normal.

Figure 3.2. ET1-ET_BR signaling promotes a RasGRP3-dependent Ras-ERK signaling to promote changes in endothelial cell behavior and vessel morphology – a model. A schematic of proposed ET1 signaling to regulate proliferation and migration in endothelial cells. In this model, ET1 promotes DAG generation through $G\alpha_q$ or $G\beta/G\gamma$ subunits. DAG binds the C1 domain of PKC and RasGRP3 to recruit them to the plasma membrane. PKC phosphorylates RasGRP3 to promote Ras activation. RasGRP3 activates Ras, which signals through other proteins to activate ERK. These signals promote changes in endothelial cell behavior (proliferation, migration) to promote endothelial cell activation. Elevated ET1 signaling can thus contribute to endothelial dysfunction and aberrant vessel morphogenesis, which requires RasGRP3.


ET1
ETBR
Gαq
Gβ
Gγ
PLC
PIP2
DAG
PKC
RasGRP3
C1
P
Ras
GTP
ERK
Migration
Proliferation
Endothelial Dysfunction
and
Aberrant Vessel Morphogenesis

B. References

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APPENDIX A

Supplemental Material and 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^{o}C, and whole mount stained for PECAM as described [1]. Briefly, embryos were washed 15 min in PBS, dehydrated through a methanol:PBS

series, blocked in 5% H_2O_2/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 described [2] 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 modifications [3]. 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 μM 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% O_2 and 5% CO_2 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 pBSIISK+/CAAX vector (kindly provided by Dr. Channing Der), and verified by sequence analysis. This version was shuttled into pEGFP-C1 (Clonetech, 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 x 10^6 cells in HUVEC optimized buffer (Amaxa) were transfected with 5 mg 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, Downington 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 Figure 2.3 J.

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 37^0C, 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 10^7 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^oC in 5% CO_2. Growth curves were generated by plating 5 x 10^3 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 37ºC. Cells were incubated in appropriate primary antibodies at 1:1000 in staining media overnight at 4ºC. 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 described [6]. 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 described [7]. 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 10^4 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-μm pore size filter membranes (Corning Incorporated, Corning NY) were immersed in medium. Cell suspensions were prepared in 1% FBS, and 2 x 10^4 cells in 100 μl were plated in the upper chamber. Cells were incubated overnight at 37˚C, then incubated with 600 μl 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) 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 mM to 20 mM 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 mM, U0126 (MEK inhibitor) at 10 mM, SB203580 (p38 MAPK inhibitor) at 5 mM, SP600125 (JNK inhibitor) at 10 mM, Bisindolymalemide I (BIM; general PKC inhibitor) at 10 mM, Gö6976 (PKCα and PKCβ inhibitor) at 6 mM, and rottlerin (PKCδ inhibitor) at 10 mM. 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, 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 37ºC. 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 37ºC 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 37ºC, rinsed in staining medium 3 times, then stored in 1X PBS at 4ºC. 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 $MgCl_2$) 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 ml of agarose beads conjugated to the Ras binding domain of Raf (RBD) for 1 hr at 4ºC, briefly centrifuged to pellet the beads, then washed with lysis buffer. Samples were suspended in 50 ml 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 4ºC. 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 4ºC, and total ERK was determined by incubation with mouse anti-mouse ERK1/2 (Abcam) at 1:1000 overnight at 4ºC. 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.

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