

Sema3F (Semaphorin 3F) Selectively Drives an Extraembryonic Proangiogenic Program

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Objective—Molecular pathways governing blood vessel patterning are vital to vertebrate development. Because of their ability to counteract proangiogenic factors, antiangiogenic secreted Sema3 (class 3 semaphorins) control embryonic vascular morphogenesis. However, if and how Sema3 may play a role in the control of extraembryonic vascular development is presently unknown.

Approach and Results—By characterizing genetically modified mice, here, we show that surprisingly Sema3F acts instead as a selective extraembryonic, but not intraembryonic proangiogenic cue. Both in vivo and in vitro, in visceral yolk sac epithelial cells, Sema3F signals to inhibit the phosphorylation-dependent degradation of Myc, a transcription factor that drives the expression of proangiogenic genes, such as the microRNA cluster 17/92. In *Sema3f*-null yolk sacs, the transcription of Myc-regulated microRNA 17/92 cluster members is impaired, and the synthesis of Myc and microRNA 17/92 foremost antiangiogenic target *Thbs1* (thrombospondin 1) is increased, whereas Vegf (vascular endothelial growth factor) signaling is inhibited in yolk sac endothelial cells. Consistently, exogenous recombinant Sema3F inhibits the phosphorylation-dependent degradation of Myc and the synthesis of *Thbs1* in mouse F9 teratocarcinoma stem cells that were in vitro differentiated in visceral yolk sac epithelial cells. *Sema3f*^{-/-} mice placentas are also highly anemic and abnormally vascularized.

Conclusions—Sema3F functions as an unconventional Sema3 that promotes extraembryonic angiogenesis by inhibiting the Myc-regulated synthesis of *Thbs1* in visceral yolk sac epithelial cells.

Visual Overview—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2017;37:1710-1721. DOI: 10.1161/ATVBAHA.117.308226.)

Key Words: angiogenesis modulating agents ■ mice ■ myc ■ semaphorins ■ thrombospondins ■ yolk sac

Effective delivery of oxygen and nutrients is mandatory for proper development of mammalian embryos.¹ Although initially supported by molecular diffusion only, around day 8 of gestation (E8), the mouse embryo starts relying in full on blood circulation to further develop. By this time, heart starts beating and extraembryonic yolk sac blood islands, that is, mesoderm-derived clusters of hematopoietic cells surrounded by a layer of endothelial cells (EnCs), coalesce to form a vascular network filled with large nucleated primitive erythroblasts.² This allows vital molecules to diffuse from the maternal blood, which is contained in giant trophoblast cell-lined uterine sinuses, into visceral yolk sac blood vessels that directly connect to the embryonic vasculature. Between E12.5 and E14.5, the mature chorioallantoic placenta progressively replaces the yolk sac as main nutrient exchange organ supporting the development of the mouse embryo.³

Large embryonic blood vessels and the vasculature of several embryonic organs and extraembryonic yolk sac initially arise by vasculogenesis, namely, the differentiation of angioblasts into EnCs that self-assemble in a homogeneously-sized primitive vascular plexus.¹ The latter is then remodeled by angiogenesis into a hierarchically sized mature vascular tree that facilitates and optimizes the distribution of blood.¹ In this context, EnC mechanosensitive adhesion receptors translate blood fluid shear traction forces into biochemical signals that allow vascular remodeling and maturation.⁴ Although it is well established that extraembryonic EnCs do not contribute to intraembryonic blood vessels, the molecular mechanisms that differentially regulate yolk sac and embryonic vascularization are poorly defined.¹

Sema3 (class 3 semaphorins) are secreted guidance cues that in the developing embryo signal through the cytosolic

Received on: June 28, 2016; final version accepted on: July 7, 2017.

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The online-only Data Supplement is available with this article at <http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.117.308226/-/DC1>.

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DOI: 10.1161/ATVBAHA.117.308226

Nonstandard Abbreviations and Acronyms	
CK8/18	cytokeratin 8/18
EnCs	endothelial cells
EpCs	epithelial cells
Fbw7	F-box and WD repeat domain-containing 7
GAP	GTPase-activating protein
PYS	parietal yolk sac
rSEMA3F	recombinant semaphorin 3F
SNAIL1	snail family transcriptional repressor 1
Thbs1	thrombospondin 1
VYS	visceral yolk sac

GAP (GTPase-activating protein) domain of Plexin receptors⁵ to inhibit R-Ras, M-Ras, and Rap1, 3 small GTPases that are crucial positive regulators of integrin-mediated cell adhesion in different cell types, EnC included.^{6,7} The chemorepulsive activity of Sema3A⁸⁻¹⁰ and Sema3E¹¹ was previously reported to control the vascular patterning in chick, zebrafish, and mouse embryos.¹² In addition, thanks to their ability to inhibit angiogenesis, both Sema3A and Sema3E exert an effective anticancer activity.^{13,14} Similarly, Sema3F was also found to display powerful antiangiogenic and antitumor effects^{13,14}; however, if and how Sema3F may play a role in embryonic vascular development is unknown. Surprisingly, at odds with the antiangiogenic function of all other Sema3 proteins, here we unveil how Sema3F drives a Myc-dependent proangiogenic program that is selectively restricted to the extraembryonic vasculature. In addition to shedding light on the molecular mechanisms that selectively drive extraembryonic but not embryonic blood vessel formation, our findings also suggest how an aberrant expression of Sema3F might be involved in placenta vascular malformations.

Materials and Methods

Materials and Methods are available in the [online-only Data Supplement](#).

Results

Targeted Disruption of Mouse *Sema3f* Gene Results in Partially Penetrant Embryonic Lethality but Does Not Control Embryonic Vascular Development

To understand whether Sema3F may control both embryonic and postnatal mouse development, we characterized and

compared *Sema3f*- and, for control purposes, *Sema3a*-null mice.⁸ Heterozygous *Sema3a* and *Sema3f* mice were phenotypically normal and fertile. As expected,⁸ the percentage of born *Sema3a*-null mice was also much lower than the Mendelian ratio (5.7% *Sema3a*^{-/-}, 39.1% *Sema3a*^{+/-}, 55.2% *Sema3a*^{+/+}; n=460). Similarly, Walz et al¹⁵ already described that *Sema3f*^{-/-} mice were born significantly less (14%) than that predicted by Mendelian ratio. As shown in Table 1, we confirmed that the frequency of born *Sema3f*-knockout pups is indeed lower than expected (5.7% *Sema3f*^{-/-}, 29.4% *Sema3f*^{+/-}, 64.9% *Sema3f*^{+/+}; n=465). The higher embryonic lethality we observed in our *Sema3f*^{-/-} mice compared with that reported by Walz et al¹⁵ might be likely because of differences in the percentage of C57Bl/6 and 129P2/OlaHsd genetic backgrounds between the 2 colonies (see Materials and Methods and Figure I in the [online-only Data Supplement](#)). Thus, similarly to *Sema3a*^{-/-} mice, most of *Sema3f*-null mice also die in utero.

To assess the timing of embryo lethality, we genotyped embryos collected at different developmental stages. Between E9.5 and E10.5, a clear significant decrease in *Sema3f*- and *Sema3a*-null embryos emerged. In particular, the *Sema3f*-null embryos were 19.6% at E9.5 (n=249) and 13.5% at E10.5 (n=81), whereas those of *Sema3a*-null embryos were 18.6% at E9.5 (n=285) and 15.2% at E10.5 (n=105). No gross phenotypic differences were present in the few outliving *Sema3f*- or *Sema3a*-knockout mice. However, as previously reported,¹⁶ if matched with wild-type mice of the same litter, 3-week-old *Sema3a*^{-/-} mice were smaller in size, their weight was halved, and they had difficulty maintaining an upright posture.

Sema3A signaling was previously reported to control vascular morphogenesis in zebrafish,⁹ chick,¹⁰ and mouse embryos.^{8,17-19} We hence investigated whether, similar to Sema3A, Sema3F may also regulate mouse embryonic angiogenesis. We examined by fluorescence confocal microscopy the vasculature of whole-mount endomucin-stained wild type (Figure 1A through 1D), *Sema3f*-null (Figure 1F through 1I), and *Sema3a*-null (Figure 1K through 1N) embryos at E9.5. Compared with control littermates (Figure 1E), *Sema3f*-knockout embryos (Figure 1J) displayed retarded growth (E9.5 wild-type embryos: 21.2±0.5 somites; E9.5 *Sema3f*^{-/-} embryos: 18.8±0.5 somites; Table 2) but not major vascular defects (Figure 1A through 1D and 1F through 1I). In agreement with previous studies⁸ and differently from

Table 1. Genotypes of the Offspring Generated From *Sema3f* and *Sema3a* Heterozygous Intercrosses on a C57Bl/6 Background

	<i>Sema3f</i>				<i>Sema3a</i>				
	Total	+/+	+/-	-/-	Total	+/+	+/-	-/-	
Term pups	465	29.4% (137)	64.9% (302)	5.7% (26)	Term pups	460	39.1% (180)	55.2% (254)	5.7% (26)
E9.5	249	27.7% (69)	32.1% (80)	19.6% (49)	E9.5	285	30.2% (86)	51.2% (146)	18.6% (53)
E10.5	81	40.7% (33)	45.6% (37)	13.5% (11)	E10.5	105	32.4% (34)	52.4% (55)	15.2% (16)

Heterozygous *Sema3a* and *Sema3f* mice were intercrossed, and the progeny was analyzed. Only 5.7% adult knockout mice were observed after genotyping the offspring of both *Sema3f*^{+/-} (n=465) and *Sema3a*^{+/-} (n=460) mating, respectively. Timed mating revealed an embryonic lethality between E9.5 and E10.5 in both *Sema3f* and *Sema3a* mutants. A percentage of 19.6 (n=249) at E9.5 and 13.5 (n=81) at E10.5 was observed in the progeny of *Sema3f* intercrosses and a percentage of 18.6 (n=285) at E9.5 and 15.2 (n=105) at E10.5 was observed in the progeny of *Sema3a*. Sema3 indicates class 3 semaphorin.

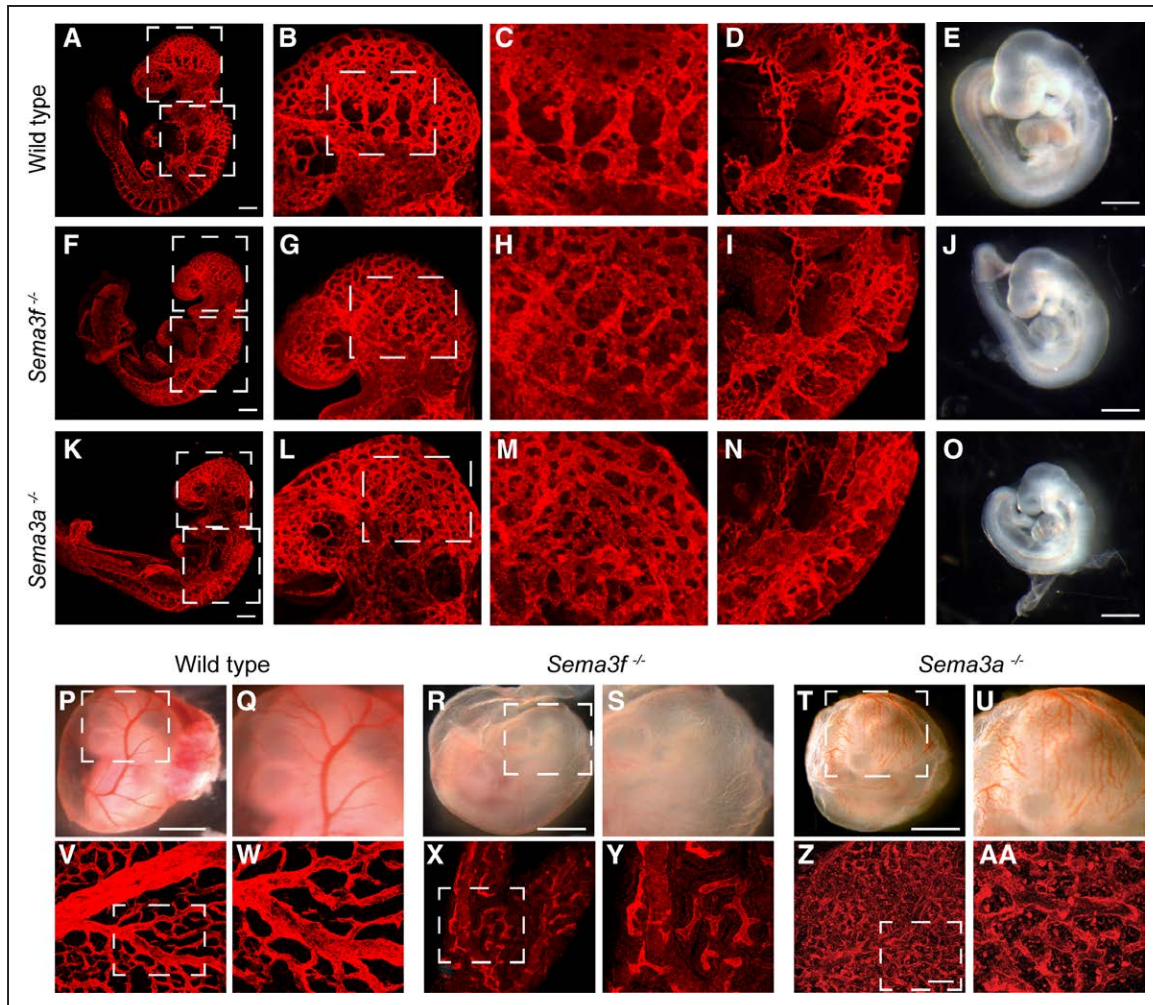


Figure 1. Sema3 (class 3 semaphorin)-A is necessary for embryonic angiogenic remodeling, whereas Sema3F is required for yolk sac blood vessel formation. Whole-mount endomucin stained E9.5 wild-type (A through D), *Sema3f*^{-/-} (F through I), and *Sema3a*^{-/-} (K through N) embryos. The cephalic plexus of wild-type (B and C) and *Sema3f*^{-/-} (G and H) but not *Sema3a*^{-/-} (L and M) embryos is remodeled to give rise to easily distinguishable cephalic veins. The cephalic perineural vascular plexus develops through remodeling of the dorsal longitudinal anatomical vessel in wild-type (D) and *Sema3f*^{-/-} (I) but not in *Sema3a*^{-/-} embryos (N). E, J, and O, Stereomicroscopic analyses reveals how, when compared with age-matched wild-type embryos (E), the development of both *Sema3f*^{-/-} (J) and *Sema3a*^{-/-} (O) E9.5 embryos is delayed. Developmental delay is more severe in *Sema3a*^{-/-} (O) than that in *Sema3f*^{-/-} (J) embryos. B, G, and L, Magnifications of the top boxed areas in (A), (F), and (K), respectively. C, H, and M, Magnifications of the boxed areas in (B), (G), and (L), respectively. D, I, and N, Magnifications of the bottom boxed areas in (A), (F), and (K), respectively. Scale bars, 300 μ m (A, F, and K) and 100 μ m (E, J, and O). Stereomicroscopy (P through U) and endomucin-staining confocal microscopy (V through AA) analyses of E10.5 wild-type (P, Q, V, and W), *Sema3f*^{-/-} (R, S, X, and Y) and *Sema3a*^{-/-} (T, U, Z, and AA) yolk sacs. When compared with wild-type yolk sacs (P, Q, V, and W), a dramatic decrease in blood vessels is observed in *Sema3f*^{-/-} yolk sacs (R, S, X, and Y), while a poorly remodeled primary capillary plexus and the persistence of some blood islands characterize *Sema3a*^{-/-} (T, U, Z, and AA) yolk sacs. Q, S, U, W, Y, and AA, Magnifications of (P), (R), (T), (V), (X), and (Z), respectively. Scale bars, 100 μ m (P, R, and T) and 150 μ m (V, X, and Z).

Sema3f-null embryos (Figure 1J), *Sema3a*-knockout embryos (Figure 1O) displayed an even stronger growth retardation (E9.5 *Sema3a*^{-/-} embryos: 17 ± 1.2 somites, $n=7$; Figure 1O) that was associated with angiogenic remodeling defects (Figure 1K through 1N). The cephalic plexus of wild-type (Figure 1B and 1C) and *Sema3f*^{-/-} (Figure 1G and 1H), but not *Sema3a*^{-/-} E9.5 embryos (Figure 1L and 1M), was stereotypically reshaped into a pattern wherein cephalic veins were discernible. Moreover, in E9.5 wild-type (Figure 1D) and *Sema3f*-null (Figure 1I) but not *Sema3a*-null (Figure 1N) embryos, the dorsal longitudinal anatomical vessel started to be remodeled into a mature perineural capillary plexus.

Sema3F Drives Extraembryonic Angiogenesis

In quest of explanations for the growth retardation associated with an essentially normal vascular development of *Sema3f*-knockout embryos, we reasoned that it may be due to defective angiogenesis in extraembryonic organs, namely, yolk sac and placenta. Accordingly, we observed that, although yolk sacs of E10.5 wild-type embryos contained a dense hierarchically ordered vascular network (Figure 1P, 1Q, 1V, and 1W), *Sema3f*-null yolk sacs unexpectedly displayed a dramatic reduction in blood vessel number and organization into a mature vascular tree (Figure 1R, 1S, 1X, and 1Y). *Sema3f*^{-/-} mice placentas were also highly anemic and abnormally vascularized (Results section; Discussion section, and Figure II in the [online-only](#)

Table 2. Number of Intersomitic Vessels Evaluated in *Sema3f*, *Sema3a*, and *Sema3a/Sema3f*-Knockout Embryos (E9.5)

Genotype	Intersomitic Vessels	Genotype	Intersomitic Vessels	Genotype	Intersomitic Vessels
<i>Sema3f^{+/+}</i>	21.2±0.5	<i>Sema3a^{+/+}</i>	21.7±0.6	<i>Sema3a^{+/+} Sema3f^{+/+}</i>	21.0±1.2
<i>Sema3f^{-/-}</i>	18.8±0.5	<i>Sema3a^{-/-}</i>	17.0±1.2	<i>Sema3a^{-/-} Sema3f^{-/-}</i>	11.0±0.6
<i>P</i> =0.0062 (n=6)		<i>P</i> =0.0039 (n=7)		<i>P</i> =0.0015 (n=3)	

All data are expressed as mean±SEM. *Sema3* indicates class 3 semaphorin.

Data Supplement). A not remodeled primary vascular plexus and occasional scattered blood islands were instead present in *Sema3a*-null yolk sacs (Figure 1T, 1U, 1Z, and 1AA).

The mouse yolk sac consists of mesoderm-derived blood islands and vessels that are positioned between endoderm-derived visceral yolk sac (VYS) epithelial cell (EpC) layer and parietal yolk sac (PYS) EpC layer.²⁰ VYS EpCs control nutrient and gas exchanges and the development of blood islands and vessels; PYS EpCs instead protect the embryo.²⁰ To understand which cell types synthesize Sema3F and

express the major Sema3F coreceptor Nrp2, we performed confocal microscopy analyses on E9.5 yolk sacs that were double stained with either anti-Sema3F or anti-Nrp2 and antibodies recognizing vascular EnC or VYS EpC or PYS EpC markers, respectively, represented by endomucin, cytokeratin 8/18 (CK8/18), and Snail1 proteins.²¹ Sema3F protein was present in VYS EpCs and in part also in vascular EnCs of *Sema3f^{+/+}* but not in *Sema3f^{-/-}* yolk sacs (Figure 2A and 2C). PYS EpCs essentially do not produce Sema3F (Figure 2A and 2C). We next analyzed the expression of Sema3F in E9.5 embryos. As illustrated in Figure IIIC and IIID in the online-only Data Supplement, Sema3F was mainly expressed in vascular EnCs of both wild-type and *Sema3a^{-/-}*, but not in *Sema3f^{-/-}*, embryos. Similarly, Sema3A was expressed in endomucin⁺ vessels of *Sema3f^{-/-}* yolk sacs (Figure IIIA and IIIB in the online-only Data Supplement).

We also observed that Nrp2, the major Sema3F coreceptor, is expressed in VYS EpCs and vascular EnCs but not in PYS EpCs (Figure 2B and 2D). Thus, in the yolk sac, Nrp2-expressing EnCs and VYS EpCs may be stimulated by paracrine/autocrine Sema3F.

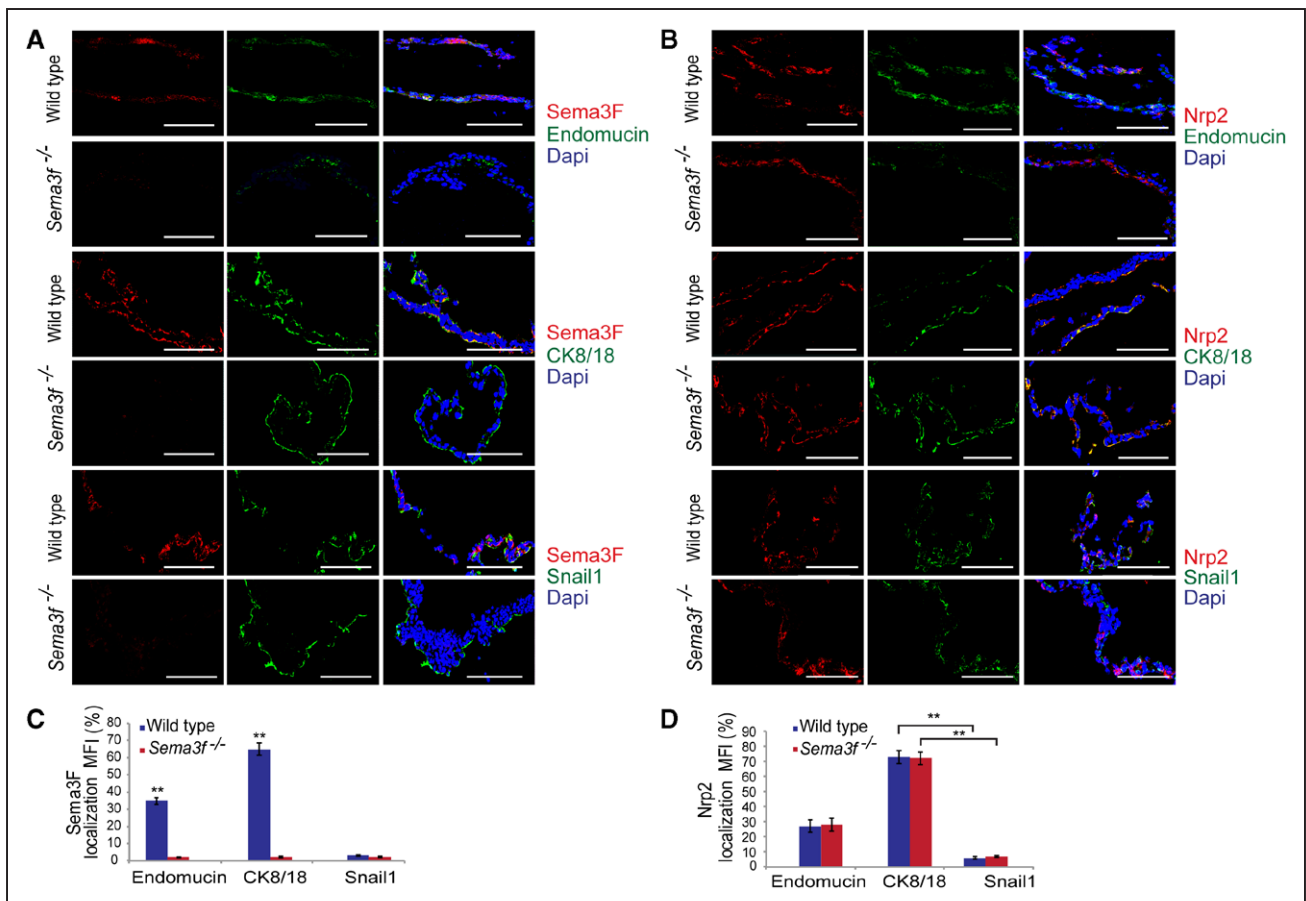


Figure 2. Sema3 (class 3 semaphorin)-F and Nrp2 are mainly expressed by the CK8/18 (cytokeratin 8/18)⁺ epithelial cells of the yolk sac. Frozen sections of yolk sacs were decorated for Sema3F (red) in costaining alternatively with endomucin, CK8/18, or Snail1 antibodies (green) and Dapi (4',6-diamidino-2-phenylindole; blue). **A**, Sema3F largely colocalized with the CK8/18 visceral epithelial cells marker. As expected, no Sema3F expression was detected in *Sema3f^{-/-}* samples. **B**, Confocal analysis showed that in both *Sema3f^{+/+}* and *Sema3f^{-/-}* mice, Nrp2 is greater expressed by the visceral yolk sac as assessed by Nrp2-CK8/18 costaining. Confocal analysis was performed on tissue sections from 6 mice per group; scale bars, 100 μm. **C** and **D**, Graphs show the percentage of Sema3F and Nrp2 colocalization with endomucin, CK8/18, and Snail1 in both *Sema3f^{+/+}* and *Sema3f^{-/-}* yolk sacs (***P*<0.01, Student *t* test).

Sema3F Signaling Inhibits the Degradation of Myc Transcription Factor in Visceral Yolk Sac Epithelial Cells

Our data indicate that, at odds with its well-characterized anti-angiogenic effects reported in the adult animal,¹³ Sema3F plays a proangiogenic role in extraembryonic tissues. Because it is well documented that in EnCs Sema3F elicits canonical anti-angiogenic signals,²² we considered the option that Sema3F might exert its proangiogenic activity at least in part by controlling gene transcription in extraembryonic tissues, for example, by acting on Nrp2-expressing VYS EpCs. In this regard, the basic helix-loop-helix transcription factor Myc is a well-established regulator of vascular development,²³ and in *Caenorhabditis elegans*, a Myc-like network was found to cooperate with semaphorin signaling in the control of cell migration.²⁴ Hence, we first assessed whether the loss of *Sema3f* was associated with the modulation of yolk sac Myc protein expression. Confocal immunofluorescence analysis on E9.5 wild-type and *Sema3f*^{-/-} yolk sacs revealed how Myc protein was decreased by 0.73-fold in *Sema3f*-knockout animals (Figure 3A and 3B).

Myc protein stability is regulated by phosphorylation and ubiquitin-dependent degradation. In particular, GSK3

(glycogen synthase kinase 3)-mediated phosphorylation of Myc at Thr58 gives rise to a binding site that is directly recognized by the E3 ubiquitin ligase Fbw7(F-box and WD repeat domain-containing 7), resulting in ubiquitination followed by proteasomal degradation of Myc.²⁵ Consistent with previous in vitro data,²⁵ we found that in E9.5 *Sema3f*^{-/-} yolk sacs the reduction in Myc protein amount was associated with a selective increase of Myc Thr58 phosphorylation in VYS EpCs, but neither in PYS EpCs nor in EnCs (Figure 3C and 3D). Altogether, our findings support a model in which in the mouse yolk sac paracrine/autocrine Sema3F signals to inhibit the (GSK3-dependent) phosphorylation on Thr58 and ensuing degradation of the proangiogenic Myc transcription factor in VYS EpCs. Hence, paracrine/autocrine Sema3F signals inhibit the phosphorylation on Thr58 and ensuing degradation of Myc in VYS EpCs.

Sema3F Promotes the Transcription of Myc-Regulated AngiomiRs in the Mouse Yolk Sac

Myc may drive yolk sac blood vessel development downstream of Sema3F signaling by promoting the transcription of different proangiogenic genes.^{23,26} In particular, it was

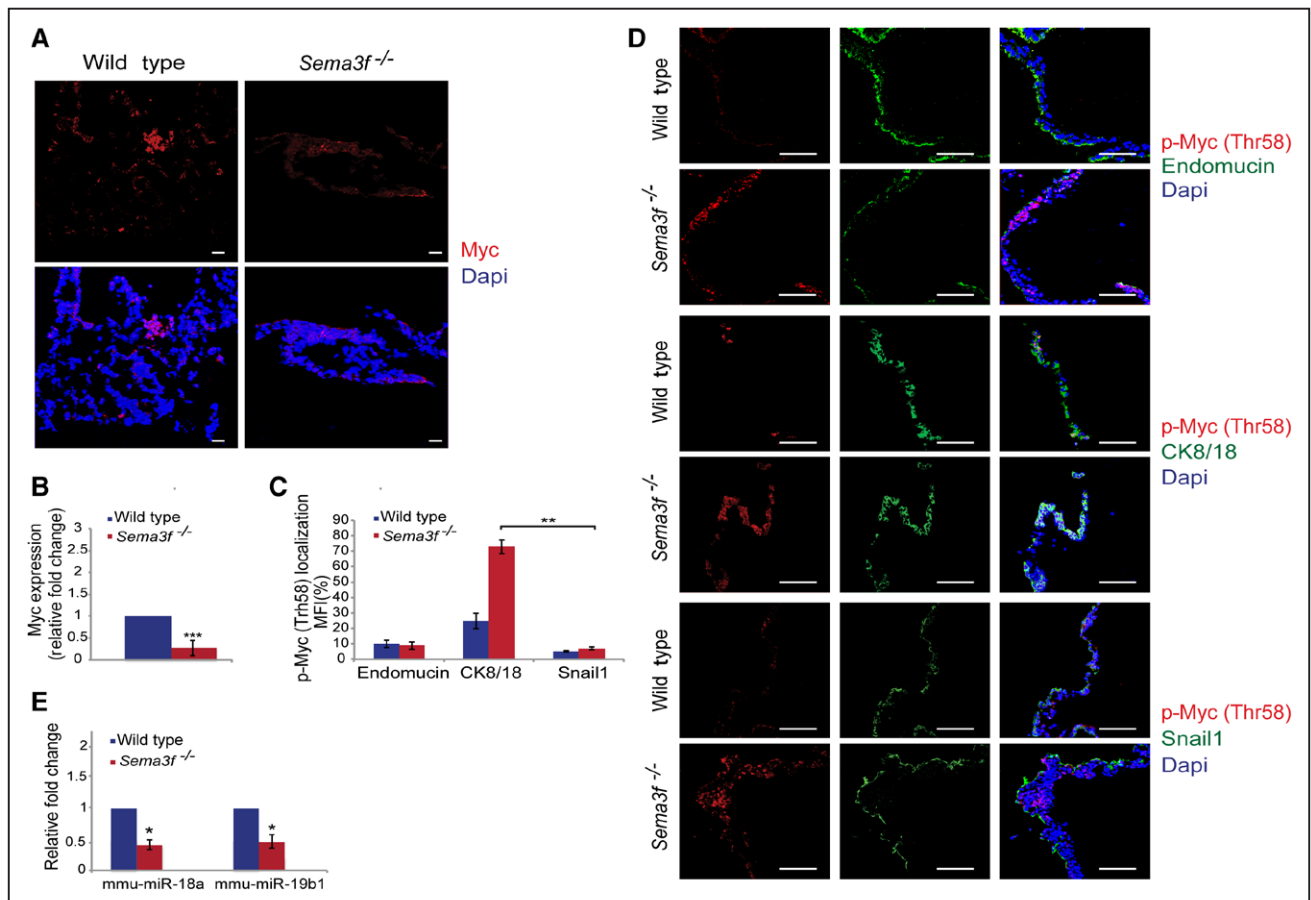


Figure 3. Sema3 (class 3 semaphorin)-F activates a Myc- and miR-18a/miR-19b-dependent proangiogenic pathway in the mouse yolk sac. **A** and **B**, Quantification of immunofluorescence analysis of E9.5 yolk sacs using Myc antibody (red) counterstained with Dapi (4',6-diamidino-2-phenylindole; blue) showed that Myc expression decreases by 0.73-fold in *Sema3f*^{-/-} yolk sacs. **C** and **D**, Myc phosphorylation at Thr58 significantly increased in E9.5 *Sema3f*^{-/-} yolk sacs compared with the wild type. Furthermore, a double staining with anti-p-Myc (Thr58) and either anti-endomucin or anti-cytokeratin 8/18 (CK8/18), or anti-Snail1 revealed that p-Myc (Thr58) is mainly expressed in CK8/18+ visceral yolk sac epithelial cells, whereas only few amounts of Thbs1 were associated with endomucin+ vascular endothelial cells. **E**, Real-time polymerase chain reaction revealed how, compared with wild-type yolk sacs, proangiogenic miR-18a and miR-19b diminished in *Sema3f*^{-/-} yolk sacs. (***)*P*<0.0001; (**)*P*<0.01; (*)*P*<0.05, Student *t* test). Scale bars, 100 μm.

reported that in cultured embryonic stem cells, Myc loss impairs *Vegfa* (vascular endothelial growth factor A) gene expression.²³ However, even if Myc protein abundance was dramatically decreased (Figure 3A and 3B), we did not detect any significant reduction of *Vegfa* mRNA in *Sema3f*-null yolk sacs, as evaluated by real-time quantitative polymerase chain reaction (Figure IVA in the [online-only Data Supplement](#)). These findings indicate that in the mouse yolk sac, Sema3F should induce the transcription of additional Myc-dependent proangiogenic genes other than *Vegfa*.

Specific miRNA genes, dubbed angiomiRs, are master regulators of developmental angiogenesis and transcription of the key proangiogenic miR-17/92 cluster²⁶ lies under the control of Myc.²⁷ Thus, we considered the possibility that Myc protein downmodulation in *Sema3f*^{-/-} yolk sacs may impair miR-17/92 gene cluster transcription. By real-time quantitative polymerase chain reaction, we analyzed the transcription of genes belonging to miR-17/92 cluster in E9.5 wild-type and *Sema3f*^{-/-} yolk sacs. Among miR-17/92 gene cluster members, miR-18a and miR-19b-1 emerged as the most modulated miRNAs in *Sema3f*^{-/-} yolk sacs. Compared with wild type,

miR-18a and miR-19b-1 levels, respectively, decreased by 0.5- and 0.8-fold in *Sema3f*^{-/-} yolk sacs (Figure 3E). Hence, Sema3F signaling drives the Myc-mediated transcription of the proangiogenic miR 17/92 cluster members miR-18a and miR-19b-1.

Sema3F Inhibits the Expression of the Antiangiogenic Factor Thbs1 (Thrombospondin 1) in Mouse Visceral Yolk Sac Epithelial Cells

Thbs1 (thrombospondin 1) is an effective angiogenesis inhibitor²⁸ whose expression is inhibited by Myc protein²³ and Myc-induced miR-17/92 cluster miRNAs,²⁹ in particular, miR-18a and miR-19b-1.³⁰⁻³² To assess whether Thbs1 expression was modulated in E9.5 *Sema3f*^{-/-} yolk sacs compared with their wild-type counterpart, we first analyzed *Thbs1* gene transcription by real-time quantitative polymerase chain reaction (Figure IVB in the [online-only Data Supplement](#)). Compared with wild-type yolk sacs, *Thbs1* mRNA levels increased by 1.5-fold in *Sema3f*^{-/-} yolk sacs. Next, we assessed Thbs1 protein expression by confocal immunofluorescence microscopy. Quantitative analysis revealed that, in comparison to wild-type

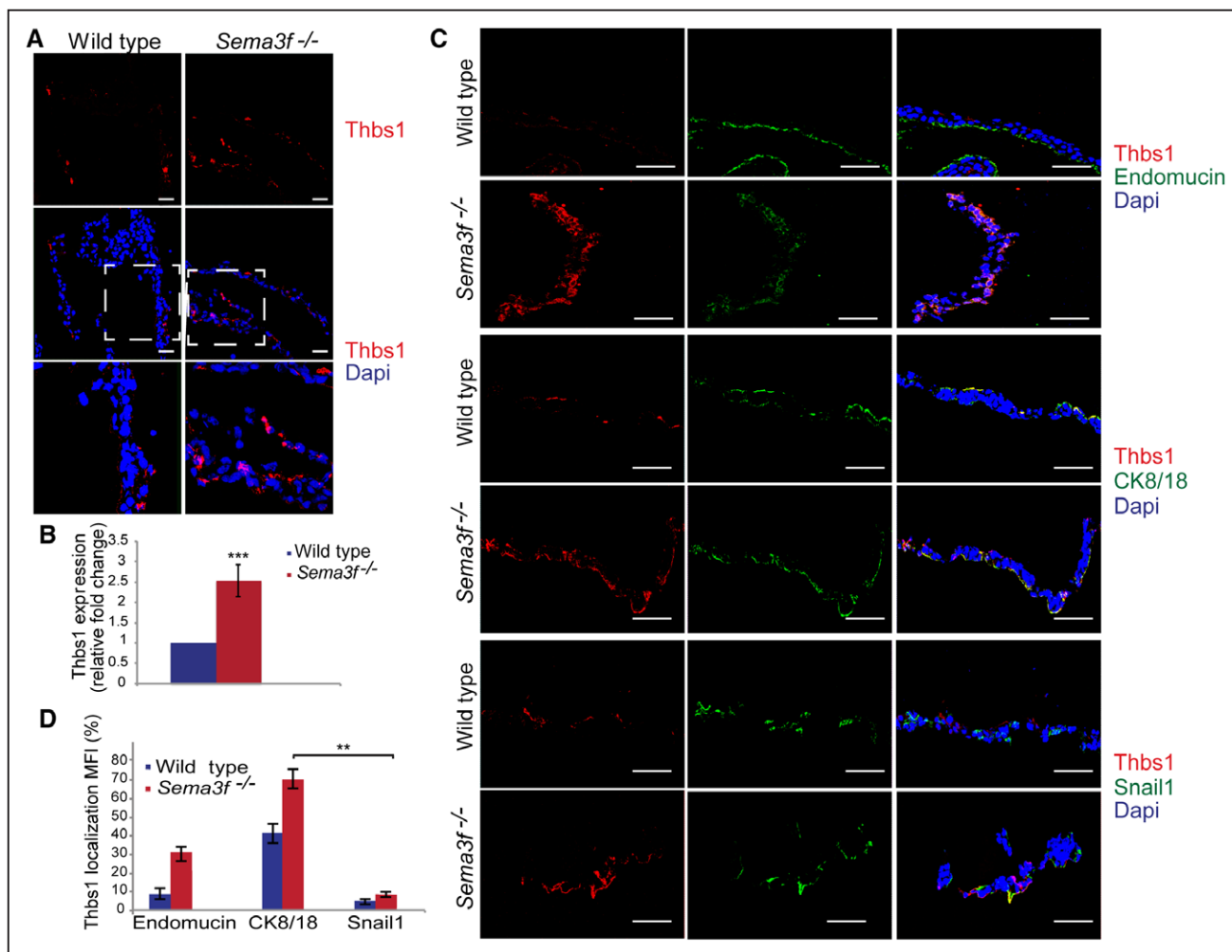


Figure 4. Sema3 (class 3 semaphorin)-F signaling inhibits Thbs1 (thrombospondin 1) protein expression. **A** and **B**, Quantification of immunofluorescence analyses of E9.5 yolk sacs showed that Thbs1 expression increases by 2.5-fold in *Sema3f*^{-/-} yolk sacs. Scale bars, 300 μ m (** P <0.001; *** P <0.0001, Student t test). **C**, Confocal analysis showed that Thbs1 protein expression is enriched in CK8/18 (cytokeratin 8/18)⁺ visceral yolk sac epithelial cells compared with the other cell types. Scale bars, 100 μ m. **D**, Bar graph shows the percentage of Thbs1 colocalization with endomucin, CK8/18, and Snail1 in both wild-type and *Sema3f*^{-/-} yolk sacs (** P <0.001, Student t test).

yolk sacs, Thbs1-positive areas increased by 2.5-fold in *Sema3f*^{-/-} yolk sacs (Figure 4A and 4B).

To understand which cell type(s) synthesize Thbs1, we analyzed by confocal microscopy E9.5 yolk sacs that were double stained with anti-Thbs1 and either anti-endothelium or anti-CK8/18, or anti-Snail1. We detected Thbs1 protein mainly in CK8/18⁺ VYS EpCs, whereas only a few amount of Thbs1 was associated with endothelium⁺ vascular EnCs (Figure 4C and 4D). Of note, we did not observe any difference in the expression of Thbs2 in the different cell types of *Sema3f*^{-/-}, compared with wild-type yolk sacs (Figure V in the online-only Data Supplement).

It has been clearly documented how Thbs1 effectively impairs VEGFA function mostly by inhibiting VEGF-R2 (VEGF receptor 2 phosphorylation).^{28,33,34} Therefore, we investigated whether endothelial VEGF signaling may be affected in *Sema3f*-knockout yolk sacs and uncovered that indeed tyrosine phosphorylation VEGF-R2 was reduced dramatically in vascular EnCs of E9.5 *Sema3f*^{-/-} yolk sacs (Figure 5). Hence, in *Sema3f*-null yolk sacs, VYS EpCs synthesize high amount of Thbs1 protein that in turn effectively impairs VEGF-R2 activation in vascular EnCs.

In In Vitro Differentiated VYS EpCs, Exogenous Recombinant Sema3F Inhibits the Myc-Dependent Expression of the Antiangiogenic Factor Thbs1

To directly assess whether Sema3F may stimulate Myc protein accumulation and the decrease of the antiangiogenic factor Thbs1, we set up an in vitro system to create differentiated CK8/18⁺ VYS Eps that may then be treated with recombinant Sema3F (rSema3F). To this aim, we exploited the well-characterized in vitro model of F9 testicular teratocarcinoma stem cells that, when cultured as aggregates in suspension and

stimulated with retinoic acid, differentiate in VYS Eps.^{35,36} Fluorescent confocal microscopy confirmed that, similar to what observed in the mouse yolk sac (Figure 2B and 2D), in vitro differentiated CK8/18⁺ VYS EpCs expressed Sema3F receptor Nrp2 as well (Figure 6A). Of note, quantitative analysis of immunofluorescent staining revealed how exogenously added rSema3F effectively inhibits the degradative GSK-dependent phosphorylation of Myc on Thr58 (Figure 6B and 6D) and increases total Myc protein levels (Figure 6B and 6D), while significantly decreasing Thbs1 (Figure 6C and 6D), whose expression is known to be inhibited by Myc.²³ Thus, direct in vitro stimulation of cultured VYS Eps with rSema3F promotes the accumulation of Myc and impairs the expression of the antiangiogenic factor Thbs1.

Sema3a Sema3f Double Knockdown Results in Early Mouse Embryonic Lethality and Severely Impairs Both Embryonic and Extraembryonic Angiogenesis

To understand whether Sema3A and Sema3F may cooperate in regulating embryonic development, we first mated *Sema3a*^{+/-} and *Sema3f*^{-/-} mice to create double *Sema3a*^{+/-} *Sema3f*^{-/-} mice that in F1 progeny were born in a normal Mendelian ratio. Then, to generate double *Sema3a/Sema3f*-knockout mice, *Sema3a*^{+/-} *Sema3f*^{-/-} animals were mated. Among 405 weaned mice, no double *Sema3a*^{-/-} *Sema3f*^{-/-} mice were born. These data indicate that all double *Sema3a Sema3f*-knockout mice died in utero. To assess the time of embryo lethality, double *Sema3a*^{-/-} *Sema3f*^{-/-} embryos were genotyped at different gestation times, observing a large loss of double *Sema3a*^{-/-} *Sema3f*^{-/-} embryos around E9.5 (Table 3).

Compared with wild-type animals (Figure 7D), *Sema3a*^{-/-} *Sema3f*^{-/-} embryos were significantly smaller (Figure 7H),

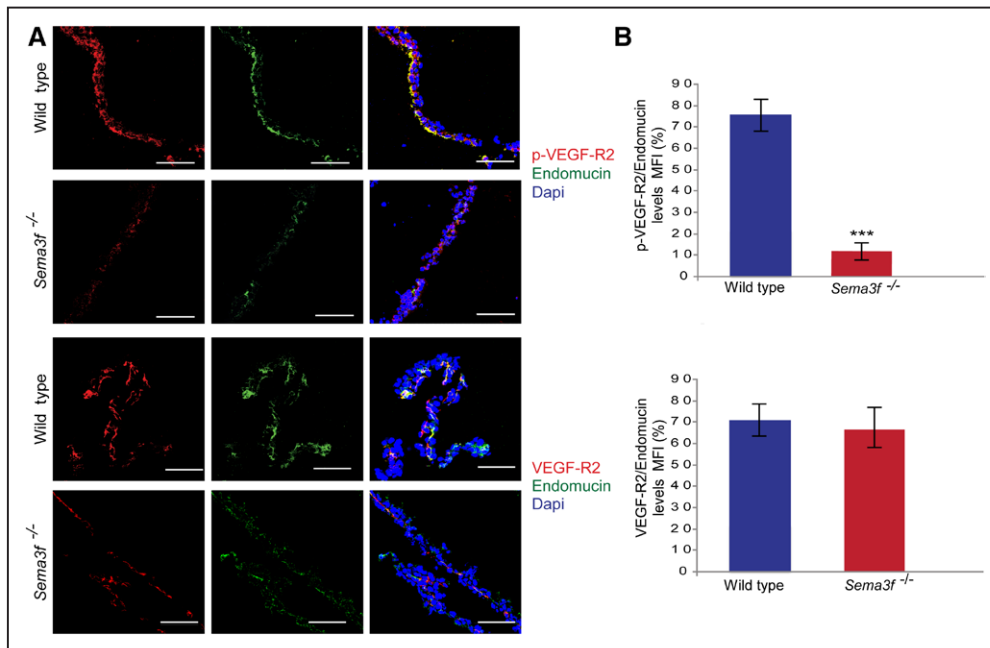


Figure 5. *Sema3f*-knockout impairs VEGF-R2 (vascular endothelial growth factor receptor 2) phosphorylation in the yolk sac. **A**, Confocal microscopy analysis showed that VEGF-R2 phosphorylation is strongly reduced in *Sema3f*^{-/-} yolk sacs compared with wild-type controls, whereas VEGF-R2 expression is not affected. Scale bars, 100 μ m. **B**, Percentage of p-VEGF-R2 and total VEGF-R2 relative levels in endothelium⁺ endothelial cell. (***) $P < 0.0001$, Student *t* test). Sema3 indicates class 3 semaphorin.

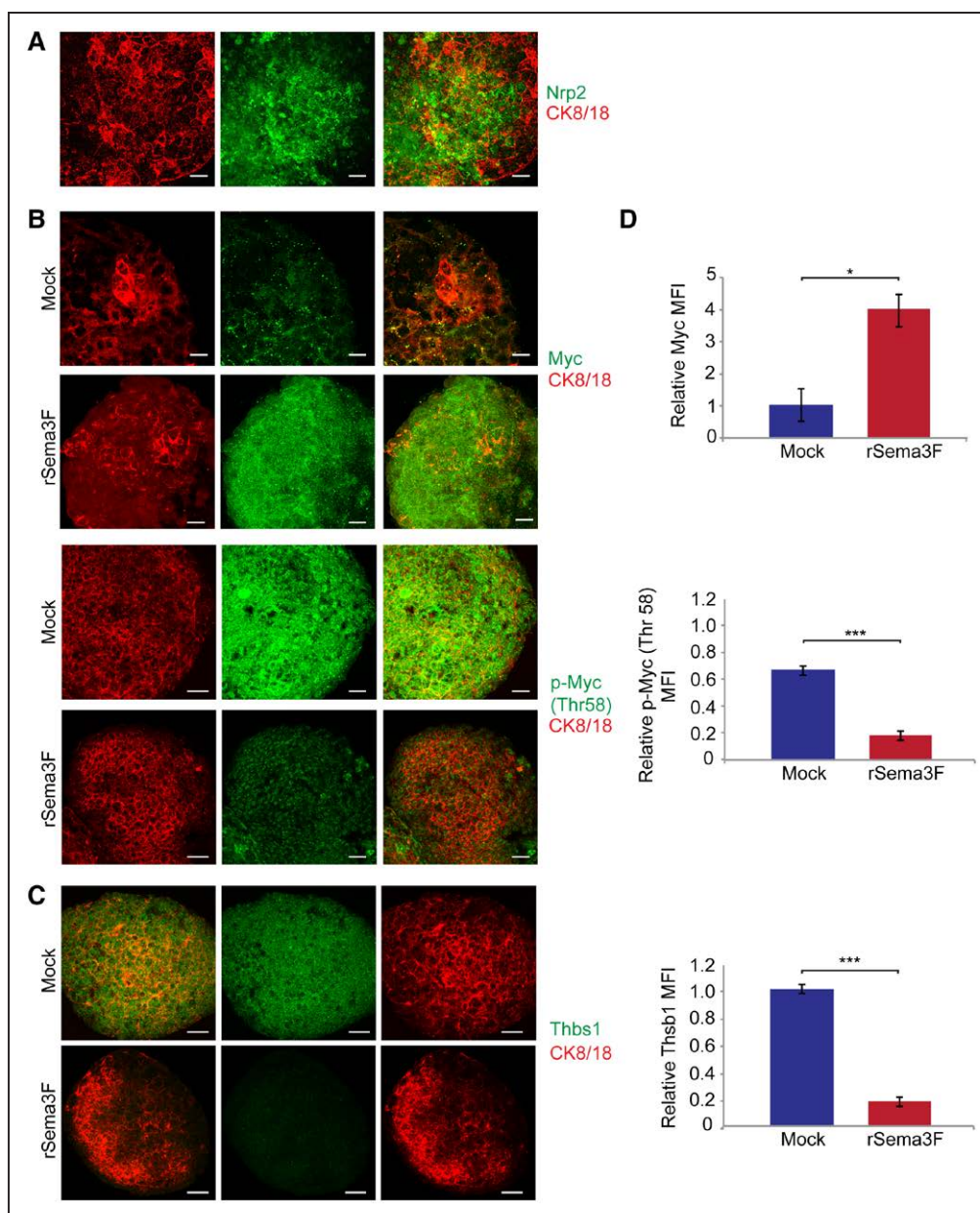


Figure 6. Exogenous recombinant Sema3 (class 3 semaphorin)-F impairs Myc degradation and inhibits Thbs1 (thrombospondin 1) expression in in vitro differentiated visceral yolk sac (VYS) epithelial cells (EpCs). **A**, Mouse F9 teratocarcinoma stem cells differentiated in VYS EpCs express Sema3F receptor Nrp2, as revealed by immunofluorescence staining. **B**, Stimulation with exogenous rSema3F increases Myc expression and reduces Myc Thr58 phosphorylation in in vitro differentiated VYS EpCs derived from mouse F9 teratocarcinoma stem cells. **C**, rSema3F stimulation also decreases the expression of antiangiogenic Thbs1 protein in VYS EpCs derived from mouse F9 teratocarcinoma stem cells. **D**, The relative fluorescence intensity of Myc, Thr58 phosphorylated Myc, and Thbs1 was measured in VYS EpCs derived from mouse F9 teratocarcinoma stem cells stimulated or not with rSema3F. Data are mean \pm SEM. n=15 cell aggregates per condition pooled from 3 independent experiments. (*** P <0.0001; * P <0.05, Student t test). Scale bars, 25 μ m.

and their growth delay at E9.5 was more important than that observed in *Sema3a*^{-/-} embryos (Figure 1O). In fact, on average, at E9.5, *Sema3a*^{-/-} *Sema3f*^{-/-} embryos displayed 11 somites compared with the 21 somites of wild-type littermates (Table 2). Confocal immunofluorescence microscopy on whole-mount endomucin-stained *Sema3a*^{-/-} *Sema3f*^{-/-} embryos (Figure 7E through 7G) unveiled how vascular abnormalities were considerably more severe than those observed in *Sema3a*-null mice (Figure 1K through 1N). In particular, compared with wild-type embryos (Figure 7A through 7C),

both cephalic vascular plexus (Figure 7E and 7F) and intersomitic blood vessels (Figure 7E and 7G) were poorly formed and, when formed, they were not remodeled. Short noninterconnected sprouts enclosed wide avascular spaces. Next, we evaluated the impact of *Sema3a* *Sema3f*-double knockout on yolk sac vascularization. Because of the difficulties in recovering viable double *Sema3a*^{-/-} *Sema3f*^{-/-} mutant embryos at E10.5, we decided to analyze the vasculature of E9.5 yolk sacs, which, differently from wild-type yolk sacs (Figure 7I), appeared extremely pale and essentially avascular (Figure 7J).

Table 3. Genotypes of Progeny from *Sema3a^{+/-}/Sema3f^{+/-}* Intercrosses

	Total	<i>3a^{+/-}/3f^{+/-}</i>	<i>3a^{+/-}/3f^{-/-}</i>	<i>3a^{-/-}/3f^{+/-}</i>	<i>3a^{-/-}/3f^{-/-}</i>	<i>3a^{+/-}/3f^{-/-}</i>	<i>3a^{-/-}/3f^{+/-}</i>	<i>3a^{-/-}/3f^{-/-}</i>	<i>3a^{-/-}/3f^{+/-}</i>	<i>3a^{-/-}/3f^{-/-}</i>
Term Pups	405	10.1% (41)	35.8% (145)	21.4% (87)	17.3% (70)	3.5% (14)	3.5% (14)	4.9% (20)	3.5% (14)	0% (0)
E9.5	387	6.46% (25)	21.44% (83)	11.1% (43)	13.9% (54)	9.8% (38)	5.2% (20)	9.6% (37)	5.6% (22)	3.6% (14)
Expected Mendelian ratio		6.25%	25%	12.5%	12.5%	12.5%	6.25%	12.5%	6.25%	6.25%

Heterozygous *Sema3a^{+/-}/Sema3f^{+/-}* mice were intercrossed, and the progeny was analyzed. No adult double *Sema3a* *Sema3f*-knockout mice were observed after genotyping the offspring (n=405). Timed mating revealed an embryonic lethality until E9.5, and only the 3.6% (n=387) were *Sema3a^{-/-}/Sema3f^{-/-}* at E9.5. This percentage is the half than the Mendelian expected ratio. *Sema3* indicates class 3 semaphorin.

Thus, abnormalities of *Sema3a^{-/-} Sema3f^{-/-}* yolk sac blood vessels (Figure 7J) were considerably more serious than those observed in either *Sema3f^{-/-}* (Figure 1R, 1S, 1X, and 1Y) or *Sema3a^{-/-}* (Figure 1T, 1U, 1Z, and 1AA) yolk sacs.

Discussion

The formation of properly patterned intraembryonic and extraembryonic blood vessel networks is crucial to development of vertebrate organisms.¹ By counteracting the activity of proangiogenic factors such as VEGFA, antiangiogenic *Sema3* proteins, eg, *Sema3A* and *Sema3E*, play key roles in shaping blood vascular patterns of developing embryos.¹² Here, we unveil how, as a notable exception, *Sema3F* selectively exerts an effective proangiogenic activity during extraembryonic, but not intraembryonic, vascular development. The defective vascularization of *Sema3f*-null yolk sacs phenocopies the defects of *Nrp1^{-/-} Nrp2^{-/-}* embryos that have nearly avascular yolk sacs.³⁷ In addition, we observed that *Sema3f^{-/-}* placentas are also anemic (Results section and Discussion section in the [online-only Data Supplement](#)). *Sema3F* signaling mainly relies on *Nrp2* coreceptor.⁶ However, in the absence of *Nrp2*, *Sema3F* can also signal via *Nrp1*³⁸ and dissociation constants for *Sema3F* binding to *Nrp1* and *Nrp2* are 1.1 and 0.09 nmol/L, respectively.³⁹ Altogether, our and previous data suggests that both *Nrp1* and *Nrp2* may be required to allow *Sema3F*-promoting extraembryonic vascular development.

In *Sema3f^{-/-}* yolk sacs, we observed a significant downregulation of *Myc*, a transcription factor that regulates the expression of several gene sets, such as those promoting developmental²³ and tumor²⁹ angiogenesis. *Myc* protein levels are regulated by GSK3 that, by phosphorylating *Myc* on Thr58, generates a phosphodegron that enables Fbw7 ubiquitin ligase binding, *Myc* ubiquitylation, and proteasomal degradation.²⁵ Notably, as previously reported in cultured cells,²⁵ we also found that in *Sema3f^{-/-}* yolk sacs, the drop in *Myc* protein abundance was combined with an increased *Myc* Thr58 phosphorylation in *Nrp2*-expressing VYS EpCs. Fittingly, we also observed that the treatment of in vitro differentiated *Nrp2⁺* VYS EpCs with exogenous *Sema3F* decreases *Myc* Thr58 phosphorylation, thus promoting *Myc* accumulation. In EnCs, *Sema3F* inhibits the phosphatidylinositol 3 kinase-dependent activation of *Akt*²² that is known to phosphorylate and inactivate GSK3.⁴⁰ It seems that instead in VYS EpCs auto-crine/paracrine *Sema3F*, by either activating a GSK3 kinase (such as *Akt*, *p90RSK*, *p70S6K*, and *PKA*) or inhibiting a

GSK3-phosphatase (eg, *PP2A*), impairs the GSK3-dependent phosphorylation of *Myc* on Thr58 and its ensuing degradation. Further work is needed to establish the diverse molecular mechanisms by which *Sema3F* may activate or inhibit GSK3 function in different cell types.

MiRNAs are central regulators of cardiovascular development, and *Myc* promotes the transcription of the miR-17/92 cluster,^{26,27} whose members miR-18a and miR-19b-1 target the angiogenesis inhibitor *Thbs1* hence promoting blood vessel formation.^{30,31} We revealed how lack of *Sema3F* results in reduced miR-18a and miR-19b-1 gene transcription, increased *Thbs1* protein in VYS EpCs, and dramatically reduced VEGF-R2 tyrosine phosphorylation in vascular EnCs of E9.5 *Sema3f^{-/-}* yolk sacs. Consistently, we also observed that exogenous *Sema3F* strongly inhibits the expression of *Thbs1* protein in in vitro differentiated *Nrp2⁺* VYS EpCs.

Our findings unveil a reciprocal cellular cross-talk that supports extraembryonic angiogenesis in the yolk sac (depicted in Figure VI in the [online-only Data Supplement](#)). In VYS EpCs, *Sema3F*, which is produced by both VYS EpCs and EnCs, signals to counteract the GSK3-driven degradative phosphorylation of *Myc* on Thr58, thus fostering the *Myc*-dependent transcription of proangiogenic miR-17/92 cluster members that in turn inhibit the synthesis of the antiangiogenic protein *Thbs1* in VYS EpCs themselves, finally allowing EnCs to be elicited by VEGFA, which is also secreted by VYS EpCs and required for yolk sac vascularization.⁴¹ In sum, we provide evidence that the extraembryonic proangiogenic activity of *Sema3F* relies on the *Myc*-dependent inhibition of the antiangiogenic protein *Thbs1*, which in turn is an effective inhibitor of VEGF signaling.³³ Indeed, the *Thbs1* receptor CD36 has been shown to physically interact with VEGF-R2 and hamper its ligand-induced phosphorylation and biological activity on EnCs.^{28,33} Mechanistically, it was reported that *Thbs1* binding to CD36 recruits the SHP-1 (Src homology 2 domain-containing protein tyrosine phosphatase-1) to the CD36-VEGF-R2 complex, where SHP-1 dephosphorylates and inactivates VEGF-R2 proangiogenic signaling.³⁴

In light of the fact that another *Nrp2*-binding semaphorin, that is, *SEMA3B*, was already suggested to be involved in human preeclampsia,⁴² our finding that *Sema3f* knockdown severely impairs placentas development and vascularization suggests that an abnormal expression of *Sema3F* might be implicated in placenta vascular malformations and malignant tumors (Results section, Discussion section, and Figure II in the [online-only Data Supplement](#)).

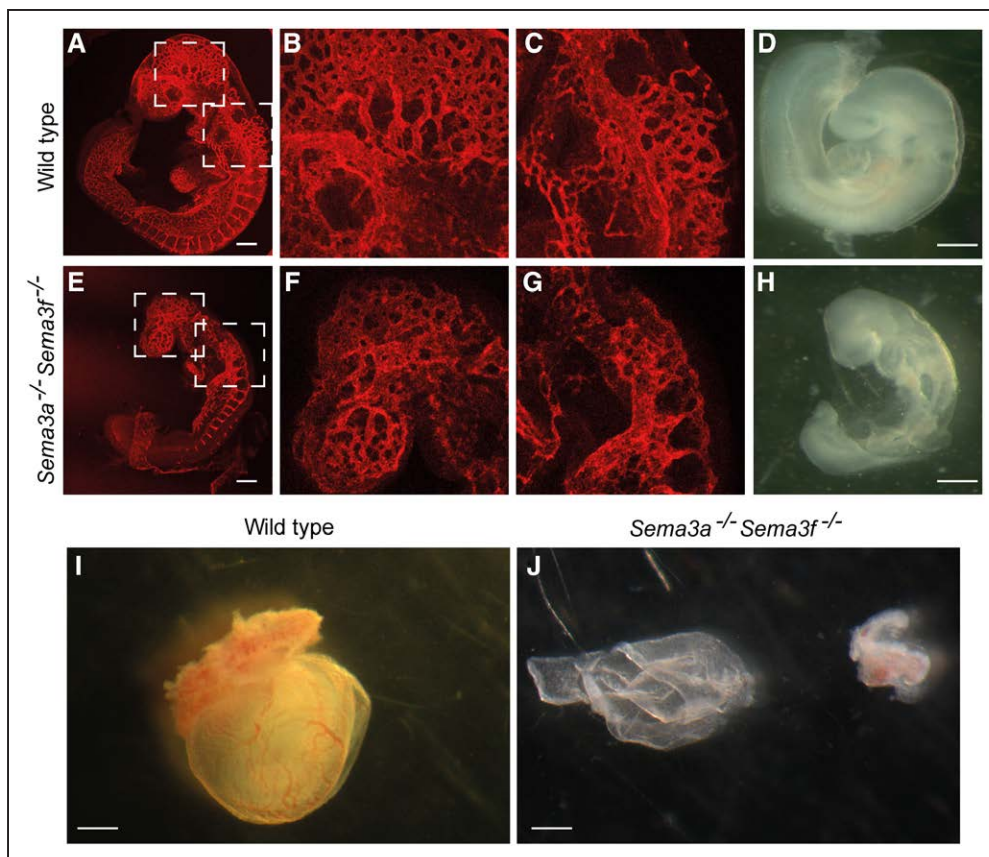


Figure 7. Sema3 (class 3 semaphorin)-F and Sema3A cooperate to control developmental angiogenesis. Whole-mount endomucin-stained E9.5 wild-type (**A**, **B**, and **C**) and *Sema3a*^{-/-} *Sema3f*^{-/-}-double knockout (**E**, **F**, and **G**) embryos. Remodeling of the cephalic plexus into veins occurs in wild type (**A** and **B**) but not in *Sema3a*^{-/-} *Sema3f*^{-/-} embryos (**E** and **F**). Similarly, the dorsal longitudinal anatomical vessel is remodeled in the cephalic perineural vascular plexus in wild type (**A** and **C**) but not in *Sema3a*^{-/-} *Sema3f*^{-/-} embryos (**E** and **G**). Stereomicroscopic analyses revealed how, when compared with age-matched wild-type embryos (**D**), the development of *Sema3a*^{-/-} *Sema3f*^{-/-} (**H**) E9.5 embryos is severely delayed. Stereomicroscopy analysis (**I** and **J**) of E10.5 wild-type (**I**) and *Sema3a*^{-/-} *Sema3f*^{-/-} (**J**) yolk sacs. *Sema3a*^{-/-} *Sema3f*^{-/-} yolk sacs (**J**) are essentially avascular. **B** and **F**, Magnifications of the top boxed areas in (**A**) and (**E**), respectively. (**C**) and (**G**) are magnifications of bottom boxed areas in (**A**) and (**E**), respectively. Scale bars, 300 μ m (**A** and **E**) and 100 μ m (**D**, **H**, **I**, and **J**).

As previously reported in zebrafish,⁹ chick,¹⁰ and mouse embryos,^{8,17-19} here we further substantiate how, differently from Sema3F, Sema3A is a crucial driver of embryonic vascular morphogenesis. Our findings that $\approx 80\%$ of *Sema3a*-knockout embryos die in utero and display a strong growth retardation compared with wild-type embryos reconcile previous apparently contradicting observations that, based on age and stage matching between wild-type and *Sema3a*-null embryos, concluded that Sema3A does not influence embryonic angiogenesis.⁴³ Furthermore, we establish how Sema3A similarly controls the remodeling of the yolk sac vasculature.

We found that in *Sema3a*-knockout mice, embryonic and extraembryonic blood vessels develop but display severe defects remodeling into an optimized hierarchically branched mature vascular tree. On the contrary, in *Sema3f*-knockout mice, embryonic blood vessels are effectively remodeled into a mature and functional tree, whereas vascularization of extraembryonic tissues is strongly reduced and poorly branched. It seems that, compared with *Sema3f*^{-/-} yolk sacs, the simultaneous knockdown of *Sema3a* and *Sema3f* gene further impairs the vascularization of the yolk sac that, until E12.5, is the primary means of nutrient, gas, and waste

exchange for the mouse embryo.³ Therefore, our findings suggest that the further dramatic impairment of embryonic vascular remodeling observed in *Sema3a*/*Sema3f*-double knockout embryos, compared with *Sema3a*^{-/-} embryos, is conceivably because of the severe disruption of the vasculature of the yolk sac, which constitutes the main nutrient and gas exchange organ between mother and embryo. Our data are in agreement with the fact that the yolk sac constitutes the first main nutrient and gas exchange organ between mother and embryo and with previous observations that mutations causing severe disruption yolk sac blood vessel formation are lethal and result in severe defects in embryo development.⁴⁴⁻⁵¹ Finally, the severe disruption of the embryonic vasculature observed in *Sema3a*/*Sema3f*-double knockout phenocopy the previously described vascular phenotype of double targeted *Nrp1*^{-/-} *Nrp2*^{-/-} embryos.³⁷

Acknowledgments

E. Giraudo, and G. Serini conceived the project; E. Giraudo, G. Serini, F. Bussolino, and F. Maione designed the experiments; D. Regano, F. Maione, A. Visintin, F. Clapero, and D. Valdembrì performed the experiments; E. Giraudo, G. Serini, D. Regano, F. Maione, A. Visintin, F. Clapero, and D. Valdembrì analyzed the data;

E. Giraudo, G. Serini, D. Regano, F. Maione, A. Visintin, F. Clapero, and D. Valdembrì interpreted the results; D. Regano, F. Maione, F. Clapero, D. Valdembrì, G. Serini, and E. Giraudo wrote the paper; and all authors read and approved the article.

Sources of Funding

This work was supported by grants from Italian Association for Cancer Research (AIRC-IG grant number: 15645 to E. Giraudo; grant numbers 13016 and 16702 to G. Serini; grant number 10133 to F. Bussolino; and AIRC 5x1000 grant number 12182 to F. Bussolino); FPRC-ONLUS grant MIUR 2010 Vaschetto - 5 per mille 2010 MIUR (to E. Giraudo and G. Serini); FPRC 5xmille 2014 Ministero Salute (to E. Giraudo and G. Serini); Swiss National Science Foundation, Sinergia grant (number CRSII3 160742/1 to E. Giraudo); Fondi di Ricerca Locale (ex 60% 2015, 2016), University of Turin (to E. Giraudo and G. Serini); Fondo Investimenti per la Ricerca di Base RBAP11BYNP (Newton to F. Bussolino); European Community-FP7, contract 318035 (Biloba to F. Bussolino). Telethon Italy (GGP09175 to G. Serini); and Associazione Augusto per la Vita (to G. Serini). D. Regano was supported by FPRC-ONLUS grant MIUR 2010 Vaschetto-Chiodo Fellowship; and F. Maione was supported by postdoctoral fellowships 2014 granted by Fondazione Umberto Veronesi.

Disclosures

None.

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Highlights

- Sema3 (class 3 semaphorin)-F is a novel extraembryonic proangiogenic factor.
- Sema3F inhibits the degradative phosphorylation of Myc on Thr58 in visceral yolk sac epithelial cells, both in vivo and in vitro.
- In the mouse yolk sac, Sema3F fosters the transcription of members of the Myc-dependent proangiogenic miR-17/92 cluster.
- Sema3F inhibits the synthesis of the Myc target and antiangiogenic protein thrombospondin 1 in visceral yolk sac epithelial cells, both in vivo and in vitro.
- Sema3F promotes the phosphorylation of vascular endothelial growth factor receptor 2 in yolk sac vascular endothelial cells.