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Signals Transduced by Ca^{2+}/Calcineurin and NFATc3/c4 Pattern the Developing Vasculature

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Summary

Vascular development requires an orderly exchange of signals between growing vessels and their supporting tissues, but little is known of the intracellular signaling pathways underlying this communication. We find that mice with disruptions of both *NFATc4* and the related *NFATc3* genes die around E11 with generalized defects in vessel assembly as well as excessive and disorganized growth of vessels into the neural tube and somites. Since calcineurin is thought to control nuclear localization of NFATc proteins, we introduced a mutation into the *calcineurin B* gene that prevents phosphatase activation by Ca^{2+} signals. These *CnB* mutant mice exhibit vascular developmental abnormalities similar to the *NFATc3/c4* null mice. We show that calcineurin function is transiently required between E7.5 and E8.5. Hence, early calcineurin/NFAT signaling initiates the later cross-talk between vessels and surrounding tissues that pattern the vasculature.

Introduction

The development of the vertebrate vascular system requires an ordered series of signals that direct the differentiation of endothelial cells and formation of a primitive endothelial vascular plexus by a process called vasculogenesis. The subsequent stages of vascular development, summarized as angiogenesis, involve a series of morphogenetic events which give rise to the anatomically highly stereotyped hierarchical network of mature vessels composed of endothelial cells and perivascular supporting cells (Folkman and D'Amore, 1996; Yancopoulos et al., 2000). Vascular endothelial growth factor (VEGF) (Carmeliet et al., 1996a; Ferrara et al., 1996) and its tyrosine kinase receptors VEGF-R2/Flk-1 (Shalaby et al., 1995),VEGF-R1/Flt-1(Fong et al., 1995), and VEGF-R3/Flt-4 (Dumont et al., 1998) are essential for the earliest phases of endothelial cell differentiation from mesodermally derived precursors as well as for vasculogenesis and angiogenesis. Angiopoietin-1 (Ang-1) and its tyrosine kinase receptor Tie-2/Tek and Tie-1, which function later than VEGF and its receptors during embryonic development, are critical regulators of angiogenesis (Dumont et al., 1994; Puri et al., 1995; Sato et al., 1995; Suri et al., 1996). During the angiogenic process, blood vessels are remodeled to follow highly specific anatomic paths that meet and probably anticipate the oxygenation needs of the growing tissues of the embryo. The mechanisms underlying these patterning events are poorly understood, and many of the mutations that interfere with angiogenesis do not affect the patterning of vessels. For example, in *Tie-2* and *Ang-1* mutant mice, growing vessels continue to observe somitic boundaries even in the face of fatal defects in angiogenesis. The determination of arterial and venous identity and the essential interconnections between them are established before the heart begins to move blood effectively and occurs by the selective expression of and signaling by ephrin-B2 on arteries and Eph-B4 on veins (Adams et al., 1999, 2001; Gerety et al., 1999; Wang et al., 1998).

Despite these major advances in the identification of crucial growth factors and their receptors, the intracellular signaling pathways critical for vascular development are still largely obscure. The paucity of information on signaling mechanisms in vascular development and angiogenesis under pathological conditions in the adult is surprising in light of the fact that most of the receptors involved are tyrosine kinases, which should activate both MAP kinase cascades and Ca^{2+} signaling. In a number of tissues, Ca^{2+} and ras signals are integrated in the nucleus by the assembly of NFAT transcription complexes from nuclear components (NFATn), which are dependent upon ras or PKC signaling, with the cytoplasmic components (NFATc), which are dependent on Ca^{2+} and calcineurin signals. (Crabtree, 1999; Flanagan et al., 1991; Rao et al., 1997). Ca^{2+} signals lead to the activation of calcineurin and the rapid dephosphorylation and nuclear import of the products of the four *NFATc* genes (*NFATc1-c4*, Hugo Nomenclature Committee).

Although the Ca^{2+}/calcineurin/NFAT signaling pathway was one of the first to be defined, genetic redundancy has made it difficult to study. In addition, calcineurin phosphatase activity is induced by a complex process. Upon reaching a threshold of about 400 nM, Ca^{2+} binds to both calmodulin and CnB displacing the inhibitory C-terminal peptide from the active site of CnA and activating phosphatase function (Klee et al., 1979, 1998). CnB thus acts as a sensor for changes in intracellular Ca^{2+}. The catalytic component CnA is encoded by three genes, while CnB is encoded by two genes. One of the *CnB* genes is ubiquitously expressed, while the other is testis specific. The immunosuppressive drugs FK506 and cyclosporin A have been helpful in dissecting this pathway. These chemically distinct natural products bind at subnanamolar affinity to intracellular receptors, forming inhibitory complexes. The resultant drug/protein composite surface binds tightly to calcineurin and prevents substrate access (Clipstone and Crabtree, 1992; Liu et al., 1991).

We have made null mutations of the *NFATc4* gene and show that mice doubly mutant for the *NFATc3* and *c4* genes have defects in vascular patterning and angiogenesis. In addition, we have introduced a germline mutation into mice which blocks the Ca^{2+}-dependent phosphatase activity of calcineurin and find that the phenotype of these *CnB* mutant mice closely resembles

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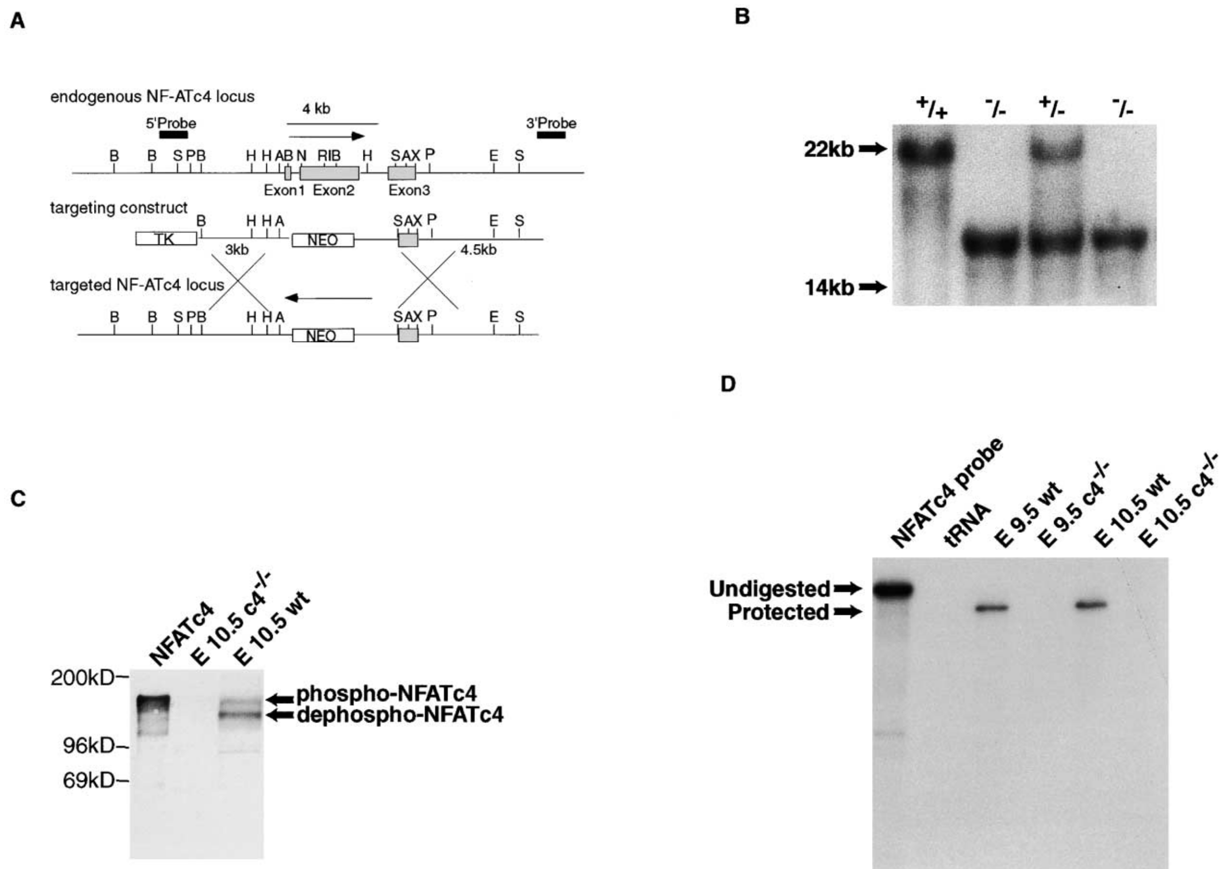


Figure 1. Targeted Disruption of the *NFATc4* Gene

(A) Diagram of the wild-type *NFATc4* allele, the targeting vector, and the recombined *NFATc4* allele and 5′ and 3′ probes used for Southern analysis.

(B) Southern blot probed with the 3′ outside probe.

(C) Western blot of $NFATc4^{+/+}$ and $NFATc4^{-/-}$ E10.5 embryos. Lane 1: recombinant *NFATc4*. Lane 2: E10.5 $NFATc4^{-/-}$ whole-cell extract. Lane 3: E10.5 $NFATc4^{+/+}$ extract.

(D) RNase protection assay of E9.5 and E10.5 RNA from $NFATc4^{+/+}$ and $NFATc4^{-/-}$ mice.

that of the *c3/c4* nulls. Our studies indicate that signals transduced by Ca^{2+}, calcineurin, and *NFATc3/c4* between E7.5 and E8.5 in the tissues surrounding vessels promote the proper anatomical patterning of the developing vascular system.

Results

Generation of Mice Bearing Null Mutations in *NFATc4*

NFATc4 is one of four genes that encode the Ca^{2+}/calcineurin-dependent subunits of NFAT transcription complexes (Graef et al., 2001). We disrupted the *NFATc4* gene by homologous recombination, removing the translational start site, the regulatory domain that controls calcineurin-dependent cytoplasmic-to-nuclear translocation and part of the DNA binding domain (Figure 1A). Southern blot analysis confirmed germline transmission of the targeted allele (Figure 1B). No expression of endogenous protein (Figure 1C) or mRNA (Figure 1D) can be detected in $NFATc4^{-/-}$ tissue, indicating that these animals bear a null mutation.

Defects in Vascular Patterning but Not Endothelial Differentiation in *NFATc4/c3* Double Mutant Mice

$NFATc4^{-/-}$ mice were viable and fertile and showed no major macroscopic or microscopic abnormalities after 36 months of observation. In situ hybridization indicated that NFATc4 expression significantly overlaps with that of NFATc3 (data not shown), which is the closest homolog of *NFATc4*, suggesting that the two genes might have redundant functions. Hence, we generated double knock-out mice (*c3/c4* null) by crossing the $NFATc4^{-/-}$ mice with $NFATc3^{-/-}$ mice (Oukka et al., 1998).

We found that 98% of *c3/c4* null embryos died in utero around E11.5. At E 9.5, most of the *c3/c4* null embryos exhibited no major morphological defects. At E10.5, the *c3/c4* null mice were alive, as indicated by the presence of a heartbeat. These mutants were smaller, pale, and showed an underdeveloped yolk sac vasculature and 20% had enlarged pericardial sacs (data not shown). We sometimes observed the presence of blood in the exocoelomic cavity, suggesting that the anemia seen in the embryos might be caused by extravasation of blood

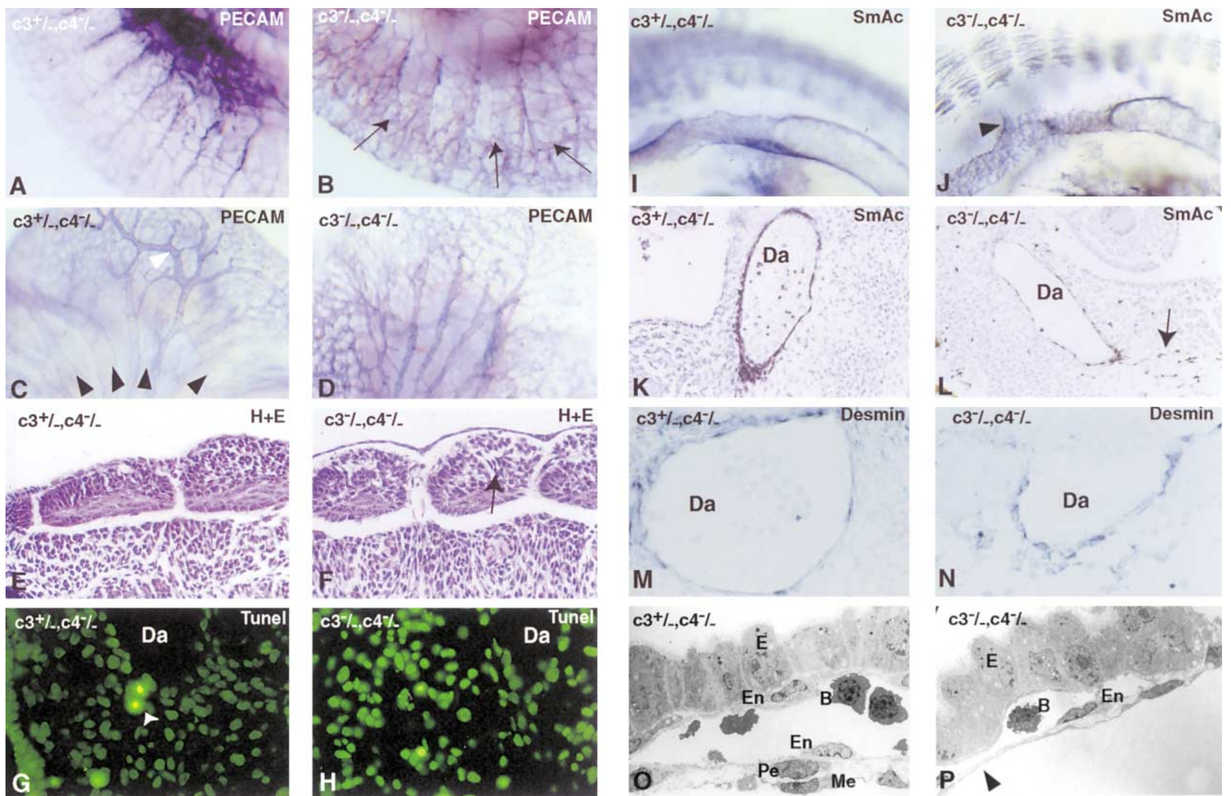


Figure 2. Disruption of Vascular Development in *c3/c4* Null E10.5 Embryos

(A–D) Whole-mount immunostaining with anti-PECAM antibody. Arrows in (B) and (F) show abnormal branching of vessels into the somites. The internal carotid artery seen in the wild-type (white arrowhead in [C]) is not fully developed in the *c3/c4* null embryos (D). Branches of the anterior cardinal vein (black arrowheads in [C]) are underdeveloped in *c3/c4* null embryos and show abnormal communicating sprouts between the main branches (D).

(E and F) H&E staining.

(G and H) Tunel staining shows no increase in apoptotic cell death .

(I and J) Whole-mount stains for smooth muscle actin. The *c3/c4* null embryos show a failure of VSMCs to tightly associate with the dorsal aorta (arrowhead in [J]).

(K and L) Sagittal sections at the level of the dorsal aorta (Da) stained with anti-smooth muscle actin antibody and (M–N) anti-desmin antibody show a reduction of the number and a lack of tight association of VSMCs with endothelial cells in the dorsal aorta of the *c3/c4* null embryo (arrow in [L]).

(O and P) Electron micrographs of E10.5 yolk sacs. The cell layer adjacent to the mesothelium (Me = mesothelium) does not contain pericytes (Pe, pericyte) in the *c3/c4* null yolk sac (P) when compared to $NFATc3^{+/-}c4^{-/-}$ yolk sac (O). Both yolk sacs contain blood cells (B, blood cells). The endodermal and mesothelial layer show a lack of contact in certain regions of the *c3/c4* null yolk sac (arrowhead in [P]). (En, endothelial cell; E, endoderm).

from the abnormal yolk sac vessels. Additionally, differentiation of E8.5 and E10.5 *c3/c4* null hematopoietic precursors into erythroid and myeloid lineages in vitro appeared to be normal, and expression of early hematopoietic markers such as GATA-1 was comparable to heterozygous littermates (data not shown).

The absence of organized vessels in the yolk sac was confirmed by PECAM staining, which revealed an enlarged and disordered capillary plexus and poorly developed vitelline vessels (data not shown). At E 9.5, the vasculature of *c3/c4* null embryos showed only mild perturbations (data not shown), while a pronounced disorganization was apparent at E10.5. Although there was sprouting and branching of the initial vascular plexus in E10.5 embryos, the major vessels including the intersomitic vessels, branchial arch arteries, and cranial vessels were severely disorganized (Figures 2A–2D and data not shown).

In the trunk region, *c3/c4* null embryos showed poorly organized, dilated, intersomitic vessels that ignored somitic boundaries (arrows in Figures 2B and 2F). At E10.5, the heads of null embryos exhibited a more primitive vasculature. The branches of the internal carotid artery failed to develop in the *c3/c4* null embryos. However, branches of the anterior cardinal vein were formed, but were underdeveloped and showed abnormal communicating sprouts between the branches (Figure 2D). In severe cases, the development of the cranial vessels was arrested at the primary capillary plexus stage and branches of the anterior cardinal vein appeared fused (data not shown).

The differentiation and proliferation of mutant endothelial cells was not impaired as suggested by the normal expression of endothelial markers such as PECAM, Flk-1, Tie-1, and Tie-2 (Figures 2A–2D and Figure 7). In addition, mutant endothelial cells expressed the vascu-

lar adhesion molecule VE-cadherin and, in contrast to the VE-cadherin knock-out endothelial cells (Carmeliet et al., 1999), were also firmly attached to the basement membrane (Supplemental Figures S2C–S2F, available online at http://www.cell.com/cgi/content/full/105/7/863/DC1). This indicated that NFATc3/c4 are not required for endothelial cell differentiation and vasculogenesis but are required for remodeling of the initial vascular plexus and the ability of the developing vasculature to respond to patterning cues.

A Selective Defect in Smooth Muscle Cell Recruitment to the Endothelium of *c3/c4* null Mice

A crucial stage of angiogenesis is the recruitment and differentiation of mesenchymal cells into pericytes and vascular smooth muscle cells (VSMC) to form a stable vascular wall. This process is dependent upon reciprocal signaling between endothelial cells and mesenchymal cells and is thought to involve angiopoietins and their receptors, Tie-1 and Tie-2 (Sato et al., 1995; Suri et al., 1996) as well as Flt-1 (Fong et al., 1995), PDGF-B (Lindahl et al., 1997), tissue factor (Carmeliet et al., 1996b), the TGF-β pathway (Dickson et al., 1995; Oh et al., 2000; Urness et al., 2000; Yang et al., 1999), and the transcription factor MEF2C (Bi et al., 1999; Lin et al., 1998). To test whether NFATc3/c4 were critical for this step in angiogenesis, we examined the expression of smooth muscle actin and desmin in the mutant embryos.

Whole-mount stains of *c3/c4* null E10.5 embryos showed poor association of smooth muscle actin positive cells with the aortic wall and irregular dilations of the aorta (arrowhead in Figure 2J), while smooth muscle staining within the somites appeared normal. Immunostaining revealed that the developing vessels of *c3/c4* null embryos had fewer cells expressing smooth muscle actin and desmin (Figures 2K–2N) and that these cells were usually distributed in the space around the vessel rather than directly adjacent to the vessel (arrow in Figure 2L). The reduction of supporting cells was not due to apoptosis or a defect in cell proliferation since there was no difference between mutant embryos and their littermates in Tunel assays or immunostains for Ki-67, a nuclear antigen expressed in proliferating cells (Figures 2G and 2H and data not shown).

Microscopically, both arteries and veins were affected and showed thin, sometimes broken vessel walls with extravasation of erythrocytes into the surrounding tissues (data not shown). Pericytes were absent in the mesothelial layer and defective formation of contacts between endodermal and mesothelial layers in the yolk sacs of *c3/c4* null embryos was observed by electron microscopy (Figures 2O and 2P). These observations indicated that NFATc3/c4 are required for the recruitment of vascular smooth muscle and pericyte precursors to the developing vessel wall and for the tight interaction between endothelial and perivascular supporting cells.

Generation of an Allele of *CnB* that Cannot Activate the Phosphatase Activity of Calcineurin A in Response to Ca^{2+} Signaling

In vitro studies have indicated that calcineurin directly controls the activity of the cytoplasmic subunits of NFAT complexes by Ca^{2+}-dependent dephosphorylation of residues within the SP repeat motifs and the serine rich region within the translocation domain at the N terminus of NFATc proteins. Dephosphorylation of these residues is thought to unveil two nuclear localization sequences within NFATc, which leads to rapid nuclear localization. To determine if calcineurin regulates NFATc3 and c4 function in vivo, we prepared a mouse strain in which the signaling function (but not other possible functions of CnB) was disrupted. This was accomplished by adding a peptide tag (FRB*) (see Supplemental Figure S1) to the C terminus of CnB (Griffith et al., 1995) (shown in yellow in Figure 3A), which prevents the interaction of CnB with CnA and hence specifically interferes with the Ca^{2+}-dependent activation of CnA catalytic activity. This allele is referred to as the *CnB** allele. The basis of this mutation is evident from the X-ray structure of the CnA-CnB complex in which the C terminus of CnB (shown in yellow) has extensive interactions with residues 345 to 420 of the A chain (shown in gray in Figure 3A). The FRB* tag was fused to the last codon of the *CnB* gene by homologous recombination (Figure 3B). The resultant heterozygous ($CnB^{+/*}$) mice were found to have both the predicted DNA rearrangement and the expected increase in size of the CnB subunit (Figure 3C and data not shown). In addition, antibodies to the FRB* tag detected the slower migrating band, indicating that the protein was in the correct reading frame (data not shown). In embryo extracts, the protein from the mutated allele was expressed at near wild-type levels and, as predicted from the structure, did not interact with the catalytic A subunit. (Figures 3C and 3D). In $CnB^{*/*}$ mice, there was no detectable Ca^{2+}-dependent dephosphorylation of NFATc4, indicating a complete loss of calcineurin phosphatase activity (Figure 3E).

In vitro experiments and assays with calcineurin inhibitors indicated that the dephosphorylation of NFATc by calcineurin induces cytoplasmic-to-nuclear translocation of these transcription factors (Clipstone and Crabtree, 1992; Flanagan et al., 1991). The $CnB^{*/*}$ mice, however, provided the first opportunity to genetically examine this in vivo. Endocardial cells of wild-type embryos expressed NFATc1 at E9.5 and the protein was nuclear in the majority of cells (Figure 3F). In contrast, in $CnB^{*/*}$ embryos, NFATc1 was cytoplasmic in all cells (Figure 3G). Thus, under physiological conditions, calcineurin is indeed necessary for the nuclear localization of NFATc1 in endocardial cells, as first demonstrated with biochemical reconstitution studies using the inhibitors FK506 and CsA (de la Pompa et al., 1998; Flanagan et al., 1991). This data is consistent with the thought that NFATc family members are both signal transducers and transcription factors.

Vascular Defects in the *CnB* Mutant Embryos Resemble Those of the *c3/c4* Null Mice

Heterozygous $CnB^{+/*}$ mice were indistinguishable from wild-type littermates and have been followed for 6 generations over 22 months with no detectable abnormalities. These results indicate that the FRB* tag on the CnB protein did not have a dominant-negative effect on calcineurin function, nor was it likely to produce a gain-of-function. No viable adult $CnB^{*/*}$ mice were found. About

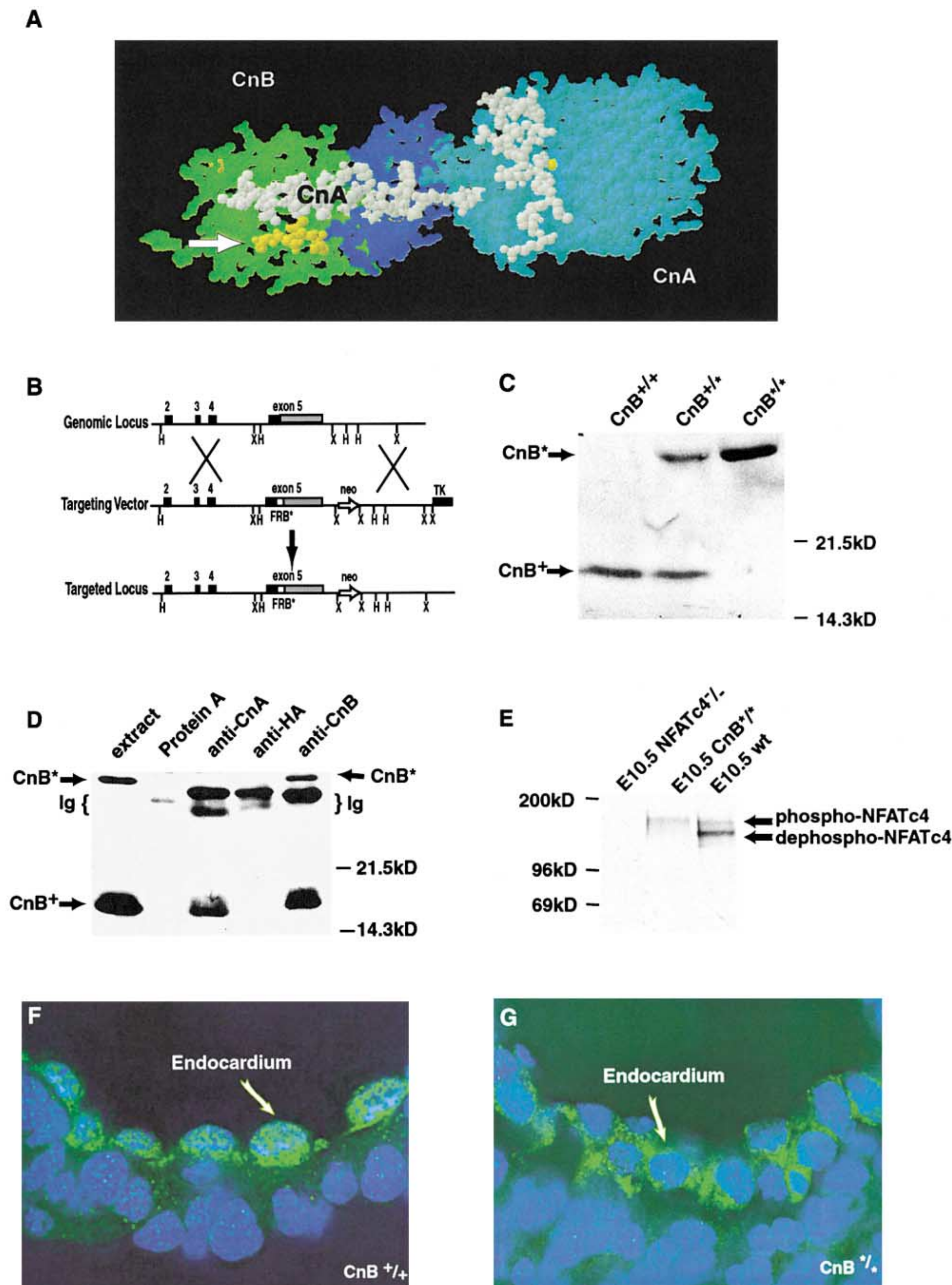


Figure 3. Generation of a Mouse Strain Carrying a *CnB* Mutant Allele that Is Unable to Interact with CnA

(A) Crystal Structure of the calcineurin complex showing the area of interaction between the C-terminal loop of the A chain (gray and light blue) and the B chain (green and dark blue). The arrow indicates the site (shown in yellow) of addition of the FRB* tag.

(B) Genomic locus, targeting vector, and the targeted CnB locus showing the insertion of the FRB* sequence at the C terminus of the CnB gene. H, HindIII; X, XbaI.

(C) Western blot of wild-type, $CnB^{+/*}$ and $CnB^{*/*}$ embryo extracts with anti-CnB antibody.

(D) Failure of the mutant CnB subunit to associate with the CnA subunit. Brain extracts of a $CnB^{+/*}$ mouse were prepared and immunoprecipitated with the indicated antibodies. Precipitates were Western blotted with the anti-CnB antibody.

(E) Absence of calcineurin activity in $CnB^{*/*}$ mice. Anti-NFATc4 Western blot of extracts from the wild-type and $CnB^{*/*}$ mice. Note that the dephosphorylated band in the wild-type extract is absent from the $CnB^{*/*}$ extract.

(F andG) NFATc1 fails to translocate to the nucleus in the endocardium of $CnB^{*/*}$ embryos. At E9.5, NFATc1 protein is nuclear in $CnB^{+/+}$ endocardium (F) but is cytoplasmic in $CnB^{*/*}$ embryos (G). Green, NFATc1 staining. Blue, DAPI.

50% of the E10.5 $CnB^{*/*}$ embryos and all of the E11.5 $CnB^{*/*}$ embryos examined were not viable. $CnB^{*/*}$ mice were macroscopically indistinguishable from wild-type littermates at E8.5, except in some rare cases where the pericardial sacs of the $CnB^{*/*}$ embryos were slightly enlarged (data not shown).

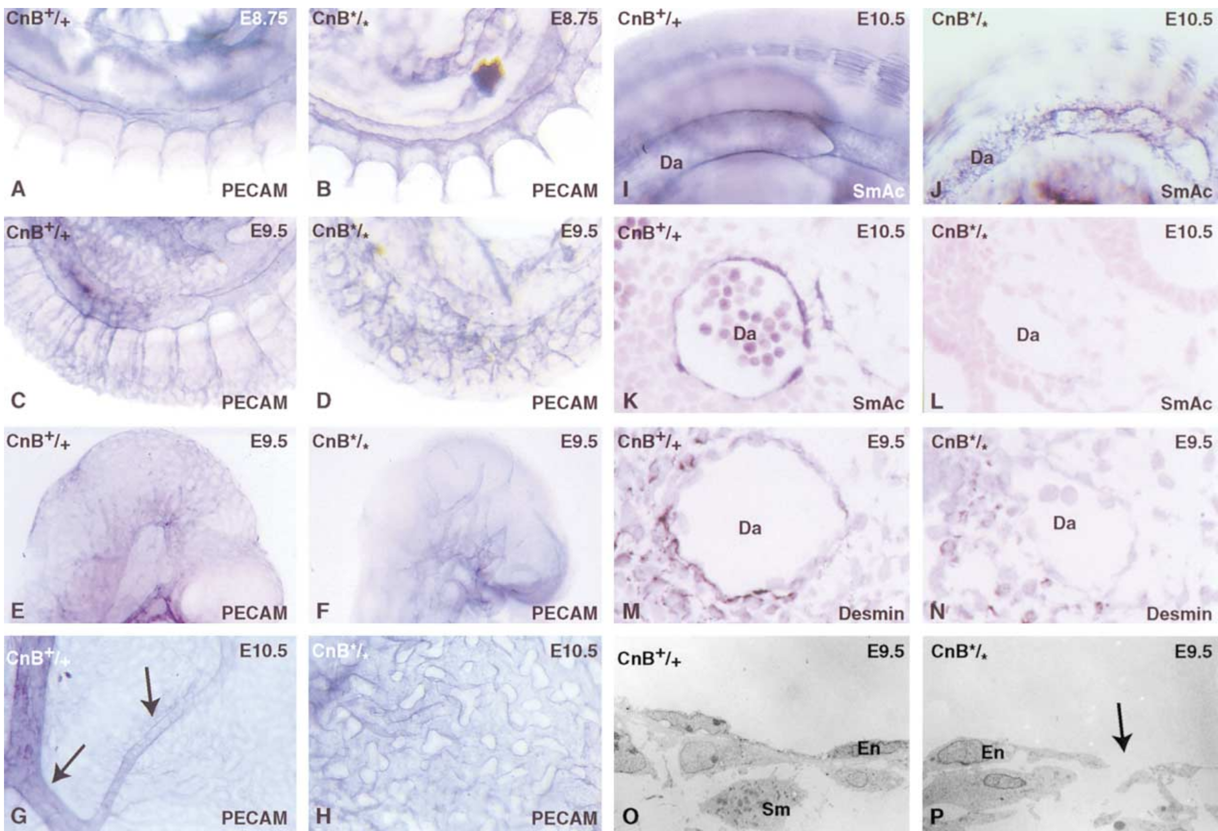


Figure 4. Defective Vascular Organization in $CnB^{*/*}$ Embryos

(A–H) Whole-mount immunostaining with anti-PECAM1 antibody: At E8.5-8.75, most $CnB^{*/*}$ embryos (B) are indistinguishable from wild-type controls (A). At E9.5, $CnB^{*/*}$ embryos have highly disorganized, syncitial intersomitic (D) and head (F) vessels compared to the organized vascular network seen in the wild-type embryo (C and E). The yolk sacs of E10.5 $CnB^{*/*}$ embryos (H) appear arrested at the stage of the primary vascular plexus and lack the vitelline vessels seen in the wild-type yolk sac (arrows in [G]).

(I and J) Whole-mount stains for smooth muscle actin (SmAc) at E10.5.

(K and L) Transverse sections at the level of the dorsal aorta stained with anti-SmAc at E10.5 show a layer of VSMCs around the wild-type dorsal aorta at E10.5 and a severe reduction of the number of VSMCs adjacent to the wall of the dorsal aorta in the $CnB^{*/*}$ embryo.

(M and N) Desmin stains of the dorsal aorta.

(O and P) Electron micrographs of E9.5 dorsal aorta (magnification 700×). There is a reduction in perivascular supporting cells surrounding the dorsal aorta and breaks in the wall of the dorsal aorta in the $CnB^{*/*}$ embryo (arrow in [P]). (En, endothelial cell; Sm, vascular smooth muscle cell; Da, dorsal aorta).

Beginning at E9.5, the $CnB^{*/*}$ embryos start to develop defects similar to those observed in the *c3/c4* null embryos. E9.5 $CnB^{*/*}$ embryos were alive, but smaller than their littermates. However, the number of somites in the mutants was only reduced by 1 or 2. By E9.5, about 40% of the homozygous embryos had significantly enlarged pericardial sacs. Although the primary vasculature was developed in $CnB^{*/*}$ embryos, it failed to remodel to the structured pattern of the wild-type vasculature (Figures 4A–4F). The dorsal aorta and intersomitic vessels were formed at E8.75 (Figures 4A and 4B), indicating that vasculogenesis occurred normally. At later stages, the vessels became progressively disorganized, characterized by excessive fusion into irregular dilated vessels and a failure to follow normal anatomical pathways as in the *c3/c4* null mice (Figures 4D and 4F). This indicates that patterning cues that normally guide vessels and prevent vessel fusion are defective in the $CnB^{*/*}$ embryos. Proper angiogenesis of the yolk sac vasculature of $CnB^{*/*}$ embryos never occurred, resulting in a primitive capillary plexus lacking the vitelline vessels (Figures 4G and 4H). As in the *c3/c4* null embryos, expression of endothelial differentiation markers, endothelial cell morphology, as well as adhesion to the basement membrane, was normal in the $CnB^{*/*}$ embryos (Figures 4O–4P, Figure 7, and Supplemental Figure S2). These vascular defects appeared similar to, but more severe, than the defects found in the *c3/c4* null embryos, suggesting that other NFATc family members and/or other calcineurin substrates contribute to the phenotype.

Similar to the *c3/c4* null embryos, whole-mount stains of $CnB^{*/*}$ embryos showed poor association of smooth muscle actin positive cells with the aortic wall and irregular dilations of the aorta, while the somites appeared normal (Figures 4I and 4J). Immunohistochemical analysis of desmin and smooth muscle actin expression showed severe reduction of perivascular supporting cells (Figures 4K–4N).

The endothelial cells lining the major vessels appeared ultrastructurally normal (Figures 4O and 4P). Consistent

with the light microscopic analysis, there was a marked reduction of pericytes and vascular smooth muscle cells surrounding the aorta and discontinuities of the vascular wall (Figures 4O and 4P), explaining the frequently observed hemorrhage in the mutant embryos. These observations indicated that calcineurin/NFAT signaling is required for the recruitment of vascular smooth muscle and pericyte precursors to the developing vessel wall. This defect is unlikely to be intrinsic to smooth muscle cells since, in contrast to the MEF2C mutant mice (Lin et al., 1998), the organization of the somitic smooth muscle was not disturbed.

Calcineurin Phosphatase Activity Is Essential between E7.5 and E8.5 for Vascular Development

To determine the time during development when calcineurin signaling is essential, we made use of the highly specific, rapidly acting and reversible inhibitor of calcineurin phosphatase activity, cyclosporin A. Injection of CsA into pregnant mice between days 7.5 and 8.5 but not earlier or later reproduced the vascular developmental defects seen in *CnB**/* and *c3/c4* null embryos (Figures 5A–5C). These embryonic CsA levels completely blocked the ability of calcineurin to dephosphorylate embryonic NFATc4, assayed by Western blots of whole embryo extracts (Figure 5D). Similar results were obtained with FK506 (data not shown). PECAM staining of E10.5 embryos taken from these mothers indicated that while endothelial cells did differentiate, there was a failure of vascular organization with defects similar to those of the *CnB**/* embryos. The temporally selective action of CsA could not be due to degradation of the drug, failure of placental transfer, or embryonic metabolism of the drugs, since similar levels of CsA were achieved during the critical period and after it. In addition, we found that even at the time that CsA administration had no developmental effect, it still produced hyperphosphorylation of *NFATc4*, indicating that the drug effectively gained access to the embryo and blocked calcineurin activity (data not shown). The observation that CsA administration mimics the phenotype of the *CnB**/* mutants indicates that in early mammalian development, CsA is a highly specific inhibitor of calcineurin function and is unlikely to have other developmentally critical targets.

Angiogenic Defects in the *c3/c4* Null and *CnB**/* Mice Are Not Related to Defective Myocardial Development

Calcineurin and NFATc4 have been shown to play important roles in mediating the response of the adult heart to hypertrophic stimuli (Molkentin et al., 1998). Since the hypertrophic response often reiterates embryonic cardiac development (Olson and Williams, 2000), we considered the possibility that the angiogenic defects seen in the *c3/c4* null and *CnB**/* mice could be secondary to a defect in cardiac development. However, the *c3/c4* null and *CnB**/* embryos showed a normal heart beat, and no apparent defects in cardiac morphology were visible (Figure 6). The expression of myocardial markers such as smooth muscle actin, desmin, and MLC-2V in the *c3/c4* null and *CnB**/* mutant hearts is comparable to control littermates (Figures 6C–6H and 6K–6P). Since the *c3/c4* null and *CnB**/* mice show severe angiogenic abnormalities at the time that the heart appears morphologically normal, we conclude that defects in heart development do not account for the angiogenic defects. These studies, however, do not preclude a critical role for NFATc3/c4 and CnB in later cardiac development or in the myocardial hypertrophic response.

Identification of Calcineurin/NFAT Target Genes in the E10.5 Embryo

Suprisingly, most of the identified regulators of vascular development were expressed at normal levels in the *c3/c4* null and *CnB**/* mice (Figure 7). Exceptions included VEGF-A, Flt-1, and adrenomedullin, which were overexpressed by several-fold in *c3/c4* null and *CnB**/* embryos (Figure 7). In addition, administration of CsA during the critical E7.5 to E8.5 period led to overexpression of the same genes. Induction of VEGF-A mRNA appears not to be caused by hypoxia, since other hypoxia-induced genes such as erythropoietin, HIF1-α, and Tie-2 were not induced (Figure 7B). We also examined the expression of VEGF-A by in situ hybridization and found that the increase was most pronounced in the neural tube, somites and heart (Supplemental Figures S2G–S2K). These results suggest that NFAT signaling either directly represses the VEGF-A gene or activates a repressor of VEGF-A production.

Since ephrins and their receptors also play critical roles in vascular development, we assayed the expression of all ephrins and Eph-receptors important in angiogenesis (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). Again these were expressed at normal levels (Supplemental Figure S3). We also monitored the expression pattern of ephrinB2 by whole-mount staining for β-galactosidase in CsA-treated *ephrinB2^{taulacZ}* embryos (Wang et al., 1998) and found that, while the overall expression of ephrinB2^{taulacZ} was unaltered, the pattern of vascular expression reiterated the CsA-induced vascular abnormalities (Supplemental Figure S3B).

Thus, in the absence of NFATc3/c4 signaling, most of the known vascular growth and differentiation factors and receptors were expressed normally.

NFATc4 Is Expressed in the Neural Tube and Somites during the Time that It Is Critical to Angiogenesis

Determination of the developmental window during which Ca^{2+}/calcineurin/NFAT signaling is necessary for angiogenesis (Figure 5), opened the unusual opportunity to ask where the protein was expressed at the time that it was performing its critical developmental functions. By both in situ hybridization and by immunoflourescence, NFATc4 was expressed in the neural tube and somites during the critical E7.5 to E8.5 period (Figure 8A). Whole-mount fluorescent staining of E8.5 embryos also showed that NFATc4 was nuclear at this time, indicating that the pathway was active in these cells (Figure 8A). This observation is consistent with the biochemical observation that the protein is dephosphorylated at E8.5 (data not shown). To help determine if the NFAT signaling occurs in the vascular endothelium or the surrounding tissues, embryos were costained for Tie-2 and NFATc4. Although NFATc4 in the trunk regions seemed to be

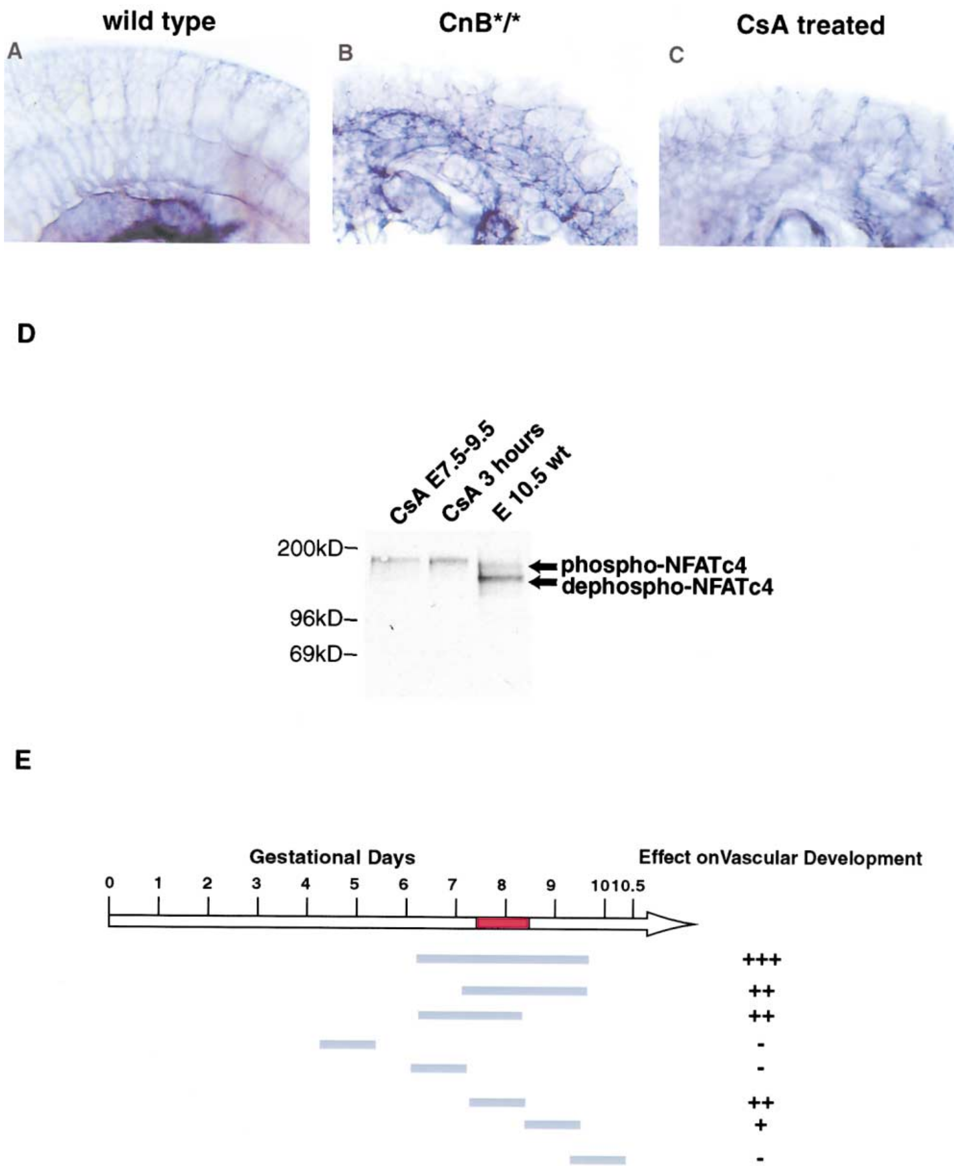


Figure 5. Calcineurin Functions Between E7.5 and E8.5 to Transduce Signals Required for Vascular Development

PECAM staining showing similar abnormalities in vascular development in E10.5 *CnB*/** embryos (B) and embryos from mothers treated with 25 mg/kg CsA twice per day at E7.5 and E8.5 (C) when compared to untreated E10.5 embryos (A). (D) Maternal administration of CsA either from E7.5–E9.5 (lane1) or for 3 hr at E10.5 (lane2) induced hyperphosphorylation of the *NFATc4* protein compared to nontreated embryos (lane3). (E) Experimental strategy to identify the window in which calcineurin is required for angiogenesis. The gray bars denote the time of CsA administration and the red region is the critical period for Cn/NFATc signaling.

located predominantly in the neural tube and somites rather than the endothelial cells, some degree of overlap in fluorescence was observed (Figure 8A). To further clarify the localization of NFATc4 in embryos, we dissociated E8.5 embryos and used fluorescence activated cell sorting (FACS) to separate endothelial cells based on PECAM and Flk-1 expression. The amount of NFATc4 in the double positive cells (PECAM and Flk-1 positive) was then compared to the amount of NFATc4 in the double negative cells by semiquantitative RT-PCR. The double negative cells expressed a 5- to 10-fold higher level of NFATc4 than the double positive endothelial population. Thus, while NFATc4 is preferentially expressed in surrounding cells, there is probably some low degree of expression in endothelial cells.

In the *c3/c4* null and *CnB*/** embryos, we found that vessels invade the neural tube and somites to an extent that greatly exceeds the normal pattern (Figures 2 and 4; data not shown), suggesting that NFAT signaling prevents abnormal growth of vessels into these tissues. Tie-2 fluorescent whole-mount staining of CsA-treated embryos at E9.0 also showed aberrant branches of intersomitic vessels invading the somites (Figure 8C). In situ hybridization of embryos for VEGF-A mRNA shows that its expression was enhanced (possibly derepressed) in somites and neural tube (Supplemental Figures S2G–

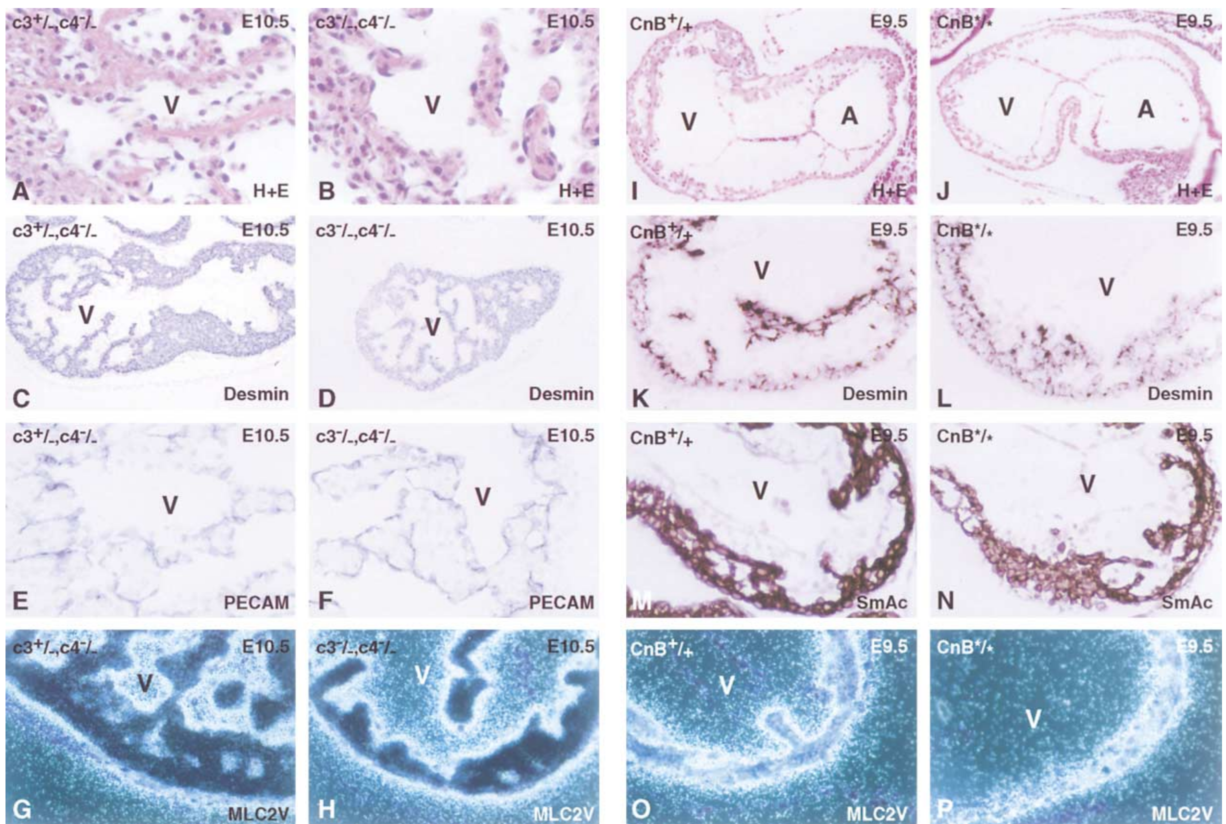


Figure 6. Early Cardiac Development Appears Normal in *c3/c4* null and $CnB^{*/*}$ Embryos

(A and B) Ventricular wall of $c3^{+/-}c4^{-/-}$ and *c3/c4* null embryos at E10.5, H&E; (C and D) Desmin-immunostaining; (E and F) PECAM-immunostaining; and (G and H) ^{35}[S] in situ hybridization of myosin light chain (MLC2V).

(I–P) Cardiac morphology in $CnB^{+/+}$ and $CnB^{*/*}$ embryos at E9.5. (I and J) H&E; (K and L) Desmin-immunostaining; (M and N) SmAc-immunostaining; and (O and P) ^{35}[S] in situ hybridization of MLC2V.

S2J). This finding suggests that regionally specific NFAT signaling (in the somites and neural tube) directly or indirectly represses VEGF-A expression, thereby preventing uncontrolled growth of vessels into regions such as the neural tube, where vessels must observe very specific pathways. (Figure 8D).

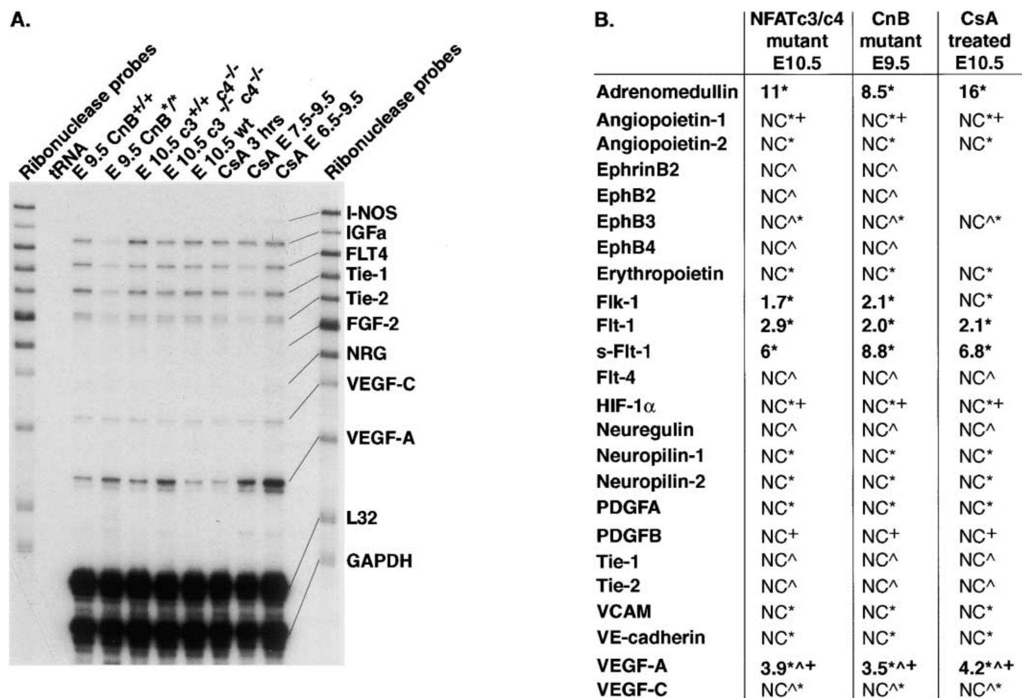
Discussion

NFAT Signaling Appears to Suppress Local Vascular Development

Arteries and veins of adult mammals follow precise anatomical paths that show relatively little variation between individuals. This stereotyped anatomy suggests that some form of guidance must be provided to the growing vessel. Such guidance cues are likely to involve both positive cues that direct vessels into specific regions and negative cues that prevent major vessels from developing within structures that require a very specific pattern of vascularization. Our results indicate that signaling through Ca^{2+}/calcineurin /NFATc3,c4 mediates a negative signal preventing aberrant growth of vessels. When signaling by this pathway is prevented, either by mutation of *CnB* or *NFATc3/c4* or by inhibition of calcineurin with CsA, vessels grow into regions where NFATc3/c4 are expressed at highest levels. These results indicate that Ca^{2+}/calcineurin/NFAT signaling patterns the vascular system by controlling regional expression of VEGF-A and probably other regulators of vessel development (Figure 8D). This hypothesis is based on the often-made assumption that proteins have their function in locations of highest expression and will have to be tested by regional specific deletion of the components of this signaling pathway.

The relationship between NFAT signaling and VEGF-A expression may well be indirect since the lag time between CsA administration to the mother and induction of VEGF-A in the embryo is in excess of 3 hr. This delay is probably not related to pharmacological barriers, since 30 min after CsA administration to the mother, we were able to detect complete inhibition of calcineurin phosphatase activity measured on endogenous embryonic NFATc4 (results not shown). Thus, a possible scenario is that NFAT signaling suppresses an inducer of VEGF-A expression or alternatively regulates the expression of a negative vascular guidance cue.

A vascular patterning defect reminiscent of the one seen in the *c3/c4* null and $CnB^{*/*}$ mice has been described in mice having mutations in *ephrinB2* as well as *EphB2/EphB3* double mutants (Adams et al., 2001, 1999). Eph receptors and their ligands are probably involved in paracrine signaling between the mesenchyme and the



	NFATc3/c4 mutant E10.5	CnB mutant E9.5	CsA treated E10.5
Adrenomedullin	11*	8.5*	16*
Angiopoietin-1	NC*+	NC*+	NC*+
Angiopoietin-2	NC*	NC*	NC*
EphrinB2	NC^	NC^	
EphB2	NC^	NC^	
EphB3	NC^*	NC^*	NC^*
EphB4	NC^	NC^	
Erythropoietin	NC*	NC*	NC*
Flk-1	1.7*	2.1*	NC*
Flt-1	2.9*	2.0*	2.1*
s-Flt-1	6*	8.8*	6.8*
Flt-4	NC^	NC^	NC^
HIF-1α	NC*+	NC*+	NC*+
Neuregulin	NC^	NC^	NC^
Neuropilin-1	NC*	NC*	NC*
Neuropilin-2	NC*	NC*	NC*
PDGFA	NC*	NC*	NC*
PDGFB	NC+	NC+	NC+
Tie-1	NC^	NC^	NC^
Tie-2	NC^	NC^	NC^
VCAM	NC*	NC*	NC*
VE-cadherin	NC*	NC*	NC*
VEGF-A	3.9*^+	3.5*^+	4.2*^+
VEGF-C	NC^*	NC^*	NC^*

Figure 7. Definition of a Cluster of Genes Regulated by *c3/c4* null, *CnB**/*, and CsA

(A) RNase protection assay shows selective overexpression of VEGF-A (determined by phosphoimaging) and normal expression of iNOS, IGF-a, Flt-4, Tie-1, Tie-2, FGF-2, neuregulin, and VEGF-C in *c3/c4* null, *CnB**/*, and CsA-treated embryos (three hours after the 50 mg/kg injection the concentration of CsA in maternal blood was 5.15 μg/ml of whole blood and the CsA concentration in the embryos was 1.94 μg/ml of embryo extract). The *CnB**/* lane was underloaded, as judged by L32 and GAPDH levels.

(B) A cluster of coregulated genes was defined by analysis of transcript arrays (Affymetrix), RPA assays, and quantitative RT-PCR. Specific attention has been given to those genes required for cardiovascular development. All results are expressed as a fold change compared to the age-matched, wild-type sample. "^" denotes RPA result, "*" denotes Affymetrix transcript array result, "+" denotes RT-PCR result, and "NC" denotes no change.

growing blood vessel and hence, EphB2, which is expressed in the mesenchyme adjacent to endothelial cells, seems a possible candidate for a receptor that regulates NFATc3/c4 at this time in development.

Defects in Cardiac Development Do Not Account for the Early Embryonic Lethality in the CnB and *c3/c4* Null Mice

Calcineurin and NFATc4 have been shown to be regulators of myocardial fiber hypertrophy in response to stress in the adult murine heart (Molkentin et al., 1998). However, myocardial dysfunction does not seem to account for the phenotypes we observed in either the *CnB**/* or the *c3/c4* null mice. Heart morphology and heartbeat appeared normal even when the angiogenic defects in the *c3/c4* null and *CnB**/* mice were far advanced (Figure 6 and data not shown). In addition, the expression of a number of cardiac markers was normal. The development of relatively normal cardiac trabeculations in the *NFATc3/c4* and *CnB* mutant mice contrasts with the severe defects in trabeculation observed in mice lacking neuregulin and its receptors (Erickson et al., 1997; Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995), or angiopoietin and its receptors (Dumont et al., 1994; Puri et al., 1995; Sato et al., 1995; Suri et al., 1996), or MEF2C (Lin et al., 1997). Although our observations indicate that defects in myocardial function do not underlie the early angiogenic defects observed in *CnB**/*, *c3/c4* null mice, present evidence strongly indicates that these molecules have critical roles later in myocardial development and function (Olson and Williams, 2000).

Signaling by NFATc1 in the endocardium is essential for development of heart valves (de la Pompa et al., 1998; Ranger et al., 1998). These defects also have no role in generating the angiogenic phenotypes we observed, since the mice completely lacking NFATc1 function do not die until E13.5, while the *CnB**/* and *c3/c4* null mice die before cardiac valve function becomes necessary. In addition, the *NFATc1* mutant mice have no defects in angiogenesis detectable by PECAM staining.

Defective Angiogenesis in *CnB* and *c3/c4* Null Mice

Several observations indicate that NFAT signaling may be necessary for both vascular organization as well as vascular assembly. Our analysis of the *c3/c4* null and *CnB**/* embryos revealed that both the yolk sac and the embryo initiate vasculogenesis properly, but fail to recruit pericytes and VSMCs to form a stable vascular wall. This process is dependent upon interactions of endothelial cells with mesenchymal cells. Light microscopic and ultrastructural analysis showed that calcineurin/NFAT signaling was required for the recruitment

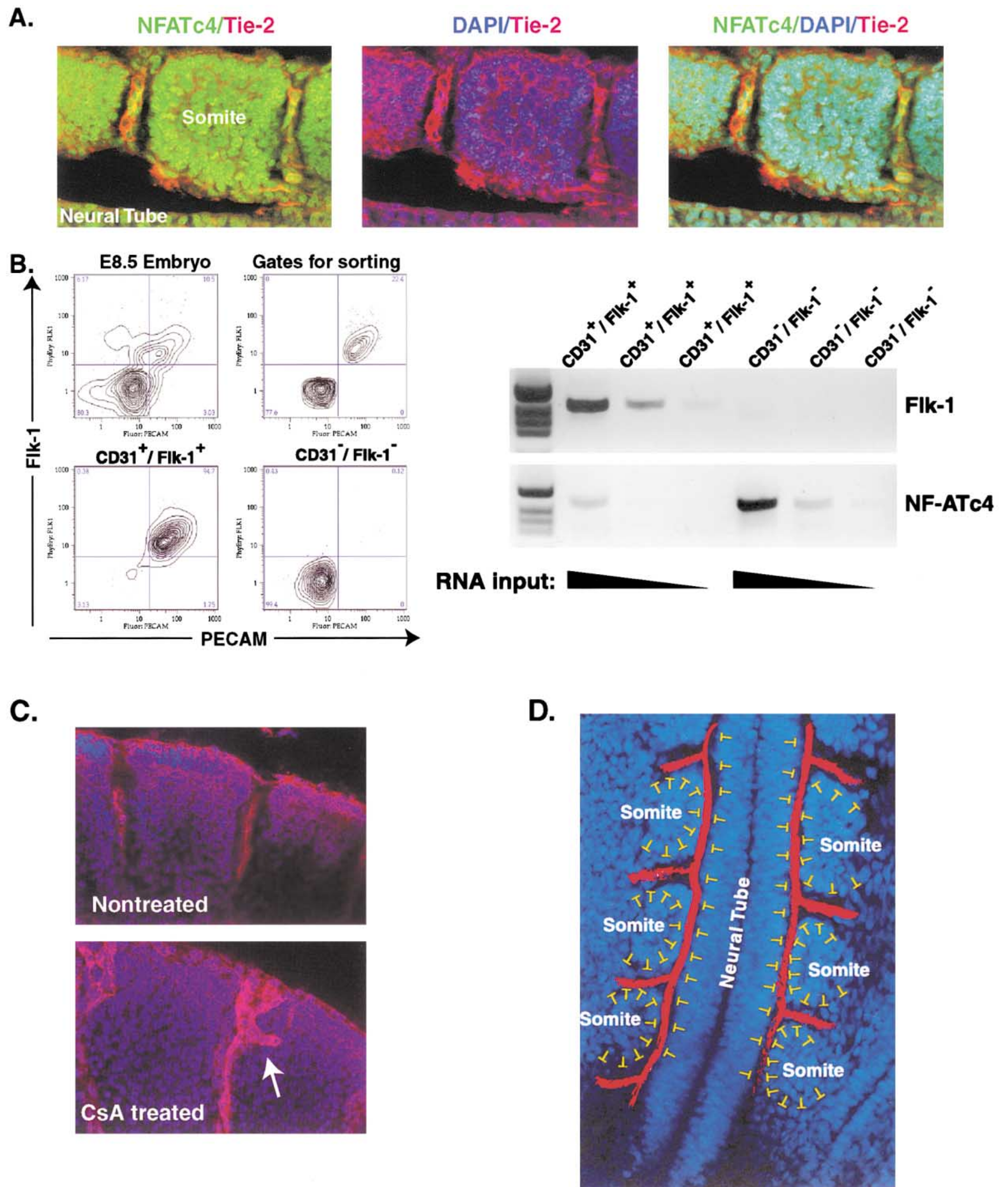


Figure 8. NFATc4 Shows Nuclear Localization in Somites and Neural Tube of E8.5 Embryos and Is Expressed at Very Low Levels in Endothelial Cells

(A) Fluorescent whole-mount immunohistochemistry with anti-NFATc4 (green) and anti-Tie-1 (red) antibodies on E8.5 embryos. Blue, DAPI. The staining in the somites and neural tube is nuclear (A) and is absent in the control samples with the secondary antibody alone (not shown).

(B) FACS analysis of dissociated cells from whole embryos using antibodies to Flk-1 and PECAM. The right panel shows the results of semi-quantitative RT-PCR for NFATc4 and Flk-1 on the PECAM/Flk-1 double-positive cells and PECAM/Flk-1 double-negative cells.

(C) Whole-mount Tie-2 staining of CsA-treated (E7.5+8.5) embryos at E9.0 vessels shows invasion of the somites by aberrant branches of intersomitic vessels (white arrow in [C]).

(D) Localized NFAT signaling in the somites and neural tube leads to a suppression of vessel growth into the somites and neural tube.

of vascular smooth muscle and pericyte precursors to the developing vessel wall without disrupting endothelial or smooth muscle cell specification. However, the defect that we see is not intrinsic to smooth muscle cells since somitic smooth muscle was correctly patterned and differentiation markers were expressed.

The angiogenic defects in the *c3/c4* null and *CnB*/** embryos differ from the defects observed in MEF2C and PDGF pathway mutant mice. In *MEF2C* mutant embryos, differentiation of smooth muscle cells is completely blocked in somites, heart ventricle, and in perivascular cells (Lin et al., 1998). The defects in *PDGF-B*$^{-/-}$ and *PDFGR*$\beta^{-/-}$ embryos occur at a later stage in embryonic development (Hellström et al., 1999) and PDGF-B is expressed at normal levels in the *c3/c4* null and *CnB*/** embryos. Since perivascular mesenchyme expresses high levels of NFATc4 (Figure 8), NFAT signaling may also be necessary in the perivascular supporting cells to assemble the vessel wall. Several features distinguish the *c3/c4* null and *CnB*/** phenotype from the *Tie1*, *Tie2*, and *Ang1* mutant mice. In these mice, vessels remodel around the somites and neural tube and patterning of vessels is relatively normal even when the angiogenic defects are far advanced and the embryo is hypoxic. Since the *c3/c4* null, *CnB*/**, *Tie2*, and *Ang1* mutant mice all die about E11.5, induction of VEGF alone could not be responsible for the patterning defects observed in the *CnB*/** and *c3/c4* null mice. Rather, the abnormalities in vessel pathfinding, remodeling, and integrity suggest a distinct role of NFAT signaling in the guidance, extension, and assembly of the vascular network.

Experimental Procedures

Generation of *NFATc4* Knockout Mice

The murine *NFATc4* gene was isolated and fully sequenced (GenBank:AF309388-AF309389). The targeting construct deletes exons 1, 2, and part of exon 3, encoding aa 1–413. Double knockout mice were generated by intercrossing of *NFATc4*$^{-/-}$ mice with *NFATc3*$^{-/-}$ mice (Oukka et al., 1998).

Generation of the *CnB* Mutant Mice

A targeting vector was designed to replace the stop codon of the ubiquitously expressed CnB with the FRB* tag. Correct targeting was confirmed by Southern analysis and long-range genomic PCR and Western blot analysis.

Immunoprecipitation

150 μg brain extract from a *CnB*$^{+}$/* mouse was immunoprecipitated with anti-CnB (clone CN-B1, Sigma), anti-CnA (clone CN-A1, Sigma), and anti-HA (clone 12CA5) and analyzed by Western blot with the anti-CnB antibody (1:3000).

Immunohistochemistry

Antibodies used for whole-mount staining are: a rat anti-PECAM-1 monoclonal antibody (clone MEC 13.3, Pharmingen) and a mouse anti-α SmAc monoclonal antibody (clone 1A4, Sigma). NFATc4 immunostaining of E8.5 embryos was performed with the affinity-purified anti-NFATc4 rabbit polyclonal antibody against aa 70–245 of human NFATc4, which had been preabsorbed with NFATc4$^{-/-}$ embryo powder and costained with anti-Tie-2 antibody. Embryos were scanned using a two-photon microscope (Zeiss LSM510 with a Coherent MIRA laser, Stanford Imaging Facility). Antibodies used on sections were; anti-NFATc1 antibody (clone 7A6), anti-αSmAc, anti Ki-67 antibody (clone B56 Pharmingen), anti-desmin antibody (Sigma), anti-laminin antibody (Sigma), and anti VE-cadherin antibody (Esser et al., 1998).

RNA Expression Analysis

The NFATc4 probe for ribonuclease protection was an EcoRI/BamHI fragment containing part of exon 2 of the murine *NFATc4* gene. Transcript arrays were done with biotinylated cRNA prepared by standard techniques using the Affymetrix murine 11k and 36k gene chips. Quantitative RT-PCR was done by standard techniques on a Bio-Rad Icycler.

Flow Cytometry

E8.5 embryos and yolk sacs were dissociated and stained with FITC-conjugated anti-PECAM (CD31, clone MEC 13.3, Pharmingen) antibody and PE-conjugated anti Flk-1 antibody (clone Avas 12α1, Pharmingen). All sorts and analyses were performed on a FACS Vantage flow cytometer (Becton-Dickinson). Dead cells were excluded by gating on forward and side scatter as well as by eliminating propidium iodine positive cells and 6.5×10^4 CD31^{+}/Flk-1^{+} and CD31^{-}/Flk-1^{-} cells were sorted. RNA was prepared by standard techniques and one-step RT-PCR for NFATc4 and Flk-1 was performed on RNA from 812, 162, and 32 cells. NFATc4 sense:gaagctaccctc cggtacagag; antisense:gcttcatagctggctgtagcc; Flk-1 sense: cagaacag taagcgaaagagccggccag antisense:aagcagcacctctctcgtgatttccaggag

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Accession Numbers

The murine *NFATc4* gene reported in this paper has been deposited in GenBank with accession numbers AF309388–AF309389.