E-Tmod capping of actin filaments at the slow-growing end is required to establish mouse embryonic circulation

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E-Tmod capping of actin filaments at the slow-growing end is required to establish mouse embryonic circulation. Am J Physiol Heart Circ Physiol 284: H1827–H1838, 2003. First published January 23, 2003; 10.1152/ajpheart.00947.2002.—Tropomodulins are a family of proteins that cap the slow-growing end of actin filaments. Erythrocyte tropomodulin (E-Tmod) stabilizes short actin protofilaments in erythrocytes and caps longer sarcomeric actin filaments in striated muscles. We report the knockin of the β-galactosidase gene (LacZ) under the control of the endogenous E-Tmod promoter and the knockout of E-Tmod in mouse embryonic stem cells. E-Tmod−/− embryos die around embryonic day 10 and exhibit a noncontractile heart tube with disorganized myofibrils and underdevelopment of the right ventricle. Accumulation of mechanically weakened primitive erythroid cells in the yolk sac, and failure of primary capillary plexuses to remodel into vitelline vessels, all required to establish blood circulation between the yolk sac and the embryo proper. We propose a hemodynamic “plexus channel selection” mechanism as the basis for vitelline vascular remodeling. The defects in cardiac contractility, vitelline circulation, and hematopoiesis reflect an essential role for E-Tmod capping of the actin filaments in both assembly of cardiac sarcomeres and of the membrane skeleton in erythroid cells that is not compensated for by other proteins.

Erythrocyte tropomodulin; cardiomorphogenesis; hematopoiesis; LacZ; yolk sac; vasculogenesis

ACTIN IS AN ABUNDANT PROTEIN in eukaryotic cells, where actin filaments form one of the three major cytoskeletal networks. Actin networks carry out cellular functions such as contraction, adhesion, and migration, which are important for normal physiology and embryonic development. In sarcomeres, six actin (thin) filaments interdigitate with each myosin (thick) filament, and, in other cases, actin filaments are organized into arrays such as contractile bundles, gellike networks, or tight parallel bundles. The polarized actin filaments undergo polymerization and depolymerization at both ends (38, 47, 61). The fast-growing (barbed) end polymerizes at up to 10 times the rate of the slow-growing (pointed) end, suggesting that the fast-growing end was more dynamic and important in assembly. The dynamics of actin networks are regulated by a number of actin-associated proteins, including those that cap either end of the filaments. The fast-growing end-capping proteins include adducin (25, 26) and CapG (33, 48). Interestingly, their null mutations are not lethal, only causing spherocytosis in erythrocytes (17) and impaired phagocytosis in macrophages (63), respectively. Knockout of gelsolin, which encodes a fast-growing end-capping and severing protein, also is not lethal (63). It is not known whether mice with targeted disruption of genes encoding slow-growing end-capping proteins would survive.

Tropomodulin was first isolated as a tropomyosin (TM)-binding protein (13) and later shown to be the long sought after slow-growing end-capping protein of the actin filaments (15, 18, 59). It increases the critical concentration of actin filaments, whose fast-growing end was capped by gelsolin, by converting ADP.P(i)-actin to ADP-actin at all slow-growing ends (60). The first tropomodulin identified was human erythrocyte tropomodulin (E-Tmod) (13), and several other tropomodulins have since been characterized. E-Tmod is highly conserved among humans (50), mice (19), rats (58), and chickens (3). E-Tmod is expressed not only in erythrocytes, but also in other tissues including lens, muscle, and embryonic stem (ES) cells (2, 8, 19, 64).

Several E-Tmod homologs encoded by distinct genes exist in humans, mice (10), and other species. These include N-Tmod in the rat brain (58), Sk-Tmod in chicken fast skeletal muscle (1), and ubiquitously expressed Tmod (U-Tmod) in various tissues (10). In mice, the identity between the amino acid sequences of E-Tmod and Sk−, N−, and U-Tmod is 56%, 57%, and 59%, respectively. Leiomedin in human extraocular muscles, sanpodo in Drosophila, and Tmod-like proteins in Caenorhabditis elegans also belong to this family (9, 12, 37, 62).

Tropomodulins may be targeted to different locations in cells to serve different functions. In chicken fast...
skeletal muscle fibers, the predominant Sk-Tmod is present at the pointed (free) end of the thin actin filaments in sarcomeres, whereas E-Tmod is recruited to costameric subsarcolemmal domains of the same cells (1). In human erythrocytes, E-Tmod is located at the junctional complex, which is the center of hexagonal lattices in the actin-spectrin-based membrane skeletal network (56).

E-Tmod binds to one end of the rodlke erythrocyte TM (14), specifically, to the NH2-terminal end of TM5 between residues 7 and 14 (52, 57). The complex formed by E-Tmod and TM5/5b functions as a molecular ruler for actin protofilaments (51). Protofilaments, uniformly 37 nm long (44), in turn define the hexagonal geometry of spectrin lattices in the membrane skeletal network, which supports the mechanical stability of the lipid bilayer and provides the elastic deformability of erythrocytes (51).

Several experiments have demonstrated a role for tropomodulin in striated muscle. In isolated skeletal myofibrils, antibodies against E-Tmod were localized to the free end of the thin (actin) filaments in sarcomeres (15). In cultured embryonic chick cardiac myocytes, microinjected antibodies against E-Tmod led to marked elongation of actin filaments and reduction in the number of beating cells (18). Long actin filament bundles also formed when antisense E-Tmod mRNA was induced in fetal cardiomyocytes in culture (53) and peripheral myofibrils were disordered and lacked Z-lines. Conversely, overexpressing E-Tmod in cultured cardiomyocytes resulted in shorter thin filaments (28, 53), whereas overexpression in the hearts of transgenic mice led to dilated cardiomyopathy (54).

To reveal the consequences in mammals of a knock-out of E-Tmod on embryonic viability and development, particularly in erythroid cells, which have never been reported, and the heart, we disrupted the E-Tmod gene in mouse ES cells and obtained mice that were null for the protein. We also knocked in a β-galactosidase reporter gene (LacZ) under the control of the endogenous E-Tmod promoter that allows visualization of those cells and tissues normally expressing E-Tmod, even in the absence of the protein. E-Tmod−/− embryos die around embryonic (E) day 10 (E10) due to abnormalities in cardiac contraction, vascular morphogenesis, and hematopoiesis. The lethality of the E-Tmod−/− mutation demonstrates the importance of the slow-growing end of actin filaments in cellular functions, and that the capping by E-Tmod during embryonic development is not replaced by other proteins, including members of the Tmod family.

**EXPERIMENTAL PROCEDURES**

Construction of E-Tmod targeting vector. Established gene targeting protocols were followed (20). A 1.7-kb 5′ genomic fragment and a 5-kb 3′ fragment of E-Tmod flanking exon 1 (8), isolated from a mouse 129/SvJ genomic library (Stratagene; La Jolla, CA), were linked to a LacZ-PGKneo cassette (6) at 5′ (Xhol site) and 3′ (BglII site) positions, respectively, to construct the targeting vector (Fig. 1A). A thymidine kinase (tk) gene was also linked to the 3′ end of the targeting cassette. In Fig. 1A, the tk gene in the targeting vector is in gray and downstream of the 3′ end of the homologous region in black. The purpose of the tk gene was to allow for negative selection to eliminate clones that had acquired neomycin resistance (NeoR) through nonhomologous recombination. However, because the positive selection by G418 had allowed us to obtain two E-Tmod−/− ES cell clones through homologous recombination, negative selection was never implemented.

Generation of E-Tmod knockout mice. The linearized targeting construct was electroporated into cultured ES cells
(R1) (30), and G418-resistant colonies were screened for homologous recombination by PCR and Southern blot analysis. The $Tmod^{+/−}$ ES clones expanded in culture were injected into normal blastocysts of C57/Black, 3 days after their fertilization, to create chimeric mice. The interbreeding of $Tmod^{+/−}$ mice was used to generate $Tmod^{−/−}$ mutants.

Genotyping of $Tmod$ knockout ES cells and mice. Genomic DNA was isolated from ES cells, yolk sacs, or tails from the embryo proper or mice (DNasey Tissue Kit, QiGen). Genotypes were examined first by PCR with a 5′ primer screen-P1 (5′-ATGCTCCGGTTGACTAAGTG-3′) and a 3′ primer mTmod140R (5′-CAGCCTCCTCTCTTGAGG-3′) for wild type or a 3′ primer Lac541R (5′-CAGGTCAAATTCA-GACGCCA-3′) for the disrupted gene. Samples containing disrupted genes were confirmed by Southern hybridization with both 5′ and 3′ probes marked in Fig. 1A.

Phenotype analyses. Whole mount LacZ staining, in situ hybridization, and immunohistological analysis were performed according to established methods (29, 42, 65), respectively.

Transmission electron microscopy. E9.5 wild-type and $Tmod^{−/−}$ embryos were immersed in a glutaraldehyde-osmium tetroxide mix containing 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 3 hours, and then postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 hour. The embryos were dehydrated through graded series of ethanol and embedded in araldite. Three thin sections of the araldite-embedded blocks were stained with KmnO4/Pb, as described by Nassar et al. (31).

Cytospin. A cytocentrifuge (Cytopsin 2, Thermo Shandon; Pittsburgh, PA) was used to spin (at 28 $g$) mouse primitive erythroid cells collected from yolk sacs.

Micropipette aspiration technique. The micropipette aspiration technique has been reported and used extensively to characterize mechanical properties of individual human erythrocytes, white blood cells, and nuclei (5, 11, 49). Deformation tests for primitive reticulocytes and erythroblasts were performed with modifications for their larger sizes and/or the presence of the cytoskeleton.

RESULTS

Generation of $Tmod$ knockout mice. A targeting vector was constructed by replacing exon 1 of mouse $E-Tmod$, which contains the translation initiation codon (8), with bacterial LacZ and NeoR genes (Fig. 1A).

Targeted disruption of $E-Tmod$ was accomplished by homologous recombination in mouse ES cells (20). After electroporation and G418 selection, 288 surviving ES cell colonies were genotyped by PCR screening, followed by Southern blot analysis (Fig. 1B). In PCR, the wild-type gene generated a 1.7-kb fragment, whereas the disrupted gene generated a 2.2-kb fragment. In Southern blot analysis, the 5′ probe (position shown in Fig. 1A) hybridized to a 4.7-kb fragment in the wild-type gene and a 7.2-kb fragment in the disrupted gene (Fig. 1B).

Two independent $E-Tmod^{+/−}$ ES cell clones, A20 and B9, were established. Microinjecting $E-Tmod^{+/−}$ ES cells into normal blastocysts created seven male chimeric mice. Two (both derived from A20) had a germ line transmission of the disrupted $Tmod$ gene and gave rise to several $E-Tmod^{+/−}$ mice, which survived to adulthood and were fertile.

Earlier in culture, G418 was increased from 0.25 to 4 and 6 mg/ml to establish two $E-Tmod^{−/−}$ ES cell lines. Although $E-Tmod^{−/−}$ ES cells were not chosen in creating chimeric mice because of the concern that they may not contribute to the germ line, their establishment proved the viability of $E-Tmod^{−/−}$ ES cells.

$E-Tmod$ null mutation is embryonically lethal. Screening of 177 offspring produced by intercrossing $E-Tmod^{−/−}$ mice revealed no $E-Tmod^{−/−}$ littermates, suggesting that an $E-Tmod$ null mutation is embryonically lethal. Because $E-Tmod^{−/−}$ ES cells are viable, we investigated the timing of lethality by genotyping embryos from E8.5 to E13. Whereas wild-type, $E-Tmod^{+/−}$, and $E-Tmod^{−/−}$ embryos exhibited ~1:2:1 ratios, respectively, in the early stages, no $E-Tmod^{−/−}$ embryos were found alive after E10.5, indicating that they had died ~E10.

Complete block of $E-Tmod$ protein synthesis in $E-Tmod^{−/−}$ embryos. Western blot analysis with the use of E-Tmod monoclonal antibody 204 (Fig. 1C) revealed that, whereas $E-Tmod^{+/−}$ embryos had about one-half of E-Tmod protein present in the wild type, $E-Tmod^{−/−}$ embryos had no detectable E-Tmod protein. Note that only exon 1 and small portion (0.8 kb) of intron 1 were replaced and the resulted sequence was confirmed by sequencing the PCR fragment derived from this mutated region. Because no upstream promoter sequence is affected, cells are able to transcribe the 5′ untranslated region of $E-Tmod$ (8) and the LacZ sequence, which has a stop codon. Furthermore, the downstream $E-Tmod$ sequence is out of the correct reading frame after the insertional mutation.

However, in heterozygous adults, the amount of E-Tmod incorporated into the erythrocyte membrane skeleton was about the same as that in the wild type (data not shown). The relative molecular weight (~43,000) of E-Tmod expressed in E9.5 embryos was similar to that in ghost membranes purified from wild-type adult mouse erythrocytes.

$E-Tmod$ expression reported by knocked-in bacterial LacZ gene. β-Galactosidase, the gene product of LacZ, generates blue signals by hydrolyzing 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-Gal). Therefore, the knocked-in LacZ under the control of the endogenous $E-Tmod$ promoter (Fig. 1A) signals the pattern of expression of $E-Tmod$ protein in heterozygotes as well as the promoter activity of $E-Tmod$ in $E-Tmod^{−/−}$ cells, even in the absence of the expression of $E-Tmod$ protein.

In $E-Tmod^{−/−}$ embryos (Fig. 1D), the whole mount X-Gal staining (29) revealed no detectable LacZ expression at E7.5. A high-level expression of LacZ was first detected at E8.0 in blood islands or primary capillary plexuses in the yolk sac and the developing heart tube in the embryo proper. The expression of LacZ revealed the detailed organization of primary capillary plexuses and fused linear heart tubes, from the developing atri (two arches at the bottom of the heart tube) to fused ventricles. The normal rightward looping of the heart tube was obvious in the $E-Tmod^{−/−}$ embryo at E8.5. Later, at E9.5, the heart of heterozygotes...
continued to express lacZ, whereas no detectable signals were found in developing somites (20–29 somite pairs stage). At E9.5 in heterozygotes, circulating LacZ-expressing blood cells also marked dorsal intersegmental (or intersomite) arteries, anterior cardinal veins, and other blood vessels in the embryo proper. In E-Tmod\(^{-/-}\) embryos, even in the absence of E-Tmod protein, the expressed LacZ reporter gene under the control of the E-Tmod promoter also highlighted the “null” tissues that normally express E-Tmod. The blue staining facilitated the analyses of disease phenotypes in cardiomorphogenesis, vascular morphogenesis, and hematopoiesis in E-Tmod\(^{-/-}\) embryos.

Disease phenotype in heart. We examined \(>50\) E-Tmod\(^{-/-}\) embryos for the null phenotype presented. The E-Tmod\(^{-/-}\) embryo was grossly normal at E8.5. Thereafter, significant growth retardation was observed. Figure 2a shows left views of a wild-type embryo and an E-Tmod\(^{-/-}\) embryo at E9.5. The abnormal heart with a massive pericardial effusion in the E-Tmod\(^{-/-}\) embryo is apparent and consistent with embryonic heart failure. The decreased growth of the facial and brain primordial is also noticeable, which may be caused by the complete deficiency of E-Tmod or the secondary effect resulting from the lack of circulation into those regions.

Contractility of E-Tmod\(^{-/-}\) heart tube. When E9.5 embryos were removed from the uterus, the developing hearts of wild-type embryos had peristaltic contractions (Fig. 2a, top). In contrast, those of E-Tmod\(^{-/-}\) embryos had barely noticeable tremors, initiated from the region corresponding to atria (Fig. 2a, bottom).

Longitudinal sections along the bilateral midline of LacZ-stained embryos (Fig. 2b, same orientation as in Fig. 2a) show that the E-Tmod\(^{-/-}\) heart tube at E9.5 looped normally, with atria, ventricular chambers, and an outflow tract in place. But E9.5 E-Tmod\(^{-/-}\) mutant had a single, fused heart chamber with overdeveloped trabeculae. A higher magnification reveals individual cardiomyocytes expressing LacZ (Fig. 2c). The trabecular myocytes were increased and more discontinuous in the Tmod\(^{-/-}\) mutants. It would be of interest to investigate why this is the case.

Looping of E-Tmod\(^{-/-}\) heart tube. The E-Tmod\(^{-/-}\) heart tube was distorted after initial looping at E8.5. Sequential front views of X-Gal-stained heart tubes of E-Tmod\(^{+/+}\) (Fig. 2d) and E-Tmod\(^{-/-}\) (Fig. 2e) embryos were compared. Between E9.0 and E10.5, E-Tmod\(^{-/-}\) embryos showed abnormal looping. Without the outgrowth of the right ventricle, the single ventricle was abruptly connected to the outflow tract.

Expression of cardiac-specific markers. To assess the degree of development and specification of the E-Tmod\(^{-/-}\) heart, four cardiac-specific markers, myosin light chain (MLC2a, MLC2v, dHAND, and eHAND), were used in whole mount in situ hybridization. E-Tmod\(^{-/-}\) embryos at E9.0 (Fig. 2, f–i, bottom), which began to exhibit retarded growth, were compared with wild-type embryos (Fig. 2, f–i, top). The images have the same right side views except for Fig. 2i, which has a left-front side view for left ventricles.

Fig. 2. The E-Tmod\(^{-/-}\) disease phenotype in cardiac morphogenesis. In a–c, the hearts of E-Tmod\(^{+/+}\) or E-Tmod\(^{-/-}\) (top) and E-Tmod\(^{-/-}\) (bottom) were compared. E9.5 embryos (a, see http://ajpheart.physiology.org/cgi/content/full/284/5/H1830/DC1 for a video showing peristaltic contractions of a normal heart tube and tremors of an E-Tmod\(^{-/-}\) heart tube), the longitudinal midline sections of whole mount LacZ-stained tissue showing the developing heart (b), and cardiomyocytes in the ventricular wall (c) are compared. Initial looping of the heart tube and later development between E8.5 and E10.5 in E-Tmod\(^{-/-}\) (d) and E-Tmod\(^{-/-}\) (e) are compared (front view). In f–i, whole mount in situ hybridization on E9.0 embryos using antisense cRNA probes was presented in E-Tmod\(^{+/+}\) (top) and \(^{-/-}\) (bottom): myosin light chain (MLC2a (f), MLC2v (g), dHAND (h), and eHAND (i)). All images are right views except for eHAND, which is a left front view. a, Atrium; h, heart; v, ventricle; lv, left ventricle; rv, right ventricle; ot, outflow tract; pe, pericardial effusion; t, trabeculae. Bars = 0.5 mm (a, d, and e, and f–i), 0.1 mm (b), and 0.01 mm (c).

Normally, MLC2a (Fig. 2f, top) is expressed in both atria and ventricles, and MLC2v (Fig. 2g, top) is expressed only in ventricles (24). The restricted expression of MLC2v relative to that of MLC2a indicates the atrioventricular specification in the developing heart tube. In the E-Tmod\(^{-/-}\) embryo, MLC2a was expressed...
in the entire heart (Fig. 2f, bottom), with a near-normal expression level, although the entire heart was slightly smaller. MLC2v (Fig. 2g, bottom) showed a restricted expression, indicating that atrioventricular chambers were specified in the E-Tmod−/− heart tube. But the area of MLC2v expression was much smaller compared with the wild-type heart tube (Fig. 2g, top), suggesting that perhaps only one ventricular chamber was developed.

To further assess the development of right and left ventricular chambers in the E-Tmod−/− heart, the expression of two transcription factor genes was analyzed. Normally the expression of dHAND (46) and eHAND (39) is predominantly restricted to the region of the looping heart tube that gives rise to the right and left ventricle, respectively. The lack of dHAND expression (Fig. 2h) is concomitant with the single ventricular chamber expression pattern of MLC2v (Fig. 2g) and is consistent with the lack of right ventricle in E-Tmod−/− mutants. Although the expression of eHAND was detectable, it appeared downregulated in the E-Tmod−/− heart tube (Fig. 2i), suggesting that even the left ventricle was not fully specified.

Transmission electron microscopy analysis of E-Tmod−/− cardiomyocytes. To assess whether the abnormal looping and the lack of contraction of the E-Tmod−/− heart reflected abnormal organization of actin filaments in cardiomyocytes in the absence of E-Tmod, we examined thin sections (30–40 nm) of wild-type and E-Tmod−/− embryonic hearts by transmission electron microscopy. At E9.5, sarcomeric myofibrils had been organized in the wild type (Fig. 3A) with normal M lines and Z lines. Most of the actin filaments were already interdigitated with thick filaments and incorporated into regular sarcomeres. Filaments had normal and uniform lengths and attached to the membrane at nascent intercalated disk junctions (Fig. 3E).

In E-Tmod−/− cardiomyocytes, in the most ordered areas (Fig. 3B), filaments were gathered into myofibril bundles with Z lines being replaced by elongated, stress fiberlike dense bodies (Zdb). In many areas, numerous thin and thick filaments formed very disordered arrays with amorphous dense Z bodies scattered around them; only a few thin and thick filaments were interdigitated (Fig. 3C). Sometimes the thin and thick filaments were segregated into separate bundles (Fig. 3B), which is rare in wild-type embryos. Intercalated disks and gap junctions were also developing in E-Tmod−/− cardiomyocytes. However, more dense material was associated with the nascent intercalated disks in the E-Tmod−/− cells compared with that of wild-type embryos (Fig. 3E).

Disease phenotype in vascular morphogenesis in yolk sacs. The primary capillary plexuses in yolk sacs were first revealed at E8.0 in E-Tmod+/− embryos (Fig. 1D) by LacZ-expressing erythroblasts (see below). Before then, at E7.5 when blood islands normally form, there was no detectable LacZ expression to reveal the structure of blood islands. At 8.5, E-Tmod+/− and E-Tmod−/− yolk sacs had similar organization of primary capillary plexuses (Fig. 4a). The heart tube was also visible, which should have initiated the rightward looping (Fig. 2, d and e), normally been beating (40), and connected to primary capillary plexuses as normal vitelline circulation starts at E8.5 (16, 21). At E9.0, the E-Tmod−/− yolk sac had noticeable remodeling of blood vessels (Fig. 4b). The process appeared to have direction because all developing vessels radiated from the largest vessel, and the diameter of each vessel tapered from large to small. At E9.5, mature vitelline vessels (Fig. 4c) had been formed.

In E-Tmod−/− mutants, the remodeling of the primary capillary plexuses into treelike mature blood vessels was arrested (Fig. 4, bottom). At E9.0, primary capillary plexuses grew to occupy a larger area but remained a honeycomb-like network of channels (Fig. 4b, bottom). These plexuses fused around E9.5, forming large blood pockets (Fig. 4c, bottom). Cross sections of
shows (from left) primitive lineages to the developing fetal liver (hematopoietic activity in mice shifts from the yolk sac outlined primary capillary plexuses and blood vessels). Sections of yolk sacs (d) revealed individual blood cells (stained blue) with their nuclei stained pink by X-Gal counterstain. Bars are 0.5 mm for a–c and 0.1 mm for d.

The X-Gal-stained yolk sac of E-Tmod−/− (Fig. 4d) revealed packed primitive erythroblasts expressing high levels of lacZ. Therefore, it is the blood cells that outlined primary capillary plexuses and blood vessels when stained by X-Gal.

**Disease phenotype in hematopoiesis.** Normally, the hematopoietic activity in mice shifts from the yolk sac (primitive lineages) to the developing fetal liver (definitive lineages) between E10 and E12, with the seeding of hematopoietic stem/precursor cells derived from the yolk sac and/or the pleuroplanchnic (P-Sp)/aortagonsads-mesonephros region (7, 34, 22). At E9.5, wild-type and E-Tmod−/− embryos had a large number of blood cells circulating in the cardiac chambers, dorsal aorta, and blood vessels in both the embryo proper and yolk sac (Fig. 5a–d, top, and Fig. 1D). The insets in Fig. 5b, top, also show some “blue” cells outside of chambers. The E-Tmod−/− embryo proper, however, was “anemic” (Fig. 5a and b, bottom). Often, the entire transverse sections of an X-Gal-stained embryo proper (Fig. 5b, bottom) revealed no visible blue blood cells. In one case, some blood cells were found inside the atria (Fig. 2b, bottom), and in another case, a small pink region was noticed near the heart chambers of an unstained embryo (Fig. 5a, bottom). The anemia suggests limited hematopoiesis in the E-Tmod−/− embryo proper, lack of vitelline circulation, and/or a defect in primitive blood cells in the yolk sac. The cross sections of the embryo proper also revealed that vascular chambers in the E-Tmod−/− embryo proper appeared to be lacking or minimized compared with the normal (Fig. 5b).

**Light microscopy of blood cells from yolk sacs.** Blood cells from E9.5 yolk sacs were examined under light microscopy and several abnormalities were found in E-Tmod−/− cells. Figure 5e, left, for wild-type cells shows (from left to right) one primitive reticulocyte or erythrocyte, one erythroid cell undergoing enucleation, and one erythroblast. Their large size (~29 μm in diameter) indicated the primitive origin, because a definitive erythrocyte in adult mice was only ~6 μm. Figure 5e, right, for E-Tmod−/− cells shows (from left to right) one of very few primitive reticulocytes or eryth-

![Figure 4](http://ajpheart.physiology.org/)  
*Fig. 4. The E-Tmod−/− disease phenotype in yolk sac vascular morphogenesis. E-Tmod+/− (top) and −/− (bottom) yolk sacs at E8.5 (a), E9.0 (b), and E9.5 (c) were stained for LacZ to reveal the remodeling of primary capillary plexuses. Sections of yolk sacs (d) revealed individual blood cells (stained blue) with their nuclei stained pink by eosin counterstain. Bars are 0.5 mm for a–c and 0.1 mm for d.

![Figure 5](http://ajpheart.physiology.org/)  
*Fig. 5. The E-Tmod−/− disease phenotype in hematopoiesis and mechanical strength of erythroid cells. The hematopoiesis in E-Tmod+/− (top) and −/− (bottom) embryo proper (a) and yolk sac (c), unstained, are compared. Transverse sections of whole mount LacZ-stained embryos (b) and yolk sacs (d) are also presented. Top inset in b shows some “blue” E-Tmod−/− cells outside the chambers; bottom inset in b shows no “blue” E-Tmod−/− cells outside the chambers. e: Individual primitive erythrocyte cells isolated from E9.5 yolk sacs. For wild type (left), a reticulocyte (~29 μm in diameter; far left), an erythroblast in the process of enucleation (middle left), and a nucleated erythroblast (near left) are shown. For E-Tmod−/− mutant (right), a partially hemolysed reticulocyte (near right), a binucleated erythroblast (middle right), and a nucleated erythroblast (far right) with membrane hernias are shown. f: E9.5 yolk sac blood cells after cytospin and Giemsa stain: E-Tmod+/− (left) and E-Tmod−/− (right). g and h: Mechanical properties of primitive erythroid cells tested by the micropipette aspiration technique. Deformation test on enucleated erythrocytes (g) and fragmentation test on nucleated erythroblasts (h) from E9.5 E-Tmod+/− (top) and −/− (bottom) yolk sacs were performed. Arrows in g indicate the length of cell membrane deformed into the pipette (pipette radius, Re = 0.75 μm; aspiration pressure, ΔP = 30,000 dyn/cm2; duration, t = 1,300 ms). In the fragmentation test (h, Re = 0.75 μm, aspiration force = 22.5 dyn/cm), the cell membrane showed progressive deformation with a nodule (arrow 1), a “neck” (narrowing membrane segment, arrow 2), a cell body (arrow 3), and a partially deformed nucleus membrane (arrow 4, not seen in wild type). Bars = 0.5 mm for a–c and 20 μm for d–f.*
erythrocytes, one binucleated primitive erythroblast, and one nucleated erythroblast that had a membrane hernia. Often, E-Tmod−/− erythroid cells were partially hemolyzed. Binucleated erythroblasts were also found in cross sections of yolk sacs (Fig. 5d, bottom) and on cytospin slides (see below; Fig. 5f, right). In fact, ~2% of the primitive blood cells examined were binucleated (5 of 246), whereas none was observed in the similar cell count of the wild type. This finding suggests that cytokinesis in E-Tmod null mutants may either be slowed or sometimes incomplete.

Cytospin of yolk sac blood cells. Blood cells collected from yolk sacs were further examined by clinical cytocentrifuge, followed by Giemsa staining. E-Tmod−/− blood cells (Fig. 5f, left) were flattened on the slides but remained intact. At E9.5, most of the cells were differentiating erythroblasts, as reported by Palis and Yoder (35). One was seen in the process of enucleating, ~3 days earlier than E13, as reported by Bethenfalvay and Block (4). In contrast, no E-Tmod−/− cell membranes or cytoplasm remained associated with the nuclei of erythroblasts. The cytospin (Fig. 5f, right) captured several erythroblast nuclei, a binuclear remnant, and a broken membrane away from the cell body. All of these findings suggest that mechanical strength of E-Tmod−/− erythroid cells is weakened.

Mechanical strength of blood cells tested by micropipette aspiration. To test the mechanical strength of E-Tmod−/− erythrocytes, membrane deformation and fragmentation analyses were performed with the micropipette aspiration technique (5, 49). Primitive erythrocytes retain nuclei during most of their lifespan and at E9.5; only very few enucleated cells were found from the yolk sac. The membrane deformation under a microscope in response to a step-negative pressure. This finding suggests an overextension of the membrane nodule had been aspirated into the pipette, and the work for fragmentation was 11.25 dyn·s·cm−1. At this level of work, 64% of E-Tmod−/− erythroblasts tested had membrane fragmentation. Therefore, both deformation and fragmentation analyses indicated that E-Tmod−/− erythroid cell membranes had less mechanical strength than those of the wild type. The erythroid cells without E-Tmod are easier to overextend (by ~2x) and to form nodules (by ~4x), leading to partial hemolysis and fragmentation in response to mechanical stress.

DISCUSSION

The E-Tmod knockout and LacZ knockin strategy provides the expression pattern of E-Tmod with single cell resolution and disease phenotypes in null mutants without the concerns of cross hybridization and cross reactivity. The cell-specific expression pattern of E-Tmod reported by LacZ agreed with the tissue-specific expression patterns detected by in situ hybridization (19), supporting the assumption that the knocked-in LacZ expression pattern in E-Tmod−/− embryos reflected the endogenous E-Tmod expression pattern. The only exception was somites at E9.5, where the expression of the knocked-in LacZ was not detectable, but a very weak signal was detected by in situ hybridization. Three possibilities for the discrepancy are the following: 1) the disruption of a portion of the endogenous promoter for E-Tmod required for the expression in somites or skeletal muscle; 2) the different sensitivities between these two techniques; or 3) the cross hybridization of the E-Tmod cDNA probe to transcripts of E-Tmod homologs. It is known that Sk-Tmod is expressed in certain types of skeletal muscles and U-Tmod is expressed ubiquitously, at least in adult tissues. To minimize the disruption of endogenous promoters, no sequence upstream from exon 1 was disrupted in our construct. Introns 0, which is upstream from exon 1, and exon 0, which is further upstream and potentially contains the transcription initiation site (8), were all intact, with their sequences confirmed. Only exon 1, which does not contain the transcription initiation site but does contain the translation initiation codon ATG, and a small portion of the downstream intron 1 (0.8 kb), were replaced by LacZ. Therefore, unless there were promoter elements specific for somites that are located within exon 1 or the small region of intron 1, the LacZ expression pattern may represent that of the endogenous E-Tmod. We are in the process of examining the spatial-temporal expression pattern of LacZ later than E9.5, to determine the exact timing of LacZ expression in somites (and other tissues) because a strong LacZ expression has been detected in skeletal muscles in the adult (data not shown).

Whether cells from E-Tmod−/− heterozygotes are totally equal to those of true wild types raises an interesting question, and we are systematically exami...
ining tissues/cells in adult E-Tmod<sup>−/−</sup> mice and comparing them with wild-type mice. Our preliminary results on the definitive erythrocyte deformation by micropipette aspiration suggest no significant difference between E-Tmod<sup>−/−</sup> heterozygotes and wild-type cells (C. Vera, K.-L. P. Sung, and L. A. Sung, unpublished results).

In the complete absence of E-Tmod capping of the slow-growing end of actin filaments, the processes of cardiac and vascular morphogenesis and hematopoiesis are arrested. These disease phenotypes in developing embryos may be better understood by discussing the role of E-Tmod in the erythrocyte membrane skeleton, where the molecular organization and mechanical properties have been characterized most extensively.

In definitive human erythrocytes, a complex formed by globular E-Tmod and rodlike TM5/5b (33–35 nm long) caps and protects a short protofilament of 37 nm (51, 57). Each of these short protofilaments is knitted by six spectrins to form a thin, continuous, elastic protein network that supports the lipid bilayer during deformation. Although the detailed organization of the membrane skeleton may not be identical in primitive and definitive erythroid cells, or before and after enucleation, the role for E-Tmod may be similar. Without E-Tmod to cap short protofilaments, the network may become thicker (some actin filaments may be longer than 37 nm), patchy (some longer actin filaments may be bundled by >6 spectrins), and discontinuous (some other actin filaments may not be connected to neighboring actin filaments by spectrins). The remnants of E-Tmod<sup>−/−</sup> membrane skeletons collected by cytopsin fit the predicted discontinuity of the network. Such disorganization results in a weaker tensile strength, allowing the membrane skeletal network to overextend (hemolysis) and break (fragmentation) in response to mechanical stress.

Primitive erythroblasts and cardiomyocytes are the two cell types that show upregulation of E-Tmod at E8.0, when they begin passive and active deformation, respectively. This finding agrees with the previous report by in situ hybridization, which located the E-Tmod gene transcript in blood islands and myocardium (19). The heart tube normally starts peristaltic contraction at E8.0 (40) and the onset of circulation is at E8.5 (16, 21). The upregulation of transcription probably coincides with a functional requirement for E-Tmod and may be mechanically regulated. Examples of genes whose promoter elements bind to transcription factors in response to mechanical signals, such as shear stress, have been reported (45, 67). Increasing E-Tmod protein concentration may ensure sufficient number of short protofilaments to build a membrane skeletal network with suitable mechanical strength in erythroblasts, enabling them to survive the deformation induced by cardiac contractions. In cardiomyocytes, E-Tmod may serve a dual role, capping and stabilizing both sarcomeric actin filaments and those associated with the membrane cytoskeleton, thereby conferring mechanical strength during contraction cycles.

The phenotype of blood cells and cardiomyocytes derived from E-Tmod null ES cells differentiated in vitro is one of the interesting projects we have proposed to do. ES cell-derived blood cells and cardiomyocytes may be characterized and compared with those derived in vivo. Along this line, we have been able to culture contractile cardiomyocytes from normal and E-Tmod<sup>−/−</sup> E9.5 embryos, but not from E-Tmod null embryos (I. Lian, X. Chu, and L. A. Sung, unpublished results).

The E-Tmod<sup>−/−</sup> heart tube was not contractile at E9.5, which is consistent with the lack of organized sarcomeres in cardiomyocytes. It was less obvious why the myofilaments were in such disarray in the absence of E-Tmod. In cultured chick cardiomyocytes, E-Tmod was reported to function late in myofibrillogenesis, maintaining the final length of thin filaments after they have been assembled (18). Therefore, if thin filaments in E-Tmod<sup>−/−</sup> cardiac myocytes were organized in sarcomeres, but longer than normal in the absence of E-Tmod, it would be consistent with a late function in sarcomere assembly. In contrast, in cultured chick skeletal myocytes, E-Tmod was reported to function early, appearing at the earliest nonstriated myofibrils and capping actin filaments even before the filaments are cross-linked into Z-bodies by α-actinin (1). Z-bodies and actin filament bundles near the sarcolemma have been proposed to serve as scaffolds for the development of mature sarcomeres (32, 43). If E-Tmod functions early in mouse cardiac myofibrillogenesis, lack of E-Tmod could interfere with the assembly of sarcomeres: actin filament length cannot be regulated and Z-bands are not assembled. The scattered Z bodies and disordered myofilaments we observe in E-Tmod null hearts may be the remnants of this disrupted process. Consistent with this idea is the “abnormal” coincidence of E-Tmod and α-actinin at Z-disks observed in cardiomyocytes of E-Tmod-overexpressing transgenic mice (54). Furthermore, in chicken skeletal muscles, E-Tmod and spectrin have been colocalized to costameres overlying the Z-band with flanking I bands, or I-Z-I bands (1). Costameres are riblike cytoskeletal structures attached to the sarcolemma that broaden and narrow in concert with the underlying I bands in contractile cycles (36). If E-Tmod stabilizes the actin-spectrin based membrane skeleton in costameres, it may allow the sarcolemma to sustain deformation. Without E-Tmod, this membrane skeleton may be mechanically weakened and cardiomyocytes may be damaged during contractions.

E-Tmod is not required for the initial rightward looping of the heart tube, but is needed for the outgrowth of right ventricle. This may be due to a potential role for E-Tmod in cell fate decisions or the consequence of heart failure. The former is suggested by the finding that sanpodo (a homolog of tropomodulin) is a lineage gene in Drosophila (37) and asymmetric cell divisions may play a major role in cardiac and somatic muscle patterning. The latter is suggested by the finding that the “diminished right ventricle” is shared by several null mutants of...
cardiac-specific transcription factors, such as MEF2C (27), dHAND (46, 66), and Csx/Nkx2.5 (55), and the Ncx1-null mouse models. Ncx1 encodes a sodium-calcium exchanger; the null mice lacked spontaneous heartbeats but showed normal cardiac morphogenesis (up to E11) except that the future right ventricle region was severely underdeveloped (23).

Several of these mutants (e.g., of dHAND, Csx/Nkx2.5, and Ncx1) also exhibited arrests in vascular morphogenesis. Vasculogenesis is the process by which primary capillary plexuses form channels of similar diameters in situ. Angiogenesis that follows is thought to involve multiple phases, including new channel formation (by sprouting and nonsprouting), segregation of arteries and veins, establishment of endothelial cell-cell junctions, and regression of vascular channels (see Ref. 41 for a review). The arrest of vascular remodeling after vasculogenesis concomitant with cardiac failure due to completely unrelated defects (transcription factors, Na/Ca exchanger, and structural protein) suggests that cardiac contraction-derived pressure gradient and blood flow may be critical. Previously, without real-time scale and single cell resolution, it has been difficult to document how the primary capillary plexuses remodel (41). Here, the LacZ reporting allows documentation of remodeling at the single cell level between E7.5 and E10, although not on a real-time scale.

We propose a plexus channel selection mechanism based on our observation that the zigzag contours of forming blood vessels (Figs. 4b and 6a) resemble the geometry of and are continuous with the channels of primary capillary plexuses (Fig. 4a). We define a “capillary channel” at this stage as the space occupied by blood cells and not that defined by an endothelium. Our hypothesis suggests that vitelline blood vessels form by selecting some preexisting channels and abandoning some others within the orthogonal network of channels, depending on their alignment with the pressure gradient and flow established by the cardiac output (Fig. 6b). With time, the blood flow smooths out sharp angles and the blood pressure increases diameters of blood vessels (Fig. 6, c and d), after endothelial junctions seal the vessels.

How are some capillary channels selected and others abandoned? Because primary capillary plexuses formed in situ are remodeled into endothelial cell-lined vessels with blood cells flowing inside, stationary blood cells grown in situ must become mobile. It is reasonable to assume that at this point, differentiated erythroblasts (stained blue in Fig. 6a, also see Fig. 5) have lost adhesive contacts with each other, whereas other cells in “white islands” (mesoderm/mesenchymal cells not expressing LacZ) still adhere to each other. Initially, blood cells in capillary channels near the outflow tube and those aligned approximately with the pressure gradient generated by cardiac output (marked by long arrows) would be pushed downstream by the positive pressure. These capillary channels would stay open and develop into mature blood vessels, thus being selected. Others that connect selected channels are often approximately perpendicular to the pressure gradient and may only experience a relatively small pressure gradient between two ends. Blood cells in these channels (flanked by short arrows in Fig. 6a) may move slowly toward the two selected channels at the two opposite ends. As the space left behind is taken up or overgrown by adjacent nonblood cells, these capillary channels would disappear and thus be abandoned (Fig. 6). At the other (future venous) end, the blood flow in capillary channels toward contractile atria keeps them open, and they subsequently develop into veins. E-Tmod<sup>−/−</sup> embryos without effective peristaltic contraction of the heart may, therefore, be unable to select and abandon channels, thus remodeling primary capillary plexuses (a network of channels) into a mature ch
vasculature (a treelike organization of channels) that consists of arteries, capillaries, and veins.

Figure 1D shows no X-Gal staining between the heart and yolk sac in normal E8.0 embryos, suggesting little, if any, blood cells are present in this region at this stage. At E8.5, the X-Gal staining has become continuous between the heart and the capillary plexuses (at least grossly) in both E-Tmod+/− heterozygotes and null mutants (Fig. 4a), suggesting this region is “patent” even in the null mutant. It is after E8.5 that the process of conversion of a primary capillary plexus into blood vessels in the E-Tmod null mutant is disrupted.

It is not clear from this study whether there are physical structures or endothelial cells in the capillary channels separating blood cells and surrounding mesodermal cells, like in the mature blood vessels. Our finding that flattened cells along the internal walls of mature vessels (data not shown) did not express detectable E-Tmod by X-Gal and that several other mutations have the same phenotype (i.e., fail to remodel), even though they do not affect vessel cells, further support the notion that pressure gradient-derived blood flow provides the mechanism to select capillary channels from a network and convert them into mature blood vessels.

Although we hypothesized that the arrest of vascular morphogenesis is mainly due to the lack of pressure gradient across the field of primary capillary plexuses because of the failure of cardiac contraction, we have not excluded the possibility that the complete deficiency of E-Tmod in endothelial cells and/or smooth muscle cells may also contribute to the arrest. Immunochemistry using an endothelial cell antibody (e.g., that against platelet endothelial cell adhesion molecule) and anti-smooth muscle actin antibody on embryos between E7.5 and E10 would provide useful information. Our preliminary analysis on E9.5 embryos showed that the signals for platelet endothelial cell adhesion molecule in the wild type corresponded to the walls of the developing vasculature, but no signals corresponding to the walls of the primary capillary plexuses were found in the E-Tmod−/− yolk sac. The signals for smooth muscle cell actin, however, were similar between wild-type and null mutants (X. Chu and L. A. Sung, unpublished data). We have not looked at the placenta to see whether the vasculature of the null mutants is normal.

In conclusion, E-Tmod null mice whose actin filaments are not capped by E-Tmod at the slow-growing end develop disease phenotypes in each of the three key components of the circulatory system: blood cells, the heart, and blood vessels. The end result is lack of circulation. Lacking circulation at this developing stage, embryos would not survive. Lacking circulation, even though they do not affect vessel cells, further suggest the notion that pressure gradient-derived blood flow provides the mechanism to select capillary channels from a network and convert them into mature blood vessels.

E-Tmod−/− cells at E9.0 are capable of reconstituting the hematopoietic system in newborn mice (68), is of great interest. If E-Tmod−/− yolk sac/P-Sp stem cells do reconstitute the hematopoietic system, there would be a large number of definitive E-Tmod−/− erythrocytes circulating in wild-type mice. This would allow their membrane skeletal network to be examined by electron microscopy and mechanical properties characterized by micropipette aspiration. The role of E-Tmod in cell membrane mechanics with the use of the simplest cell model would then be established.

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REFERENCES


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55. Tanaka M, Chen Z, Bartunkova S, Yamashita N, and Izumo S. The cardiac homeobox gene Csx/Nkx2.5 lies genetically up-


