Formation of the Venous Pole of the Heart From an Nkx2–5–Negative Precursor Population Requires Tbx18

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Abstract—The venous pole of the mammalian heart is a structurally and electrically complex region, yet the lineage and molecular mechanisms underlying its formation have remained largely unexplored. In contrast to classical studies that attribute the origin of the myocardial sinus horns to the embryonic venous pole, we find that the sinus horns form only after heart looping by differentiation of mesenchymal cells of the septum transversum region into myocardium. The myocardial sinus horns and their mesenchymal precursor cells never express Nkx2–5, a transcription factor critical for heart development. In addition, lineage studies show that the sinus horns do not derive from cells previously positive for Nkx2–5. In contrast, the sinus horns express the T-box transcription factor gene Tbx18. Mice deficient for Tbx18 fail to form sinus horns from the pericardial mesenchyme and have defective caval veins, whereas the pulmonary vein and atrial structures are unaffected. Our studies define a novel heart precursor population that contributes exclusively to the myocardium surrounding the sinus horns or systemic venous tributaries of the developing heart, which are a source of congenital malformation and cardiac arrhythmias. (Circ Res. 2006;98:1555-1563.)

Key Words: sinus horns ■ congenital heart defect ■ Nkx2–5 ■ Tbx18 ■ morphogenesis ■ recruitment

The systemic venous return of the heart consists of multiple anatomical components including the proximal myocardial part of the right superior and inferior caval veins, the coronary sinus (the persisting left caval vein in the mouse), and the sinus venarum. These structures are thought to be the mature counterparts of the right and left sinus horns in the embryo, which are the myocardial parts of the common cardinal veins upstream of the venous valves that bulge into the pericardial cavity. These, in turn, are presumed to derive from the embryonic venous pole or inflow tract of the forming heart and the common cardinal veins.1,2 Developmental disorders of the heart, which include malformations of the pulmonary and systemic venous returns,3,4 represent the most common human birth defects.5,6 In addition, several specific components of the venous returns are found to be the origin of arrhythmias.7–9 Recent 3D reconstruction and genetic analyses have greatly improved our insight into the morphogenesis of the systemic and pulmonary venous returns.2,3,10 Nevertheless, the cellular origin of the components of the systemic and pulmonary venous return and the genetic mechanisms underlying their formation are not known.

The embryonic heart of amniotes initially represents a tube consisting of precursor cells for most of the left ventricle and a small portion of the atria. Outflow tract, right ventricle, and large portions of the atria are only subsequently recruited from a second lineage of mesenchymal cells.11–13 Nkx2–5, which encodes a homeobox transcription factor, is expressed in both the first and second lineages of the heart and plays pivotal roles in early and late steps of cardiogenesis.13,14 Using genetic lineage analysis, we show that the myocardial sinus horns and their precursor cells do not express Nkx2–5. The sinus horns form only after heart looping in the presence of Tbx18. We demonstrate that Tbx18 is essential for the formation of the sinus horns from the mesenchyme of the pericardial wall and for their myocardial differentiation but not for formation of the atria or pulmonary veins.

Materials and Methods

Mice

The Nppa-Cre,15 Nkx2–5-IRES-Cre,16 R26R,17 Nkx2–5GFP,18 and Tbx18lacZ/lacZ transgenic mouse lines have been described previously. Construction of the GFP-null allele of Tbx18 (Tbx18GFP) will be described elsewhere. Tbx18GFP/GFP and Tbx18GFP/gfp mice are phenotypically indistinguishable. For timed pregnancies, vaginal plugs were checked in the morning after mating, noon was taken as embryonic day (E) 0.5. Embryos and fetuses were dissected in PBS and fixed in 4% paraformaldehyde overnight. Genomic DNA prepared from amnion or tail biopsies was used for genotyping by
β-Galactosidase Activity Detection and Immunohistochemical Analyses

Detection of β-galactosidase activity on 20-μm cryostat sections was performed as described. For immunohistochemistry on 5- to 10-μm embryo sections, the following primary antibodies were used: monoclonal antibody against proliferating cell nuclear antigen (PCNA) (1:2000) (Santa Cruz Biotechnology); monoclonal antibody against Desmin (1:50; Monosan Clone D33); rabbit polyclonal antibody against GFP (1:150; Santa Cruz Biotechnology). Proliferating cells were marked with an antibody against phosphorylated histone H3 (Ser10; 1:50; Cell Signaling). Adjacent sections were stained for α-tubulin to define the Tbx18-positive region. Mitotic indices were calculated as fraction of labeled cells to the total number of DAPI-positive nuclei within a GFP-positive region. Quantification of proliferation by detection of 5-bromodeoxyuridine (BrdU) incorporation was performed as described. TUNEL staining for apoptotic cells was performed on 10-μm sections of embryos according to the protocol supplied with the in situ cell death detection kit POD (Roche).

Nonradioactive In Situ Hybridization and Three-Dimensional Reconstruction

Nonradioactive in situ hybridization analysis of 10- to 12-μm embryo sections was performed as described. The following probes were kindly provided: Isl1 isoform 1 (Isl11) and Hcn4 by S. Evans (Skaggs School of Pharmacy, University of California, San Diego) and B. Santoro (Center for Neurobiology and Behavior, Columbia University, New York). Probes for chicken Tbx18 and Isl1 were derived from ChEST 519c15 and 861p11 (BBSRC ChickEst databank, MRC geneservice). Three-dimensional visualization and geometry reconstruction of patterns of gene expression determined by in situ hybridization was performed as described previously. Files with reconstructions are available on request.

Results

Definitions

Systemic Venous Tributaries/Return

The whole of veins that return the systemic blood to the developing or mature heart.

Inflow Tract/Venous Pole

Generic terms for most upstream (caudal) component of the heart, regardless of developmental stage.

Venous Valves

Valvular folds guarding the sinoatrial orifice (entrance to the right atrium). They form after E9.5 from Nkx2–5' caudal inflow tract myocardium (this study).

Sinus Horns

The myocardial parts of the common cardinal veins upstream of the venous valves that bulge into the pericardial cavity. They form after mouse E9.5 and express Tbx18 (this study).

Expression and Three-Dimensional Reconstruction Analysis of Sinus Horn Development

In a previous study, 3D reconstruction analyses were performed to gain insight into the morphologically complex area of the developing atria and venous pole. To get a clearer picture of the formation of the sinus horns, we extended this analysis here. At E9.5, when the inflow tract was a bilaterally symmetrical structure, a very small Cx40- myocardial component formed (Figure 1). At subsequent stages, the walls of the left and right common cardinal vein bulged into the pericardial cavity, forming reflections at the pericardial lining. By E10.5, the Cx40- myocardial volume of the sinus horns gained 5-fold in size. The common entrance shifted to the right because of the stronger increase of Cx40- myocardium at the left compared with the right sinus horn. The sinus horns continued to expand until the intrapericardial component formed (Figure 1).10 The mesenchyme lining the pulmonary vein in the dorsal mesenchyme became visible from E11.5 onward. It was surrounded by Cx40+ mediastinal myocardium at the border of the Cx40- sinus horn component (Figure 1). The mesenchyme lining the pulmonary vein lumen would become Cx40+ myocardium from E12.5 to 13.5 (not shown).

At E9.5, all myocardium (cTnI+) expressed Nkx2–5 (Figure 2A). In addition, Nkx2–5- was expressed in the Isl1 isoform 1 (Isl1+) second field of cardiac precursors (Figure 2A and 2B). At this stage, Tbx18 was expressed in mesenchyme abutting the Nkx2–5- myocardium at the venous pole of the heart (Figure 2A, left panels). Thus, in the region of the developing sinus horns, 2 distinct cell populations were present, the Nkx2–5- caudal myocardium and the adjacent Tbx18+ mesenchyme. Surprisingly, the sinus horn myocardium forming from E9.5 on failed to express Nkx2–5 but was positive for Tbx18.

Figure 1. Dorsal views of 3D reconstructions of hearts of consecutive stages of development. The Cx40-positive atrial myocardium is shown in green, the Cx40-negative myocardium of the sinus horns in gray, the lumen of the caval veins in orange, the lumen of the forming pulmonary vein in red, and the atrioventricular cushion mesenchyme in yellow. The mesenchyme to which the sinus horns are attached has been removed to expose the embedded veins. The volume of the reconstructed Cx40-negative myocardium of the sinus horns is given underneath the stage. The size bar represents 300 μm. icv indicates inferior caval vein; lra, left/right atrium; lrcv, left/right cardinal vein; lrs, left/right sinus horn; mm, mediastinal myocardium.
The expression domains of \textit{Nkx2–5} and \textit{Tbx18} continued to be adjacent and mutually exclusive. At E9.5 and E10.5, the transcription factor gene \textit{Islet1} (\textit{Isl1}) was expressed in the dorsal mesenchyme that includes the second lineage but also in the myocardial component of the inflow tract and outflow tract (Figure 2B). However, the \textit{Isl1}/H11001 and \textit{Tbx18}/H11001 domains were largely exclusive, with a marginal overlap in the myocardial–mesenchymal border zone (Figure 2B). To assess whether the genetic mechanism of sinus horn formation was evolutionary conserved, the expression patterns of \textit{Tbx18}, \textit{Nkx2–5}, and \textit{Isl1} were analyzed in chicken hearts. The patterns of these genes were conserved between mouse and chicken (Figure 2C), indicating a lack of contribution of \textit{Tbx18}$^+$ cells to the atria or venous valves (E). Sinus horn derived caval veins express \textit{Tbx18}, pulmonary veins do not (F). \textit{a} indicates atrium; \textit{am}, atrial myocardium; \textit{Cra}, cranial; \textit{Cau}, caudal; \textit{D}, dorsal; \textit{dm}, dorsal mesocardium; \textit{ep}, epicardium; \textit{L}, left; \textit{Lscv}, left superior caval vein; \textit{oft}, outflow tract; \textit{pe}, proepicardium; \textit{pv}, pulmonary vein; \textit{R}, right; \textit{sh(m)}, sinus horn (myocardium); \textit{V}, ventral. For other abbreviations, see the legend of Figure 1.

(Figure 2A and 2D). The expression domains of \textit{Nkx2–5} and \textit{Tbx18} continued to be adjacent and mutually exclusive. At E9.5 and E10.5, the transcription factor gene \textit{Islet1} (\textit{Isl1}) was expressed in the dorsal mesenchyme that includes the second lineage but also in the myocardial component of the inflow tract and outflow tract (Figure 2B). However, the \textit{Isl1}$^+$ and \textit{Tbx18}$^+$ domains were largely exclusive, with a marginal overlap in the myocardial–mesenchymal border zone (Figure 2B). To assess whether the genetic mechanism of sinus horn formation was evolutionary conserved, the expression patterns of \textit{Tbx18}, \textit{Nkx2–5}, and \textit{Isl1} were analyzed in chicken hearts. The patterns of these genes were conserved between mouse and chicken (Figure 2C), indicating that the formation of the sinus horns may be governed by a general and conserved mechanism. Because only 2 sinus horn precursor populations are present in the inflow tract region, the \textit{Nkx2–5}$^+$ myocardial precursors, on the one hand and the \textit{Tbx18}$^+$ mesenchymal precursors on the other hand, formation of the sinus horns is likely to follow 1 of 2 possible mechanisms. The sinus horns may form from the inflow tract myocardium by proliferation, losing \textit{Nkx2–5} expression but gaining \textit{Tbx18} expression. Alternatively, the sinus horns may expand by recruitment and myocardial differentiation of the adjacent \textit{Tbx18}$^+$ mesenchyme, maintaining \textit{Tbx18}, and not initiating \textit{Nkx2–5} expression.

**Lineage Analysis of Sinus Horn Development**

We addressed the origin of the sinus horn cells by using the 2-component Cre-loxP system. The first component is a transgene expressing \textit{Cre} in a cell-type specific manner. The second component is a conditional \textit{lacZ} reporter, \textit{R26R}, which is irreversibly activated by \textit{Cre}.\textsuperscript{17} Progeny of labeled cells continue to express \textit{β}-galactosidase even after \textit{Cre} expression has been terminated, permitting the mapping of...
the fate of these cells. We first analyzed the contribution of atrial (Cx40⁺, Nppa⁺) myocardium to the Cx40⁻ Nppa⁻ sinus horns during development. The Nppa-Cre line drives Cre expression specifically in Cx40⁺ atrial myocardium from E9.5 onward, but not in the inflow tract myocardium, resulting in detectable β-galactosidase activity at E10.5 and robust labeling at E11.5 (Figure 3A). At E17.5, we observed almost complete recombination within the atria of Nppa-Cre/R26R double transgenic animals, whereas the myocardial cells of the caval veins bordering the atria remained almost free of recombined cells (Figure 3B). The few recombination events detected in the caval veins were probably attributable to sporadic activity of the Nppa promoter. In line with the expression analysis, this experiment shows that the sinus horns are not derived from atrial myocardium.

We next deployed the Nkx2–5-IRES-Cre knock-in line, which drives expression of Cre in a pattern identical to that of Nkx2–5/− (Figure 3C). Because all myocardial cells express Nkx2–5 at the start of sinus horn formation at E9.5 (Figure 2A), we could test whether the Nkx2–5⁻ sinus horn myocardium is derived from the Nkx2–5⁺ caudal myocardium. In Nkx2–5-IRES-Cre/R26R double knock-in embryos of E10.5, all myocardium except that of the sinus horns was labeled by β-galactosidase (Figure 3D and 3E). These results show that the sinus horns are not derived from Nkx2–5⁻ cells, suggesting that they are derived from the adjacent Tbx18⁻ posterior pericardial mesenchyme instead. This is supported by the pattern of β-galactosidase activity from the lacZ reporter gene in the Tbx18 locus that recapitulated Tbx18 expression in the myocardial sinus horns at E12.5 (Figure 2E). In contrast, atrial cells, venous valve cells, and atrial septal cells failed to express β-galactosidase, a lineage marker because of its longevity, indicating that they are not derived from Tbx18⁻ precursors.

The complementary expression of Nkx2–5 and Tbx18 may suggest mutual transcriptional repression. However, neither Tbx18 nor Nkx2–5 were derepressed in Nkx2–5 and Tbx18 mutants, respectively, indicating that upstream factors and/or patterning processes are responsible for the formation of the border between the sinus horns and atria (Figure 3G through 3N).

We analyzed the presence of PCNA, a marker for proliferating cells, in the sinus horns at a stage of recruitment,
E11.5, and after recruitment, E14.5 (Figure 3P through 3U). At E11.5, almost all atrial cells proliferated, but only a few of the cells of the myocardial sinus horns revealed PCNA expression. In contrast, a large fraction of the nonmyocardial cells of the mesenchyme bordering the sinus horns was PCNA-positive (Figure 3S). At E14.5, PCNA staining was detected in a large fraction of the sinus horn cells (Figure 3T and 3U).

Collectively, these results exclude that the sinus horns are formed by proliferation of existing Nkx2–5–/− caudal myocardium. They suggest rather that the sinus horns are formed by myocardial differentiation (recruitment) and subsequent proliferation of Tbx18+ mesenchyme (Figure 3O).

Systemic Venous Defects in Tbx18-Deficient Mice

The specific expression of Tbx18 in the systemic venous return suggested a functional involvement of the gene in the formation of this heart region. To investigate the role of Tbx18 in this process, we analyzed Tbx18-deficient mice for phenotypic alterations in this region. Heterozygous Tbx18+/− and Tbx18GFP/+ fetuses were unaffected. Hearts of Tbx18−/− embryos appeared grossly normal. Similarly, the morphology and gene expression profiles of the pulmonary veins appeared unchanged in Tbx18-deficient mice, consistent with the absence of Tbx18 expression in the mesenchyme and the myocardium of the developing pulmonary veins (Figure 2F). We detected GFP expression around the common cardinal veins both in Tbx18GFP/+ and Tbx18GFP/GFP embryos (the GFP signal being stronger in the Tbx18GFP/GFP mutants), indicating that Tbx18 is not required for the formation of the Tbx18+ precursor population (Figure 4A and 4B). At E9.5, the inflow tracts of mutant and control embryos appeared similar on sections, but at E10.5, all mutants had failed to form a left sinus horn in the pericardial cavity (Figure 4A and 4B and the Table). Instead, the left and right common cardinal veins remained embedded in the laterodorsal mesenchyme, and until E12.5, their walls did not express myocardial markers (Figure 4C and 4D and the Table). The malformation was restricted to the venous GFP-expressing domain.

In some E12.5 embryos, primary myocardial cells protruded into the walls of the common cardinal veins, indicating they had initiated differentiation into the myocardial phenotype (Type), as is illustrated by the 3D reconstructions of a Tbx18−/− and a Tbx18GFP/GFP heart (Figure 4E and 4F). The caudal myocardium, including the sinus horn myocardium of mutants and littermate controls expressed Bmp4, Tbx5, Serca2a, cTnI, aMyh, Mlc2a, Cx43, aSMA, and Desmin at comparable levels (not shown).

From E14.5 on, Tbx18 mutant hearts featured thinner and smaller veins that were abnormally positioned within the membranous wall that separates the pericardial and pleural cavities (Figure 4G through 4L). This membrane derives from the mesenchyme that holds the common cardinal veins before sinus horns formation. At this stage, the venous walls had differentiated into myocardium like in the wild-type and acquired a small and deformed Hcn4+ sinoatrial nodal region (Figure 4I and 4J). At a more cranial level, the veins were shifted laterally and were surrounded by lung tissue (Figure 4K and 4L). The defect was restricted to the sinus horns, as the pulmonary vein and dorsal atrial wall (mediastinal wall) were normal (Figure 4M and 4N). Taken together, these results indicate that in Tbx18 mutants, the sinus horns fail to develop from the pericardial mesenchyme. Myocardial differentiation of the walls of the systemic veins is delayed, but not completely blocked, in Tbx18−/− embryos.

Because all Tbx18 mutant embryos failed to form sinus horns, we investigated whether proliferation of the Tbx18-expressing sinus horn precursor cells was decreased at E10.5, when the defect became first visible. Serial sections of E10.5 Tbx18GFP/+ and Tbx18GFP/GFP embryos were alternately stained for GFP to identify the Tbx18-positive area and for BrdU reflecting proliferation rates. No differences in proliferation rates were noticed (Tbx18GFP+, 0.147±0.022, versus Tbx18GFP/GFP, 0.157±0.022; P=0.11, Figure 5A through 5E). Both heterozygous and Tbx18-deficient embryos displayed comparable fractions of cells in M-phase of the cell cycle within the GFP-positive mesenchyme (Figure 5F through 5I; Tbx18GFP+, 2.2%, versus Tbx18GFP/GFP, 2.6%; P=0.396), confirming the BrdU analysis. These results indicate that sinus horn defects were not caused by decreased proliferation in mutants. To address whether Tbx18 deficiency affects programmed cell death, apoptosis was examined by TUNEL analysis and cleaved-caspase 3 staining on serial sections of E9.5 to 11.5 Tbx18GFP/+ and Tbx18GFP/GFP embryos. Apoptosis was not detected in the GFP-positive region of either genotype (Figure 5J through 5M).

Discussion

Sinus Horns Form After E9.5 From a Novel Cardiac Precursor Population

Previous studies have suggested that all components of the multichambered mammalian heart derive from 2 fields of cardiac precursor cells. The left ventricle that is already represented in the early linear heart tube forms from a first field, whereas all other cardiac components are subsequently added from a second field that lies anterior and dorsal to the linear heart tube. These fields, or lineages, are marked by expression of the homeobox transcription factor genes Nkx2–5 and Isll.13 Thus far, the cell lineages of the systemic venous tributaries have only been investigated at stages earlier than E9.5. Our present study indicates that Nkx2–5–/− caudal cardiac cells constitute the venous pole myocardium up to E9.5 but are then taken up into the forming atria and venous valves (Figure 6). Thus, the “sinus horns” of the early heart tube consist of atrial chamber and venous valve precursors, whereas the “real” sinus horns of the systemic venous return only form after E9.5. These sinus horns will subsequently form the sinus venarum and myocardial components of the right superior and inferior caval vein and persistent left caval vein (coronary sinus in human).

The mesenchymal precursor population of the sinus horns not only fails to express Nkx2–5, the majority of the precursors also lack Isll expression before their differentiation. Instead, they uniquely express Tbx18 and will give rise to myocardium that is distinguished from the atrial myocardium by its Tbx18− Nkx2–5− Cx40− expression profile (Figure 6).
In addition, the Tbx18+ population is positioned ventral-caudal-lateral to the second heart field, meeting the Isl1+ precursors at the site where the venous pole myocardium is recruited. Therefore, we conclude that the sinus horns are formed from a precursor population that is genetically distinct from the first and second heart fields. Alternatively, the sinus horn precursor population may be considered as a molecular distinct sublineage of the second field.

**Tbx18 Regulates Morphogenesis and Myocardial Differentiation of the Sinus Horns**

Recruitment and myocardial differentiation of pericardial mesenchymal precursor cells are processes common to the formation of sinus horns and the development of cardiac components like the outflow tract from the anterior heart field. The genetic control of the latter relies on the function of Nkx2–5, Isl1, and other factors including Mef2C, Foxh1, Fgf8,
and Tbx1, the mutation of which primarily results in defects in the anterior portion of the tubular heart.\textsuperscript{12} In contrast, defects in Tbx18 mutants are restricted to the sinus horns and derived caval veins, indicating that the molecular program underlying the development of the sinus horns is profoundly distinct from that of other components of the heart.

Gata4, Gata6, Raldh2, Coup-TFII, and Tbx5 mutants show defects of the atrial and venous pole region, suggesting...
Genotype and Sinus Horn Morphology of Embryos From Matings of Tbx18+/− Mice

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<th>Myocardium (Total)*</th>
<th>No. Analyzed</th>
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Assessment of the formation of a left sinus horn structure, defined as a venous structure bulging into the intracardiac cavity. Each embryo in the mutant group (−/−) has one or more literates in the control (+/+ , +/−) group. Only embryos were included, of which the entire region of interest (sinus horns or common cardinal veins, venous valves, dorsal atrial wall, pulmonary vein) was analyzed on serial sections. NA indicates not applicable. No. of embryos with myocardium in the wall of the sinus horns (+/+ , +/−) or embedded common cardinal veins (−/−) per no. of total embryos analyzed. †Limited myocardium was observed in the walls of the systemic veins in these embryos. One of these E12.5 embryos has been 3D reconstructed (Figure 4). ‡The systemic veins were embedded in the pleuro-pericardial membrane. These veins had a myocardial wall. §The pulmonary vein was normal and the dorsal atrial wall intact in these fetuses. ¶One representative fetus was 3D reconstructed (Figure 4).

Congenital Malformations and Arrhythmias of the Venous Pole

Recent studies indicated that the development of the systemic and pulmonary venous returns involves physically separated and phenotypically distinct types of myocardium. However, not all studies fully agree on this issue because the systemic and pulmonary venous returns also share phenotypic properties. To address these issues, however, insight into the position and nature of the mesenchymal precursors of the venous returns is required. Our study now provides some of these insights. We found that Tbx18 is critical for the formation of the sinus horns but not for the pulmonary venous return. Moreover, the systemic venous returns and their precursors express Tbx18 and not Nkx2–5, whereas the pulmonary venous return and its precursors show the opposite profile. These observations show that distinct regulatory mechanisms are required for each structure and indicate that the precursors have a distinct phenotype. Nevertheless, because systemic and pulmonary vein precursors at the caudal pole of the heart are in close proximity, it is conceivable that errors in patterning and misdirection of fate of either population may result in malformations such as communications between the sinus venosus and pulmonary vein, as seen in abnormal venous return. It will be interesting to identify additional genes involved in the specification of the precursor subpopulations to test this hypothesis.

Foci of ectopic beats that initiate atrial arrhythmias are typically found in myocardium surrounding the pulmonary vein. However, a fraction of these ectopic beats arises from the sinus horn-derived structures. Although the mechanism behind the formation of these ectopic foci has not been solved, the molecular pathways may be distinct for the pulmonary vein and systemic venous return structures, the latter involving small regions with nodal, spontaneously depolarizing activity. We observed that at late fetal stages, the primary myocardial sinus horns (caval veins) initiate Cx40 expression and lose expression of Hcn4, which is essential for embryonic and adult cardiac pacemaking. Therefore, the sinus horns lose their nodal phenotype. It is conceivable that some of the primary myocardium of the sinus horns does not sufficiently mature and persists. This would provide a molecular explanation for the formation and the nonrandom distribution of these foci.

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Disclosures

None.
References

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