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## Reptilian heart development and the molecular basis of cardiac chamber evolution

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**Author Contributions.** K.K-T. performed reptile histology and gene expression studies; A.D.M., B.K., T.S., and B.G.B. performed mouse experiments; J.C.-T. and S.F.G. obtained turtle specimens and isolated *T. scripta Tbx5* cDNA; S.L. and L.B. isolated *Anolis* specimens under direction of J.W.; B.K. acquired and reconstructed OPT images; R.O.G. performed *Tbx5* immunohistochemistry under direction of M.N.; R.M.H. directed initial mouse embryo OPT; J.K.T. obtained chick and mouse specimens; R.O.G., M.N., B.L.B., and E.N.O. provided genetically modified mice prior to publication; B.G.B. conceived and directed the project, and wrote the paper. All authors contributed to the written manuscript.

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## Abstract

The emergence of terrestrial life witnessed the need for more sophisticated circulatory systems. This has evolved in birds, mammals, and crocodilians into complete septation of the heart into left and right sides, allowing separate pulmonary and systemic circulatory systems, a key requirement for the evolution of endothermy<sup>1–3</sup>. However, the evolution of the amniote heart is poorly understood. Reptilian hearts have been the subject of debate in the context of the evolution of cardiac septation: do they possess a single ventricular chamber or two incompletely septated ventricles<sup>4–7</sup>? We examined heart development in the red-eared slider turtle, *Trachemys scripta elegans* (a chelonian), and the green anole, *Anolis carolinensis* (a squamate), focusing on gene expression in the developing ventricles. Both reptiles initially form a ventricular chamber that homogeneously expresses the T-box transcription factor gene *Tbx5*. In contrast, in birds and mammals, *Tbx5* is restricted to left ventricle precursors<sup>8,9</sup>. In later stages, *Tbx5* expression in the turtle (but not anole) heart is gradually restricted to a distinct left ventricle, forming a left-right gradient. This suggests that *Tbx5* expression was refined during evolution to pattern the ventricles. In support of this hypothesis, we show that loss of *Tbx5* in the mouse ventricle results in a single chamber lacking distinct identity, indicating a requirement for *Tbx5* in septation. Importantly, misexpression of *Tbx5* throughout the developing myocardium to mimic the reptilian expression pattern also results in a single mispatterned ventricular chamber lacking septation. Thus, ventricular septation is established by a steep and correctly positioned *Tbx5* gradient. Our findings provide a molecular mechanism for the evolution of the amniote ventricle, and support the concept that altered expression of developmental regulators is a key mechanism of vertebrate evolution.

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Amphibians have a three-chambered heart, while mammalian, crocodilian, and avian hearts have four chambers, two each for pulmonary and systemic circulations. The acquisition of a fully septated ventricle has evolved independently in birds, mammals, and crocodilians<sup>10</sup>, and is an important example of convergent evolution. Non-crocodilian reptiles (squamates, chelonians, and rhynchocephalians) hold a unique place in the evolution of the heart, as their ventricular chambers are apparent intermediates between these forms<sup>4–7</sup>. In reptiles, shunting can produce functional separations between left and right circulatory systems, but only complete septation allows a dual pressure system required for endothermy. Therefore, the evolutionary status of the reptilian ventricles is controversial<sup>7</sup>. Is it a primitive arrangement presaging the septated heart of crocodilians, birds, and mammals? Or is it an adaptation to particular circulatory requirements? Development of reptilian hearts has not been addressed in over 100 years<sup>11</sup>, and thus the developmental basis of reptilian heart formation is not known. Furthermore, clear insight into the evolution of cardiac septation has not emerged from molecular studies of heart development<sup>3</sup>.

Transcription factors of the T-box family are important regulators of heart formation<sup>12</sup>. One T-box gene, *Tbx5*, has an expression pattern that suggests a role in the evolution of cardiac septation (see Supplementary note 1). In amphibians, *Tbx5* is expressed throughout the developing heart<sup>13</sup>. In birds and mammals, there is a steep gradient of *Tbx5* expression from high levels in the prospective left ventricle (LV) to low levels in the prospective right ventricle (RV)<sup>8,9</sup>. Reduced dosage of *Tbx5* in humans and mice leads to defects in interventricular septum (IVS) formation and patterning<sup>14–17</sup>, suggesting that a steep gradient of *Tbx5* is critical for IVS formation. The evolutionary role of *Tbx5* in septation is unknown.

We examined cardiac embryology of the red-eared slider turtle, *T. scripta elegans* (a chelonian), and the green anole, *A. carolinensis* (a squamate), focusing on the ventricles. Although the phylogenetic relationship of turtles to other reptiles is controversial based on anatomical considerations<sup>18,19</sup>, molecular phylogenies consistently group turtles with the archosaurs (birds and crocodiles)<sup>20,21</sup>. Anoles are considered to be more basal than archosaurs<sup>19–21</sup>. The post-hatching anole heart has a thick muscular ridge (Fig. 1a–d and Supplementary Fig. 1) that separates a proximal outflow tract, or cavum pulmonale<sup>6,11</sup>, from the main ventricular chamber. Turtles have a smaller muscular ridge and are thought to have a primitive IVS-like structure<sup>4,6,11</sup>, as we determined by three-dimensional reconstructions revealing a dense coalescence of trabeculae spanning the full depth of the heart (Fig. 1e–h and Supplementary Fig. 1). Initially, developing turtle and anole hearts showed no clear evidence of ventricular septation (Fig. 1i, and Supplementary Figs. 2–4). In contrast, the chick has a well-developed IVS at comparable early stages (Figs. 1i and Supplementary Fig. 3). In the turtle, a structure resembling an IVS appears only at stage 21 (Fig. 1i). Alligator embryos (Fig. 1j) have a muscular ridge and a distinct IVS. The muscular ridge has been interpreted as analogous to the IVS, leading to the impression that reptiles have multiple septa<sup>4–6</sup>. We speculate that the development of the muscular ridge in reptiles reflects persistent growth of the proximal outflow tract<sup>11</sup>, as seen transiently in chick heart (Fig. 1i–k and Supplementary Fig. 3).

To observe molecular patterning of reptile ventricles, we examined expression of *Tbx5*. In mammals and birds, *Tbx5* mRNA and protein are highly enriched in the prospective LV (Fig. 2b,c and Supplementary Fig. 5)<sup>8,9</sup>. At looping heart tube stages, *Tbx5* was broadly expressed throughout the embryonic turtle and anole hearts (Fig. 2a,d), similar to *Xenopus Tbx5* (Ref 13), but unlike its early restricted expression in chick and mouse (Fig. 2b,c). In the anole, *Tbx5* expression extended to the boundary of the ventricle and outflow tract, where the muscular ridge forms. At later stages, *Tbx5* expression in turtle (stage 15) and anole (stage 13) remained homogeneous throughout the ventricle (Fig. 2e,h and data not shown). In comparable stages in chick, it was sharply restricted to LV primordium. At stages 17–18 in the turtle, *Tbx5* mRNA levels decreased in RV primordium, remaining enriched in LV primordium, creating a steep left-right gradient, although not as sharply defined as in chick (Fig. 2f,g,h,i and Supplementary Figs. 6,7). This gradient was maintained at stage 21 (Fig. 2h,i). *Tbx5* expression in *Anolis* was not restricted in the ventricle (Fig. 2f,i and Supplementary Fig. 7). We examined expression of *Tbx5* target genes expressed in trabeculae but excluded from mammalian IVS myocardium<sup>14,16,17,22</sup>. *Bmp10* was

expressed throughout the early turtle and anole trabeculae, but was excluded in turtles at Stage 17–18 from an expansion of the compact myocardium corresponding to presumptive IVS precursors, correlating with the boundary of *Tbx5* expression (Fig. 2j and Supplementary Fig 6). This suggests a conserved molecular transition in the trabeculae that form the IVS. Turtle *Nppa* (not found in anoles<sup>23</sup>), formed a gradient similar to *Tbx5* (Fig. 2k). Thus, turtle ventricles, but not those of *Anolis*, acquire distinctions between left and right components late in development.

A steep *Tbx5* gradient in chick and mouse may have evolved to pattern the ventricles. Reducing *Tbx5* levels supports this<sup>14,16,17</sup>. To address a potential role for *Tbx5* in septation, we deleted *Tbx5* from segments of developing mouse ventricles, using a conditionally deletable *Tbx5* allele (*Tbx5*<sup>LDN</sup>)<sup>16</sup>, and ventricular myocyte-specific *Nkx2.5::Cre* mice<sup>24</sup> (Fig. 3a). These mice (*Nkx2.5::Cre*<sup>tg/0</sup>; *Tbx5*<sup>LDN/LDN</sup> mice, or *Tbx5*<sup>V-del</sup> mice) lacked morphological distinctions between the LV and RV that were obvious in wild-type embryos by embryonic day (E) 9.5 (Fig. 3b). Embryos with this univentricular phenotype persisted until E11.5 (Fig. 3c). Expression of *Nppa* and *Bmp10*, normally excluded from the interventricular groove, was expanded throughout the single ventricle of *Tbx5*<sup>V-del</sup> embryos (Fig. 3e,f). *Hand1* was expressed at lower levels, but in its normal domains, the LV and RV primordia (Fig. 3g). Thus, loss of *Tbx5* from developing ventricles results in a single mispatterned ventricle.

To determine if a steep *Tbx5* gradient at the interventricular midpoint is critical for IVS formation, we deleted *Tbx5* with *Mef2cAHF::Cre* mice<sup>25</sup> (Fig. 3h). Since *Mef2cAHF::Cre* is active in RV and IVS precursors, but not in the LV free wall, the *Tbx5* expression boundary is shifted leftward. *Tbx5*<sup>LDN/LDN</sup>; *Mef2cAHF::Cre* (*Tbx5*<sup>AHF-del</sup>) mice lacked an IVS (Fig. 3i–k). Gene expression analysis showed that a distinction between LV and RV was maintained (Fig. 3l,m), but a clear absence of IVS-enriched markers (*Irx2*, *Dkk3*) at the ventricular midpoint, while maintained in the adjacent trabeculae, emphasize the absence of ventricular septation (Fig. 3n, Supplementary Fig. 8). Thus, a boundary of cells expressing high *Tbx5* levels is necessary within a segment of myocardium where IVS outgrowth will occur. This implies a prepattern within which *Tbx5* must function; the nature of this prepattern is unknown (See supplementary note 2). *Tbx5* expression and additional patterning cues may have co-evolved, or the prepattern may exist in all amniotes. Regardless, IVS formation requires a sharp *Tbx5* boundary indicating that *Tbx5* patterning was a major factor in evolution of septation.

Our loss-of-function experiments demonstrate a requirement for *Tbx5* in IVS formation distinct from a more global role in differentiation. These results do not address the evolutionary role of *Tbx5* patterning; in particular, whether the broad expression of *Tbx5* observed in anole and turtle would preclude IVS formation. Previous misexpression attempts yielded variable results ranging from no effect to severely malformed hearts (Ref. 9 and J.K.T., unpublished data). We misexpressed *Tbx5* in the ventricles by crossing a mouse line bearing a stable Cre-activatable transgene expressing moderate *Tbx5* levels upon induction (*CAT-Tbx5*)<sup>26</sup> with *Mef2cAHF::Cre* or *Nkx2.5::Cre* mice (Fig. 4). *CAT-Tbx5*; *Mef2cAHF::Cre* embryos survived until E11 and had a single ventricle at E10.25. Molecular analysis revealed expanded expression of *Tbx5*, *Nppa*, and *Bmp10* across the interventricular

groove of *CAT-Tbx5;Mef2cAHF::Cre* embryos (Fig. 4b). *CAT-Tbx5;Nkx2.5::Cre* embryos survived longer (until E12), presumably because this manipulation avoided secondary effects of *Tbx5* overexpression in cardiac progenitors. *CAT-Tbx5;Nkx2.5::Cre* embryos at E11.5 also had defective ventricular septation and mispatterned gene expression (Fig. 4c and Supplementary Fig. 9). Interestingly, due to the mosaic expression of *Tbx5* by *Nkx2.5::Cre*, some embryos had no septum at all, while others with a more graded expression of *Tbx5* had a rudiment of a septum in which not all genes were mispatterned (Fig. 4c and Supplementary Fig. 9). Thus, misexpression of *Tbx5* in a pattern reminiscent of the reptilian heart leads to loss of IVS patterning and morphogenesis, further supporting a role for *Tbx5* patterning in the evolution of septation.

Our results provide evidence that the reptilian heart, although evolved to function physiologically under conditions particular to reptilian life<sup>7</sup>, is an evolutionary intermediate between amphibian and avian/crocodylian hearts in its ventricular development. The dynamic expression of *Tbx5* and its leftward restriction suggest a temporal refinement model in which early restriction of *Tbx5* expression to LV precursors, as seen in chick and mouse, provides a robust patterning cue for ventricular septation. In this model (Fig. 4d), a quantitative gradient of *Tbx5* is essential for proper formation and patterning of the IVS. Our mouse genetic analyses, including decreased dosage<sup>14,16</sup>, are consistent with an important role for a steep gradient of *Tbx5* in chamber patterning and IVS formation. In the reptilian heart, the delayed and less pronounced establishment of this patterning may contribute to varying degrees of septation. Therefore patterning of *Tbx5*, in the archosaurian and synapsid lineages, is likely to be an important mechanism in the convergent evolution of septation. Our findings generally support the concept that altered expression of developmental regulators is an important aspect of morphological evolution<sup>27</sup>.

## METHODS SUMMARY

Embryos were isolated from *T. scripta elegans* eggs (Kliebert Turtle and Alligator Farm, Hammond, LA). Green anole (*A. carolinensis*) embryos were collected in captivity. Mouse strains were described<sup>16,24–26</sup>. Whole-mount and section in situ hybridizations were performed using standard protocols. Immunohistochemistry and OPT were performed as previously described<sup>26,28</sup>. For all mouse experiments, at least 3 embryos were examined for each genotype at each stage, all with comparable results.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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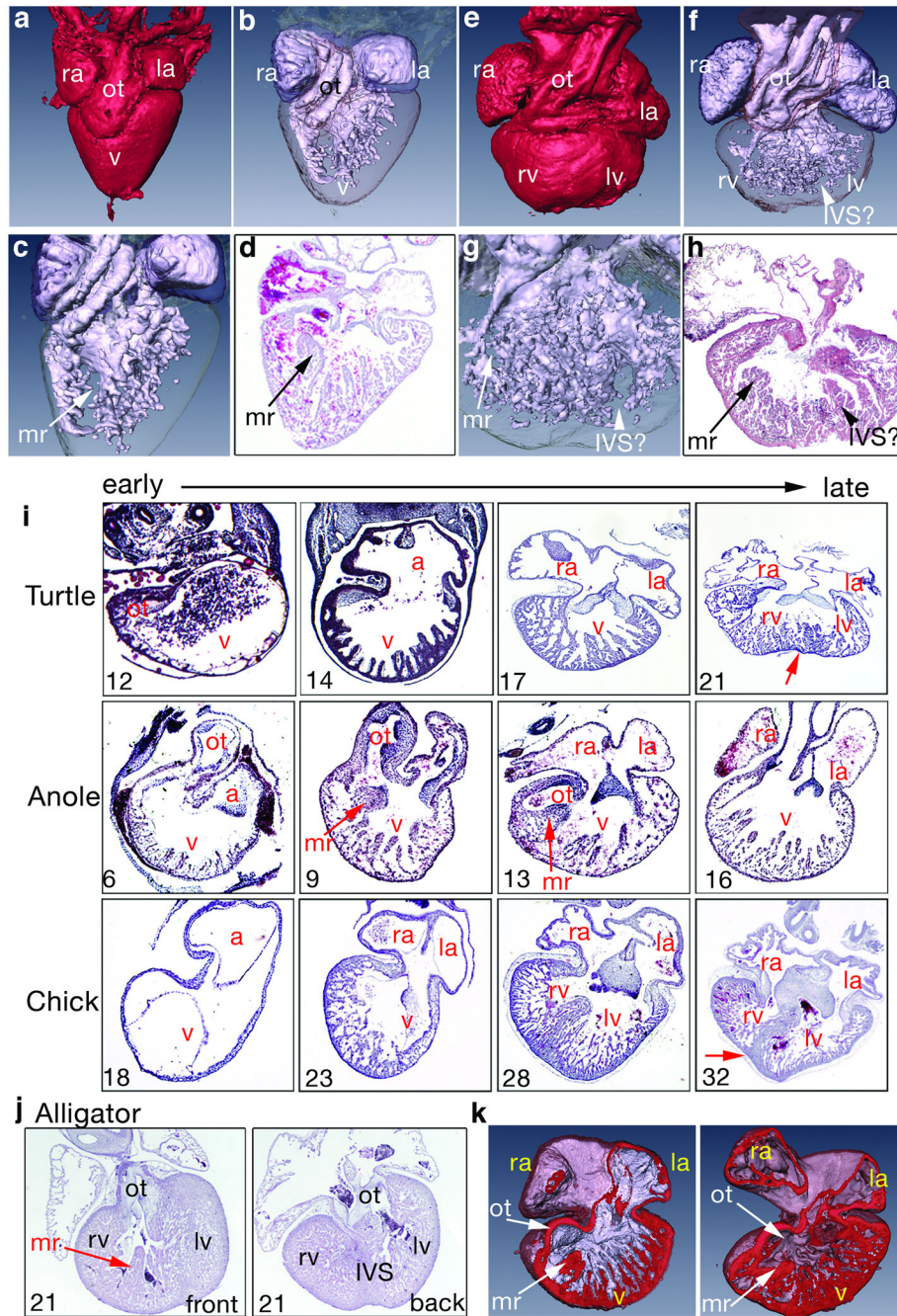
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### Figure 1. Reptilian heart development

**a–h.** Optical projection tomography of post-hatching anole (**a–d**) and turtle (**e–h**) hearts. **a,e** external view, **b,c,f,g** chamber fill; **d,h** histology. **i.** Histological analysis of heart development in turtle, anole, and chick embryos. Four representative stages shown are equivalent between species. arrow: interventricular groove. **j.** Histology of stage 21 embryonic alligator heart. **k.** OPT of st. 17 (left) and st. 21 (right) turtle hearts. In all reptile embryos (**i–k**), note close apposition of mr and ot. a: atrium, IVS?: IVS-like structure, la:

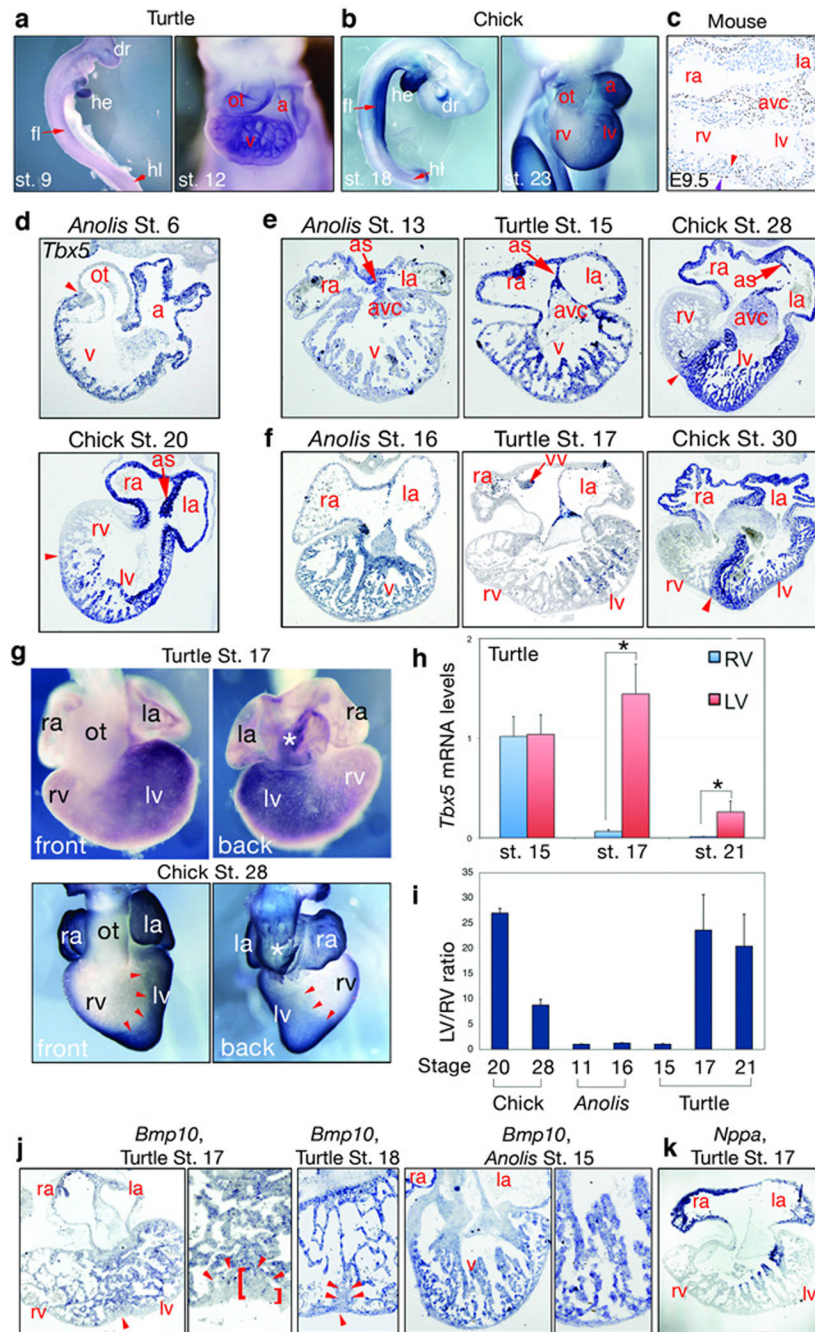
left atrium, lv: left ventricle, mr: muscular ridge, ot: outflow tract, ra: right atrium, rv: right ventricle.

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**Figure 2. Gene expression in amniote embryos**

**a–c**, *Tbx5* expression in turtle, chick, and mouse. Left panels: whole-embryo views. He: heart, fl: forelimbs, dr: dorsal retina, hl: hindlimbs. Right panels: closeup ventral views of embryonic hearts. In **c**, bottom panel shows *Tbx5* immunohistochemistry; red arrowheads: rv/lv junction; purple arrowhead: epicardium. la: left atrium, lv:left ventricle, ot: outflow tract, ra: right atrium, rv: right ventricle. **d–g**, Expression of *Tbx5*. As: atrial septum; avc: atrioventricular cushion. Arrowheads mark the boundary between lv and rv, or V and OT for Anole in **d**. **h**. Quantitation of *Tbx5* mRNA levels in turtle LV and RV; data are mean  $\pm$  SD

normalized to St 15 RV. \* $P < 0.005$  by t-test. **i.** Ratio of *Tbx5* mRNA levels between the LV and RV. **j.** *Bmp10* expression in turtle and Anole hearts. Arrowheads: interventricular groove and septum. Brackets: thickness of *Bmp10*-negative area. **k.** *Nppa* expression in the turtle is in a left-right gradient similar to *Tbx5*.

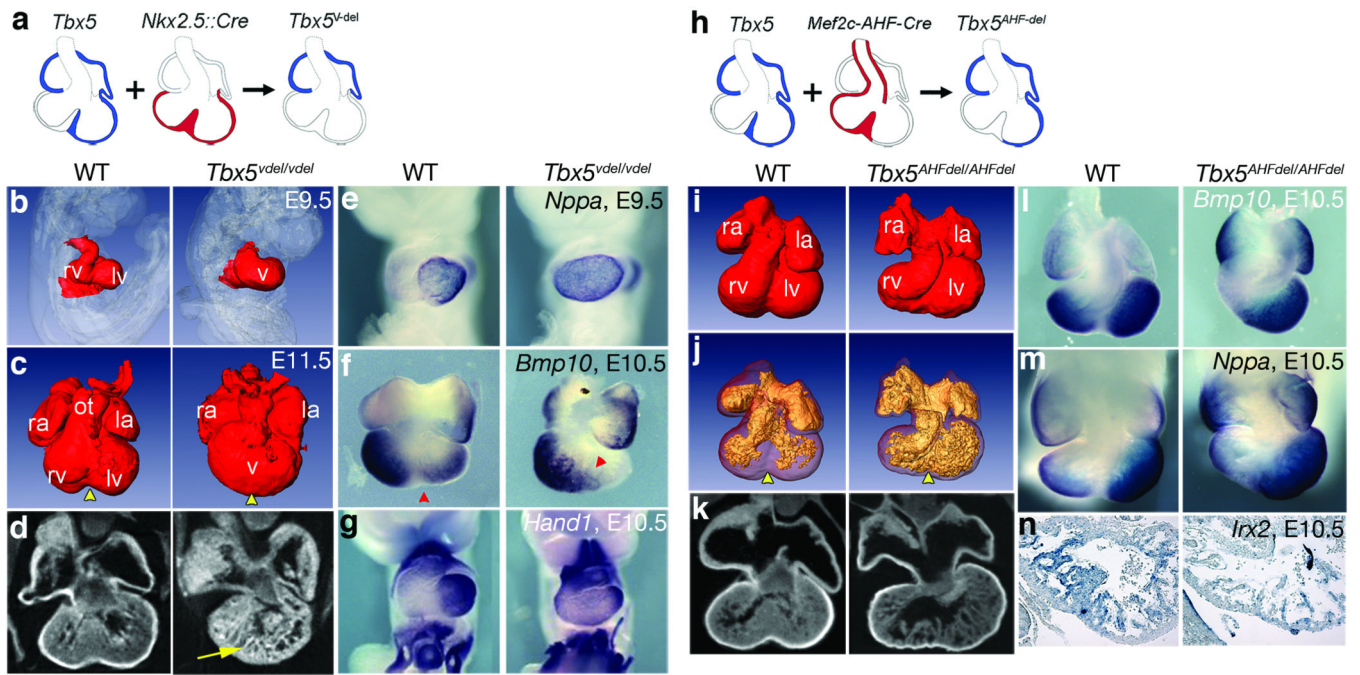
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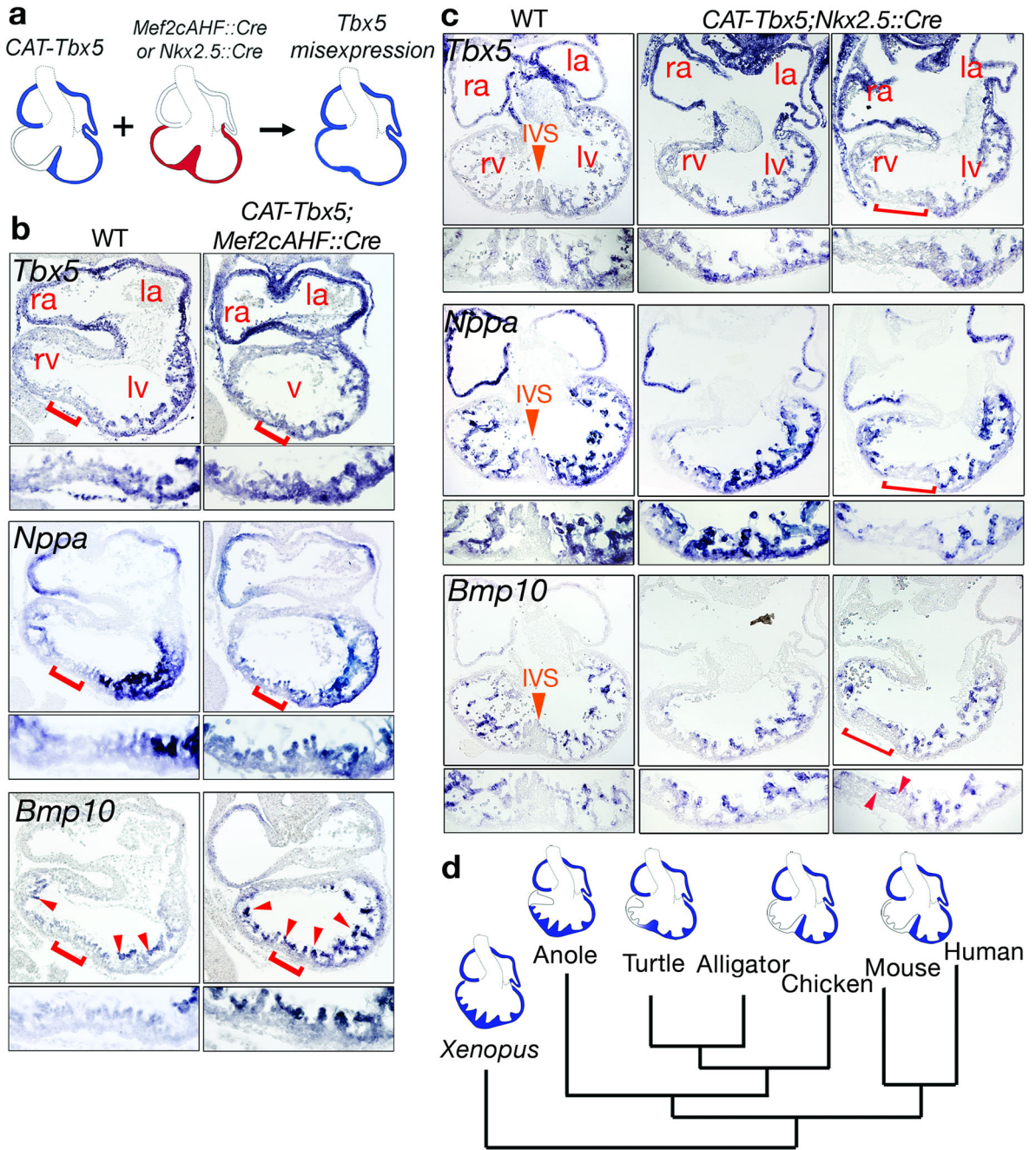
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**Figure 3. Ventricule-restricted deletion of mouse *Tbx5***

**a**, Strategy for ventricular deletion of *Tbx5*. **b–d**, OPT of wild-type (WT) and *Tbx5<sup>vdel/vdel</sup>* embryos and hearts at E9.5 (**b**) and E11.5 (**c**, **d**). Arrowheads indicate position of the IVS. **e,f,g**: Gene expression for indicated transcripts. **h**, Strategy for *Tbx5* deletion in anterior heart field derivatives. **i–k**, OPT of wild-type (WT) and *Tbx5<sup>AHFdel/AHFdel</sup>* hearts at E10.5. **i**: external view, **j**: chamber fill, **k**: virtual sections. **l,m,n**: Gene expression for indicated transcripts.



**Figure 4. Misexpression of *Tbx5* results in loss of IVS patterning**

**a.** Strategy for ventricular misexpression of *Tbx5*. **b.** Morphology and gene expression in *CAT-Tbx5; Mef2cAHF::Cre* embryos for indicated transcripts. Brackets: IVS region, magnified in lower panels. Arrowheads: trabeccular *Bmp10* expression. **c.** Morphology and gene expression in *CAT-Tbx5; Nkx2.5::Cre* embryos at E11.5. Orange arrows: interventricular septum region (IVS). Brackets show a rudimentary septum in a mutant embryo. **d.** Diagrammatic representation of embryonic heart structures and patterns of *Tbx5*



expression (blue) in vertebrate evolution. la: left atrium, lv: left ventricle, ot: outflow tract, ra: right atrium, rv: ventricle, v: ventricle.

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