LETTERS

Reptilian heart development and the molecular basis of cardiac chamber evolution

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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[1–3]. 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[4–7]? Here we examine 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 homogenously expresses the T-box transcription factor gene *Tbx5*. In contrast, in birds and mammals, *Tbx5* is restricted to left ventricle precursors[8,9]. 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.

Amphibians have a three-chambered heart, whereas 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[10], 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[4–7]. 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[7]. 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[11], 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[3].

Transcription factors of the T-box family are important regulators of heart formation[12]. 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[13]. In birds and mammals, there is a steep gradient of *Tbx5* expression from high levels in the prospective left ventricle to low levels in the prospective right ventricle[8,9]. Reduced dosage of Tbx5 in humans and mice leads to defects in interventricular septum (IVS) formation and patterning[14–17], 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[18,19], molecular phylogenies consistently group turtles with the archosaurs (birds and crocodiles)[20,21]. Anoles are considered to be more basal than archosaurs[19–21]. 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[6,11], from the main ventricular chamber. Turtles have a smaller muscular ridge and are thought to have a primitive IVS-like structure[4,6,11], 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 (Fig. 1i and Supplementary Fig. 3). In the turtle, a structure resembling an IVS appears only at stage 21 (Fig. 1i). Alligator

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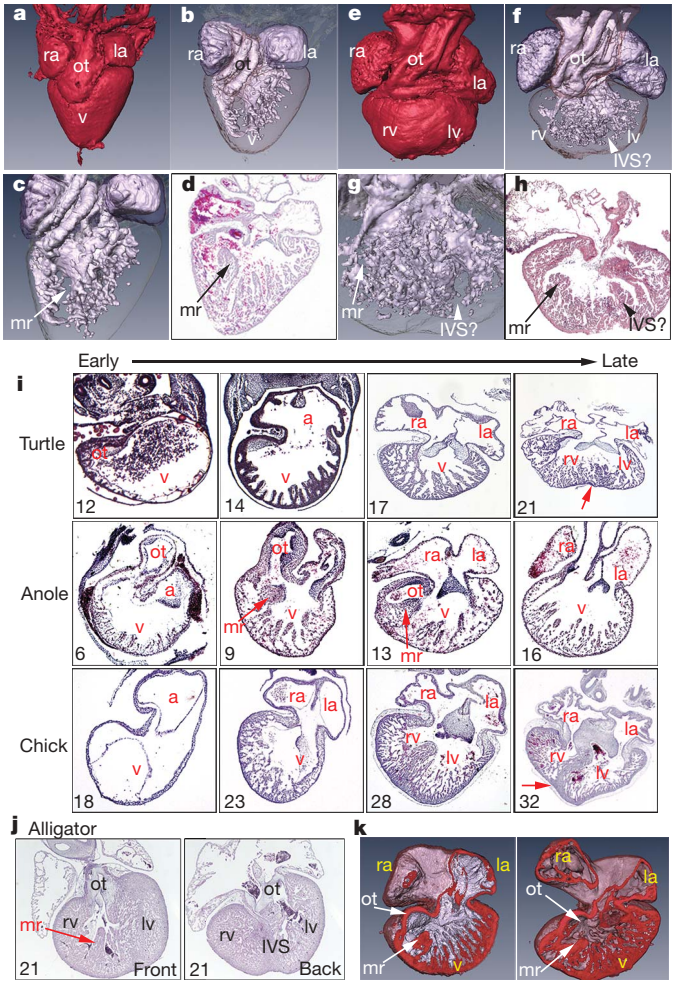

Figure 1 | Reptilian heart development. **a**–**h**, OPT of post-hatching anole (**a**–**d**) and turtle (**e**–**h**) hearts. **a, e**, External view; **b**, **c**, **f**, **g**, chamber fill; **d**, **h**, histology. **i**, Histological analysis of heart development in turtle, anole and chick embryos. Four representative stages shown are equivalent between species (arrow, interventricular groove). **j**, Histology of stage 21 embryonic alligator heart. **k**, OPT of stage 17 (left) and stage 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.

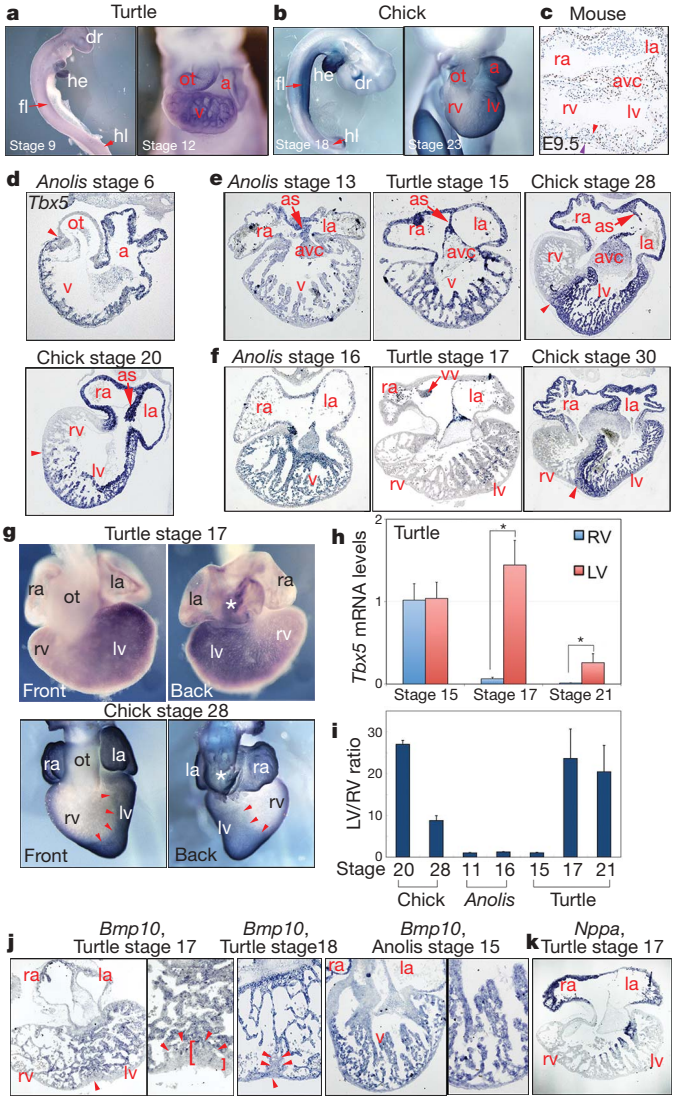

Figure 2 | Gene expression in amniote embryos. **a**–**c**, *Tbx5* expression in turtle, chick and mouse. **a**, **b**, mRNA expression. Left panels, whole-embryo views. he, heart; fl, forelimbs; dr, dorsal retina; hl, hindlimbs. Right panels, close-up ventral views of embryonic hearts. **c**, Tbx5 immunohistochemistry; red arrowheads, right ventricle/left ventricle 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 left ventricle and right ventricle, or ventricle (v) and ot for anole in **d**. **h**, Quantification of *Tbx5* mRNA levels in turtle left ventricle and right ventricle; data are mean ± s.d. normalized to stage 15 right ventricle. $^{*}P < 0.005$ by *t*-test. **i**, Ratio of *Tbx5* mRNA levels between the left ventricle and right ventricle. **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*.

embryos (Fig. 1j and Supplementary Fig. 1) 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[4–6]. We speculate that the development of the muscular ridge in reptiles reflects persistent growth of the proximal outflow tract[11], as seen transiently in the 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* messenger RNA (mRNA) and protein are highly enriched in the prospective left ventricle (Fig. 2b, c and Supplementary Fig. 5)[8,9]. 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 the 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 left ventricle primordium. At stages 17–18 in the turtle, *Tbx5* mRNA levels decreased in right ventricle primordium, remaining enriched in left ventricle primordium, creating a steep left–right gradient, although not as sharply defined as in chick (Fig. 2f–i and Supplementary Figs 6 and 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[14,16,17,22]. *Bmp10* was expressed throughout the early turtle and anole trabeculae, but was excluded in turtles at stages 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[23]) formed a gradient

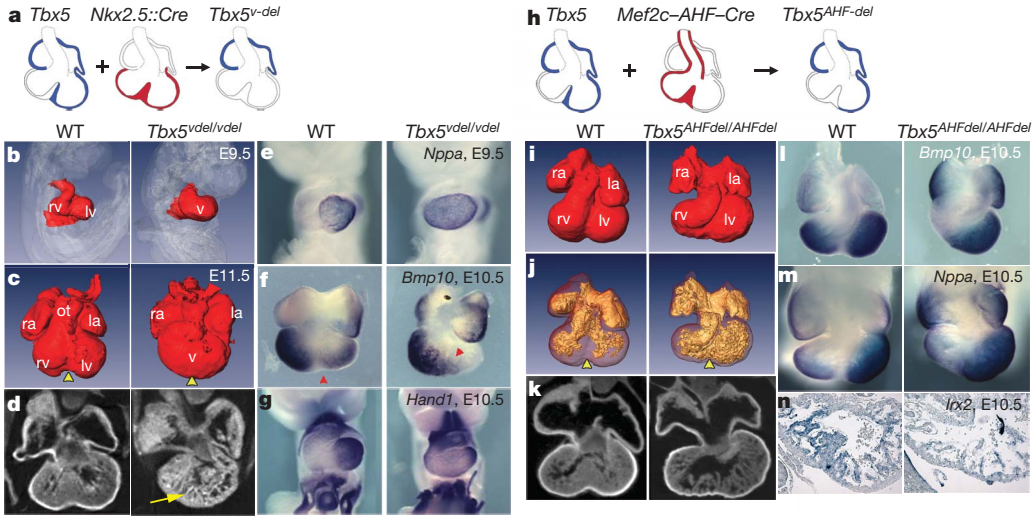

Figure 3 | Ventricle-restricted deletion of mouse Tbx5. **a**, Strategy for ventricular deletion of *Tbx5*. **b**–**d**, OPT of wild-type (WT) and $Tbx5^{vdel/vdel}$ embryos and hearts at embryonic day (E) 9.5 (**b**) and E11.5 (**c**, **d**). Arrowheads indicate position of the IVS. **e**–**g**, Gene expression for indicated transcripts. **h**, Strategy for *Tbx5* deletion in anterior heart field derivatives. **i**–**k**, OPT of wild-type (WT) and $Tbx5^{AHFdel/AHFdel}$ hearts at E10.5. **i**, External view; **j**, chamber fill; **k**, virtual sections. **l**–**n**, Gene expression for indicated transcripts.

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[14,16,17]. To address a potential role for Tbx5 in septation, we deleted *Tbx5* from segments of developing mouse ventricles, using a conditionally deletable *Tbx5* allele ($Tbx5^{LDN}$)[16], and ventricular myocyte-specific *Nkx2.5::Cre* mice[24] (Fig. 3a). These mice ($Nkx2.5::Cre^{tg/0}$;$Tbx5^{LDN/LDN}$ mice, or $Tbx5^{V\text{-}del}$ mice) lacked morphological distinctions between the left ventricle and right ventricle that were obvious in wild-type embryos by embryonic day 9.5 (Fig. 3b). Embryos with this univentricular phenotype persisted until embryonic day 11.5 (Fig. 3c, d). Expression of *Nppa* and *Bmp10*, normally excluded from the interventricular groove, was expanded throughout the single ventricle of $Tbx5^{V\text{-}del}$ embryos (Fig. 3e,f). *Hand1* was expressed at lower levels, but in its normal domains, the left ventricle and right ventricle primordia (Fig. 3g). Thus loss of *Tbx5* from developing ventricles results in a single mispatterned ventricle.

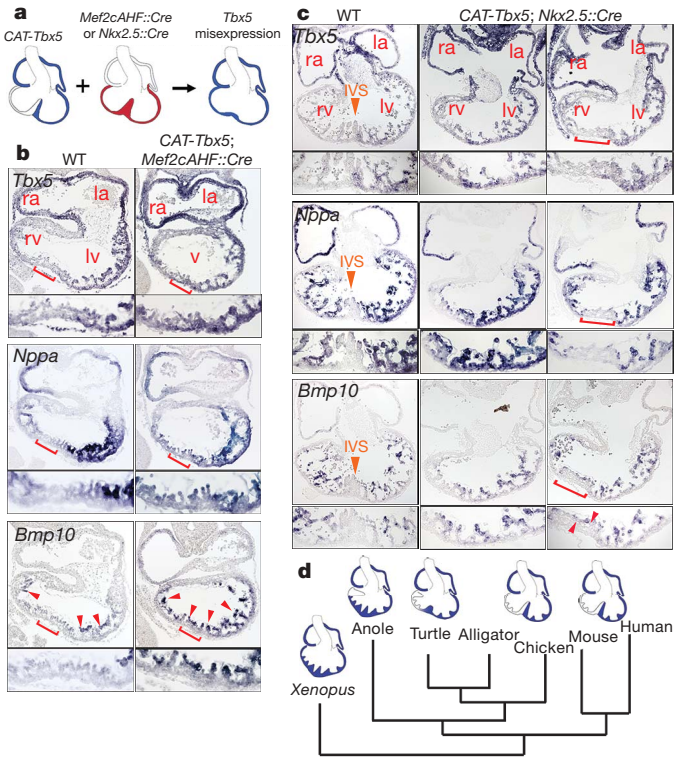

Figure 4 | Misexpression of *Tbx5* results in loss of IVS patterning. **a**, Strategy for ventricular misexpression of *Tbx5*. **b**, Morphology and gene expression in *CAT-Tbx5*;*Mef2cAHF::Cre* embryos for indicated transcripts. Brackets, IVS region, magnified in lower panels. Arrowheads, trabecular *Bmp10* expression. la, left atrium; lv, left ventricle; ra, right atrium; rv, right ventricle; v, ventricle. **c**, Morphology and gene expression in *CAT-Tbx5*;*Nkx2.5::Cre* embryos at embryonic day 11.5. Orange arrows, interventricular septal region (IVS). Brackets show a rudimentary septum in a mutant embryo. **d**, Representation of embryonic heart structures and patterns of *Tbx5* expression (blue) in vertebrate evolution.

To determine if a steep Tbx5 gradient at the interventricular midpoint is critical for IVS formation, we deleted *Tbx5* with *Mef2cAHF::Cre* mice[25] (Fig. 3h). Because *Mef2cAHF::Cre* is active in the right ventricle and IVS precursors, but not in the left ventricle free wall, the *Tbx5* expression boundary is shifted leftwards. $Tbx5^{LDN/LDN}$;*Mef2cAHF::Cre* ($Tbx5^{AHF\text{-}del}$) mice lacked an IVS (Fig. 3i–k). Gene expression analysis showed that a distinction between left ventricle and right ventricle was maintained (Fig. 3l, m); however, a clear absence of IVS-enriched markers (*Irx2*, *Dkk3*) at the ventricular midpoint, although maintained in the adjacent trabeculae, emphasizes the absence of ventricular septation (Fig. 3n and 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, which indicates that *Tbx5* patterning was a major factor in the 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 observations). 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*)[26] with *Mef2cAHF::Cre* or *Nkx2.5::Cre* mice (Fig. 4). *CAT-Tbx5;Mef2cAHF::Cre* embryos survived until embryonic day 11 and had a single ventricle at embryonic day 10.25. 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 embryonic day 12), presumably because this manipulation avoided secondary effects of Tbx5 overexpression in cardiac progenitors. *CAT-Tbx5;Nkx2.5::Cre* embryos at embryonic day 11.5 also had defective ventricular septation and mispatterned gene expression (Fig. 4c and Supplementary Fig. 9). Interestingly, owing to the mosaic expression of Tbx5 by *Nkx2.5::Cre*, some embryos had no septum at all, whereas 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[7], is an evolutionary intermediate between amphibian and avian/crocodilian 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 left ventricle 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[14,16], 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[27].

METHODS SUMMARY

Embryos were isolated from *T. scripta elegans* eggs (Kliebert Turtle and Alligator Farm). Green anole (*A. carolinensis*) embryos were collected in captivity. Mouse strains were as previously described[16,24–26]. Wholemount and section *in situ* hybridizations were performed using standard protocols. Immunohistochemistry and optical projection tomography (OPT) were performed as previously described[26,28]. For all mouse experiments, at least three embryos were examined for each genotype at each stage, all with comparable results.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions K.K.-T. performed reptile histology and gene expression studies; A.D.M., B.L.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* complementary DNA (cDNA); S.L. and L.B. isolated *Anolis* specimens under direction of J.W.; B.L.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 before publication; B.G.B. conceived and directed the project, and wrote the paper. All authors contributed to the written manuscript.

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METHODS

Reptiles. Embryos from *T. scripta elegans* eggs (Kliebert Turtle and Alligator Farm) were dissected free of extra-embryonic membranes and staged according to Greenbaum & Carr[29]. Green anole (*A. carolinensis*) embryos were collected in captivity during the spring and summer breeding season. The day the eggs were discovered in the cage was considered embryonic day 0. Eggs were incubated at 27 °C until embryo collection at embryonic days 4, 8, 11, 12, 14, 18 and 19 and on the day of hatching. Anole embryos were staged as previously described[30]. All embryos were fixed in 4% paraformaldehyde, followed by stepwise dehydration in methanol.

Mouse genetics. Mouse strains were as previously described[16,24–26].

Reptile cDNAs. Turtle total RNA was prepared from pooled day 14 *T. scripta* embryos (stages 12–15). PCR was performed from cDNA with 5′-GTTTC CCAGTTACAAAGTGAAGG (forward) and 5′-GTCTCACTGTGCTCCTGGG (reverse) primers designed against the chick *Tbx5* sequence. PCR products matching the estimated size (540 base pairs) were extracted from a 1% agarose gel, ligated into pCRII (Invitrogen) and used to transform DH5α-competent cells. Positive plasmids were identified by hybridization with digoxygenin-labelled chick *Tbx5* and by amplification using nested primers also designed against chick *Tbx5* and *Tbx4* (5′-TAYGTGCACCCGGAYTCYCCWGC and 5′-TGGTASGARGTCACAGMGATRAA). *Anolis* cDNA probes were obtained by PCR from genomic DNA, using sequences obtained at the National Center for Biotechnology Information Trace Archive (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi?).

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CORRIGENDUM

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In this Letter, author Laurel Beck was incorrectly listed as Laural Beck.