Supplementary note 1: Expression patterns of Tbx5, Tbx20, and others relating to septum formation.

Tbx5 and Tbx20 have patterns of expression that suggest potential roles in regulating chamber identity and the boundaries between left and right ventricular chambers. Tbx5 is initially expressed in a posterior-anterior gradient in the heart, encompassing the entire linear heart tube, which will give rise to the atria and left ventricle. As the heart tube loops and chamber promordia arise, expression of Tbx5 in mouse and chick is rapidly refined to be predominantly expressed in the left ventricle, with barely detectable expression in the right ventricular chamber; this creates a steep gradient of Tbx5-high cells on one side of the prospective left ventricle, and Tbx5-low cells on the right ventricular side, as shown independently by several investigators. Later in development, Tbx5 acquires expression in right ventricular trabeculae, and following septation is eventually expressed at equivalent low levels in the ventricles, with high level atrial expression being retained. We have confirmed these mRNA expression patterns at the protein level, as shown in Suppl. Fig. S5, indicating a steep and sharp gradient of Tbx5 between the left and right ventricular precursors immediately preceding and during septation.

The expression of Tbx20 is highly variable between species, and thus is not a particularly good candidate for an evolutionarily important regulator of septation. In the chick, it is clearly restricted to the RV precursors in a pattern complementary to that of Tbx5. This
pattern is not well conserved in the mouse \(^{11}\), and in turtle hearts Tbx20 expression partly overlaps with that of Tbx5, with some regions of the heart expressing only Tbx5 or Tbx20 (Fig S6).

Other genes such as the Hand basic helix-loop-helix family of transcription factors have been suggested as potential regulators of left versus right ventricle identity or morphogenesis \(^{6,12-14}\), but their chamber-specific expression patterns are mammalian-specific, and they are expressed throughout chicken hearts \(^{12}\); thus these are not compelling candidates in the evolution of ventricular morphogenesis.

**Supplementary note 2: Tbx5 and IVS formation**

The deletion with *Mef2cAHF::Cre* is predicted to shift the boundary of Tbx5 leftward, which might be expected to shift the IVS leftward as well. Instead, this deletion eliminated the formation of the IVS. The fact that *Tbx5\(^{AHF-d}\) hearts developed a distinct LV chamber, but not an IVS, suggests that a molecularly distinct LV alone is insufficient to direct IVS development. Rather, Tbx5 expression in anterior heart field derivatives is necessary both for the proper positioning of the IVS and for its subsequent outgrowth. One possibility is that the interface between the first and second heart lineages might contribute to forming this prepattern. In the chick, the IVS develops exclusively from a narrow, prepatterned band of myocardium (400–500 \(\mu\)m long) at the interventricular midpoint \(^{15}\). Consistent with this view, fate-mapping studies in mouse show that the IVS has an oligoclonal origin, deriving from only a few precursor cells, which then invade the ventricular lumen through oriented cell growth from both the left and right ends \(^{16}\). However, the left- and right-sided IVS
lineages remained distinct, suggesting that patterning events within the IVS prevent the intermingling of cells from the LV and RV. These lineage studies would argue that IVS formation is secondary to second heart field migration. Importantly, in the chicken embryo, the contribution of second heart field cells to the RV is limited; only the proximal outflow tract contributes any cells to the outer free wall of the RV, and no contribution to the IVS can be determined. Since the chicken has a very sharp boundary of Tbx5 expression that correlates with IVS formation, the notion that the IVS patterning is dependent on the apposition of the first and second heart fields is not clear. Additional mechanisms might exist, including signaling from the adjacent cell layer. In mice, for example, Notch signaling is active in endocardial cells that line the trabeculae but not in the endocardium overlying the nascent IVS. Alternatively, additional mechanisms may also include the patterned expression of partners of Tbx5 that are also important for septation, such as Sall4.

Our ventricle-specific deletions of Tbx5 led to grossly normal chambers, in contrast to the severely malformed chambers in the Tbx5-null mice. Indeed, the expression of most markers of the developing chambers was not significantly disturbed, and key morphological landmarks such as trabeculae formed normally, suggesting that the loss of Tbx5 from the ventricular myocardium after E8.5 does not result in significant defects in chamber formation. Particularly intriguing was the persistent expression of Nppa, which is reduced in Tbx5 haploinsufficient hearts, and is absent in embryos completely lacking Tbx5. This indicates that Tbx5 is important for the initiation and high-level expression of Nppa, but is dispensable for its quantitative expression later on. The expanded expression of Nppa in embryos lacking Tbx5 in the ventricles clearly shows that Tbx5 is also a key regulator of the patterning of Nppa. The altered patterns of expression of Bmp10, Irx2, and Dkk3 point to a
consistent role for Tbx5 in patterning ventricular gene expression. These results show that Tbx5 has an early role in cardiac differentiation and diverse later roles in chamber patterning, including the formation of the IVS.

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Supplementary Figure 1: Reptile heart histology. Serial sections through a post-hatching *Anolis carolinensis* heart (top), a post-hatching *Trachemys scripta elegans* heart (middle), and a late embryonic *Alligator mississippiensis* heart (bottom).
Supplementary Figure 2: Developing turtle heart histology. Sections of *Trachemys scripta elegans* embryos at various indicated developmental stages, at the level of the heart. All are transverse sections, from anterior to posterior.
Supplementary Figure 3: Developing chicken heart histology. Sections of embryonic chicken hearts at various indicated developmental stages. All are transverse sections, from anterior to posterior.
Supplementary Figure 4: Developing anole heart histology. Sections of *Anolis carolinensis* embryos at various indicated developmental stages, at the level of the heart. All are transverse sections, from anterior to posterior.
Supplementary Figure 5: Immunohistochemistry for Tbx5 and Nkx2-5 protein in sections from mouse embryos at E9.75 (a,b), 10.25 (c,d), 12 (e,f), 13.25 (j,k), 13.5 (l,m), and 14.5 (n,o). Boxed regions in a,c,e are shown at higher magnification in g,h,i, respectively. Note the localization in the ventricles is initially restricted to the left ventricle (IV), and is excluded from the right ventricle (RV) at E9.75, and E10.25 (red arrowheads in g,h show expression in IV nuclei), but beginning at E12 ventricular expression is predominantly restricted to trabeculae of both ventricles (asterisks in i), although compact myocardium of the LV (LVM) but not RV (RVM) still express Tbx5 at E12 and E13.25 (e,i,j). Purple arrowhead in h shows epicardial expression. Immunostaining against Nkx2-5 is shown as a control for all cardiomyocytes.
Supplementary Figure 6: In situ hybridization for Tbx5, Tbx20, and Bmp10 on serial adjacent sections from a Stage 17 turtle heart. Bottom row shows Bmp10 mRNA from a stage 18 heart, and from a stage 15 heart. All are transverse sections, from anterior (front) to posterior (back).
Supplementary Figure 7. Quantitation of Tbx5 transcripts in microdissected heart tissues. Top row shows experimental stages and dissection strategy to isolate LV and RV tissue from chick, anole, and turtle embryonic hearts at the indicated stages. Bottom graphs show quantitation of Tbx5 mRNA levels relative to the earliest stage RV sample. Blue bars: RV, red bars: LV. Data are shown as mean +/- SEM; n=3-7 for each sample. *P<0.01 vs RV.

Supplementary Figure 8: Expression of Dkk3 and Slit2 in wild-type (WT) and Tbx5^{AHFdel/AHFdel} hearts at E10.5.
Supplementary Figure 9: External morphology (a) and In situ hybridization (b) for Slit2 and Irx2 in wild-type (WT) and CAT-Tbx5;Nkx2.5::Cre embryonic hearts at E11.5.