Figures and figure supplements

A calcium transport mechanism for atrial fibrillation in Tbx5-mutant mice

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Figure 1. Atrial fibrillation in Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup> mice is associated with altered expression of genes important to cellular calcium handling. (A) Tbx5<sup>fl/fl</sup>; R26<sup>CreERT2</sup> mice developed spontaneous AF as assessed by surface ECG compared to R26<sup>CreERT2</sup>. Traces are representative of 15 animals per group. Figure 1 continued on next page.
genotype. (B) Poincaré plot shows irregularly irregular rhythm in \textit{Tbx5}^{fl/fl};\textit{R26}^{CreERT2}, consistent with AF, compared to normal sinus rhythm in \textit{R26}^{CreERT2} mice. Poincaré plots are each from one animal, and representative of 15 animals per genotype. (C) Simultaneous AP and [Ca$^{2+}$] recordings show prolonged AP duration and slowed [Ca$^{2+}$] transient decay in \textit{Tbx5}^{fl/fl};\textit{R26}^{CreERT2} atrial cardiomyocytes compared to \textit{R26}^{CreERT2}. Recordings are representative of simultaneous [Ca$^{2+}$] and \textit{E}_m recordings (myocytes/mice; dual \textit{E}_m and [Ca$^{2+}$] from 5/5 \textit{R26}^{CreERT2} and 17/5 \textit{Tbx5}^{fl/fl};\textit{R26}^{CreERT2}, \textit{E}_m only from 23/9 \textit{R26}^{CreERT2} and 20/9 \textit{Tbx5}^{fl/fl};\textit{R26}^{CreERT2}, and [Ca$^{2+}$] only from 27/6 \textit{R26}^{CreERT2} and 28/6 \textit{Tbx5}^{fl/fl};\textit{R26}^{CreERT2}). (D) Quantitative PCR was performed on RNA isolated from left atrial tissue of 3–5 animals per genotype. mRNA expression of a panel of calcium handling genes potentially important for rhythm regulation was determined. \textit{Ryr2} and \textit{Atp2a2} expression were decreased and \textit{Pln} expression was increased in \textit{Tbx5}^{fl/fl};\textit{R26}^{CreERT2} relative to \textit{R26}^{CreERT2}. (***p<0.001, **, p<0.01, *, p<0.05).

DOI: https://doi.org/10.7554/eLife.41814.003
## PCR primers

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*Figure 1—figure supplement 1. PCR primers.*

DOI: https://doi.org/10.7554/eLife.41814.004
Figure 2. Calcium current blockade dramatically shortened the AP in Tbx5^{5fl};R26^{CreERT2} atrial cardiomyocytes, consistent with the [Ca]$_i$ dependence of AP prolongation following TBX5 loss. (A) Representative recording of an AP from R26^{CreERT2} atrial cardiomyocytes before and after 30 μM nifedipine treatment. (B) Representative recording of a Tbx5^{5fl};R26^{CreERT2} atrial cardiomyocytes before and after nifedipine treatment. (C) Paired APD properties before and after treatment with 30 μM nifedipine (myocytes/mice; n = 8/3 Tbx5^{5fl};R26^{CreERT2} and n = 6/4 R26^{CreERT2}). In R26^{CreERT2} cardiomyocytes, the effect of nifedipine on APD90 was small, but significant 19 ± 4%. A much larger nifedipine effect was observed in Tbx5^{5fl};R26^{CreERT2} cardiomyocytes.

Figure 2 continued on next page.
APD50 decreased by 16 ± 6% and APD90 decreased by 61 ± 6% in the presence of nifedipine. (D) Western blot of atrial tissue in five animals for each genotype showed protein expression for the alpha 1C subunit of the L-type calcium channel (Ca$_{\text{v}}$1.2) was unchanged. (normalized to GAPDH) (E,F) Representative $I_{\text{CaL}}$ recordings show Peak L-type calcium current was increased in Tbx5$^{fl/fl}$;R26$^{CreERT2}$ cardiomyocytes compared to R26$^{CreERT2}$. (G) Average IV relationship of L-type calcium current (myocytes/mice, n = 22/7 R26$^{CreERT2}$ and 20/5 Tbx5$^{fl/fl}$;R26$^{CreERT2}$). (***p<0.001, **, p<0.01, *, p<0.05). DOI: https://doi.org/10.7554/eLife.41814.006
Figure 2—figure supplement 1. 30 µM Nifedipine blocks L-type calcium current and calcium-induced calcium release in R26CreERT2 and Tbx5fl/fl;R26CreERT2 cardiomyocytes.

DOI: https://doi.org/10.7554/eLife.41814.007
Figure 2—figure supplement 2. Steady state inactivation of $I_{CaL}$ was unchanged in $Tbx5^{fl/fl};R26^{CreERT2}$ atrial cardiomyocytes. (A) Protocol used to assess steady state inactivation of $I_{CaL}$ (representative trace from $Tbx5^{fl/fl};R26^{CreERT2}$ cardiomyocytes) (B) Steady state inactivation of $I_{CaL}$ was the same in $R26^{CreERT2}$ and $Tbx5^{fl/fl};R26^{CreERT2}$ cardiomyocytes. (myocytes/mice; Representative of 4/9 $R26^{CreERT2}$ and 3/10 $Tbx5^{fl/fl};R26^{CreERT2}$).

DOI: https://doi.org/10.7554/eLife.41814.008
Figure 3. Spark frequency is reduced in Tbx5\textsuperscript{fl/fl};R26\textsuperscript{CreERT2} atrial cardiomyocytes. (A) Western blot from atrial tissue from 10 animals per genotype was used to measure RyR2 expression. RyR2 was significantly decreased in Tbx5\textsuperscript{fl/fl};R26\textsuperscript{CreERT2} atria compared to R26\textsuperscript{CreERT2} atria (normalized to GAPDH). (B) Fluo-4 loaded cardiomyocytes demonstrated reduced spark frequency in Tbx5\textsuperscript{fl/fl};R26\textsuperscript{CreERT2} compared to R26\textsuperscript{CreERT2} atrial cardiomyocytes (representative recordings). (C) Spark frequency was reduced at rest and after steady state pacing at different frequencies (myocytes/mice; n = 12/4 Tbx5\textsuperscript{fl/fl};R26\textsuperscript{CreERT2} and n = 12/3 R26\textsuperscript{CreERT2}). (D) Ryanodine binding assay (without normalization) demonstrated no significant difference over the physiologic range of [Ca\textsuperscript{2+}] in Tbx5\textsuperscript{fl/fl};R26\textsuperscript{CreERT2} compared to R26\textsuperscript{CreERT2} (Weng et al., 2018). Each measure corresponds to an assay performed on pooled atria from 8 to 10 mice with three independent measures per condition (*p<0.05, **p<0.01, ***p<0.001).

DOI: https://doi.org/10.7554/eLife.41814.012
Figure 4. SERCA function is decreased while NCX function is increased in Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup> atrial cardiomyocytes. (A) Expression of SERCA2 was significantly decreased (normalized to GAPDH) while (B) expression of NCX1 was significantly increased in Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup> atria compared to R26<sup>CreERT2</sup> atria as measured by western blot in 10 animals per genotype. (normalized to GAPDH) (C) Application of Na<sup>+</sup> free caffeine solution after pacing to steady state at 1 Hz provided a measurement of SR load. In the absence of extracellular Na<sup>+</sup>, [Ca<sup>2+</sup>]<sub>i</sub> plateaud at high levels due to negligible role of non-NCX mediated extrusion in R26<sup>CreERT2</sup> and Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup> atrial cardiomyocytes. Removal of caffeine in the absence of external Na<sup>+</sup> provided a measure of SERCA mediated SR calcium uptake (representative traces). (D) Restoration of external Na<sup>+</sup>, in the presence of sustained extracellular caffeine provided a measure of NCX mediated calcium efflux (representative traces). (E) The peak of steady state twitch [Ca<sup>2+</sup>]<sub>i</sub> transient was similar but (F) tau of [Ca<sup>2+</sup>]<sub>i</sub> decay, determined from twitch [Ca<sup>2+</sup>]<sub>i</sub> transients, was increased in Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup> compared to R26<sup>CreERT2</sup> cardiomyocytes (myocytes/mice; n = 27/6 R26<sup>CreERT2</sup>, n = 28/6 Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup>). (G) SR load, determined from peak caffeine transients was decreased in Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup> compared to R26<sup>CreERT2</sup> cardiomyocytes (myocytes/mice; n = 34/6 R26<sup>CreERT2</sup>, n = 32/6 Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup>). (H) SERCA activity, determined from the maximal rate of calcium decay was diminished in Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup> compared to R26<sup>CreERT2</sup> cardiomyocytes (myocytes/mice; n = 27/6 R26<sup>CreERT2</sup>, n = 28/6 Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup>). Figure 4 continued on next page.
n = 29/3 R26CreERT2, n = 32/3 Tbx5fl/fl;R26CreERT2). (I) NCX activity (decay slope), was increased at all levels of calcium in Tbx5fl/fl;R26CreERT2 cardiomyocytes (myocytes/mice; n = 35/3 R26CreERT2, n = 21/3 Tbx5fl/fl;R26CreERT2). (*p<0.05, **p<0.01, ***p<0.001).

DOI: https://doi.org/10.7554/eLife.41814.016
Figure 4—figure supplement 1. [Ca$^{2+}$]$_i$ transients recorded using 40 ms voltage clamp pulses demonstrate 23 ± 4% (p=0.02) reduction in peak calcium in Tbx5$^{fl/fl}$;R26$^{CreERT2}$ atrial cardiomyocytes. While the decay kinetics of Tbx5$^{fl/fl}$;R26$^{CreERT2}$ are slowed, there no differences in the initial rate of [Ca$^{2+}$]$_i$ rise (R26$^{CreERT2}$ rise slope = 107 ± 11 F/Fo•s; Tbx5$^{fl/fl}$;R26$^{CreERT2}$ rise slope = 108 ± 18 F/Fo•s, p=0.96). Average of all myocytes is shown in black with grey indicating standard error of mean (myocytes/mice; n = 5/3 R26$^{CreERT2}$, n = 13/5 Tbx5$^{fl/fl}$;R26$^{CreERT2}$).

DOI: https://doi.org/10.7554/eLife.41814.017
Figure 5. Phospholamban knockout normalized SERCA function in Tbx5\(^{fl/fl}\);R26\(^{CreERT2}\). (A) PLN expression was increased in Tbx5\(^{fl/fl}\);R26\(^{CreERT2}\) compared to R26\(^{CreERT2}\) as measured by western blot with five animals per genotype. PLN expression was normalized to GAPDH. The proportion of PLN S16, but not T17 phosphorylation was also increased (normalized to PLN). (B) Representative SR load and SERCA measurements in R26\(^{CreERT2}\), Tbx5\(^{fl/fl}\);R26\(^{CreERT2}\), Pln\(^{-/-}\);R26\(^{CreERT2}\), Tbx5\(^{fl/fl}\);Pln\(^{+/+}\);R26\(^{CreERT2}\) and Tbx5\(^{fl/fl}\);Pln\(^{-/-}\);R26\(^{CreERT2}\) atrial cardiomyocytes were collected as described in Figure 4. (C, D) SR load and SERCA function were significantly higher in Tbx5\(^{fl/fl}\);Pln\(^{+/+}\);R26\(^{CreERT2}\) and Tbx5\(^{fl/fl}\);Pln\(^{-/-}\);R26\(^{CreERT2}\) compared to Tbx5\(^{fl/fl}\);R26\(^{CreERT2}\) cardiomyocytes and comparable to R26\(^{CreERT2}\) cardiomyocytes. (E) [Ca\(^{2+}\)]\(_{i}\) transient peaks were unchanged in Tbx5\(^{fl/fl}\);Pln\(^{+/+}\);R26\(^{CreERT2}\), Tbx5\(^{fl/fl}\);Pln\(^{-/-}\);R26\(^{CreERT2}\) and Tbx5\(^{fl/fl}\);Pln\(^{-/-}\);R26\(^{CreERT2}\) cardiomyocytes, but increased in Pln\(^{-/-}\);R26\(^{CreERT2}\) cardiomyocytes. (F) [Ca\(^{2+}\)]\(_{i}\) transient decay rate in Tbx5\(^{fl/fl}\);Pln\(^{+/+}\);R26\(^{CreERT2}\) and Tbx5\(^{fl/fl}\);Pln\(^{-/-}\);R26\(^{CreERT2}\) cardiomyocytes were normalized to that of R26\(^{CreERT2}\) cardiomyocytes (myocytes/mice; n = 34/3 R26\(^{CreERT2}\), n = 36/3 Tbx5\(^{fl/fl}\);R26\(^{CreERT2}\), n = 30/3 Pln\(^{+/+}\);R26\(^{CreERT2}\), n = 21/3 Tbx5\(^{fl/fl}\);Pln\(^{-/-}\);R26\(^{CreERT2}\), n = 27/3 Tbx5\(^{fl/fl}\);Pln\(^{-/-}\) atrial cardiomyocytes). (*p<0.05, **p<0.01, ***p<0.001).

DOI: https://doi.org/10.7554/eLife.41814.022
Figure 6. PLN knockout normalized AP duration and prevented triggered activity in Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup>. Representative APs recorded from (A) R26<sup>CreERT2</sup>, (B) Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup>, (C) Pln<sup>-/-</sup>;R26<sup>CreERT2</sup>, (D) Tbx5<sup>fl/fl</sup>;Pln<sup>-/-</sup>;R26<sup>CreERT2</sup>, (E) Tbx5<sup>fl/fl</sup>;Pln<sup>-/-</sup>;R26<sup>CreERT2</sup> atrial cardiomyocytes as described previously in Figure 2. (F) TBX5-loss dependent AP prolongation and frequency of triggered activity was normalized by phospholamban knockout (myocytes/mice: n = 18/7 R26<sup>CreERT2</sup>, n = 24/12 Tbx5<sup>fl/fl</sup>;R26<sup>CreERT2</sup>, n = 12/5 Pln<sup>-/-</sup>;R26<sup>CreERT2</sup>, n = 9/3 Tbx5<sup>fl/fl</sup>;Pln<sup>-/-</sup>;R26<sup>CreERT2</sup>, and n = 22/3 Tbx5<sup>fl/fl</sup>;Pln<sup>-/-</sup>;R26<sup>CreERT2</sup>), (*p<0.05, **p<0.01, ***p<0.001).

DOI: https://doi.org/10.7554/eLife.41814.025
Figure 7. PLN deficiency protected against TBX5-loss associated AF. Intra-atrial pacing was used to induce AF. Representative intracardiac atrial electrogram recordings and corresponding surface ECG are shown from (A) Figure 7 continued on next page
Figure 7 continued

R26CreERT2, (B) Tbx5fl/fl;R26CreERT2, (C) Pln−/−;R26CreERT2, (D) Tbx5fl/fl;Pln−/+;R26CreERT2, (E) Tbx5fl/fl;Pln−/+;R26CreERT2 atrial cardiomyocytes. A, atrial electrical signal; V, far field ventricular electrical signal. (F) AF was reproducibly demonstrated in 6/6 Tbx5 knockouts in contrast to 1/11 Pln/Tbx5 double knockouts, indicating rescue of atrial arrhythmogenesis. P values were determined by Fisher's exact test (n = 5 R26CreERT2, n = 6 Tbx5fl/fl;R26CreERT2, n = 7 Pln−/−;R26CreERT2, and n = 5 Tbx5fl/fl;Pln−/+;R26CreERT2, n = 11 Tbx5fl/fl;Pln−/− mice).

DOI: https://doi.org/10.7554/eLife.41814.027
Figure 8. Model of TBX5-dependent calcium regulation in atrial cardiomyocytes. (A) Excitation-contraction coupling of atrial cardiomyocytes is achieved through regulation of intracellular calcium handling. (B) Adult-specific Tbx5 knockout leads to decreased expression of SERCA2 and increased expression of PLN, leading to decreased SR Ca^{2+} load. In addition, removal of Tbx5 is associated with increased NCX1 expression and activity, thereby increasing Ca^{2+} extrusion, which is balanced by increased L-type calcium entry. (C) Combined Tbx5/Pln knockout relieves repression of SERCA2. This results in normalization of SERCA activity and rescue of cardiomyocyte ectopy, triggered activity, and AF observed with Tbx5 deficiency.

DOI: https://doi.org/10.7554/eLife.41814.028