ESC European Society of Cardiology

Cardiovascular Research (2022) **118**, 2805–2818
https://doi.org/10.1093/cvr/cvab325

Genetic inhibition of nuclear factor of activated T-cell c2 prevents atrial fibrillation in CREM transgenic mice

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Received 18 December 2018; editorial decision 5 October 2021; accepted 11 October 2021; online publish-ahead-of-print 14 October 2021

Time for primary review: 33 days

Aims Abnormal intracellular calcium (Ca^{2+}) handling contributes to the progressive nature of atrial fibrillation (AF), the most common sustained cardiac arrhythmia. Evidence in mouse models suggests that activation of the nuclear factor of activated T-cell (NFAT) signalling pathway contributes to atrial remodelling. Our aim was to determine the role of NFATc2 in AF in humans and mouse models.

Methods and results Expression levels of NFATc1–c4 isoforms were assessed by quantitative reverse transcription–polymerase chain reaction in right atrial appendages from patients with chronic AF (cAF). NFATc1 and NFATc2 mRNA levels were elevated in cAF patients compared with those in normal sinus rhythm (NSR). Western blotting revealed increased cytosolic and nuclear levels of NFATc2 in AF patients. Similar findings were obtained in CREM-IbΔC-X transgenic (CREM) mice, a model of progressive AF. Telemetry ECG recordings revealed age-dependent spontaneous AF in CREM mice, which was prevented by NFATc2 knockout in CREM:NFATc2$^{-/-}$ mice. Programmed electrical stimulation revealed that CREM:NFATc2$^{-/-}$ mice lacked an AF substrate. Morphometric analysis and histology revealed increased atrial weight and atrial fibrosis in CREM mice compared with wild-type controls, which was reversed in CREM:NFATc2$^{-/-}$ mice. Confocal microscopy showed an increased Ca^{2+} spark frequency despite a reduced sarcoplasmic reticulum (SR) Ca^{2+} load in CREM mice compared with controls, whereas these abnormalities were normalized in CREM:NFATc2$^{-/-}$ mice. Western blotting revealed that genetic inhibition of Ca^{2+}/calmodulin-dependent protein kinase II-mediated phosphorylation of S2814 on ryanodine receptor type 2 (RyR2) in CREM:RyR2-S2814A mice suppressed NFATc2 activation observed in CREM mice, suggesting that NFATc2 is activated by excessive SR Ca^{2+} leak via RyR2. Finally, chromatin immunoprecipitation sequencing from AF patients identified Ras and EF-hand domain-containing protein (Rasef) as a direct target of NFATc2-mediated transcription.

Conclusion Our findings reveal activation of the NFAT signalling pathway in patients of Chinese and European descent. NFATc2 knockout prevents the progression of AF in the CREM mouse model.

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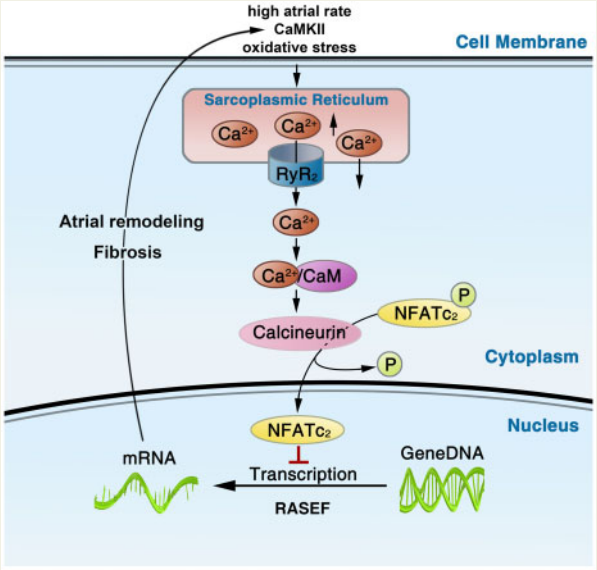
Graphical Abstract



Keywords Atrial fibrillation • Atrial remodelling • Calcium handling • NFAT • RASEF

1. Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia disorder associated with high cardiovascular morbidity and mortality rates.[1] Current pharmacological treatment options or ablation procedures remain ineffective in up to 25–30% of AF patients.[2] Therefore, there is a great need to improve our understanding of the mechanisms underlying AF, such that new therapeutic targets can be identified for drug development efforts.

Recent studies suggest that abnormal intracellular calcium (Ca^{2+}) handling contributes to the progressive nature of AF. First, enhanced diastolic Ca^{2+} release via ryanodine receptor type 2 (RyR2) can promote cellular afterdepolarizations and triggered activity in the heart.[3,4] Secondly, our group demonstrated that RyR2-mediated SR Ca^{2+} leak causes the development of atrial structural remodelling required for the progress of the substrate permissive of spontaneous AF (sAF) in a mouse model.[4] In these CREM-IbΔC-X transgenic (CREM) mice,[5] we found evidence for activation of the Ca^{2+}-dependent hypertrophic pathway involving nuclear factor of activated T-cell (NFAT)/regulator of calcineurin 1-4 (Rcan1-4) signalling, suggesting a role for this signalling pathway in the development of a substrate for long-lasting AF in CREM mice.[4]

The NFAT family of inducible transcription factors includes five members (NFATc1–c4 and NFAT5), each with distinct expression patterns. NFAT5 has DNA-binding specificity and regulatory interactions that are distinct from NFATc1–c4.[6] Calcineurin, also known as protein phosphatase 2B, dephosphorylates NFATc1–c4 in response to increased intracellular Ca^{2+} levels, which leads to nuclear translocation of NFAT.[7] In the nucleus, NFAT transcription complexes assemble on DNA to activate downstream genes.[7] A growing body of evidence shows the importance of the calcineurin/NFAT pathway in heart disease including cardiac hypertrophy and heart failure.[8–10] Moreover, enhancement of NFAT activity has been associated with AF and atrial tachycardia remodelling of the L-type Ca^{2+} current.[11] Sustained elevation of cytosolic Ca^{2+} occurs in the early stages of AF,[12] and this can lead to calcineurin–NFAT pathway activation.[13] Tachypacing of atrial cardiomyocytes was shown to initiate NFATc3/c4 translocation into the nucleus, to suppress transcription of the Cav1.2 Ca^{2+} channel subunit encoded by CACNA1C, to reduce the I_{CaL} current, to shorten the action potential duration, and to promote re-entry.[11]

Prior studies revealed that NFATc2 is the most abundantly expressed NFAT isoform in murine heart ventricle.[10] On the other hand, little is known about the relative levels of NFAT isoforms and their nuclear targets in the atrial myocardium. Therefore, we examined expression levels of NFAT isoforms in right atrial tissue samples from patients with chronic (persistent) AF compared with patients in normal sinus rhythm (NSR). Fractionation of nuclear and cytosolic fractions revealed increased nuclear translocation of NFATc2 in patients with AF. Similarly, increased nuclear translocation of NFATc2 was observed in CREM-IbΔC-X transgenic (CREM) mice, an animal model of spontaneous-onset AF showing similarities to human AF.[14] Chromatin immunoprecipitation sequencing (ChiP-seq) was performed on human atrial samples, and bioinformatics analysis was carried out to identify possible binding sites of NFATc2. The candidate genes were further verified by fluorescent quantitative PCR in clinical samples. Expression of the '*Ras and EF-hand domain-containing protein*' (*RASEF*) gene was significantly increased in atrial samples from patients with AF. Genetic inhibition of NFATc2 in CREM mice (CREM:NFATc2$^{-/-}$) prevented *RASEF* gene expression, as well as the development of sAF, atrial dilatation, and fibrosis. Moreover, intracellular Ca^{2+} handling abnormalities observed in CREM mice were normalized in CREM:NFATc2$^{-/-}$ mice.

Together, these findings suggest that enhanced NFATc2 nuclear translocation may be a key early event in AF progression through the

regulation of RASEF expression. Therefore, preventing NFATc2 activation and suppressing RASEF expression may be novel strategies to prevent AF development.

2. Methods

A detailed description of all methods is provided in the Supplementary material online.

2.1 Human atrial samples

Collection of human tissue samples was approved by the Institutional Review Board of the Medical Faculty Mannheim, Heidelberg University, and the Ethics Committee of Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology. All studies were performed according to the principles outlined in the Declaration of Helsinki. Following informed consent, right atrial appendages were obtained during open-heart surgery and flash-frozen in liquid nitrogen.

2.2 Animal studies

All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine conforming to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). CREM-IbΔC-X (CREM) transgenic mice were on an FVB/N background,[5] and NFATc2-deficient mice on a B6Jx129F1 background were provided by Dr. L. de Windt.[10] We intercrossed CREM mice with Nfatc2$^{-/-}$ mice for at least eight generations to obtain the CREM:NFATc2$^{-/-}$ mice. Male and female wild-type (WT), Nfatc2$^{-/-}$, CREM, and CREM:NFATc2$^{-/-}$ mice on a mixed genetic background were used in the current study.

2.3 Western blotting

Tissues were snap-frozen in liquid nitrogen. Nuclear and cytoplasmic cell fractionation and western blotting were performed as detailed in the Supplementary material online.

2.4 Histology

Mice were anaesthetized with 2% isoflurane and humanely euthanized by cervical dislocation. Mouse hearts were excised, washed in normal saline, fixed in 10% buffered formalin, and dehydrated in an ethanol series. Longitudinal 5 μm sections were cut, placed on slides, and deparaffinized. Sections were stained with Masson's trichrome (Thermo Fisher Scientific, Waltham, MA, USA) for fibrosis. Fibrosis was quantified as described.[15]

2.5 Telemetry ECG recordings

Mice were anaesthetized using 2% isoflurane in 0.5 L/min 100% O_2. ECG telemeters (Data Sciences International, New Brighton, MN, USA) were implanted in the abdominal cavity with subcutaneous electrodes in lead II configuration as described.[16] Twenty-four-hour ambulatory ECGs were recorded in conscious mice at 3, 5, and 7 months of age. Recordings were analysed by using ECG-Auto software (EMKA, Middletown, PA, USA). Spontaneous atrial ectopic events were quantified by manual analysis of ECG tracings obtained between 12 p.m. and 1 p.m. to exclude confounding effects of circadian variation. When a mouse exhibited at least one atrial ectopic complex during this 1 h recording, it was considered as atrial ectopy positive. When a mouse exhibited one episode of AF, defined as absence of P-waves and irregular R–R intervals, lasting longer than 10 s during a 24 h recording, it was considered positive for sAF.

2.6 Intracardiac electrophysiology studies

In vivo electrophysiology studies were performed in mice at 4–5 months of age, as described.[17] Mice were anaesthetized using 2% isoflurane in 0.5 L/min 100% O_2. Inducible AF was defined as at least two out of three burst pacing trains resulting in AF episodes lasting >1 s. A detailed description is provided in the Supplementary material online.

2.7 Transthoracic echocardiography

Cardiac function was assessed using a VisualSonics VeVo 770 Imaging System (VisualSonics, Toronto, Canada) equipped with high-frequency 30 MHz probe, as described.[18] Mice were anaesthetized using 1.5±0.5% isoflurane maintaining a heart rate >400 bpm while measurements were made. In addition, body temperature was maintained in a range (37.0 ± 1.0°C) to avoid confounding effects of hypothermia.

2.8 Atrial myocyte Ca^{2+} imaging

Atrial myocytes were isolated with collagenase as described.[3] Briefly, mice were anaesthetized with 2% isoflurane and humanely euthanized by cervical dislocation. Atrial myocytes were loaded with 2 mmol/L Fluo-4-AM (Invitrogen, Carlsbad, CA, USA) in normal Tyrode solution containing 1.8 mmol/L Ca^{2+} for 30 min at room temperature.[17] Cells were subsequently imaged by confocal microscopy. Line scans were used to obtain Ca^{2+} spark data and transient amplitudes. Caffeine was used to determine the SR Ca^{2+} load. Data were analysed using Image J and the Spark Master plug-in. A detailed description is provided in the Supplementary material online.

2.9 Chromatin immunoprecipitation sequencing

ChIP-seq library generation was performed as described.[19] Human atrium samples (50 mg each) from patients in AF ($n = 2$) and NSR ($n = 2$) were used for these studies. A detailed description is provided in the Supplementary material online.

2.10 Statistical analysis

Continuous variables are presented as mean ± standard error of the mean and categorical data are presented as percentages. Statistical analysis was performed using SPSS Statistics (IBM, New York, NY, USA). The generalized estimating equation approach was performed using a binomial distribution to study the dichotomous spontaneous SR Ca^{2+} release event and fibrosis outcomes. One-way ANOVA followed by the *post hoc* Bonferroni *t*-test was used for multiple group non-repeated measures data and paired *t*-test for single repeated measures. Fisher's exact test was used to compare categorical data. To compare continuous variables with a skewed distribution, the Mann–Whitney *U* test and Kruskal–Wallis test were applied. *P*-value <0.05 was considered statistically significant.

3. Results

3.1 Increased NFATc2 expression and translocation in patients with chronic AF

To examine whether there is increased NFAT activity in the atria of patients with chronic AF (cAF), we first determined the relative

abundance of all four calcineurin-regulated members of the NFAT family (NFATc1–c4) in right atrial appendage samples of cAF patients from Germany and China. Patient characteristics are provided in Supplementary material online, *Tables SI and SII*. In German patients, who are all of the Caucasian descent, NFATc1–c3 mRNA levels were increased compared with patients in normal sinus rhythm (NSR) (Supplementary material online, *Table S III* and *Figure 1A*). To determine whether similar changes occurred in patients of East Asian descent, we perform the same analysis on right atrial appendage samples of cAF patients from China. In these patients with a Han ethnic background, similar amounts of NFATc1 and NFATc2 upregulation were observed compared with NSR patients (*Figure 1B*).

Next, we determined the protein levels of NFAT isoforms in both cytosolic and nuclear fractions of right atrial samples from Chinese patients. Western blotting confirmed proper fractionation since GAPDH was exclusively detected in cytosolic fractions, while histone H3 was only found in nuclear samples (*Figure 1C–F*). Protein levels of NFATc2 and NFATc4 were increased in the cytosolic fraction, but only NFATc2 levels were increased in the nuclear fractions of cAF patients compared with patients in NSR (*Figure 1G and H*). These findings suggest that only NFATc2 nuclear translocation is significantly enhanced in patients with cAF.

3.2 Upregulation of NFAT expression and translocation in CREM mice

Transgenic mice with cardiomyocyte-restricted overexpression of transcriptional repressor CREM-IbΔC-X (CREM) have been used as an animal model to study the progressive nature of AF.[4] Here, we measured mRNA expression levels of NFAT isoforms in atrial samples from 3- and 7-month-old CREM mice and WT littermates (Supplementary material online, *Table S III* and *Figure 2A and B*). Expression of NFATc2–4 isoforms was modestly enhanced in 3-month-old CREM mice, but only NFATc2 and NFATc4 were increased in 7-month-old CREM mice compared with WT littermates. Next, we determined the protein levels of NFAT isoforms in both cytosolic and nuclear fractions of mouse atrial samples. For subsequent studies, we focused on the NFATc2 isoform since it was the most consistently altered isoform in both human AF patients and CREM mice, but used the NFATc1 isoform as a control. In 3-month-old mice, NFATc1 and NFATc2 levels were increased in the cytosolic fraction of atrial tissue from CREM mice, whereas only NFATc2 was significantly enhanced in the nuclear fraction (*Figure 2C–F*). At 7 months of age, both NFATc1 and NFATc2 protein levels were increased in the cytosolic and nuclear fractions of atrial tissue from CREM mice (*Figure 2G–J*). These studies suggest that the NFAT signalling pathway, in particular NFATc2, is activated prior to development of cAF in CREM mice.

3.3 Genetic inhibition of NFATc2 prevents development of atrial ectopy and sAF in CREM mice

To determine whether NFATc2 is required for the progression of atrial arrhythmias in CREM mice, we crossed CREM mice with NFATc2$^{-/-}$ mice to generate CREM:NFATc2$^{-/-}$ mice. Western blots confirmed the absence of NFATc2 expression in atrial samples from NFATc2$^{-/-}$ and CREM:NFATc2$^{-/-}$ mice (Supplementary material online, *Figure SI*). Next, 24 h ECG telemetry recordings were performed in cohorts of WT, NFATc2$^{-/-}$, CREM, and CREM:NFATc2$^{-/-}$ mice at 3, 5, and 7 months of age (*Figure 3A and B* and Supplementary material online, *Figure SII*). CREM mice began to exhibit premature atrial contractions (PACs) at ~3 months of age. Quantification of PACs revealed a higher incidence (95.7 ± 70.2 events/h) in 3-month-old CREM mice compared with WT and NFATc2$^{-/-}$ mice (*Figure 3C*). In contrast, CREM:NFATc2$^{-/-}$ mice showed a lower incidence of PACs (2.5 ± 1.2 events/h). At 3 months of age, 31% (4 of 13) of CREM mice exhibited at least one episode of sAF during the 24 h monitoring period (*Figure 3B*) compared with no episodes in WT or NFATc2$^{-/-}$ controls (*Figure 3C*). The average duration of the longest sAF episode in CREM mice was 60.4 ± 40.5 min (*Figure 3C*).

By 5 months of age, all of the CREM mice showed PACs (2745 ± 1238 events/h), while CREM:NFATc2$^{-/-}$ mice exhibited fewer PACs (11.9 ± 5.1 events/h) (*Figure 3D*). Genetic NFATc2 deficiency prevented the development of sAF in CREM:NFATc2$^{-/-}$ mice. By 5 months of age, 54% of CREM mice (7 of 13) showed at least one episode of sAF compared with none of the CREM:NFATc2$^{-/-}$ mice ($P < 0.05$; *Figure 3D*). The average duration of the longest sAF episode in CREM mice was 153.5 ± 48.6 min (*Figure 3D*). Because of the high incidence of sAF, PAC frequency was not quantified in 7-month-old CREM mice. By 7 months of age, 83% of CREM mice (10 of 12) experienced frequent and long-lasting episodes of sAF (*Figure 3E*), while only 11% (1 of 9) CREM:NFATc2$^{-/-}$ mice exhibited sAF. The average duration of the longest sAF episode was 678.1 ± 158.1 min for CREM mice and 120.0 ± 120.0 min for CREM:NFATc2$^{-/-}$ mice (*Figure 3E*). These results suggest that genetic inhibition of NFATc2 prevents the progression of atrial ectopic activity to sAF in CREM mice.

3.4 Inhibition of NFATc2 prevents the development of a substrate for AF in CREM mice

To evaluate whether NFATc2$^{-/-}$ plays a critical role in the formation of a substrate for AF, we performed programmed electric stimulation (PES) in cohorts of 5-month-old mice (*Figure 4A*). Under baseline conditions, there were not significant differences in heart rate, QRS and QTc intervals (Supplementary material online, *Table SIV*). However, CREM mice exhibited a small but significant prolongation in the PR interval compared with WT mice. PES studies were performed when the CREM mice were in sinus rhythm. Although (7 of 12) 58% of CREM mice developed AF after atrial burst pacing, only 1 of 10 (10%) of CREM:NFATc2$^{-/-}$ mice developed pacing-induced AF (*Figure 4B*). In addition, the average duration of the longest inducible AF episodes was much longer in CREM mice than in CREM:NFATc2$^{-/-}$ mice ($P < 0.05$) (*Figure 4C*). These findings suggest that genetic inhibition of NFATc2 prevents the development of a substrate permissive of sAF development in CREM mice.

3.5 Inhibition of NFATc2 attenuates atrial remodelling in CREM mice

To determine whether inhibition of NFATc2 blunts structural remodelling associated with the development of sAF,[20] hearts were examined from cohorts of 7-month-old mice. Consistent with our prior studies,[4] there was bilateral atrial enlargement in CREM mice compared with WT and NFATc2$^{-/-}$ mice (*Figure 5A*), with no changes in left ventricular ejection fraction or dimensions (Supplementary material online, *Table SV*). The ratio of atrial weight to tibia length (AW/TL) was increased in CREM mice (2.42 ± 0.36 mg/mm) compared with WT mice (0.36 ± 0.13 mg/mm; $P < 0.001$) and NFATc2$^{-/-}$ mice (0.39 ± 0.12 mg/mm; *Figure 5B*). In contrast, the AW/TL ratio was normalized in CREM:NFATc2$^{-/-}$ mice (0.48 ± 0.11 mg/mm; $P < 0.001$ vs. CREM) (*Figure 5B*).

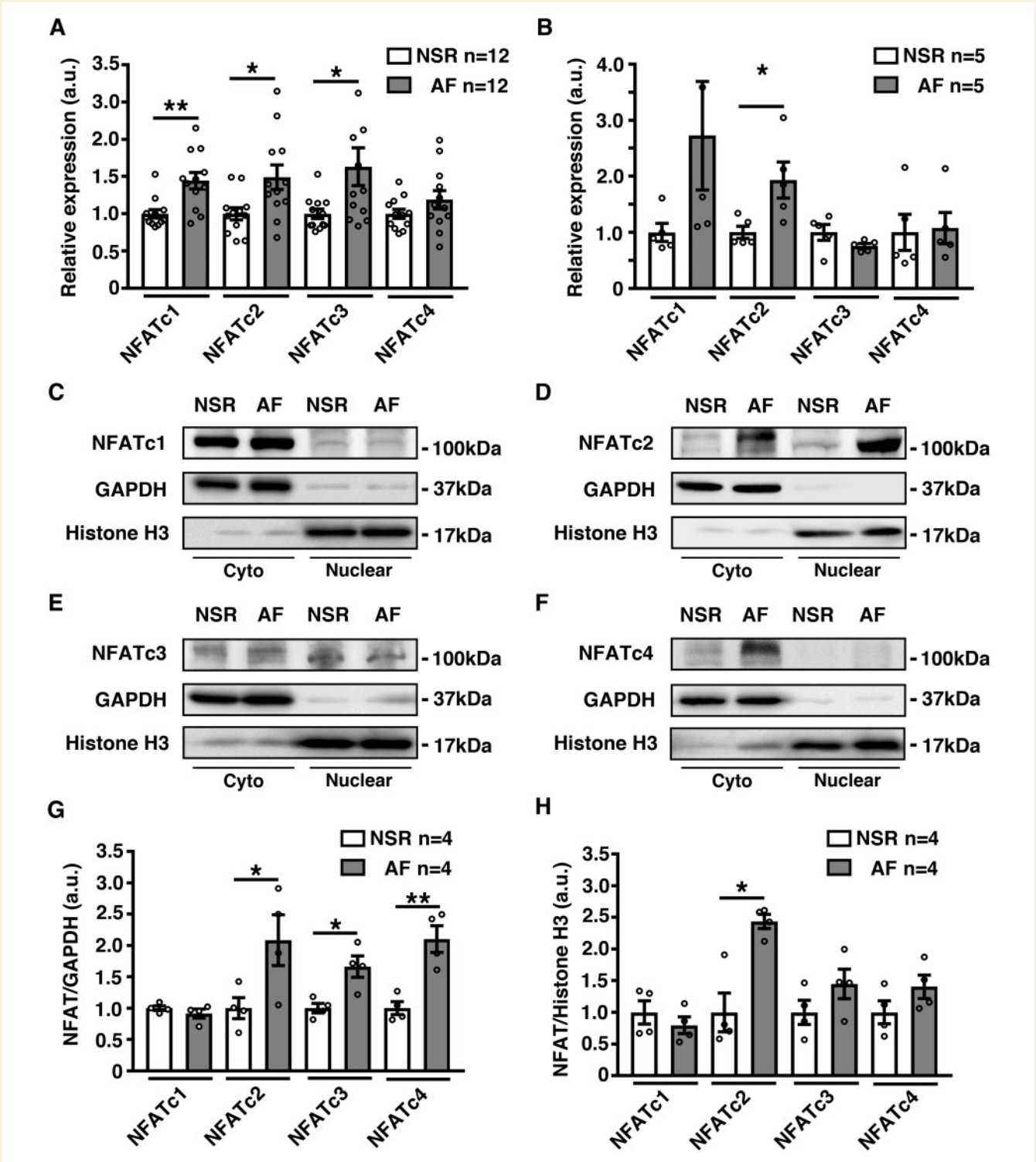


Figure 1 Upregulation of NFAT signalling pathway in atrial fibrillation (AF) patients. (*A*) NFATc1–c4 mRNA levels normalized to GAPDH levels in right atrial samples from chronic AF patients in Germany (NSR: $n = 12$, AF: $n = 12$) and (*B*) China (NSR: $n = 5$, AF: $n = 5$). (*C–F*) Western blots showing NFATc1–c4 in cytosolic and nuclear fractions from in right atrial samples from Chinese patients in normal sinus rhythm (NSR) or chronic AF. (*G* and *H*) quantifications of NFATc1–c4 proteins levels normalized to GAPDH (cytosolic fraction) or histone H3 (nuclear fraction), respectively (NSR: $n = 4$, AF: $n = 4$). AF, atrial fibrillation; NSR, normal sinus rhythm. Unpaired Student's *t*-test was used, $^{*}P < 0.05$, $^{**}P < 0.01$.

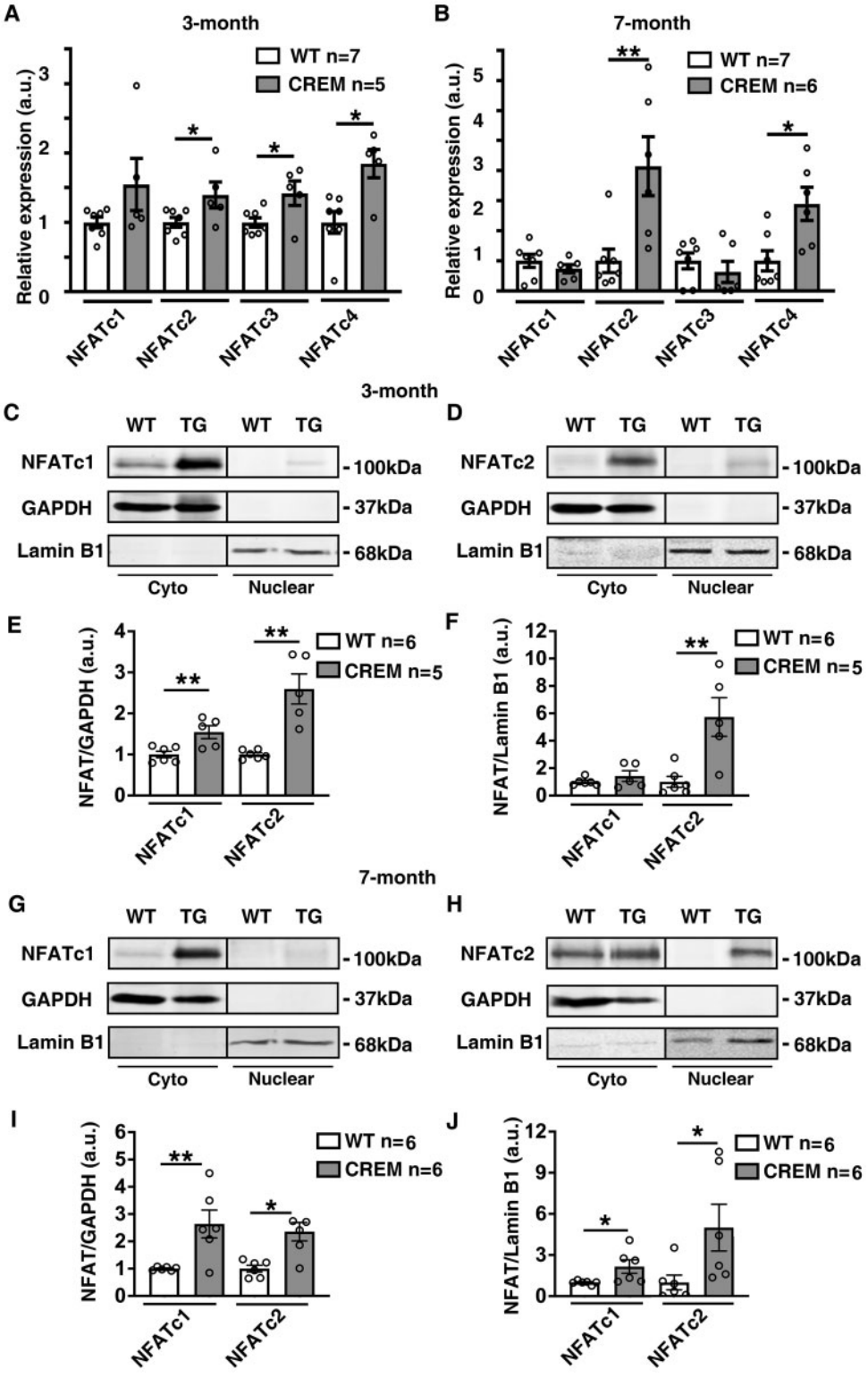


Figure 2 Upregulation of NFAT in CREM mice. (*A*) and (*B*) shows NFATc1--c4 mRNA normalized to L7 in atrial samples from CREM WT and TG mice at 3 months (WT: $n = 7$, CREM: $n = 5$) and 7 months (WT: $n = 7$, CREM: $n = 6$), respectively. Western blots showing NFATc1 and NFATc2 in cytosolic and nuclear fractions of the atria from 3-month-old (*C–F*) (WT: $n = 4$, CREM: $n = 4$) and 7-month-old (WT: $n = 4$, CREM: $n = 4$) mice (*G–J*), respectively. Cytosolic levels were normalized to GAPDH, nuclear fractions to lamin B1. Unpaired Student's *t*-test was used, $^*P < 0.05$.

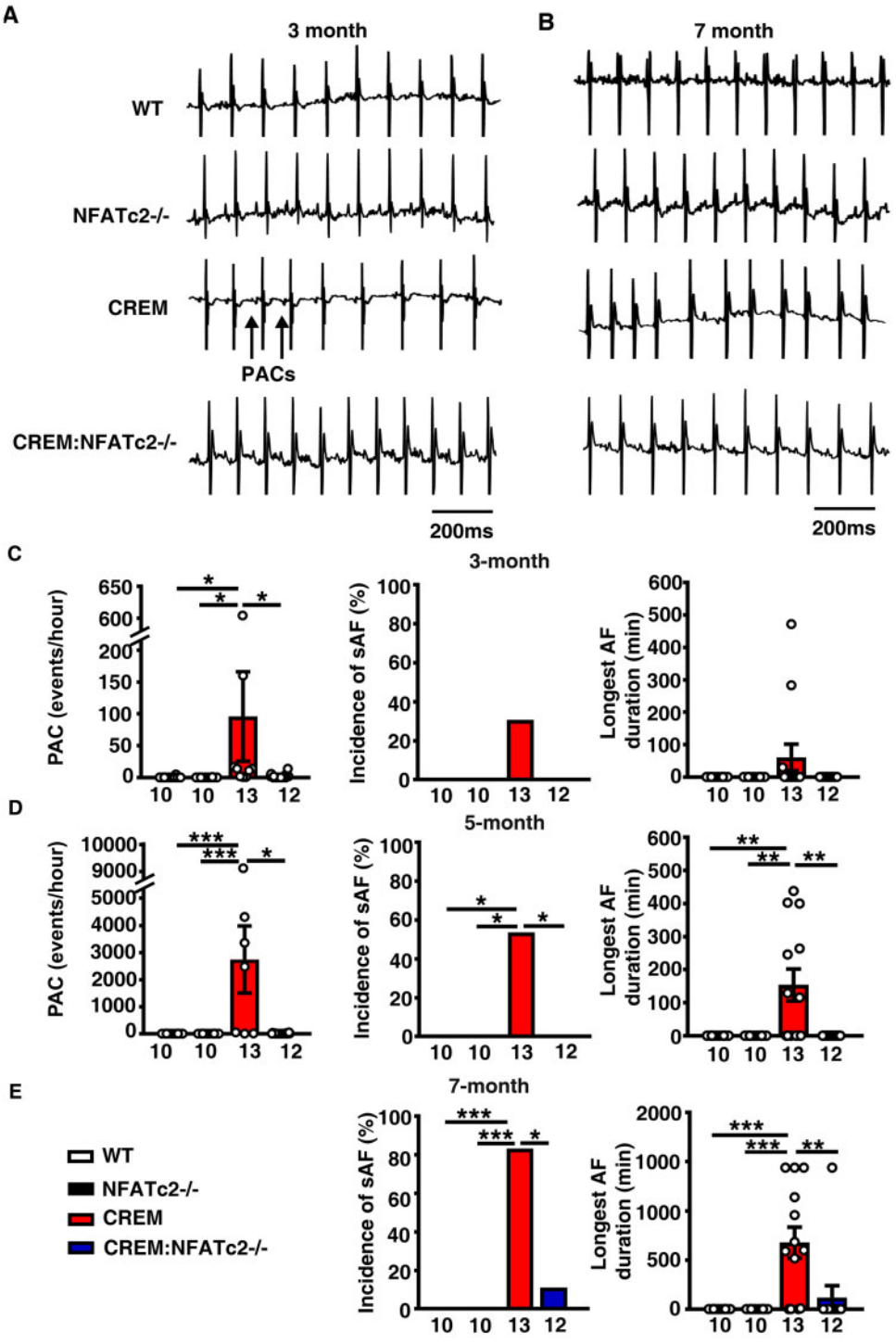


Figure 3 CREM:NFATc2$^{-/-}$ mice are protected from age-dependent development of spontaneous AF. (*A*) Representative ECG telemetry recordings showing premature atrial contractions (PAC) in 3-month-old CREM mice. (*B*) Representative ECG telemetry recordings showing spontaneous AF in 7-month old CREM mice, whereas the other genotypes were in sinus rhythm. (*C* and *D*) Left: the number of PACs per hour in mice that exhibited atrial ectopy at the age of 3 and 5 months. Middle: the incidence of sAF (as a fraction of the number of mice) at the age of 3 and 5 months. Right: the duration of longest episodes of sAF (in minutes) at the age of 3 and 5 months. (*E*) Middle: the incidence of sAF at the age of 7 months (as a fraction of the number of mice). Right: the duration of longest episodes of sAF (in minutes) at the age of 7 months. Numbers below the bars indicate the number of animals studied (WT: $n = 10$, NFATc2$^{-/-}$: $n = 10$, CREM: $n = 13$, CREM:NFATc2$^{-/-}$: $n = 12$). One-way ANOVA followed by the Kruskal–Wallis test was applied. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

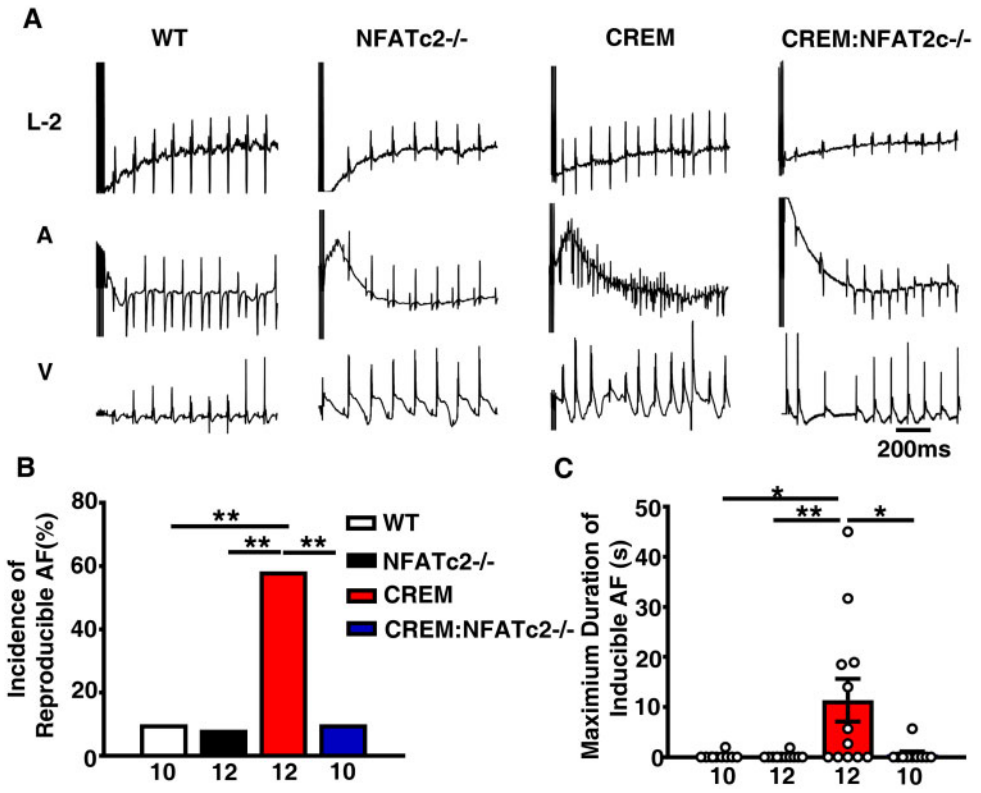


Figure 4 Programmed electrical stimulation reveals absence of an AF substrate in CREM:NFATc2$^{-/-}$ mice. (*A*) Representative lead 2 (L2) surface ECG, intracardiac atrial (*A*) and ventricular (*V*) electrograms at the end of an atrial burst pacing protocol, showing AF in the CREM mouse and sinus rhythm in the other genotypes at 5 months of age. (*B*) Percentage of mice with reproducible AF following programmed electric stimulation (PES) (WT: $n = 10$, NFATc2$^{-/-}$: $n = 12$, CREM: $n = 12$, CREM:NFATc2$^{-/-}$: $n = 10$), Fisher's exact test was used. $^{**}P < 0.01$. (*C*) Average duration of the longest AF episode induced by pacing in each group (WT: $n = 10$, NFATc2$^{-/-}$: $n = 12$, CREM: $n = 12$, CREM:NFATc2$^{-/-}$: $n = 10$). One-way ANOVA followed by the Kruskal–Wallis test was applied. $^{*}P < 0.05$; $^{**}P < 0.01$.

Next, the amount of atrial fibrosis was examined using Masson's trichrome staining of longitudinal cardiac sections (*Figure 5C*). Quantification revealed an increased amount of interstitial fibrosis in atria from CREM (8.8 ± 1.4%) compared with WT mice (2.3 ± 0.7%; $P < 0.001$) and NFATc2$^{-/-}$ mice (1.5±0.3%; $P < 0.01$; *Figure 5D*). In contrast, fibrosis levels were significantly reduced in CREM:NFATc2$^{-/-}$ mice (1.5 ± 0.5%; $P < 0.001$ vs. CREM; *Figure 5D*). The amount of collagen I and III mRNA levels was measured using RT–PCR to confirm the increased fibrosis in CREM mice. We found that collagen I and III were both upregulated in CREM mice compared with WT and NFATc2$^{-/-}$ (Supplementary material online, *Table SIII* and *Figure 5E and F*). In contrast, collagen I and III levels were normalized in atrial tissues from CREM:NFATc2$^{-/-}$ mice (*Figure 5E and F*).

3.6 Inhibition of NFATc2 normalizes SR Ca^{2+} handling in CREM mice

Our prior studies revealed that CREM mice exhibit enhanced SR Ca^{2+} leak as a result of enhanced RyR2 activity, which can promote atrial ectopy.[4] Confocal imaging was performed to assess the effects of NFATc2 deficiency on intracellular Ca^{2+} handling (Supplementary material online, *Table SVI*). Atrial myocytes from 5-month-old CREM mice exhibited a higher Ca^{2+} spark frequency (CaSpF; 3.22 ± 0.21 sparks/100 μm/s) compared to cells from WT mice (1.10 ± 0.21; $P < 0.001$; *Figure 6A and B*). In addition, myocytes from CREM mice exhibited a reduced SR Ca^{2+} load (1.88 ± 0.90) compared with WT myocytes (3.11 ± 0.56; $P < 0.05$; *Figure 6C*). The CaSpF normalized to SR Ca^{2+} load was also increased in myocytes from CREM mice compared with cells from WT mice (*Figure 6D*). Interestingly, genetic inhibition of NFATc2 in CREM:NFATc2 normalized both SR Ca^{2+} leak and SR Ca^{2+} load to levels similar to those seen in WT and NFATc2$^{-/-}$ mice (*Figure 6B–D*). Analysis of the frequency of spontaneous SR Ca^{2+} waves revealed an increased incidence in atrial myocytes from CREM mice compared with WT and NFATc2$^{-/-}$ controls, while there was a trend towards a lower incidence in CREM:NFATc2$^{-/-}$ mice (Supplementary material online, *Figure SIII*). These findings suggest that inhibition of NFATc2 prevents the development of aberrant intracellular Ca^{2+} leak associated with AF development in CREM mice.

3.7 Reduced SR Ca^{2+} handling prevents NFATc2 translocation in CREM mice

To investigate the upstream regulation of the NFATc2 signalling pathway, we crossed CREM mice with RyR2-S2814A knockin mice in which CaMKII phosphorylation of RyR2 is prevented, to generate CREM:S2814A (DM) mice.[4,21] In prior studies, we demonstrated that genetic inhibition of RyR2 phosphorylation normalizes SR Ca^{2+} leak in CREM mice.[4] As above, we assessed NFATc2 protein levels in cytosolic

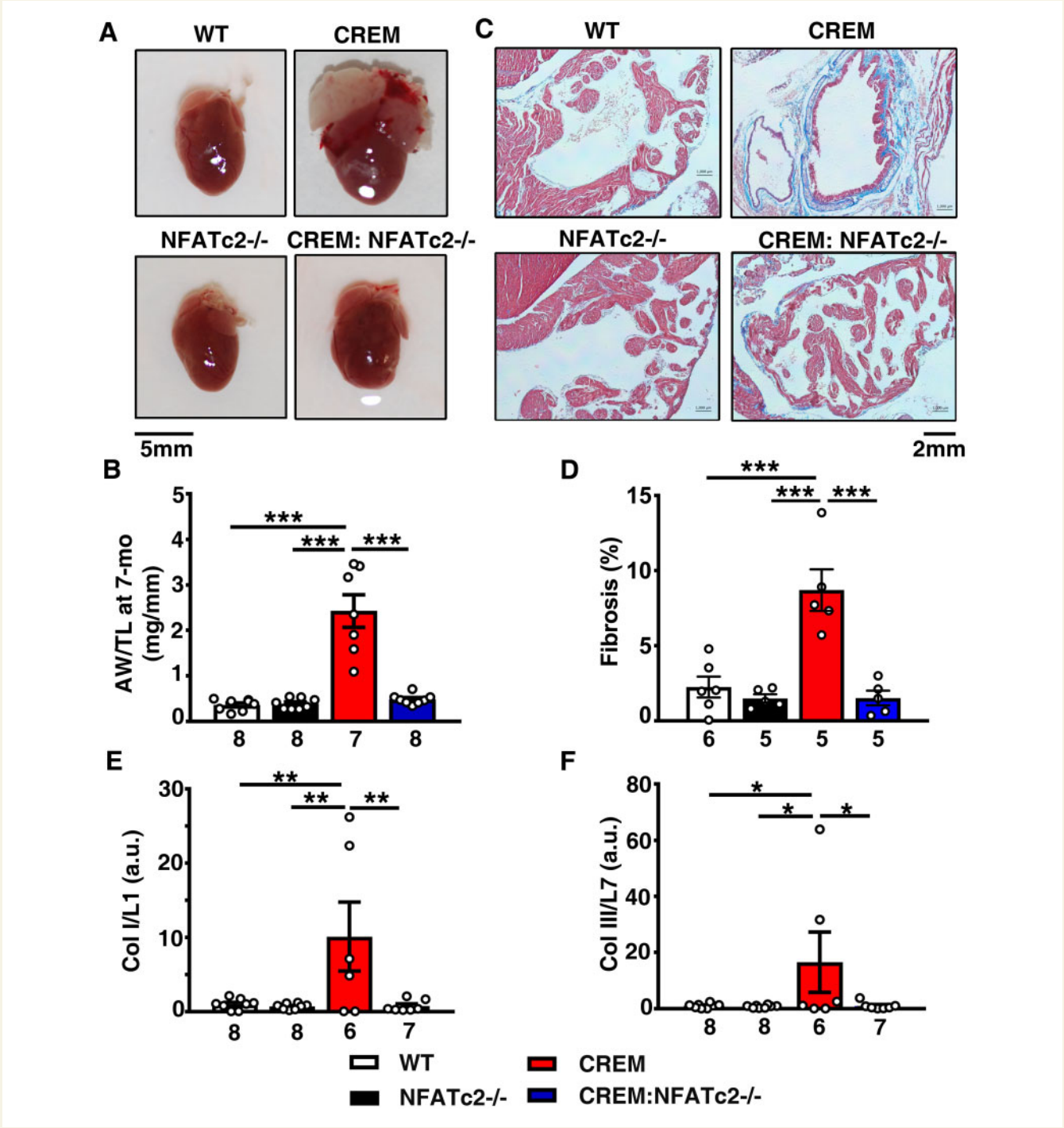


Figure 5 Reversal of atrial hypertrophy and atrial fibrosis in CREM:NFATc2$^{-/-}$ mice. (*A*) Whole-mount photographs of hearts from 7-month-old mice. (*B*) The atrial weight to tibial length ratio (AW/TL) of 7-month-old mice. Numbers below graphs indicate number of mice per group. (*C*) Masson trichrome staining of fibrosis in atrial sections. (*D*) Quantification of atrial fibrosis. (*E* and *F*) The level of collagen I and III mRNA normalized to L7. Numbers in the bars indicate the number of animals studied. The generalized estimating equation approach was performed by the use of the binomial distribution to study the fibrosis outcomes. One-way ANOVA followed by the Holm–Sidak's multiple comparisons test was applied to study the other data. $^{*}P < 0.05$; $^{**}P < 0.01$, $^{***}P < 0.001$.

and nuclear fractions from atrial tissues of WT, CREM, and CREM:S2814A mice. While NFATc2 levels were increased in both cytosolic and nuclear fractions from CREM mice compared to WT mice, genetic inhibition of RyR2 phosphorylation prevented such NFATc2 activation and nuclear translocation (Supplementary material online, *Figure SIV*). These findings suggest that enhanced RyR2-mediated SR Ca^{2+} leak may be an upstream activator of NFATc2 signalling in CREM mice, responsible for the progression of atrial remodelling leading to sAF.

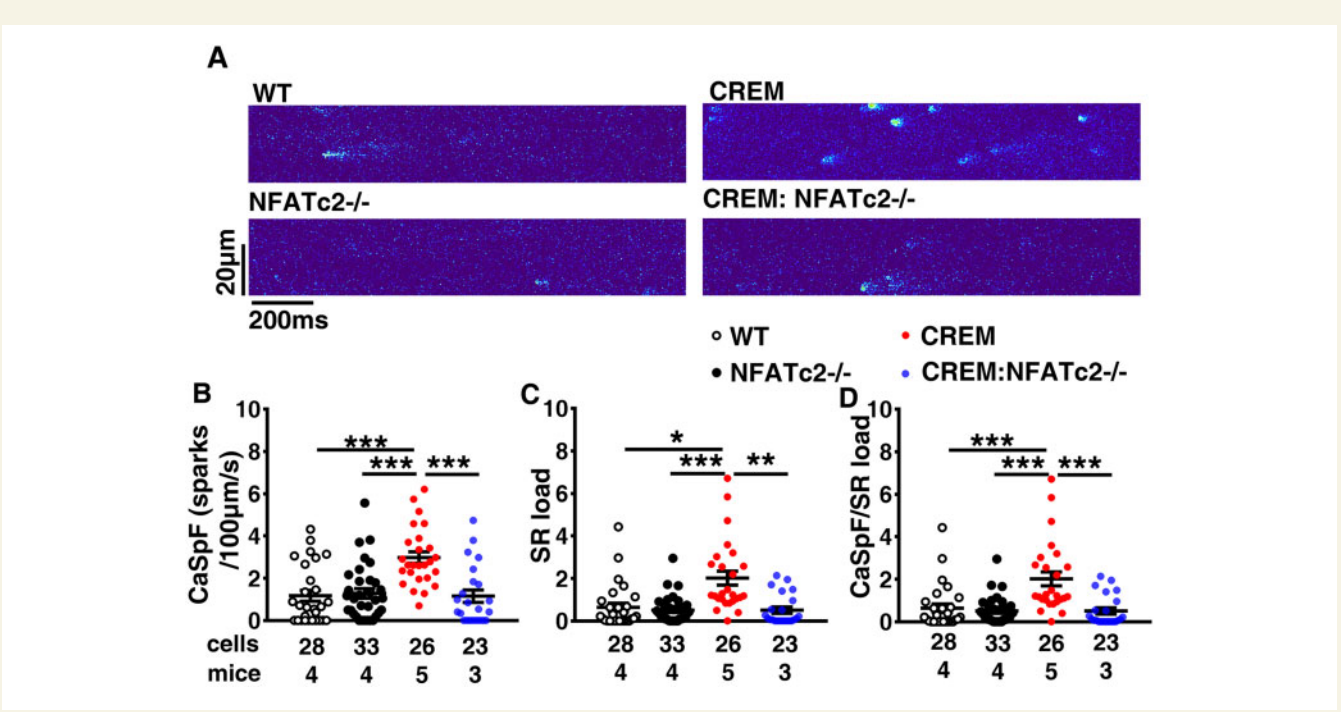


Figure 6 Genetic inhibition of NFATc2 in CREM mice normalizes SR Ca^{2+} release. (*A*) Confocal line-scan images showing more spontaneous Ca^{2+} sparks in atrial myocytes from CREM mice compared with WT and CREM:NFATc2$^{-/-}$ mice. (*B*) Bar graphs showing quantification of Ca^{2+} spark frequency (CaSpF). (*C*) Total sarcoplasmic reticulum Ca^{2+} content (SR load) and (*D*) CaSpF normalized to SR Ca^{2+} load. The number of mice and cells studies for each group is indicated in the graphs. The generalized estimating equation approach was performed by the use of the binomial distribution to study the dichotomous spontaneous SR Ca^{2+} release event. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

3.8 NFATc2 regulates *RASEF* gene expression

ChiP-seq was performed on atrial samples from AF patients ($n = 2$) and controls in NSR ($n = 2$) to identify NFATc2 target genes. Analysis revealed 214 peaks that were detected in both AF samples but not in those from NSR controls. The 54 peaks located in promoter regions were analysed further. Literature research revealed that 27 of these peaks occurred in 21 NFATc2-target genes previously implicated in cardiovascular disease development. qPCR analysis revealed that *NFATc2* binding to the *RASEF* gene promoter region was increased in AF patients (*Figure 7A*). Seventeen predicted *NFATc2*-target sites were selected and their expression levels in human AF samples were analysed by qPCR (Supplementary material online, *Table SVII*). The expression of *RASEF* mRNA in AF patients ($n = 6$) was significantly higher than that in patients in NSR ($n = 17$) (Supplementary material online, *Table* III and *Figure 7B*). Furthermore, *Rasef* mRNA levels were increased in CREM mice compared to WT and CREM:NFATc2$^{-/-}$ mice (*Figure 7C*). These findings suggest that activation of the NFATc2 signalling pathway can activate *RASEF* gene expression, which may contribute to atrial remodelling in AF.

4. Discussion

Our findings show for the first time a direct causal role of NFATc2 activation in atrial structural remodelling, which is necessary for the development of a substrate underlying sAF in CREM mice. Increased nuclear translocation of NFATc2 preceded the transition from atrial ectopy to sAF in CREM mice, suggesting that NFATc2 activation is a cause rather than a consequence of AF progression. Conversely, inhibition of NFATc2 signalling by genetic deletion of NFATc2 prevented age-dependent development of sAF in CREM mice. Moreover, our results demonstrate that inhibition of NFATc2 prevented atrial enlargement and atrial fibrosis in CREM mice. These findings have translational relevance as patients with persistent AF also exhibit enhanced NFAT signalling, with NFATc2 being the only isoform with significantly increased nuclear expression levels.

4.1 Mechanisms underlying atrial remodelling in CREM transgenic mice

CREM transgenic mice represent one of the few mouse models that exhibit an age-dependent progression from atrial ectopy to long-lasting AF, thereby mimicking the natural disease evolution typically seen in AF patients.[4,14] Our findings in the present study are similar to our prior work in that 7-month-old CREM mice spent the majority of the time in AF, with many episodes lasting for hours at a time.[4] CREM mice exhibited key features of atrial remodelling observed in patients and large animal models of AF, including atrial dilatation, slowed conduction, and increased amounts of fibrosis.[22,23] Our work reveals that genetic inhibition of NFATc2 attenuated the development of atrial enlargement and fibrosis, and normalized the levels of collagen-1 and -3 in the atria of 7-month-old CREM mice, the effects on collagen-1 being more pronounced consistent with observations in AF patients showing a more dominant role for collagen 1.[24] These findings suggest that NFATc2 is required for structural remodelling processes that lead to the development of a substrate for AF in CREM mice.

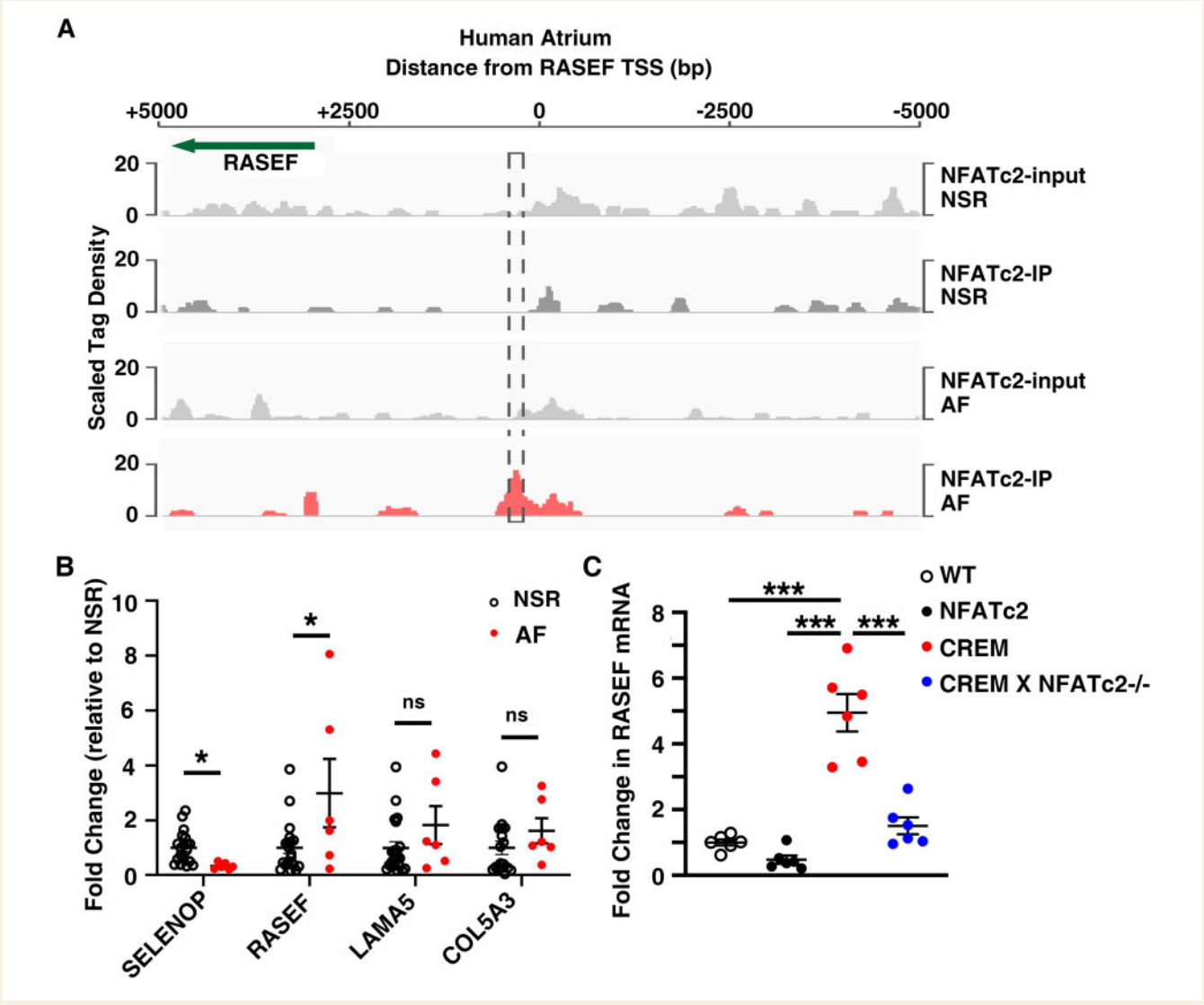


Figure 7 RASEF transcription is the potentially modulated by NFATc2. (*A*) ChiP-seq analysis of RASEF. Scaled tag density represents the abundance of the corresponding sequence combined with NFATc2. TSS is the transcription start site. The arrow indicates the direction of gene transcription. (*B*) qPCR shows that the expression of RASEF mRNA is upregulated in AF patients ($n = 6$) vs. NSR controls ($n = 17$). *$P < 0.05$ compared with NSR. AF, atrial fibrillation; NSR, normal sinus rhythm. (*C*) RASEF mRNA normalized to L7 in mouse atrial samples. The number of mice was six for each group.

4.2 Role of altered NFAT signalling in AF

In the nucleus, NFAT regulates transcription by binding to NFAT-binding elements in the promoter region of target genes.[25] NFAT was first identified as a key regulator of IL-2 gene expression.[26] In the heart, NFAT was shown to regulate cardiac troponin-I and cardiac troponin-T, among other genes.[25] It is conceivable that the regulation of these cytoskeletal proteins could be involved in structural remodelling associated with atrial arrhythmias. There is some evidence from prior studies that NFAT signalling plays a role in the pathogenesis of AF in both animal models and patients with AF.[11,27,28] For example, NFAT was shown to regulate mRNA transcription of two key atrial ion channels, which is linked to the downregulation of the α-subunit of $I_{Ca,L}$,[11] and microRNA-26-mediated upregulation of the Kir2.1 encoded subunit of the I_{K1} inward rectifier K^+ channel.[29] Interestingly, Pluteanu *et al.*[30] recently demonstrated decreased transient outward current, inward rectifier K^+ current, and acetylcholine-activated K^+ current, and reduced protein expression of several K^+ channel subunits including Kir2.1, in the CREM mouse model. Thus, NFAT-dependent changes could contribute to changes in the atrial myocardium which facilitate the maintenance of AF by altering cardiac conduction, among other things.[20]

Our study is the first to demonstrate which NFAT isoforms are upregulated in patients with AF. Only NFATc2 was significantly upregulated at the mRNA level in right atrial samples from both Chinese and German patients (*Figure 1*). Additional analysis of the Chinese samples revealed that only NFATc2 protein levels were significantly increased in the nuclear fraction of these samples, suggesting that the NFATc2 isoform is responsible for enhanced NFAT activity in the atria of AF patients. One interesting observation is the

differential expression of NFATc3 mRNA levels between Chinese and German patients, suggesting potential ethnic determinants of factors involved in atrial remodelling. Recent Genome-Wide Association Studies (GWAS) studies suggest that there may be ethnicity-dependent differences in genetic risk factors, but it is difficult at this time to correlate these population-based studies with our small-scale human studies.[31]

Given that NFATc2 was identified as the main isoform upregulated in patients with AF, and prior studies showing that NFATc2 is most abundantly expressed in the heart among NFAT isoforms,[10] we decided to evaluate the role of NFATc2 in the CREM mouse model of AF progression. We found that genetic deletion of NFATc2 prevented the development of sAF despite the presence of atrial ectopic activity, thereby provide strong evidence that NFATc2 is required for the progression of AF. To the best of our knowledge, our study is the first to demonstrate a direct causal relationship between augmented activity of NFATc2 and AF progression.

4.3 Possible upstream mechanisms for NFAT activation in the atria

Activation of the NFAT pathway requires dephosphorylation, which leads to its nuclear translocation and increased DNA-binding activity.[10] This process is regulated by Ca^{2+}- and calcineurin-dependent mechanism,[32] as it was shown that NFAT activation can be elicited by Ca^{2+} ionophore ionomycin. Conversely, NFAT can be blocked by immunosuppressants cyclosporin, cyclosporine A, and FK506.[33] In prior studies, we reported that CREM mice exhibit an increased frequency of Ca^{2+} sparks and enhanced SR Ca^{2+} leak.[4,14] We found that mice with constitutively active RyR2-S2814 phosphorylation (S2814D mice) exhibited enhanced NFAT activation (based on elevated RCAN1-4 levels), suggesting that increased RyR2 activity and the related increases in diastolic SR Ca^{2+} leak are sufficient to activate NFAT in CREM mice.[4] Phosphorylation of RyR2 at S2814 in turn is caused by enhanced CaMKII activity, which can be the result of a faster heart rhythm, enhanced oxidative stress, or higher glucose levels, among other things.[34–36] Studies in atrial myocytes isolated from patients in paroxysmal and persistent AF also revealed enhanced SR Ca^{2+} leak, suggesting that this finding is also clinically relevant. Increased Ca^{2+} levels as a result of the faster atrial rate can activate calcineurin, which promotes NFAT dephosphorylation associated with nuclear translocation.[37] The regulator of calcineurin 1 (RCAN1) isoform 4 (RCAN1.4) is transcriptionally upregulated by calcineurin activation.[38] We previously demonstrated that increased SR Ca^{2+} leak via RyR2 leads to increased RCAN1.4 mRNA levels, consistent with enhanced calcineurin activity in the atria of CREM mice.[4] Our current study demonstrates that the frequency of Ca^{2+} sparks and SR Ca^{2+} leak were both normalized by genetic inhibition of NFATc2 in CREM mice. To determine whether RyR2-mediated Ca^{2+} leak is the cause of NFATc2 activation, we crossed CREM mice with RyR2-S2814A knockin mice, in which CaMKII phosphorylation of RyR2 is prevented.[4] We found greatly reduced nuclear NFATc2 levels in these CREM:S2814A mice compared with CREM mice, suggesting that aberrant RyR2 Ca^{2+} leak is upstream of the NFATc2 activation. Finally, prior studies showed that constitutive RyR2 phosphorylation in RyR2-S2814D knockin mice exhibit enhanced RyR2 Ca^{2+} leak and activation of the calcineurin–NFAT–RCAN1.4 pathway. These findings suggest a direct causal link between RyR2-mediated SR Ca^{2+} leak, NFATc2 activation in the nucleus, and structural remodelling.[4]

4.4 Possible downstream effects of NFAT activation in the atria

ChiP-seq revealed enhanced binding of NFATc2 to the *RASEF* gene promoter region in patients with AF. Similarly, *Rasef* expression levels were increased in CREM mice, compared to WT littermates, whereas genetic inhibition of NFATc2 prevented *Rasef* activation. *RASEF* is a GTPase gene encoding Rab45 protein.[39] Rab proteins are the largest subfamily of the Ras superfamily of the small GTP-binding protein family with about 80 members. Rab can regulate cell differentiation, proliferation, apoptosis, DNA replication, and transcription, and plays an important role in cell signal transduction.[39] Recent studies have shown that *RASEF* promotes cigarette-induced pulmonary artery remodelling and pulmonary hypertension in rats by regulating the Akt pathway.[40] Thus, our results suggest that AF-related remodelling may depend on NFATc2-mediated upregulation of *RASEF* gene expression.

4.5 Clinical relevance

AF is the clinical outcome of a multifactorial, progressive disease progress involving the atria. Slowly progressive electrical and structural alterations result in a worsening atrial substrate and lead to persistent and permanent forms of AF.[41] It is believed that early restoration and maintenance of sinus rhythm can influence the progressive nature of AF. However, current treatment of AF using antiarrhythmic drugs, catheter ablation, and anti-thrombotic therapy remain unsatisfactory. The present findings suggest that pharmacological or gene therapy-mediated targeting of NFATc2 may prevent the development or worsening of AF by disrupting the structural remodelling process that underlies the slowly progressive nature of AF. This approach is based on the clinical observations that NFATc2 protein levels and nuclear translocation are increased in patients with persistent AF. Moreover, genetic inhibition of NFATc2 prevented the development of an atrial substrate and sAF in the CREM transgenic mouse model of progressive AF. The CREM transgenic model is also relevant because CREM expression levels are enhanced in patients with AF.[4] Thus, NFATc2 is a clinically relevant therapeutic target for AF progression in humans.

4.6 Potential limitations

Clinical AF is complex and it is unlikely that our findings apply to all forms of AF. It is possible that there are differences in expression levels between different parts of the atria. Given that atrial biopsies were obtained from living patients, we were only able to obtain right atrial appendage samples during cardiac surgery procedures. Our group and several other groups have done extensive prior research using the same type of tissue samples and have been able to obtain valuable insights into the pathogenesis of AF.[42–44] For some analyses, the sample size of human tissue samples was too low to draw definitive conclusions. For example, we analysed the relationship between NFAT isoform expression levels and patient age, LVEF, and selected medications. Although no significant correlations were observed, the limited sample size of our patient cohorts should be considered when interpreting these results. The CREM mouse model exhibits many similarities to progressive AF observed in patients, with an initial presentation of atrial ectopy that over time evolves into progressively longer episodes of sAF.[4] Moreover, CREM levels are increased in patients with paroxysmal and cAF,[4] and the downregulation of CREB/CREM target genes was linked to an increased susceptibility to AF in patients.[45] However, mouse models do have some limitations and extrapolation of our findings to human AF needs to be done with caution.[46,47] A limitation of the mouse model is that NFATc2

germline knockout mice were used as opposed to cardiac or atrial-specific knockout mice.[5] Therefore, the potential extra-cardiac effects of NFAT in immune cells was not studied separately. On the other hand, echocardiography studies revealed normal ventricular contractility and dimensions in the NFATc2-deficient mice, suggesting that changes in ventricular function did not confound our studies. We recently developed a new mouse model of sAF development as a result of atrial-specific knockdown of liver kinase B1 (*Lkb1*).[48,49] It would be interesting to determine whether AF progression in these mice also depends on NFATc2 signalling. Additional studies are needed to uncover other genes that are modulated by NFATc2 in addition to RASEF in fibrillating atria compared with those in sinus rhythm. Finally, pharmacologic inhibition of the calcineurin–NFAT pathway using cyclosporine A or FK506 was not performed in the present study. Such studies might provide proof-of-principle evidence whether this pathway can be targeted using a pharmaceutical approach.

5. Conclusions

NFATc2 activation was shown to be an essential mechanism for the development of sAF in CREM mice. Our data revealed that NFATc2 is activated by enhanced SR Ca^{2+} leak via RyR2 in CREM mice with sAF (Supplementary material online, *Figure* S*V*). Inhibition of NFATc2 in CREM mice prevented atrial remodelling necessary for the development of sAF. Increased activation of NFATc2 was also observed in AF patients of Chinese and German descent. Inhibition of NFATc2 signalling pathway can regulate RASEF gene expression and improve atrial remodelling. These findings show for the first time that activation of the NFATc2 isoform is necessary for sAF development in an experimental model of AF.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

Authors' contributions

L.N., S.K.L., and X.H.T.W. designed the study; L.N., S.K.L., J.N., X.P., I.A.-T., J.O.R., and H.M.C. performed experiments and analysed results; N.L. drafted the manuscript; and S.K.L., H.W., M.K., W.S., F.U.M., N.L., X.W., D.W.W., D.D., and X.H.T.W. revised the manuscript for important intellectual content.

Acknowledgements

We are grateful to Dr L.J. de Windt for kindly providing the NFATc2$^{-/-}$ mice.

Conflict of interest: X.H.T.W. is a co-founder and Scientific Advisory Board member of Elex Biotech, a drug development company focused on novel compounds for the cardiac arrhythmia disorders and heart failure. All other authors declared no conflict of interest.

Funding

This work is supported by National Natural Science Foundation of China (NSFC, No. 82070354 and 81470519) (L.N.), Program for HUST Academic Frontier Youth Team (2019QYTD08) (L.N.), National Institutes of Health grants R01-HL089598, R01HL147188, R01HL153358 (X.H.T.W.), R56-HL131649, and R01-HL136389 (N.L.), an American Heart Association grant 14SDG20080008 (N.L.), and a grant from the Saving Tiny Hearts Foundation (X.H.T.W.).

Data availability

All original data used for this study are available from the corresponding authors upon reasonable request.

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Translational perspective

Atrial fibrillation (AF) is a progressive disease characterized by electrical and structural remodelling which promotes atrial arrhythmias. This study provides evidence for increased 'nuclear factor of activated T-cell' (NFAT) signalling in patients with chronic AF. Studies in the CREM transgenic model of progressive AF revealed that the NFATc2 isoform mediates atrial remodelling associated with AF substrate development. Chromatin immunoprecipitation sequencing of atrial biopsies from AF patients identified 'Ras And EF-Hand Domain-Containing Protein' (RASEF) as a downstream target of NFATc2-mediated transcription, suggesting that targeting these factors might be beneficial for curtailing AF progression.