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ORIGINAL ARTICLE

NFAT5-mediated *CACNA1C* expression is critical for cardiac electrophysiological development and maturation

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Abstract
Entry of calcium into cardiomyocyte via L-type calcium channel (LTCC) is fundamental to cardiac contraction. *CACNA1C*, a type of LTCC and a hallmark of a matured ventricular myocyte, is developmentally regulated. Here, we identified 138 potential transcription factors by a comparative genomic study on 5-kb promoter regions of *CACNA1C* gene across eight vertebrate species, and showed that six factors were developmentally regulated with the expression of *Cacna1c* in mouse P19cl6 in vitro cardiomyocyte differentiation model. We further demonstrated that the nuclear factor of activated T cells 5 (Nfat5) bound to a consensus sequence TGGAAGCGTTC and activated the transcription of *Cacna1c*. The siRNA-mediated knockdown of *Nfat5* suppressed the expression of *Cacna1c* and decreased L-type calcium current in mouse neonatal cardiomyocytes. Furthermore, morpholino-mediated knockdown of *nfat5* in zebrafish prohibited the expression of *cacna1c* and resulted in a non-contractile ventricle, while over-expression of either *cacna1c* or *nfat5* rescued this impaired phenotype. Thus, NFAT5-mediated expression of *CACNA1C* is evolutionarily conserved and critical for cardiac electrophysiological development and maturation of cardiomyocyte.

Key message
- Nfat5 binds to a consensus sequence TGGAAGCGTTC in the promoter of *Cacna1c.*
- Nfat5 activates the transcription of *Cacna1c.*
- *Nfat5* knockdown suppresses *Cacna1c* expression, decreases L-type calcium current, and results in non-beating ventricle.
- NFAT5-mediated expression of *CACNA1C* is evolutionarily conserved.
- NFAT5-mediated *CACNA1C* expression is critical for cardiac electrophysiological development and maturation.

Keywords L-type calcium channel · NFAT5 · Cardiac development · Comparative genomic · Transcriptional factor

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Introduction

As an electrical signal and second messenger, calcium (Ca^{2+}) plays an essential role in cardiac automaticity, excitation-contraction (E-C) coupling, and regulation of a number of functions, such as electrophysiological signaling, growth,

migration, and secretion [1–3]. Cardiomyocyte is possessed of various distinct calcium channels that are dynamically regulated during development [4–10]. For instance, *CACNA1C*, *CACNA1D*, *CACNA1G*, and *CACNA1H* are expressed in embryonic cardiac tissues, encoding the L-type voltage-gated calcium channel subunits α1C and α1D as well as the T-type voltage-gated calcium channel subunits α1G and α1H, respectively [11, 12]. The temporal and spatial expression of these channels is crucial to cardiac electrophysiological function as well as structural development and maturation [13–19].

Among these channels, CACNA1C produces L-type calcium current ($I_{Ca,\ L}$) that allows Ca^{2+} to enter myocytes and trigger calcium-induced calcium release, which is essential for E-C coupling and maintenance of calcium homeostasis in an adult cardiomyocyte. Initially, *CACNA1C* is not expressed in cardiomyocyte, but in later developmental stage, it presents one of the hallmarks for cardiac electrophysiological maturation [19, 20]. It has been shown that a disruption of *CACNA1C* causes non-beating heart and embryonic lethality in mouse and mutations in *CACNA1C* cause life-threatening arrhythmias in human [21–23]. These findings suggest that the function of *CACNA1C* is indispensable and that the temporal and spatial expression of *CACNA1C* is imperative to cardiogenesis and cardiac electrophysiological maturation [24]. However, it is largely unknown on how the expression of *CACNA1C* is precisely controlled during development.

Sequential bindings of *trans*-acting factors (transcription factors) to DNA consensus sequences (*cis*-regulatory elements or transcription-binding sites) are pivotal to ensure the correct expression of target genes during the developmental stages temporally and spatially [25]. On the other hand, comparative genomics approach allows to the identification of evolutionarily conserved DNA sequences (DNA consensus sequence) across species, providing a systemic tool widely applied to predict genes, define critical motifs and functional domains, and screen candidate genes [26, 27]. Here, we performed comparative genomic analysis on 5.0-kb promoter region of *CACNA1C*, together with functional assessment, comprehensively searching for novel *trans*-acting factors critical for the developmental expression of *CACNA1C*.

Materials and methods

Comparative genomics study of promoter region of *CACNA1C*

The DNA consensus sequences and putative *trans*-acting factors were screened with Discovery Studio Gene (version 2.5, Accelrys) on the 5.0-kb promoter region (upstream of transcription-start site) of *CACNA1C* across eight species including human, rhesus, rat, mouse, opossum, cow, chick, and zebrafish, and selected by a perl-based script if they existed in all the species. A hash table was applied to count the number of motifs occurrences in each species. The text version of user interface outputted the list of consensus sequences and factors in the species, while a graphic illustration of their locations in promoter from each species was exported as well.

The consensus sequences and factors were further selected if they met the following criteria: (1) they were conserved in their copy numbers and positions across all analyzed species (conserved), (2) they have been reported to be expressed in vertebrates previously (experimentally proved), and (3) there are few reports on whether they are expressed in cardiac tissues (novel).

The novel *trans*-acting factors were then tested to determine if they were expressed in mouse cardiac tissues and regulated in P19cl6 in vitro cardiomyocyte differentiation model (see below). The promising candidates were subject to functional characterization.

The analysis process was outlined in electronic supplementary material, Fig. S1.

Plasmids, probes, and antibodies

Plasmid TY-YFP-VSNL pcDNA4 that carries rat *Cacna1c* cDNA was kindly provided by Ricardo Dolmetsch (University of Stanford, USA). The probe to detect the expression of *nfat5a* in situ in zebrafish was cloned from zebrafish by inserting a reverse-transcription polymerase chain reaction (RT-PCR) product into pGEM T-vector with the following primers (5′-CCCCAGTTCTCCCAAATCTCTCTAT-3′ and 5′-GCTCTTCGCGCTTGACCATCGGT-3′). The mouse *Cacna1c* promoter (−797 to +120 bp) was amplified from genomic DNA and cloned into pGL3B luciferase reporter plasmid for the promoter activity assay. Human *NFAT5* cDNA plasmid was purchased from OPEN BIOSYSTERMS and verified by sequencing. Nfat5 antibody is purchased from Santa Cruz. MF20 antibody is purchased from DSHB.

P19cl6 cell culture, differentiation, and transfection

P19cl6 cells were cultured and induced to differentiate into cardiomyocytes as described previously [28, 29]. The expressions of the markers for successful cardiomyocytes differentiation, alpha myosin heavy chain (*αMhc*), myosin light chain-2v (*Mlc2v*), *Gata4*, and *Nkx2.5*, were quantified by RT-PCR with primers listed in the Supplementary material online, Table S1. To obtain P19cl6 cells over-expressing *NFAT5*, pAsRed2-C1-NFAT5 was transfected using lipofectamine 2000 (Invitrogen) on day 0 (before induction). To knock down the expression of *Nfat5*, P19cl6 cells were infected by adenovirus expressing siRNA on day 0. Sequences of *Nfat5* siRNA were as follows: 5′-GGCACAACAGCAGTTATCG-3′ (siRNA1), 5′-GCAACACAGTTTCAGACAA-3′ (siRNA2), and 5′-GGGCTCCTACACCACAGAA-3′ (scrambler). A

real-time PCR was used to quantify the expression of *Nfat5* (5′-CTGGCTCATCTCAGCAGACT-3′ and 5′-CATGTTCTGACTGCTGTTCA-3′, PCR product: 123 bp) and *Cacna1c* (5′-GAGCATCACAATCAGCAGGCT-3′ and 5′-GGTGCACATACGCACATACTAT-3′, PCR product: 162 bp) at 0, 4, 8 and 12 days after induction based on the previously reported methods [30]. The standardized CT (threshold cycle) at the single time point for *Nfat5* = 1 / [CT(*Nfat5*) − CT(*18S*)], similar to *Cacna1c*.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (Ch-IP) was performed as described previously [31]. Proteins were sampled from the induced P19cl6 cells on day 12 and cross-linked to DNA with 1 % paraformaldehyde for 10 min at room temperature. DNA-protein complex was immunoprecipitated by Nfat5 antibody and protein A + G sepharose after sonication. The DNA strands were released and amplified by PCR with the primers: *Cacna1c* promoter forward 5′-GCTCCACTCGGGCTCC GGT-3′; *Cacna1c* promoter reverse, 5′-CTGGCAGGGAG CTGTTTCT-3′; *Cacna1c* last exon forward 5′-GAGCATCA CAATCAGCAGGCT-3′; *Cacna1c* last exon reverse, 5′-GGT GCACATACGCACATACTAT-3′.

Luciferase assay

A mutated promoter was produced by the removal of two bases in Nfat5 motif in wild-type *Cacna1c* promoter by PCR-directed mutagenesis. The expression of luciferase was under the control of wild-type *Cacna1c* or mutated promoter. Plasmid pGL3B was used as indicated. They were transfected into P19cl6 cells together with pRL-TK (an internal control plasmid) using lipofectamine 2000 (Invitrogen). The proteins were collected and subject to the dual luciferase assay with kit from Promega in Synergy HT luminometer (BioTek) at 48 h after transfection.

Recordings of L-type Ca^{2+} currents

To isolate neonatal cardiomyocytes, four litters of newborn C57BL/6 mice at postnatal day 1 (9–16 mice per litter) were euthanized by pentobarbital. The hearts were taken, and the ventricles were incised, minced, and dispersed mechanically in solution 2 for 5 min. The rest of the procedures and cell culture for calcium current recording as described previously [32, 33]. L-type Ca^{2+} current was recorded at the holding voltage of −40 mV. The experimental protocol on mice was approved by the Institutional Animal Care and Use Committee (IACUC) of Peking University accredited by AAALAC International (IACUC No.: IMM-Tian XL-03).

Morpholino injection, rescue, and heart isolation of zebrafish

Zebrafish morpholino specific to the 5′-untranslated region of *nfat5a* was designed and synthesized by Gene Tools (5′-AGCTCAGAGGAAGCGACTCTCTCAC-3′). It was injected into embryos at one-cell stage from *nppa:GFP* transgenic or wild-type zebrafish [34]. In the rescue experiment, a rat full-length cDNA of *Cacna1c* was co-injected with zebrafish *nfat5a* morpholino. The zebrafish were raised on 14:10-h light:dark cycle at 28.5 °C. The embryos were incubated in petri dishes at 28.5 °C The heart beating was video-typed from treated zebrafish at 72 hpf. The experimental protocol on mice was approved by IACUC of Peking University accredited by AAALAC International (IACUC No.: IMM-Tian XL-05). The real-time PCR was carried out to determine the expression of *cacna1c* (5′-GCACCAATCTCCACTGCGAGCT-3′ and 5′-GTAGACAGCTAAGGCCACACAGT-3′) at 24, 48, and 72 h following knockdown of *nfat5* by morpholino. Primers for zebrafish *b-actin* are 5′-TCCCCTTGTTCACAATA ACCTAC-3′ and 5′-GGTCACAATACCGTGCTCAAT-3′. The standardized CT (threshold cycle) at the single time point for *cacna1c* = 1 / [CT(*cacna1c*) − CT(*b-actin*)].

Histochemical staining

Zebrafish embryos at 72 hpf were fixed in 4 % paraformaldehyde overnight at 4 °C. In lateral view, 5-μm sections of paraffin-embedded 72-hpf zebrafish embryos were stained with hematoxylin and eosin (H&E) followed by photographed using an Olympus DP71 microscope.

Statistical analysis

Data were presented as means ± SE and compared by Student's *t* test in promoter assay, and by ANOVA in calcium current measurement. Differences were considered statistically significant if $P < 0.05$. The Pearson coefficient and two-tailed *P* value were calculated to evaluate the correlation between the expression of *Nfat5* and *Cacna1c*. Two-way ANOVA was used to determine the difference between *cacna1c* and *serca2* in *nfat5*-morphant and wild-type zebrafish at different time points, and the *P* value was calculated. The numbers (*N*) of related experiments were listed in the figure legends.

Results

Identification of consensus sequences and transcription factors in promoter region of *CACNA1C* gene

To identify evolutionarily conserved DNA consensus sequences and their putative *trans*-acting factors, the upstream

regions from −5000 to −1 bp of *CACNA1C* gene from the eight species were first screened individually. The analysis strategy was outlined (Supplementary Fig. S1). 612, 597, 593, 576, 557, 609, 577, and 534 putative *trans*-acting factors were found in the promoter regions of *CACNA1C* gene from human, rhesus, mouse, rat, opossum, cow, chicken, and zebrafish, respectively (Supplementary Table S2, each species has its own named sheet). After filtering, 138 *trans*-acting factors were found to be present in all the species and considered as "conserved *trans*-acting factors" (Supplementary Table S2, sheet name: conserved factors).

Of these conserved factors, 38 (Supplementary Table S2, see the sheet "Tested results") were previously reported in vertebrate organisms and subjected to experimental screening as follows: first, we tested whether these factors were expressed in mouse heart by RT-PCR and found that *Nfat5 (TonE_CS)*, *Grl*, *Etv4*, *Ap4b1*, *Lyf*, *Hnf3a*, *Ap1b1*, *Ptf1*, *CdxA*, *Tcf1*, *Ibp1*, *Ctcf*, and *Hstf* were expressed in adult (atria and ventricles) and neonatal heart (Supplementary Fig. S2A, B). The *trans*-acting factors, such as Nkx2.5, Ap-2, and Pol II complex that are known to be present in cardiac tissues, were not further considered in this study. Then, we examined whether the expressions of those factors found in mouse cardiac tissues were developmentally regulated using the P19cl6 in vitro cardiomyocyte differentiation model. The successful in vitro differentiation was demonstrated by the expressions of a set of cardiac marker genes (*Nkx2.5*, *α-Mhc*, *Ryr2*, *Scn5a*, and *Serca2*) over the time courses (Fig. 1a). As shown in Fig. 1a, the expression of *Nfat5* and *Cacna1c* gene was upregulated during the cardiomyocyte differentiation. Furthermore, we demonstrated that *Ap1b1*, *Ctcf*, *Grl*, and *Hstf* were significantly upregulated while *CdxA* was downregulated in differentiated cardiomyocytes compared with non-differentiated P19cl6 (Supplementary Fig. S2C). The others remained unchanged (not shown). Thus, we identified six novel genes that were predicted to become the transcriptional factors, whose expression patterns were significantly associated with that of *Cacna1c*, indicating that they may potentially regulate the expression of *Cacna1c* gene during cardiomyocyte differentiation.

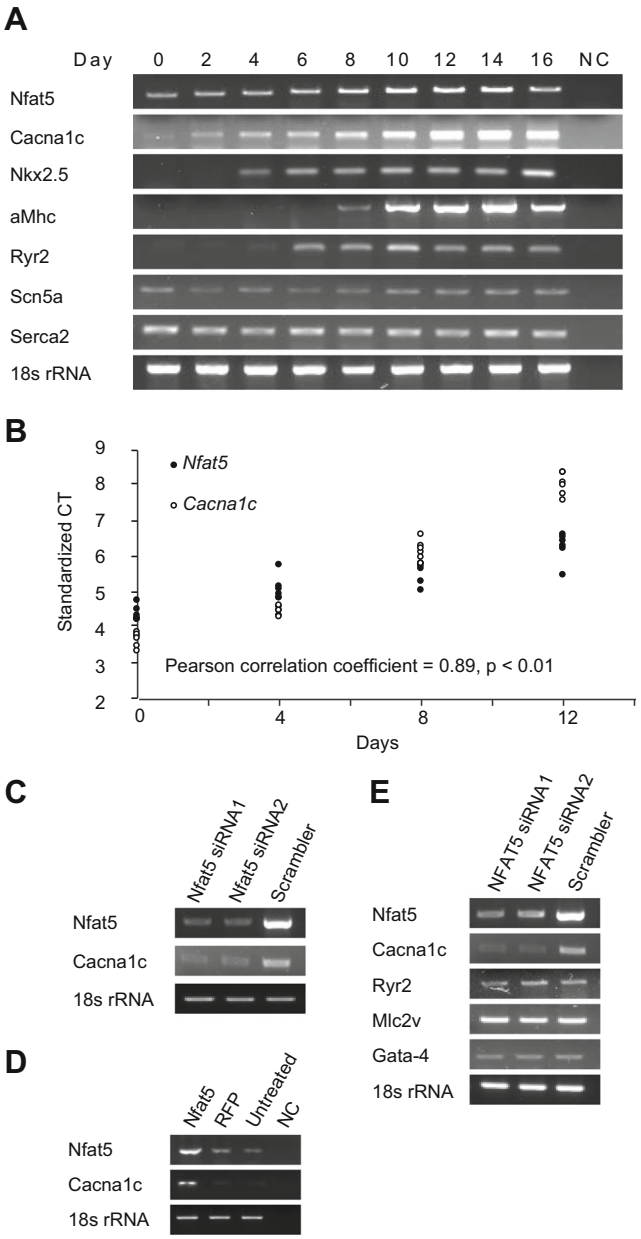

Fig. 1 Nfat5 regulates the expression of *Cacna1c* gene. **a** Expressions of *Nfat5*, *Cacna1c*, *αMhc*, *Ryr2*, *Scn5a*, *Serca2*, *Nkx2.5*, and *18s rRNA* during cardiomyocyte differentiation in P19cl6 in vitro model (from day 0 to day 16) were determined by semi-quantitative PCR. **b** Expressions of *Nfat5* and *Cacna1c* measured by real-time PCR (at day 0, 4, 8, and 12) were presented as plot of standardized CT against days after induction ($N = 6$, the P value for the Pearson coefficient was less than 0.01). **c** Knockdown of *Nfat5* in P19cl6 cells attenuated *Cacna1c* expression during P19cl6 differentiation into cardiomyocyte. **d** Transfection of *NFAT5* into P19cl6 cells induced *Cacna1c* expression in absence of DMSO compared with vector (RFP) and no treatment (untreated). **e** Knockdown of *Nfat5* in C57BL/6 mouse neonatal cardiomyocyte attenuated *Cacna1c* expression and had no effects on *Mlc2v*, *Gata-4*, and *Ryr2*. *NC* negative control for RT-PCR

Nfat5 regulates expression of *Cacna1c* gene during cardiomyocyte differentiation

Of the six *trans*-acting factors identified in this study, the Nfat5 was functionally evaluated in the regulation of *Cacna1c* transcription, as the mice with null the function of either *Nfat5* or *Cacna1c* die on the same developmental stage (14.5 p.c.), suggesting that they may be potentially linked [21, 45, 46].

For this purpose, we first determined the expression of *Nfat5* and *Cacna1c* in P19cl6 differentiation model and found that the transcripts of *Nfat5* and *Cacna1c* were increased synchronously with the induction, which was demonstrated by semi-quantitative PCR and real-time quantitative PCR (Fig. 1a, b). The expression of the two genes was significantly

correlated with 0.89 of Pearson coefficient ($P < 0.01$). *Ryr2* was also upregulated while *Serca2* remained not changed (Fig. 1a).

Second, the knockdown of *Nfat5* mediated by siRNA suppressed the expression of *Cacna1c* in differentiated P19cl6 cells at day 4 (Fig. 1c) and in primary cardiomyocytes from neonatal mice (Fig. 1e), as was validated by the siRNAs that targeted different regions of *Nfat5* gene. On the other hand, the over-expression of *NFAT5* upregulated *Cacna1c* expression in P19cl6 cells (Fig. 1d). Notably, the knockdown of *Nfat5* did not have significant effects on channels or transporters including *Ryr2* or cardiomyocyte markers such as *Mlc2v*, *Gata4* (Fig. 1e).

The putative Nfat5-binding sequences have been known as TGGAARMGYKY (R represents A or G, M for A or C, Y for C or T, and K for T or G). The sequences, which present in the promoter region of *Cacna1c* from the eight species and meet the criteria for the putative Nfat5-binding sequences, were shown in Fig. 2a. The putative binding sequence is high conserved from fish to human, and it is identical from chick to human (TGGAAGCGTTC). In order to test whether the putative Nfat5-binding sequence (TGGAAGCGTTC) influences the expression of *Cacna1c* gene, a promoter activity assay was performed. We deleted two critical bases (GG) in the putative Nfat5-binding sequence (TGGAAGCGTTC to T▵▵AAGCGTTC). It was found that the transcriptional activity of *Cacna1c* was significantly decreased when Nfat5-binding site in *Cacna1c* promoter region was mutated without GG, compared with wild-type promoter (Fig. 2b). To establish whether Nfat5 indeed binds to *Cacna1c* gene promoter region, Ch-IP assay carried out. A 215-bp PCR fragment that harbors Nfat5-binding site (TGGAAGCGTTC) was amplified from the chromatin fragments immunoprecipitated with Nfat5 antibody and input sample but not from IgG control (Fig. 2c).

To examine if Nfat5 affects L-type Ca^{2+} currents, whole-cell patch clamp was performed. Knockdown of *Nfat5* by siRNAs apparently suppressed L-type Ca^{2+} currents (Fig. 3a–c).

Over-expression of *Cacna1c* cDNA rescued non-contractile ventricle caused by morpholino knockdown of *nfat5* in zebrafish

We tested the expression of *cacna1c* at various developmental stages, and found that the expression of *cacna1c* was upregulated at 24, 48, and 72 hpf in wild-type fish embryos (Fig. 4a).

To confirm the regulatory effect of *nfat5* on *cacna1c* gene in vivo, *nfat5a* was knocked down by injecting morpholino oligonucleotides (MO) into zebrafish eggs at one-cell stage. The efficiency and specificity of MO against *nfat5a* was assessed and validated by co-injection of both MO and GFP report vectors that contained either *nfat5a* MO target sequences or mismatched sequences (Supplementary Fig. S3A, B). The *nfat5a* MO suppressed the expression of *cacna1c* significantly at all time points tested (Fig. 4a, lower panel, tested by two-way ANOVA), but the expression of *serca2* remained not significantly changed (from WT 24 48 72 to MO 24 48 72, the densities of the bands in comparison with *b-actin* were 0.023 ± 0.0012, 0.019 ± 0.0022, 0.024 ± 0.0016 to 0.023 ± 0.0022, 0.025 ± 0.002, 0.021 ± 0.0019. Two-way ANOVA analysis showed no significant difference, $N = 3$ in each group).

At 72 hpf, *nfat5a*-morphant zebrafish exhibited pericardial edema (Fig. 4b), thin ventricle walls (Fig. 4c), non-contractile ventricle, and lacking of normal blood flow, compared with wild-type fish embryos (Supplementary Video S2 vs S1). The severities of the phenotypes were MO dosage-dependent, and the incidence of non-contractile ventricle was 75 or 90 % when 4 and 8 ng of MO were applied, respectively (Fig. 4d). The atrial beating seemed not disturbed. Because the dose of 4 ng MO was sufficient to produce phenotypes therefore used in the further experiments.

To demonstrate that the phenotypes were caused by the suppressed expression of *cacna1c* gene in *nfat5*-morphant zebrafish embryos, a full-length cDNA of rat *Cacna1c* or human *NFAT5* was co-injected with *nfat5a* MO. The incidence of non-beating ventricle was decreased to 40 and 10 % in *NFAT5*- and *Cacna1c*-injected fish embryos, respectively (Fig. 4d). The incidence of healthy heart was increased significantly (Supplementary S3 and S4 vs S2) at 72 hpf.

Discussion

In present study, we identified the six novel *trans*-acting factors, which exhibited transcriptional correlations with the expression of *CACNA1C* gene. We further demonstrated that Nfat5 bound to the consensus sequence (TGGAAGCGTTC) of *CACNA1C* promoter and showed that this binding was critical for the initiation and developmental regulation of *CACNA1C* expression. Knockdown of *nfat5* prohibited the expression of *CACNA1C* and terminated ventricular contraction. Our studies, for the first time, demonstrate that Nfat5 is an important transcriptional regulator for the expression of *CACNA1C*, and that *NFAT5*-*CACNA1C* cascade regulates cardiac electrophysiological development and maturation.

A number of calcium channels are developmentally expressed in cardiomyocytes of various model organisms [4–10]. Corrected switches of their expression patterns temporally and spatially are essential to ensure cardiac electrophysiological development. For instance, L-type calcium channel expression and current are increased during cardiac electrophysiological maturation [8, 9, 35], and after birth, the expression of *CACNA1C* is continuously upregulated, whereas *CACNA1D* is decreased significantly in adult myocytes [36]. Knockout of *CACNA1C* leads to failure for the

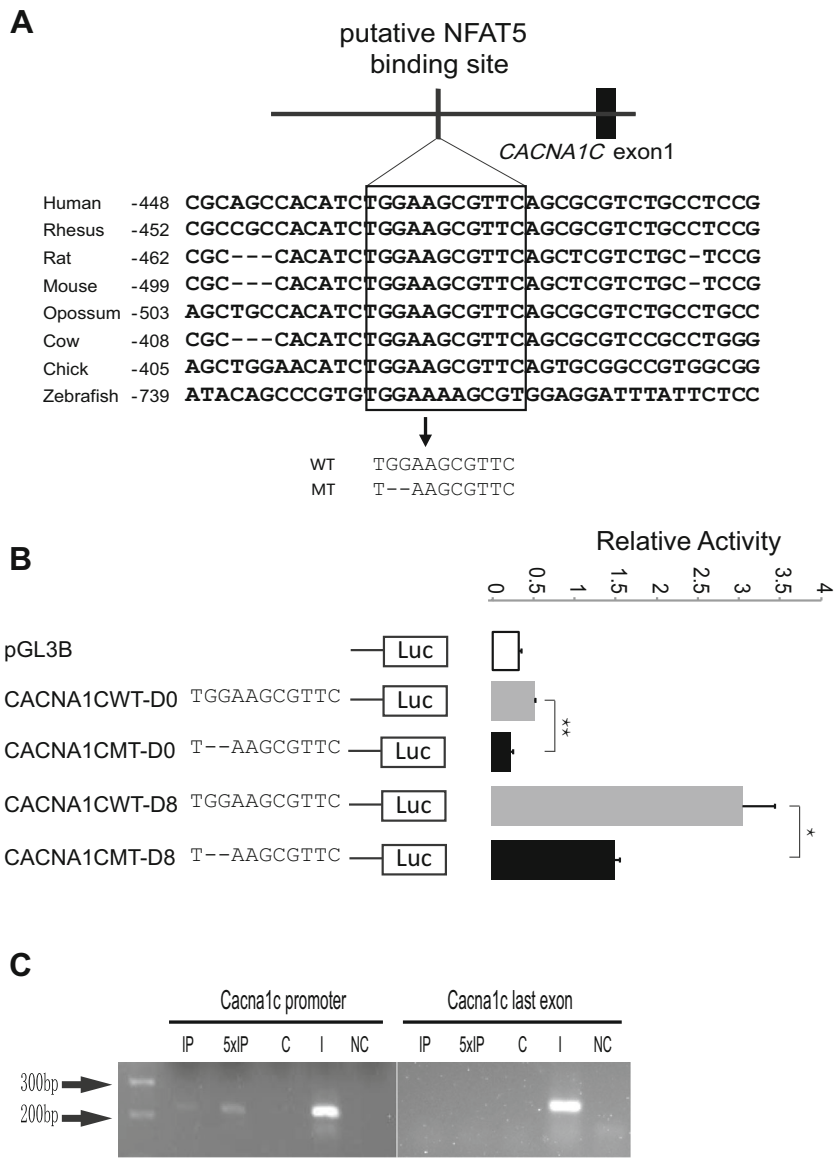

Fig. 2 Nfat5 binds to *Cacna1c* promoter and increases its transcriptional activity. **a** Conservative analysis of Nfat5 *cis*-element sequence in *Cacna1c* promoter in the eight species. **b** *Cacna1c* promoter assay. Cacna1cWT-D0, Cacna1cWT-D8, Cacna1cMT-D0, and Cacna1cMT-D8 represented wild-type *Cacna1c* promoter (WT) and mutated *Cacna1c* promoter (MT) at day 0 (D0) and day 8 (D8) in P19cl6 following DMSO induction (*$P < 0.05$, **$P < 0.01$; $N = 5$). **c** Chromatin IP (Ch-IP) assay; *IP*, *5 × IP*, *C*, *I*, and *NC* stands for one dose, a fivefold dose of Nfat5 antibody, IgG control, input DNA, and negative control for PCR, respectively. *Cacna1c* last exon served as a control for specificity

expression transition from *CACNA1D* to *CACNA1C*, which causes embryonic lethality, demonstrating the precise expression and transition of *CACNA1C* is pivotal to cardiac development [20]. Previously, it has been found that the promoter region of *CACNA1C* harbored several known consensus sequences that allow Nkx2.5, GATA-4, cAMP response factor, HA-Ras, AP-1, DCT, Sox6, and TGF-beta1 to bind and to regulate the expression of *CACNA1C* [37–42]. Among these known factors, cAMP response factor and AP-1 are required for basal expression of *CACNA1C* whereas the others suppress the expression of *CACNA1C*. Thus, the critical regulator that produces an upstroke of *CACNA1C* in the expression of remains unclear in the previous studies.

To comprehensively search for the transcriptional factors that regulate *CACNA1C*, comparative genomic analysis on 5-kb promoter of *CACNA1C* was carried out. We identified 138 *trans*-acting factors that conservatively present on 5-kb promoter of *CACNA1C* from all the eight species. Of these factors, 38 were reported to be expressed in vertebrates, and several motifs, such as NKx2.5, AP-1, and Sox6, which have been reported to regulate *CACNA1C*, served as positive controls for the successful comparative genomic analysis in this study [37–39]. We eventually identified six transcriptional factors whose expressions were developmentally regulated during cardiomyocyte differentiation from 15 transcriptional factors that are not well characterized previously in cardiac tissues. Among the six transcription factors identified in our study, five factors, including *NFAT5*, *Ap1b1*, *CTCF*, *GRL*, and *HSTF* were upregulated, whereas *CdxA* was downregulated during cardiomyocyte differentiation. We functionally proved that NFAT5 was critical to enhance the expression of *CACNA1C*.

NFAT5 is also referred to as NFATL1 or TonEBP, a transcription factor binding to tonicity-responsive enhancer

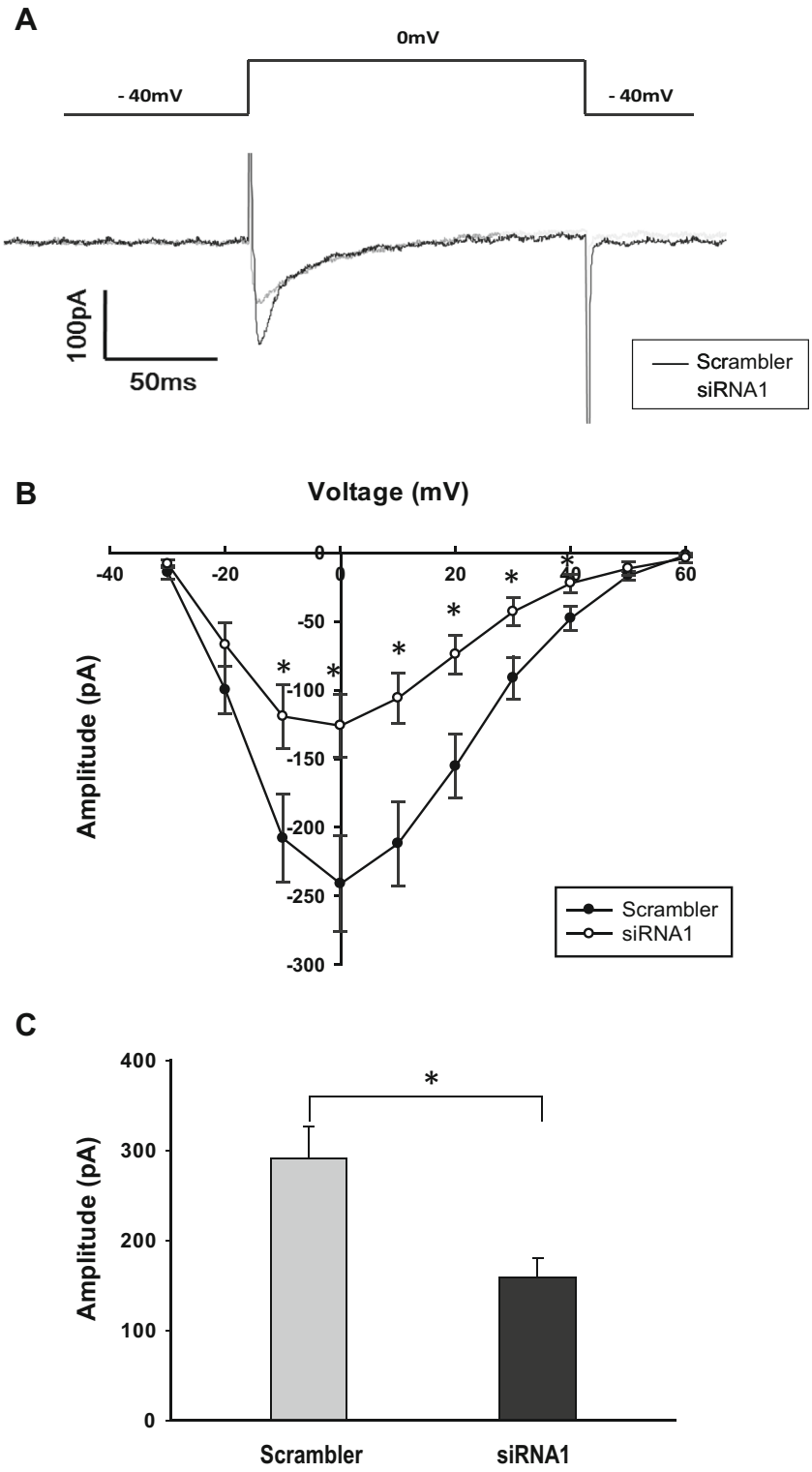

Fig. 3 Knock down of *Nfat5* gene decreases the L-type Ca^{2+} currents in neonatal mouse ventricular myocytes. **a** Typical trace of a depolarization pulse induced whole-cell $I_{Ca,L}$ from scrambler (*black*) and Nfat5 siRNA1 (*grey*) in cardiomyocytes ($N = 20$). **b** Voltage dependence of relationships of the L-type Ca^{2+} current amplitude in scrambler ($N = 20$) and Nfat5 siRNA1 group ($N = 22$), respectively. **c** Average data of amplitude of one pulse induced whole-cell L-type Ca^{2+} currents in scrambler ($N = 20$) and Nfat5 siRNA1 groups (*$P < 0.05$, $N = 22$)

(TonE) [43], which translates the tonicity-stress into transcriptional signals, and is essential for normal cell proliferation under hyperosmotic conditions as well as optimal adaptive immunity [44]. Null function of *Nfat5* led to embryonic and perinatal lethality with incomplete penetrance that has been previously linked to a disturbed kidney homeostasis and function [45]. A recent study, however, demonstrated that mice lacking *Nfat5* exhibited impaired cardiac development [46]. Further characterization of those mice revealed that the cardiomyocytes from *Nfat5* (−/−) embryos exhibited a

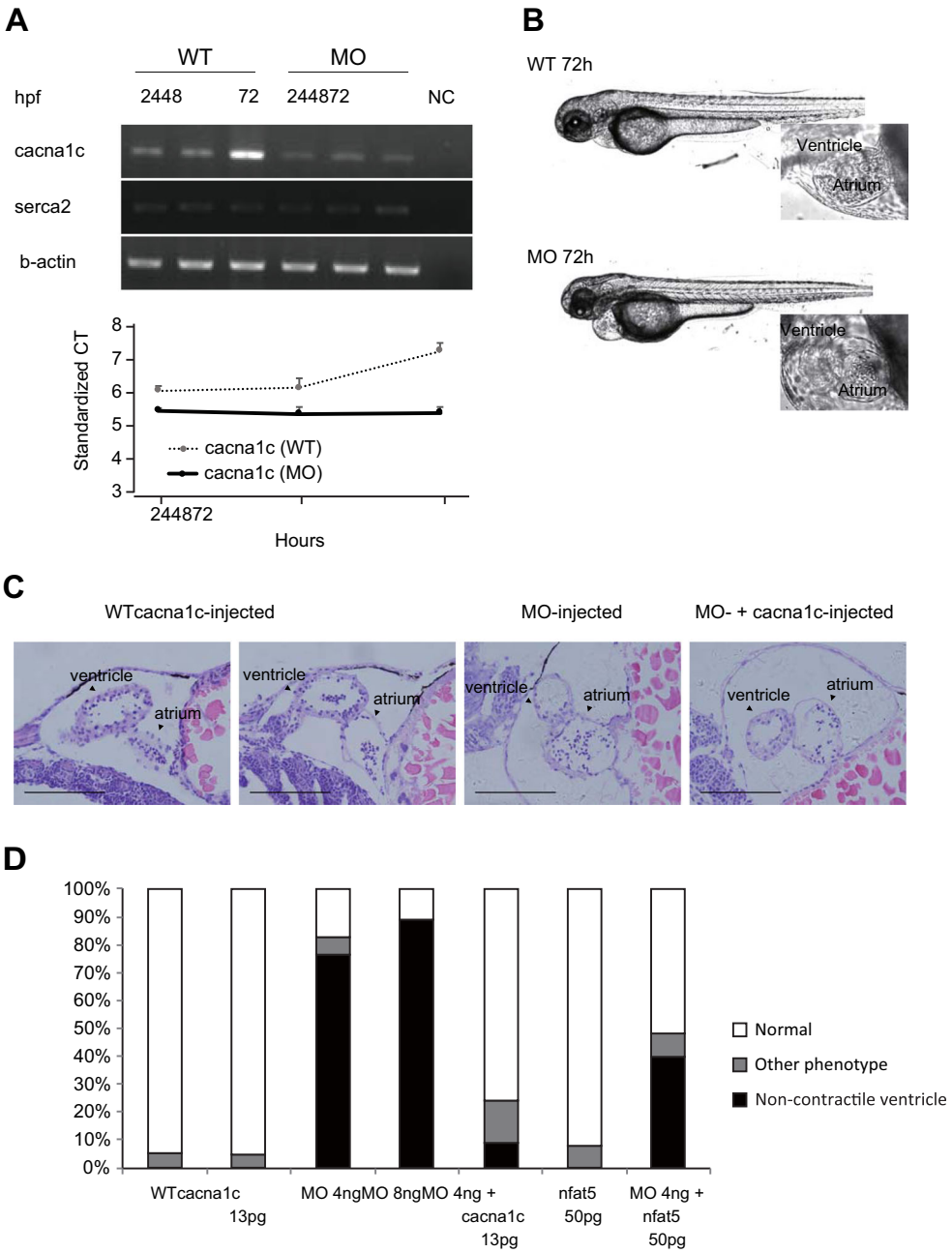

Fig. 4 The *nfat5* regulates ventricular beats and affects cardiac morphogenesis in zebrafish. **a** Semi-quantitative RT-PCR to determine the expression of genes *cacna1c*, *serca2*, and *b-actin* in cardiac tissues from wild-type (WT) and MO-injected zebrafish at 24, 48, and 72 hpf. *MO*: the embryos were injected with morpholino against *nfat5* (*nfat5a*, *upper panel*); the *lower panel* was the expression of cacna1 in WT and MO by a real-time PCR. A plot of standardized CT against hours following introducing morpholino to present the results ($N = 3$ for each group; the least significant difference was used for the post hoc test, $P < 0.01$ between WT and MO). **b** Gross morphology of zebrafish and heart at 72 hpf following injection of MO. **c** Histochemical staining of sections (5 μm) from zebrafish at 72 hpf. *WT*, *Cacna1c-injected*, *MO-injected*, and *Cacna1c- + MO-injected* represented the fish embryos that were wild-type, injected with *Cacna1c*, injected with MO against *nfat5*, and co-injected with both nfat5-MO and *Cacna1c*, respectively. Sections were stained with hematoxylin/eosin. **d** Statistical analysis of cardiac phenotypes at 72 hpf after injection of *Cacna1c*, MO, MO plus *Cacna1c*, *NFAT5*, and MO plus *NFAT5* ($N \geq 60$)

reduction of heart beating rate and developmental defects of ventricular wall that were associated with the disturbed calcium handling [46]. Coincidently, knockout of either *Nfat5* or *Cacna1c* gene leads to embryonic lethality and the embryos die on the same day (E14.5) [21, 45, 46], suggesting that *NFAT5* and *CACNA1C* may have intrinsic relationships associated with cardiac developments. The detailed mechanisms, however, remain unknown.

We provided several lines of evidence to demonstrate that NFAT5 regulates the expression of *CACNA1C*. First, NFAT5 binds to the promoter region (TGGAAGCGTTC) of *CACNA1C* and upregulates its expression. If the NFAT5-binding site is mutated, the expression of *CACNA1C* is dramatically suppressed, as were evident by Ch-IP and promoter activity analysis. Second, morpholino knockdown of *NFAT5* caused a similar phenotype as loss-of-function of *CACNA1C*, and the impaired phenotypes in zebrafish were rescued by over-expression of *CACNA1C*. Finally, knockdown of *NFAT5* dramatically decreased L-type calcium current in neonatal cardiomyocytes. Thus, our study demonstrates NFAT5 as a novel transcriptional regulator of *CACNA1C*.

It has been shown that cardiomyocytes with null function of NFAT5 presented abnormal calcium handling,

presumably attributed to a reduced expression of *RyR2* [46]. To investigate whether NFAT5 regulates other calcium handling-related channels or transporters, we analyzed 5-kb promoters of *RyR2* and *SERCA2* from the eight species, including human, chimpanzee, rat, mouse, chick, dog, zebrafish, and xenopus, and found one potential NFAT5-binding site in *SERCA2* but none in *RyR2* promoter region. The sequence of NFAT5-binding site in mouse *SERCA2* differs from that in *CACNA1C* (TGGTGCTTTA vs TGGAAGCGTTC). Interestingly, both the previous and present studies show that knockdown or knockout of *NFAT5* does not alter the expression of *SERCA2* significantly [46]. On the other hand, there are three copies of NFAT5-binding site in the promoter of *CACNA1C* rather one in that of *SERCA2*. These may explain why the NFAT5-binding site in the promoter of *SERCA2* is not as strong as in that of *CACNA1C*; while the reduction in *RyR2* expression in *NFAT5*-knockout mice is unlikely caused directly by null function of *NFAT5*.

The regulation of NFAT5 on the transcription of *CACNA1C* is evolutionarily conserved. The conserved NFAT5-binding sequence motif does not exist in the promoter of *CACNA1D*. In our study, we showed that NFAT5 significantly enhanced the transcription of *CACNA1C* in both the zebrafish and mouse. This is a critical event during heart development, since functional shift from *CACNA1D* to *CACNA1C* is important for cardiac development and electrophysiological maturation. Our study shows that the developmental expression of *CACNA1C* is due to the increased expression of its regulatory factor NFAT5.

One limitation of our study was that we did not fully characterize other motifs identified; therefore, it is not known how they contribute to the developmental expression of *CACNA1C*. In addition, P19cl6 is derived from P19 embryonic carcinoma cells, thus may not fully present cardiomyocyte. Although we have validated our findings in fish and mouse cardiomyocyte, it should be cautious when considering it as differentiation model for cardiomyocyte in vitro. The other limitation is co-injection of *CACNA1C* did not completely rescue all defective phenotypes in fish; the reasons remain unclear. It is possible that the amount of injected *CACNA1C* was not adequate or that CACNA1C does not cover all functions of NFAT5. Finally, whether NFAT5 has any effects of sarcomeric structures, which are also critical to cardiac contraction, remains to be investigated.

In summary, our study demonstrates that the expression of *CACNA1C* initiated by NFAT5 is essential for cardiac electrophysiological development and maturation.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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