LncRNA ZFAS1 as a SERCA2a Inhibitor to Cause Intracellular Ca\(^{2+}\) Overload and Contractile Dysfunction in a Mouse Model of Myocardial Infarction

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Rationale: Ca\(^{2+}\) homeostasis—a critical determinant of cardiac contractile function—is critically regulated by SERCA2a (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a). Our previous study has identified ZFAS1 as a new lncRNA biomarker of acute myocardial infarction (MI).

Objective: To evaluate the effects of ZFAS1 on SERCA2a and the associated Ca\(^{2+}\) homeostasis and cardiac contractile function in the setting of MI.

Methods and Results: ZFAS1 expression was robustly increased in cytoplasm and sarcoplasmic reticulum in a mouse model of MI and a cellular model of hypoxia. Knockdown of endogenous ZFAS1 by virus-mediated silencing shRNA partially abrogated the ischemia-induced contractile dysfunction. Overexpression of ZFAS1 in otherwise normal mice created similar impairment of cardiac function as that observed in MI mice. Moreover, at the cellular level, ZFAS1 overexpression weakened the contractility of cardiac muscles. At the subcellular level, ZFAS1 deleteriously altered the Ca\(^{2+}\) transient leading to intracellular Ca\(^{2+}\) overload in cardiomyocytes. At the molecular level, ZFAS1 was found to directly bind SERCA2a protein and to limit its activity, as well as to repress its expression. The effects of ZFAS1 were readily reversible on knockdown of this lncRNA. Notably, a sequence domain of ZFAS1 gene that is conserved across species mimicked the effects of the full-length ZFAS1. Mutation of this domain or application of an antisense fragment to this conserved region efficiently canceled out the deleterious actions of ZFAS1. ZFAS1 had no significant effects on other Ca\(^{2+}\)-handling regulatory proteins.

Conclusions: ZFAS1 is an endogenous SERCA2a inhibitor, acting by binding to SERCA2a protein to limit its intracellular level and inhibit its activity, and a contributor to the impairment of cardiac contractile function in MI. Therefore, anti-ZFAS1 might be considered as a new therapeutic strategy for preserving SERCA2a activity and cardiac function under pathological conditions of the heart. (Circ Res. 2018;122:1354-1368. DOI: 10.1161/CIRCRESAHA.117.312117.)

Key Words: calcium ■ myocardial infarction ■ RNA, long noncoding ■ sarcoplasmic reticulum calcium-transporting ATPases

The heart beats rhythmically to drive blood circulating throughout the body, and each single heartbeat begins with the initial phase with Ca\(^{2+}\) influx into cells and Ca\(^{2+}\) release from sarcoplasmic reticulum (SR) through RyR2 (ryanodine receptor 2) to trigger contraction of cardiac muscles, followed by the second phase

DOI: 10.1161/CIRCRESAHA.117.312117

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Novelty and Significance

What Is Known?

- Cardiac function depends on properly controlled concentration of calcium within the myocardium, and excess calcium (or calcium overload) in cardiac muscles is deemed to cause severe impairment of cardiac function.
- SERCA2a (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a) is the major regulator of calcium concentration in cardiac muscles, and reduction of SERCA2a level or dysfunction of this protein can result in calcium overload and cardiac dysfunction.
- The levels and activity of SERCA2a are tightly regulated in cardiac muscles; however, the mechanisms regulating SERCA2 remain unclear.

What New Information Does This Article Contribute?

- ZFAS1—a member of long nonprotein-coding RNAs—is increased in the mouse myocardium after infarction, and artificial mitigation of this increase restores the impaired contractile dysfunction after myocardial infarction.
- ZFAS1 acts as a natural inhibitor of SERCA2a. It reduces the level and function of SERCA2a, causing calcium overload and cardiac dysfunction through direct RNA–protein interactions.
- A specific sequence motif of ZFAS1 was identified as the functional domain responsible for the anti-SERCA2a action of the full-length ZFAS1.

An important mechanism underlying myocardial dysfunction after infarction is abnormal calcium accumulation, or calcium overload muscles, which is often the consequence of decreased level or impaired function of SERCA2a—a key molecule regulating calcium homeostasis. We identified a lncRNA, ZFAS1, as a natural inhibitor of SERCA2a in cardiac muscles. Specifically, we found that levels of ZFAS1 were abnormally increased after myocardial infarction, and this increase was associated with impaired cardiac function. Artificial suppression of ZFAS1 abrogated these anomalies. At the subcellular level, ZFAS1 caused calcium overload, and at the molecular level, ZFAS1 decreased the level and function of SERCA2a by direct binding to this protein. These findings provide insights into the role of lncRNA in regulating SERCA2a and the associated cardiac function. Inhibition of ZFAS1 could be a viable therapy for ameliorating cardiac dysfunction associated with calcium overload.

| Nonstandard Abbreviations and Acronymse |
|----------------------------------------|
| AsZFAS1-FD | antisense fragment corresponding to the functional domain of ZFAS1 |
| CUPID2 | Calcium Upregulation by Percutaneous Administration of Gene Therapy in Patients With Cardiac Disease |
| EF | ejection fraction |
| LV | left ventricular |
| MI | myocardial infarction |
| NFATc2 | nuclear factor of activated T cells C2 |
| NFATc3 | nuclear factor of activated T cells C3 |
| NMCMs | neonatal mouse cardiomyocytes |
| PLN | phospholamban |
| RyR2 | ryanodine receptor 2 |
| SERCA2a | sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a |
| shZFAS1-V | adenine-associated virus 9 vector carrying a ZFAS1-shRNA fragment |
| SR | sarcoplasmic reticulum |
| SS | sarcocere shortening |
| ZFAS1-FD | functional domain of ZFAS1 |
| ZFAS1-V | adenine-associated virus 9 vector carrying the ZFAS1 gene |

contractile function and is delicately regulated by a large body of proteins in a highly coordinated manner. Among these regulatory proteins, SERCA2a (SR Ca\(^{2+}\)-ATPase 2a) is the key protein that governs the normal intracellular Ca\(^{2+}\) handling process and thereby cardiac contractile function by mediating Ca\(^{2+}\) reuptake into SR in cardiac muscles. SERCA2a activity is susceptible to various cellular environmental cues and is under the tight regulation by an array of factors. SERCA2a function can be impaired as a result of either expression deregulation (mostly downregulation) or activity depression leading to deficiency of Ca\(^{2+}\) reuptake back to SR leading to intracellular Ca\(^{2+}\) overload. The consequence of this is manifested by impairment of cardiac contractile function and by cardiomyocyte apoptosis as well. Indeed, SERCA2a dysfunction has been associated with a wide spectrum of cardiovascular diseases, such as myocardial infarction (MI), cardiac hypertrophy, arrhythmias, ischemia/reperfusion, and heart failure especially. Both basic scientists and clinical practitioners have shown their enthusiasm on the potential of SERCA2a overexpression/replacement as a therapeutic strategy. However, a recent randomized, double-blind, placebo-controlled clinical trial named CUPID2 (Calcium Upregulation by Percutaneous Administration of Gene Therapy in Patients With Cardiac Disease) presented negative results on the benefit of SERCA2a overexpression in patients with advanced heart failure with the lack of improvement of reduced ejection fraction (EF). The authors proposed the inadequate dose of SERCA2a as one of the explanations for the lack of benefit of SERCA2a in their study. Clearly, the benefits of SERCA2a replacement have not been well established in the clinical settings, and our understanding of the precise mechanisms for the pathological expression downregulation and functional depression of SERCA2a and the multilayer complex regulatory network for Ca\(^{2+}\) handling is still far from being complete. This constitutes an obstacle for the potential clinical applications of SERCA2a replacement to the treatment of heart disease.

MI is the worst threat to human lives, and it affects an increasing number of individuals worldwide. This is because MI is not only the main cause of sudden cardiac death but also the primary process leading to heart failure. MI is a metabolic catastrophe characterized by contractile dysfunction, arrhythmia, and cell death because of reduced supply of O\(_2\) and nutrients to the myocardium. Hypoxia—a condition of insufficient O\(_2\) supply to support metabolism—is a frequently encountered problem of the cellular microenvironment and a major factor in the pathology of MI. The contractile dysfunction occurring in MI or hypoxia is primarily caused by...
impairment of intracellular Ca\textsuperscript{2+} homeostasis because of the chaos of Ca\textsuperscript{2+} handling.\textsuperscript{19–21} SERCA2a dysfunction as a causal factor for intracellular Ca\textsuperscript{2+} overload and cardiac contractile dysfunction in the setting of MI has been well documented by many research groups.\textsuperscript{22–24} Yet, how SERCA2a expression and function are regulated during MI remained poorly understood.

Human genome sequencing and GENCODE (human genome encyclopedia of DNA elements) project have revealed that only \(\approx 2\%\) of the human genome can be translated into proteins and the rest of it is primarily transcribed into ncRNAs.\textsuperscript{25,26} Recently, several IncRNAs have been reported to be involved in heart disease.\textsuperscript{27–35} In addition, IncRNAs are also emerging as biomarkers for cardiovascular diseases.\textsuperscript{29,35}

In our previous study, we identified ZFAS1—an antisense IncRNA to the 5’ end of the protein-coding gene ZNFX1—as an independent predictor of acute MI.\textsuperscript{30} Intriguingly, ZFAS1 level was found to be markedly decreased in the bloodstream but elevated in the myocardium. We have, therefore, proposed that in addition to its potential as a biomarker of MI, ZFAS1 might also contribute to the development of MI.\textsuperscript{30}

This thought prompted us to carry out the present study to exploit the role of ZFAS1 in the pathological process associated with MI and to decipher the underlying molecular and signaling mechanisms. Specifically, we investigated the effects of ZFAS1 on cardiac contractile function and intracellular Ca\textsuperscript{2+} handling with both gain- and loss-of-function approaches in a mouse model of MI and a cellular model of hypoxia. Our experimental results indicate that ZFAS1 is a detrimental factor to the heart in the setting of MI, and knockdown of this IncRNA is able to mitigate the ischemic contractile dysfunction.

Methods

The data that support the findings of this study are available from the corresponding author on reasonable request.

Human Samples

Human cardiac preparations used in this study were collected from the Tissue bank of the Heilongjiang Academy of Medical Sciences (Harbin, China). The use of human cardiac tissues for the present study was approved from the Ethics Committee of the Harbin Medical University (No. HMUIRB20170034). Our study protocols complied with the guidelines that govern the use of human tissues outlined in the Declaration of Helsinki.

Mouse Model of MI

C57BL/6 mice ranging from 8 to 10 weeks in age and weighing between 22 and 25 g each were used for animal studies, and pregnant C57BL/6 mice were used for neonatal myocyte isolation (Animal Experimental Ethical Inspection Protocol No. HMUIRB20170034). Use of animals was approved by the Animal Ethics Committee of Harbin Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (Publication No. 85-23, revised 1996).

Data Analysis

Data are expressed as means±SEM and were analyzed with SPSS 13.0 software. Statistical comparisons among multiple groups were performed using ANOVA followed by Dunnett test. Student t test was performed for comparisons between 2 groups. A 2-tailed \(P<0.05\) was taken to indicate a statistically significant difference. Nonlinear least square curve fitting of raw data points was performed with GraphPad Prism.

Detailed descriptions on the materials and methods used in this study are provided in the Online Data Supplement.

Results

Upregulation of ZFAS1 Expression in Ischemic Hearts and Hypoxic Cardiomyocytes

We have demonstrated in our recently published study that ZFAS1 expression is markedly elevated in the myocardium of mice with acute MI (from 1 to 24 hours post-MI).\textsuperscript{30} Here, we verified this result in MI mice: compared with the sham group, the expression level of ZFAS1 was increased by 1.8-fold in mouse myocardium 12 hours after MI (Figure 1A, left). Similar increase in ZFAS1 level was also observed in human myocardium from patients with MI (Figure 1A, right).

![Image: Figure 1. Upregulation of ZFAS1 expression in ischemic heart and in hypoxic cardiomyocytes. A. Elevation of cardiac ZFAS1 level in the peri-infarct areas of a mouse model of myocardial infarction (MI; left) and of myocardium from patients with MI (right). Mouse MI was created by ligation of the left descending coronary artery, and the measurements were conducted 12 h after MI. ZFAS1 levels were determined by real-time polymerase chain reaction (same below). *\(P<0.05\), MI mice vs Sham mice (n=6) or patients with MI vs without MI (n=3). B, Increase in ZFAS1 expression in cultured neonatal mouse cardiomyocytes (NMCMS; left) and in AC16 cells (adult human ventricular cardiomyocyte cell line; right) after hypoxia treatment for 12 h, relative to the cells kept under normoxic conditions. **\(P<0.01\), hypoxia vs control; n=4–6. Above data are presented as mean±SEM. C, Representative images of In Situ Hybridization (ISH) in NMCMS showing an increase in ZFAS1 expression after hypoxia treatment for 12 h. Note that ZFAS1 was distributed evenly in both cytosol and nucleus. Red arrows pointing to the nucleus stained in light blue and green ones pointing to ZFAS1 stained in brown. Images are presented with a magnification of ×100 for the (top) and ×500 for the (bottom). Similar results were consistently observed in another 3 batches of cells.](Image312x251 to 531x569)
Figure 2. Impairment of cardiac contractile function by ZFAS1 in myocardial infarction (MI) mice. A, Amelioration of impairment of contractile function by ZFAS1 overexpression produced by the recombinant adeno-associated virus (serotype 9; AAV9) vector carrying the shRNA (shZFAS1-V) to knock down endogenous ZFAS1 in MI mice. The viral constructs were intravenously injected into mice. Note that shZFAS1-V abrogated the ischemia-induced decreases in ejection fraction (EF) and fractional shortening (FS), and enlargement of left ventricular internal dimension at end diastole (LVIDd) and left ventricular internal dimension at systole (LVIDs). shNC-V: the negative control shRNA engineered into the AAV9 vector. **P<0.01 vs Sham, #P<0.05 vs MI, §P<0.05 vs shZFAS1-V; n=≈12–18. B, Impairment of contractile function induced by ZFAS1 overexpression generated by AAV9 vector carrying the full-length ZFAS1 gene (AAV9 vector carrying the ZFAS1 gene [ZFAS1-V]) in healthy mice. Note that ZFAS1-V significantly decreased EF and FS, and increased LVIDd and LVIDs, similar to those seen in MI mice, and these effects were essentially reversed by coinjection of shZFAS1-V. NC-V: the empty AAV9 vector as a negative control for ZFAS1-V. **P<0.01 vs control or NC-V, ##P<0.01 vs ZFAS1-V; n=≈7–10. C, Decreased maximum rate of rise of left ventricular pressure during contraction (+dp/dtmax) and the maximum rate of drop of left ventricular pressure during relaxation (−dp/dtmax) in MI mice. **P<0.01 vs Sham. ZFAS1 overexpressed by ZFAS1-V delivery showing reduced ±dp/dtmax. *P<0.05 vs control, #P<0.05 vs ZFAS1-V; n=3. D, Raw traces (left) showing the changes of sarcomere shortening (SS) as an index of contractility of cardiac muscles isolated from MI hearts, and mean values of SS in the presence of shZFAS1-V (middle) or ZFAS1-V (right). **P<0.01 vs control or NC-V, #P<0.05 vs MI, §§P<0.01 vs ZFAS1-V, §§§P<0.01 vs shZFAS1-V; n=≈20–43. E, Representative cardiac sections showing the successful delivery of shZFAS1-V and ZFAS1-V into mouse myocardium in vivo (Continued)
Hypoxia is a crucial event in the setting of MI, and it alone can cause many of the detrimental alterations of ischemic myocardium. We thought that hypoxia might be sufficient to cause upregulation of ZFAS1 as MI did. Our experiments conducted with cultured neonatal mouse cardiomyocytes (NMCMs) indeed generated the data showing a significant increase in ZFAS1 level after exposure to hypoxic environment for 12 hours (2.6-fold up; \( P<0.01 \); Figure 1B, left). Qualitatively the same results were reproduced in adult human ventricular cardiomyocyte cell line AC16 cells cultured under hypoxic condition (ZFAS1 upregulated by 2-fold; \( P<0.01 \); Figure 1B, right), relative to that in cells kept under normoxic condition.

Moreover, in situ hybridization in NMCMs showed markedly increased abundance of ZFAS1, as indicated by enhanced staining in brown, after exposure to hypoxic conditions, relative to the normoxic conditions (Figure 1C). Also noted was that ZFAS1 is distributed in both cytoplasm and nucleus.

**Impairment of Cardiac Contractile Function by ZFAS1 in MI Mice**

A question we asked was whether the upregulation of ZFAS1 in MI is a contributor to ischemic cardiac injuries or is merely a bystander or a consequence of MI. To clarify this issue, we investigated the effects of ZFAS1 on cardiac function in vivo using echocardiography. We first used a loss-of-function strategy with adeno-associated virus serotype 9 vector carrying a ZFAS1-shRNA fragment (shZFAS1-V) and investigated whether ZFAS1 knockdown could alter cardiac function. Adeno-associated virus serotype 9 was chosen because it is one of the most promising gene transfer tools for gene therapy, and it has been demonstrated to have impressively high infection efficiency and safety profile in cardiac tissue. As illustrated in Figure 2A, both EF and FS (fractional shortening) were substantially lower in the MI mice, compared with the sham-operated control counterparts. Moreover, left ventricular (LV) internal dimension at end diastole and LV internal dimension at systole were both enlarged. These changes indicated severe cardiac dysfunction in our MI model. Strikingly, in the MI mice that had received shZFAS1-V for 4 weeks, the abovementioned parameters of cardiac function were all improved with EF and fractional shortening nearly recovered back to normal levels (Figure 2A). In addition, there was a decrease in LV anterior wall thickness and dilatation, as reflected by the diminished LV internal dimension at end diastole and LV internal dimension at systole (Figure 2A). The negative control construct shNC-V (the negative control shRNA engineered into the AAV9 vector) failed to affect the ischemia-induced cardiac dysfunction.

The data presented above suggest that upregulation of ZFAS1 in MI produces deleterious effects on the heart. If this is true, then overexpression of ZFAS1 in otherwise normal mice should be able to reproduce the phenotypes of MI-induced cardiac contractile dysfunction. To examine this notion, we went on to use the gain-of-function approach for our subsequent experiments using the adeno-associated virus serotype 9 vector carrying the ZFAS1 gene (ZFAS1-V) for its overexpression under in vivo conditions. As depicted in Figure 2B, ZFAS1-V, but not the negative control NC-V (the empty AAV9 vector as a negative control for ZFAS1-V), significantly reduced EF and fractional shortening and enlarged LV internal dimension at end diastole and LV internal dimension at systole, the effects resembling those induced by MI. These deleterious effects were nearly abolished by shZFAS1-V to knock down ZFAS1, indicating the specificity of actions by ZFAS1.

The maximum rate of rise of LV pressure during contraction (+dP/dt\(_{\text{max}}\)) and the maximum rate of drop of LV pressure during relaxation (−dP/dt\(_{\text{max}}\)) have been considered the better manifestations of cardiac contraction and relaxation functions. Our results showed marked decreases in ±dP/dt\(_{\text{max}}\) in MI mice compared with the sham control animals (Figure 2C). Similarly, the healthy mice pretreated with ZFAS1-V for overexpression also exhibited substantially reduced ±dP/dt\(_{\text{max}}\) values relative to control mice. Notably, the decreases in ±dP/dt\(_{\text{max}}\) induced by ZFAS1 overexpression were nearly completely reversed by coapplication of shZFAS1-V (Figure 2C).

The effects of ZFAS1 on cardiac contractile function revealed by the results described above indicate that ZFAS1 produces a negative impact on cardiac mechanical function. To test this notion, we compared the contractility of cardiac muscles isolated from MI mice with those from healthy mice pretreated with ZFAS1-V or shNC-V and sham-operated control mice. It is well established that sarcomere shortening (SS) is an index of contraction force of cardiac muscles: the longer the SS, the greater the contraction force. This is because an increase in SS can result in shortening of interfilament lattice spacing bringing cross-bridges in closer proximity to actin monomers thereby increasing the number of force generating cross-bridges (Figure 2D, left). We, therefore, used SS to reflect contractility of cardiac muscles. As shown in Figure 2D (middle), SS was pronouncedly shortened in MI mice compared with the sham control animals, indicating the mechanical deficiency under ischemic insults. Strikingly, in the cardiac cells isolated from the MI mice infected with ZFAS1-V, but not with shNC-V, SS was restored back toward the normal level as that observed in the sham group. Similar to those occurring in MI, in the cardiac fibers isolated from the healthy mice pretreated with ZFAS1-V, SS was substantially reduced (Figure 2D, right), whereas SS was unaltered by NC-V. Moreover, the decrease of SS induced by ZFAS1 overexpression was considerably mitigated by coapplication of shZFAS1-V.

Successful delivery of shZFAS1-V and ZFAS1-V into the mouse myocardium in vivo was verified by the appreciable presence in myocardial sections of fluorescence signal elicited by GFP (green fluorescent protein) attached to the viral vectors. F. Verification of knockdown of endogenous ZFAS1 by shZFAS1-V in MI myocardium determined by quantitative real-time-polymerase chain reaction. \( *P<0.05 \) vs Sham, \( \#P<0.05 \) vs MI, \( \dagger P<0.05 \) vs shZFAS1-V; \( n=12–20 \). G. Verification of overexpression of ZFAS1 elicited by ZFAS1-V in normal mice 4 and 7 wk after infection. \( *P<0.05 \) vs control, \( \#P<0.05 \) vs ZFAS1-V; \( n=4–10 \). Data are all expressed as mean±SEM.
Figure 3. Impairment of intracellular Ca\textsuperscript{2+} homeostasis by ZFAS1 in cardiomyocytes. A, Restoration of the decreased amplitude of Ca\textsuperscript{2+} transient by adeno-associated virus serotype 9 vector carrying a ZFAS1-shRNA fragment (shZFAS1-V; left) and acceleration of the slowed time courses of the rising and decaying phases of Ca\textsuperscript{2+} transient by shZFAS1-V (right) in adult cardiac cells isolated from myocardial infarction (MI) hearts. **P<0.01 vs Sham, #P<0.05 vs MI, §P<0.05 vs shZFAS1-V; n=17–25. Middle left, Typical examples of Ca\textsuperscript{2+} transient traces recorded in Fluo-3-loaded cardiomyocytes isolated from MI mice with or without shZFAS1-V treatment. Right, Averaged data of time constants for sarcoplasmic reticulum (SR) Ca\textsuperscript{2+} release from (\(\tau_r\) for the rising phase) and Ca\textsuperscript{2+} reuptake back to SR (\(\tau_d\) for the decaying phase). The time constants were acquired by single exponential curve fitting to the data points (Continued)
the ZFAS1 transcript level in the myocardium (Figure 2F). Overexpression of ZFAS1 on administration of ZFAS1-V for 4 and 7 weeks to healthy mice was also affirmed (Figure 2G; Online Figure IB). As expected, the negative controls NC-V did not affect the ZFAS1 level, whereas coapplication of shZFAS1-V and ZFAS1-V abolished the overexpression of ZFAS1.

**Impairment of Intracellular Ca²⁺ Homeostasis by ZFAS1 in Cardiomyocytes**

Ca²⁺ homeostasis is the key determinant of cardiac contractility, and abnormal intracellular Ca²⁺ handling might account at least partially for the observed impairment of contractile function caused by ZFAS1 in the setting of MI. We, therefore, went on to study the effects of ZFAS1 on dynamic Ca²⁺ transient and resting intracellular Ca²⁺ concentration ([Ca²⁺]i), reflecting the intracellular Ca²⁺ handling process and the consequence of alteration of such a process, respectively.⁴²⁻⁴³ To this end, the adult ventricular myocytes isolated from MI hearts were loaded with fluo-3 and electrically stimulated at 1 Hz to initiate intracellular Ca²⁺ transients. The amplitude of peak systolic Ca²⁺ transient was calculated according to the equation (Fₚ – F₀)/F₀, where F represents the maximum value of a Ca²⁺ transient and F₀ the diastolic/resting Ca²⁺ level immediately before the onset of a Ca²⁺ transient, and the time courses of rising (an indication of the rate of SR Ca²⁺ release) and decaying (an indication of the rate of SR Ca²⁺ reuptake) phases were obtained by exponential curve fitting to the raw traces of Ca²⁺ transient. As shown in Figure 3A (left), the amplitude of Ca²⁺ transient was decreased by 34.5% in MI relative to that in sham controls, which was partially but significantly restored toward the normal values in the MI mice pretreated with shZFAS1-V. More strikingly, the MI-induced prolongation of time course for the decay phase of Ca²⁺ transient was remarkably accelerated by shZFAS1-V, as reflected by the reduced decay time constant (τ_r; Figure 3A, right). The rate of rising phase of Ca²⁺ transient (τ_r) exhibited a trend of decelerating in MI, and such a slowing was also effectively reversed by knockdown of endogenous ZFAS1 (Figure 3A, middle right). In all cases, the negative control construct shNC-V did not affect the deleterious alterations in MI mice.

As anticipated, overexpression of ZFAS1 by ZFAS1-V in healthy mice produced the phenotypic changes of Ca²⁺ transient, which were qualitatively the same as those seen in MI mice (Figure 3B). Specifically, ZFAS1-V slowed the kinetics of decaying phases, as the manifested by the remarkable increase of the time constants for Ca²⁺ reuptake (τ_r; Figure 3B, right), and such an effect was completely abrogated by shZFAS1-V. By comparison, the kinetics of rising phases (τ_r) exhibited a trend to slow, but the alterations did not reach statistical significance (Figure 3B, middle right). Along with the changes of the time constants was a decrease in the amplitude of Ca²⁺ transient with ZFAS1 overexpression (Figure 3B, left).

The opposite changes of Ca²⁺ transient seen between ischemia or ZFAS1-V and shZFAS1-V predicted a loss of Ca²⁺ homeostasis leading to intracellular Ca²⁺ overload in MI and a relief of such an overload by shZFAS1-V. The results acquired from the measurements of [Ca²⁺]i in adult cardiomyocytes isolated from MI mice and in cultured neonatal cardiomyocytes were indeed in line with this note. As illustrated in Figure 3C (left), the resting [Ca²⁺]i in isolated cardiomyocytes from MI mice was increased, and such an increase was abolished by shZFAS1-V to knockdown endogenous ZFAS1. On the contrary, forced expression of ZFAS1 with ZFAS1-V infection directly caused Ca²⁺ overload in healthy mice (Figure 3C, right). These effects of ZFAS1-V were effectively reversed by coinjection of shZFAS1-V, and the negative control NC-V was unable to exert any appreciable impact on [Ca²⁺]i.

As expected, the resting [Ca²⁺]i in NCMCs exposed to hypoxic environment underwent similar changes as in MI: it was increased in hypoxia, and such an increase was abolished by siZFAS1 to silence ZFAS1 expression (Figure 3D, left). Consistent with the increase in [Ca²⁺]i, the rate of Ca²⁺ reuptake into SR was tremendously slowed (τ_r increased) in hypoxic cells compared with the control group, and this anomaly was essentially rescued by siZFAS1 (Figure 3D, right). In sharp contrast to ZFAS1 silencing, forced expression of ZFAS1 with transfection of the pCDNA-ZFAS1 vector directly caused Ca²⁺ overload in nonhypoxic cardiomyocytes (Figure 3E, left). These effects of ZFAS1 were effectively reversed by cotransfection of siZFAS1, and the negative control construct of siZFAS1 was unable to exert any appreciable impact on [Ca²⁺]i. Expectedly, overexpression of ZFAS1 was able to induce slowing of τ_r—an effect abrogated by siZFAS1—whereas negative control construct of siZFAS1 did not elicit any significant changes (Figure 3E, right).

The effects of shZFAS1-V and ZFAS1-V on Ca²⁺ transient were also assessed by optical mapping techniques.
Downregulation and Dysfunction of SERCA2a

It has been reported that SERCA2a—the primary cardiac isoform and the key protein involved in sequestration of Ca\(^{2+}\) into SR during diastole—plays a major role in the regulation of Ca\(^{2+}\) homeostasis in cardiomyocytes.\(^3\) Moreover, SERCA2a has been reported to be abnormally downregulated in a variety of cardiac conditions, including MI.\(^2,24\) The delayed time course of the decaying phase of Ca\(^{2+}\) transient by ZFAS1 in our study suggests an impairment of Ca\(^{2+}\) reuptake into SR. A question raised in our mind was whether ZFAS1 is involved in the regulation of SERCA2a expression and function or whether alteration of SERCA2a mediates the effects of ZFAS1. As shown in Figure 4A, the cardiac expression of SERCA2a at the protein level was prominently decreased in MI mice relative to the sham animals. Similarly, SERCA2a level was also significantly downregulated on overexpression of ZFAS1 in normal mice (Figure 4B). In both of these situations, shZFAS1-V rescued the loss of SERCA2a. The expression of SERCA2a mRNA demonstrated qualitatively the same changes as its protein levels (Figure 4A and 4B).

Similar patterns of expression alterations of SERCA2a mRNA and protein were consistently observed in primary NMCMs cultured under hypoxic conditions and with treatments with varying constructs (Figure 4C and 4D).

With the above results indicating the significant role of ZFAS1 in regulating SERCA2a expression, we came up with a further question: Does ZFAS1 have any direct effects on SERCA2a activities in addition to expression regulation? To get insight into this issue, the first step we took was to perform theoretical analysis for RNA:protein binding using the RNA–Protein Interaction Prediction database. Our analysis revealed a high probability of ZFAS1:SERCA2a interaction (Online Figure III). This initial analysis encouraged us to verify the functional relationship between the 2 molecules. We, therefore, switched to an experimental approach using RNA-binding
protein immunoprecipitation to see whether ZFAS1 could physically bind SERCA2a. The results depicted in Figure 5A clearly indicate the presence of such an interaction: immunoprecipitation of SERCA2a carried an appreciable amount of ZFAS1; conversely, RNA pulldown of ZFAS1 also dragged down an appreciable quantity of SERCA2a (Figure 5B, right), suggesting that ZFAS1 has a strong affinity to SERCA2a.

If ZFAS1 indeed binds to SERCA2a, then it should be present in the SR and colocalize with the latter. The purity of SR was verified by the exceptionally enhanced activities of SR-specific NADPH (nicotinamide adenine dinucleotide phosphate) cytochrome C reductase (Figure 5C). The data depicted in Figure 5D show that ZFAS1 level was significantly increased in the RNA samples isolated from purified SR. Meanwhile, our data exhibited that SERCA2a protein level in SR was significantly decreased (Figure 5E). Moreover, following the same approach described by other groups,45-46 our immunofluorescent staining of SERCA2a protein in conjunction with GFP-labeling of lncRNA ZFAS1 demonstrated that ZFAS1 and SERCA2a proteins had essentially the same subcellular distribution pattern primarily around the nucleus and the 2 molecules colocalized (Online Figure IV).

As already mentioned, in addition to SERCA2a, other proteins, such as RyR2, PLN (phospholamban), and Cav1.2 (the pore-forming α-subunit of L-type Ca2+ channels), are also crucially involved in the regulation of intracellular Ca2+ homeostasis.7-40 We, therefore, evaluated the effects of ZFAS1 on the expression of these proteins. As shown in Online Figure V, no significant alterations of PLN, RyR2, and Cav1.2 protein levels are observed with ZFAS1 overexpression. Moreover, the lack of effect of ZFAS1 on L-type Ca2+ channel current density further excluded the contribution of this channel to the intracellular Ca2+ overload induced by ZFAS1 (Online Figure VI).

Identification of the Functional Domain of ZFAS1 Key to the Modulation of SERCA2a

By sequence alignment, we identified a region of high-degree conservation across varying species, including man and mouse (Online Figure VII). We contemplated that this sequence domain might be the functional region for ZFAS1 (Functional Domain of ZFAS1 [ZFAS1-FD]) to interact with SERCA2a. Our analysis using the computational docking software Hex 8.050,51 indeed provided a piece of theoretical evidence for our conjecture by showing the ability of ZFAS1-FD to bind SERCA. The docking results obtained by Pymol software revealed that the nucleotides U19, G20, G23, G25, and G26 encompassed by ZFAS1-FD are likely the core motif for specific binding to SERCA amino acids Ala241, Glu240, Glu249, Ser693, Arg667, Arg671, and Arg672 (Online Figure VIII). Further intriguing was that the SERCA domain that binds ZFAS1 identified in this study is known to be the phosphorylation domain of SERCA, which contains several potential phosphorylation sites (Ser693, Arg667, and Arg671).52,53 This information prompted us to further examine our assumption by conducting the following experiments with gain-of-function and loss-of-function approaches.

First, an oligonucleotide fragment corresponding to the conserved region of ZFAS1 gene (ZFAS1-FD; Figure 6A, top) was synthesized. ZFAS1-FD was transfected into NMCMS by using X-tremeGENE Transfection Reagent (No. 10810500; Roche) according to the manufacturer’s instructions. The ability of ZFAS1-FD to bind SERCA2a and its effects on SERCA2a and Ca2+ handling were examined. As illustrated in Figure 6B through 6D, application of ZFAS1-FD reproduced the effects of full-length ZFAS1 on SERCA2a expression and intracellular Ca2+ status in NMCMS: downregulation of SERCA2a at both protein and mRNA levels (Figure 6B), deceleration of decaying kinetics of Ca2+ transient (Figure 6C), and increase in resting Ca2+ concentration (Figure 6D). The negative control oligonucleotides fragment (NC) did not produce any of these effects.

Next, we mutated the core motif of the ZFAS1-FD by nucleotide substitution to disrupt the binding site for SERCA (Mut-ZFAS1-FD; Online Table I) and examined the effects of this construct on SERCA2a function. The computational docking results confirmed the loss of the original binding sites for SERCA in Mut-ZFAS1-FD (Online Figure IX). Compared with wild-type ZFAS1-FD, Mut-ZFAS1-FD failed to affect the expression of SERCA2a at both mRNA and protein levels in NMCMS (Figure 6E), and as anticipated, Mut-ZFAS1-FD also lost the ability to alter [Ca2+]i (Figure 6F) and Ca2+ transient (Figure 6G).

We then subsequently constructed an oligonucleotide fragment antisense to ZFAS1-FD (As-ZFAS1-FD; Figure 6A, bottom) with methylation modification to enhance stability. Introduction of As-ZFAS1-FD into the NMCMS effectively abolished the repressive effect of ZFAS1 on SERCA2a expression at both mRNA and protein levels, presumably by annealing to ZFAS1-FD (Figure 6H). Consistently, As-ZFAS1-FD also robustly reversed the ZFAS1-induced reduction of the amplitude of Ca2+ transient (Figure 6I) and delay of the time courses of Ca2+ release and reuptake (Figure 6J). By comparison, the negative control antisense fragment failed to affect the effects of ZFAS1.

Finally, to verify that the effects of As-ZFAS1-FD observed in our experiments were indeed attributable to the antisense action, we looked at the changes of ZFAS1 level in cells treated with the antisense construct. As shown in Figure 6K, the ZFAS1 level was markedly decreased on transfection with As-ZFAS1, but not with negative control antisense fragment, in NMCMS pretreated with pCDNA-ZFAS1 vector.

NFATc2 as a Transactivator of ZFAS1 Expression

Although the above data have indicated the role of ZFAS1 up-regulation in causing intracellular Ca2+ overload and the underlying downstream mechanisms in the setting of MI, it remained unknown how ZFAS1 was upregulated in MI. To shed light on this issue, we first performed computational analysis and identified several binding sites for NFATc2 (nuclear factor of activated T cells C2) that has been reported to be abundantly expressed in the cardiac tissue and participated in cardiac hypertrophy and heart failure.54,55 We, therefore, investigated the possible role of NFATc2 in the regulation of ZFAS1 expression. Our results first demonstrated that the protein level of NFATc2 was robustly upregulated in MI and in NMCMS exposed to hypoxic conditions (Online Figure XA). Application of NFATc2 inhibitor FK506 (10 nmol/L) to NMCMS caused a significant reduction of ZFAS1 expression relative to nontreated cells.
Figure 5. Interaction between IncRNA ZFAS1 and SERCA2a (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a) protein. 

A. RNA-binding protein immunoprecipitation (RIP) analysis for ZFAS1:SERCA2a interaction. Note that immunoprecipitation (IP) of SERCA2a retrieved a robust amount of ZFAS1. **P<0.01 vs anti-IgG; n=4. 

B. RNA pulldown of ZFAS1 dragged down an appreciable quantity of SERCA2a. The band for the binding between ZFAS1 and SERCA2a protein disappeared when treated with an antisense fragment to ZFAS1 (AsZFAS1). Additionally, an unrelated IncRNA PLSCR4 (phospholipid scramblase 4) as a negative control, could not drag down SERCA2a, indicating the specific of the ZFAS1:SERCA2a interaction. **P<0.01, ###P<0.01, §§P<0.01 vs ZFAS1; n=3. 

C. Verification of the purity of isolated sarcoplasmic reticulum (SR) by the enhanced activity of SR-specific NADPH (nicotinamide adenine dinucleotide phosphate) cytochrome C reductase determined by colorimetry assay. **P<0.01 vs total protein samples; n=4. 

D. Upregulation of ZFAS1 expression in SR isolated from myocardial infarction (MI) hearts relative to Sham hearts, determined by quantitative real-time-polymerase chain reaction. *P<0.05 vs Sham; n=3. 

E. Downregulation of SERCA2a protein in SR of MI myocardium relative to sham control, determined by Western blot analysis. *P<0.05 vs Sham; n=3.
Figure 6. Dysfunction of SERCA2a (sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a) produced by ZFAS1. A, Top, An oligonucleotide fragment corresponding to the conserved region of ZFAS1 gene (functional domain of ZFAS1 [ZFAS1-FD]). Bottom, An oligonucleotide fragment antisense to ZFAS1-FD (AsZFAS1-FD). B, Downregulation of SERCA2a expression at both protein (left) and mRNA (right) levels in neonatal mouse cardiomyocytes (NMCMs) transfected with ZFAS1-FD. *P<0.05 vs control, #P<0.05 vs ZFAS1-FD; n=≈5–7. C, Deceleration of decaying kinetics of Ca\(^{2+}\) transient in NMCMs transfected with ZFAS1-FD. *P<0.05 vs control, #P<0.05 vs ZFAS1-FD; n=≈17–22. D, Increase in resting Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in NMCMs transfected with ZFAS1-FD. **P<0.01 vs control, ##P<0.01 vs ZFAS1-FD; n=≈14–30. E, Effects of Mut-ZFAS1-FD and ZFAS1-FD on the expression of SERCA2a at both protein (left) and mRNA (right) levels in NMCMs. *P<0.05 vs control or NC; n=≈6–8. F, Resting Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in NMCMs transfected (Continued)
(Online Figure XI). Notably, the effects of were found consistent with the lack of NFATc3 binding sites in the pro-

**Discussion**

The aims of this study were to elucidate the pathophysiologi-

The decaying kinetics of Ca\(^{2+}\) transient in NMCMs ~0.05 vs As\(\)P, §~0.05 vs \(-\)FD; \(n=6\).

**Verification of the efficacy of As\(\) overexpression.**

**Comparison With Published Studies on ZFAS1**

ZFAS1—a new member of IncRNAs—was initially identified in patients with breast tumors and subsequently characterized in other cancers.\(^{56-59}\) Our recently published study suggested circulating level of ZFAS1 as a new biomarker for MI be-

An interesting finding in the present study is that the se-

a research article on ZFAS1 in the heart was published.\(^{60}\) In agreement with our finding with MI mice, the authors showed that ZFAS1 is upregulated in a rat model of acute MI, and such an increase induces cardiomyocyte apoptosis. They fur-

Here, we presented the experimental data with both an animal model of MI and a cellular model of hypoxia clearly indicating the pathophysiological function of ZFAS1 in the regulation of cardiac contractile function. Specifically, abnormal upregulation of ZFAS1 in MI or artificial over-

Comparison With Published Studies on ZFAS1

ZFAS1—a new member of IncRNAs—was initially identified in patients with breast tumors and subsequently characterized in other cancers.\(^{56-59}\) Our recently published study suggested circulating level of ZFAS1 as a new biomarker for MI be-

**Norms**, \(P\text{-}\)FD. **P<0.01 vs control, §~0.05 vs As\(\)P, #~0.05 vs As\(\)P-FD. *~0.05 vs control or NC; \(n=6\). The decaying kinetics of Ca\(^{2+}\) transient in NMCMs ~0.05 vs As\(\)P-FD; \(n=5–6. G,\) The driving kinetics of Ca\(^{2+}\) transient in NMCs transfected with Mut-ZFAS1-FD. **P<0.01 vs control or NC; \(n=9–12. H,\) Uprégulation of SERCA2a expression at both protein (left) and mRNA (right) levels by As\(\)ZFAS1-FD in NMCs pretreated with ZFAS1-P for ZFAS1 overexpression. **P<0.05 vs ZFAS1-P, \#P<0.05 vs As\(\)ZFAS1-FD; \(n=5–9. I,\) Reversal of ZFAS1-induced reduction of Ca\(^{2+}\) transient amplitude by As\(\)ZFAS1-FD. **P<0.05 vs ZFAS1-P, \#P<0.05 vs As\(\)ZFAS1-FD; \(n=10–18. J,\) Reversal of the time delay of Ca\(^{2+}\) release and reuptake by As\(\)ZFAS1-FD in NMCs pretreated with ZFAS1-P for ZFAS1 overexpression. **P<0.05 vs As\(\)ZFAS1-FD (left) or ZFAS1-P (right), \#P<0.05 vs As\(\)ZFAS1-FD; \(n=12–18. K,\) Verification of the efficacy of As\(\)ZFAS1-FD in reducing ZFAS1 level in NMCs pretreated with ZFAS1-P for ZFAS1 overexpression. **P<0.01 vs control, \#P<0.05 vs ZFAS1-P, §P<0.05 vs As\(\)ZFAS1-FD; \(n=6.**
the core motif with specific nucleotides contained in ZFAS1-FD being responsible for binding to a SERCA domain containing amino acids Ala241, Glu243, Glu89, Ser693, Arg677, Arg671, and Arg672. Most strikingly, the SERCA domain that binds ZFAS1 identified in this study is right within the phosphorylation domain of SERCA, which contains several potential phosphorylation sites (Ser693, Arg677, and Arg672). It is known that SERCA pumps are P-type ion motive ATPases that in their sequence structures contain 3 major domains on the cytoplasmic face: the phosphorylation domain (or phosphorylation domain), nucleotide-binding domain, and the actuator domain.25,35 Although PLN phosphorylation to increase the affinity of SERCA2 for Ca²⁺ is the most important modulation of this protein, direct CaMKII (calmodulin-dependent protein kinase II)-dependent phosphorylation of SERCA2 has also been documented to be an important route to control the enzyme function,61,62 particularly when considering the fact that PLN was unaffected by ZFAS1 in our models. Direct phosphorylation at residue Ser28 in SERCA2a activates enzyme function and enhances Ca²⁺ reuptake into SR and cardiac contractility.63 It is possible that SERCA2a can also be activated with its phosphorylation domain being phosphorylated, and when bounded by ZFAS1, the phosphorylation sites of phosphorylation domain are masked by the latter. Such masking would prevent SERCA2a from being phosphorylated by CaMKII or other kinases thereby preventing SERCA2a from being activated. At present, such a view is highly speculative and requires rigorous studies to clarify. Nonetheless, these facts together with our findings would suggest that we have identified the ZFAS1-FD responsible for the effects of this lncRNA on SERCA2a and its associated intracellular Ca²⁺ handling process.

Potential Implications of Our Findings

The findings in the present study, together with the published studies from our own laboratory29 and from other research groups,60 indicate that ZFAS1 is not only a biomarker for, or predictor of, MI but also a determinant of cardiac function in the setting of MI and might be in other cardiovascular pathological processes as well. In particular, the fact that knockdown of ZFAS1 is able to rescue the deleterious actions of ZFAS1 on cardiac function opens up an opportunity for correcting the functional impairment of heart caused by ischemia/hypoxia. This would imply that ZFAS1 might be considered a novel therapeutic target for maintaining cardiac function in MI. Or in other words, shZFAS1-V or other forms of ZFAS1 inhibitor could be developed into novel therapeutic agent for ameliorating cardiac dysfunction.

In addition, our finding that AsZFAS1-FD was also able to eliminate the detrimental action of ZFAS1 on SERCA2a and cardiac function suggests that antisense is another valid strategy for the management of MI-induced cardiac impairment. Such an antisense strategy might be even superior to the knockdown approach because it targets directly to the functional domain of the lncRNA, and it does not induce breakdown of ZFAS1 but merely reduces the functional availability of it. One of the advantages of these properties is that it is not on withdrawal of the antisense, the function of ZFAS1 could readily recover.

Possible Limitations of Our Study

We are well aware that our study contains several limitations. First, despite that ZFAS1 elicited remarkable suppressive effects on SERCA2a and the associated Ca²⁺ transient decaying kinetics and contractility, it did not cause proportional depression of cardiac function (EF and fractional shortening). One possible explanation for this is that ZFAS1 might also be able to evoke some other actions in addition to SERCA2a inhibition, which could relieve its detrimental effects and partially preserve the cardiac function. Second, although we have identified a sequence motif that is evidently important to the role of ZFAS1 in regulating SERCA2a and is reasonably well conserved between mouse and human, the conclusion drawn from our animal study may not be extrapolated directly to man. And third, the present study does not answer the question how ZFAS1 altered the mRNA level of SERCA2a. There are a couple of possible explanations for the observation. First, ZFAS1 might act as transcriptional repressor by modifying the chromatin structure/methylation or indirectly by regulating relevant transcriptional factors or RNA maturation via epigenetic modifications. In addition, lncRNAs can also interact with mRNAs through direct antisense binding to either stabilize or induce degradation of the targeted mRNAs. We found that ZFAS1 carried several sequence stretches that are complementary to the 3'-end untranslated region of SERCA2a, and such an antisense relationship might impose a direct interaction between ZFAS1 and SERCA2a mRNA to alter the expression levels mutually. Yet, rigorous future studies are required to clarify these issues.

Sources of Funding

This work was supported, in part, by the grants from the Funds for National Key Research and Development Program of China (2017YFC1307403 to Dr Yang), the Key Program of National Natural Science Foundation of China (81730012 to Dr Yang), and the National Natural Science Foundation of China (81470490 to Dr Ying Zhang and 81570399 to Dr Yong Zhang).

Disclosures

None.

References

1. Luo M, Anderson ME. Mechanisms of altered Ca²⁺ handling in heart failure. Circ Res. 2013;113:690–708. doi: 10.1161/CIRCRESAHA.113.301651.
2. Li XC, Wei L, Zhang GQ, Bai ZL, Hu YY, Zhou P, Bai SH, Chai Z, Lakatta EG, Hao XM, Wang SQ. Ca²⁺ cycling in heart cells from ground squirrels: adaptive strategies for intracellular Ca²⁺ homeostasis. PLoS One. 2011;6:e24787. doi: 10.1371/journal.pone.0024787.
3. Park WJ, Oh JG. SERCA2a: a prime target for modulation of cardiac contractility during heart failure. BMB Rep. 2013;46:237–243.
4. Xin W, Lu X, Li X, Niu K, Cai J. Attenuation of endoplasmic reticulum stress-related myocardial apoptosis by SERCA2a gene delivery in ischemic heart disease. Mol Med. 2011;17:201–210. doi: 10.2119/molmed.2010.00197.
5. Younce CW, Burmeister MA, Ayala JE. Exendin-4 attenuates high glucose-induced cardiomyocyte apoptosis via inhibition of endoplasmic reticulum stress and activation of SERCA2a. Am J Physiol Cell Physiol. 2013;304:C508–C518. doi: 10.1152/ajpcell.00248.2012.
6. Kawai Y, Hajiar RJ. The cardiac sarcoplasmic/endoplasmic reticulum calcium ATPase: a potent target for cardiovascular diseases. Nat Clin Pract Cardiovasc Med. 2008;5:554–565. doi: 10.1038/ncpcardio1301.
7. Faria Tde O, Costa GP, Almenara CC, Angeli JK, Vassallo DV, Stefanon I, Vassallo PF. Chronic exposure to low doses of HgCl₂ avoids calcium handling impairment in the right ventricle after myocardial infarction in rats. PLoS One. 2014;9:e95639. doi: 10.1371/journal.pone.0095639.
Transl Res 2016;173:38–54. doi: 10.1111/bph.12472.

Br J Cardiovasc Dis 2013;8:e79374. doi: 10.1371/journal.pone.0079374.

Circ Res 2015;6:6779. doi: 10.1038/ncomms7779.

Zhang Y, Sun L, Xuan L, et al. Reciprocal changes of long non-coding RNAs ZFAS1 and CDR1AS predict acute myocardial infarction. Sci Rep. 2016;6:22384. doi: 10.1038/srep22384.

Wang K, Liu F, Zhou LY, Long B, Yuan SM, Wang Y, Liu CY, Sun T, Zang XJ, Li P. The long non-coding RNA CHRF regulates cardiac hypertrophy by targeting miR-485. Circ Res. 2014;114:1377–1388. doi: 10.1161/CIRCRESAHA.114.302476.

Wang K, Long B, Zhou LX, Liu F, Zhou QY, Liu CY, Fan YY, Li P. CARL IncRNA inhibits anoxia-induced mitochondrial fission and apoptosis in cardiomyocytes by impairing miR-539-dependent PHB2 downregulation. Nat Commun. 2014;5:3596. doi: 10.1038/ncomms4596.

Zhang X, Sha M, Yao Y, Da J, Jing D. Increased B-type-natriuretic peptide promotes myocardial cell apoptosis via the B-type-natriuretic peptide/long non-coding RNA LIPCAR non-coding RNA signaling pathway. Mol Med Rep. 2015;12:6761–6767. doi: 10.3892/mmr.2015.4247.

Han P, Li W, Lin CH, et al. A long non-coding RNA protects the heart from pathological hypertrophy. Nature. 2014;514:102–106. doi: 10.1038/ nature13596.

Liu L, An X, Li Z, Song Y, Li L, Zuo S, Liu N, Yang G, Wang H, Cheng X, Zhang Y, Yang X, Wang J. The H19 long non-coding RNA is a novel negative regulator of cardiomyocyte hypertrophy. Cardiovasc Res. 2016;111:56–65. doi: 10.1093/cvrcel/ocv007.

Xuan L, Sun L, Zhang Y, et al. Circulating long non-coding RNAs NRON and MHRT as novel predictive biomarkers of heart failure. J Cell Mol Med. 2017;21:1803–1814. doi: 10.1111/jcmm.13101.

Aurora AB, Mahmoud AI, Luo X, Johnson BA, van Rooij E, Matsuzaki S, Humphries KM, Hill JA, Bassel-Duby R, Sadek HA, Olson EN. MicroRNA-214 protects the mouse heart from ischemic injury by controlling Ca2+ overload and cell death. J Clin Invest. 2012;122:1222–1232. doi: 10.1172/JCI59327.

Regula KM, Ens K, Kirshenbaum LA. Inducible expression of BNP3 provokes mitochondrial defects and hypoxia-mediated cell death of ventricular myocytes. Circ Res. 2002;91:226–231.

Inagaki K, Fuess S, Nandakumar A, Fliegauf M, Mei C, Moser M, Riva M, Nakai K. Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. Mol Ther. 2006;14:45–53. doi: 10.1038/sj.mt.6300349.

Parekh K, Kolias TJ. A novel noninvasive method to assess left ventricular -dP/dt using diastolic blood pressure and isovolumic relaxation time. EchoCardiography. 2015;30:267–270. doi: 10.1111/eco.12042.

Buchanan LY, Warner WA, Arthur SR, Gleeson CR, Lewis GW, Levesque PC, Gill MW. Evaluation of cardiac function in unrestrained dogs and monkeys using a left ventricular dp/dt. J Pharmacol Toxicol Methods. 2016;80:51–58. doi: 10.1016/j.jptm.2016.03.006.

Hobai IA, Morse JC, Siwak DA, Colucci WS. Lipo polysaccharide and cytokines inhibit rat cardiomyocyte contractility in vitro. J Surg Res. 2015;193:888–901. doi: 10.1016/j.jss.2014.09.017.

Kobayashi S, Susa T, Ishiguchi H, et al. A low-dose β1-blocker in combination with milrinone improves intracellular Ca2+ handling in failing cardiomyocytes by inhibition of milrinone-induced diastolic Ca2+ leak- age from the sarcoplasmic reticulum. PLoS One. 2015;10:e0141341. doi: 10.1371/journal.pone.0141341.

Gómez-Viquez NL, Guerrero-Serna G, Arvizu F, García U, Guerrero-Hernández A. Inhibition of SERCA2a pump induces desynchronized RyR activation in overloaded internal Ca2+ stores in smooth muscle cells. Am J Physiol Cell Physiol. 2010;298:C1038–C1046. doi: 10.1152/ajpcell.00222.2009.

Zalvidea S, André L, Loyer X, Cassan C, Sainte-Marie Y, Thieau J, Sjaastad I, Heymes C, Pasquié JL, Cazorla O, Aimond F, Richard S, ACE inhibition prevents diastolic Ca2+ overload and loss of myocardial Ca2+ sensitivity after myocardial infarction. Circulation. 2015;132:206–217.

Nguyen BR, Makarewich CA, Anderson DM, Winders BR, Troapea CD, Wu F, Reese AL, McNally JR, Chen X, Kavalali ET, Cannon SC, Houser
SR, Bassel-Duby R, Olson EN. A peptide encoded by a transcript annotated as long noncoding RNA enhances SERCA activity in muscle. Science. 2016;351:271–275. doi: 10.1126/science.aad4076.

46. Anderson DM, Anderson KM, Chang CL, Makarewich CA, Nelson BR, McAnally JR, Kasaragod P, Shelton JM, Liu J, Bassel-Duby R, Olson EN. A micropeptide encoded by a putative long noncoding RNA regulates muscle performance. Cell. 2015;160:595–606. doi: 10.1016/j.cell.2015.01.009.

47. Zhao G, Li T, Brochet DX, Rosenberg PB, Lederer WJ. STIM1 enhances SR Ca²⁺ content through binding phospholamban in rat ventricular myocytes. Proc Natl Acad Sci USA. 2015;112:E4792–E4801. doi: 10.1073/pnas.1423295112.

48. Yang Y, Guo T, Oda T, Chakraborty A, Chen L, Uchinoumi H, Knowlton AA, Fruen BR, Cornea RL, Meissner G, Bers DM. Cardiac myocyte Z-line calmodulin is mainly RyR2-bound, and reduction is arrhythmogenic and occurs in heart failure. Circ Res. 2014;114:295–306. doi: 10.1161/CIRCRESAHA.114.302857.

49. Weiss S, Oz S, Bennmocha A, Dascal N. Regulation of cardiac L-type Ca²⁺ channel CaV1.2 via the β-adrenergic-cAMP-protein kinase A pathway: old dogmas, advances, and new uncertainties. Circ Res. 2013;113:617–631. doi: 10.1161/CIRCRESAHA.113.301781.

50. Rahim AH, Setiawan B, Dewi FR, Noor Z. Regulation by phloroglucinol of Nrf2/MAF-mediated expression of antioxidant enzymes and inhibition of osteoclastogenesis via the RANKL/RANK signaling pathway: in silico study. Acta Inform Med. 2014;23:332–337. doi: 10.5455/aim.2014.23.332-337.

51. Sørensen TL, Møller JV, Nissen P. Phosphoryl transfer and calcium ion occlusion in the calcium pump. Science. 2004;304:1672–1675. doi: 10.1126/science.1099366.

52. Zhang P, Toyoshima C, Yonekura K, Green NM, Stokes DL. Structure of the calcium pump from sarcoplasmic reticulum at 8-A resolution. Nature. 1998;392:835–839. doi: 10.1038/33959.

53. Bourajjaj M, Armand AS, da Costa Martins PA, Weijts BR, van der Nagel R, Ahmadi R, Li Y, Cheng Y. Knockdown of long non-coding RNA ZFAS1 protects ovarian cancer cell malignancy. Oncotarget. 2017;8:19534–19546. doi: 10.18632/oncotarget.14663.

54. Wang Y, Ou X, Wang Z, Wang Z, Liu M, Lou G. Long non-coding RNA ZFAS1 interacts with miR-150-5p to regulate Sp1 expression and ovarian cancer cell malignancy. Oncotarget. 2017;8:38227–38238. doi: 10.18632/oncotarget.9611.

55. Chaudhry A, Carthan KA, Kang BY, Calvert J, Sutliff RL, Michael Hart C. PPARγ attenuates hypoxia-induced hypertrophic transcriptional pathways in the heart. Palm Circ. 2017;7:98–107. doi: 10.18632/oncotarget.14663.
LncRNA ZFAS1 as a SERCA2a Inhibitor to Cause Intracellular Ca\textsuperscript{2+} Overload and Contractile Dysfunction in a Mouse Model of Myocardial Infarction

Ying Zhang, Lei Jiao, Lihua Sun, Yanru Li, Yuqiu Gao, Chaoqian Xu, Yingchun Shao, Mengmeng Li, Chunyan Li, Yanjie Lu, Zhenwei Pan, Lina Xuan, Yiyuan Zhang, Qingqi Li, Rui Yang, Yuting Zhuang, Yong Zhang and Baofeng Yang

*Circ Res.* 2018;122:1354-1368; originally published online February 23, 2018;
doi: 10.1161/CIRCRESAHA.117.312117

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Material

LncRNA ZFAS1 as a SERCA2a inhibitor to cause intracellular Ca\(^{2+}\) overload and contractile dysfunction in a mouse model of myocardial infarction

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METHODS

Human Cardiac Samples

Left ventricular samples were obtained from three patients received heart surgery who were diagnosed with myocardial infarction (MI; coronary stenosis stages III-IV) and three were diagnosed without any ventricular disorders. The tissues were stored until use.

Animal Care

C57BL/6 mice ranging from 8 to 10 weeks in age and weighing between 22-25 g each used for animal studies and pregnant C57BL/6 mice for neonatal myocyte isolation were provided by Liaoning changsheng biotechnology co. LTD and the animal center at the Second Affiliated Hospital of Harbin Medical University (Animal Experimental Ethical Inspection Protocol No. HMUIRB20170034). Use of animals was approved by the Ethic Committees of Harbin Medical University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Mouse Model of Myocardial Infarction (MI)

We followed the same procedures for developing a mouse model of MI as described previously in detail. Briefly, mice were anesthetized with averitn (0.2 g/kg, ip; T48402; Sigma-Aldrich Corporation, St. Louis, MO, USA) and their chests were opened to expose the hearts. The left descending coronary artery (LAD) was ligated with a 7/0 nylon suture at 2 mm below the border between left atrium and ventricle to create MI. Myocardial ischemia was confirmed by significant elevation of S-T segment in electrocardiograph (ECG). The sham-operated mice for control underwent the same experimental procedures as the MI group but without ligation of LAD. Standard lead II ECG was recorded during surgery and 1 h after surgery. The mice were anesthetized for echocardiographic and hemodynamic measurements, and were sacrificed by cervical dislocation at 12 h after MI. Randomization and blinding were adopted in animal experiments and dead mice were excluded.

Echocardiographic Assessment of Cardiac Function

Left ventricular (LV) function was assessed by an echocardiographic system with an ultrasound machine Vevo2100 (Visualsonics, Toronto, Ontario, Canada) equipped with a 10-MHz phased-array transducer with the M-mode recordings as described previously. Echocardiographic parameters measured included LV internal dimension at end-diastole (LVIDd), LV internal dimension at systole (LVIDs). Ejection fraction (EF) was determined automatically by the machine, and fractional shortening (FS) was calculated according to the equation: ((LVIDd−LVIDs)/LVIDd) × 100.

Construction of Viral Vectors for ZFAS1 Overexpression and Knockdown

Adeno-associated virus (AAV) have been identified as the most promising gene therapy vehicle due to the advantages including efficient infection, non-pathogenicity, broad tissue transduction and long-term gene expression. Among various serotypes, AAV9 is the most efficient vector for myocardial transduction. AAV9 vectors carrying a short RNA fragment for silencing ZFAS1 (shZFAS1-V, “V” representing virus) and a CAG promoter conjugated with green fluorescent protein (GFP) were constructed (Lederer biological technology Co., Ltd., Guangzhou, Guangdong, China). AAV9 vector carrying ZFAS1 sequence and enhanced GFP gene for ZFAS1 overexpression (ZFAS1-V) was constructed. Vector titers were determined by quantitative polymerase chain reaction (qPCR) with primer pairs corresponding to the specific region of AAV vectors.

In Vivo Gene Delivery

C57BL/6 mice with body weights ranging from 18~22 g were randomized to receive the virus
solution \((2\times10^{11})\) genome containing particles (GC)/animal; shZFAS1-V or ZFAS1-V or control constructs diluted to a total volume of 150 µl saline) via tail vein injection. For data collection following single-gene transfer, experimental measurements were carried out four weeks following \textit{in vivo} injection of ZFAS1-V for ZFAS1 overexpression or shZFAS1-V to silence ZFAS1 expression\(^5\). For acquiring data with co-transfer of ZFAS1-V and shZFAS1-V, experimental measurements were first taken in the first four weeks after ZFAS1-V injection and then in the following three weeks after shZFAS1-V injection, respectively.

\textit{Isolation and Primary Culturing of Adult Mouse Cardiomyocytes}

Adult mouse cardiomyocytes were isolated as described previously\(^6\). Animals were anesthetized by injection of avertin (0.2 g/kg, ip) and 0.1 ml heparin (50 mg/ml, ip; H31022051; Biochemical & Pharmaceutical, Shanghai, China). Hearts were rapidly excised and the aorta was cannulated on a constant-flow Langendorff apparatus. The heart was digested by perfusion of Tyrode’s solution containing 1 mg/ml Type II collagenase powder (\#17101-015; Invitrogen, Carlsbad, CA, USA) and 0.75 mg/ml bovine serum albumin V (\#A8020; Solarbio, Beijing, China). Tyrode’s solution contained (mM): CaCl\(_2\) 0.02, glucose 10, HEPES 5, KCl 1.2, NaCl 150, and sodium pyruvate 2 (pH 7.4). After the tissue had become softened, LV was dissected and gently minced into small chunks which were then equilibrated in Tyrode’s solution with 200 μM CaCl\(_2\) and 1% bovine serum albumin at room temperature.

Cardiomyocytes were isolated from 1-3-day-old neonatal mice (C57BL/6) according to the procedures identical to those described in our previous study\(^7\). In brief, after dissection and washes, the hearts were finely minced and the chunks were placed in 0.25% trypsin. Pooled cell suspension was centrifuged and resuspended in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The resuspension was plated onto a culture flask for 90 min at 37°C, allowing for preferential attachment of fibroblasts to the bottom. The on-adherent and weakly attached cells, mainly cardiomyocytes, were removed and seeded into plates. 5-Bromo-2-deoxyUridine (5-BrdU, 10 nM; #B5002; Sigma, Saint Louis, USA) was then added into the culture medium to remove fibroblasts. The cells were incubated at 37°C in a humidified atmosphere of 5% CO\(_2\) and 95% air. After 48 h, cardiomyocytes that adhered onto the culture dish were used for subsequent experimental procedures. Cells were then deprived of serum and placed in an anoxic chamber for 12 h with a water-saturated atmosphere comprising 5% CO\(_2\) and 95% N\(_2\).

\textit{Culturing of Adult Human Ventricular Cardiomyocyte Cell Line AC16}

The human AC16 cell line, which possesses many of the biochemical and morphological properties of cardiomyocytes, was grown as previously described\(^8\). Briefly, non-differentiated AC16 cells were maintained in medium composed of DMEM:F12 (\#SH30023.01; HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum, 1% penicillin-streptomycin and 1% fungizone (\#C0222; Beyotime, Shanghai, China), and grown at 37°C in a humid atmosphere of 5% CO\(_2\) and 95% air until they had reached 70-80% confluence. Cells were incubated under hypoxia for 12 h and then collected for RT-PCR.

\textit{Synthesis and Transfection of ZFAS1 Constructs for Knockdown and Overexpression}

ZFAS1 specific siRNA (siZFAS1) and a negative control siRNA (siNC) were commercially synthesized by Ribobio (Guangzhou, Guangdong, China). These constructs were transfected into cells for ZFAS1 knockdown at a final concentration of 100 nM. ZFAS1 cDNA was inserted into the pCDNA3.1 (ZFAS1-P; “P” representing plasmid). Plasmid vectors (ZFAS1-P and empty vectors as a negative control construct NC-P) were transfected into cells for ZFAS1 overexpression at a final concentration of 2.5 mg/L. The transfection was performed using X-tremeGENE Transfection Reagent (\#10810500; Roche, Basle, Switzerland) according to the manufacturer’s instructions. Forty-eight h after transfection, the cardiomyocytes were
collected for total RNA isolation or protein purification. The sequences of siZFAS1 were: sense 5’-GCGUGAACUCCUGGCGAdTdT-3’ and antisense 5’-UCGCCUCAGGAGUUCACGdTdT-3’.

In addition, an oligonucleotides fragment corresponding to the conserved region of ZFAS1 gene (ZFAS1-FD) and an oligonucleotides fragment antisense to ZFAS1-FD (AsZFAS1-FD) were synthesized by Lederer Biological Technology (Guangdong, China).

**Measurements of Cardiac Contractility**

Left ventricular (LV) myocytes were freshly isolated from cardiac tissue, and placed in normal Tyrode’s solution (in mM): NaCl 137, KCl 5.4, NaH₂PO₄ 0.16, glucose 10, CaCl₂ 1.8, MgCl₂ 0.5, HEPES 5.0, and NaHCO₃ 3.0 (pH 7.4 adjusted with NaOH). Cells were paced to steady state using 1 Hz field stimulation. After 20 s of stimulation, the cells showed steady contractions and video images were acquired using Flash4.0 LT camera by line scanning (4 ms/line; C11440-42U; Hamamatsu, Japan). Image J was used to measure the cell length under contraction (systolic length) and relaxation (diastolic length). Cardiomyocytes sarcomere shortening (SS) that reflects the contractility of cardiac muscles was calculated as (diastolic length – systolic length) / diastolic length × 100%. Measurements were made in more than 20 myocytes for each group from n≥3 animals.

Cardiac contractility was also assessed with an ADVANTAGE Pressure-Volume Measurements System (#FY897B; Scisense, London, England). In brief, mice were anesthetized by avertin (0.2 g/kg, ip). The catheter was inserted into the left ventricle via the right carotid artery to assess systolic blood pressure, diastolic blood pressure, left ventricular pressure, and the peak and minimum values of dp/dt_max and dp/dt_min. After 10 min of stabilization, hemodynamic parameters were recorded by LabScribe software (Iworx, Dover, DE, USA). Measurements were obtained from n≥3 animals for each group.

**Measurements of Intracellular Ca²⁺-Transient**

For measurements of cellular Ca²⁺ transient, both freshly isolated cardiomyocytes and primarily cultured cardiomyocytes were incubated with 5 μM fluo-3 (#F1242; Invitrogen) and 0.01% Pluronic® F127 (BASF, Florham Park, NJ, USA) in Tyrode’s solution for 35 min. Resting intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) defined as the basal or diastolic Ca²⁺ level immediately prior to the onset of Ca²⁺ transient or immediately prior to the action potential upstroke in the cases where there was no obvious Ca²⁺ transient were measured by confocal microscopy (Olympus Fluoview FV1000, Osaka, Japan). For measurement of intracellular Ca²⁺ transients, cardiomyocytes were electrically paced at 1 Hz. Images were obtained by an Olympus camera. Amplitude of intracellular Ca²⁺ transient was calculated as the difference between peak and diastolic Ca²⁺ levels according to the equation (Fₘₐₓ - Fₙₐₓ)/Fₙₐₓ after subtraction of background fluorescence. The kinetics of Ca²⁺ transient (initial rising phase τᵢ and subsequent decay phase τₗ) time constants were determined using exponential curve fitting. The experiments were performed at room temperature. Measurements were made in more than 20 myocytes for each group from n≥3 animals.

**RNA Extraction and Quantitative Real-time RT-PCR (qPCR)**

Total RNA samples were extracted from cardiomyocytes and myocardial tissues (including mouse and human) by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The quality of the RNA samples was measured by NanoDrop ND-8000 (Thermo Fisher Scientific, Waltham, MA, USA) to ensure the RNA/DNA ratio of 1.8–2.0. Integrity of RNAs was assessed by standard denaturing agarose gel electrophoresis.

RNA was reversely transcribed to cDNA using Reverse Transcription Kit (Takara, Dalian, China). Real-time PCR was then performed with SYBR Green (04913914001; Roche, Basel, Basel-City, Swiss). GAPDH or β-actin was used as an internal control for ZFAS1 in myocardial tissue and myocytes. Each sample was analyzed in triplicate. The primers used
were ZFAS1 (mouse); forward 5'-AGCGTTTGCTTTTGTTCCC-3' and reverse 5'-CTCCCTCAGTGCCTTCTCT-3'; GAPDH (mouse and human); forward 5'-GCTTCGGCAGCACATATACAAAAT-3' and reverse 5'-GCAACCGAGCGGCAAAACC-3'; SERCA2a (mouse); forward 5'-TAAATGCGCGCTTGGTCTGCT-3' and reverse 5'-TTGTCACTCTGGCCAGGACC-3'; β-actin (mouse); forward 5'-ACTTCCGCATCTCTCTTCTCT-3' and reverse 5'-TCAACGTCACTCTCATGATGGA-3'; ZFAS1 (human); forward 5'-AACCAGGCTTTGATGGAACC-3' and reverse 5'-ATCCATCGCCAGTGTCT-3'.

### Isolation of Sarcoplasmic Reticulum (SR) and SR RNA

SR was isolated from cardiac tissues of MI or sham by Animal Tissue Crude ER Isolation Kit (GMS10040.1.2 v.A; GENMED SCIENTIFICS INC. U.S.A) according to the manufacturer’s protocol. Briefly, cardiac tissues weighted 200 mg was washed with clean buffer and homogenized in ice. The homogenate was centrifuged for 10 min at a speed of 1000g/min. The supernatant was placed into a new tube and centrifuged for 15 min at a speed of 12000g/min. The supernatant was then moved into a super centrifuge tube and was centrifuged for 60 min at a speed of 100000g/min to obtain the precipitate particles or SR. RNA in SR was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol.

### Isolation and identification of isolated Sarcoplasmic Reticulum (SR)

The purity of the isolated SR was examined by Tissue NADPH CytC Reductase Assay Kit (#GMS50303.2 v.A; GENMED SCIENTIFICS INC. U.S.A) according to the manufacturer’s protocol. NADPH cytochrome C reductase or NADPH-cytochrome P450 reductase (CPR) is a SR-specific protein, which can catalyze the production of reduced cytochrome C, resulting in the change of the peak value of absorption (550 nm wavelength). The absorption changes can be used to determine the activity of PCR quantitatively. The protein concentration in isolated SR was measured by Enhanced BCA Protein Assay Kit (P0012 BCA; Beyotime, Shang Hai, China). The absorption of the protein sample from isolated SR or total cardiac tissues (2 mg/ml) was detected at the wavelength of 550 nm by a spectrophotometer. The activity of CPR was calculated by the equation: [(sample reading-background reading) × system capacity in ml] × dilution times] / [0.05 (sample volume) × 21.845 x 5 (min)].

### Western Blot Analysis

Protein extraction from cardiomyocytes was performed 72 h after transfection. Whole-cell lysates from treated cardiomyocytes (80-100 μg) or from treated myocardial tissue (100-120 μg) were fractionated by SDS-PAGE (10–15% polyacrylamide gels). Proteins were transferred to Pure Nitrocellulose Blotting Membrane (PALL, New York, USA). The samples were then incubated with a primary antibodies for SERCA2a (#A1097; ABclonal, Wuhan, China), phospholamban (#GTX109254; Genetex, Irvine, California, USA), RyR2 polyclonal (#E-AB-32840; Elabscience Biotechnology, Wu Han, Hubei, China), L-type Ca²⁺ CP α1C (Cav1.2; #sc-25686; Santa Cruz Biotechnology, Dallas, Texas, USA), and NFATc2 (#A14189; ABclonal) at 4°C overnight. After washing, the membrane was incubated with anti-IgG horseradish peroxidase-conjugated secondary antibody (Jackson Immuno Research, West Grove, PA, USA). Protein bands were quantified using Odyssey v1.2 software (LI-COR Biosciences, Lincoln, NE, USA) by measuring the band intensity for each group and normalizing to β-actin or β-tubulin as an internal control. The data presented were obtained from three independent experiments with each conducted in triplicate for each group.

### RNA-interacting Protein Immunoprecipitation (RIP) and RNA-protein Pulldown

RIP was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Cat#17-701; Millipore, Darmstadt, Germany) as described previously⁵ and according to the manufacturer’s instructions. RNA was reverse transcribed to cDNA using the ReverTra Ace qPCR RT Kit (#FSQ-101; Toyobo, Osaka, Japan). The SYBR Green PCR Master Mix Kit
(Cat#4309155; Invitrogen) was used for real-time PCR quantification of the target genes on the 7500 fast Real Time PCR system (AB Applied Biosystems, Waltham, MA, USA). ZFAS1 plasmid was constructed into pCDNA3.1 vector (Invitrogen). RNA for in vitro experiments was transcribed using the T7 RNA Polymerase Kit (Cat#D7069; Beyotime, Shanghai, China) according to the manufacturer’s instructions. The transcripts were labeled by RNA 3’ End Desthiobiotinylation Kit (Cat#20163; Oshkosh, WI, USA) according to the manufacturer’s instructions. The Pierce™ Magnetic RNA-Protein Pull-Down Kit (Cat#20164; Pierce) was used in RNA-protein pulldown experiments according to the manufacturer’s instructions. The protein pulled down by ZFAS1 was detected by Western blotting with an anti-SERCA2a antibody (Cat#ab2861; Abcam, Cambridge, UK).

**Optical Mapping**

The optical mapping system MICAM05 (Brainvision, Tokyo, Japan) was used for monitoring the real-time changes of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)), according to the procedures described previously with minor revision in detail\(^{10,11}\). Animals were anesthetized by injection of avertin (0.2 g/kg, ip) and 0.1 ml heparin (50 mg/ml, ip). Hearts were rapidly excised and the aorta was cannulated on a constant-flow Langendorff apparatus. The heart was perfused by Tyrode’s solution containing (mM) NaCl 128.2, CaCl\(_2\) 1.3, KCl 4.7, MgCl\(_2\) 1.05, NaH\(_2\)PO\(_4\) 1.19, NaHCO\(_3\) 20 and glucose 11.1 (pH 7.35). Langendorff perfusion system was oxygenated (95% O\(_2\) and 5% CO\(_2\)). When the heart reached a steady-state contraction, 0.3~0.6 ml of excitation-contraction uncoupler blebbistatin was added into the perfusate (a final concentration of 10~20 µM) under dark. After the contraction of the heart had ceased, RHOD2 AM dye (Cat#1784532; Invitrogen) loading solution was prepared by mixing 50 µl of RHOD2 AM stock solution (1 mg/ml) to 50 µl warm 20% Pluronic F127 and 2 ml warm Tyrode’s solution. RHOD2 AM loading solution was then added to the perfusion system at a rate of 0.04 ml/min. Immediately prior to completion of dye loading, the heart was positioned so that the optical mapping camera could be focused to ensure the appropriate field of view for examinations. An image capturing software (BV_MC05E; Brainvision, Tokyo, Japan) was adopted for optical recording of the heart at 4 Hz field stimulation and an image analysis software (BV-Analyze; Brainvision) was used for data analysis.

**In Situ Hybridization (ISH)**

ISH was conducted by Lederer Biological Technology (Guangzhou, Guangdong, China). The cultured cardiomyocytes were fixed in 4% polyoxymethylene in PBS at room temperature for 20 min. After digestion by proteinase K (20 µg/ml; Beyotime, Shanghai, China) for 5 min, washed for three times and pre-hybridized at 37°C for 1 h. After the coverslip had been completely cooled, the cell preparation was incubated with hybridization solution containing Digoxin (DIG) labeled ZFAS-134 probe (8 ng/µl; Dig-5’-GCTAGCTAATGGTTGTCCAG-3’) at 70°C for 5 min and then at 37°C overnight. The preparation was subsequently washed for 30 min, blocked by BSA for 30 min, and subsequently incubated with anti-DIG-HP (horseradish peroxidase) at 37°C for 40 min. After washed, coverslip was treated with chromogenic agent diaminobenzidine/DAB (Dako, Produktionsvej 42, DK-2600 Glostrup, Denmark). DAB can form a very stable, brown/claybank end-products at the site of the target antigen or nucleic acid (e.g. ZFAS1). The nuclei were stained by hematoxylin followed by dehydration through a series of increasing ethanol concentrations and the results were analyzed by Image-Pro Plus software.

**Immunofluorescent staining for co-localization of ZFAS1 and SERCA2a**

The plasmid (pCDNA3.1) containing ZFAS1 cDNA and green-fluorescent protein (GFP) was constructed and transfected into cells for ZFAS1 overexpression at a final concentration of 2.5 mg/L. The transfection was performed using X-tremeGENE Transfection Reagent (#10810500; Roche, Basle, Switzerland) according to the manufacturer’s instructions. Forty-eight hours after transfection, the expression of SERCA2a was detected with an anti-rabbit SERCA2a-specific antibody (#A1097; Abclonal) at a 1:50 dilution. The secondary
antibody was an Alexa-Fluor 594-linked anti-mouse IgG antibody (Molecular Probes, Eugene, OR, USA) at a 1:300 dilution. All blocking steps were performed with SuperBlock reagent (Biogenex, San Ramon, CA, USA). 4', 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI, #D1306, Thermo Scientific) was used for nucleus staining. Co-localization between ZFAS1 and SERCA2a was viewed under a confocal laser microscope (Olympus, Tokyo, Japan).

**Mutation of ZFAS1-FD**

Our computational docking results indicated that nucleotides U19, G20, G23, G25, and G26 encompassed by the functional domain of ZFAS1 (ZFAS1-FD) are likely the core motif for specific binding to SERCA2a. To test this issue, we mutated this sequence region by nucleotide replacement from U19 mutating to A19 (U19/A19), G20/C20, G23/C23, G25/C25, and G26/C26. The RNA sequence was constructed by Lederer biological technology (Guangzhou, Guangdong, China) and transfected into in cultured neonatal mouse cardiomyocytes (NMCs) at a final concentration of 100 nM. Forty-eight h after transfection, the cardiomyocytes were collected for total RNA isolation or protein purification or intracellular calcium detection.

**Whole-cell Patch-clamp Techniques**

Whole-cell patch-clamp techniques were applied to isolated ventricular myocytes from the mice with ZFAS1 overexpression and with negative control as well, according to the procedures described previously with minor modifications\(^\text{12}\). The pipette of patch electrodes had the tip resistance of 2-3 MΩ when filled with pipette solution. The isolated single cells were placed in a 1-ml chamber mounted on an inverted microscope (IX-70, Olympus) and perfused with Tyrode solution. Whole-cell recording were performed using an amplifier (Axopatch 700B, Axon instrument, USA). Signals were filtered at 1 kHz and data were acquired by A/D conversion (Digidata 1440, Axon Instrument). Ion currents were recorded in the voltage-clamp mode. For the recording of L-type Ca\(^{2+}\) current (I_{CaL}), the external solution for I_{CaL} recording contained (in mM): tetraethylammonium (TEA) 120, HEPES 10, MgCl\(_2\) 1.0, CsCl 10, glucose 10, CaCl\(_2\) 2.8, pH adjusted to 7.4 with CsOH. The pipette solution contained (in mM) Cs-MeSO\(_4\) 138, CsCl 5, EGTA 0.5, MgCl\(_2\) 1, MgATP 4 and HEPES 10, pH adjusted to 7.3 with CsOH. The current-voltage (I-V) relationship was obtained from a holding potential of -55 mV, starting with 0.3 s depolarizing pulses to different membrane potential (10 mV increments from -50 mV to +60 mV). The IV curves were generated from the first pulse currents and fitted by

\[
I/I_{max}=G_{max}(V-E_{rev})/[1+exp\{(V-V_{1/2})/K_I-V\}],
\]

where I is the first pulse current, I_{max} is the normalized peak current, G_{max} is maximum conductance, E_{rev} = reversal potential, V_{1/2} is the voltage at 50 % of Ca\(^{2+}\) current activation, and K_I-V is slope factor. Since our study was designed for group comparisons of the experimental results, the currents were all recorded immediately after membrane rupture and series resistance compensation in order to minimize the possible time-dependent rundown of currents. Individual currents were normalized to the membrane capacity to control for differences in cell size, being expressed as current density pA/pF.

**Synthesis and transfection of siNFAc2 and siNFAc3 constructs for knockdown of NFAc2 and NFAc3**

NFAc2 and NFAc3 specific siRNAs (siNFAc2 and siNFAc3) and a negative control siRNA (siNC) were commercially synthesized by Ribobio (Guangzhou, Guangdong, China). These constructs were transfected into cells for NFAc2 and NFAc3 knockdown at a final concentration of 100 nM. The transfection was performed using X-tremeGENE Transfection Reagent (#10810500; Roche, Basle, Switzerland) according to the manufacturer’s instructions. Forty-eight hours after transfection, the cardiomyocytes were collected for total RNA isolation or protein purification or calcium detection. The sequences of candidates siNFAc2 were: sense 1: 5'-GCTACGGATTGAGGTCAAdTdT-3', 2: 5'-GCACGCCCTTACTCCAAGTAdTdT-3', and 3: 5'-CCATTCCTCCTGCAAGCATdTdT-3, and antisense 1: 5'-TTGGACCTCAATCCGAAGTAgdTdT-3', 2: 5'-TACTTTGTCAGAAGCCTGdCdTdT-3', and 3: 5'-ATGCTTGAAGATGGGAAATGdTdT-3. The sequences of siNFAc3 were: sense 5'-GAGGCCACGAAUGAUUGUTT-3', antisense
REFERENCES

1. Bock-Marquette I, Saxena A, White MD, Dimaio JM, Srivastava D. Thymosin beta4 activates integrin-linked kinase and promotes cardiac cell migration, survival and cardiac repair. *Nature*. 2004; 432: 466-472.

2. Pan Z, Sun X, Shan H, Lu Y, Yang B. MicroRNA-101 inhibited postinfarct cardiac fibrosis and improved left ventricular compliance via the FBJ osteosarcoma oncogene/transforming growth factor-beta1 pathway. *Circulation*. 2012; 126: 840-850.

3. Zincarelli C, Soltys S, Rengo G, Rabinowitz JE. Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection. *Mol Ther*. 2008; 16: 1073-1080.

4. Inagaki K, Fuess S, Storm TA, Gibson GA, McTiernan CF, Kay MA, Nakai H. Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8. *Mol Ther*. 2006; 14: 45-53.

5. Chen BD, He CH, Chen XC, Pan S, Liu F, Ma X, Li XM, Gai MT, Tao J, Ma YT, Yang YN, Gao XM. Targeting transgene to the heart and liver with AAV9 by different promoters. *Clin Exp Pharmacol Physiol*. 2015; 42: 1108-1117.

6. Makarewich CA, Zhang H, Davis J, Molkentin JD, Houser SR. Transient receptor potential channels contribute to pathological structural and functional remodeling after myocardial infarction. *Circ Res*. 2014; 115: 567-580.

7. Lu Y, Xiao J, Lin H, Bai Y, Luo X, Wang Z, Yang B. A single anti-microRNA antisense oligodeoxyribonucleotide (AMO) targeting multiple microRNAs offers an improved approach for microRNA interference. *Nucleic Acids Res*. 2009; 37: e24.

8. Davidson MM, Nesti C, Palenzuela L, Walker WF, Hernandez E, Protas L, Hirano M, Isaac ND. Novel cell lines derived from adult human ventricular cardiomyocytes. *J Mol Cell Cardiol*. 2005; 39: 133-147.

9. Liu Y, Zhao J, Zhang W, Gan J, Hu C, Huang G, Zhang Y. IncRNA GAS5 enhances G1 cell cycle arrest via binding to YBX1 to regulate p21 expression in stomach cancer. *Sci Rep*. 2015; 5: 10159.

10. Wang L, De Jesus NM, Ripplinger CM. Optical mapping of intrasarcoplasmic reticulum Ca\(^{2+}\) and transmembrane potential in the Langendorff-perfused rabbit heart. *J Vis Exp*. 2015; 103: 53166.

11. Salama G, Hwang SM. Simultaneous optical mapping of intracellular free calcium and action potentials from Langendorff perfused hearts. *Curr Protoc Cytom*. 2009; Chapter 12: Unit 12.17.

12. Lu Y, Zhang Y, Wang N, Pan Z, Gao X, Zhang F, Zhang Y, Shan H, Luo X, Bai Y,
Sun L, Song W, Xu C, Wang Z and Yang B. MicroRNA-328 contributes to adverse electrical remodeling in atrial fibrillation. *Circulation*. 2010; 122: 2378-2387.

13. Macindoe G, Mavridis L, Venkatraman V, Devignes MD and Ritchie DW. HexServer: an FFT-based protein docking server powered by graphics processors. *Nucleic Acids Res*. 2010; 38: W445-449.

**FIGURE LEGENDS**

**Online Figure I.** Verification of the efficiencies of AAV9-shZFAS1 vector (shZFAS1-V) in knocking down endogenous ZFAS1 (A) and of AAV9-ZFAS1 vector (ZFAS1-V) for ZFAS1 overexpression (B) in mouse myocardial tissues, determined by qRT-PCR. *P*<0.05, **P*<0.01 vs Control; n=3-4.

**Online Figure II.** Impairment of intracellular Ca\(^{2+}\) homeostasis by ZFAS1 assessed by optical mapping techniques in MI mice. (A) Restoration of the decreased rate of Ca\(^{2+}\) reuptake into SR by silencing endogenous ZFAS1 by shZFAS1-V in MI mice. The color bar indicates increasing [Ca\(^{2+}\)], in cytoplasm from blue to red. Decreasing [Ca\(^{2+}\)], or red color with time suggests gradual reuptake of Ca\(^{2+}\) back to sarcomere reticulum (SR). Note that ischemia slowed the dissipation of red color in MI mice as compared to sham animals, and such a slowing was completely abolished by shZFAS1-V to silence endogenous ZFAS1. *P*<0.05 vs Sham; *P*<0.05 vs MI; n=3. (B) Slowing of Ca\(^{2+}\) reuptake back into SR by forced expression of ZFAS1 with ZFAS1-V in healthy mice. Note that ZFAS1-V mimicked the damaging effects of MI on intracellular Ca\(^{2+}\) handling process. Note that ZFAS1 overexpression upon infection with ZFAS1-V delayed Ca\(^{2+}\) reuptake in healthy mice, mimicking the effects of MI. *P*<0.05 vs Control; *P*<0.05 vs ZFAS1-V; n=3.

**Online Figure III.** Computational analysis for RNA:protein binding using the RNA-Protein Interaction Prediction (RPISeq) database showing a high probability of ZFAS1:SERCA2a interaction.

**Online Figure IV.** Immunofluorescent staining of SERCA2a protein and GFP-labelling of lncRNA ZFAS1 in NMCMs. Note that ZFAS1 and SRRC2a proteins had essentially the same subcellular distribution pattern primarily around the nucleus and the two molecules co-localized each other.

**Online Figure V.** Effects of ZFAS1 on expression of Ca\(^{2+}\)-handling regulatory proteins other than SERCA2a. (A) Downregulation of SERCA2a protein level by ZFAS1 overexpression in the myocardium of mice treated with AAV9-ZFAS1 vector (ZFAS1-V), determined by Western blot analysis. *P*<0.05 vs Control or NC; n=8. (B-D) The lack of effects of ZFAS1 on the protein levels of phospholamban (PLN), ryanodine receptor 2 (RyR2) and the pore-forming α-subunit of L-type Ca\(^{2+}\) channel (Cav1.2) in the myocardium of mice treated with ZFAS1-V (*P*<0.05, n=5-8).

**Online Figure VI.** Lack of effects of ZFAS1 on L-type Ca\(^{2+}\) current (I\(_{Cal}\)) measured by whole-cell patch-clamp techniques. (A) Representative raw traces of I\(_{Cal}\) in isolated mouse ventricular myocytes infected with negative control construct for ZFAS1 (left panel) and with ZFAS1-carrying AAV9-ZFAS1 (ZFAS1-V; right panel). (B) Current-voltage relationship (I-V curve) of I\(_{Cal}\) treated with ZFAS1-V and NC-V.

**Online Figure VII.** A region of high degree conservation across varying species including human and mouse.

**Online Figure VIII.** Structural cartoons showing the computational docking between ZFAS1-FD (the putative functional domain of lncRNA ZFAS1 represented by sticks) and SERCA protein (represented by ribbons) by Hex 8.0. Hex 8.0 is an interactive protein docking and molecular superposition program for calculating and displaying feasible docking modes.
of pairs of protein and nucleic acid molecules. Left panels give top views and right panels show the side views.

Online Figure IX. The computational docking showing the loss of the binding sites for SERCA within the reserved sequence motif or functional domain (ZFAS1-FD) with nucleotide substitution mutation of ZFAS1-FD (Mut-ZFAS1-FD).

Online Figure X. Nuclear factor of activated T-cells C2 (NFATc2) as a transactivator of ZFAS1. (A) Upregulation of the protein level of NFATc2 in MI myocardium and in neonatal mouse cardiomyocytes (NMCs) exposed to hypoxic conditions. *P<0.05 MI or hypoxia vs Control; n=3. (B) Downregulation of ZFAS1 expression by NFATc2 inhibitor FK506 (10 nmol/L) in NMCs. *P<0.05 FK506 vs non-treated control; n=4–6. (C-D) Downregulation of ZFAS1 expression upon silence of endogenous NFATc2 by NFATc2 siRNA (siNFATc2). *P<0.05, **P<0.01 siNFATc2 vs Control or NC; n=3–10. (E) Increases in NFATc3 expression in MI myocardium and hypoxic NMCs. *P<0.05 vs Sham, **P<0.01 vs Control; n=4–6. (F) Silence of NFATc3 by siRNAs. **P<0.01 siNFATc3 vs siNC; n=4. (G) Lack of effects of siNFATc3 on ZFAS1 expression in NMCs.

Online Figure XI. Schematic diagram depicting the proposed signaling mechanisms underlying the effects of ZFAS1 in the setting of MI. MI → NFATc2↑ → ZFAS1↑ → SERCA2a↓ → [Ca^{2+}]i↑/Ca^{2+} overload → contractility↓ → contractile function↓. PLN: phospholamban, a protein that regulates Ca^{2+} through regulating SERCA2a activity in muscle cells; RyR2: ryanodine receptor-2 located in the sarcoplasmic/endoplasmic reticulum membrane being responsible for the release of Ca^{2+} from intracellular stores during excitation-contraction coupling in cardiac muscles; SUMO-1: small ubiquitin-like modifier protein-1 that regulates expression and activity of SERCA2a via SUMOylation.
Supplementary Tables

Online Table I. Nucleotide replacement mutations to the putative binding sites within the functional domain of ZFAS1.

| Name       | Sequence                                           |
|------------|----------------------------------------------------|
| Mut-ZFAS1-FD| UGCGUGCCCAAGCGGACAAACGCCCCCAAGCGAGAA              |
|            | GCCCGGGAGGCCC                                      |
FIGURES

Online Figure I
Online Figure III

RNA-Protein Interaction Prediction (RPiSeq)

Protein:

RNA:

| Interaction probabilities |
|---------------------------|
| Prediction using RF classifier 0.75 |
| Prediction using SVM classifier 0.91 |
Online Figure IV

![Immunofluorescence images showing the localization of DAPI, SERCA2a, GFP-ZFAS1, and their merge.](image)

Scale bar: 20 µm
Online Figure V

A

B

C

D

SERCA2a
β-Tubulin

PLN
β-Tubulin

RYR2
β-Tubulin

Cav1.2
β-Tubulin

Relative Level

0.0
0.5
1.0
1.5

Control
ZFAS1-V
NC-V

Relative Level

0.0
0.5
1.0
1.5

Control
ZFAS1-V
NC-V

Relative Level

0.0
0.5
1.0
1.5

Control
ZFAS1-V
NC-V

Relative Level

0.0
0.5
1.0
1.5

Control
ZFAS1-V
NC-V

225kDa
190kDa
110kDa
24kDa
50kDa
50kDa
Online Figure VI
Online Figure VII
Online Figure IX
Online Figure XI
Long In Vivo Checklist

Circulation Research - Preclinical Animal Testing: A detailed checklist has been developed as a prerequisite for every publication involving preclinical studies in animal models. Checklist items must be clearly presented in the manuscript, and if an item is not adhered to, an explanation should be provided. If this information (checklist items and/or explanations) cannot be included in the main manuscript because of space limitations, please include it in an online supplement. If the manuscript is accepted, this checklist will be published as an online supplement. See the explanatory editorial for further information.

This study involves use of animal models:
Yes

Study Design

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study. Yes

An overall study timeline is provided. Yes

The protocol was prospectively written. Yes

The primary and secondary endpoints are specified. Yes

For primary endpoints, a description is provided as to how the type I error multiplicity issue was addressed (e.g., correction for multiple comparisons was or was not used and why). (Note: correction for multiple comparisons is not necessary if the study was exploratory or hypothesis-generating in nature). N/A

A description of the control group is provided including whether it matched the treated groups. Yes

Inclusion and Exclusion criteria

Inclusion and exclusion criteria for enrollment into the study were defined and are reported in the manuscript. Yes

These criteria were set a priori (before commencing the study). Yes

Randomization

Animals were randomly assigned to the experimental groups. If random assignment was not used, adequate explanation has been provided. Yes

Type and methods of randomization have been described. Yes

Allocation concealment was used. N/A

Methods used for allocation concealment have been reported. N/A

Blinding

Blinding procedures with regard to masking of group/treatment assignment from the experimenter were used and are described. The rationale for nonblinding of the experimenter has been provided, if such was not performed. Yes

Blinding procedures with regard to masking of group assignment during outcome assessment were used and are described. Yes

If blinding was not performed, the rationale for nonblinding of the person(s) analyzing outcome has been provided. N/A

Sample size and power calculations

Formal sample size and power calculations were conducted before commencing the study based on a priori determined outcome(s) and treatment effect(s), and the data are reported. Yes

If formal sample size and power calculation was not conducted, a rationale has been provided. N/A

Data Reporting
Baseline characteristics (species, sex, age, strain, chow, bedding, and source) of animals are reported.  

The number of animals in each group that were randomized, tested, and excluded and that died is reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided for all experimental groups.

Baseline data on assessed outcome(s) for all experimental groups are reported.  

Details on important adverse events and death of animals during the course of the experiment are reported for all experimental groups.

Numeric data on outcomes are provided in the text or in a tabular format in the main article or as supplementary tables, in addition to the figures.  

To the extent possible, data are reported as dot plots as opposed to bar graphs, especially for small sample size groups.  

In the online Supplemental Material, methods are described in sufficient detail to enable full replication of the study.

**Statistical methods**

The statistical methods used for each data set are described.  

For each statistical test, the effect size with its standard error and \( P \) value is presented. Authors are encouraged to provide 95% confidence intervals for important comparisons.  

Central tendency and dispersion of the data are examined, particularly for small data sets.  

Nonparametric tests are used for data that are not normally distributed.  

Two-sided \( P \) values are used.  

In studies that are not exploratory or hypothesis-generating in nature, corrections for multiple hypotheses testing and multiple comparisons are performed.  

In "negative" studies or null findings, the probability of a type II error is reported.

**Experimental details, ethics, and funding statements**

Details on experimentation including formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring are described.  

Both male and female animals have been used. If not, the reason/justification is provided.  

Statements on approval by ethics boards and ethical conduct of studies are provided.  

Statements on funding and conflicts of interests are provided.

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