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The *TNNI3* p.R186Q mutation is responsible for hypertrophic cardiomyopathy via promoting FASN-stimulated abnormal fatty acid metabolism

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Abstract

Introduction: The *TNNI3* gene encodes the protein of cardiac troponin I (cTnI), which is an inhibitory subunit of sarcomeres. Mutations in this gene account for 3% of hypertrophic cardiomyopathy (HCM) and the molecular mechanism is complex. Recently, lipid metabolism has been revealed to be involved in HCM.

Aim: The purpose of this work is to identify whether the pathological mechanism of the hotspot mutation *TNNI3* p.R186Q in HCM is related to abnormal lipid metabolism.

Methods and Results: A knock-in (KI) mouse model carrying the *Tnni3* p.R186Q homozygous mutation (*Tnni3*^{R186Q/R186Q}) was novelty generated by CRISPR/Cas9 technology and successfully constructed a typical phenotype of cardiac-myopathy. Likewise, neonatal rat cardiomyocytes (NRCMs) transfected with a mutant plasmid with the *TNNI3* p.R186Q mutation showed the same phenomenon. In-depth experiments on related



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functions and molecular mechanisms were conducted, and *Tnni3*^{R186Q/R186Q} mice exhibited abnormal fatty acid metabolism, which was induced by the activation of epidermal growth factor receptor (EGFR)-dependent high expression of fatty acid synthase (FASN) *in vivo* and *in vitro*. Specifically, the direct binding of EGFR and cTnI was destroyed by *TNNI3* p.R186Q mutation, as observed through bioinformatics, Co-IP and GST-pull down analysis.

Conclusion: In the present study, we successfully engineered *Tnni3*^{R186Q/R186Q} mice with the typical phenotype of myocardial hypertrophy. We demonstrated that the *TNNI3* p.R186Q mutation could induce HCM by the dissociation of EGFR and cTnI, which further led to EGFR-dependent increased expression of FASN and abnormal lipid metabolism.

Keywords: *TNNI3*, gene mutation, FASN, hypertrophic cardiomyopathy, abnormal fatty acid metabolism

INTRODUCTION

Hypertrophic cardiomyopathy (HCM), characterized by asymmetrical hypertrophy of the left ventricle and ventricular septum, is a familial autosomal dominant hereditary cardiomyopathy^[1,2]. Generally, there are five common pathogenic genes: β -myosin heavy chain (*MYH7*), cardiac myosin binding protein C3 (*MYBPC3*), Troponin T2 (*TNNT2*), Troponin I3 (*TNNI3*) and Tropomyosin 1 (*TPM1*)^[3]. Among them, the *TNNI3* gene encoding the protein of cardiac troponin I (cTnI) protein is an inhibitory subunit of the sarcomeres. To date, more than 55 mutations of *TNNI3* have been linked to cardiomyopathy and account for approximately 3% of HCM^[4]. Several studies have also reported that the *TNNI3* p.R186Q mutation is associated with HCM and a high risk of sudden death^[4,5]. Similarly, we previously discovered the *TNNI3* p.R186Q mutation in a 39-year-old proband from an HCM family of Han Chinese individuals^[6]. Nevertheless, to date, the molecular mechanisms responsible for the HCM pathogenesis of *TNNI3* p.R186Q, which is a hotspot, are poorly understood.

It is well known that much of the ATP necessary for heart contraction mainly depends on the oxidation of fatty acids (FAs)^[7], and oxidation and synthesis of FAs maintain the dynamic balance of FAs under physiological conditions. However, the imbalance of FAs can result in many different cardiovascular diseases. Previous studies have found that increased fatty acid synthesis leads to lipid accumulation and lipo-toxicity by disrupting FAs balance, eventually developing into HCM^[8,9]. Fatty acid synthase (FASN), a key metabolic multi-enzyme, is responsible for the *de novo* synthesis of long-chain FAs, which play an essential role in fatty acid metabolism. Studies have shown that the overexpression of FASN is related to the occurrence, evolution, invasion and prognosis of tumors^[10,11]. Nevertheless, until now, the influence of FASN on the heart remains unclear. Strikingly, our preliminary work showed that the expression of FASN was significantly enhanced in the hearts of mice carrying the *Tnni3*-R186Q mutant allele (*Tnni3*^{R186Q/R186Q}). Thus, the purpose of this study was to further explore the influence of FASN on *TNNI3*-R186Q in HCM.

MATERIALS AND METHODS

Animals

Neonatal SD rats at 3 days were purchased from Shanghai Laboratory Animal Centre (*Shanghai, CAS*). C57BL/6J Knock-in (KI) mice were engineered through homologous recombination with help from Shanghai Biomodel Organism Science & Technology Development. DNA sequencing confirmed the successful construction of KI mice and the germline transmission of the *TNNI3* p.R186Q point mutation. Standard rodent sterile chow and water were given ad libitum in individually ventilated cage systems (IVC systems). All animal care procedures and experiments were approved by the Animal Ethics and Experimentation Committee of Nanchang University and conformed to the "Guide for the Care and Use of Laboratory Animals" (revised 1996). According to the genotype, the experimental KI mice were divided into

Tnni3^{R186Q/R186Q} and *Tnni3*^{R186Q/+} mice and the littermates of *Tnni3*^{+/+} mice were matched by age and sex. Adult mice of both genders were used in the present study. There were no significant differences in the littering period, litter size, growth and behavior of the *Tnni3*^{R186Q/R186Q}, *Tnni3*^{R186Q/+} and *Tnni3*^{+/+} mice, respectively.

TNNI3 protein interaction network analysis

A protein-protein interaction (PPI) network of TNNI3 was constructed using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database Available from: <https://www.string-db.org/>.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

Proteins were extracted from *Tnni3*^{R186Q/R186Q} and *Tnni3*^{+/+} mice heart tissues and analyzed by label-free tandem mass tag (TMT) and LC-MS/MS. The KEGG analysis was done with the help of Shanghai Applied Protein Technology Co., Ltd., who provided technological assistance.

Cell preparation and plasmid construction

The neonatal rat cardiomyocytes (NRCMs) used in the experiments were derived from newborn rats within three days, according to a previously published protocol^[12]. The adenoviral vectors (adenovirus *TNNI3* p.R186Q mutation, Ad-Mut; adenovirus wild type, Ad-WT) were obtained from Invitrogen. All cells were seeded into 6-well plates 1 day prior to transfection at a density of 1×10^5 cells/well. The cells were infected with adenovirus at an MOI of 50 and re-incubated with DMEM supplemented with 10% FBS 8 h following transfection. After 48 h, the transfection effects were confirmed by fluorescence microscopy and western blot analysis. Plasmids and reagents are described in the [Supplementary Materials and Methods](#).

Doppler echocardiography and histopathological analysis

Transthoracic echocardiography was performed as previously described^[13] (*Vevo 2100; Visual Sonics, Canada*). Wheat germ agglutinin-fluorescein isothiocyanate-staining (WGA), hematoxylin and eosin (HE) and Masson's trichrome staining were performed as previously described^[13,14]. Please refer to the expanded materials and methods in the supplement for details.

Tandem mass tag-based quantitative proteomic analysis

Tandem mass tag-based quantitative proteomic analysis was performed at Shanghai Applied Protein Technology Co., Ltd.

Statistical analysis

All experimental results and experimental data were statistically analyzed and processed by SPSS 17.0 software. Continuous data were presented as the means \pm standard error of the mean (SEM). Comparison data between the two groups were performed using unpaired Student's *t*-tests (equal variance), or unpaired Student's *t*-tests with Welch's correction (unequal variance). Comparison data between multiple groups were performed using one-way analysis of variance (one-way ANOVA) with post hoc Tukey's tests. GraphPad Prism 5.0 software was used to plot all statistical data. Statistical significance was defined as $P < 0.05$.

RESULTS

The *TNNI3* p.R186Q mutation facilitates cardiac hypertrophy *in vivo* and *in vitro*

The *TNNI3* p. R186Q mutation found in a 39-year-old female HCM proband with a family history of sudden cardiac death was reported by the Hong group^[6]. High pathogenicity possibility was predicted for the mutation by multiple bioinformatics tools [[Supplementary Table 1](#)].

To explore the effect of the *TNNI3* p.R168Q mutation on myocytes *in vivo*, we engineered a KI mouse model harboring the mutation (*Tnni3*^{R186Q/R186Q}, *Tnni3*^{R186Q/+}) by CRISPR/Cas9 technology in point mutation in C57BL/6 mice [Supplementary Figure 1]. Compared with age- and sex-matched *Tnni3*^{+/+} littermates, the cTnI transcriptional and posttranslational expression levels were no different among the *Tnni3*^{+/+}, *Tnni3*^{R186Q/+} and *Tnni3*^{R186Q/R186Q} mice, respectively [Figure 1A, Supplementary Figure 2]. First of all, the *Tnni3*^{R186Q/R186Q} mice exhibited a higher mortality rate [Figure 1B]. Further, echocardiographic monitoring was performed monthly beginning at 3 months of age, and the *Tnni3*^{R186Q/R186Q} mice displayed significant anatomical hallmarks of HCM at 10 months when compared with age- and sex-matched *Tnni3*^{+/+} mice littermates [Supplementary Figure 3, Figure 1C and D]. As shown in Table 1, *Tnni3*^{R186Q/R186Q} mice displayed a thicker left ventricular posterior wall (LVPW); however, the left ventricular internal dimension (LVID) was significantly reduced. Moreover, *Tnni3*^{R186Q/R186Q} mice showed cardiac myocyte (CM) hypertrophy by WGA staining [Figure 1E and F], cardiomyocyte necrosis, disordered arrangement, and fibrosis in the heart by HE and Masson's trichrome staining [Figure 1G and H] and increased expression of hypertrophy markers [Figure 1I]. However, none of the above hypertrophic signs were observed in *Tnni3*^{R186Q/+} mice [Supplementary Figure 4, Supplementary Table 2].

In vitro, the adenovirus expressing the *TNNI3* p.R168Q mutation (Ad-Mut) was transfected into NRCMs. Consistent with the above data *in vivo*, increased expression of hypertrophy markers was observed in the Ad-Mut group [Supplementary Figure 5]. Overall, these results suggested that the *TNNI3* p. R168Q mutation in myocytes can induce the HCM phenotype.

***Tnni3*^{R186Q/R186Q} mice exhibit aberrant fatty acid metabolism**

To elucidate the mechanism of the *TNNI3* p. R168Q mutation in HCM, the TMT coupled with LC-MS/MS was used to screen for differential proteins and the KEGG enrichment analysis was performed in *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mouse hearts at 10 months. The differentially expressed proteins from the KEGG pathways were mainly related to fatty acid metabolism [Figure 2A], which suggested that abnormal fatty acid metabolism might be involved in the pathogenesis of *TNNI3* p.R168Q -related HCM. Consistent with this assumption, the results from Oil Red O assays showed that *Tnni3*^{R186Q/R186Q} mouse hearts had a higher level of lip droplets [Figure 2B]. Furthermore, through an enzyme-linked immunosorbent assay, the contents of total triglycerides (TG) and total cholesterol (TC) were increased in *Tnni3*^{R186Q/R186Q} mouse hearts [Figure 2C and D], the content of the corresponding indicators in the serum did not increase [Figure 2E and F]. In addition, there were no differences in the contents of high-density lipoprotein (HDL) or low-density lipoprotein (LDL) in the heart and serum between *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice [Figure 2G and H, and Supplementary Figure 6].

The expression level of FASN is up-regulated in the hearts of *Tnni3*^{R186Q/R186Q} mice

FASN is a key metabolic multi-enzyme, which plays an essential role in fatty acid metabolism. To explore the involvement of FASN in *TNNI3*^{R186Q/R186Q} related fatty acid metabolism, both mRNA and protein expressions were determined in *Tnni3*^{R186Q/R186Q} mice at 10 months. Consistent with our expectation, FASN expressions in both mRNA level and protein level were significantly increased in *Tnni3*^{R186Q/R186Q} mice compared with those of *Tnni3*^{+/+} mice of the same age [Figure 3A-C]. As we know, the balance of fatty acid metabolism is jointly determined by fatty acid synthesis and beta-oxidation. Thus, we detected the expression of other related fatty acid synthesis (such as ACLY, SCD and PPAR- γ) and fatty acid β -oxidation genes (such as HADHB, ACAA2, ACOX-1, PPAR-a). However, we failed to observe any expression differences among these genes between *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mouse hearts [Figure 3D and E]. The above results suggested that the *TNNI3* p.R168Q mutation leads to aberrant fatty acid metabolism by increasing the expression of FASN.

Table 1. Parameters of heart function and structure detected by Echocardiography

ECHO	<i>Tnni3</i> ^{+/+} (n = 8)	<i>Tnni3</i> ^{R186Q/R186Q} (n = 8)
LVIDd (mm)	3.92 ± 0.26	3.27 ± 0.26***
LVIDs (mm)	2.46 ± 0.29	1.92 ± 0.40*
IVSd (mm)	0.80 ± 0.06	1.02 ± 0.18*
IVSs (mm)	1.28 ± 0.08	1.54 ± 0.13*
LVPWd (mm)	0.86 ± 0.02	1.00 ± 0.13
LVPWs (mm)	1.18 ± 0.11	1.46 ± 0.16*
EF (%)	67.91 ± 4.11	72.89 ± 9.19
FS (%)	37.39 ± 3.17	41.68 ± 8.34

ECHO: Echocardiography; LVIDd: left ventricular internal diameter at diastolic phase; LVIDs: left ventricular internal diameter at systolic phase; IVSd: interventricular septal thickness at diastolic phase; IVSs: interventricular septal thickness at systolic phase; LVPWd: left ventricular posterior wall thickness at diastolic phase; LVPWs: left ventricular posterior wall thickness at systolic phase; EF: ejection fraction; FS: left ventricular fractional shortening. * $P < 0.05$ *Tnni3*^{R186Q/R186Q} vs. *Tnni3*^{+/+}; *** $P < 0.001$ *Tnni3*^{R186Q/R186Q} vs. *Tnni3*^{+/+}.

FASN inhibition attenuates *TNNI3 p.R186Q*-induced aberrant fatty acid metabolism and hypertrophy

To address whether *TNNI3 p.R186Q*-induced aberrant fatty acid metabolism and hypertrophy were dependent on FASN. Mice were treated with a suitable dose of C75^[15], a FASN selective inhibitor by food intake for 2 months [Figure 4A]. Echocardiography detection showed that C75 did not affect cardiac structural changes [Figure 4B, Supplementary Table 3]. Furthermore, inconsistent with the above date, Oil Red O assays demonstrated that the increased lip droplets were profoundly reduced [Figure 4C], and the levels of TC and TG in mouse hearts were reduced [Figure 4D], and cardiac fibrosis [Figure 4E and F] and myocyte hypertrophy were attenuated [Figure 4G and H] by C75 administration in *Tnni3*^{R186Q/R186Q} mice.

In vitro, when cells were treated with C75, the *TNNI3 p. R168Q* mutation-stimulated higher expression of hypertrophic marker was largely inhibited [Figure 4I and J]. Taken together, these results suggested that *TNNI3 p.R186Q* related HCM is dependent on FASN.

EGFR is essential for *TNNI3 p.R168Q* mutation mediated up-regulation of FASN

To explore the effect of *TNNI3 p.R168Q* on FASN, we first conducted a co-IP assay and found that there was no interaction between cTnI and FASN [Supplementary Figure 7], which imply that this mutation might regulate the expression of FASN indirectly. Furthermore, we used bioinformatics methods to scan potential partners for cTnI. Consequently, an interaction between cTnI and EGFR was observed [Figure 5A], which suggested that the *TNNI3 p.R168Q* mutant might regulate FASN by affecting EGFR. To confirm the transactivation of EGFR, we detected the phosphorylation of AKT *in vivo* and *in vitro*. As expected, the expression of PI3K/AKT was increased in the Ad-Mut than in the Ad-WT group [Supplementary Figure 8A], and also increased in *Tnni3*^{R186Q/R186Q} mouse hearts compared with that in *Tnni3*^{+/+} mouse hearts [Supplementary Figure 8B] which suggested that the EGFR-AKT pathway is activated and involved in *Tnni3*^{R186Q/R186Q} induced HCM. In addition, the expression of EGFR was increased in *Tnni3*^{R186Q/R186Q} mouse hearts compared with that in *Tnni3*^{+/+} mouse hearts [Figure 5B]. In addition, an EGFR inhibitor gefitinib attenuated the increase in FASN expression in NRCMs transfected with Ad-Mut adenovirus [Figure 5C]. These findings verified that EGFR is a key pathway for *TNNI3 p.R168Q* mutation mediated up-regulation of FASN. Moreover, Co-IP and GST pull-down assays demonstrated that cTnI could interact with EGFR directly [Figure 5D and E]. However, the interaction of cTnI and EGFR was decreased when cTnI was mutated with p.R168Q *in vivo* and *in vitro* [Figure 5F and G]. These data suggested that the *TNNI3 p. R168Q* mutation leads to a decrease in binding to EGFR, which in turn up-regulates the expression of FASN.

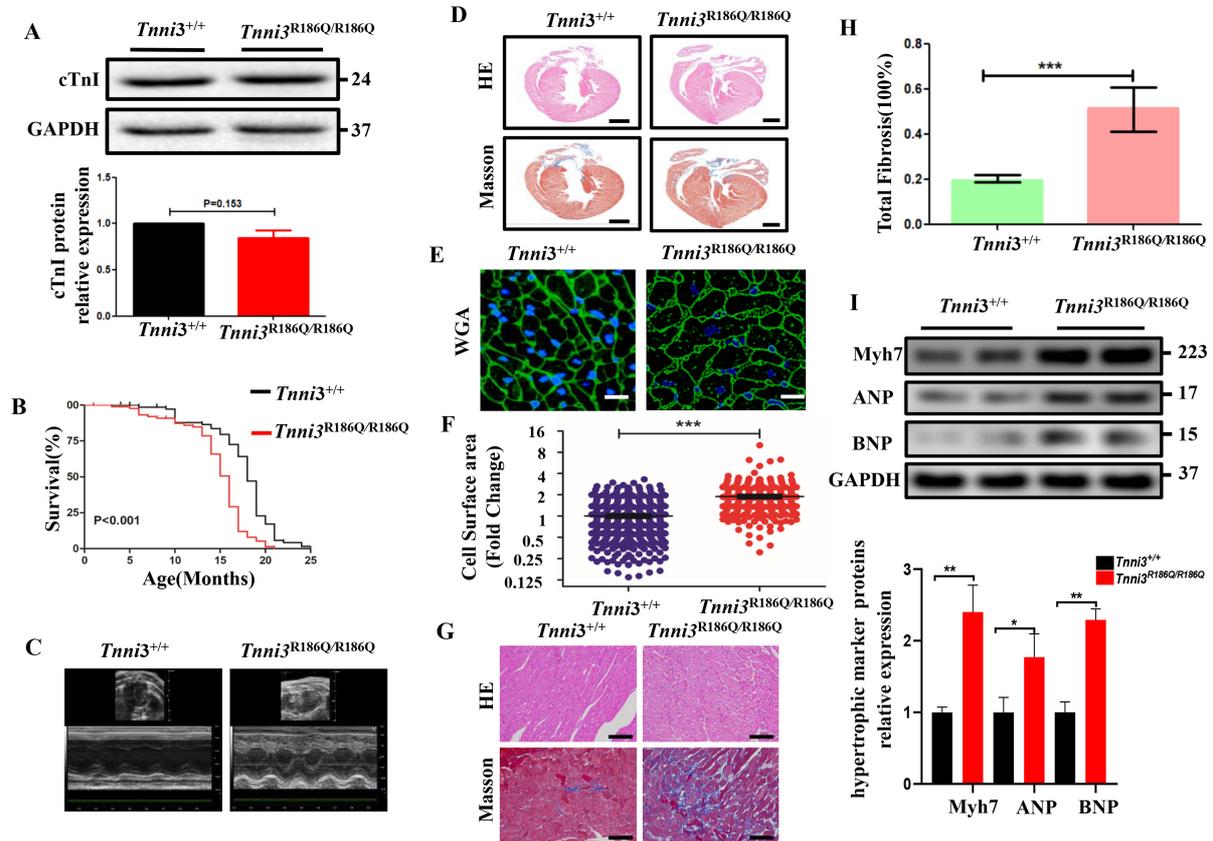


Figure 1. *Tnni3*^{R186Q/R186Q} mice exhibit cardiomyocyte hypertrophy. (A) The cTnI protein expression level in sections of the ventricles of *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} littermates (n = 3 per group). (B) The survival curve of *Tnni3*^{+/+} (n = 91) and *Tnni3*^{R186Q/R186Q} (n = 78) mice. *P < 0.05, Log-Rank (Mantel-Cox) tests. (C) Representative M-mode echocardiographic images of *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice at 10 months old. (D) Gross histological view (longitudinal section) of *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ventricular myocardium stained with HE (pink) and Masson's trichrome staining (red) to reveal myocardial structure (100×). Scale bar 2 mm. (E) Representative images of WGA staining in *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice hearts, showing cardiac myocyte (CM) cross-sectional area. Scale bar 20 μm. (F) Quantitative data of CM hypertrophy assessed by cross-sectional areas; (n = 3 per group with 100 CMs analyzed). ***P < 0.001. (G) HE (pink) and Masson's trichrome staining (red) of *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ventricular myocardium (cross-section). With HE staining (400×), blue represents the nucleus, and pink represents the cytoplasm. With Masson's trichrome staining (400×), the collagen fiber is shown blue and cardiomyocytes are red. Scale bar 100 μm. (H) Quantification of total fibrosis for Masson's trichrome staining of *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ventricular myocardium (cross-section). (I) Western blot analysis of the hypertrophic markers MYH7, ANP and BNP in whole heart tissue of *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice (n = 3 per group). The results are expressed as the means ± SEM and a representative experiment is shown. (*P < 0.05, **P < 0.01, ***P < 0.001). The statistical test was done with unpaired T-test. GAPDH was used as the loading control.

DISCUSSION

In the present study, we reported important novel findings demonstrating the role of the *TNNI3* p.R186Q mutation and its detailed mechanism in HCM. First, using different approaches, we demonstrated that the *TNNI3* p.R186Q mutation can induce the phenotype of cardiac hypertrophy *in vivo* and *in vitro*. In addition, KI mice bearing this mutation displayed aberrant fatty acid metabolism. Mechanistically, the *TNNI3* p.R186Q mutation reduced the interaction between cTnI and EGFR, activated EGFR and increased the expression of FASN, which likely bring about aberrant fatty acid metabolism and the development of HCM. Finally, the FASN selective inhibitor C75 appeared to be effective in the management of *TNNI3* p.R186Q-related HCM [Figure 6].

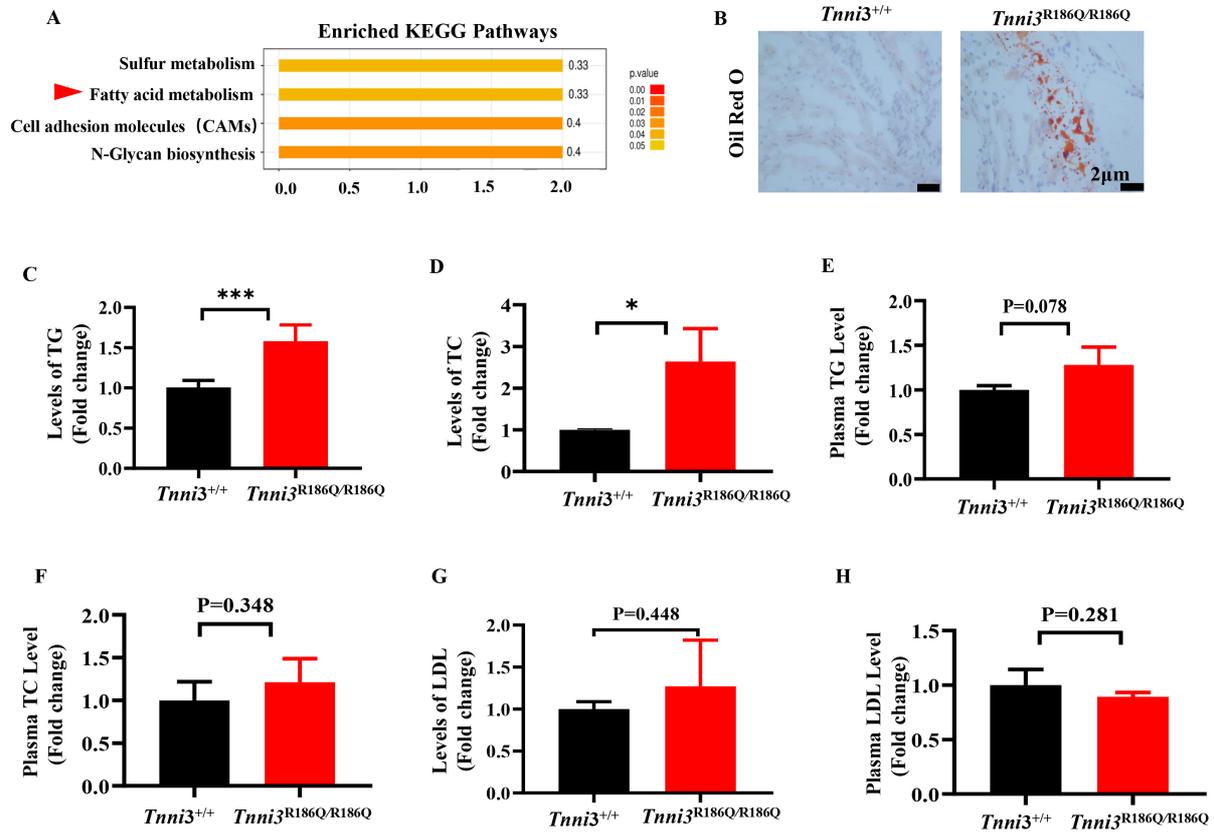


Figure 2. *Tnni3*^{R186Q/R186Q} mice exhibit aberrant fatty acid metabolism. (A) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of differentially expressed genes in *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ventricular myocardium ($n = 3$ per group). (B) Lipogenesis in the ventricular myocardium from *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice was determined by Oil Red O staining using frozen sections ($n = 3$ per group). (C-E) The levels of TG, TC and LDL were individually measured using enzyme-linked immunosorbent assay in the ventricular myocardium of *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ($n = 3$ per group). (F-H) The levels of TG, TC and LDL were individually measured using enzyme-linked immunosorbent assay in the serum of fasting *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ($n = 3$ per group). * $P < 0.05$, *** $P < 0.001$ and ns: not significant. The statistical test was performed with an unpaired T -test.

In recent decades, several phenotypes of HCM have been caused by mutation-related landmarks, including thickening of the left ventricle or septal wall, disordered arrangement of myocardial cells, increased myocardial necrosis/fibrosis or inflammatory cell infiltration, and increased expression of hypertrophy markers (ANP, BNP, MYH6, MYH7)^[16]. In this study, the KI mice at baseline showed thickening of the left ventricle and septal wall, obvious myocardial fibrosis, increased myocardial cell size, and increased expression of hypertrophy markers, which indicated that the mouse model was successfully established. It has been reported that most pathogenic mutations of the *TNNI3* gene are located in the cTnI mobile domain region, which plays a key role in myocardial diastolic function. In view of the high-frequency occurrence of mutations in this region, some studies have suggested that there is a strict structure-function relationship in this domain^[17], but others have indicated that these mutations are highly tolerated, thereby avoiding embryonic lethality and allowing these mutations to remain in the population^[18]. The KI mice in this study survived normally to adulthood without embryonic lethality, further confirming the latter view.

FAs are the most important energy source in the heart^[19], and abnormal FAs metabolism is critical to the pathogenesis of cardiac disease. Previous studies have demonstrated that some diseases including ischemic heart disease, heart failure, metabolic syndrome and type 2 diabetes all exhibit reduced oxidation of free FAs, which leads to the accumulation of FAs and corresponding changes in the structure of myocardial

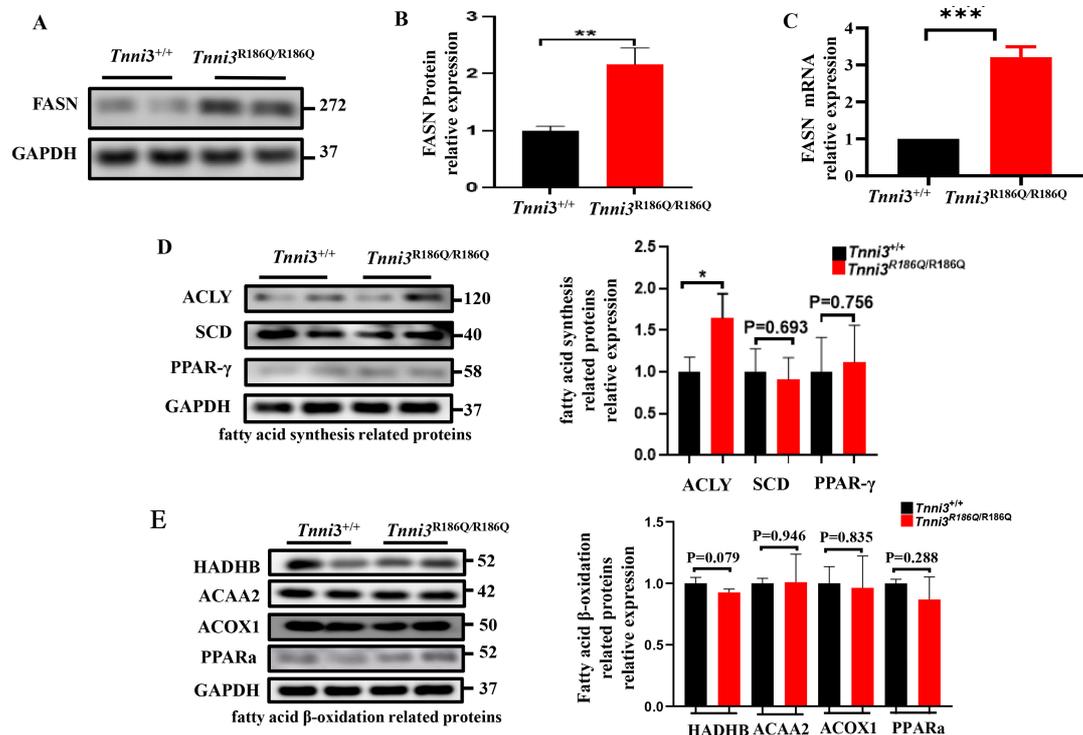


Figure 3. FASN expression is upregulated in *TNNI3*^{R186Q/+} mouse hearts. (A) FASN protein levels measured by Western blot were detected in *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ventricular myocardium and a representative experiment is shown ($n = 3$ per group). (B) The Western blot of FASN protein levels is expressed as the means \pm SEM. (C) The expression levels of FASN mRNA were detected by qRT-PCR in the ventricular myocardium of *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ($n = 3$ per group). (D) Protein expression levels of fatty acid synthesis related proteins were detected by Western blot in *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ventricular myocardium ($n = 3$ per group). (E) Protein expression levels of fatty acid β -oxidation related proteins were detected by Western blot in *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mouse ventricular myocardium ($n = 3$ per group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ and ns: not significant. The statistical test was done with unpaired T-test.

cells, such as myocardial fibrosis^[20], decreased cardiac function, and pressure overload in cardiac hypertrophy^[21-24]. In the present study, there was no change in the β -oxidation of FAs, but the synthesis of FAs was significantly increased in the myocardial tissue of *Tnni3*^{R186Q/R186Q} mice, which also resulted in the accumulation of FAs in cardiomyocytes, and eventually triggered cardiac hypertrophy. Previous studies have found that mutations in sarcomere-related genes, as well as in metabolism-related energy-sensitive enzyme genes, can cause HCM^[25,26]. Unexpectedly, this study found that *TNNI3* sarcomere gene mutations can both directly cause HCM, and also cause HCM by further changing the metabolism of cardiomyocytes. Moreover, no change was observed in the TC, TG, LDL or HDL levels in the serum of KI mice, while the corresponding indicators of TC and TG in the myocardial tissue increased, further confirming the myocardial specificity of cTnI^[27].

Previous studies have found that changes in protein-protein interactions between troponin complexes increase the severity of the disease^[28]. Consistent with their findings, this study found that the *TNNI3* p.R186Q mutation did not directly affect the structure of cTnI protein, but had a weakened effect on the interaction between cTnI and EGFR. EGFR plays an important role in regulating signaling pathways in cellular activities, such as proliferation, differentiation, and death^[29]. Among them, FASN is one of the downstream targets of EGFR for regulating cell proliferation. FASN is a key enzyme involved in the de novo fatty acid synthesis pathway, which plays a very important role in cell FAs accumulation and affects many metabolic processes of cells. Many studies have shown that under pathological conditions, the

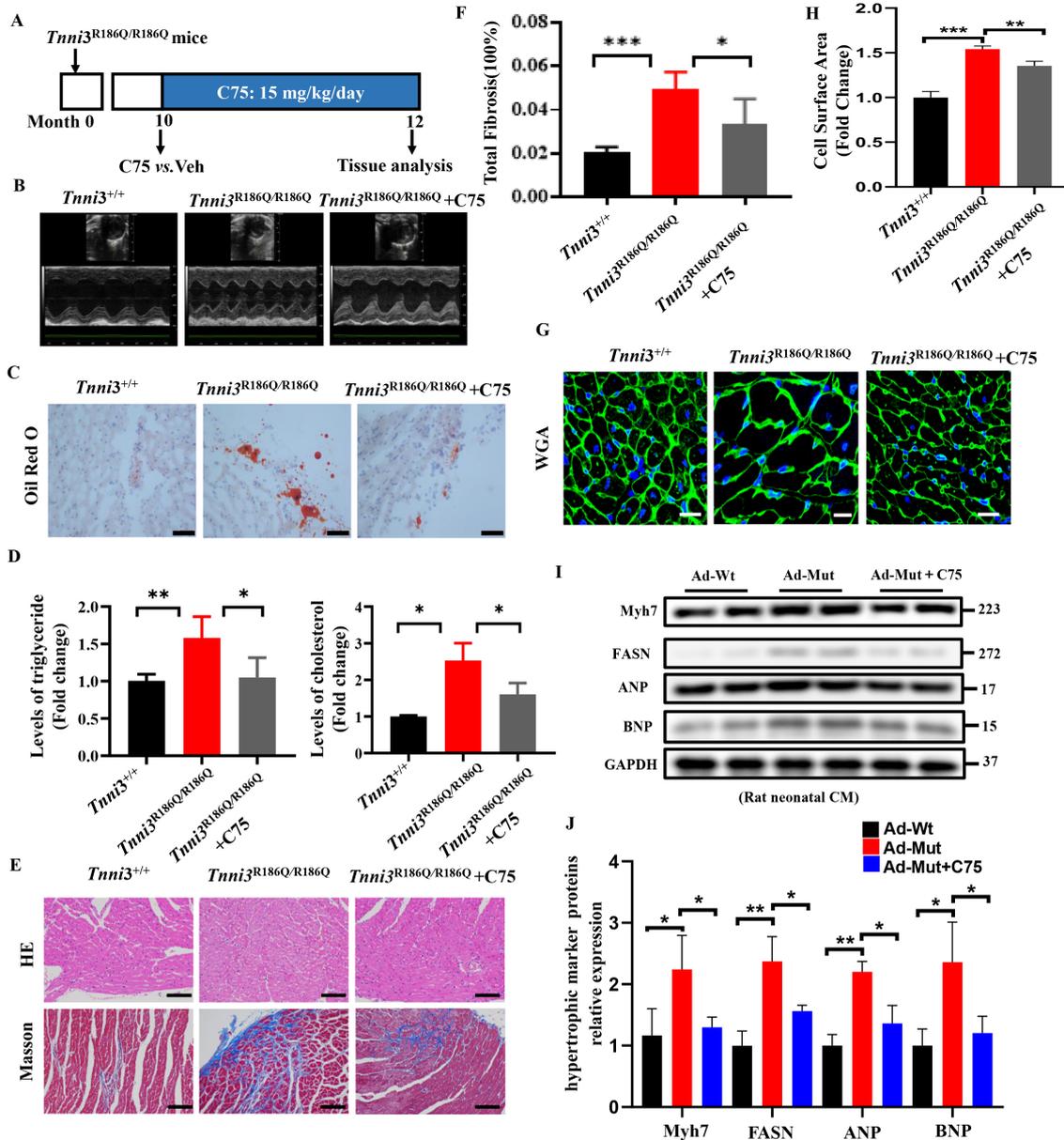


Figure 4. FASN inhibition attenuates *TNNI3* p.R186Q-induced aberrant fatty acid metabolism and cardiac hypertrophy. *Tnni3*^{R186Q/R186Q} mice at 10 months of age were administered saline or C75 (15 mg/kg/day) daily for 8 weeks. (A) Experiment timeline for *Tnni3*^{R186Q/R186Q} mice. (B) Representative M-mode echocardiographic images of *Tnni3*^{+/+}, *Tnni3*^{R186Q/R186Q} and *Tnni3*^{R186Q/R186Q} + C75 mice. (C) Lipogenesis in the ventricular myocardium of *Tnni3*^{+/+}, *Tnni3*^{R186Q/R186Q} and *Tnni3*^{R186Q/R186Q} + C75 mice was determined by Oil Red O staining using frozen sections (*n* = 3 per group). (D) The levels of TG and TC were individually measured using enzyme-linked immunosorbent assay in the ventricular myocardium of *Tnni3*^{+/+}, *Tnni3*^{R186Q/R186Q} and *Tnni3*^{R186Q/R186Q} + C75 mice (*n* = 3 per group). (E) HE (pink) and Masson's trichrome staining (red) of *Tnni3*^{+/+}, *Tnni3*^{R186Q/R186Q} and *Tnni3*^{R186Q/R186Q} + C75 mice ventricular myocardium (cross-section). With HE staining (400×), blue represents the nucleus, and pink represents the cytoplasm. With Masson's trichrome staining (400×), the collagen fiber is shown blue and the cardiomyocytes are red. Scale bar 100 μm. (F) Quantification of total fibrosis by Masson's trichrome staining in *Tnni3*^{+/+}, *Tnni3*^{R186Q/R186Q} and *Tnni3*^{R186Q/R186Q} + C75 mice ventricular myocardium (*n* = 3 per group). (G-H) Representative images of WGA staining in *Tnni3*^{+/+}, *Tnni3*^{R186Q/R186Q} and *Tnni3*^{R186Q/R186Q} + C75 mice ventricular myocardium, showing the cardiac myocyte (CM) cross-sectional area. Scale bar 20 μm. Quantitative data of CM hypertrophy assessed by cross-sectional area for WGA staining in *Tnni3*^{+/+}, *Tnni3*^{R186Q/R186Q} and *Tnni3*^{R186Q/R186Q} + C75 mice ventricular myocardium (*n* = 3 per group with 100 CMs analyzed). (I) Western blot analysis of the hypertrophic markers MYH7, ANP and BNP in ventricular myocardium heart tissue of *Tnni3*^{+/+}, *Tnni3*^{R186Q/R186Q} and *Tnni3*^{R186Q/R186Q} + C75 mice, and a representative experiment is shown (*n* = 3 per group). (J) The Western blot of the hypertrophic markers MYH7, ANP and BNP protein levels is expressed as the means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. The statistical test was done with unpaired *T*-test. GAPDH was used as the loading control.

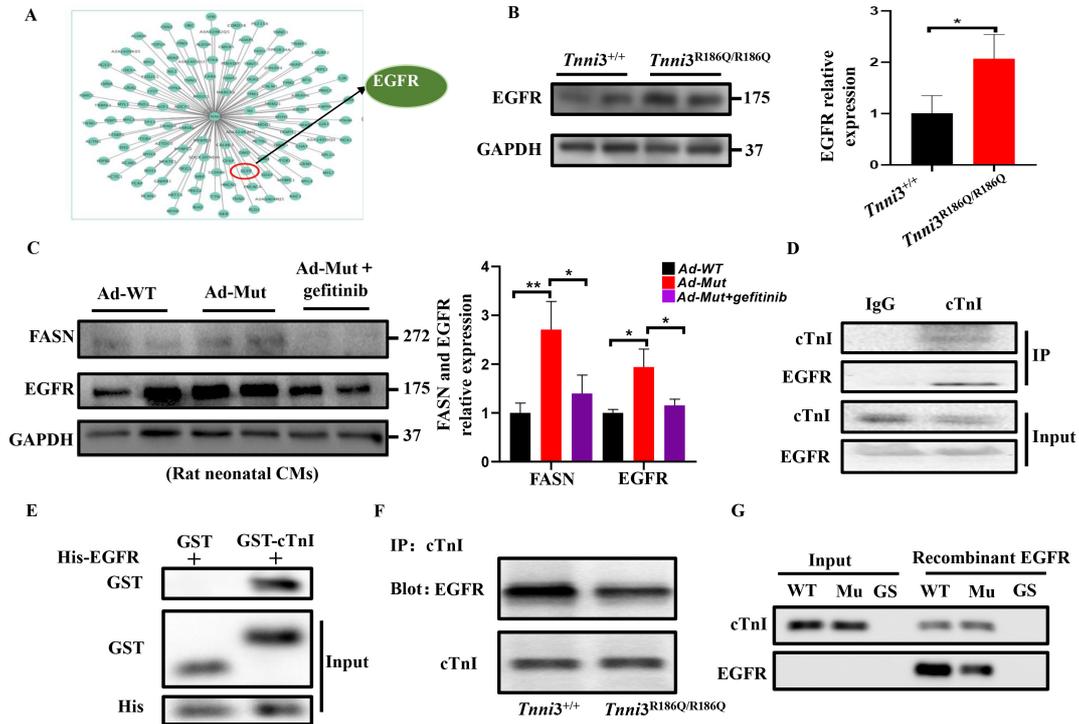


Figure 5. *TNNI3* p.R168Q mutation regulates FASN expression through EGFR. (A) PPI network of *TNNI3* generated using STRING 11.0. Nodes represent proteins. Edges represent PPIs. (B) EGFR protein levels measured by Western blot were detected in *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice ventricular myocardium (*n* = 3 per group). The results are expressed as the means ± SEM and a representative experiment is shown. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. The statistical test was done with unpaired *T*-test. GAPDH was used as the loading control. (C) Western blot analysis of FASN and EGFR in NRCMs of the Ad-WT, Ad-Mut and Ad-Mut + gefitinib groups (*n* = 3 per group). GAPDH was used as the loading control. (D) Co-IP for endogenous cTnI and EGFR in cultured cardiomyocytes of the Ad-WT and Ad-Mut groups (*n* = 3 per group). (E) Direct interaction of EGFR with cTnI by GST-pull down assay of Ad-WT and Ad-Mut groups (*n* = 3 per group). (F) Direct interaction of EGFR with cTnI by CO-IP assay in *Tnni3*^{+/+} and *Tnni3*^{R186Q/R186Q} mice (*n* = 3 per group). (G) Representative GST pull-down assays showing that GST-labelled WT-cTnI pulled down recombinant EGFR, but GST-labelled Mut-cTnI showing a decreased binding to EGFR. The results are expressed as the means ± SEM and a representative experiment is shown. **P* < 0.05, ***P* < 0.01. The statistical test was done with unpaired *T*-test. For Western blot analysis, GAPDH was used as the loading control.

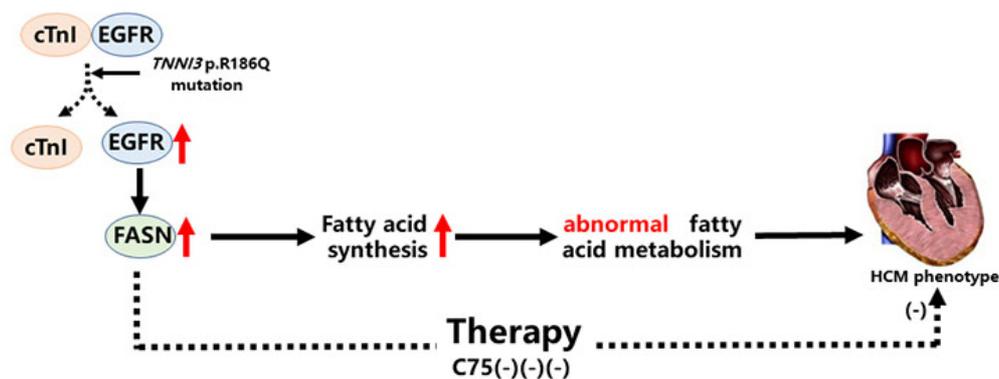


Figure 6. Schematic model of *Tnni3*^{R186Q/R186Q} mouse related HCM.

overexpression of FASN is closely related to the development of cardiovascular disease, type 2 diabetes, many types of cancer and fibroproliferative actions^[30-32]. Similarly, in this study, the overexpression of FASN directly caused the accumulation of FA in myocardial tissue. This is probably the main cause of HCM.

Several groups have investigated that the activation of EGFR can directly increase the levels of intracellular FAs, and at the same time, if EGFR inhibitors are used, the content of intracellular free FAs can be significantly reduced^[33,34]. Other studies also found that EGFR has a positive feedback regulatory effect on the expression of FASN. The activation of EGFR can promote an increase in FASN expression, and the increased expression of FASN can in turn promote the activation of EGFR, which strongly induces cell growth^[35]. Likewise, in our KI hearts, the *TNNI3* p.R186Q mutation may weaken the interaction between cTnI and EGFR, activate EGFR and further increase the expression of FASN, leading to FA accumulation.

Until now, there has been little evidence that drug treatment alone can fundamentally change the natural history of HCM or cause left ventricular remodeling^[36]. For more than 30 years, no other HCM treatment drugs have been introduced^[37]. In this study, we found that FASN inhibition of C75 can improve cardiomyocyte hypertrophy and fibrosis, which will help open more clinical options for HCM treatment in the future, and might represent a new treatment strategy for *TNNI3* p.R186Q related HCM.

Several limitations should be considered in this study. First, most of the pathogenic variants identified in HCM patients are heterozygous mutations, but interestingly, the heterozygous mice in this study did not have an overt HCM phenotype. Therefore, although the mouse model provides insight into the mechanisms of human disease, inherent differences between human and mouse physiology must ultimately be considered when interpreting our findings. Second, we mainly focused on the effects of FASN inhibitors in the mouse model. Previous studies have shown that EGFR inhibitors can prevent myocardial hypertrophy and heart damage through a variety of pathways, and there is no specific inhibitor of the EGFR-FASN pathway thus far^[38,39]. Therefore, EGFR inhibitors were not studied in this study and whether inhibition of EGFR could affect *TNNI3* p.R186Q related HCM is still unknown. Further investigations will provide insight into these fascinating questions. The primary phenotype was analyzed in a very large number of mice, including 78 *Tnni3*R186Q/R186Q mice [Figure 1B]. Likewise, cardiac function was analyzed in 8 mice per genotype. However, some of the secondary phenotypes, such as myocardial histology and western blotting were analyzed only in 3 mice per genotype. Thus, the secondary data are considered provisional pending replication in additional studies.

In conclusion, this study reveals a novel mechanism in the development of *TNNI3* p.R186Q related HCM by promoting abnormal fatty acid metabolism through FASN. Importantly, FASN inhibition is an interesting target for future research on *TNNI3* p.R186Q related to HCM.

DECLARATIONS

Authors' contributions

Conceived, designed and coordinated the study: Hong K, Wan R

Conducted the experiments, analyzed the data and wrote the paper: Guo L, Su Y, Chen C

Contributed to manuscript revisions: Guo L, Su Y, Chen C, Zhou Q, Shen Y, Jiang Z, Yan X, Li X, Zhuo W, Peng X, Wan R, Hong K

All authors have read and approved the final version of the manuscript and agree to be responsible for its content.

Availability of data and materials

The data supporting the results of this study can be obtained from the corresponding author upon reasonable request. For detailed methods, please refer to the online [Supplementary Materials](#).

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

All animal care procedures and experiments were approved by the Animal Ethics and Experimentation Committee of Nanchang University and conformed to the “Guide for the Care and Use of Laboratory Animals” (revised 1996).

Consent for publication

Not applicable.

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