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UNIVERSITY OF CALIFORNIA SAN DIEGO

Investigating Filamin C's Function in Cardiomyopathy

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Bioengineering

by

Yujun Xu

Committee in charge:

Professor Ju Chen, Chair
Professor Andrew McCulloch, Co-Chair
Professor Pedro Cabrales

2020

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University of California San Diego

2020

DEDICATION

I would like to dedicate my thesis work to my family. I sincerely appreciate my parents supporting me to finish my academic. They keep encouraging me, pushing me and giving me power.

I would like to dedicate my thesis work to my friends in UCSD. They taught me experimental techniques and gave precious suggestions to my research work.

I would like to give special thanks to my psychological therapist, Julie Badaracco. I learnt many skills to help myself get rid of the negative emotions. Her warm advice is a treasure to me.

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Acknowledgements

I would like to thank Dr. Ju Chen, who allowed me to be a part of his lab without any prior research experience. He shared his knowledge with every lab member and inspired us, caring about our success. It is my pleasure to be able to work in this collaborative environment and have this precious opportunity to work with every lab member.

I would like to thank Tongbin Wu, my supervisor, who provided this interesting project as my thesis. He shared all his research experiences to me, training me and helping me to be a researcher. Without his dedicated guidance and advice, it would be very difficult for me to finish this thesis.

I would like to thank other lab members, who supported me and teaching me throughout my thesis project. It would be my pleasure to be able to have connection with them.

This project had already started before I joined the lab. After I entered the lab, Tongbin and I both carried on this project. At the end of the introduction, in the preliminary works section, in order to provide a more comprehensive background and better understanding of my parts, some figures generated by Tongbin Wu were used. I sincerely thank Tongbin willing to share those unpublished data with me.

This paper is coauthored with Tongbin Wu. The thesis author was the primary author of this paper.

ABSTRACT OF THE THESIS

Investigating Filamin C's Function in Cardiomyopathy

by

Yujun Xu

Master of Science in Bioengineering

University of California San Diego, 2020

Professor Ju Chen, Chair
Professor Andrew McCulloch, Co-Chair

Filamin C (FLNc) is a multi-domain, actin-binding and actin-crosslinking protein, specifically expressed in cardiac and skeletal muscles. Multiple point mutations within FLNc have be identified and related to cardiac diseases, such as dilated cardiomyopathy, hypertrophic cardiomyopathy and restrictive cardiomyopathy. As one

of the cytoskeleton proteins in the cardiomyocytes, FLNc has also been reported to play an important role in maintaining the integrity and stability of the sarcomere structure and a linkage to the sarcolemma. However, the precise role of FLNc in the cardiomyocytes and the underlying mechanisms by which FLNc deficiency causes cardiomyopathy is still unclear. Previous studies have revealed that truncation mutation of FLNc in mouse model resulted in embryonic lethality without significant cardiac phenotypes, and loss of FLNc in fish model resulted in heart ruptures. In this study, by generating a true null FLNc mouse model, we located that at myocardium layer is where the cardiac structural integrity was largely alternated. We also visualized the rupture by 3D model. We will look into the specific mechanism which leads to the rupture in the future.

Introduction

Background

Cardiomyopathy is a group of diseases which will affect the human heart muscle (Mayo Clinic, 2019). In most of the cases, those diseases will result in the stiffness of heart muscle and difficulties of pumping blood from heart to the other body parts. With the aggravation of the cardiomyopathy, it can result in heart failure and other complications. According to NHANES data, there were about 6.2 million American adults (≥ 20-years-old, 2.2% of the population) suffering from heart failure between 2013 and 2016 (Benjamin et al., 2019). Multiple reasons, such as family history and long-term high blood pressure, can contribute to the diseases. There are 5 main types of cardiomyopathy: dilated cardiomyopathy (DCM), hypertrophic cardiomyopathy (HCM), restrictive cardiomyopathy (RCM), arrythmogenic right ventricular dysplasia (ARVD), and transthyretin amyloid cardiomyopathy (ATTR-CM) (American Heart Association, 2016). In DCM, the left ventricle is enlarged and not able to pump blood out of the heart efficiently. In HCM, the ventricular cardiac muscle becomes abnormally thicken, which in turn affecting the contractile function of the heart. In RCM, the cardiac muscle becomes more rigid, in other words less elastic. Consequently, the contractile and relaxation function of the heart is reduced. As for ARVD, it is a relative rare type of cardiomyopathy. In this case, the heart muscle is replaced by scar tissue, leading to cardiac arrhythmia problems (Mayo Clinic, 2019). Whereas ATTR-CM is either caused by genetic mutation of transthyretin or by aging, resulting in arrhythmia etc. (American Heart Association, 2016).

It can be clearly seen that, especially in DCM, HCM and RCM, the cardiac structure and the contractile/relaxation function are largely alternated because of cardiomyopathy. Sarcomere, as one of the key structures in the cardiomyocyte, is in charge of the contraction and relaxation ability of the muscle cell, cardiomyocyte signaling and mechanotransduction. It has been reported that the proper organization and the stabilization of the sarcomere are essential to maintaining normal cardiac structure and contractile function (Frank et al., 2007). Within each individual sarcomere unit, according to Chen and Chien, the force-generating contractile sarcomeric cytoskeleton plays an important role in constructing the arranged structure of sarcomere (1999). Besides, in order to performing the contraction/relaxation while adapting the cyclic changes of the cardiomyocyte geometry because of the repetitive cardiac cycle, there are two main cytoskeleton structures that also need to be considered: the intrasarcomeric cytoskeleton and extrasarcometic cytoskeleton. Intrasarcomeric cytoskeleton is responsible for regulating the displacement of myofilaments and anchoring those myofilaments and sarcomeric units during cardiac cycle. Extrasarcomeric cytoskeleton, on the other hand, provide linkages between sarcomere to the extracellular matrix (Chen & Chien, 1999). Therefore, by summing up all the factors above, the proper arrangements of cardiomyocyte cytoskeleton and sarcomere, and the connection of extracellular matrix-cytoskeleton-sarcomeric network are crucial for the maintenance of normal cardiac structure and contractile function.

Mutations in cardiac cytoskeleton proteins have been being investigated throughout these years. They are identified to playing increasingly significant roles in the cardiomyopathies and linking to some mechanisms of pathogenesis (Frank et al.,

2007; Clark et al., 2002). Whereas, there are several pivotal molecules have not been deeply understood, which allowing the heart to accommodate the repetitive and intense mechanical forces during cardiac cycle. Not only in the direction of the details of their functions/mechanisms, but also how have they connected to other cytoskeleton structures.

Significance

Filamins are a family of multi-domain, actin-binding and actin-crosslinking proteins (Razinia et al., 2012). In which, Filamin C (FLNc) is specifically expressed in cardiac and skeletal muscles. It localizes to Z-disc, sarcolemma, intercalated disc (ICD), and costamere. Generally speaking, FLNc is involved in the activities such as mechanical stabilization, mechanosensation and intracellular signaling. It also serves as a connection bridge between myofibrils and the sarcolemma by interacting with actin and β1-integrin (ITGB1) (Dalkilic et al., 2006). FLNc is composed of an actin-binding domain followed by 24 immunoglobulin (Ig) -like domains and a dimerization domain (Figure 1). The Ig-like domain 20 is very different from those in Filamin A and B. There are 82 amino acids uniquely inserted in Ig-like domain 20, interacting with several muscle-specific ligands (Fürst et al., 2013). The dimerization domain involves in the interactions of FLNc with ITGB1 and sarcoglycans at the costamere (Thompson et al., 2000). The diverse interactions between FLNc and different proteins indicated that FLNc, as one of the cytoskeleton members, can play an important role in maintaining the integrity and stability of the sarcomere structure and a linkage to the sarcolemma (Chen & Chien, 1999; Clark et al., 2002; Dalkilic et al., 2006; Frank et al).

Identified mutation positions within FLNc are shown in Figure 1 as well. Several dominant mutations in FLNc have been found to be closely related to the pathogenesis of DCM, HCM and RCM, which in turn emphasize the importance of the role of FLNc in cardiomyopathy (Fürst et al., 2013). Whereas, the specific role of FLNc in cardiomyocytes and the underlying mechanisms by which FLNc deficiency causes cardiomyopathy are still need to be explored.

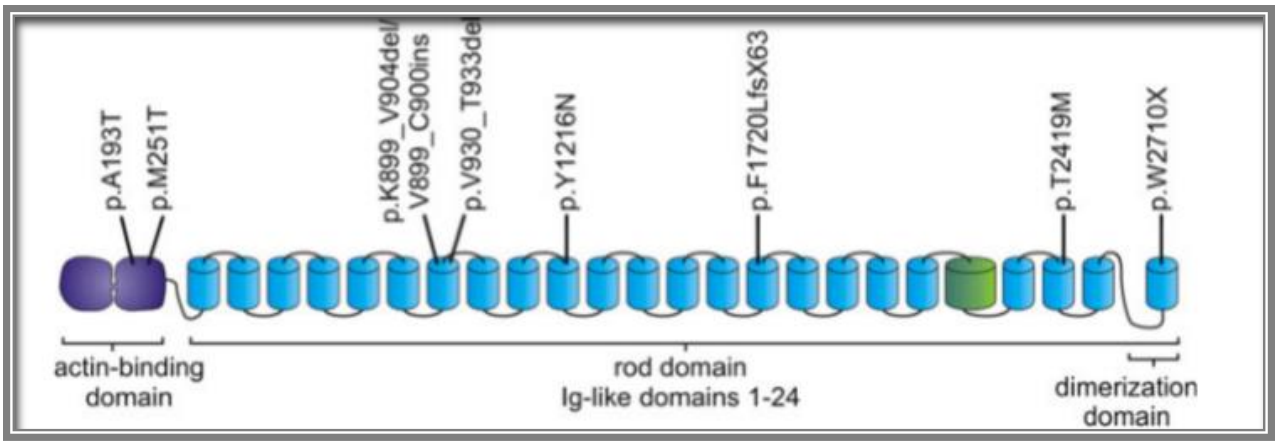

Figure 1: Stucture of Filamin C (Fürst et al., 2013).

Previous Studies

Cell models and gene-engineered animal models were established to understand these aspects. FLNc have been shown to be able to bind with β1-integrin, such as connecting Z-disc proteins to sarcolemma (Gontier, 2005). It has also been reported that, as one of the inactivators of the integrin, filamin is able to inhibit cell migration and cell spreading in certain cell types, and to stabilize the focal adhesion. These could possibly because upon the filamin's expression, the activity of integrin is decreased (Bouvard et al., 2013). In FLNc knocking-down C2C12 cell line, no elongated myotubes could form (Thompson et al., 2000). In a zebrafish model, loss of FLNc resulted in

catastrophic myofiber disintegration and myosin aggregates, which in turn caused muscle weakness (Ruparelia et al., 2016). Mouse model was also constructed to investigate FLNc's function. In this case, the last eight exons of FLNc were deleted, however, it was not a true null FLNc mouse model. Instead, there was still a truncated FLNc protein expressed. Those FLNc -deficient mice died shortly after birth due to the respiratory failure and severe primary myogenesis defects (Thompson et al., 2000). Whereas the specific cardiac phenotypes that was caused by missing of FLNc remained unclear. Therefore, it is important to generate a true null FLNc mouse model, the FLNc global knockout (gKO) mouse model, and an FLNc cardiomyocyte-specific knockout (cKO) mouse model to comprehensively investigate the actual role of FLNc in cardiomyocytes and in the heart and the mechanisms of it.

Interestingly, a heart rupture phenotype was observed in medaka mutant --- *zacro (zac)*, a fish model, which had a nonsense mutation in FLNc. At embryonic stage 27, ruptures were found at the myocardium layer of the ventricle. Moreover, the muscle structure was largely disrupted in zac mutants; the ventricular cardiomyocytes were not in proper organization. Additionally, comparing to the wild-type, they had rougher surfaces and developed some lamellipodia-like and filopodia-like structures on the surfaces. At stage 28, pericardial edema and blood congestion were observed (Fujita et al., 2012). Therefore, we hypothesized that FLNc plays an essential role in maintaining the muscle structure, the integrity of the cardiomyocyte's sarcomere and costamere, and normal cardiac functions. As the heart rupture phenotype was not found in the previous mouse model, it would be worthwhile to generate a global knock (gKO) and a cardiac-specific knockout (cKO) mouse model, without truncated FLNc proteins

expressed, and investigated whether the rupture phenotype also shows up. These *in vivo* animal models will help us to understand the mechanisms of the cardiomyopathy caused by FLNc mutations at the molecular basis

Preliminary Works

Tongbin Wu, my supervisor, generated both gKO and cKO mice by deleting 9 to 13 exons of FLNc using Cre-Lox recombination system. According to the western blot as shown in Figure 2, FLNc protein was not detected in gKO mice. The quantity of FLNc in largely decreased in the heterozygous. Homozygous offspring from gKO and cKO mice heterozygous matings developed severe chest edema at E 10.5, and died at E 11.5. By comparing the chest edema and ventricular shapes between the gKO model and cKO model, all of the observed phenotypes are very similar. Therefore, FLNc gKO was decided to be used for all of the future experiments. As the chest edema phenotype was observed in the medaka mutant *zacro*, which had a nonsense mutation in FLNc, it would be interesting to figure that whether the heart rupture also occurs in the FLNc - gKO mouse model.

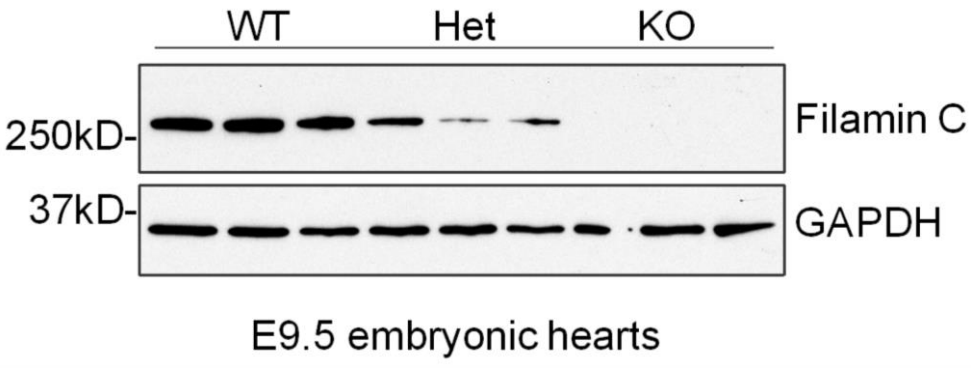

Figure 2: Western Blot for E 9.5 Embryonic Hearts (Tongbin Wu).

Tongbin then observed the FLNc gKO mice' embryonic heats at E 9 under normal light microscope. By comparing to the WT mice, rupture sites were clearly found as shown in Figure 3. The rupture sites were pointed out with red circles. Through immunofluorescent analysis, the rupture sites were actually located at the embryonic ventricular myocardium (Figure 4).

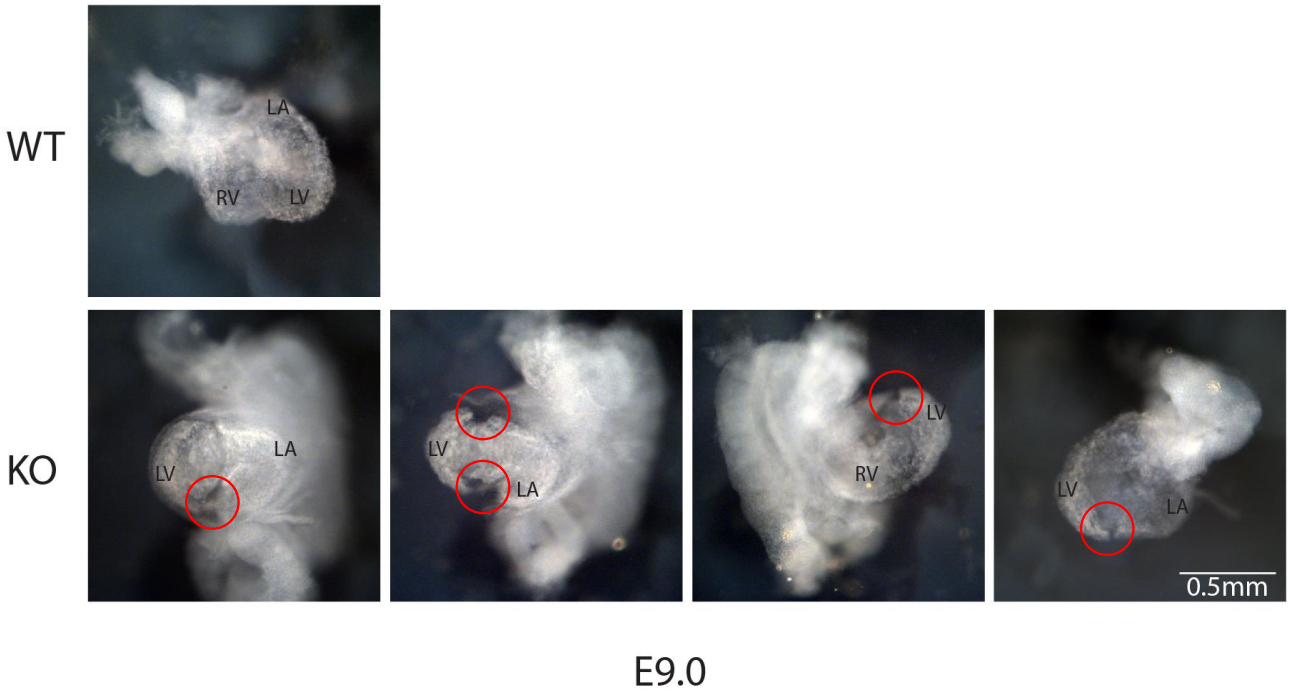

Figure 3: Heart Ruptures in FLNc gKO at E 9.0 (Tongbin Wu).

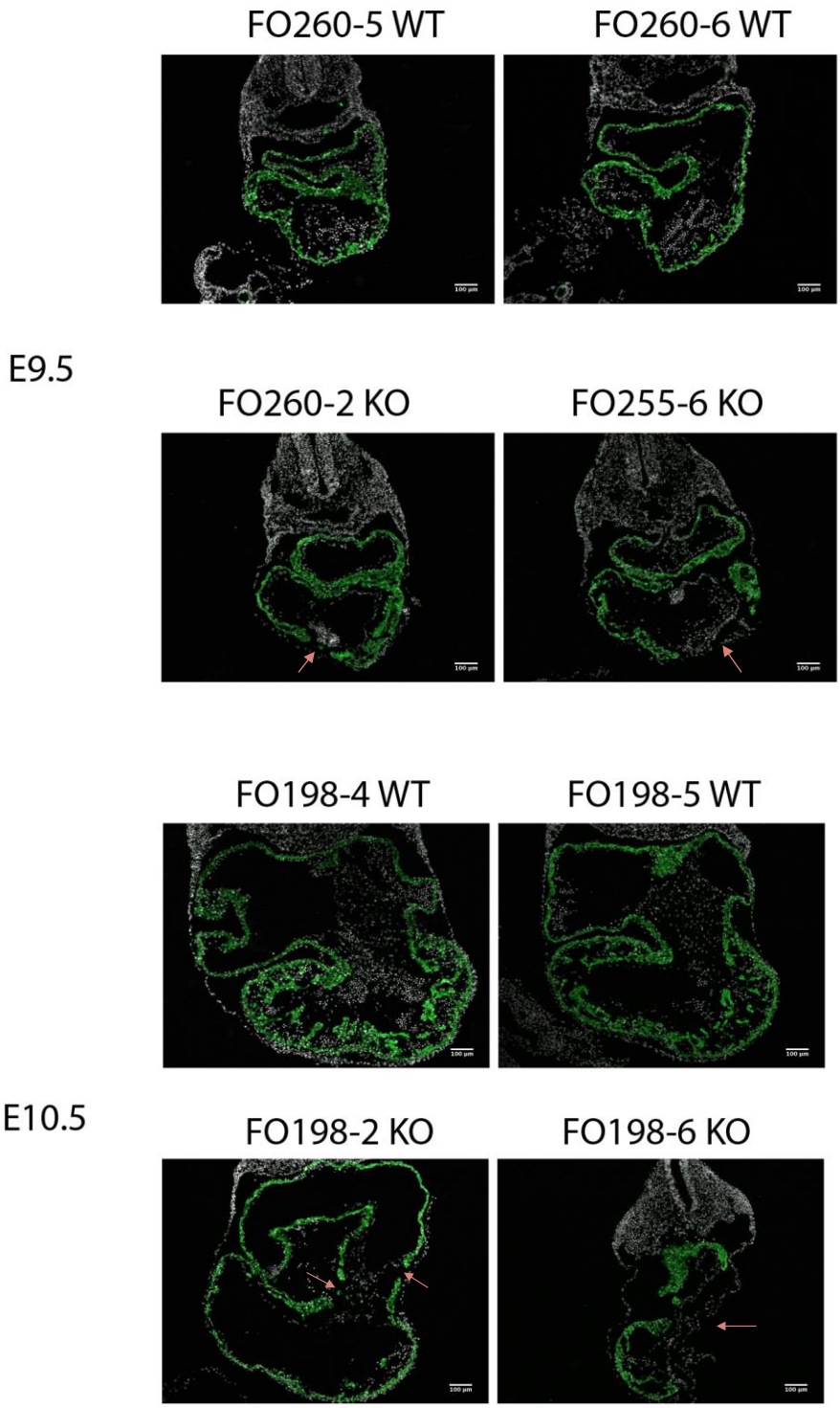

Figure 4: Immunofluorescence analysis of transverse sections of hearts from wild-type and FLNc gKO embryos at E 9.5 and E 10.5, staining for Cardiac Troponin T (green) and DAPI (gray) (Tongbin Wu). Red arrows indicate the rupture site.

This phenotype was not found in the previous mouse model (Thompson et al., 2000), which had a truncated FLNc protein expressed. Immunofluorescence has already showed the rupture was at the myocardium (cTnT-positive layer), our next question moves to, specifically, at exactly where does the rupture happens?

Materials and Methods

Animal Models

The FLNc-gKO mice was generated as stated above and acquired from the lab. The mice used for light sheet microscopy were acquired by crossing FLNc gKO mice with ROSA26 td-tomato indicator mice. Thus, the cardiomyocytes expressing Cre will be specifically labeled. Mice used for future experiments included FLNc gKO (FLNc -/-, Rosa26-TdTomato+) and control (FLNc +/-, Rosa26-TdTomato+ or FLNc +/+, Rosa26-TdTomato+). The genotype of the acquired mice' hearts were verified by polymerase chain reaction (PCR) analysis using the corresponding embryonic yolk sac and FLNc primers, Cre primers and ROSA primers. All procedure were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of California, San Diego.

Cubic Clearing & Light Sheet Microscopy

The embryos of ROSA26 td-tomato indicator mice were isolated at E 9.5 and were immediately placed in fixative (2% paraformaldehyde, 2% glutaraldehyde in PBS). After 24 hours, the embryos were stored in 1X PBS at 4℃. Before performing the light

sheet scanning, the embryos were perfused in Reagent-1, composed of N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine, urea and Triton X-100 in water (Susaki & Ueda, 2016) for 30 minutes. The perfused embryos were then transferred into 1:1 v/v Reagen-1:PBS solution, preparing to be loaded in the Zeiss light sheet microscopy. The objective set used for this experiment is 5x. The light sheet microscopy was performed at the National Center for Microscopy and Imaging Research, University of California, San Diego.

Quantitative Real-Time PCR

The embryonic heats from the offspring of gKO mice heterozygous matings were isolated at E 9.5, immediately frozen in liquid nitrogen and stored in -80℃. The hearts were treated with TRIzol (Invitrogen) to extract total RNA. Super Script III cDNA Synthesis Kit (Invitrogen) was used to reverse transcribe the total RNA to cDNA. Complementary DNA amplicons were quantified by SYBR Green probe (Biorad). Samples were losaded in a 96-well low profile PCR plates and placed in CFX96 Biorad Thermocycler to carry on the RT-PSR reactions. 18s RNA was used for adjusting and samples were compared after the adjustment.

RNA Sequencing

The embryonic heats from the offspring of gKO mice heterozygous matings were isolated at E 9.5, immediately frozen in liquid nitrogen and stored in -80℃. The hearts were homogenized with TRIzol (Invitrogen) and total RNA was extracted. cDNA libraries were acquired with Illumina TruSeq stranded mRNA kit and subjected to deep sequencing.

Results

Localization of the Rupture Site

By using the ROSA26 td-tomato indicator mice, one more detailed microscopic analysis, we identified that the rupture happened on the myocardium layer (Figure 5). The integrity of the heart was severely disrupted because of loss of FLNc.

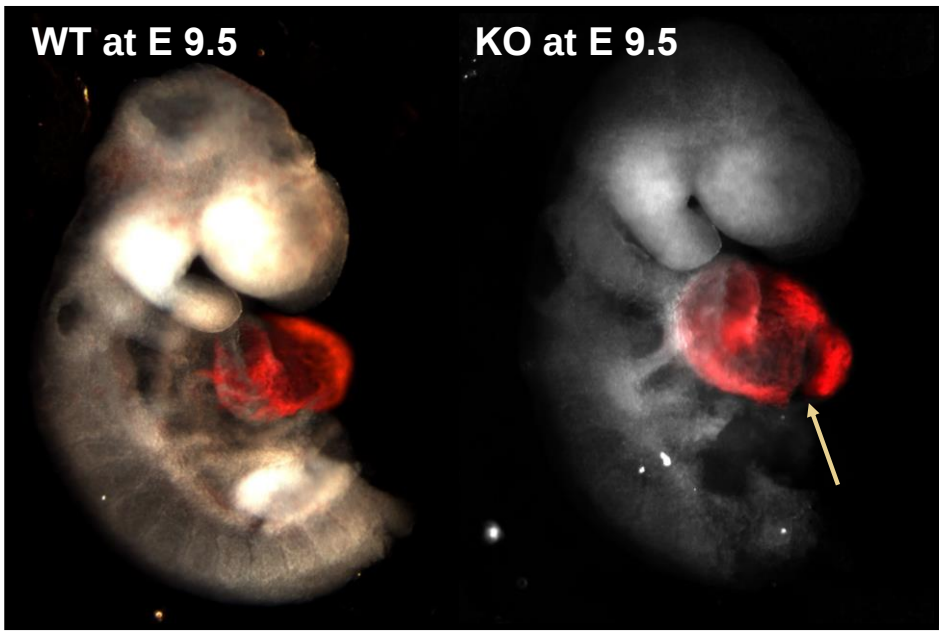

Figure 5: Whole-mount microscopic assessment (right lateral view) of wildtype (WT) and FLNc gKO embryos at E 9.5. The arrow indicates the rupture site.

The 3D imaging from light sheet microscopy was shown in Figure 6. At this stage, the heart hadn't develpoed in to four chambers, but it is still be able to tell the outflow track and inflow track. The rupture almost splited the heart into two pieces. Thus, loss of FLNc from cardiomyocytes would largely affect the integrity of the myocardium in the developing heart.

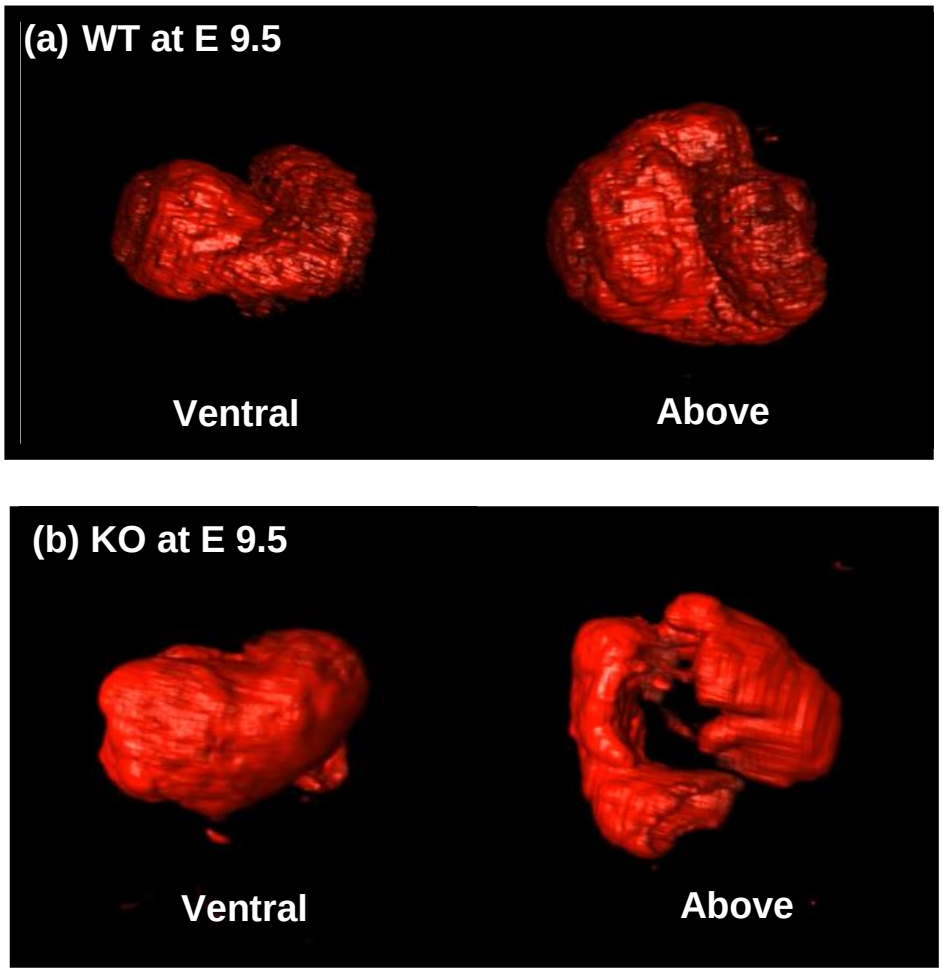

Figure 6: 3D heart structure of wildtype (WT) and FLNc gKO embryos at E 9.5 rendering from light sheet microscopy.

RNA Sequencing Analysis

The plot on principle component analysis (PCA) was shown in Figure 7, with FC ≥ 1.2 and Padjust < 0.05. The PCA analysis demonstrated control and FLNc gKO groups were clearly separated, indicating the transcriptome was significantly altered in FLNc gKO mice.

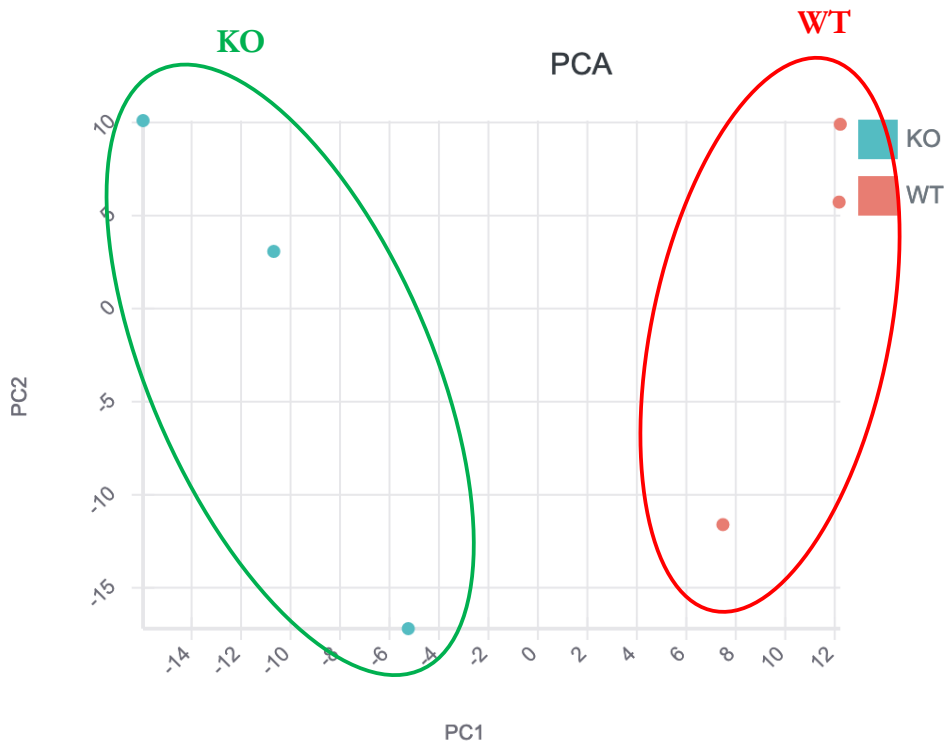

Figure 7: PCA Plot. Green indicating gKO group and red indicating WT group.

As expected, FLNc mRNA level was greatly reduced in FLNc gKO mice. Some genes related to wound healing pathways are extremely up-regulated in the FLNc gKO mice and are indicated by red circles in volcano plot (Figure 8), whereas genes related to integrin signaling pathways are indicated by green circles (Figure 8).

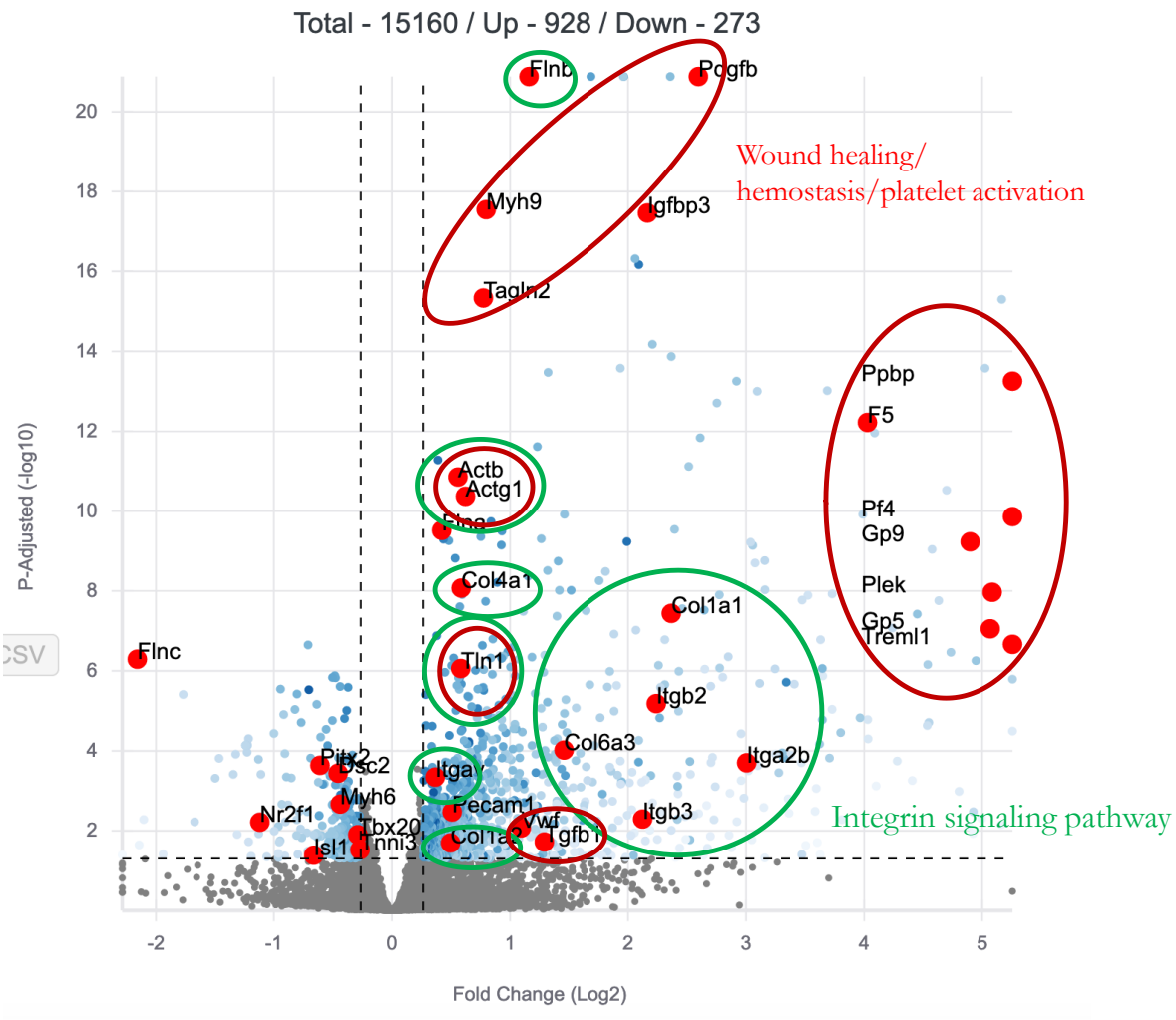

Figure 8: Volcano Plot of RNA-Sequencing. Red indicates genes related to wound healing. Green indicates genes related to integrin signaling pathway.

Discussion

In this study, we confirmed that loss of FLNc in cardiomyocytes causes myocardial rupture at E9.5 during cardiac development in the gKO mouse model. The defect in maintaining the cardiac integrity will result in early embryonic lethality. Thus, FLNc is essential in maintaining the integrity of the cardiac structure.

Along with the rupture formation, the wound healing process is activated according to the RNA-sequencing analysis, as the body would like to repair the damaged tissue. It has been reported that FLNc directly interacts and inhibits integrin activity (Bouvard et al., 2013). Loss of FLNc could in turn result in the upregulation of other genes, which are on the integrin signaling pathway. This is also ensured by the RNA-sequencing.

It has been reported that FLNc functions as linkage between the myofibril cytoskeleton and the extracellular matrix, playing a dynamic role in regulating and maintaining the cardiac structural integrity (Zhang et al., 2007; Clark et al., 2002; Dalkilic et al., 2006; Frank et al). Moreover, FLNc truncating mutations were found to alter the cell-cell adhesion structures, with decreased levels of desmoplakin in the myocardium (Begay et al., 2018). Due to the loss of FLNc, the changes within the interactions of cell-extracellular matrix and cell-cell adhesion can contribute to the formation of the rupture. In the previous immunofluorescent analysis, it is notable that the cardiomyocyte's cell-cell junction were not affected. The total amount of collagen-I, laminin and β1-integrin in gKO mouse at E 9.5 were not changed comparing to the WT's. Whereas, the active form of ITGB1 increased at the rupture site and but were relatively decreased at the other place of gKO mouse at E 9.5 (Figure 9). This is an interesting finding. As we

mentioned before, FLNc, along the integrin signaling pathway, works as an inactivator, inhibiting cell migration and cell spreading (Bouvard et al., 2013). Therefore, Tongbin and I we are generating an FLNc /ITGB1 double knockout mouse line to see if eliminating integrin activation in FLNc KO will rescue the heart rupture phenotype. The flow of creating FLNc -ITGB1 double conditional KO mouse model is shown in Figure 10.

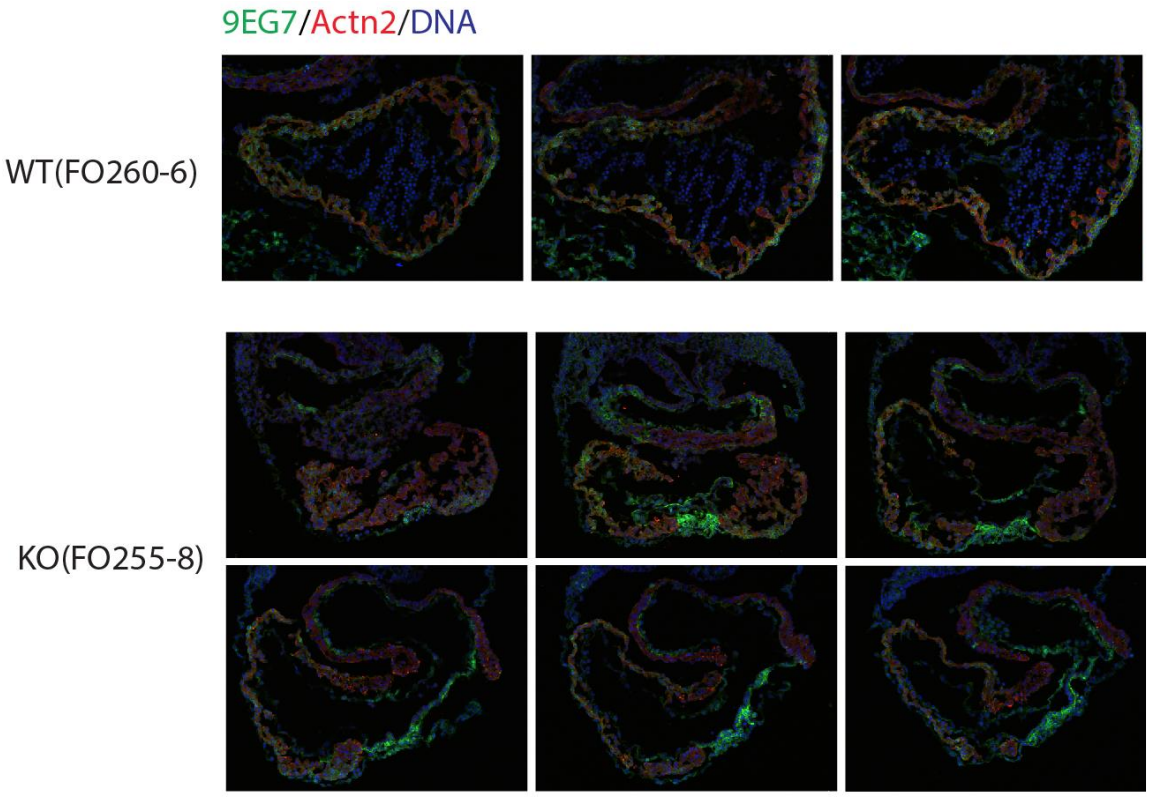

Figure 9: Immunofluorescence analysis of the active form of β1-integrin at E 9.5 (Tongbin).

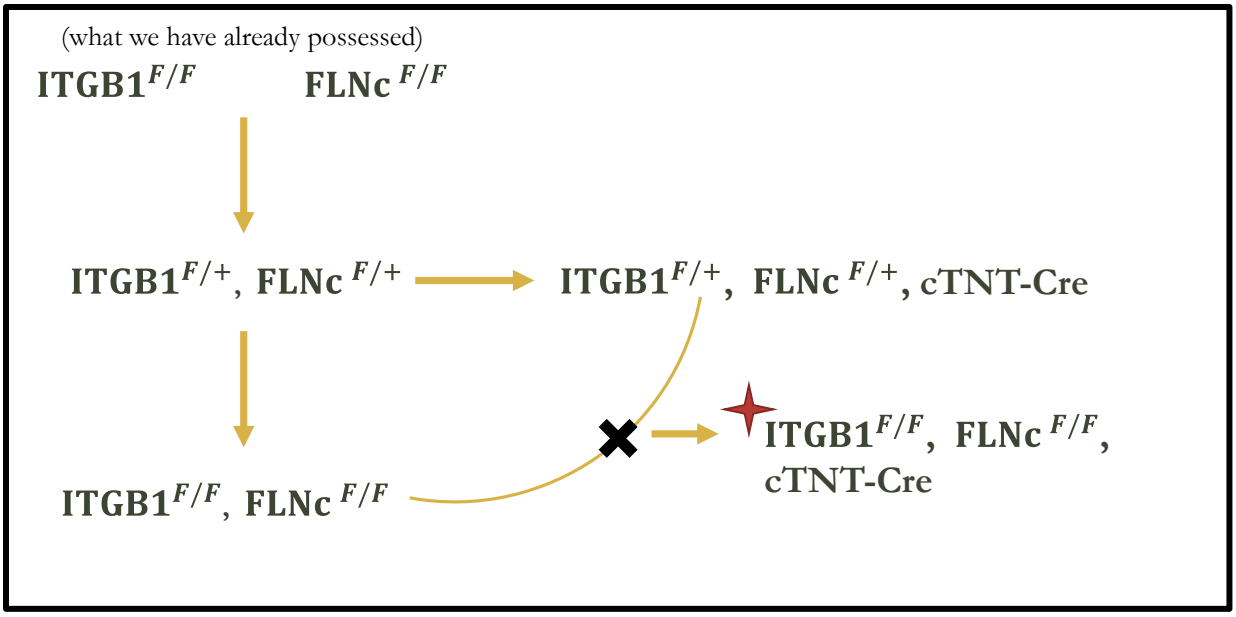

Figure 10: Flow of setting up FLNc-ITGB1 double cKO mouse model.

Future Perturbations

After acquired the double cKO mice, we will first characterize the phenotypes of them at E 8.5, E 9.5 and E 10.5, such as survival rate, chest edema ventricle ruptures etc. If those embryos are rescued, this indicates that the activation of ITGB1 is responsible for the myocardium ruptures. We will also perform immunoprecipitation to identify the physical interactions between FLNc and ITGB1. This could possibly give us more insights of the functional interactions between FLNc and ITGB1. If those embryos are not rescued, then we will shift our focus on the cell-cell junctions, such as adherens, desmosomes, tight and gap junctions.

Acknowledgement

This paper is coauthored with Tongbin Wu. The thesis author was the primary author of this paper.

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