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TITIN M-LINE INSERTION SEQUENCE 7 IS REQUIRED FOR PROPER CARDIAC FUNCTION IN MICE

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ABSTRACT

Titin is a giant sarcomeric protein that is involved in a large number of functions, with a primary role in skeletal and cardiac sarcomere organization and stiffness. The titin gene (*TTN*) is subject to various alternative splicing events, but in the M-line region the only exon that can be spliced out is Mex5, which encodes for the insertion sequence 7 (IS7). Interestingly, in the heart, the majority of titin isoforms are Mex5+, suggesting a cardiac role for is7. Comprehensive functional, histological, transcriptomic, microscopic, and molecular analyses of a mouse model lacking the *Ttn* Mex5 exon (Δ Mex5) revealed that the absence of the is7 is causative for dilated cardiomyopathy. Δ Mex5 mice show altered cardiac function accompanied by increased fibrosis and ultrastructural alterations. Abnormal expression of excitation-contraction coupling proteins was also observed. The results reported here confirmed the importance of the C-terminal region of titin in cardiac function and are the first to suggest a possible relationship between the is7 and excitation-contraction coupling. Finally, these findings give important insights for the identification of new targets in the treatment of titinopathies.

INTRODUCTION

Titin is the largest described protein with a molecular mass of 3 to 3.7 MDa, and is the third most abundant protein in striated muscle (Jackel et al., 1997). A single molecule of titin spans half a sarcomere from the Z-disc to the M-line, stretching over 1.2 μm (Labeit and Kolmerer, 1995) and interacts with numerous protein partners through this length (Chauveau et al., 2014). This giant protein plays a major role in sarcomere stability and organization, and also works as a molecular spring responsible for the passive elasticity of muscle (Gregorio et al., 1998, Granzier and Labeit, 2004). In relation to the sarcomere, titin is organized into four distinct regions that differ in structure and function: the N-terminal segment that anchors titin to the Z-disc through binding to multiple proteins; the I-band, which constitutes the molecular spring region of titin; the A-band region, involved in thick filament length control; and a final C-terminal portion bound to thick filaments in the M-line, where two adjacent titin molecules overlap in an antiparallel orientation (Chauveau et al., 2014, Martonfalvi et al., 2014).

In humans, the titin gene *TTN* (MIM #188840) is located on chromosome 2q31 and consists of 294 kb containing 364 exons (the first non-coding plus 363 coding exons), which can theoretically generate over a million splice variants (Bang et al., 2001, Guo et al., 2010). Traditionally, titin isoforms are divided in 3 main categories depending on presence of N2A and N2B elements within the I-band region of the protein. N2A isoforms (which contain only N2A elements) are mainly expressed in skeletal muscles, while N2B and N2BA isoforms (which contain only N2B elements, or both N2B and N2A, respectively) are specific to the heart (Linke et al., 1999, Freiburg et al., 2000, Labeit et al., 2006). In addition to this “splicing hotspot”, alternative splicing can also occur in the Z-disc and M-line regions of titin. The latter is encoded by exons 359 to 364 or Mex1 to Mex6. It is composed of a serine/threonine kinase (TK) domain followed by an alternation of immunoglobulin domains (M1 to M10) and

unique insertion sequences (is1 to is7) (**Fig. 1A**). The TK domain is an inactive pseudokinase scaffold and acts as a strain sensor (Puchner et al., 2008, Bogomolovas et al., 2014).

There are numerous binding partners for titin in the M-line: M-protein, FHL2 and Myomesin 1 (Hu et al., 2015, Lange et al., 2002, van der Ven and Furst, 1997). Alignment of the M4 domain with myomesin stabilizes the sarcomere during contraction (Lange et al., 2009). In its most terminal region, M10, titin interacts with obscurin (Obscn), obscurin-like 1 (Obsl1), cardiomyopathy-associated protein 5 (Cmya5) and alpha-synemin (Synm) (Fukuzawa et al., 2008, Prudner et al., 2014) (Sarparanta et al., 2010). Interestingly, the only exon of this region that can be spliced out is Mex5 (Kolmerer et al., 1996). The fraction of fibres expressing a Mex5+ titin isoform is dependent on the muscle, with a higher Mex5+/Mex5- ratio in muscles undergoing aerobic exercises (Guo and Sun, 2018). The splicing of Mex5 exon depends on the species, the phase of development and the M-line structure of the muscle (Kolmerer et al., 1996, Pask et al., 1994). The Mex5 exon encodes for the insertion sequence 7 (is7), situated between two Ig domains, M9 and M10 (**Fig. 1A**). The is7, which is part of a binding site for calpain 3, an enzyme implicated in limb girdle muscular dystrophy R1 that is nearly absent in the heart (Richard et al., 1995, Charton et al., 2010). Fascinatingly, is7+ titin is the major isoform expressed in the heart (Charton et al., 2016, Guo and Sun, 2018).

Considering its huge size, it is not surprising that *TTN* is subject to numerous mutations. About one percent of the general population carries a heterozygous truncating variation in *TTN* with no consequences (Schafer et al., 2017). When pathological, variations in *TTN* are responsible for titinopathies presenting with muscular and/or cardiac phenotypes. Titinopathies have highly variable phenotype and prognosis depending on the nature and position of the mutations. Among them, *TTN* mutations are responsible for more than twenty-five percent of all dilated cardiomyopathies (DCMs) (Tayal et al., 2017). The majority of reported variants associated with DCM are frameshift-causing mutations in high percentage

spliced in (PSI) exons located in the A-band region (Roberts et al., 2015, Stohr et al., 2018). Animal models also confirmed the importance of several titin regions for the heart function. A canine naturally occurring missense N2B-*TTN* variant in Doberman pinscher dogs leads to DCM and sudden cardiac death (Meurs et al., 2019). Several murine models presenting large

5 *Ttn* deletions have been developed, all presenting with cardiac abnormalities: homozygous deletion of the PEVK domain (Granzier et al., 2009), of the N2B (Radke et al., 2007); of the N2BA (Nusayr et al., 2018); in the A-band region (Tonino et al., 2017); in the M-line segment (Charton et al., 2016, Gotthardt et al., 2003, Radke et al., 2019). Notably, the M-line region of titin seems to play a determinant role in cardiac function; in fact, the loss of this protein

10 segment is causative of cardiac lethality in mice. The absence of Mex1 and Mex2 exons reduces the mechanical stability of the sarcomere by preventing the formation of a continuous titin filament (Weinert et al., 2006). In cardiomyocytes, the homozygous full deletion of M-band abrogates sarcomere formation (Musa et al., 2006). In addition, while mutations in Mex5 are responsible of Tibial Muscular Dystrophies (TMD) in humans (Hackman et al.,

15 2008) deletion of the C-terminal end of titin segment is causative of a myopathy with fatal cardiomyopathy (Carmignac et al., 2007).

Our laboratory previously reported that CRISPR/Cas9-driven homozygous deletion of the Mex5 exon leads to a dystrophic phenotype of all the muscles that in non-mutant mice mainly express Mex5+ *Ttn* isoforms (Charton et al., 2016). This mouse model (Δ Mex5) also showed

20 a progressive cardiac fibrosis, but previous publications were more focused on the skeletal muscle phenotype or the role of the is7 region in the interaction with calpain 3 (Charton et al., 2016, Lostal et al., 2019). Here, we further characterized the cardiac phenotype in Δ Mex5 mice, showing that absence of titin is7 region leads to DCM with decreased cardiac function, also confirming the presence of a progressive fibrosis. Our data showed sarcomere alterations

25 and mislocalization of the C-terminal end of titin in Δ Mex5 cardiomyocytes. In addition,

molecular analyses revealed that the deletion of *is7* caused a decreased expression of the M10-binding partners *Obscn* and *Cmya5*. A decreased expression of the two most important excitation-contraction coupling proteins (cardiac ryanodine and dihydropyridine receptors, or *Ryr2* and *Dhpr*) was also observed. Altogether, these results explain how the deletion of the *is7* region is responsible for the appearance of the cardiomyopathy in Δ Mex5 mice, by destabilizing the sarcomeric organization in cardiomyocytes, suggesting also a possible alteration of the calcium-release machinery. To our knowledge, the results reported here are the first demonstration of the potential molecular mechanisms underlying DCM resulting from Mex5 alterations, providing useful insights for future therapeutic targets.

RESULTS

Mex5 homozygous deletion leads to dilated cardiomyopathy with altered cardiac function and fibrosis in mice.

5 To gain a better understanding of the is7 role in titin function, we previously generated a mouse model homozygous for a deletion of the Mex5 region (Δ Mex5) (**Fig. 1A**) (Charton et al., 2016). The Δ Mex5 mice were reported to show a dystrophic phenotype in the skeletal muscle, with the soleus being the most affected muscle and with an involvement of the heart as observed by the presence of fibrotic tissue (Charton et al., 2016). To better understand the
10 role of is7 within the titin molecule, we studied the consequences of its deletion in the heart. Of note, the cardiac tissue is the only muscle where titin isoforms containing this domain are expressed by the majority of the cells, suggesting the particular importance of is7 in this organ.

To evaluate whether the absence of the is7 impacts cardiac function, transthoracic
15 echocardiographic measurements on wild type (WT) and Δ Mex5 mice were performed at two, four and thirteen months of age (**Fig. 1B-E; Table S1**). Echocardiography in Δ Mex5 mice showed a progressive increase in left ventricular dimension in both, diastole and systole compared to WT controls, with significant differences starting from four months of age (**Fig. 1B, C; Table S1**). The change in left ventricular volume is not associated with significant
20 differences in wall thickness, or in the heart weight/body weight ratio (**Table S1**). Chamber dimension abnormalities were accompanied by decreased cardiac function, as shown by reduction in the fractional shortening percentage (**Fig. 1D, E; Table S1**).

In view of the significantly reduced fractional shortening of the left ventricular chamber in Δ Mex5 mice starting at four months of age, we assessed systolic and diastolic ventricular

properties via pressure-volume (PV) analysis at this time point. PV loops showed (**Fig. 1F; Table S2**) that, compared to WT mice, the end systolic pressure volume relationship (ESPVR) of the Δ Mex5 mice LV chamber was shifted rightward, as a consequence of the increase in end systolic and end diastolic volumes (**Table S2**). Although with no significant difference, the End-systolic elastance (Ees), which provides an index of myocardial contractility, tends to be decreased in Δ Mex5 mice (**Fig. 1H**) as well as the maximal left ventricular pressure (dP/dt max; mmHg/s) (**Table S2**). The end diastolic pressure volume relationship (EDPVR) and the relative coefficient β , which describes the passive properties of the myocardium, remain unchanged (**Fig. 1I**). In agreement with echography data, the ejection fraction was also significantly decreased in Δ Mex5 hearts (**Fig. 1G; Table S2**). Overall, the PV analysis revealed a decreasing trend in cardiac inotropy in Δ Mex5 mice, as shown by the reduced Ees, consistent with the ventricular dilatation and dysfunction, but no change in diastolic stiffness, since no alterations in the coefficient- β were present.

To establish the impact of the Mex5 deletion on heart morphology, light sheet imaging, a technique that allows three-dimensional visualization of organs, was performed on hearts from four-month-old mice. Longitudinal, sagittal and transversal sections confirmed the left ventricular dilation in Δ Mex5 hearts (**Fig. 2A**). Three-dimensional imaging allowed for a deeper analysis of the heart dimensions that, in contrast with the echocardiography measurements, revealed an observable difference in the LV free-wall between mutant and controls. The light sheet image quantification showed an increase in the volume of all the heart chambers also confirming the left ventricle dilation observed in echocardiography (**Fig. 2B, C**).

Histological and molecular characterizations of Δ Mex5 and control hearts were performed at the same time point. Heart tissue sections from four-month-old mice were stained with hematoxylin phloxin saffron (HPS) and Sirius red (**Fig. 2D**). HPS-stained cross-sections of

Δ Mex5 hearts displayed large areas of damaged tissue with increased cellularity when compared to WT. Sirius red staining showed an extended presence of fibrotic tissue in Δ Mex5 hearts, whereas normal connective tissue was found in WT hearts (**Fig. 2D**). Additional analyses, at different time points, indicated that the fibrotic tissue was already present in Δ Mex5 hearts at 1 month of age (albeit with no significant increase in respect of WT controls), and that the fibrotic condition proportionally worsened with aging (**Fig. 2E; Fig. S1A**). Immunofluorescence analyses of Collagens 1a1 and 3a1 (**Fig. S1B**) and quantitative RT-PCR analysis of fibrosis markers (Collagen 1a1, Fibronectin, Collagen 3a1, Vimentin) (**Fig. 2F, G; Fig. S1C, D**) confirmed the observed fibrotic phenotype.

Different cardiac stress and inflammation markers, known to be associated with pathological cardiac conditions, were also measured by quantitative RT-PCR (**Fig. 2H-J; Fig. S1E, F**). In particular, the balance between the two Myosin heavy chains Myh7 and Myh6 was altered in Δ Mex5 hearts as already reported in pathological heart remodeling (Fatkin et al., 2000, Gupta, 2007, Vanderheyden et al., 2008, Montgomery et al., 2011): Myh7 was increased, whereas Myh6 was decreased with respect to WT controls (**Fig. 2H, I**). Natriuretic peptide A (Nppa), whose expression is reactivated in cardiovascular pathologies (Houweling et al., 2005), transforming growth factor beta 1 (Tgf- β 1), a key player in cardiac fibrosis and pathological remodeling and TIMP metalloproteinase inhibitor 1 (Timp1), known to be upregulated in deteriorating heart failure, were all upregulated, indicating a typical dilated cardiomyopathy (DCM) pattern in the mutant mice (**Fig. 2J; Fig. S1E, F**).

To identify the impact of the *is7* deletion on sarcomere organization, electron microscopy on heart samples from four-month-old mice was performed (**Fig. 2K**). Ultrastructural analysis of Δ Mex5 mice cardiac tissue revealed the presence of altered sarcomeres with irregular Z-disc, less defined A- and I-bands, and absence or disruption of the M-line, although occasional normal sarcomere structures were found (**Fig. 2K i-ii**). Abnormal condensed mitochondria

(Mit), autophagic vacuoles, lipids, inflammatory infiltrate cells and collagen fibrils were also observed, further confirming the fibrotic condition (**Fig. 2K iii-iv**). Mitochondria quantitative analyses showed a statistically significant increased area and fractional area, and reduced perimeter in Δ Mex5, while no significant differences on Feret's diameter was found. Shape parameters were significantly different in Δ Mex5 mitochondria compared to WT, including the increase in roundness accompanied by the reduction of aspect ratio, and an increase in circularity and solidity, indicating a reduction in the elongation of Δ Mex5 mitochondria (**Fig. 2L**).

We then compared Δ Mex5 sarcomere length to WT controls. A huge variation in Δ Mex5 sarcomere length was observed compared to controls, with a higher number of shorter sarcomeres in Δ Mex5 hearts (**Fig. 2M**). These data demonstrated that the homozygous deletion of Mex5 in titin is causative of a DCM phenotype accompanied by cardiac fibrosis, inflammation and mitochondria morphological alterations, as well as altered sarcomere length.

Sarcomere alterations and titin “ultra-localization shift” in Δ Mex5 hearts.

To investigate the underlying mechanisms leading to the cardiac abnormalities, a first characterization of the consequences of the Mex5 deletion on the expression of *Titin* at the RNA and protein levels was performed. Quantitative RT-PCR showed a slight (albeit not significant) reduction in *Titin* relative expression in Δ Mex5 with respect to WT controls, suggesting that the deletion does not drastically affect titin mRNA level (**Fig. 3A**). At the protein level, Coomassie blue stained gels using lysates from WT controls and Δ Mex5 hearts were performed to visualize the presence of the different titin isoforms. The results showed no increase in the degradation product T2 in Δ Mex5 heart samples compared to controls, a slight decrease in N2BA/N2B ratio was observed, although not statistically significant (**Fig. S2A, B**). A

more detailed analysis was conducted via Western Blot (WB) using a set of antibodies raised against all different domains of the C terminus titin (M1, is5, is6, M8M9, is7 and M10), while using an antibody specific for titin N-terminus (Z1Z2) as comparative control (**Fig. 3B**). No differences in Z1Z2, M1, is5 and is6 were observed between Δ Mex5 and WT controls, indicating that the protein is present at similar levels with or without the is7. As expected, no specific labeling was detected for is7 on Δ Mex5, confirming the deletion. Interestingly, a reduction of intensity was also observed for the two adjacent domains M8M9 and M10 in Δ Mex5 compared to controls, suggesting that the deletion of the is7 affects the adjacent titin Ig domains (**Fig. 3C**).

Immunostainings of cardiac tissue from four-month-old mice were then performed to determine the presence of the different C-terminal end of titin domains and to assess the localization of titin in Δ Mex5 mice (**Fig. 3D**). Is7 specific staining showed the complete loss of the domain, whereas M8M9 and M10 staining confirmed the presence of these domains on each side of the Mex5 deletion in WT tissues, although not all Δ Mex5 sarcomeres were positive for M8M9 and M10 staining. Despite a reduced intensity of the staining on WB, immunofluorescence images showed a correct integration of titin in Δ Mex5 cardiomyocytes, and no apparent change in localization with respect to WT controls (**Fig. 3D**).

Immunoelectron microscopy was performed with antibodies raised against is7 and M8M9. As expected, titin is7 was completely absent in Δ Mex5 but present in WT sarcomeres, while titin M8M9 domain epitopes were labeled in a spotty pattern in both mutants and controls (**Fig. 3E**). However, the M8M9 staining is not present in all the sarcomeres and the pattern is less regular in Δ Mex5 samples. Measurement of the distance between M8M9 epitopes across the M-line was increased in Δ Mex5 compared to WT (88 ± 1 nm vs 94 ± 3 nm), indicating a modification in the organization of the domains and suggesting that the mutation alters titin localization at a sarcomeric level, shifting it more toward the Z-disc side (**Fig. 3F**).

These results suggest that the absence of Mex5⁺ titin in the heart causes sarcomere abnormalities.

Deletion of is7 leads to a classical molecular signature of a DCM.

To understand the molecular mechanisms underlying the cardiac phenotype in Δ Mex5 mice, a
5 RNAseq analysis on samples from WT and mutant hearts at four months of age was performed using Illumina sequencing. The reads were aligned onto the mm10 mouse genome and DESeq2 analysis was performed. A total of 3473 genes were identified as significantly dysregulated with a log2 fold change higher than 0.5 in Δ Mex5 hearts compared to controls with 2116 up-regulated and 1356 down regulated (**Fig. 4A**). The top dysregulated genes at
10 four months of age sorted by log2 fold change are presented in **Tables S3 and S4**. These included transcripts encoding for proteins implicated in cardiac fibrotic processes (e.g. Spp1, Cilp, Ltbp2, Sfrp2, Thbs4), fibrotic tissue structure (e.g. Tnc, Col12a1, Col8a2) and cardiac protection upon stress (e.g. Mpeg1, Gpnmb) for the up-regulated, and in mitochondria for the down regulated (e.g. mt-Ts1, mt-Ti). Gene Set Enrichment Analysis (GSEA) using the Gene
15 Ontology Biological Process collection revealed that the most up-regulated genes are related to immune response activation (e.g. GO leukocytes migration, regulation of cell activation, leukocytes differentiation and adaptive immune response) (**Fig. 4B, C; Tables S5 and S6**). The GSEA also confirmed that down-regulated genes were significantly enriched in the Gene Ontology Mitochondrial pathways (e.g. GO mitochondrial respiratory chain complex
20 assembly, respiratory electron transport chain, mitochondrial electron transport NADH to ubiquinone) (**Fig. 4B, D; Tables S5 and S6**). Ingenuity Pathway Analysis (IPA) software was used to identify enriched pathways. We used the IPA toxicology analysis to obtain a picture of the negative effects of the is7 removal. Most of the significantly dysregulated genes are implicated in known cardiac diseases pathways as cardiac enlargement (86 genes), cardiac
25 dysfunction (45 genes), cardiac dilatation (38 genes), cardiac fibrosis (27 genes), cardiac

necrosis/cell death (35 genes), etc. (**Fig. 4E**). These results confirmed that deletion of *Mex5* in titin is causative of DCM associated with severe inflammation, cardiac fibrosis and mitochondrial damage.

Interestingly, two genes, *Cmya5* and *Obscn*, encoding for proteins that bind to titin at the M10 region were present among the down-regulated group with a high significance (adjusted pValues: 4.04e-4 and 0.001, respectively), although not the most dysregulated genes (**Table S7**) (Benian and Mayans, 2015, Sarparanta et al., 2010) . A close homolog of *Obscn* (i.e. *Obsl1*) is also slightly dysregulated (Adjusted pValue 0.028). On the other hand, the two other proteins known to interact with titin in M10, alpha-synemin (*Synm*) and calpain 3 (*Capn3*), which are present at a low expression level, remained unchanged (**Table S7**). *Cmya5* encodes for the cardiomyopathy-associated protein 5, a 500kDa tripartite motif (TRIM)-related protein, highly expressed in striated muscles and that serves as an anchoring protein that mediates subcellular compartmentation of intracellular signaling (Benson et al., 2004, Benson et al., 2017, Sarparanta, 2008). *Obscn* is a 720 kDa sarcomeric protein with an essential structural role in positioning the longitudinal sarcoplasmic reticulum at the M-line (Lange et al., 2009). We also noticed that two genes, *Ryr2* and *Atp2a2* encoding for Ryr2 and SERCA2, two proteins known to play a central role in Ca^{2+} -handling, were down-regulated with high significance (Adjusted pValue 2.5e-05 and 1.6e-6, respectively; **Table S8**). RyR2 and SERCA2, are two of the main components of the cardiac Ca^{2+} -release/uptake machinery present at the level of the sarcomeric reticulum, and alterations in their expression levels are a hallmark of cardiac disease (Liu et al., 2019, Liu et al., 2020). Interestingly, Ryr2 clustering in striated muscle involves *Cmya5* and these two proteins form an oligomeric protein complex in cardiac muscle (Benson et al., 2017). Genes encoding for two additional proteins involved in Ca^{2+} -handling were also slightly dysregulated: *Jph2* and *Pln* (**Table S8**). *Jph2* encodes for junctophilin2, which is a protein determinant in dyads formation between junctional SR and

T-tubules (Takeshima et al., 2000, Spinozzi et al., 2020, Feng et al., 2020). *Pln* encodes for phospholamban, a protein that is involved in SERCA2 regulation (Koss et al., 1997).

Deletion of the *is7* alters the expression of titin M10-binding partners and of junctional membrane complex proteins in Δ Mex5 mice.

5 The data obtained with the RNAseq analysis guided us in further investigating the dysregulations in titin M10 binding partners and in assessing whether the Ca^{2+} -release/uptake machinery was altered in the Δ Mex5 mice. In agreement with RNAseq results, quantitative RT-PCR measurements showed a reduced mRNA expression for *Cmya5*, *Obscn*, *Ryr2*, *Atp2a2* and *Pln*, whereas *Synn*, *Cacna1c* (encoding for Dhpr) and *Jph2* levels were not
10 significantly changed (**Fig. 5A-H, 4A and Table S8**). At the protein level, WB analyses showed a decreased expression only for *Cmya5*, *Obscn* and *Ryr2* in Δ Mex5, in agreement with the reduction at the transcription level (**Fig. 5I-L**). On the other hand, no significant change in *Jph2* or *Serca2* was detected between mutant and WT hearts (**Fig. 5M, N**). Surprisingly, Dhpr protein levels were significantly reduced in Δ Mex5 hearts, in contrast with
15 transcriptomic and qPCR data (**Fig. 5O**). These results suggested a specific alteration in Ca^{2+} -release, with abnormal expression of the two main proteins involved in this process, but no apparent alteration in the organization of the junctional membrane complex architecture (no change in *Jph2* expression), or in the Ca^{2+} -uptake machinery (no alterations in *Serca2* protein levels were observed). Next, we investigated whether *Cmya5* and/or *Obscn* localization was
20 impacted by the destabilization of the M-line portion of titin in mutant mice. Immunofluorescence analyses on heart sections from four-month-old Δ Mex5 and WT mice showed no altered pattern for either *Cmya5* or *Obscn* in mutant mice. α -actinin staining (Z-disc pattern) was used as sarcomeric localization control (**Fig. 6A, B**).

Abnormal Ryr2 and Dhpr levels are usually followed by alterations in Ca^{2+} -release, one of the most common underlying mechanisms in heart failure. Often, DCM and Ca^{2+} -handling defects are associated with T-tubules remodeling, which in turns reflects on the localization of Dhprs. This delocalization creates orphaned Ryr2 on the SR, impeding their activation
5 (Spinozzi et al., 2020, Lipsett et al., 2019). To understand if the reduction in the expression of these two proteins could be the result of a “feedback mechanism” due to a Ryr2/Dhpr uncoupling, immunofluorescence analyses using antibodies against Ryr2 and Dhpr were performed. The data showed no apparent mislocalization of either Ryr2, or Dhpr in ΔMex5 hearts when compared to WT (**Fig. 6C, D**), suggesting absence of remodeling in either the T-
10 tubules or the SR. Therefore, these results demonstrated a specific decrease in Ryr2 and Dhpr expression with no reorganization of either T-tubules or junctional SR.

DISCUSSION

In this study, we characterized the cardiac phenotype induced by the deletion of the is7 in titin. Physiological and histological analyses revealed a progressive dilation of the left ventricle with decreased function and presence of extensive fibrosis. Morphological observations by electron microscopy showed alterations in the sarcomere and in mitochondrial shape and organization. Finally, analyses of the molecular mechanisms responsible for the cardiac defects in Δ Mex5 demonstrated that the absence of the is7 alters the sarcomeric organization of the molecule and perturbs interactions with titin partners, ultimately leading to the abnormal expression of Excitation/Contraction (EC)-coupling proteins.

Heart contraction and relaxation cycles are possible owing to a perfect balance of mechanical force and elasticity. At the sarcomere level, the protein that most contributes to the elasticity of the cardiomyocyte is titin. Analysis of Δ Mex5 hearts indicated no change in heart stiffness, consistent with the fact that the corresponding region is not involved in titin elastic properties compared to other regions of the molecule. The large sarcomere length inhomogeneity and the observed sarcomere alterations may be due to modifications of the spatial organization of the M-line segment of titin, known to have a role as a shock absorber (Lange et al., 2020), making the sarcomere more susceptible to contraction-induced damages. The decrease in sarcomere length could also be due to fibrosis that makes sarcomeres resistant to stretch. In addition, there is evidence of reorganization of the topology of titin domains with respect to the sarcomere. It is unlikely that the removal of the is7 would be directly responsible for the observed reduction in sarcomere length because of its small size. It is more plausible that the presence or absence of the is7 determines the modality of interaction between titin and other proteins at the M-band (Lange et al., 2020). If this is the case, the altered interaction, when the

is7 is absent, may change the anchoring of the titin molecules in the M-line, explaining the wider distance between M8M9 epitopes. Another possible explanation is that destabilization of the C-terminal part of titin, induced by the loss of the is7, bends the titin filament toward the N-terminal part, preventing it to span straight across the sarcomere.

5 WB analyses showed also that the absence of the is7 destabilizes the is7-adjacent domains. We previously reported that a pathological cleavage can occur in the is4–is5 regions of titin in some titinopathies (Charton et al., 2015, Sarparanta et al., 2010). WB analyses performed on different cells fractions did not show any pathological cleavage of titin C-terminus (data not shown), suggesting that a similar event is not happening here. Most likely, the destabilization
10 might be due to partial degradation of the domains, rather than a “total” cleavage. Intriguingly, molecular analyses showed that the is7 deletion impacts the expression of M10 partners of titin, Obscn and Cmya5, at both the RNA and protein levels, as demonstrated by their down-regulation in Δ Mex5 hearts. Since the decrease happens at both levels, the defect could originate solely from a transcriptional dysregulation with no change in protein stability
15 due to the lack of protein interactions. Therefore, these data suggest a transcriptional control that adapts the level of transcript to the level of the binding domain. Interestingly, Cmya5 was previously shown to be down-regulated in several muscle and cardiac disorders such as tibial muscular dystrophy, limb-girdle muscular dystrophy 2J and 2A (Sarparanta et al., 2010) and Duchenne muscular dystrophy (Tkatchenko et al., 2001). Mutations in *CMYA5* have also been
20 associated with cardiac pathologies (Xu et al., 2015, Nakagami et al., 2007). Different mutations in *OBSCN* were identified to be responsible for dilated cardiomyopathy (Marston et al., 2015). Besides the sarcomere abnormalities, alterations in the expression levels of these proteins may also contribute to the phenotype, considering the role of the proteins and their implication in muscle diseases. In fact, it is well known that alterations in the interaction
25 between Obscn and titin may results in cardiac defects (Bang et al., 2001, Grogan et al.,

2020), while alterations in Obscn expression are associated with defects in SR organization and dysregulated Ca^{2+} -cycling (Lange et al., 2009, Hu et al., 2017). In addition, Cmya5 interacts with Ryr2, also participating in the clustering of the channel in the dyads (Benson et al., 2017). It is important to mention that Cmya5 is mainly, but not exclusively, localized in the Z-disc (Sarparanta, 2008). However, since an interaction with titin M10 domain has been demonstrated (Sarparanta et al., 2010), it cannot be excluded that variations in this interaction may reflect on the function in the Cmya5 localized in the Z-disc.

Interestingly, WB data showed that Ryr2 and Dhpr protein levels were decreased in ΔMex5 hearts. These two proteins are strictly associated to each other in the dyad and are the main ion channels responsible for Ca^{2+} -release during EC-coupling. The loss of is7^+ titin in cardiomyocytes decreases Obscn and Cmya5 expression, and we speculated that this alteration in turn induces a down-regulation of Ryr2 that sequentially alters Dhpr protein levels. Notably, it is known that reduction in Ryr2 and Dhpr protein levels can also be the result of their uncoupling, induced by abnormal organization of the dyads due to T-tubules remodeling (Zhang et al., 2013). However, in our mouse model, this was not the case. In fact, additional molecular analyses of ΔMex5 cardiac tissue lysates at the protein level showed no change in the junctional SR protein Jph2 or in the longitudinal SR protein Serca2, two proteins that are well-known to be reduced in severe DCM where T-tubule remodeling is present (Liu et al., 2019, Spinozzi et al., 2020, Liu et al., 2020, Wu et al., 2012, Takeshima et al., 2000, Lipskaia et al., 2010). Moreover, confocal microscopy on ΔMex5 heart samples showed no localization abnormalities of either Ryr2 or Dhpr, indirectly demonstrating that dyads and T-tubules organization was not altered. Altogether, these findings allow us to assume that Ryr2 and Dhpr reduced protein expression is most likely a direct consequence of Cmya5 and Obscn abnormal levels induced by the absence of the is7 in titin, rather than just a subsequent effect of the DCM phenotype.

Conclusion

In conclusion, preventing the cardiomyocytes from expressing their preferred titin isoforms (Mex5⁺) leads to a severe cardiomyopathy, confirming that the presence of the is7 is essential for titin function in the heart. Most likely, the absence of this part of the protein destabilizes the surrounding domains and leads to the described DCM phenotype. Our data are the first to suggest a possible involvement of titin M-line region in EC-coupling regulation. Further studies on this mouse model are necessary to gain a better understanding of the potential role of titin is7 in this physiological mechanism. Altogether, these results further stress how important the integrity of the M-line region of titin is for cardiac function and provides important insights for the identification of possible novel therapeutic targets.

MATERIAL AND METHODS

Animal

The Δ Mex5 (C57BL/6N-*Tn^{gnt1}*) mouse model used in this study has been previously described (Charton et al., 2016). This model was bred and housed in a barrier facility with 12-h light, 12-h dark cycles and provided food and water ad libitum. C57BL/6N (WT) mice, strain-matched control, were obtained from Charles River laboratories. Males were included in the study at different ages according to the experiment. All mice were handled according to the European guidelines for the human care and use of experimental animals, and animal experimentations were approved by the Ethical Committee for Animal Experimentation C2AE-51 of Evry under number APAFIS#1720 and APAFIS#19736.

For histological and molecular analysis of mouse tissues, specimens were collected immediately after sacrifice, snap frozen in liquid-nitrogen-cooled isopentane and stored at -80°C .

15 *Echocardiography*

Conventional echocardiography was performed on anesthetised mice using a Vevo 770 (Visual sonics) with a 30 MHz cardiac probe (RMV707B). During the procedure, heart rate (HR) and temperature were monitored. For echocardiography recording, sweep speed, depth, focus and gain settings were optimized to obtain the most defined acquisitions. Two-D and M-mode echocardiography were obtained manually from long parasternal axis view at the level of the largest LV diameter (at the level of the papillary level). LV dimensions (LV end-diastolic diameter = LVEDD, LV end-systolic diameter = LVESD), posterior wall (PW) and interventricular septum (IVS) wall thickness were measured using the leading edge

convention of the American Society of Echocardiography (ASE). SF and EF of the left ventricle, PW thickening (PWth) and LV mass were calculated from the above dimensions.

Pressure Volume loops

In vivo pressure-volume (PV) analysis was performed in four-month-old mice using a SciSense Advantage Admittance Derived Volume Measurement System and 1.2F catheters with 4.5 mm electrode spacing (SciSense, London, Ontario, Canada). Mice were anesthetized and ventilated with 1.5-3% isoflurane using a Physiosuite Rovent (Kent Scientific). Body temperature under anesthesia was maintained at 37°C using a Physiosuite RightTemp (Kent Scientific). A lateral incision through the skin and muscle was made below the ribcage and the diaphragm was cut in order to expose the apex of the heart. A small puncture was made in the apex of the left ventricle using a 28G needle and the 1.2F catheter was inserted into the LV. Baseline functional parameters were assessed during a pause in ventilation to avoid respiratory influences. For load-independent indices, including the end-systolic and end-diastolic pressure-volume relationships, the inferior vena cava was temporarily occluded to vary the preload conditions. Data acquisition and analysis was performed using LabScribe 3 (iWorx, Dover, NH) and curve fitting was performed with MATLAB (MathWorks, Natick, MA). Diastolic PV data was analyzed using a monoexponential fit with constant ($P = Ae^{\beta V} + C$) with the exponent (β) reported as the stiffness.

Electron Microscopy and Immunoelectron Microscopy

Intact LV papillary muscles from four-month-old male Δ Mex5 mice (n= 4) and littermate WT controls (n= 4) were stretched from the slack length and processed for transmission electron microscopy (TEM). Briefly, fixation of cardiac tissue was performed with a mixture of 3.7 % paraformaldehyde, 3% glutaraldehyde and 0.2 % of tannic acid in 10 mM PBS, pH 7.2, for 1 h at 4 ° C. This step was followed by a postfixation in 1 % OsO₄ in the same buffer for 30 min

at 4 ° C. Samples were then dehydrated in an ethanol graded series, infiltrated with propylene oxide, and transferred to a mix of propylene oxide/Araldite 502/Embed 812 resin polymerized for 48 h at 60 ° C. Ultrathin, 90 nm longitudinal sections were obtained with a Reichert-Jung ultramicrotome and contrasted with 1 % potassium permanganate and lead citrate. Specimens were observed in a TECNAI Spirit G2 transmission electron microscope (FEI, Hillsboro, OR) with a side-mounted AMT Image Capture Engine V6.02 (4Mpix) digital camera operated at 100 kV. Digital images were stored for quantitative analyses of mitochondria morphology and measurements of sarcomere length with ImageJ 1.49v (NIH-USA).

Labeling of titin M-line epitopes by immunoelectron microscopy (IEM) was performed on bundles of skinned papillary muscle from WT (n= 4) and Δ Mex5 (n= 4) mice, stretched to ~ 25 % in relaxing solution and processed by the pre-embedding technique previously described (Tonino et al., 2017). Cardiac tissue bundles were fixed in 3.7 % paraformaldehyde in 10 mM PBS for 30 min at 4° C and after rinsed in PBS were incubated with glycine 50 mM in the same buffer and then blocked with 0.5 % bovine serum albumin (BSA) in PBS containing protease inhibitors and 0.05 % Tween 20, 1 h at 4° C. After blocking, muscle bundles were incubated 48 h at 4° C with the following polyclonal titin domains antibodies from rabbit: anti-M8M9 (0.35 mg/mL, BioGenes, Germany) and anti-is7 (0.724 mg/mL, (Charton et al., 2016)), then followed by overnight incubation with secondary antibody AlexaFluor-568 goat anti-rabbit IgG (ab175471, Invitrogen, 2mg/mL). Controls were performed in muscle bundles by replacing each primary antibody with PBS/0.5% BSA solution containing protease inhibitors. Afterward, bundles of cardiac tissue were fixed with 3 % glutaraldehyde and processed for TEM as explained above. Digital images were obtained and M8M9 and is7 epitope distances were measured across the M-line from density plot profiles using ImageJ 1.49v (NIH-USA). These values were then corrected for shrinkage (caused by the TEM procedure) using the A-band width known value of 1.6 μ m (Sosa et al., 1994).

Histology and Immunohistochemistry

Transverse cryosections (8 μm thickness) were prepared from frozen muscles, air dried, and stored at -80°C . Mouse sections were processed for Hematoxylin-Phloxine-Saffron (HPS) or Sirius red histological staining. Sections were visualized on an Axioscan Z1 automated slide scanner (Zeiss, Germany), using a Plan APO 10X / 0.45 NA objective. Quantification was performed by using ImageJ software.

Sections were immunostained overnight at 4°C with primary antibodies listed in **Table S9**. After washing three times with PBS, muscle sections were incubated with a goat -secondary antibody conjugated with Alexa Fluor 594 or 647 dye (Molecular probe, dilution 1:1000) for 1 hour at room temperature (RT). Sections were mounted with DAPI-fluoromount-G (SouthernBiotech) and visualized on a LEICA TCS-SP8 confocal microscope (Leica, Germany) using a 63X APO CS2 1.4NA objective.

Light Sheet Imaging

Clarification of heart samples was performed following iDISCO clearing protocol (Renier et al., 2014). Images were acquired using an Alpha3 (α^3) Light Sheet Microscope (PhaseView, France) equipped with a XLFLUOR objective, 2X magnification, wd 340mm, 0.14 NA (Olympus, France), and with an ORCA FLASH 4.0 SCMOS camera (Hamamatsu, Japan). Samples were immersed in a 10mL sample chamber containing Dibenzyl Ether solvent (DBE). Sidewalls of the sample holder are glass coverslips (0,17mm). Illumination arms located at each side of the sample holder create the excitation light sheet using two Plan-Neofluar 2.5X magnification objectives (Zeiss, Germany). The sample is laid down on a motorized sample-holder, within the DBE-filled sample chamber, allowing for the three-dimensional scan of the sample. To assess heart tissue volume, heart autofluorescence was used: images were acquired using a 488nm Laser line and to maximize the signal, no emission

filters were used except for a notch filter cutting the laser wavelengths. Voxel size: 2.5x2;5x2 μm .

The QTSPIM software (PhaseView, France) was used for image acquisition. To cover the full XY-surface of the heart, a 2x2 tile scan was manually performed for each sample. Tiles stitching was performed with IMARIS Stitcher software (Bitplane – Oxford Instruments, UK). The reconstructed stacks for each heart were processed with IMARIS software (Bitplane – Oxford Instruments, UK), which automatically created a three-dimensional object for volume calculations.

RNA extraction

10 Total RNA extraction was performed from frozen tissues by the Trizol™ method (Thermo Fisher Scientific, Waltham, MA). Extracted RNA was dissolved in 20 μl of RNase-free water and treated with Free DNA kit (Ambion) to remove residual DNA. Total RNA was quantified using a Nanodrop spectrophotometer (ND8000 Labtech, Wilmington Delaware).

RNA-sequencing

15 RNA from 3 WT mice and 3 ΔMex5 mice heart at four months were sequenced. RNA concentration was measured on a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA quality ($\text{RIN} \geq 7$) was controlled using an Agilent RNA 6000 Pico Kit on a 2100 Bioanalyzer instrument (Agilent Technologies). The sequencing libraries were prepared using the TruSeq Stranded Total RNA Library Prep Kit (Illumina) and sequenced according
20 to the Illumina protocol. The reads were paired using Fastq-pair and aligned onto the mouse genome (mm10) using STAR aligner. The analysis was performed using DESeq2 library on R software. Genes were considered as significantly dysregulated when absolute value of log2 fold change was higher than 0.5 and adjusted p-value smaller than 0.05, genes with low read count (< 10) were filtered out. Gene Set Enrichment Analysis was performed using

GSEABase library on R with Gene Ontology Biological Process (C5) gene sets. Ingenuity Pathway Analysis (IPA, Qiagen, Redwood City, California, USA) was used to investigate enriched toxicity pathways.

qRT-PCR analyses

5 One µg of RNA was reverse-transcribed using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) and a mixture of random oligonucleotides and oligo-dT. Real-time PCR was performed using LightCycler480 (Roche, Basel, Switzerland) with 0.2mM of each primer and 0.1 mM of the probe according to the protocol Absolute QPCR Rox Mix (Thermo Fisher Scientific, Waltham, MA, USA). The
10 primers and Taqman probes (Eurogentec, Liege, Belgium) used are listed in **Table S10**. Data from the ubiquitous acidic ribosomal phosphoprotein (P0) was used to normalize the data across samples. The primer pairs and Taqman probes used for P0 amplification were: m181PO.F (5'-CTCCAAGCAGATGCAGCAGA-3'), m267PO.R (5'-ACCATGATGCG CAAGGCCAT-3') and m225PO.P (5' -CCGTGGTGCTGATGGGCAAGA A-3'). Each
15 experiment was performed in duplicate. Quantification cycle (Cq) values were calculated with the LightCycler® 480 SW 1.5.1 using 2nd Derivative Max method.

Protein extraction, Coomassie Blue gel and Western Blot

Proteins were extracted from LV tissues pulverized in liquid nitrogen by solubilisation in urea buffer (in mol/L): 8 urea, 2 thiourea, 0.05 tris-HCl, 0.075 dithiothreitol with 3% SDS and
20 0.03% bromophenol blue, pH 6.8) and 50% glycerol with protease inhibitors ((in mmol/L): 0.04 E64, 0.16 leupeptin and 0.2 PMSF) at 60 °C for 10 min. Samples were centrifuged at 13,000 RPM for 5 min and stored at -80 °C.

The titin isoform visualization was performed by loading solubilized samples on agarose gels (1%). After electrophoresis at 15 mA per gel for 3h20, the gels were stained using Coomassie

brilliant blue and scanned using a commercial scanner. For titin western blot, solubilized samples were run on a 0.8% agarose gel, then transferred onto polyvinylidene difluoride membranes using a semi-dry transfer unit (Trans-Blot Cell, Bio-Rad). Blots were stained with Ponceau S to visualize the total protein transferred. Blots were then probed with primary antibodies listed in **Table S9** followed by secondary antibodies conjugated with infrared fluorescent dyes.

For the other proteins, the samples were prepared and separated following the NuPAGE 4 to 12% Bis-Tris or 3 to 8% Tris-Acetate Gel protocol (ThermoFisher) depending on the size of the protein, and transferred with iBlot 2 Dry Blotting System (ThermoFisher). Detection of proteins were performed using standard Odyssey protocol with primary specific antibodies listed in **Table S9** followed by secondary antibodies conjugated with infrared fluorescent dyes. Blots were scanned using an Odyssey Infrared Imaging System (Li-COR Biosciences). The quantification of each protein was performed in comparison to GAPDH protein quantity in the sample, using Image Studio software (Li-COR Biosciences).

Data and Statistical Analysis

The characterization parameters were compared between WT and Δ Mex5 groups by non-parametric unpaired Mann-Whitney test. Data are expressed as mean \pm SEM. Values of $P < 0.05$ were considered as statistically significant. Prism package (GraphPad Software) and R software were used for data analysis.

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DATA AVAILABILITY

RNAseq raw data are available upon reasonable request by contacting the corresponding author.

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FIGURE LEGENDS

Figure 1: Cardiac functional characterization of Δ Mex5 mice. A) Schematic representation of titin carboxy-terminal region from exon Mex1 to Mex6, and regions encoded. M = Ig domains, IS = unique insertion sequence, Mex = exons. The penultimate Mex5 exon has been deleted in the model. B), C), D), E) Evolution of left ventricle diameter in diastole, left ventricle diameter in systole, fraction shortening and left ventricle mass compared to total body mass at two, four and thirteen months in WT and Δ Mex5. F) Representative Pressure-Volume Loops from WT and Δ Mex5 mice at four months. G), H), I) Ejection fraction, end-systolic elastance (Ees) and diastolic stiffness coefficient β of EDPVR from WT and Δ Mex5 mice at four months. Unpaired Mann-Whitney test was used to compare groups, * $P \leq 0.05$, ** $P \leq 0.01$

Figure 2: Cardiac structural characterization of Δ Mex5 mice. A) Representative Light Sheet Imaging images of hearts from four-month-old mice; from left to right: three-dimensional, longitudinal, sagittal, or transversal view. Scale bars, 1000 μ m. B) Quantification of the total heart volume. C) Quantification of the total cavities volume. D) Histological sections of heart from WT and Δ Mex5 mice by hematoxylin phloxin saffron (HPS) and Sirius Red at four months. Scale bars, 10 μ m. E) Sirius red staining quantification at four months. Amount of staining is quantified in percentage of the total tissue section (mean \pm SEM). F), G), H), I), J) qPCR results for fibrosis and heart damage makers (Colla1, Nppa, Myh7, Myh6) relative expression in WT and Δ Mex5 mice hearts at four months. K) Representative longitudinal electron microscopy images of papillary muscle from WT (i) and Δ Mex5 (ii-iv) mice at four months. Irregular Z-disc (Z) and M-line (M) (ii), abnormal

condensed mitochondria (Mit), autophagic vacuoles (white arrows), lipids (L) (iii), inflammatory infiltrate cells (red arrows) and collagen fibrils (Coll) (iv). Scale bars, 500nm.

L) Mitochondria quantitative analyses: area, fractional area, perimeter, Feret's diameter, roundness, aspect ratio, circularity and solidity. Values are expressed as the mean \pm SEM (WT: n= 270 and Δ Mex5: n= 285 measurements). **M)** Sarcomere length measurement (mean \pm SEM). Unpaired Mann-Whitney test was used to compare groups, * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. and **** $P \leq 0.0001$.

Figure 3: Sarcomeric characterization of Δ Mex5 mice. **A)** qPCR results for titin relative expression in WT and Δ Mex5 mice hearts at four months. **B)** WB analysis of full-length Titin terminal domains on cardiac samples from WT and Δ Mex5 mice at four months. Z1Z2 situated at the amino-terminal region of titin is used for normalization. Full-length titin predicted molecular weight is around 3700 kDa. **C)** Quantification of the WB analysis for titin M-line domains normalized to Z1Z2. Relative expression of Δ Mex5/WT ratio. **D)** M-line immunostaining in WT and Δ Mex5 mice hearts samples at four months: M8M9, is7 and M10 antibodies. Scale bar, 5 μ m. **E)** Example of sarcomeres from WT and Δ Mex5 mice heart at four months labeled with titin is7 and M8M9 antibodies (arrowheads) by immunoelectron microscopy. Scale bar, 500 nm. **F)** M8M9 domain epitopes distance across the M-line (mean \pm SEM) corrected for shrinkage with A-band. Unpaired Mann-Whitney test were used to compare groups, $P \leq 0.05$, ** $P \leq 0.01$ and **** $P \leq 0.0001$

Figure 4: RNAseq analysis of Δ Mex5 heart. **A)** Volcano plot representation of RNAseq analysis of Δ Mex5 mice versus WT hearts at four months. Red dots: 25 more up-regulated genes. Blue dots: 25 more down-regulated genes. Green dots: genes of interest. **B)** Volcano

plot representation of Gene Set Enrichment Analysis of GO biological process ontology. **C)**, **D)** Gene Set Enrichment Analysis (GSEA) enrichment score curves of GO leukocyte migration and GO mitochondrial respiratory chain complex assembly. **E)** Toxicity functions significantly activated in the heart of the Δ Mex5 model.

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Figure 5: Analysis of interesting dysregulated genes. **A), B), C), D), E), F), G), H)** qPCR results for Cmya5, Obscn, Ryr2, Atp2a2, Pln, Synm, Cacna1c and Jhp2 and relative expression in WT and Δ Mex5 mice hearts at four months. **I)** Western blot analysis of Ryr2, Cmya5, Obscn, Jph2, Serca2 and Dhpr on cardiac samples from WT and Δ Mex5 mice at four months. Gapdh was used for normalization (representative band showed). **J), K), L), M), N), O)** Quantification of the western blot analysis for Ryr2, Cmya5, Obscn, Jph2, Serca2 and Dhpr. Unpaired Mann-Whitney test were used to compare groups, $P \leq 0.05$.

Figure 6: Immunofluorescence staining. **A), B)** Immunostaining of M10 partners: Cmya5 and Obscn in WT and Δ Mex5 mice hearts at four months. Merge with alpha-actinin staining. Sale bar, 5 μ m. **C), D)** Immunostaining of Ryr2 and Dhpr in WT and Δ Mex5 mice hearts at four months. Merge with alpha-actinin staining. Sale bar, 5 μ m.

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