Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

OTUD6B governs cardiomyocyte cell-cycle progression and ventricular chamber development

Huabo Su

hsu@augusta.edu

Augusta University https://orcid.org/0000-0002-8475-2685

Yilang Li

Augusta University

Wenjuan Wang

Augusta University

Josue Zambrano-Carrasco

Augusta University

Jianqiu Zou

Augusta University

Yang Cui

Augusta University

Juan Ayala

Augusta University

Ning Hou

Guangzhou Medical University https://orcid.org/0000-0002-7745-4946

Jinbao Liu

Guangzhou Medical University https://orcid.org/0000-0003-2220-2407

Jie Li

Augusta University

Article

Keywords:

Posted Date: September 15th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10780838/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations:
There is no duality of interest

Figures are available in the Supplementary Files section.

OTUD6B governs cardiomyocyte cell-cycle progression and ventricular chamber development

Yilang Li, PhD[1,2*], Wenjuan Wang, MD, PhD[1,3,4*], Josue Zambrano-Carrasco[1], Jianqiu Zou, PhD[1], Yang Cui[1], Juan Ayala[1], Ning Hou, MD, PhD[2], Jinbao Liu, MD, PhD[3]†, Jie Li, MD, PhD[1,5]†, Huabo Su, PhD[1,6]†

[1]Vascular Biology Center, Medical College of Georgia, Augusta University, Augusta, Georgia 30912, United States

[2]Guangzhou Municipal and Guangdong Provincial Key Laboratory of Molecular Target & Clinical Pharmacology, the NMPA and State Key Laboratory of Respiratory Disease, School of Pharmaceutical Sciences, Guangzhou Medical University, Guangzhou, Guangdong 511436, China

[3]Guangzhou Municipal and Guangdong Provincial Key Laboratory of Protein Modification and Degradation, State Key Laboratory of Respiratory Disease, School of Basic Medical Sciences, Affiliated Cancer Hospital and Institute of Guangzhou Medical University, Guangzhou 511436, China

[4]Department of Cardiology, Shandong Key Laboratory of Digital Diagnosis and Treatment of Thoracic Oncology, The First Affiliated Hospital of Shandong First Medical University & Shandong Provincial Qianfoshan Hospital, Jinan 250014, China

[5]Department of Medicine-Cardiology, Medical College of Georgia, Augusta University, Augusta, Georgia 30912, United States

[6]Department of Pharmacology and Toxicology, Medical College of Georgia, Augusta University, Augusta, Georgia 30912, United States

*These authors contributed equally.

Running title: Otud6b knockout results in neonatal Lethality

Correspondence to Huabo Su, Jie Li, and Jinbao Liu.

Dr. Huabo Su, PhD, Vascular Biology Center, Medical College of Georgia, Augusta University, 1460 Laney Walker Blvd, Augusta, GA 30912, United States. Tel.: 706-721-9152; FAX: 706-721-9799, e-mail: hsu@augusta.edu

Dr. Jie Li, MD, PhD, Vascular Biology Center, Medical College of Georgia, Augusta University, 1460 Laney Walker Blvd, Augusta, GA 30912, United States. Tel.: 706-721-9414; FAX: 706-721-9799, e-mail: jieli@augusta.edu

Dr. Jinbao Liu, MD, PhD, Guangzhou Municipal and Guangdong Provincial Key Laboratory of Protein Modification and Degradation, State Key Laboratory of Respiratory Disease, School of Basic Medical Sciences, Affiliated Cancer Hospital and Institute of Guangzhou Medical University, Guangzhou 511436, China. e-mail: jliu@gzhmu.edu.cn

Abstract

Deubiquitinases (DUBs) regulate protein homeostasis and virtually all ubiquitin-dependent cellular processes by reversing ubiquitination. Despite their biological importance, the roles of DUBs in cardiac development remain largely unexplored. OTUD6B, a member of the ovarian tumor (OTU) family of DUBs, has emerged as a disease-associated gene, with biallelic loss-of-function variants linked to a multisystem developmental syndrome frequently accompanied by congenital heart defects (CHDs). However, the physiological function of OTUD6B in cardiac development and the mechanisms underlying OTUD6B-associated CHDs remain unknown.

Here, we identify OTUD6B as an essential regulator of ventricular chamber development and perinatal survival using complementary hypomorphic, knockout, and disease-associated knock-in mouse models. OTUD6B was ubiquitously expressed during embryogenesis. Hypomorphic *Otud6b*$^{neo/neo}$ mice, expressing approximately 10% of normal OTUD6B protein, were viable and exhibited no overt abnormalities up to 24 weeks of age. In contrast, complete loss of OTUD6B resulted in growth retardation, ventricular hypoplasia, ventricular septal defects, cardiac dysfunction, and uniform perinatal lethality. Transcriptomic profiling of embryonic hearts revealed dysregulation of pathways governing cell-cycle progression, fatty acid metabolism, myogenesis, and cardiomyocyte maturation. Mechanistically, the cardiac developmental defects were attributable to impaired cardiomyocyte cell-cycle progression, characterized by reduced expression of Cyclin D3, Cyclin E1, Cyclin A2, and CDK2, leading to S-phase arrest, diminished cardiomyocyte proliferation, and impaired binucleation. Importantly, mice harboring the pathogenic *Otud6b*$^{R116*/R116*}$ knock-in mutation faithfully recapitulated the myocardial

hypoplasia, ventricular septal defects, and perinatal lethality observed in knockout mice, mirroring the cardiac manifestations of patients carrying the corresponding OTUD6B-R115* mutation.

Together, these findings establish OTUD6B as one of the few deubiquitinases with an indispensable role in mammalian cardiac development and provide a mechanistic framework linking OTUD6B deficiency to congenital heart disease. Our study identifies OTUD6B-mediated deubiquitination as a critical regulator of cardiomyocyte cell-cycle progression and ventricular morphogenesis, highlighting the ubiquitin system as a fundamental determinant of normal heart development.

Keywords: OTUD6B, Cardiac development, Cell cycle, Deubiquitinase

Nonstandard Abbreviations and Acronyms

CDK	Cyclin-dependent kinase
CHDs	Congenital heart defects
CMs	Cardiomyocytes
DEGs	Differentially expressed genes
DUBs	Deubiquitinases
E	Embryonic day
EF	Ejection fraction
FS	Fraction shortening
GSEA	Gene set enrichment analysis
LV	Left ventricle
OTU	Ovarian tumor family
PCA	Principal component analysis
PSWT	Post-systolic wall thickening
RNA-seq	RNA-sequencing
RV	Right ventricle
SV	Stroke volume
VSD	Ventricular septal defect

Introduction

The heart is the first functional organ to develop during embryogenesis and is essential for establishing the embryonic circulation required for subsequent organogenesis [1,2]. In mice, cardiac development begins at embryonic day (E) 7.5 with formation of the cardiac crescent, followed by heart tube looping, chamber morphogenesis, trabeculation, and septation, culminating in the establishment of a four-chambered heart by mid-gestation [1]. These highly coordinated morphogenetic events require precise temporal and spatial regulation of cardiomyocyte differentiation, proliferation, migration, and maturation [3-6]. Although cardiac development is known to be orchestrated by complex networks involving lineage-specific transcription factors, chromatin remodelers and epigenetic regulators that govern cell fate determination and morphogenesis [7], considerably less attention has been paid to how post-translational mechanisms, particularly ubiquitin-dependent signaling, regulate these developmental processes.

Deubiquitinases (DUBs) constitute a family of approximately 100 enzymes that cleave ubiquitin from conjugated substrates or within the ubiquitin chain to maintain ubiquitin homeostasis and remodel ubiquitin signaling [8,9]. Through regulating protein stability, localization, and activity, DUBs govern virtually every aspect of cell physiology [10,11]. Consequently, dysregulation of DUBs has been implicated in a broad spectrum of human diseases, including cancer, neurodegenerative diseases, immune disorders, and cardiovascular diseases [8,12-14], highlighting DUBs as both disease genes and emerging therapeutic targets.

OTUD6B is an evolutionarily conserved OTU family deubiquitinase that is mostly studied in the context of tumorigenesis. OTUD6B promotes breast cancer cell division by

deubiquitinating KIFC1 [15], stabilizes the RNA-binding protein LIN28B to accelerate cell-cycle progression in multiple myeloma [6], and suppresses esophageal squamous cell carcinoma through stabilization of the E3 ubiquitin ligase β-TRCP [16]. In addition, OTUD6B also exerts deubiquitinase-independent functions by interacting with the von Hippel–Lindau (pVHL) complex to regulate protein stability in hepatocellular carcinoma [17-19]. Notably, human genetic studies have recently identified OTUD6B as a disease gene underlying a multisystem developmental syndrome. Biallelic pathogenic variants, including the recurrent nonsense mutation R115*, frameshift mutation L127Rfs*8, missense mutation Y186C, and splice-site variants, cause developmental delay, intellectual disability, and characteristic craniofacial abnormalities [20]. Importantly, congenital heart defects (CHDs), including atrial septal defects and ventricular septal defects, have been reported in multiple affected individuals, implicating OTUD6B in cardiac morphogenesis. However, the physiological role of OTUD6B in heart development and the mechanisms by which OTUD6B deficiency causes CHDs remain completely unknown.

In the present study, we addressed this question using complementary hypomorphic, constitutive knockout, and patient-relevant knock-in mouse models of Otud6b deficiency. We demonstrate that OTUD6B is indispensable for ventricular chamber morphogenesis and perinatal survival. Complete loss of OTUD6B causes ventricular hypoplasia, ventricular septal defects, cardiac dysfunction, and perinatal lethality, whereas mice carrying the pathogenic Otud6b R116* mutation faithfully recapitulate these developmental abnormalities. Mechanistically, OTUD6B deficiency disrupts cardiomyocyte cell-cycle progression by suppressing key Cyclin-CDK regulators, leading

to S-phase arrest, reduced cardiomyocyte proliferation, and impaired binucleation. Collectively, our findings identify OTUD6B as one of the first OTU family deubiquitinases required for normal mammalian cardiac development and establish a mechanistic link between OTUD6B deficiency and congenital heart disease.

Results

Complete loss of OTUD6B, but not OTUD6B hypomorphism, causes neonatal lethality

To investigate the physiological function of Otud6b *in vivo*, we obtained gene-trapped Otud6b (*Otud6b*$^{neo/neo}$) mice from the International Mouse Phenotyping Consortium. In this allele, a LacZ-Neo cassette is inserted between exons 3 and 4, permitting visualization of Otud6b expression by LacZ staining while disrupting endogenous Otud6b transcription (Figure 1A). This gene-trap allele reduced Otud6b expression by approximately 90% at both the mRNA and protein levels in the heart and other tissues in *Otud6b*$^{neo/neo}$ mice, indicating that it represents a hypomorphic rather than a null allele (Figure 1B–E). LacZ staining revealed ubiquitous Otud6b expression in E14.5 embryos and multiple adult tissues (Figures 1F and S1A). Despite the marked reduction of OTUD6B expression, *Otud6b*$^{neo/neo}$ mice generated from heterozygous intercrosses were viable, displayed normal cardiac morphology, function, and heart size at 6 months of age, and survived without premature lethality for at least 7 months (Figures 1G-I and S2B-C). These findings indicate that partial loss of OTUD6B is well tolerated and is dispensable for embryonic development and postnatal cardiac homeostasis.

These observations were inconsistent with a previous report describing VSDs and perinatal lethality in mice carrying Reporter-tagged deletion allele [20]. To definitively determine the requirement for OTUD6B during cardiac development, we generated a constitutive Otud6b knockout (*Otud6b*$^{-/-}$) by sequentially crossing *Otud6b*$^{neo/+}$ mice with FLP and CMV-Cre deleter strains (Figure 2A). OTUD6B protein was virtually undetectable in *Otud6b*$^{-/-}$ embryonic hearts, confirming successful deletion (Figure 2B-C). Although *Otud6b*$^{-/-}$ embryos survived throughout gestation, they exhibited progressive growth retardation during late embryogenesis, as reflected by reduced crown-rump length (Figure 2F–G). Strikingly, all *Otud6b*$^{-/-}$ neonates died within 1–2 hours after natural or cesarean delivery and displayed severe cyanosis (Figure 2E). Histological analysis revealed ventricular septal defects in E14.5 and P0 hearts (Figure 2H). In addition, P0, but not E18.5, lungs exhibited collapsed alveoli, consistent with impaired postnatal lung inflation (Figure 2I). Together, these findings demonstrate that complete, but not hypomorphic, loss of OTUD6B causes neonatal lethality associated with severe cardiac malformation and respiratory insufficiency.

Complete loss of OTUD6B impairs ventricular chamber development

Histological analysis revealed progressive myocardial hypoplasia in *Otud6b*$^{-/-}$ hearts. Left ventricular wall thinning was evident as early as E14.5 and progressed to severe thinning of the left ventricle (LV), right ventricle (RV), and interventricular septum by E18.5 (Figure 3A–C). Consistent with these findings, immunostaining demonstrated marked reductions in the thickness of both the compact myocardium and trabecular layer in the LV and RV of *Otud6b*$^{-/-}$ hearts (Figure 3D-E). Echocardiographic analysis of E19.5 embryos further revealed significantly reduced stroke volume (SV) and post-systolic wall thickening

(PSWT), indicative of impaired cardiac function (Figures 3F-G, and S2). Together, these findings demonstrate that OTUD6B is essential for ventricular chamber morphogenesis and that its loss leads to progressive myocardial hypoplasia accompanied by fetal cardiac dysfunction.

Transcriptomic alterations in OTUD6B-deficient hearts identify impaired cell-cycle regulation as a dominant molecular signature

To gain mechanistic insights into how OTUD6B regulates cardiac development, we performed bulk RNA sequencing (RNA-seq) of hearts isolated from E14.5 *Otud6b*$^{+/+}$ (WT), *Otud6b*$^{+/-}$ (HET) and *Otud6b*$^{-/-}$ (gKO) embryos. Principal component analysis (PCA) revealed a clear separation of gKO hearts from WT and HET hearts, whereas WT and HET samples clustered closely together (Figure 4A). Consistent with the absence of overt cardiac abnormalities in heterozygous embryos, only nine differentially expressed genes (DEGs, |fold change| > 1.5, adjusted $P < 0.05$) were identified between WT and HET hearts (Figure S3A). Therefore, WT and HET samples were combined as a single control group for subsequent analyses.

Comparative transcriptomic analysis identified 633 downregulated and 59 upregulated genes in gKO hearts (|fold change| > 1.5, adjusted $P < 0.05$) (Figure 4B). Gene Set Enrichment Analysis (GSEA) revealed significant enrichment of pathways related to oxidative phosphorylation, fatty acid metabolism, DNA repair, apoptosis, hypoxia, E2F targets, G2/M checkpoint, and p53 signaling among upregulated genes, whereas pathways associated with mitotic spindle were significantly suppressed (Figure 4C-D).

Notably, multiple enriched pathways converged on the regulation of cell-cycle progression and mitosis.

Consistently, Gene Ontology (GO) analysis showed that genes involved in chromosome segregation, G1/S transition, and cell-cycle progression were among the most significantly dysregulated biological processes (Figure 4E–G). Heatmap analysis further demonstrated coordinated dysregulation of numerous cell-cycle regulators in gKO hearts (Figure 4H). Together, these transcriptomic analyses identify dysregulated cell-cycle progression as a predominant molecular consequence of OTUD6B deficiency.

OTUD6B deficiency disrupts cardiomyocyte cell-cycle progression

To assess cardiomyocyte cell-cycle progression, E14.5-E18.5 embryonic hearts were immunostained for Ki67, EdU, and Aurora A/B [21,22], to identify proliferating cells, cells undergoing DNA synthesis, and cells entering G2/M, respectively (Figure 5A). Although the proportion of Ki67-positive cardiomyocytes was reduced in *Otud6b*$^{-/-}$ hearts at E14.5, it became significantly increased at E16.5 and E18.5 (Figure 5B-C). Likewise, EdU incorporation was consistently elevated in *Otud6b*$^{-/-}$ hearts, indicating enhanced DNA synthesis (Figure 5D-E). In contrast, Aurora B-positive cardiomyocytes, indicative of cells entering G2/M, were markedly reduced in knockout embryonic hearts. These data suggest that OTUD6B-deficient cardiomyocytes initiate DNA replication but fail to complete cell-cycle progression. To directly assess cell-cycle distribution, embryonic cardiomyocytes were isolated from E18.5 hearts and analyzed by flow cytometry. Compared with controls, *Otud6b*$^{-/-}$ cardiomyocytes accumulated in G0/G1 and S phases, whereas the proportion of cells in G2/M was significantly reduced (Figure 5F-G). Together,

these data indicate that loss of OTUD6B impairs progression through S phase and delays entry into mitosis.

To determine whether this defect is cardiomyocyte autonomous, Otud6b was knocked down in neonatal rat ventricular cardiomyocytes (NRVCs) using siRNA. Similar to the *in vivo* findings, OTUD6B knockdown increased Ki67 and EdU labeling but markedly reduced Aurora A-positive cardiomyocytes (Figure 6A-D). Flow cytometric analysis likewise demonstrated accumulation of cells in G0/G1 and S phases, accompanied by reduced binucleated cardiomyocytes (Figure 6E-G). Collectively, these findings establish that OTUD6B is required for efficient cardiomyocyte cell-cycle progression and successful completion of cell division.

OTUD6B maintains the cardiomyocyte cell-cycle machinery

To investigate the molecular basis, we examined expressions of key cell-cycle regulators. Immunoblotting demonstrated that OTUD6B knockdown markedly reduced expression of Cyclin D3, Cyclin E1, Cyclin A2, and CDK2, all of which are essential for G1/S transition and S-phase progression (Figure 6H-I). Consistent with activation of cell-cycle inhibitory pathways, p53 protein expression was significantly increased following OTUD6B depletion (Figure 6J). Together our findings from RNAseq (Figure 4H), these findings indicate that OTUD6B preserves cardiomyocyte proliferative capacity by maintaining the Cyclin-CDK machinery while suppressing cell-cycle inhibitory signaling.

The OTUD6B R116* variant leads to perinatal death and recapitulates the cardiac phenotype of Otud6b null mice

Among the five reported individuals carrying the biallelic OTUD6B nonsense mutation (R115*), two presented with congenital heart defects [20]. To investigate the pathogenic significance of this variant, we generated a knock-in (KI) mouse line harboring the corresponding Otud6b R116* mutation (equivalent to human R115*) using CRISPR/Cas9 (Figures 7A and S6A). Western blot analysis confirmed the near-complete loss of full-length OTUD6B protein with no detectable truncated protein, consistent with premature termination of translation in *Otud6b*$^{KI/KI}$ mice (Figure 7B).

Similar to *Otud6b*$^{-/-}$ mice, *Otud6b*$^{KI/KI}$ mice exhibited developmental delay and perinatal lethality (Figure 7C–E). Histological analysis further revealed marked ventricular wall thinning and VSD (Figures 7F–G and S7B–C). These structural abnormalities were accompanied by an increased number of Ki67- and EdU-positive cardiomyocytes at E18.5 (Figure 7H), indicating dysregulated cardiomyocyte cell-cycle activity during late embryonic development. Collectively, these findings demonstrate that the OTUD6B R116* loss-of-function variant phenocopies complete Otud6b deficiency, establishing this disease-associated mutation as pathogenic and further supporting an essential role for OTUD6B in regulating cardiomyocyte cell-cycle progression and ventricular chamber morphogenesis.

Discussion

In this study, we identify OTUD6B as a previously unrecognized regulator of mammalian cardiac development. Using complementary hypomorphic, constitutive knockout, and patient-derived knock-in mouse models, we demonstrate that OTUD6B is indispensable for ventricular chamber morphogenesis and perinatal survival. While partial reduction of

OTUD6B expression is well tolerated, complete loss of OTUD6B results in ventricular hypoplasia, ventricular septal defects, impaired cardiac function, and neonatal lethality. Mechanistically, OTUD6B deficiency disrupts cardiomyocyte cell-cycle progression by suppressing the Cyclin-CDK machinery, leading to defective S-phase progression, impaired mitotic entry, and reduced cardiomyocyte proliferation. Importantly, mice harboring the pathogenic OTUD6B R116* mutation faithfully recapitulate the developmental and cardiac abnormalities observed in null mice, providing genetic and mechanistic evidence linking OTUD6B deficiency to congenital heart disease. Together, these findings identify OTUD6B as a previously unrecognized developmental deubiquitinase that couples ubiquitin signaling to cardiomyocyte proliferation and ventricular morphogenesis.

Our findings establish OTUD6B as one of the few DUBs with an indispensable role in mammalian cardiac development. Although DUBs have emerged as important regulators of cardiac remodeling in pathological conditions, including cardiac hypertrophy, ischemic injury, and heart failure [23-26], their physiological functions during embryonic heart development remain largely unexplored. Compared with E3 ubiquitin ligases, whose developmental functions have been extensively characterized [27], relatively few DUBs have been genetically implicated in mammalian organogenesis. Human genetic studies previously identified biallelic OTUD6B mutations as the cause of a multisystem developmental syndrome in which a subset of affected individuals presents with congenital heart defects, including ventricular septal defects [20]. However, whether OTUD6B is required for cardiac development and how its deficiency causes these abnormalities to have remained unknown. By integrating complementary hypomorphic

(*Otud6b*$^{neo/neo}$), constitutive knockout (*Otud6b*$^{-/-}$), and patient-derived knock-in (*Otud6b*$^{KI/KI}$) mouse models, we demonstrate that OTUD6B dosage is a critical determinant of cardiac developmental outcome. Whereas hypomorphic mice retaining approximately 10% of normal OTUD6B expression exhibited normal cardiac development, function, and survival, complete loss of OTUD6B caused progressive ventricular myocardial hypoplasia, ventricular septal defects, fetal cardiac dysfunction, and uniform neonatal lethality. Importantly, *Otud6b*$^{KI/KI}$ mice faithfully phenocopied the null mice, providing compelling genetic evidence that pathogenic OTUD6B R115* mutations directly underline the congenital cardiac abnormalities observed in affected individuals. Notably, no overt cardiac abnormalities were detected before E14.5, indicating that OTUD6B is dispensable for early cardiogenic events, including cardiac chamber specification and outflow tract formation, but becomes essential during the rapid ventricular growth and maturation that occurs in late gestation. This stage-specific requirement is consistent with the temporal expansion of the ventricular myocardium and suggests that OTUD6B primarily regulates cardiomyocyte proliferative capacity rather than early cardiac lineage specification or morphogenesis. Although severe ventricular hypoplasia and impaired fetal cardiac function likely represent the primary cause of neonatal death, the presence of collapsed alveoli after birth suggests that defective cardiopulmonary adaptation or respiratory insufficiency may also contribute to the lethal phenotype. Collectively, our study identifies OTUD6B as a previously unrecognized post-translational regulator of ventricular chamber morphogenesis and provides the first mechanistic framework linking OTUD6B deficiency to congenital heart disease.

Mechanistically, our findings identify OTUD6B as a critical regulator of cardiomyocyte cell-cycle progression during ventricular chamber maturation. During mid-to-late gestation, rapid thickening of the ventricular wall is achieved through coordinated expansion of both the compact myocardium and trabecular layer, a process that depends on robust cardiomyocyte proliferation before the perinatal transition to cell-cycle withdrawal and terminal differentiation [28-30]. Our transcriptomic analysis identified cell-cycle regulation as the most profoundly affected pathway in OTUD6B-deficient hearts, whereas immunostaining, flow cytometry, and biochemical analyses demonstrated defective cell-cycle progression characterized by accumulation of cardiomyocytes in G0/G1 and S phases, reduced G2/M entry, diminished mitotic activity, and impaired binucleation. These data collectively support dysregulated cell-cycle progression as the primary mechanism underlying ventricular myocardial hypoplasia. Furthermore, similar defects were reproduced in OTUD6B-silenced neonatal rat ventricular cardiomyocytes, indicating that the proliferative defects are, at least in part, cardiomyocyte autonomous.

Our findings are consistent with previous studies demonstrating that OTUD6B promotes cell proliferation in several cancer cell types. OTUD6B has been reported to facilitate G1/S transition by stabilizing LIN28B and enhancing MYC signaling [6], regulate Cyclin D1 translation downstream of mTORC1 [31], and promote mitotic progression through stabilization of KIFC1 [15]. Extending these observations to a physiological developmental context, we found that OTUD6B deficiency resulted in coordinated downregulation of multiple cell-cycle regulators, including Cyclin D3, Cyclin E1, Cyclin A2, and CDK2, together with increased expression of the cell-cycle inhibitor p53 [32]. Interestingly, unlike previous studies that primarily implicated Cyclin D1 or MYC signaling in transformed cells,

our findings identify coordinated suppression of multiple Cyclin-CDK complexes in embryonic cardiomyocytes, suggesting that OTUD6B regulates proliferative capacity through cell type-specific mechanisms. Notably, the altered protein abundance of these regulators was not accompanied by corresponding changes in transcript levels, supporting a predominantly post-translational mechanism of regulation. Whether these proteins represent direct OTUD6B substrates remains unknown. Identification of the cardiac substrates of OTUD6B will therefore be an important next step toward understanding how deubiquitination controls cardiomyocyte proliferation and ventricular morphogenesis.

Although congenital heart defects have been reported in patients carrying biallelic OTUD6B mutations, the molecular basis underlying these cardiac manifestations has remained elusive. By demonstrating that the patient-derived R116* mutation faithfully phenocopies complete OTUD6B deficiency and disrupts ventricular morphogenesis through impaired cardiomyocyte proliferation, our study provides a mechanistic explanation for the cardiac defects associated with OTUD6B syndrome. More broadly, these findings suggest that congenital heart disease may represent an underappreciated consequence of impaired ubiquitin homeostasis during embryogenesis.

Finally, although our *in vitro* studies strongly support a cardiomyocyte-intrinsic function of OTUD6B, normal ventricular morphogenesis is coordinated through reciprocal signaling among cardiomyocytes, endocardial cells, epicardial cells, fibroblasts, and other cardiac-resident cell populations [28,33]. Future studies using lineage-specific *Otud6b* deletion models will therefore be required to define the cell type-specific functions of OTUD6B during heart development and to determine how OTUD6B-dependent ubiquitin signaling

is integrated with established developmental pathways that govern ventricular growth and maturation.

Materials and Methods

Animals

All animal experiments were approved by the Augusta University Institutional Animal Care and Use Committee. A transgenic mouse line carrying Otud6b "knockout-first" allele (*Otud6b*$^{neo/neo}$, MGI:1919451, International Mouse Phenotyping Consortium [34]) was crossed with FLP mice (Jackson Laboratory, strain #005703) and CMV^{Cre} mice (Jackson Laboratory, strain #006054) to create and *Otud6b*$^{-/-}$ mice. These mice were maintained in the C57BL/6J inbred background for our studies.

Genomic DNA from the adults and embryos were extracted from their tail tips and genotyped by PCR as previously described. Briefly, a tail sample was digested in Proteinase K solution at 55 °C overnight and lysate boiled for 15 min, centrifuged at 1200 rpm, and subjected to PCR using the following conditions: 94 °C for 5 min; followed by 34 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 45 s; and final extension at 72 °C for 5 min, then held at 4 °C. PCR products were electrophoresed on 2% agarose gels.

The nucleotide sequences of the PCR primers for genotyping were listed in Supplemental data.

Embryo isolation

Female mice crossed with their mating partners were checked for plug formation. Pregnant females were euthanized by euthanasia, and the embryos at different embryonic ages were carefully dissected from the uterus and washed in cold PBS. For immunostaining and the embryos were fixed in cold 4% paraformaldehyde overnight, 70% ethanol overnight at 4°C, and subjected to paraffin processing and embedding for paraffin sections at thickness of 5 μm.

X-Gal staining

All tissues were harvested from 8-week-old *Otud6b*$^{neo/neo}$ and *Otud6b*$^{+/+}$ mice. Mice were anesthetized with pentobarbital and then perfused with 4% PFA. The tissues were immediately dissected and placed in cold PBS, followed by fixation in 2% PFA, 0.25% glutaraldehyde, and 0.01% NP40 in PBS for 2 h with agitation at 4 °C. The tissues were then washed in rinsed buffer (2mM $MgCl_2$, 0.02% NP40, and 0.01% Na-deoxycholate in PBS) and processed for whole-mount X-Gal staining (30 mM $K_3Fe(CN)_6$, 30 mM $K_4Fe(CN)_6{\cdot}3H_2O$, 2 mM $MgCl_2$, 0.01% Na-deoxycholate, 0.02% NP40, and 1 mg/mL 5-bromo-4-chloro- 3-indolyl β-D-galactopyranoside) at 37 °C for 6–8 h or until the desired intensity was observed. Stained tissues were then washed in PBS, postfixed in 4% PFA, and stored in 70% glycerol at 4°C.

The mouse embryos were harvested at E14.5 and cut along the longitudinal axis. The staining procedure is the same as described previously.

Echocardiography

Mice were anesthetized by inhalation of isoflurane (2.5% for induction and 1.5-2% for maintenance) via a nose cone. The adequacy of anesthesia was monitored by toe pinch.

The echocardiography of conscious adult was performed by gently securing the mice on the station with tapes. Images were recorded using a VEVO 3100 echocardiography system with a 30MHz transducer (Visual Sonics). The LV morphometric and functional parameters were analyzed off-line using VEVO LAB software (FUJIFILM Visual Sonics).

RNA Isolation and Real-Time PCR

Isolation of total RNA by TRIzol reagent (Thermo Fisher Scientific) and reverse transcription into single-stranded cDNA by RevertAid RT Reverse Transcription Kit (Thermo Fisher Scientific) was performed as previously described [35]. For quantitative real-time PCR analysis, an equal amount of cDNA was mixed with Power SYBR Green PCR master mix (Thermo Fisher Scientific) and real-time PCR was performed using a QuantStudio 3 Real-Time PCR System (Applied Biosystems). All primers used for qPCR are listed in Supplemental data. The 2ΔΔCt method was used to analyze the relative changes in gene expression normalized against mouse *Hprt* expression.

RNA-seq analysis

Mouse embryonic ventricles were minced and treated with RNAlater (Thermo Fisher Scientific) according to the manufacturer's protocol at −80°C. The isolated RNA was subjected to RNA-sequencing analysis performed by Novogene Corporation Inc. (Sacramento, CA, USA). Genotyping information of the mice was pre-coded and blind during RNA-seq analysis and data analysis.

Mouse embryonic cardiomyocytes isolation

Pregnant mice were anesthetized with isoflurane, and embryonic hearts were dissected individually. The atria were removed in PBS, and the ventricles were torn and digested with 0.25% trypsin (containing EDTA, Gibco, USA) in 37 ºC water bath with flicking every 10-15 min for a total of three times to gently disperse the cells. When no obvious tissue was observed, the digestion process was stopped with DMEM containing 10% FBS. The cell suspension was filtered with a 100-μm strainer and centrifuged at 300 ×g for 5 min. The supernatant was aspirated carefully. After removing the red blood cells, the CMs were separated using the gradient settlement method and harvested [36].

Culture of neonatal rat ventricular cardiomyocytes

Neonatal rat ventricular cardiomyocytes (NRVCs) were isolated using Neonatal Cardiomyocyte Isolation System (Worthington Biochemicals, Cat#LK003245) following the manufacturer's protocol [35]. Briefly, hearts from neonatal rats or mice were excised with the removal of the atrium, minced into ~1 mm diameter, and digested in 0.05% Trypsin in 4°C overnight on a slow-speed rocker. The next day, the heart tissues were washed 4 times and subjected to collagenase digestion at 37°C for 40min. CMs were next separated by pipet flushing and through 70-μm filter, preplated for 2 hour to exclude cardiac fibroblasts, and finally plated in 60-mm dishes in DMEM containing 10% FBS, 1% P/S and for one days in 37°C incubator with 5% CO2 prior to use for subsequent experiments.

For genetic knockdown experiments, NRVCs were transfected with siRNA of Otud6b using Lipofectamine RNAiMAX (Thermo Fisher Scientific) following the manufacturer's protocol. The small interference RNA (siRNA) used in this study were designed and synthesized by Integrated DNA Technologies (IDT, USA).

Histology and immunohistochemistry analysis

For histology analysis, 5-μm paraffin-embedded sections were subjected to hematoxylin-eosin (H&E) staining. For immunohistochemistry analysis, these sections were incubated in preheated sodium citrate buffer (pH 6.0, 98 °C) for 10 minutes with the PT Link system (Dako). After pre-incubation with 10% non-immune goat serum (Thermo Fisher Scientific) to prevent non-specific binding, tissue sections were incubated with primary antibodies (listed in Supplemental data) at 4°C overnight and subsequently with appropriate AlexaFluor conjugated secondary antibodies (Thermo Fisher Scientific) for 1 hour at room temperature. Finally, sections were stained with DAPI (Sigma) and mounted in VECTASHIELD antifade mounting medium (Vector Laboratories). Images were captured with Leica STELLARIS Confocal Microscope (Leica).

EdU labeling

EdU was intraperitoneally injected into pregnant mice at a dose of 20 mg/kg, 2h before embryo dissection. The embryos were fixed immediately after dissection, followed by sectioning and staining. For labeling NRVCs with EdU, cells were treated with 20 μM EdU for 2h, washed with PBS, and then fixed with 4% PFA. The Click-iT™ EdU Cell Proliferation Kit (Thermo Fisher Scientific) was used according to the manufacturer's protocol for both *in vivo* and *in vitro* experiments. After EdU assay, blocking and primary antibodies incubation were performed according to the immunostaining protocol.

Cell cycle assays

Mouse embryonic cardiomyocytes or NRVCs were harvested and fixed at -20°C for 10 minutes with 70% ethanol. According to the manufacturer's protocol (Thermo Fisher Scientific), the fixed cells were incubated with FxCycle™ PI/RNase Staining Solution (Thermo Fisher Scientific) for 15-30 min at room temperature (25°C) in the dark and analyzed by flow cytometry (NovoCyte Quanteon Flow Cytometer Systems 4 Lasers, Agilent). NovoExpress flow cytometry software (version 1.6.2, Agilent, CA, USA) was used to analyze cell cycle distribution.

Protein extraction and Western blot analysis

Protein was extracted from ventricular myocardium tissues or cultured NRVCs, concentration determined with BCA reagents (Thermo Fisher Scientific), and SDS-PAGE, immunoblotting, and densitometry analysis were performed as previously described [37]. Antibodies information was listed in the supplementary.

Quantification and statistical analysis

Statistical analyses were performed with GraphPad Prism software 10.3. All values are presented as mean ± SEM.

Acknowledgements

We gratefully acknowledge Dr. Lin Gan, Ms. Xiaoling Xie, and the Genome Editing Core at Augusta University (RRID:SCR 027269) for generating the *Otud6b*^R116*^ knock-in mouse line and for their outstanding technical expertise and support. We also acknowledge the Flow and Mass Cytometry Core Facility at the Georgia Cancer Center (RRID: SCR 025747) for their support with flow cytometry experiments.

Supplemental Materials

Figure S1–S6

Major Resources Table

Figure Legends

Figure 1. OTUD6B hypomorphism is dispensable for embryonic development and postnatal cardiac homeostasis. A. Schematic of the gene-trapped *Otud6b* allele (*Otud6b*^{neo}) and breeding strategy. **B.** qPCR analysis of *Otud6b* mRNA in 2-month-old hearts. **C-D.** Representative immunoblots (**C**) and quantification (**D**) of OTUD6B protein in 2-month-old hearts. **E.** OTUD6B immunoblots in indicated tissues from 2-month-old mice. **F.** X-gal staining of E14.5 embryos (upper) and corresponding myocardial sections (lower). **G-H.** Temporal left ventricular ejection fraction (**G**) and representative M-mode echocardiograms at 6 months of age (**H**). **I.** Kaplan-Meier survival curves. Data are presented as mean ± SEM. One-way ANOVA was used in (**B**, **D**). $^{**}P < 0.01$, $^{****}P < 0.0001$.

Figure 2. Complete loss of OTUD6B causes neonatal lethality. A. Generation of constitutive *Otud6b*$^{-/-}$ mice. **B-C.** Representative immunoblots (**B**) and quantification (**C**) of OTUD6B protein in E18.5 embryonic hearts (n=5/group). **D.** Genotype distribution of offspring from *Otud6b*$^{+/-}$ intercrosses. Numbers of individual genotypes are indicated within each bar. **E-F.** Gross morphology of neonates (**E**) and embryos at the indicated developmental stages (**F**). **G.** Quantification of embryonic body length (n=3-10/group). **H.** Representative H&E-stained embryo sections showing ventricular septal defects (arrowheads) at E14.5 and P0. **I.** Representative H&E-stained lung sections at E18.5 and P0. Data are presented as mean ± SEM. Mann–Whitney test was used in (**C**, **G**). Chi-square test was used in (**D**). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$.

Figure 3. OTUD6B is required for ventricular chamber maturation. A-B. Representative H&E-stained embryonic heart sections (**A**) and higher-magnification

views (**B**). **C.** Quantification of ventricular wall and interventricular septum thickness. **D.** Representative heart sections stained for Endomucin and cTnT at E18.5. **E.** Quantification of compact and trabecular layer thickness. **F-G.** Echocardiographic analysis of left ventricular stroke volume (**F**) and post-systolic wall thickness (**G**). Data are presented as mean ± SEM. Nested t-test was used in (**C**). Unpaired t-test was used in (**E**). Mann-Whitney test was used in (**F**, **G**). *P < 0.05, **P < 0.01, ****P < 0.0001.

Figure 4. Transcriptomic profiling identifies cell-cycle dysregulation in OTUD6B-deficient hearts. A. Principal component analysis of RNA-seq samples. **B.** Volcano plot showing differentially expressed genes in *Otud6b*$^{-/-}$ hearts compared with combined wild-type and heterozygous controls (|Fold Change| > 1.5, adjusted P < 0.05). **C.** Hallmark gene set enrichment analysis (GSEA). **D.** Enrichment plot of the mitotic spindle gene set. **E.** Gene Ontology enrichment analysis of cell-cycle-related biological processes. **F-G.** Enrichment plots of chromosome segregation (**F**) and G1/S phase transition (**G**). **H.** Heatmap showing relative expression of representative cell-cycle genes.

Figure 5. OTUD6B deficiency disrupts cardiomyocyte cell-cycle progression *in vivo*. **A.** Schematic of cell-cycle markers used in this study. **B.** Representative heart sections stained for Ki67 (green), cTnT (red), and DAPI (blue) at the indicated embryonic stages. **C.** Quantification of Ki67-positive cardiomyocytes. **D.** Representative heart sections stained for EdU (green), cTnT (red), and DAPI (blue). **E.** Quantification of EdU-positive cardiomyocytes. **F.** Representative heart sections stained for Aurora B (green), cTnT (red), and DAPI (blue) at E18.5. **G.** Quantification of Aurora B positive nuclei per 100 μm^2 of cTnT positive area. **H-I.** Representative flow cytometry plots (**H**) and quantification (**I**) of cell-cycle distribution in E18.5 embryonic cardiomyocytes. Data are presented as mean

± SEM. Nested t-test was used in (**C**, **E**). Mann-Whitney test was used in (**G**). Two-way ANOVA was used in (**I**). *P < 0.05, **P < 0.01.

Figure 6. OTUD6B knockdown impairs cardiomyocyte cell-cycle progression. Neonatal rat ventricular cardiomyocytes (NRVCs) following *Otud6b* knockdown were analyzed. **A-B.** Representative immunofluorescence images (**A**) and quantification (**B**) of Ki67- and EdU-positive NRVCs. **C-D.** Representative Aurora A immunostaining (**C**) and quantification (**D**). **E.** Quantification of binucleated cardiomyocytes. **F-G.** Representative flow cytometry plots (**F**) and quantification (**G**) of cell-cycle distribution of NRVCs. **H-I.** Representative immunoblots (**H**) and quantification (**I**) of OTUD6B and cell-cycle regulators. **J.** Representative immunoblot and quantification of p53 expression. Data are presented as mean ± SEM. Unpaired t-test was used in (**B**). Mann-Whitney test was used in (**D**, **I**, **J**). Two-way ANOVA was used in (**E**, **G**). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 7. Patient-derived OTUD6B R116* mutation recapitulates the null phenotype. **A.** Generation of *Otud6b*$^{KI/KI}$ mice. **B.** Representative immunoblots and quantification of OTUD6B protein expression in E18.5 embryonic hearts. **C.** Genotype distribution of offsprings from heterozygous intercrosses. Numbers of individual genotypes are indicated within each bar. **D.** Gross morphology of E18.5 embryos. **E.** Quantification of embryonic body length. **F.** Representative H&E-stained heart sections showing ventricular septal defects (arrowhead). **G.** Representative H&E-stained embryonic heart sections and quantification of ventricular wall and septum thickness. **H.** Representative Ki67 (upper) and EdU (lower) staining of E18.5 embryonic hearts and corresponding quantification. Data are presented as mean ± SEM. Chi-square test was used in (**C**).

Mann-Whitney test was used in (**E**). Nested t-test was used in (**G**, **H**). *P < 0.05, **P < 0.01, ****P < 0.0001.

References

1. Knutson AK, Williams AL, Boisvert WA, Shohet RV. HIF in the heart: development, metabolism, ischemia, and atherosclerosis. *J Clin Invest*. 2021;131. doi: 10.1172/jci137557
2. Zaffran S, Frasch M. Early signals in cardiac development. *Circ Res*. 2002;91:457–469. doi: 10.1161/01.res.0000034152.74523.a8
3. Pagan J, Seto T, Pagano M, Cittadini A. Role of the Ubiquitin Proteasome System in the Heart. *Circulation Research*. 2013;112:1046–1058. doi: doi:10.1161/CIRCRESAHA.112.300521
4. Rana MS, Christoffels VM, Moorman AF. A molecular and genetic outline of cardiac morphogenesis. *Acta Physiol (Oxf)*. 2013;207:588–615. doi: 10.1111/apha.12061
5. Kelly RG, Buckingham ME, Moorman AF. Heart fields and cardiac morphogenesis. *Cold Spring Harb Perspect Med*. 2014;4. doi: 10.1101/cshperspect.a015750
6. Paulmann C, Spallek R, Karpiuk O, Heider M, Schäffer I, Zecha J, Klaeger S, Walzik M, Öllinger R, Engleitner T, et al. The OTUD6B-LIN28B-MYC axis determines the proliferative state in multiple myeloma. *Embo j*. 2022;41:e110871. doi: 10.15252/embj.2022110871
7. Kathiriya IS, Nora EP, Bruneau BG. Investigating the transcriptional control of cardiovascular development. *Circ Res*. 2015;116:700–714. doi: 10.1161/CIRCRESAHA.116.302832
8. Park J, Cho J, Kim EE, Song EJ. Deubiquitinating Enzymes: A Critical Regulator of Mitosis. *Int J Mol Sci*. 2019;20. doi: 10.3390/ijms20235997
9. Clague MJ, Urbe S, Komander D. Breaking the chains: deubiquitylating enzyme specificity begets function. *Nat Rev Mol Cell Biol*. 2019;20:338–352. doi: 10.1038/s41580-019-0099-1
10. Wang X, Pattison JS, Su H. Posttranslational modification and quality control. *Circ Res*. 2013;112:367–381. doi: 10.1161/circresaha.112.268706
11. Lim KH, Song MH, Baek KH. Decision for cell fate: deubiquitinating enzymes in cell cycle checkpoint. *Cell Mol Life Sci*. 2016;73:1439–1455. doi: 10.1007/s00018-015-2129-2
12. Komander D, Clague MJ, Urbé S. Breaking the chains: structure and function of the deubiquitinases. *Nature Reviews Molecular Cell Biology*. 2009;10:550–563. doi: 10.1038/nrm2731
13. Dewson G, Eichhorn PJA, Komander D. Deubiquitinases in cancer. *Nat Rev Cancer*. 2023;23:842–862. doi: 10.1038/s41568-023-00633-y
14. Xian Y, Ye J, Tang Y, Zhang N, Peng C, Huang W, He G. Deubiquitinases as novel therapeutic targets for diseases. *MedComm (2020)*. 2024;5:e70036. doi: 10.1002/mco2.70036
15. Marotta VE, Sabat-Pospiech D, Fielding AB, Ponsford AH, Thomaz A, Querques F, Morgan MR, Prior IA, Coulson JM. OTUD6B regulates KIFC1-dependent centrosome clustering and breast cancer cell survival. *EMBO Rep*. 2025. doi: 10.1038/s44319-024-00361-w
16. Li L, Zhu R, Zhou H, Cui CP, Yu X, Liu Y, Yin Y, Li Y, Feng R, Katz JP, et al. All-Trans Retinoic Acid Promotes a Tumor Suppressive OTUD6B-β-TrCP-SNAIL Axis

in Esophageal Squamous Cell Carcinoma and Enhances Immunotherapy. *Adv Sci (Weinh)*. 2023;10:e2207458. doi: 10.1002/advs.202207458
17. Dai X, Liu J, Wei W. DUB-independent regulation of pVHL by OTUD6B suppresses hepatocellular carcinoma. *Protein Cell*. 2020;11:546–548. doi: 10.1007/s13238-020-00721-x
18. Liu X, Zhang X, Peng Z, Li C, Wang Z, Wang C, Deng Z, Wu B, Cui Y, Wang Z, et al. Deubiquitylase OTUD6B Governs pVHL Stability in an Enzyme-Independent Manner and Suppresses Hepatocellular Carcinoma Metastasis. *Adv Sci (Weinh)*. 2020;7:1902040. doi: 10.1002/advs.201902040
19. Guo K, Wei Y, Wang Z, Zhang X, Zhang X, Liu X, Wu W, Wu Z, Zhang L, Cui CP. Deubiquitylase OTUD6B stabilizes the mutated pVHL and suppresses cell migration in clear cell renal cell carcinoma. *Cell Death Dis*. 2022;13:97. doi: 10.1038/s41419-021-04135-3
20. Santiago-Sim T, Burrage LC, Ebstein F, Tokita MJ, Miller M, Bi W, Braxton AA, Rosenfeld JA, Shahrour M, Lehmann A, et al. Biallelic Variants in OTUD6B Cause an Intellectual Disability Syndrome Associated with Seizures and Dysmorphic Features. *Am J Hum Genet*. 2017;100:676–688. doi: 10.1016/j.ajhg.2017.03.001
21. Carmena M, Earnshaw WC. The cellular geography of aurora kinases. *Nat Rev Mol Cell Biol*. 2003;4:842–854. doi: 10.1038/nrm1245
22. Yan M, Wang C, He B, Yang M, Tong M, Long Z, Liu B, Peng F, Xu L, Zhang Y, et al. Aurora-A Kinase: A Potent Oncogene and Target for Cancer Therapy. *Med Res Rev*. 2016;36:1036–1079. doi: 10.1002/med.21399
23. Zhao GJ, Shi HT, Tian X, Chen XX, Kong L, Li H, Wang J, Liu Y, Liu M, Li X, et al. USP10 Deubiquitinates MTX2 to Suppress cGAS-STING Signaling in MI. *Circ Res*. 2026;138:e326551. doi: 10.1161/circresaha.125.326551
24. Xie SY, Liu SQ, Zhang T, Shi WK, Xing Y, Fang WX, Zhang M, Chen MY, Xu SC, Fan MQ, et al. USP28 Serves as a Key Suppressor of Mitochondrial Morphofunctional Defects and Cardiac Dysfunction in the Diabetic Heart. *Circulation*. 2024;149:684–706. doi: 10.1161/circulationaha.123.065603
25. Ye B, Zhou H, Chen Y, Luo W, Lin W, Zhao Y, Han J, Han X, Huang W, Wu G, et al. USP25 Ameliorates Pathological Cardiac Hypertrophy by Stabilizing SERCA2a in Cardiomyocytes. *Circ Res*. 2023;132:465–480. doi: 10.1161/circresaha.122.321849
26. Wang M, Han X, Yu T, Wang M, Luo W, Zou C, Li X, Li G, Wu G, Wang Y, et al. OTUD1 promotes pathological cardiac remodeling and heart failure by targeting STAT3 in cardiomyocytes. *Theranostics*. 2023;13:2263–2280. doi: 10.7150/thno.83340
27. Cruz Walma DA, Chen Z, Bullock AN, Yamada KM. Ubiquitin ligases: guardians of mammalian development. *Nat Rev Mol Cell Biol*. 2022;23:350–367. doi: 10.1038/s41580-021-00448-5
28. Guo Y, Pu WT. Cardiomyocyte Maturation: New Phase in Development. *Circ Res*. 2020;126:1086–1106. doi: 10.1161/circresaha.119.315862
29. Samsa LA, Yang B, Liu J. Embryonic cardiac chamber maturation: Trabeculation, conduction, and cardiomyocyte proliferation. *Am J Med Genet C Semin Med Genet*. 2013;163c:157–168. doi: 10.1002/ajmg.c.31366

30. Moorman AF, Christoffels VM. Cardiac chamber formation: development, genes, and evolution. *Physiol Rev*. 2003;83:1223–1267. doi: 10.1152/physrev.00006.2003
31. Sobol A, Askonas C, Alani S, Weber MJ, Ananthanarayanan V, Osipo C, Bocchetta M. Deubiquitinase OTUD6B Isoforms Are Important Regulators of Growth and Proliferation. *Mol Cancer Res*. 2017;15:117–127. doi: 10.1158/1541-7786.Mcr-16-0281-t
32. Engeland K. Cell cycle regulation: p53-p21-RB signaling. *Cell Death Differ*. 2022;29:946–960. doi: 10.1038/s41418-022-00988-z
33. Tian Y, Morrisey EE. Importance of myocyte-nonmyocyte interactions in cardiac development and disease. *Circ Res*. 2012;110:1023–1034. doi: 10.1161/circresaha.111.243899
34. Birling MC, Yoshiki A, Adams DJ, Ayabe S, Beaudet AL, Bottomley J, Bradley A, Brown SDM, Bürger A, Bushell W, et al. A resource of targeted mutant mouse lines for 5,061 genes. *Nat Genet*. 2021;53:416–419. doi: 10.1038/s41588-021-00825-y
35. Wang W, Li E, Zou J, Qu C, Ayala J, Wen Y, Islam MS, Weintraub NL, Fulton DJR, Liang Q, et al. Ubiquitin Ligase RBX2/SAG Regulates Mitochondrial Ubiquitination and Mitophagy. *Circulation Research*. 2024;135:e39–e56. doi: doi:10.1161/CIRCRESAHA.124.324285
36. Feng J, Li Y, Nie Y. Methods of mouse cardiomyocyte isolation from postnatal heart. *J Mol Cell Cardiol*. 2022;168:35–43. doi: 10.1016/j.yjmcc.2022.04.007
37. Zou J, Ma W, Li J, Littlejohn R, Zhou H, Kim I-m, Fulton DJR, Chen W, Weintraub NL, Zhou J, et al. Neddylation mediates ventricular chamber maturation through repression of Hippo signaling. *Proceedings of the National Academy of Sciences*. 2018;115:E4101–E4110. doi: doi:10.1073/pnas.1719309115

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Figure.pdf
- SupplementaryMaterials.pdf
- Originalwesternblot.pdf