Cyclin A2 Induces Human Adult Cardiomyocyte Cytokinesis and Elicits Cardiomyocyte Reprogramming and Dedifferentiation

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Abstract

Background: Cyclin A2 (CCNA2), the master regulatory gene of the cell cycle is commonly silenced in postnatal mammalian cardiomyocytes. We have previously demonstrated that it can induce significant cardiac repair in both small and large animals when delivered to the heart via a viral vector. To date, whether CCNA2 gene delivery can induce cytokinesis in isolated cardiomyocytes from adult human hearts has not been investigated. Here we report that CCNA2 delivery can induce cytokinesis in cardiomyocytes isolated from adult human hearts.

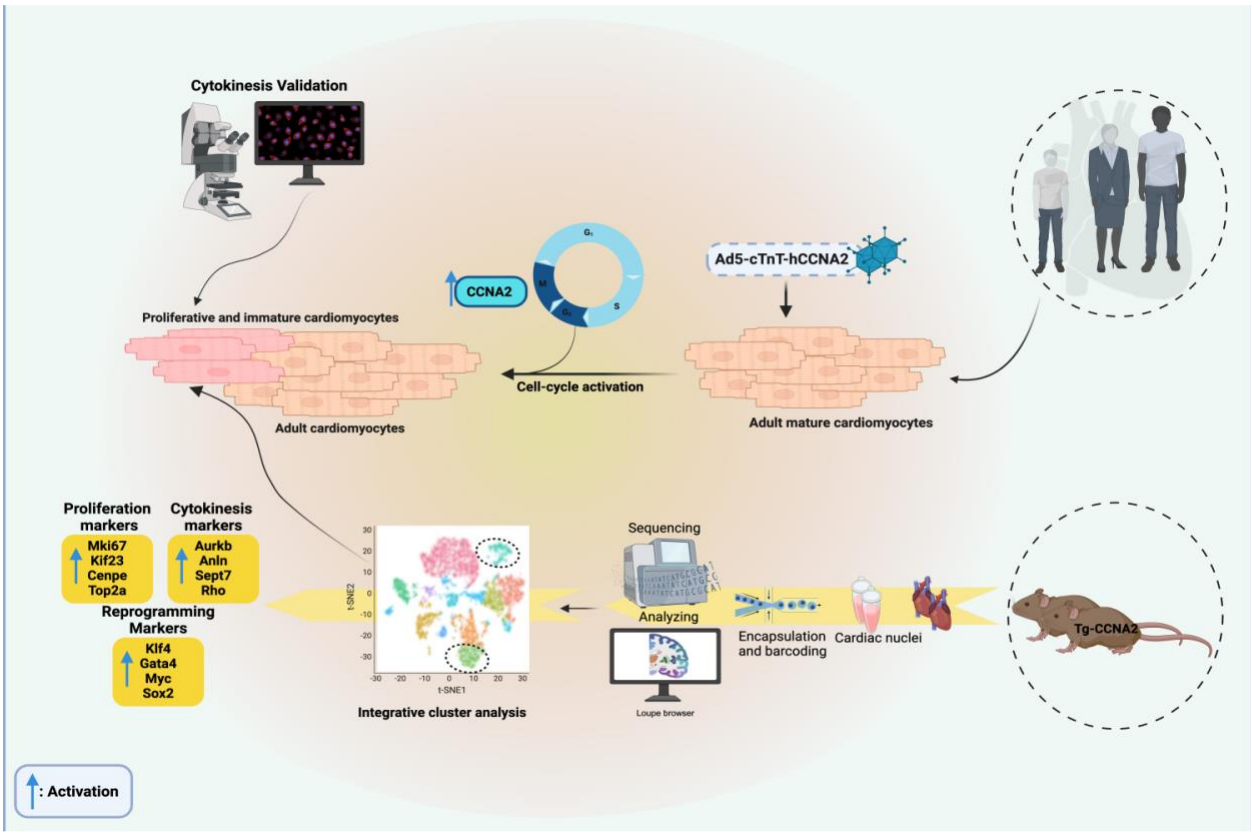
Methods: We designed a human gene therapy vector featuring a replication-deficient, E1/E3-deleted human adenovirus five encoding human CCNA2 driven by the cardiac Troponin T promoter to enable the expression of CCNA2 in freshly isolated human cardiomyocytes. We utilized time-lapse microscopy with live imaging to study cultured adult human cardiomyocytes isolated from a 21-year-old male, a 41-year-old female, and a 55-year-old male. To elucidate the mechanistic underpinnings of CCNA2-dependent gene regulation in governing cardiomyocyte cytokinesis, we conducted single nucleus transcriptomics (snRNA-seq, 10X Genomics) analysis of hearts isolated from adult transgenic mice that constitutively express CCNA2 in cardiomyocytes (CCNA2-Tg) and non-transgenic mice (nTg).

Results: We now report that human adult cardiomyocytes can be induced to undergo complete cytokinesis in response to CCNA2 gene delivery with preservation of sarcomere integrity in the resulting daughter cells and maintaining active calcium mobilization in redifferentiated cardiomyocytes. Remarkably, snRNA-seq analysis revealed a subpopulation of cardiomyocytes enriched with cytokinesis, proliferative and reprogramming genes in hearts obtained from CCNA2-Tg mice as compared to hearts obtained from nTg mice. Additionally, bulk RNA sequencing of human adult and fetal hearts identified key reprogramming genes relevant for understanding the mechanisms of CCNA2-induced effects observed in our experimental models.

Conclusion: These results provide a compelling path forward for the clinical development of cardiac regenerative therapy based on strategic manipulation of CCNA2 in cardiomyocytes.

Key Words: CCNA2; cytokinesis ■ single-nucleus RNA-Seq ■ cell cycle ■ heart regeneration.

Graphical Abstract



CCNA2 gene expression in primary adult cardiomyocytes induces cytokinesis while eliciting expression of proliferative and reprogramming genes in both humans and mice.

Nonstandard Abbreviations and Acronyms	
Actn2	Actinin Alpha 2
Atp2a2	ATPase, SERCA2
Atp5b	ATP Synthase, Mitochondrial F1 Complex, Beta Polypeptide ATP
Atp5g3	Synthase, Mitochondrial C Subunit, Gamma Polypeptide 3
Bub1	BUB1 Mitotic Checkpoint Serine/Threonine Kinase
C1qa	Complement C1q A Chain
Ca^{2+}	Calcium ion
Calm1	Calmodulin 1
Calm2	Calmodulin 2
CCNA2	Cyclin A2
CCNA2-Tg	CCNA2- transgenic mice

Cdc20	Cell Division Cycle 20
Cdk1	Cyclin-Dependent Kinase 1
Cdk2	Cyclin-Dependent Kinase 2
CGC	Cardiac glial cells
CM	Cardiomyocytes
CPM	Counts per million
CTnT	Cardiac Troponin T
CTnT-hCCNA2	Cardiac Troponin T- human cyclin A2
E1/E2	Early region 1/Early region 2
EC	Endothelial cells
EC-derived FB	Endothelial-derived fibroblasts
EDC	Endocardial cells
Efnb2	Ephrin B2
eGFP	Enhanced Green Fluorescent Protein
EPC	Epicardial cells
EP-derived FB	Epicardial derived fibroblasts
ES	Embryonic stem cells
FB	Fibroblasts
FDR	False discovery rate
Gata4	GATA Binding Protein 4
H2B	Histone 2B
Igfbp3	Insulin-Like Growth Factor Binding Protein 3
iPS	Induced pluripotent
Kcnd2	Potassium Voltage-Gated Channel Subfamily D Member 2
Kif23	Kinesin Family Member 23
Klf4	Kruppel Like Factor 4
MI	Myocardial infarction
Mki67	Marker of Proliferation Ki-67
MOI	Multiplicity of Infection
Mono	Monocytes
Myc	MYC Proto-Oncogene, BHLH Transcription Factor
Myh7	Myosin Heavy Chain 7
Myl2	Myosin Light Chain 2
Myob	Myoblasts
NC	Neuronal cells
Neb	Nebulin
nTg	Non-transgenic mice
PEC	Pericytes
Pkp2	Plakophilin 2
Pln	Phospholamban
Pou5f1	POU Class 5 Homeobox 1, Oct4
Prc1	Protein Regulator of Cytokinesis 1
Pro-vCM	Proliferative ventricular cardiomyocyte
Ryr2	Ryanodine Receptor 2

Scn5a	Sodium Voltage-Gated Channel Alpha Subunit 5
SMC	Smooth muscle cells
SnRNA-seq	Single nucleus RNA sequencing
TC	T-Cells
Tnnc1	Troponin C1
Tnni3k	Troponin I Interacting Kinase
Top2a	Topoisomerase (DNA) II Alpha
t-SNE	T-distributed Stochastic Neighbor Embedding
Ttn	Titin
UMI	Unique molecular identifier
UnkC	Unknown cells
vCM	Ventricular cardiomyocyte
VEC	Vascular endothelial cells
α-act	Alpha-actinin

Introduction

The significant morbidity and mortality associated with heart disease largely results from the limited proliferative capacity of adult human cardiomyocytes, preventing sufficient cellular regeneration for clinical recovery [1]. Scar formation via fibrosis is, therefore, the primary response to cardiac injury. Multitudes of molecular and cellular approaches have been investigated over the past 20 years aimed at regenerating the myocardium in various states of heart disease [2-4]. Use of stem/progenitor cell therapies for myocardial regeneration have demonstrated limited efficacy in clinical trials with no conclusive evidence of actual cardiomyocyte differentiation arising from the cell types used previously in these trials. Human embryonic stem (ES) cells and induced pluripotent (iPS) cells have been evaluated in non-human primate models of heart disease with the demonstrated ability of these cell types to differentiate, at the very least, to immature cardiomyocytes with the hope that they would undergo maturation *in vivo.* However, it remains to be seen whether these approaches may be translated for human therapy [3, 5]. Aside from the differentiation and generation of new cardiomyocytes, some technical concerns still need to be addressed, such as the propensity for arrhythmias noted in primate hearts treated with ES-derived and iPS-derived cardiomyocytes [5, 6].

There has been evidence of low-level cardiomyocyte turnover in the healthy human heart, but it is very limited, and this ability declines with age [7, 8]. Thus, cardiac regeneration in response to injury such as myocardial infarction (MI) remains a clinical challenge. Evolution may not have favored adult mammals in this regard, but certain members of metazoan species are capable of heart regeneration, and these examples may perhaps serve to enlighten us. Urodele amphibians, such as the newt, retain a remarkable capacity to replace lost anatomical tissues through epimorphic regeneration [9]. This process relies on the local plasticity of differentiated cells near the region of injury and involves reentry to the cell cycle with loss of differentiated characteristics to generate a 'local progenitor cell' of restricted potentiality [9].

The adult zebrafish heart can regenerate up to 20% of its volume via a mechanism largely dependent on the proliferation of cardiomyocytes adjacent to the area of injury [10, 11]. However, zebrafish harboring a temperature-sensitive mutation in *mps1*, a gene encoding a mitotic checkpoint kinase [10], cannot regenerate the heart, and scarring and fibrosis are noted in the excised areas in a manner similar to the response of the human myocardium after injury [10, 12]. In summary, the loss of a single gene can avert cardiomyocyte mitosis and mitigate the normal regenerative process, thus permitting fibrosis to proceed unimpeded.

Previously, we have reported that CCNA2 is a 'master regulator' of the cardiomyocyte cell cycle [13]. Unlike other cyclins, cyclin A2 complexes with its cyclin-dependent kinase partners to

regulate both key transitions of the cell cycle: G1-S and G2-M [14-16], and is silenced shortly after birth in mammalian cardiomyocytes [1, 17, 18]. Subsequently, we have also shown that Ccna2 mediates cardiac repair by inducing cardiomyocyte mitoses after myocardial infarction (MI) in two small animal models of MI [19-21]. As molecular mechanisms may be widely divergent across species, we performed a therapeutic efficacy study of CCNA2-mediated cardiac repair in a porcine model of MI as it closely mimics human anatomy and physiology [22]. Therapeutic delivery of CCNA2 one week after MI in the porcine heart induced cardiomyocyte cell cycle activation *in vivo* with marked enhancement of cardiac function as noted with multimodality imaging, including magnetic resonance imaging. The experimental pigs also exhibited significantly decreased fibrosis, lack of cardiomyocyte hypertrophy, and a 55% increase in cardiomyocyte cell numbers in the peri-infarct zones. Using the same viral vector encoding CCNA2 that was delivered *in vivo*, cultured porcine adult cardiomyocytes were induced to undergo cytokinesis with preservation of sarcomere structure and captured via live imaging [22]. For the purposes of clinical translation, we designed the next-generation gene therapy vector using a cardiomyocyte-specific promoter, cardiac Troponin T (cTnT), driving human CCNA2 expression. We then sought to determine whether this cTnT-CCNA2 vector would induce human adult cardiomyocytes to undergo cytokinesis. We employed live imaging microscopy after dynamically labeling the sarcomere within the adult human cardiomyocytes. Additionally, we use single nucleus RNA-seq (snRNA-seq, 10x Genomics) to characterize the transcriptional landscape and molecular signaling mechanisms associated with Ccna2 expression in cardiomyocytes.

Methods

Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki. This study used cardiomyocytes from adult human heart tissues provided by Anabios, San Diego, CA. The specimens used in this research are fully de-identified and therefore are exempt from ethics approval.

Culture of Adult Human Cardiomyocytes and *In Vitro* Cytokinesis Study

Cardiomyocytes from adult human (21-year-old male, 41-year-old female, and 55-year-old male) heart tissue were isolated after enzymatic digestion at Anabios, San Diego, CA, and were shipped to our laboratory within 24 hours of isolation. Adult human cardiomyocytes were cultured

according to our previously published adult porcine cardiomyocyte culture technique [22] with slight modification. In brief, upon arrival, cells were washed with serum-free Dulbecco's modified Eagle's medium (DMEM) media twice, and 105 cells were seeded in 100mm untreated polystyrene plates. Non-adherent cells were collected every 24 hours and centrifuged at 20Xg for 2 min at room temperature. Cell pellet was washed with serum-free DMEM and seeded on new polystyrene plates in modified Cardiomyocyte Culture Media (mod CMC) [22] formulated by adding 13% fetal bovine serum (FBS), 2.5% horse serum, 1X nonessential amino acid, 1mM sodium pyruvate, penicillin (100 U/ml), streptomycin (100 mg/ml), and fungizone (0.5 mg/ml) to DMEM/F12 (50:50). Cells were washed every day with serum-free DMEM, re-seeded in new polystyrene plates and cultured for 3 days. On day 4, they were seeded in glass-bottom 24-well tissue culture plates for 20 days, with the medium changed every 4th day. The wells with cardiomyocytes exhibiting adhesion and spreading were selected. Cells in these wells were trypsinized and counted, and 10^3 cells per well were seeded in new glass bottom tissue culture plates. After 2 days of culture, cells were divided into two groups (test and control) and were transduced with adenoviruses. The test group was transduced with cTnT-hCCNA2 along with cTnT-eGFP and/or cTnT-H2B-GFP and CMV-α-act-mCherry adenoviruses while the control group was transduced with only cTnT-eGFP and/or cTnT-H2B-GFP and CMV-α-act-mCherry adenoviruses. The multiplicity of infection (MOI) of adenoviruses were adjusted to 180 in each well of test (with cTnT-hCCNA2; MOI 100, CMV-α-act-mCherry; MOI 40 and cTnT-eGFP or cTnT-H2B-GFP; MOI 40) and control (cTnT-eGFP; MOI 140 and CMV-α-act-mCherry; MOI 40) group. After 48 hours of incubation, transduction was confirmed by observing the desired fluorescence in live cell imaging with Zeiss AxioVision Observer Z1 inverted microscope (Carl Zeiss). We also tested the differentiation potential (active calcium (Ca^{2+}) flux) of cardiomyocytes after cytokinesis *in vitro* by culturing them in an agarose-based semi-solid medium for 2 weeks. Cardiomyocytes were subjected to Ca^{2+} flux imaging with pacing of 0.5 Hz and 1 Hz current.

Time-lapse microscopy

To capture cell division events in cardiomyocytes *in vitro*, we carried out live cell epifluorescence time-lapse microscopy as described in [22]. In brief, Zeiss AxioVision Observer Z1 (Carl Zeiss, Thornwood, NY, USA) inverted epifluorescence microscope in a humidified chamber in the presence of 5% CO_2 at 37°C was used to carry out time-lapse microscopy. Multiple random points with cells expressing eGFP (green) and mCherry (red) were selected in the test and control groups. The positions were marked with the "position-list" tool in the AxioVision microscopy software (AxioVision Release 4.7, Carl Zeiss). After the first cycle of imaging, only the channel for

Texas red was used (for detection of mCherry) to acquire images for 72 hours. The fluorescein isothiocyanate (green) channel of the microscope was kept closed during the time-lapse imaging to avoid cell death from exposure to ultraviolet rays in this channel. Images were taken at intervals of 30 min. The 10X objective was used for all time-lapse imaging. Time-lapse movies were generated after the end of each experiment and exported as MOV files. The time-lapse movies were analyzed, and cells that underwent successful cytokinesis were enumerated in each group. The percentage (%) of cytokinesis events was calculated for each position, and the graph was plotted.

Cell fixation and nuclear staining

After time-lapse microscopy, cells in the glass-bottom plate were fixed with 4% paraformaldehyde at room temperature for 20 min and were stored at 4°C. For nuclear staining, cells were washed with 1XPBS then permeabilized with 0.5% Triton X-100 solution for 20 min at room temperature. Cells were washed three times with 1XPBS and incubated in DAPI solution (2.5 µg/ml) for 5 min. Cells were washed twice with 1X PBS, and imaging was carried out using a Zeiss AxioVision Observer Z1 inverted epifluorescence microscope.

Real-time quantitative PCR

Quantitative PCRs were performed in cultured cardiomyocytes transduced with cTnT-hCCNA2 (test) or cTnT-eGFP (control) adenovirus with similar MOI (MOI 100). “SYBR Green quantitative PCR protocol” on the “StepOnePlus” real-time PCR system (Applied Biosystems, CA) was used. The PCR protocol consisted of one cycle at 95°C for 2 min, 39 cycles at 95°C for 15 sec + 60°C for 1 min, and then 4°C on hold. Gene expression was determined by using the formula 2^(39-ΔΔCT) with consideration of CT value 39 for a single transcript and with normalization to the endogenous control *GAPDH*.

Animal

Cyclin A2 transgenic mice, adult males and females 8-12 weeks old (n=4), were maintained in a B6CBA background [13, 19]. Non-transgenic (wild-type) littermates were used as controls (n=4). All manipulations were performed according to Institutional Animal Care and Use Guidelines.

Collection of CCNA2-Tg mouse hearts and Sample preparation for Single Nucleus RNA-sequencing (snRNA-seq)

Frozen heart tissues from CCNA2-Tg- and nTg adult mice, male and female, were minced and transferred into a Dounce homogenizer containing ice-cold lysis buffer (Benthos Prime Central, TX, USA). Samples were homogenized gently, and nuclei were isolated. Nuclei were ensured to be free of clumps and debris by trypan blue staining under a microscope. Nuclei were then counted, and concentration was adjusted. 10x library concentration, insert size, and quantification were checked using Qubit, Agilent bioanalyzer, and q-PCR, respectively. For data Processing and Quality Control of 10x Sequencing of snRNA-seq, the reads were processed with 10X Genomics CellRanger software (v.3.1.0) with the default parameters for each sample. Cell Ranger employs the STAR aligner for splicing-aware mapping of reads to the genome. It utilizes a transcript annotation GTF file to categorize the reads into exonic, intronic, and intergenic regions based on whether the reads align confidently with the genome. Only confidently mapped, non-PCR duplicates with valid barcodes and UMIs were used to generate a gene-barcode matrix. This involved various steps, including alignment to a reference, collapsing of unique molecular identifiers (UMIs), UMI counting, and initial quality control procedures. The output of this process was the generation of filtered gene expression matrices that exclusively contained cellular barcodes. Subsequently, the Seurat R package (v.3.1.1) was used for data quality control and downstream processing. The filtered_feature_bc_matrix generated by Cell Ranger was used for Seurat processing. Post-sequencing quality control was performed using Seurat. Cells were excluded from downstream processing in each sample if they met any of the following criteria: fewer than 200 genes were detected per cell, genes had non-zero counts in at most three cells, total feature counts exceeded 8000 (suggesting potential multiplets), more than 50% of the feature count was attributable to mitochondrial genes, or more than 5% of the feature count was attributable to hemoglobin-related genes, and doublets from all the clusters were filtered during QC using DoubletFinder version 2.0.3 **(Figure S2)**. Raw read counts were log-normalized, cells were clustered and marker for each cluster were identified by using the Seurat Findmarkers and FindAllMarker functions [23]. The cell clusters were annotated by using iRhythmics FairdomHub instance (https://fairdomhub.org/studies/739) and prior knowledge.

Human Fetal and Adult Hearts and RNA sequencing

Human fetal heart RNA (n=3 pooled, Cat #636532, Takara) and human adult heart RNA (n=4 pooled, Cat #636583, Takara) were used for bulk RNA sequencing with an increased read depth (Ultra-deep sequencing), generating approximately 300 million reads per sample. This high sequencing depth enabled the detection of rare transcripts, improved quantification accuracy, and

enhanced the resolution of transcript isoforms, including novel and unannotated RNAs. For library preparation, we employed a ribosomal RNA (rRNA) depletion strategy instead of poly(A) enrichment to capture both polyadenylated (polyA$^+$) and non-polyadenylated (polyA$^-$) RNAs. While polyA$^+$ selection primarily enriches for mRNAs and some lncRNAs, rRNA depletion allows for the inclusion of a broader spectrum of regulatory RNAs, including enhancer RNAs (eRNAs), small nuclear RNAs (snRNAs), and uncharacterized lncRNAs that may lack poly(A) tails. Additionally, this approach reduces transcript bias, preserves isoform diversity, and is better suited for degraded RNA samples.

By combining rRNA depletion with ultra-deep sequencing, we achieved a comprehensive and unbiased profile of the cardiac transcriptome. The sequence reads were mapped to the human genome (GRCh38.110) using STAR [24] (version 2.7.9a). Using the mapped reads, the R package edgeR [25] (version 3.30.3) was employed to calculate counts per million (CPM) values and false discovery rate (FDR)-adjusted p-values obtained through the Benjamini–Hochberg method (Benthos Prime Central, TX, USA).

Pathway enrichment analysis

Pathway enrichment analysis was performed using the Reactome pathway analysis tool [26]. The differentially expression genes among nTg and CCNA2-Tg condition (log2FC > / < 1 and a P value < 0.05) were used for pathway analysis. Fold enrichment was calculated to indicate the gene expression observed in our list compared to the expected list. Categories with a fold enrichment greater than 1 were considered overrepresented, while those with a fold enrichment less than 1 were considered underrepresented in our experiment. Additionally, pathway enrichment analysis was conducted using BioPlanet via the EnrichR suite [27], following a previously described approach [28].

Data availability

The processed single-nucleus data and the Deep bulk RNA sequencing, including raw expression matrices and raw sequence files that support this research study's findings, are available on the Gene Expression Omnibus GSE249433 and GSE256519, respectively.

Statistical analysis

Statistical analyses were carried out using the R package (version 3.30.3), GraphPad Prism (version 8.2.1, GraphPad), and Python (via Google Colab). Two-tailed unpaired t-tests or one-/two-way analysis of variance (ANOVA) were used for normally distributed data. Chi-square tests for proportions were performed using Python libraries, including *Scipy*. Data are presented as means ± SEM unless otherwise indicated. Statistical significance was defined as $P < 0.05$.

Results

cTnT-CCNA2 adenovirus vector designed for therapeutic use induces expression of CCNA2 in cultured adult human cardiomyocytes

The therapeutic use grade human CCNA2 adenovirus vector was designed to express human cyclin A2 (CCNA2) specifically in cardiomyocytes by cloning human cDNA (NCBI Reference Sequence: NM_001237.4; 374-1672 bp) downstream to the cTnT promoter **(Figure 1A)**. The cultured adult human cardiomyocytes were transduced with cTnT-hCCNA2 (test) versus cTnT-eGFP (control) adenoviruses with MOI of 100 each for assessing the induced expression of CCNA2. We observed significantly increased expression of CCNA2 in the cultured adult human cardiomyocytes transduced with the test compared to the control adenovirus (**Figure 1B**). We utilized cTnT-H2B-GFP to confirm the nuclei of the daughter cells belonged to cardiomyocytes.

Adenoviral vector-mediated expression of CCNA2 induces cytokinesis in cultured adult human cardiomyocytes

To assess the effect of induced CCNA2 expression on cell division in adult human cardiomyocytes, we cultured adult human cardiomyocytes and studied cytokinesis *in vitro* by using live cell epifluorescence time-lapse microscopy. The adult human cardiomyocytes were plated at equal densities and transduced with cTnT-hCCNA2, cTnT-eGFP and/or cTnT-H2B-GFP and CMV-α-act-mCherry adenoviruses (test) or with cTnT-eGFP and/or cTnT-H2B-GFP and CMV-α-act-mCherry adenoviruses (control). cTnT-eGFP and/or cTnT-H2B-GFP adenovirus was transduced in both groups to confirm the initial tracking of cardiomyocytes (green) during live cell epifluorescence microscopy. For live visualization of sarcomeric structure in cardiomyocytes, cells from both groups were co-transduced with adenovirus containing α-act-mCherry, which we had constructed to allow for proper folding of the virally delivered α-actinin into the live cardiomyocyte sarcomere (CMV-α-act-mCherry) and was successfully used in cultured adult porcine cardiomyocytes [22]. This strategy enabled us to confirm cardiomyocyte identity by assessing the expression of eGFP before and after cytokinesis and tracking sarcomere dynamics during live

cell imaging. This approach provides a significant advantage over antibody-based identification, which is susceptible to artifacts and restricted to a single time point after cell fixation. We observed co-expression of eGFP (green) and α-actinin (red) in cultured adult cardiomyocytes **(Figure 1C** and **1H**; first panel). We performed time-lapse microscopic imaging of live cells to capture cardiomyocyte cytokinesis **(Figure 1C, 1F**, and **1H;** still images from **movie S1)**. Cytokinetic index of adult human cardiomyocytes was calculated by counting the cytokinetic events observed in 42 regions of interest (ROIs) **(Please see source Data Figure 1I)**. The cytokinetic index was significantly higher in the test samples with cTnT-CCNA2 adenovirus transduction compared to control samples in the 41- and 55-year-olds **(Figure 1I).** In contrast, no significant change in the cytokinetic events between test and control samples was observed in the 21-year-old patient's cardiomyocytes **(Figure 1I)**. Most remarkably, sarcomere structure was preserved in the daughter cells after cytokinesis **(Figure 1D** and **Figure 1G**; upon magnification of a daughter cell, the presence of sarcomeric structure is easily noted). The daughter cells were further identified with the expression of H2B-GFP (as they were originally also transduced with cTnT-eGFP and/or H2B-GFP) and noted to be mononuclear after they had been fixed and stained with DAPI **(Figure 1E** and **1H)**. Clusters of other cardiomyocytes with expression of eGFP could be seen adjacent to the daughter cells **(Figure 1E)**. To assess the functional differentiation of cardiomyocytes, Ca^{2+} transients were measured in CCNA2-expressing cardiomyocytes using live-cell calcium imaging under pacing conditions (0.5 Hz and 1 Hz; **Figure 2A**). Cardiomyocytes transduced with Ad5-cTnT-hCCNA2 exhibited active Ca^{2+} transients **(Figure 2B)**. Furthermore, we carried out ultra-deep bulk RNA-sequencing of fetal and adult human hearts to provide a developmental reference framework, allowing us to compare the molecular signatures of CCNA2-induced cardiomyocyte reprogramming with naturally occurring fetal-like states. Our ultra-deep sequencing approach enhanced our ability to detect low-abundance transcripts, improve quantification accuracy, and provide a more comprehensive transcriptomic profile. This comparison helps contextualize how CCNA2 expression modulates gene networks associated with dedifferentiation, proliferation, and calcium handling, reinforcing its role in cardiac plasticity and regeneration. Our transcriptomic analysis revealed upregulation of calcium handling and contractility-related genes, as well as sarcomere assembly genes (*RYR2, ATP2A2, SLC8A1, CALM1, CALM2, NEB, TTN*, and *TNNC1*) in adult human hearts compared to fetal human hearts **(Figure 2C)**. Additionally, bulk RNA-seq analysis of mouse hearts with constitutive CCNA2 expression in cardiomyocytes demonstrated downregulation of key calcium handling genes (*Ryr2, Atp2a2, Slc8a1*) and sarcomere assembly genes (*Neb, Ttn,* and *Tnnc1*) in CCNA2-Tg hearts. Conversely, genes involved in intracellular calcium release and store-operated calcium

entry, such as *Itpr1, Orai2,* and *Stim2*, were upregulated in CCNA2-Tg mouse hearts, further aligning with the fetal gene expression profile **(Figure 2D and S1)**. This suggests that CCNA2 may regulate sarcomere assembly genes during cell division **(Figure S1)**, facilitating cardiomyocyte proliferation [24]. Furthermore, CCNA2 appears to influence calcium mobilization post-cytokinesis, hinting at a broader role beyond cell cycle regulation, potentially impacting cardiomyocyte maturation and function. Live-cell imaging of CCNA2-overexpression in adult human cardiomyocytes undergoing cytokinesis revealed that daughter cells retain sarcomeric integrity post-division. Despite RNA-seq data showing a significant downregulation of thin filament genes (*Neb, Tnn, Tnnc1*), structural sarcomere proteins such as *Tnnt2* and *Actn2* remained stable **(Figure S1)**, suggesting that sarcomere remodeling may occur transiently to accommodate cell division without leading to complete dedifferentiation. Interestingly, transcriptomic profiling revealed upregulation of *ZEB1* in both fetal hearts and CCNA2-Tg mouse hearts, suggesting a conserved role in cardiomyocyte plasticity **(Figure S1)**. While *SNAI1* was not consistently upregulated, *ZEB1*'s expression aligns with transient mesenchymal activation, which has been implicated in neonatal heart regeneration [29].

Single nucleus transcriptomic (snRNA-seq) analysis of CCNA2-Tg mouse hearts identifies CCNA2-induced reprogramming genes in a selective subset of cardiomyocytes

To explore the full spectrum of CCNA2-induced cardiomyocyte cytokinesis in an unbiased manner, we performed snRNA-seq on hearts isolated from control non-transgenic (nTg) and CCNA2 constitutively expressing transgenic (CCNA2-Tg) adult mice. We included both male and female mice at 8-12 weeks old for this purpose. This dataset helped in the identification of key differentially expressed genes in cellular clusters throughout the heart of both nTg and CCNA2-Tg mice, in which CCNA2 expression is high **(Figure 3A**, **3B**, and **Figure S1)**. Another significant advantage of snRNA-seq is its capability to capture reads from diverse cell types that are typically underrepresented or lost during enzymatic tissue digestion.

Data visualization using a t-distributed Stochastic Neighbor Embedding (t-SNE) plot of combined nuclei profiles from both nTg and CCNA2-Tg mice enabled us to identify and classify 16 major cell populations, including cardiomyocytes, endothelial cells, fibroblasts, cardiac, immune cells, neuronal cells **(Figure 3C, 3D and 3E)**. Cell type clusters were annotated based on canonical marker genes, validated by their differential expression and reference to publicly available (iRhythmics FairdomHub) **(Figure 3F, 3G)**. Additionally, we identified several common clusters between nTg and CCNA2-Tg mice **(Figure 3E, Figure S3)**. The distribution of these clusters

differs between the nTg and CCNA2-Tg heart tissues **(Figure 3H, and 3I)**. For instance, endothelial-derived fibroblasts are decreased while fibroblasts are increased in CCNA2-Tg mice as compared to controls **(Figure 3H)**. Cell identities were confirmed through the expression of cell-specific marker genes [30]. Cardiomyocyte populations were identified by classical marker genes (*Myh7*, *Actn2,* and *Tnnc1*) in both nTg and CCNA2-Tg hearts. Cardiomyocytes that express an enhanced level of contractility genes, including the *Ryr2*, are designated as mature cardiomyocytes [31].

Ventricular cardiomyocyte (vCMs) populations were identified by the enrichment of *Myl2* and *Myh7*. We identified 11 subclusters of the vCM population common to both nTg and CCNA2-Tg hearts; however, each had different proportions of the nuclei profiles. Notably, the vCM1 and vCM5 were more prevalent in nTg hearts, whereas the vCM2, vCM3, vCM4, vCM6, vCM7, vCM8, vCM9, pro-vCM10, and the vCM11 were more abundant in CCNA2-Tg hearts **(Figure 3J).**

The populations vCM1, vCM2, vCM4, vCM5, and vCM11 exhibit elevated expression of genes associated with more mature cardiomyocytes, particularly those involved in sarcomere development such as *Ttn*, and Ca^{2+} handling and cardiac contractility genes like *Pln, Ryr2*, and *Pkp2, Kcnd2*, and *Tnni3k,* which regulates cardiomyogenesis and function [32]. Notably, vCM5 displays heightened levels of *Ebf2*, implicated in brown adipocyte differentiation [33], and *Lsamp*, which guides neuronal connections [34]. In contrast, vCM3 and vCM7 show increased expression of ATP synthase genes *Atp5b* and *Atp5g3. Efnb2* expression is prominent in vCM4 and vCM8, indicating a shared gene expression signature with endothelial cells. Additionally, vCM8 expresses *Tnni3, Ahsa1*, and *Tagln2, which are* involved in cardiomyocyte structure, function, and cellular regulation. Exclusive expression of *Igfbp3* characterizes vCM9 as mid-phase cardiomyocytes.

Conversely, analyzing the transcriptomic profile of the Pro-vCM10 population reveals elevated expression of early-phase proliferative markers (thus the name “Pro”) and cell cycle genes, including *Mki67*, *Kif23, Top2a, Cdc20, Cdk2d, Cdk1, Prc1,* and *Bub1*. Additionally, it exhibits elevated expression of *Stmn1*, which is implicated in mitotic cytokinesis pathways [35], and *Cd44,* a regulator of adhesion complex assembly [36]. This subcluster also displays immune markers such as *C1qa.* Remarkably, Pro-vCM10 is increased in CCNA2-Tg mouse hearts compared to nTg mouse hearts **(Figure 3J)**. Next, we scrutinized the transcriptomic profiles of the distinct cardiomyocyte subsets, with a particular focus on the proliferative vCM population, analyzing individual features, both in nTg and CCNA2-Tg hearts. We confirmed CCNA2 expression in cardiomyocytes, notably within the enriched proliferative subset identified in CCNA2-Tg hearts,

along with the expression of cell cycle genes **(Figure 4A** and **4B)**. This expression pattern remained consistent across the combined transcriptomic profiles of all subclusters **(Figure S4)**. Therefore, the identification of this pro-vCM subcluster in CCNA2-Tg mice shows lower expression levels of *Ryr2* **(Figure 5A)** and an increased expression of proliferative markers (*Mki67* and *Kif23*) **(Figure 5B)**. In mature CCNA2-Tg mice, *Ryr2* expression is decreased compared to nTg mice, where the cardiomyocytes express higher *Ryr2* **(Figure 5A)**. This implies greater cardiomyocyte maturation in nTg mice compared to CCNA2-Tg mice, where cardiomyocytes appear less mature and may be primed for dedifferentiation processes to facilitate proliferation and regeneration [37]. These findings correlate with our bulk RNA-seq results, wherein CCNA2-Tg cardiomyocytes show decreased *Ryr2* expression as well as enhanced expression of proliferation markers as compared to nTg cardiomyocytes **(Figure S5A** and **S5B**). The observed reduction in *Ryr2* and sarcomeric gene expression in CCNA2-Tg cardiomyocytes aligns with a dedifferentiation-like state, which facilitates cell cycle re-entry and cytokinesis. This transient immaturity may enable the proliferative potential necessary for myocardial regeneration.

To further probe CCNA2-induced cytokinesis events, we examined whether distinct cardiomyocyte subclusters exhibit gene expression associated with cytokinesis and/or reprogramming in CCNA2-Tg mice. Intriguingly, the pro-vCM population demonstrated upregulation of key cytokinesis genes (such as *Aurkb, Anln, Sept7,* and *Rho,* among others), notably significantly higher expressions of *Rho* and *Aurka* in CCNA2-Tg compared to nTg mice **(Figure 5C)**. Furthermore, pro-vCM subcluster shows upregulation of reprogramming genes (including *Pou5f1, Klf4, Gata4, Myc, Sox2,* and *Tbx5*), with *Pou5f1* and *Tbx5* expression significantly increased in CCNA2-Tg hearts compared to nTg hearts **(Figure 5D)**. Consistent with the observations derived from bulk RNA-seq analysis, the expression levels of genes associated with cytokinesis and reprogramming were increased in CCNA2-Tg cardiomyocytes compared to nTg cardiomyocytes **(Figure S5C** and **S5D)**.

Pathway enrichment analysis of CCNA2-Tg cardiomyocytes shows significant activation of cell cycle, cytokinesis, and mitotic regulation pathways compared to nTg controls. Notably, Rho GTPase signaling, spindle checkpoint regulation, and cytokinesis pathways were highly enriched **(Figure 5E)**, indicating increased proliferation and cytoskeletal remodeling in CCNA2-Tg hearts. In contrast, nTg cardiomyocytes showed enrichment in extracellular matrix organization, collagen biosynthesis, and muscle contraction pathways, reflecting a more differentiated and structurally stable state. These findings indicate that CCNA2 enhances proliferation and cellular plasticity, promoting a regenerative-like phenotype in cardiomyocytes.

Our bulk RNA-seq data from mouse nTg and CCNA2-Tg cardiomyocytes yielded comparable findings. We observed upregulation of *Ccnb1* and *Ccnb2* in CCNA2-Tg cardiomyocytes compared to nTg cardiomyocytes, along with slight increases in the expression of *Cdk1* in CCNA2-Tg cardiomyocytes **(Figure 5F)**. *Cdk1* is the sole essential cyclin-dependent kinase (*CDK*), when complexed to Cyclin A2 promotes entry into mitosis, whereas Cyclin A2/Cdk2 is critical for the G1/S transition [13, 38, 39]. Key cytokinesis and proliferation genes including *Aurka, Aurkb, Anln, Mki67,* and *Kif23* were upregulated in CCNA2-Tg cardiomyocytes, reinforcing the role of CCNA2 in cell cycle reactivation **(Figure 5F)**. In contrast, adult human hearts exhibited downregulation of proliferative and cytokinesis-related genes **(Figure 5G)**, whereas CCNA2-Tg cardiomyocytes retained a transcriptional profile favoring cell cycle progression and cytokinesis. These findings support the hypothesis that CCNA2 reactivates a fetal-like transcriptional program in adult cardiomyocytes, promoting cell cycle re-entry without complete dedifferentiation.

Differentially expressed genes in the adult heart were downregulated as compared to the fetal human heart (**Figure 5I)**. Pathway analysis showed the enrichment of several pathways upregulated in the adult human heart, including metabolism, Ca^{2+} mobilization, and ion homeostasis, and downregulated pathways, including mitotic telophase/cytokinesis, regulation of the mitotic cell cycle, and myogenesis **(Figure 5H, and 5I).**

Furthermore, pathway enrichment analysis revealed activation of key signaling pathways associated with cardiomyocyte plasticity, proliferation, and reprogramming in CCNA2-Tg cardiomyocytes. Specifically, the NOTCH signaling pathway and its downstream effectors (HES/HEY) were upregulated, reinforcing CCNA2's role in progenitor-like gene activation and cardiac reprogramming. Additionally, developmental pathways such as TGF-beta regulation and Wnt interactions suggest a shift toward a more plastic, regenerative state. Increased signaling through MAPK, PDGF, and Jak-STAT pathways further supports enhanced proliferation and cytoskeletal remodeling. Furthermore, upregulation of focal adhesion, integrin interactions, and actin cytoskeleton regulation pathways indicate structural adaptations that facilitate cardiomyocyte dedifferentiation and division. Conversely, mitochondrial metabolism and oxidative phosphorylation, hallmarks of mature cardiomyocyte function, were significantly downregulated, aligning with a metabolic reversion to a fetal-like, proliferative phenotype in CCNA2-Tg cardiomyocytes. **(Figure S5F).** Moreover, adult CCNA2-Tg cardiomyocytes exhibit a greater number of upregulated genes compared to nTg cardiomyocytes in mice **(Figure S5E).**

On the other hand, it is noteworthy to mention that in the adult murine context, approximately 85–90% of cardiomyocytes exist in a binucleated state [40], indicating that the nuclear count does not precisely correspond to the cell count. Furthermore, the polyploidization of cardiomyocytes

has been recognized as an impediment to heart regeneration [41]. It has been reported that binucleated cardiomyocytes re-entering the cell cycle are less likely to complete cytokinesis, thereby impeding the efficient regeneration of cardiac tissue following injury [42].

Our study establishes that CCNA2 induces the expression of reprogramming genes, which ultimately enhances cardiomyocyte cytokinesis. These results provide a compelling pathway forward for the clinical development of cardiac regenerative therapy based on the manipulation of CCNA2 expression in cardiomyocytes.

Discussion

Epimorphic regeneration is a conserved mechanism across evolutionary biology, permitting organ-specific tissue regeneration in a variety of metazoan phylogeny, including early postnatal mammals [42]. In fact, in human neonates, the ventricular myocardium responds to the pressure overload of congenital aortic stenosis through hyperplastic growth, yet the degenerative, calcific aortic stenosis of older patients elicits hypertrophic growth of the myocardium [43-45]. These clinical observations imply that the cell cycle repertoire of human neonatal cardiomyocytes is also intact, as cardiac growth is able to occur in a similar fashion to the embryonic heart. Moreover, the adult human heart is also capable of a very low rate of turnover [8], with diminishing rates of turnover past childhood and adolescence, with cardiomyocyte cytokinesis markers not found past age 20 [7].

Strategies to induce cardiomyocyte cytokinesis in adult human hearts are of critical need in order to face the growing public health crisis of congestive heart failure. To our knowledge, most cell therapy clinical trials in heart disease have provided inconsistent evidence of improvements in left ventricular ejection fraction, with a lack of evidence that cell types utilized actually differentiate into functional cardiomyocytes. To this end, we previously explored the effects of cell cycle manipulation in small and large animal models and successfully induced cardiomyocyte proliferation *in vivo* with significant enhancement of cardiac function in animal models [19-22]. In any translational plan to use cell cycle regulators for clinical use, two angles must be considered. Firstly, the wrong cell cycle regulators can induce cellular apoptosis [46-49] or be ineffective if the G2/M checkpoint is not navigated [13, 22, 50]. Secondly, precautions must be taken to prevent the expression of cell cycle activators in extra cardiac tissues due to the potential for oncogenic transformation [51]. This safety concern prompted us to explore methods of tissue-specific activation of cyclin A2. One such method was to design a next-generation viral vector in which a cardiomyocyte-specific promoter, cardiac Troponin T, drives the expression of human cyclin A2

(cTnT-hCCNA2). Human cardiomyocytes isolated from 21-, 41- and 55-year-olds who had died of noncardiac causes were cultured utilizing adult mammalian cardiomyocyte culture methods developed by our laboratory [22] and transduced with cTnT-hCCNA2. Cardiomyocytes from the 41- and 55-year-olds in the experimental wells underwent complete cytokinesis at a significantly higher frequency than the cardiomyocytes in the control wells. Based on our previous studies of rodent cardiomyocytes, we attribute the low rate of cytokinetic events we observed in the control wells to the reactivation of endogenous CCNA2 with the prolonged culture of cardiomyocytes. This is a phenomenon we have previously observed and quantified in rodent cardiomyocytes (unpublished data), and likely not correlated with the low turnover seen in human hearts as measured by 14C dating [52] as cytokinesis markers were not noted at all in human hearts over the age 20 by Kühn and colleagues [7]. Similarly, Frisén and colleagues [52] used stereological and 14C quantification techniques to measure cardiomyocyte exchange. They found that most cardiomyocytes were never exchanged and that the substantial replacement of existing cardiomyocytes was generated in the first 10 years of life, while the second decade of life focused on DNA polyploidization. Cardiomyocyte plasticity plays a key role in cardiac remodeling and repair in infarcted hearts [53]. Our snRNA-Seq also confirms heightened cardiomyocyte plasticity of CCNA2 transgenic mice as we note changes in gene expression involved in cell cycle progression and reprogramming. Ca^{2+} signaling is a fundamental pathway in the regulation of cell division, cardiac contractility, and remodeling [54-56]. The increase in the cytokinetic index in the test samples from 41- and 55-year-olds shows the ability of CCNA2 to stimulate intracellular Ca^{2+} mobilization. Of note, the transduced cTnT-hCCNA2 (test samples) cultured cardiomyocytes isolated from a 21-year-old male did not show a significant increase in cytokinetic index invoked by CCNA2 when compared to the control samples, which is consistent with reports of previous investigators [7, 52] that cell turnover and cell division can naturally occur at younger ages in humans.

SnRNA-seq has been used to identify transcriptomic changes occurring at the single-cell level, shedding light on the precise mapping of transcriptomic alterations in a cell-type-specific manner in response to cellular stimulation or gene delivery [57]. Employing snRNA-seq in intact tissues enables the capturing of cell-specific transcriptomics changes in a more precise manner while maintaining the integrity of tissue responses, compared to isolated *in vitro* transcriptomics analyses. Our strategy of using snRNA-seq is to identify cardiomyocyte-specific changes in an *in vivo* setting in a clinically relevant CCNA2-Tg model, which allowed us to map specific changes to a subpopulation of cardiomyocytes. This would not have been captured by analyzing the transcriptome of the bulk tissue of isolated cardiomyocytes using bulk RNA-seq strategies. Live-

cell imaging demonstrated that CCNA2-transfected adult human cardiomyocytes successfully undergo cytokinesis, with daughter cells retaining what appear to be intact sarcomeric structures. However, transcriptomic analyses (bulk RNA-seq and snRNA-seq) revealed significant downregulation of sarcomere-related genes. These findings suggest a dynamic remodeling process during cytokinesis, where transcriptional downregulation of sarcomeric genes may occur transiently, while structural integrity is preserved through post-transcriptional mechanisms or protein stability. This highlights the intricate balance between cellular reprogramming and maintenance of cardiomyocyte identity during division.

Furthermore, the genes induced in the CCNA2-Tg model appear to be representing a common mechanism underlying cytokinesis in both male and female mice, consistent with observations in human male and female hearts. Given that we identified a subset of cardiomyocytes being reprogrammed into a more proliferative state, our next goal is to investigate this cardiomyocyte subset population more definitively. Unlike other regenerative approaches that separately target cardiomyocyte proliferation or reprogramming, our findings suggest that CCNA2 integrates both processes, making it a unique therapeutic candidate for cardiac regeneration. Defining the transcriptomic state of cyclin A2-expressing cardiomyoyctes and their proliferative potential in greater detail can enable us to recapitulate such proliferative cardiomyocytes both *in vitro* and *in vivo* to determine whether targeting these specific subpopulations holds greater therapeutic potential than targeting the entire cardiomyocyte population. The use of computational resources can aid our comprehension of these molecular mechanisms for more precision-guided regenerative strategies for human heart disease.

The ability of CCNA2 to induce proliferation while maintaining cardiomyocyte function positions CCNA2 gene therapy as a promising clinical approach, minimizing risks associated with exogenous cell transplantation, immune rejection, and off-target differentiation. Moreover, CCNA2 appears to induce a transient mesenchymal-like state, as indicated by the upregulation of *Zeb1* and *Snai1*, key regulators of epithelial-to-mesenchymal transition (EMT). This suggests that CCNA2-Tg cardiomyocytes adopt a plastic phenotype to facilitate cell division, similar to mechanisms observed in neonatal heart regeneration. These findings highlight CCNA2 as a novel and safe strategy for cardiac regeneration, offering a balance between proliferative potential and structural preservation.

Limitations

While the findings presented above are very promising, there are a few limitations to consider. Firstly, the use of freshly isolated human adult primary cardiomyocytes offers an ideal cellular

model but presents several challenges. There is a high susceptibility to cell death in culture, which can hinder such experiments, and a fair amount of biological variability in getting the cells to adhere to the petri dishes. Adherence is necessary to perform these cytokinesis experiments. Greater consistency with very rapid isolation from human cadavers and further refinements of the culture methods may overcome these challenges. Additionally, the use of adenovirus vectors is safe, and they are being utilized in other gene therapies that have been clinically tested. Immunogenicity is a concern, and therefore, there are ongoing investigations in our laboratory to also explore alternative delivery strategies. Furthermore, *in vitro* models, though valuable, do not fully replicate the complexity of the *in vivo* cardiac environment. Nevertheless, this study is a valuable translational step forward from our previous preclinical report demonstrating that CCNA2 gene therapy induced a 55% increase in cardiomyocytes of the peri-infarct zones of the porcine heart [22] with significant enhancement of cardiac function. CCNA2-induced cytokinesis of human adult cardiomyocytes provides a compelling pathway towards human clinical trials of CCNA2 gene therapy for heart repair.

Article Information

Author Contributions

E.B. wrote the original article, contributed to experiments, and analyzed and performed snRNA-seq data with input from P.M. and A.K.; S.V.M and A.K.R contributed to cytokinesis experiments and calcium measurements; P.M. contributed to processing snRNA-seq samples and initial analysis; C.D.S. assisted with the cell biology experiments and organization of the manuscript; P.E.M., A.G., N.A.G. provided cardiomyocytes through their proprietary technology. H.W.C. was responsible for conceptualization, vector design, validation, supervision, reviewing, and funding acquisition. Review & editing- E.B, H.W.C, P.M. and A.K.

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Disclosures

H.W.C. is founder and equity holder of VentriNova, Inc. and is listed as the inventor of multiple patents regarding cyclin A2-mediated cardiac repair and caudal-related homeobox 2 cells for cardiac repair. Other authors report no conflict.

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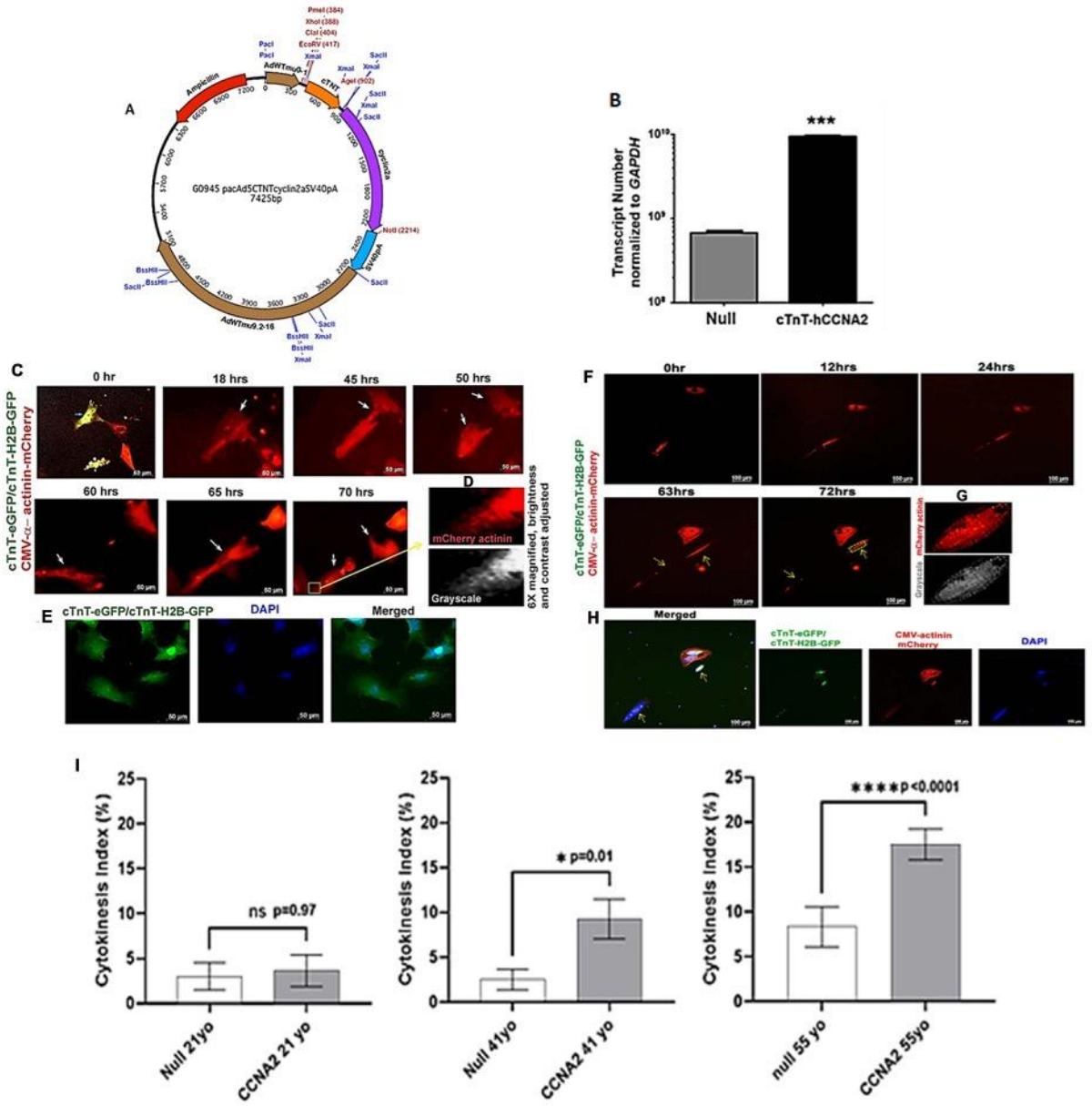

Figure 1. CCNA2 expression induces cytokinesis in adult human cardiomyocytes *In Vitro*. **A)** Clinical-use grade replication-deficient human adenovirus type 5 (Ad5) was used for this study. The vector was designed with cardiac Troponin T (cTnT) promoter to express human cyclin A2 (hCCNA2) specifically in cardiomyocytes. **B)** The cTnT-hCCNA2 adenovirus was used to transduce cultured adult human cardiomyocytes to induce the expression of CCNA2. Significantly higher CCNA2 expression was observed in cells transduced with cTnT-hCCNA2 compared to the control virus. **C-H)** Still images from representative time-lapse epifluorescence microscopy of cultured cardiomyocytes isolated from adult humans **C**) 55, and **F)** 41-year-old. Please note that still images of 55 and 41-years-old have scale bars of 50µm and 100µm as it is shown in the movies S1 and S2 respectively. Cells were transduced with cTnT-hCCNA2, cTnT-H2B-GFP, cTnT-eGFP (to label cardiomyocytes), and CMV-α-actinin-m-Cherry adenoviruses prior to the beginning of the time-lapse imaging. **C)** At time 0 hr, a cell (yellow) expressing both cTnT (green) and α-actinin (red) was followed for 70 hrs via time-lapse microscopy. The green channel was closed, and cells were only followed through the red channel to avoid UV photo-toxicity. The observed human

cardiomyocytes show the 1st cell division at 50 hrs of imaging, and one of the daughter cells subsequently undergoes division at 70 hrs of imaging. **D** and **G)** At 70/72 hrs, a daughter cell is partially magnified with a grayscale version to demonstrate the presence of an intact sarcomere structure. **E, H)** One day after live imaging ended, these wells were fixed with subsequent labeling of nuclei with DAPI. The green fluorescence of the original cTnT-eGFP and cTnT-H2B-GFP transduction are visible, further confirming that these cells are cardiomyocytes. **I)** The cytokinetic events were counted in the control and test samples from the 21-, 41- and 55-year-old subjects. A significantly higher rate of cytokinesis was observed, on the other hand, in the test samples from the 41 years old ($p=0.01$) and 55 years old ($p<0.0001$) compared to the control. The control and test samples from the 21-year-old did not exhibit a significant difference in cytokinetic events ($p=0.097$).

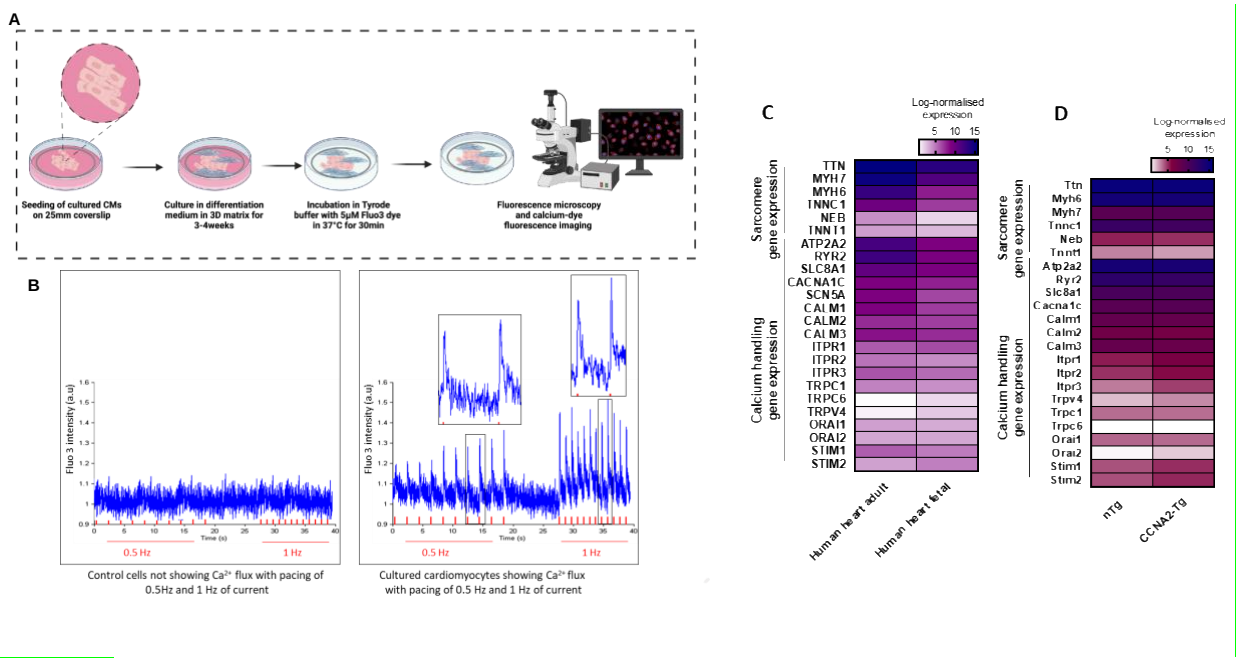

Figure 2. Differentiation of cultured adult human cardiomyocytes. Cultured adult human cardiomyocytes typically undergo dedifferentiation and were then subjected to 3D redifferentiation culture conditions and examined for Ca^{2+} flux. **A)** Overview of the 3D culture method of differentiation, Ca^{2+} flux measurement, and fluorescence microscopy in adult human cardiomyocytes. **B)** Representative Ca^{2+} flux graphs of re-differentiated cultured adult human cardiomyocytes. Ad5-cTnT-hCCNA2 transduced adult human cardiomyocytes show Ca^{2+} flux with pacing of 0.5 and 1 Hz of current, while no Ca^{2+} flux was detected in the non-Ad5-cTnT-hCCNA2 transduced cardiomyocytes. **C)** Heatmap depicting the expression of Ca^{2+} handling, contractility, and sarcomere-associated genes in human adult versus fetal hearts, **D)** Comparison of gene expression in CCNA2-Tg versus nTg mice, highlighting differences in Ca^{2+} regulatory genes and contractile machinery. Positive values indicate upregulation in adult hearts relative to fetal hearts. Data represent normalized RNA-seq expression values.

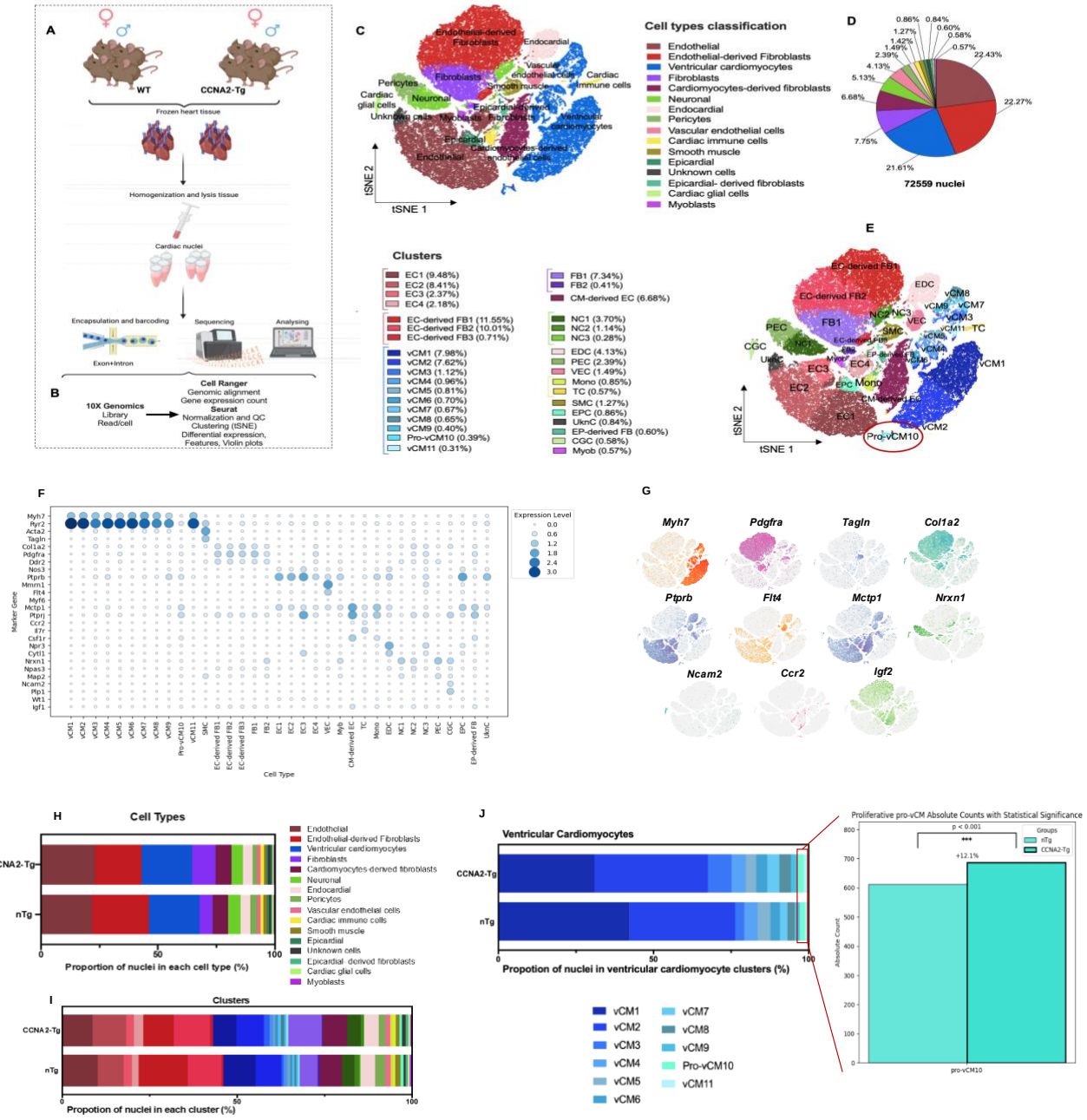

Figure 3. Single nucleus RNA sequencing identified major cell types in CCNA2-Tg mice
A) Schematic depicting the design of the snRNA-seq experiments (n=2) for each condition nTg female, CCNA2-Tg female, nTg male, and nTg male (Total: n=8). **B)** The analysis pipeline and single-cell barcoded library generation (10X Genomics), sequence alignment (Cell Ranger), and further analysis using R (SeuratR package). **C)** t-distributed Stochastic Neighbor Embedding (t-SNE) clustering the nuclei and reveals more than 16 distinct major cell types identified based on the cell-specific markers dataset provided at iRhythmics FairdomHub instance and **D)** the pie chart showing the proportion of cells within the snRNA-seq. **E** t-SNE of a total of 35 subclusters. **F)** Dot plot depicting the expression levels of some of the key marker genes used to classify cell types. Dot size represents the percentage of cells expressing each gene, while color intensity

reflects expression levels. **G)** Feature plots highlighting the expression of selected cell-type-specific markers across the t-SNE space. **H)** Stacked bar plots representing the proportion of nuclei classified into each major cell type (top) and **I)** each identified cluster (bottom) in both CCNA2-Tg and nTg mice. **J)** Stacked bar plots displaying the proportion of nuclei in vCM clusters (left), and a bar graph showing the absolute counts of Pro-vCM10 cardiomyocytes in nTg and CCNA2-Tg hearts (right). CCNA2-Tg hearts exhibit a significant increase (+12.1%) in proliferative cardiomyocytes compared to nTg ($p < 0.001$), supporting the role of CCNA2 in promoting cardiomyocyte proliferation. CM: Cardiomyocytes, vCM: ventricular cardiomyocytes, EC: endothelial cells, FB: fibroblasts, EC-derived FB: endothelial-derived fibroblasts, NC: neuronal, EDC: endocardial cells. PEC: pericytes, VEC: vascular endothelial cells, Mono: monocytes, TC: T-Cells, SMC: smooth muscle cells, EPC: epicardial cells, UnkC: unknown cells, EP-derived FB: epicardial derived fibroblasts, CGC: Cardiac glial cells, Myob: myoblasts.

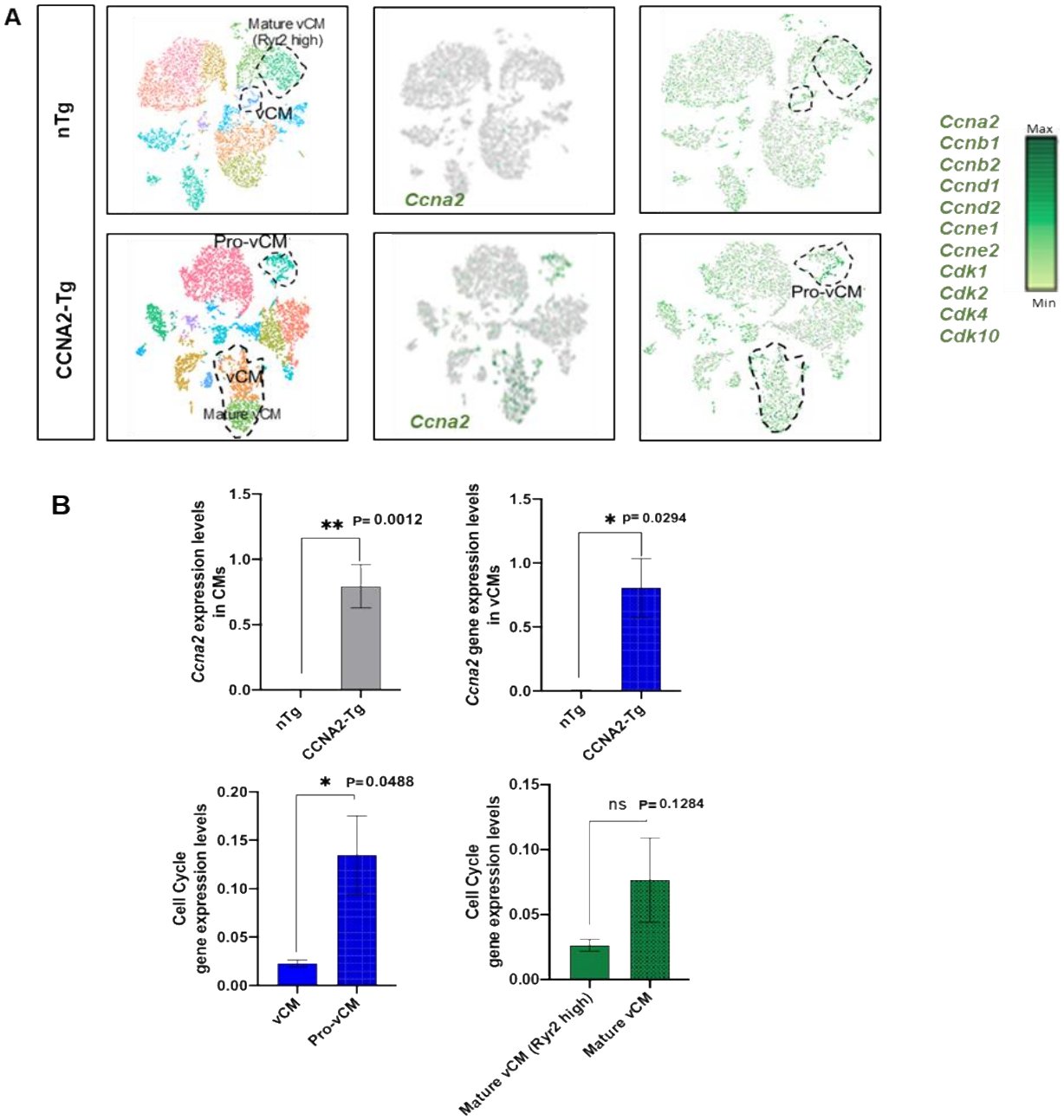

Figure 4. CCNA2 and cell cycle genes expression in CCNA2-Tg and nTg mice cardiomyocytes delineated by snRNA-seq. A) CCNA2 expressed in the vCM, specifically in the pro-vCM subset of the cardiomyocyte population in CCNA2-Tg and cell cycle genes projected on the t-SNE plot. **B)** A representative bar graph showing the expression level of CCNA2 and cell cycle genes in cardiomyocytes of CCNA2-Tg and nTg mice.

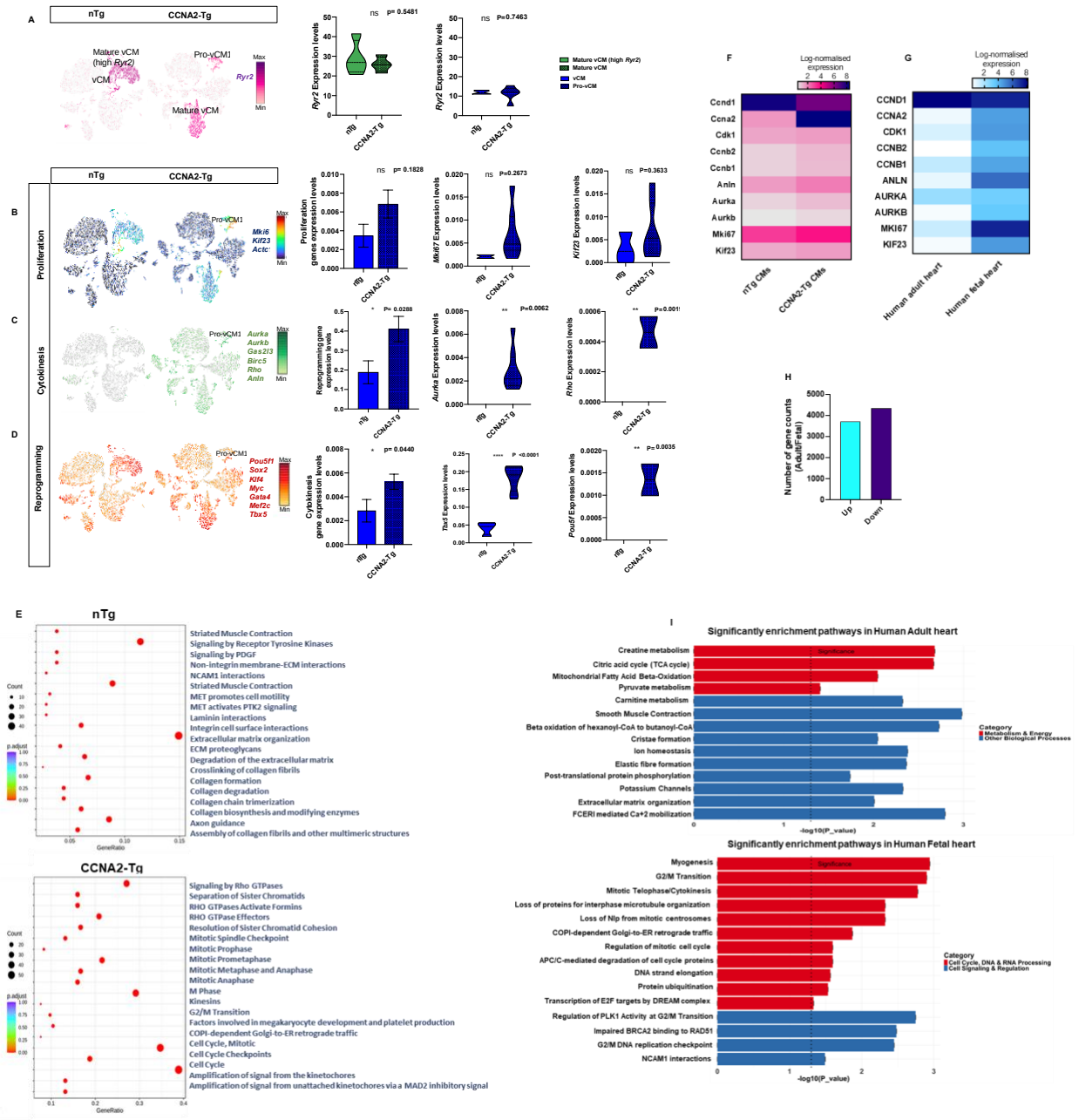

Figure 5. CCNA2 expression induces proliferation, cytokinesis, and reprogramming in mouse cardiomyocytes. A) t-SNE projection showing *Ryr2* expression across cardiomyocyte subpopulations in nTg and CCNA2-Tg mice. *Ryr2*-high cardiomyocytes correspond to a more mature ventricular cardiomyocyte (vCM) state, whereas *Ryr2*-low cardiomyocytes represent less mature or proliferative cardiomyocytes. Quantification of *Ryr2* expression levels among cardiomyocyte subpopulations is shown in the accompanying violin plot and bar graph, highlighting differences in *Ryr2* expression across conditions. **B)** t-SNE plots and representative graph and Violin plot representing the expression of key proliferation, **C)** cytokinesis, and **D)** reprogramming gene markers in both CCNA2-Tg and nTg mice. **E)** Representative dot plot of the differential gene expression analysis showing the upregulation of proliferation, cytokinesis, and reprogramming genes in CCNA2-Tg versus nTg cardiomyocytes. **F)** Heatmaps of log-transformed gene expression changes in human fetal versus adult hearts **G)** and in CCNA2-Tg versus nTg

cardiomyocytes. **H)** Bar graph representing the up (LogFC>1) or down-regulated genes (LogFC<1) in human adult versus fetal heart. **I)** Pathway enrichment analysis showing significantly enriched pathways in human adult (top) and fetal (bottom) heart. In the adult heart, pathways related to metabolism and energy are enriched, based on 4,348 downregulated genes and 3,713 upregulated genes ($P<0.05$). In the fetal heart, cell cycle, DNA/RNA processing, and signaling pathways are enriched ($P<0.05$).

Supplemental Material

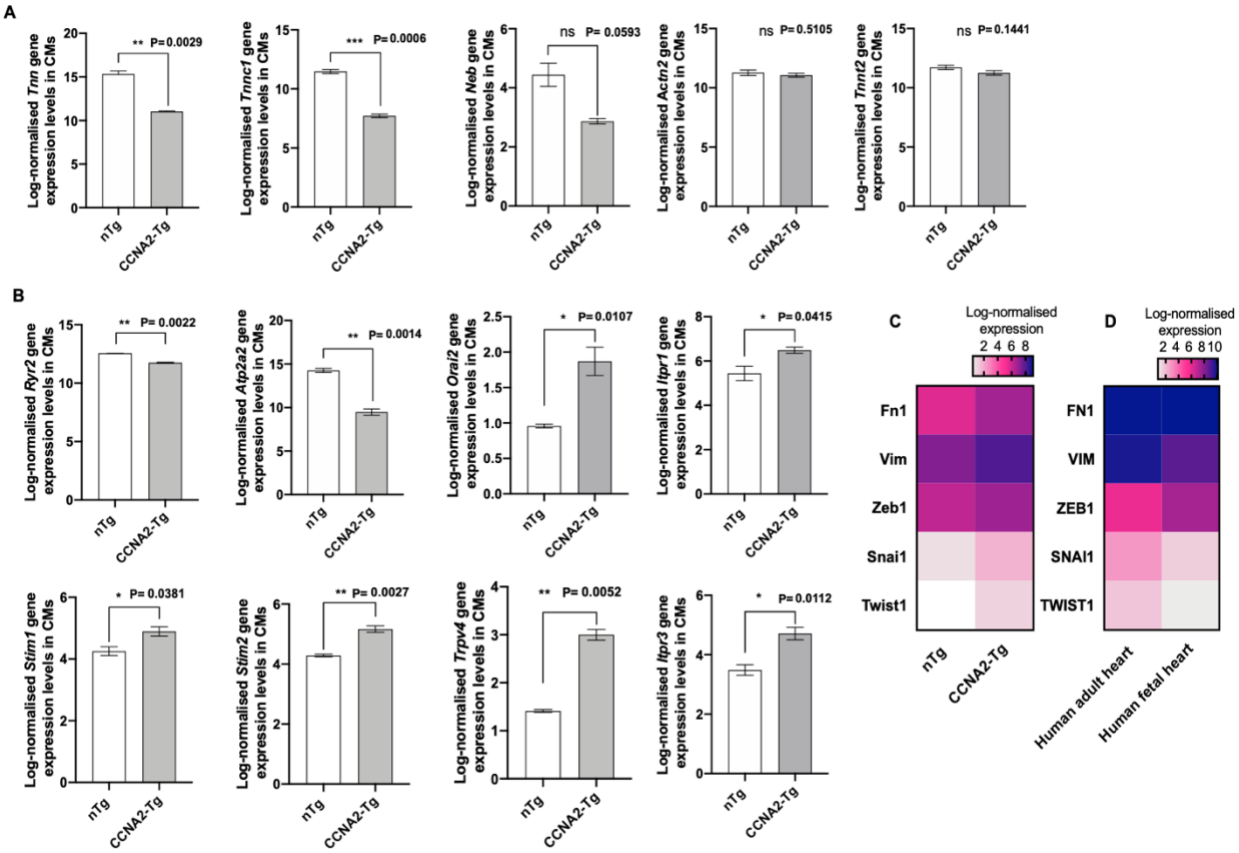

Figure S1. A) Representative graphs of sarcomere assembly genes *(Neb, Tnn, Tnnc1 Actn2,* and *Tnnt2),* and **B)** Ca^{2+} handling genes (*Ryr2, Atp2a2, Slc8a1*, and others) in nTg and CCNA2-Tg cardiomyocytes as delineated from bulk RNA seq. **C)** Heatmap of log-normalized expression of mesenchymal transition markers (*Fn1, Vim, Zeb1, Snai1, and Twist1*) in nTg and CCNA2-Tg cardiomyocytes, as well as **D)** human adult and fetal hearts. Gene expression levels are log-transformed and normalized. Data are presented as mean ± SEM, with statistical significance indicated (*P*-values). ns denotes non-significant differences.

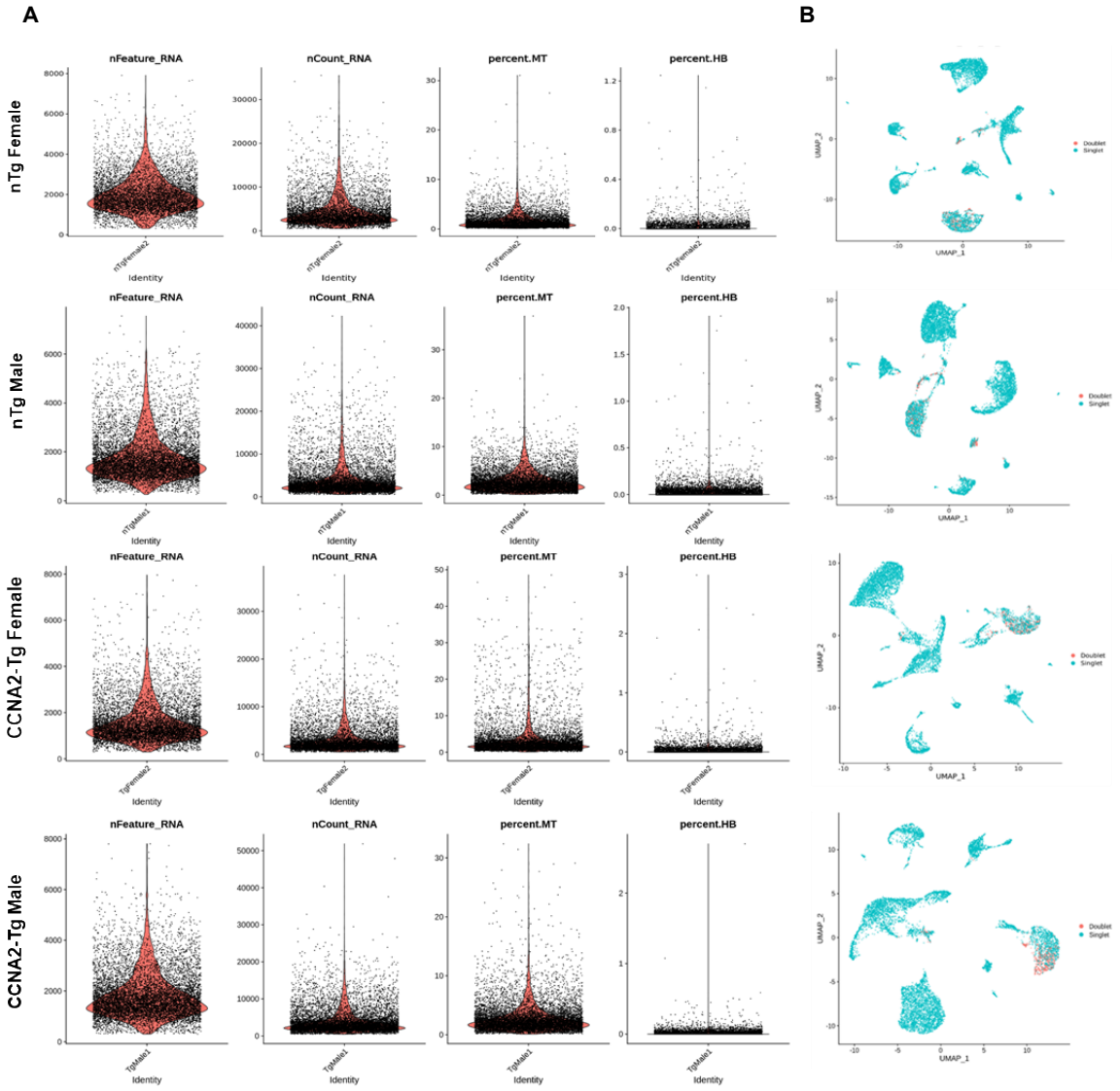

Figure S2. Quality control (QC) filtering and visualization in SnRNA-seq. **A)** "nFeature_RNA" indicates the count of gene features; "nCount_RNA" represents the count of UMIs; "percent.MT" signifies the proportion of counts originating from mitochondrial genes, and "percent.HB" signifies the proportion of counts stemming from Hemoglobin-related genes in all conditions nTg and CCNA2-Tg males and females. Each data point corresponds to a cell. **B)** The UMAP plots are generated from the top principal components within the clustered dataset. Each data point represents an individual cell and is color-coded based on whether it is categorized as a singlet or doublet.

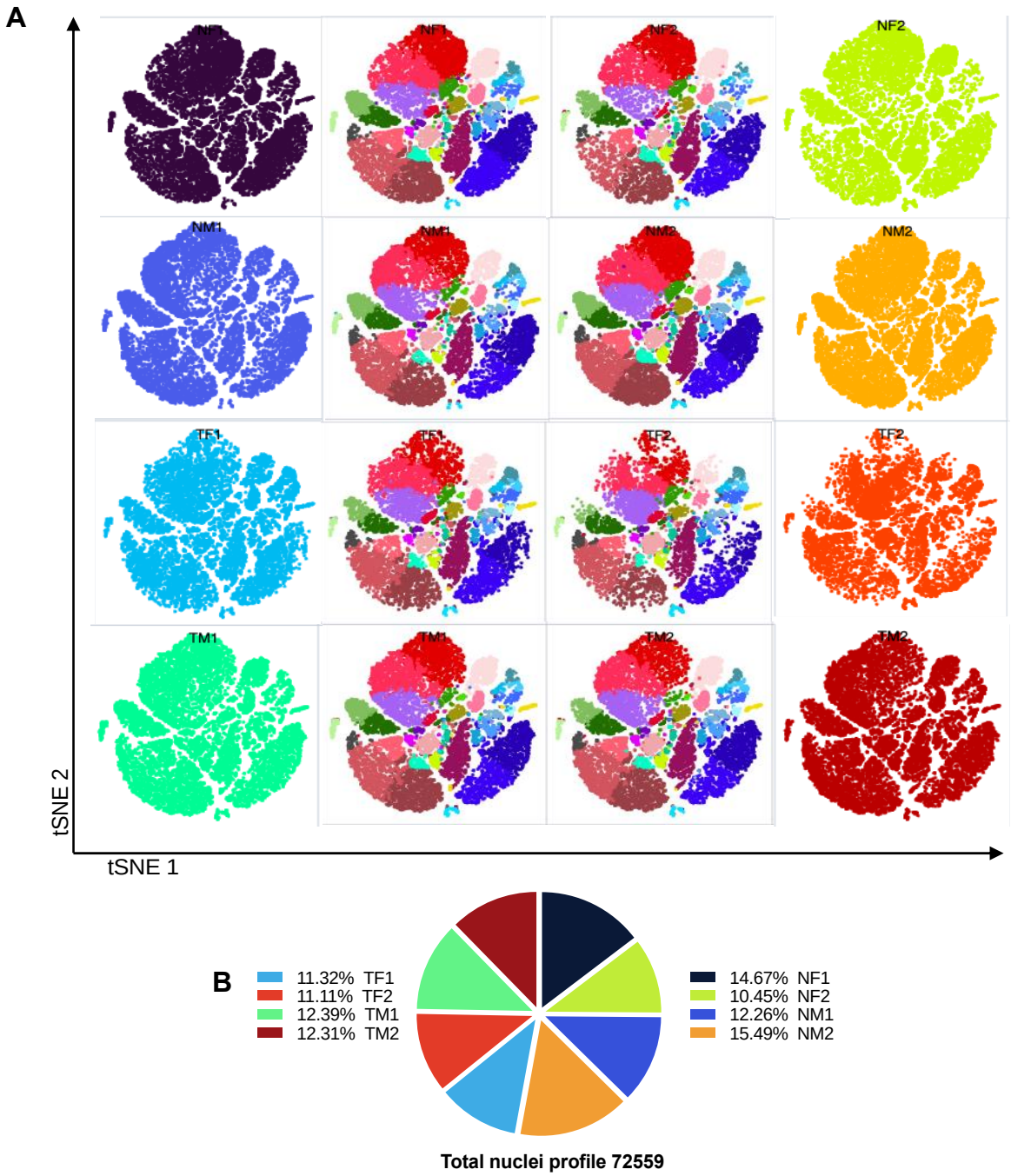

Figure S3. A) t-SNE plot clustering the nuclei of nTg and CCNA2-Tg conditions (n=8 total). **B)** Pie chart representing the percentage of nuclei profile in all conditions of nTg and CCNA2-Tg mice.

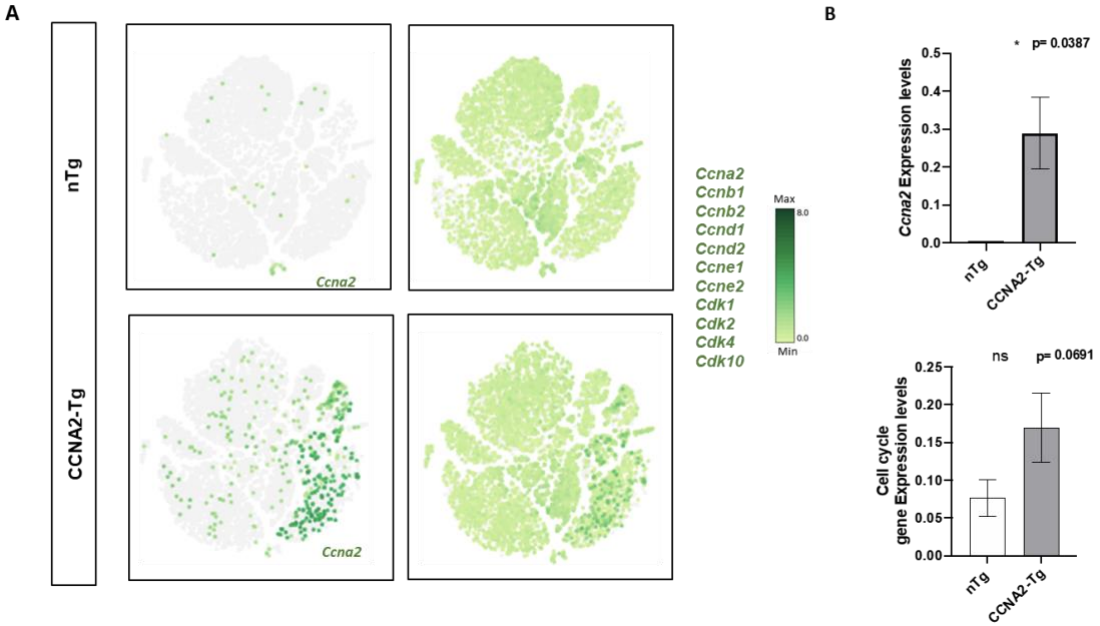

Figure S4. A) t-SNE plots and **B)** representative graphs of CCNA2 and cell cycle genes expression across the combined transcriptomic profiles of all subclusters of nTg and CCNA2-Tg mice.

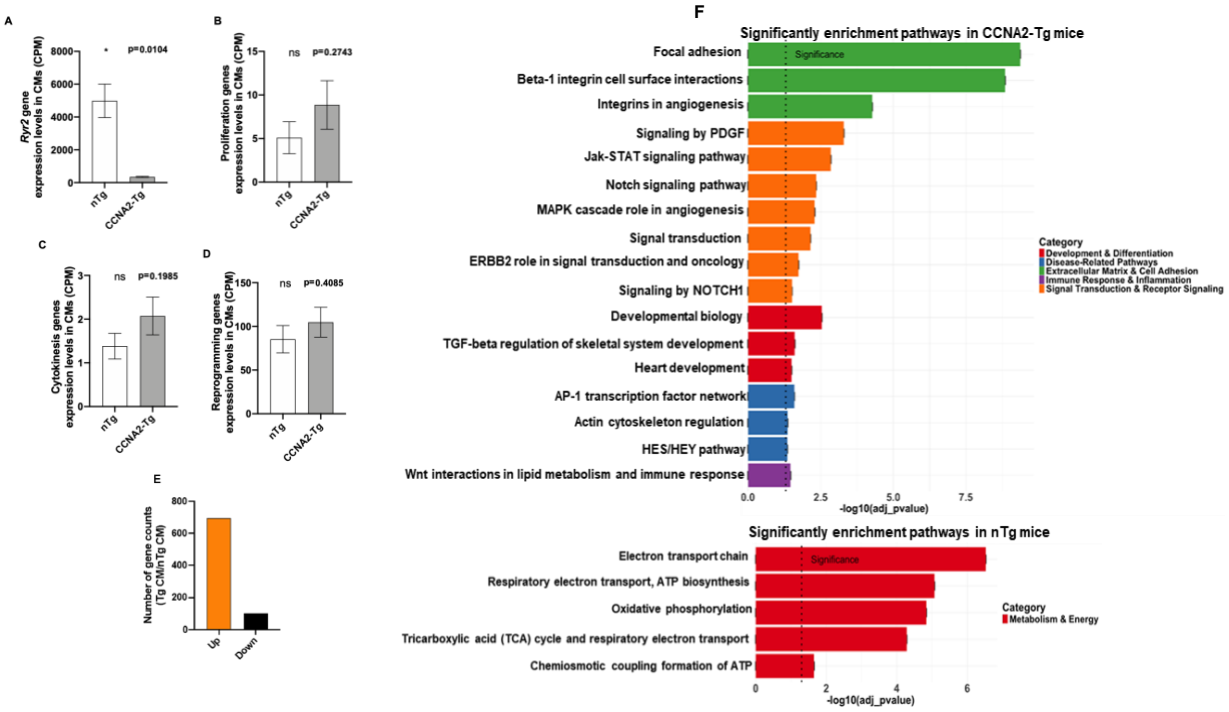

Figure S5. Representative graphs of **A)** cardiomyocytes maturation gene *Ryr2,* ***B)*** proliferation, **C)** cytokinesis, and **D)** reprogramming gene expression in nTg and CCNA2-Tg cardiomyocytes. **E)** Number of differentially expressed genes (Up or Down) in adult CCNA2-Tg mouse cardiomyocytes as compared to adult nTg mouse cardiomyocytes. **F)** Significantly enriched pathways in adult CCNA2-Tg and nTg mouse cardiomyocytes (P-value adjusted <0.05), analyzed using BioPlanet.

Source Data Figure

Movie S1. The real-time, live-imaging movie of human adult cardiomyocytes (55-year-old male) as described in **Figure 1.**
Movie S2. Real-time, live-imaging movie of human adult cardiomyocytes (41-year-old female) as described in **Figure 1.**

Source Data for Figure 1H.
Source Data for Figure 2D and S1C
Source Data for Figure 2C and S1D
Source Data for Figure 3J
Source Data for Figure 5E
Source Data for Figure 5I
Source Data for Figure S5F