

Loss of Adult Cardiac Myocyte GSK-3 Leads to Mitotic Catastrophe Resulting in Fatal Dilated Cardiomyopathy

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Rationale: Cardiac myocyte-specific deletion of either glycogen synthase kinase (GSK)-3 α and GSK-3 β leads to cardiac protection after myocardial infarction, suggesting that deletion of both isoforms may provide synergistic protection. This is an important consideration because of the fact that all GSK-3-targeted drugs, including the drugs already in clinical trial target both isoforms of GSK-3, and none are isoform specific.

Objective: To identify the consequences of combined deletion of cardiac myocyte GSK-3 α and GSK-3 β in heart function.

Methods and Results: We generated tamoxifen-inducible cardiac myocyte-specific mice lacking both GSK-3 isoforms (double knockout). We unexpectedly found that cardiac myocyte GSK-3 is essential for cardiac homeostasis and overall survival. Serial echocardiographic analysis reveals that within 2 weeks of tamoxifen treatment, double-knockout hearts leads to excessive dilatative remodeling and ventricular dysfunction. Further experimentation with isolated adult cardiac myocytes and fibroblasts from double-knockout implicated cardiac myocytes intrinsic factors responsible for observed phenotype. Mechanistically, loss of GSK-3 in adult cardiac myocytes resulted in induction of mitotic catastrophe, a previously unreported event in cardiac myocytes. Double-knockout cardiac myocytes showed cell cycle progression resulting in increased DNA content and multinucleation. However, increased cell cycle activity was rivaled by marked activation of DNA damage, cell cycle checkpoint activation, and mitotic catastrophe-induced apoptotic cell death. Importantly, mitotic catastrophe was also confirmed in isolated adult cardiac myocytes.

Conclusions: Together, our findings suggest that cardiac myocyte GSK-3 is required to maintain normal cardiac homeostasis, and its loss is incompatible with life because of cell cycle dysregulation that ultimately results in a severe fatal dilated cardiomyopathy. (*Circ Res.* 2016;118:1208-1222. DOI: 10.1161/CIRCRESAHA.116.308544.)

Key Words: cell cycle ■ dilated cardiomyopathy ■ GSK-3 ■ heart failure ■ mitotic catastrophe

Heart failure is a complex and debilitating clinical syndrome with an estimated economic burden of \$32 billion a year in the United States.^{1,2} Despite advances in treatment and increased availability of heart transplants, approximately half of the patients that develop heart failure die within 5 years of diagnosis.^{1,2} Dilated cardiomyopathy (DCM), the most common cause of heart failure, manifests itself with structural thinning and expansion of cardiac chambers with a progressive and sharp decline in cardiac function. Genetic studies have identified several genes that contribute to isolated cases

of familial DCM. However, as DCM is the result of multiple pathogeneses (both genetic and injury induced), the common underlying molecular mechanisms remain poorly defined.

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Glycogen synthase kinase-3 (GSK-3) is a highly conserved, integral regulator of numerous cellular processes including cell proliferation, metabolism, and cell death.³⁻⁶ GSK-3 consists of GSK-3 α and GSK-3 β isoforms, which our laboratory and others have shown to have both distinct and overlapping functions

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Nonstandard Abbreviations and Acronyms

Anln	anillin actin-binding protein
AurkB	aurora B kinase
BAX	BCL2-associated X protein
BCL-2	B-cell lymphoma 2
Bub1	budding uninhibited by benzimidazoles 1
CDC25	cell division cycle 25
CDK1	cyclin-dependent kinase 1
Cenpa	centromere protein A
DAPI	4',6-diamidino-2-phenylindole
DCM	dilated cardiomyopathy
DKO	double knockout
GSK-3	Glycogen synthase kinase 3
H2AX	H2A histone family, member X
Kif4	kinesin family member 4
Mad2	mitotic arrest deficient 2
Plk1	polo-like kinase 1
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling

in the heart.^{7–15} Germline homozygous deletion of *GSK3B* results in embryonic lethality because of the development of hypertrophic cardiomyopathy.¹⁶ This was the result of a hyperproliferation of cardiac myocytes that obliterated the ventricular cavity. In contrast, mice with germline homozygous deletion of *GSK-3α* are viable but develop cardiac hypertrophy with progressively deteriorating cardiac function in the nonstressed heart.^{17,18} These studies show that GSK-3 is a critical regulator of the cardiac myocyte cell cycle during embryogenesis. To better understand the mechanisms by which GSK-3 protects against cardiomyopathy, we generated mice that allow for conditional deletion of GSK-3 isoforms specifically in cardiac myocytes. Surprisingly, adult mice with cardiac myocyte-specific deletion of either *GSK3A* or *GSK3B* demonstrate preserved cardiac function and reduced progression to heart failure after myocardial infarction.^{7,14} However, it is unknown whether deletion of both isoforms may provide synergistic protection for cardiac function, and thereby reduce heart failure progression. Indeed, genetic studies assessing GSK-3 function in the heart, to date, have focused on isoform-specific models and none have explored the consequences of combined targeting of GSK-3 isoforms. Moreover, there are multiple clinical trials targeting GSK-3 isoforms for treatment of severe neurological diseases that would benefit from a clearer understanding of the cardiac effects of chronic GSK-3 inhibition.^{19–21}

Herein, we report that mice with adult cardiac myocyte-specific deletion of both isoforms of GSK-3 (double-knockout [DKO]) rapidly succumb to death. Microarray analysis of DKO hearts identified GSK-3 regulated transcriptional changes that provide insight on adult cardiac myocyte cell cycle activation. DKO adult cardiac myocytes exhibited cell cycle re-entry resulting in increased DNA content and multinucleation. However, instead of successful completion of cell cycle, cardiac myocytes accumulated severe DNA damage, activated cell cycle checkpoints, and culminated in mitotic catastrophe. The loss of cardiac myocytes severely impaired cardiac function and ultimately caused DCM and heart failure.

These findings are the first to provide evidence for mitotic catastrophe as a cell death mechanism for adult cardiac myocytes. Thus, cardiac myocyte GSK-3 is required to maintain cardiac homeostasis and overall survival.

Methods

An expanded Materials and Methods section is available in the online-only Data Supplement.

Mice

The *GSK3A^{flox/flox}*, *GSK3B^{flox/flox}*, and α -myosin heavy chain (MHC) *Mer-Cre-Mer* mice have been previously described.^{7,14} Cardiac myocyte-specific conditional GSK-3 DKO mice (*GSK3A^{flox/flox}*, *GSK3B^{flox/flox}*, and α -MHC *Mer-Cre-Mer*) were generated through several rounds of mating the above strains. All strains were maintained on the C57BL/6 background. At 12 weeks of age, male mice started getting tamoxifen (tam) chow treatment (400 mg/kg, TD.130860 Harlan) for 14 days followed by standard rodent diet (5001* LabDiet).

Statistics

Differences between data groups were evaluated for significance using unpaired 2-tailed Student *t* test or 1-way ANOVA, as appropriate and Bonferroni post-test (GraphPad Prism Software Inc, San Diego, CA). Survival analysis was performed by the Kaplan–Meier method, and between-group differences in survival were tested by the Gehan–Breslow–Wilcoxon test. For statistical assessment of double-positive pH3 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) cells, Chi χ^2 analysis was used. Data are expressed as mean $\pm$ SEM, unless noted otherwise. For all tests, a *P* value of <0.05 was considered statistically significant.

Results

Cardiac Myocyte-Specific Deletion of GSK3 Causes DCM and Death

To determine the effect of combined cardiac myocyte GSK-3 α and GSK-3 β deletion, we generated cardiac myocyte-specific knockout mice lacking both isoforms. This model allows for conditional deletion of all 4 *GSK3* alleles (2 α and 2 β) using a tamoxifen-inducible mER-Cre-mER system (herein referred to as DKO). Tamoxifen was administered using a well-established oral dosing regimen.⁷ Throughout this study, we refer to the tamoxifen-timeline (tam-timeline) to indicate the relative duration of tamoxifen-chow administration and age of mouse (Figure 1A). All results are reported as day postonset of tamoxifen administration. After administration of tamoxifen, Western blot analysis was utilized to evaluate efficiency of Cre-mediated gene excision. Results demonstrate a significant decline in GSK-3 α (85.54%) and GSK-3 β (66.84%) total protein levels within 2 weeks of tamoxifen administration (Figure 1B).

DKO mice demonstrated increased mortality versus controls with 100% death by day 42 (Figure 1C). The majority of DKO mice were found dead between days 35 to 40. Furthermore, at day 15 of the tamoxifen timeline, DKO mice demonstrated visible symptoms of heart failure as evidenced by tachypnea, labored breathing, and peripheral edema. At autopsy, the DKO mouse hearts exhibited severe DCM with multichamber enlargement. On gross examination, ascites, pleural effusions, and pericardial effusions were found. To assess the cardiac function in DKO and littermate controls, we performed serial M-mode echocardiography. At baseline, WT and DKO hearts had comparable chamber dimensions and ventricular function, but as early as 2 weeks of tamoxifen

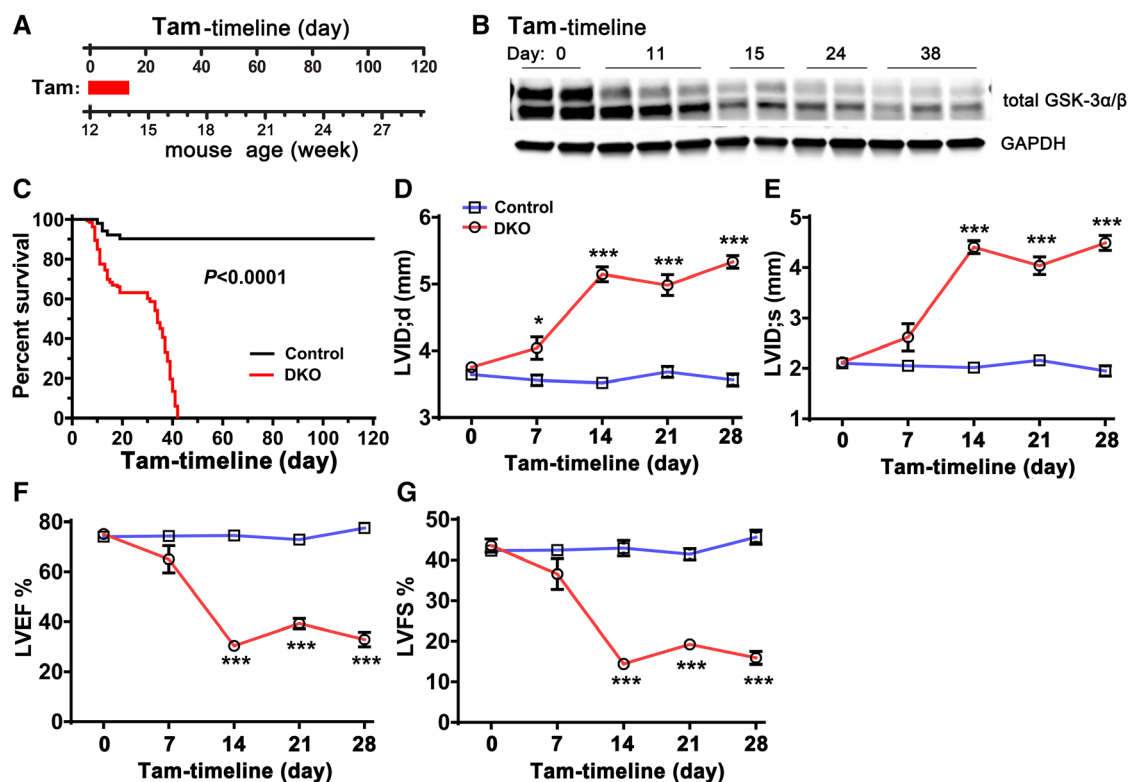


Figure 1. Cardiac myocyte-specific deletion of glycogen synthase kinase 3 (GSK3) leads to severe cardiac dysfunction and death.

A, Diagrammatic representation of the timeline for tamoxifen (tam)-induced Cre-recombinase-mediated deletion of GSK3.

B, Schematic representation of Western blot showing tam-induced deletion of GSK-3α/β (n=4). **C**, Kaplan-Meier survival curves for double knockout (DKO) mice versus controls indicate a significant reduction in lifespan within 40 days of tam-treatment. **D–G**, WT and DKO mice underwent baseline transthoracic echocardiographic examination and subjected to tam protocol. Mice were then followed with serial echocardiography at the time points shown. **D**, Left ventricular internal dimension at end-diastole (LVID; d). **E**, LVID at end-systole (LVID; s). **F**, Left ventricular ejection fraction (LVEF). **G**, LV fractional shortening (LVFS). * $P < 0.05$; *** $P < 0.005$. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.

timeline, DKO animals had a substantial increase in end-diastolic and end-systolic dimensions in comparison with littermate controls, reflecting accelerated dilatative remodeling (Figure 1D and 1E). This was associated with marked left ventricular dysfunction as reflected by significant decline in left ventricular ejection fraction and fractional shortening (Figure 1F and 1G). Left ventricular dilatation and dysfunction remained worse in the DKO throughout the study duration.

DKO Leads to Pathological Hypertrophy, Accelerated Fibrosis, and Heart Failure

Histological analysis of trichrome-stained cardiac sections at 0, 2, 3, and 4 weeks of tamoxifen timeline revealed myocardial thinning with enlarged atrial and ventricular chambers, consistent with DCM (Figure 2A). Cardiac myocytes were both elongated and widened in DKO heart sections compared with control (Figure 2B). For further assessment of cardiac hypertrophy, heart weight/tibia length ratios were compared. Hearts from DKO mice displayed 120.19% increase in heart weight/tibia length ratio compared with controls (Figure 2C). Quantification of trichrome-stained heart sections demonstrated enhanced fibrosis in the DKO hearts starting at 3 weeks time point (Figure 2D and 2E). Importantly, fibrosis was comparable between groups at 2 weeks, a critical time point at which morphological and functional changes were already evident suggesting fibrosis as a consequence not the

cause of observed phenotype. Histological assessment of hematoxylin and eosin (H&E)-stained pulmonary sections from DKO lungs demonstrated marked thickening of the alveolar interstitium, which is consistent with congestive heart failure (Figure 2F). DKO lungs demonstrated characteristic hemosiderin-laden macrophages, enhanced interstitial infiltrates, and marked thickening of the alveolar interstitium, all of which were consistent with congestive heart failure (Figure 2F). Overall, these data are the first to characterize the complete loss of cardiac myocyte-specific GSK-3 in the adult heart and reveal that cardiac myocyte GSK-3 is critical for organism survival. Loss of GSK-3 in the adult cardiac myocyte results in severe DCM and death.

Cardiac Myocyte Nuclear Enlargement and Ultrastructural Defects in DKO Hearts

Examination of H&E-stained cardiac sections from DKO mice indicated cardiac myocyte enlargement with expanded interstitium in all 4 chambers (Figure 3A). Interestingly, nuclear enlargement was clearly evident in all cardiac chambers in the DKO heart on H&E-stained cardiac sections. Nuclei in the DKO heart were irregular in size and shape, with variably distributed central and peripheral basophilic aggregates.

To further characterize the abnormalities in cardiac myocyte morphology found on H&E, cardiac sections from the DKO were evaluated using electron microscopy. Interrogation

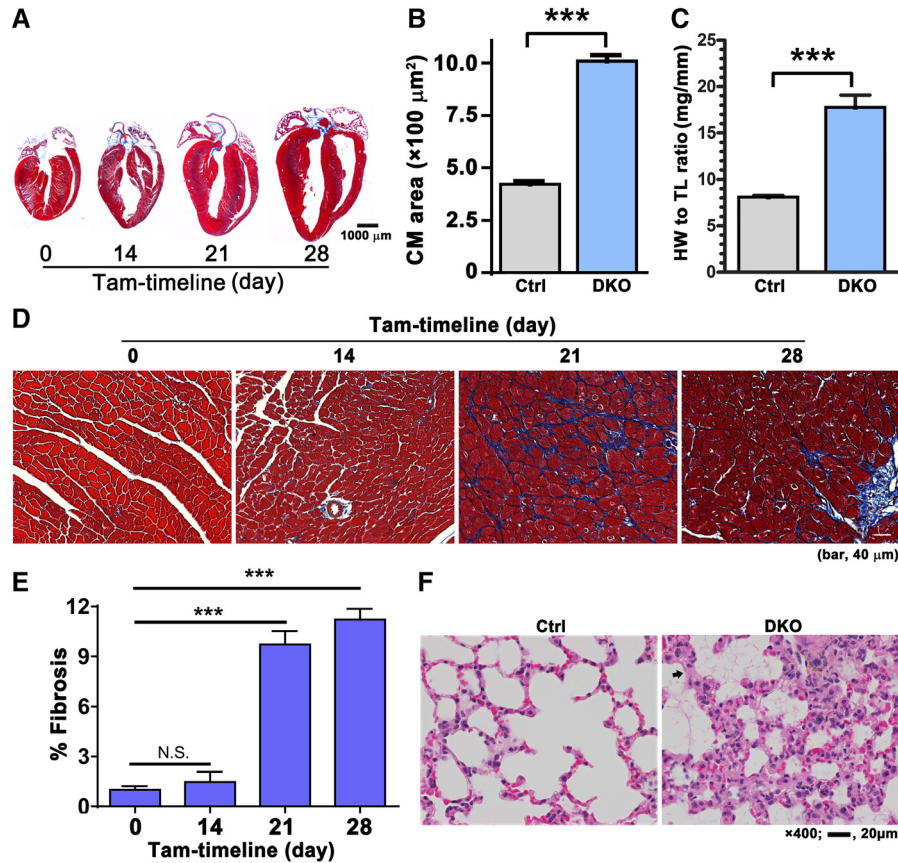


Figure 2. Cardiac myocyte-specific deletion of glycogen synthase kinase 3 (GSK3) leads to dilated cardiomyopathy, cardiac myocyte enlargement, accelerated fibrosis, and heart failure. **A**, Gross morphology of hearts from double knockout (DKO) versus control demonstrates multichamber enlargement. Schematic representation of images displaying morphological changes in a temporal manner. **B**, Cardiac myocyte cross-sectional area was significantly increased in the DKO hearts at day 21 of tamoxifen timeline ($n=4$). **C**, Morphometric analysis of cardiac hypertrophy in day 25 animals using heart weight/tibia length (HW/TL) ratio indicates significant increases in HW/TL ratio in DKO mice ($n=17$) compared with controls ($n=21$). **D**, Schematic representation of trichrome-stained heart sections. **E**, Quantification of fibrosis demonstrating increased fibrosis in DKO hearts starting from day 21 at tamoxifen timeline. **F**, Schematic representation of hematoxylin and eosin-stained lung sections demonstrate thickening of alveolar interstitium (arrow). *** $P<0.005$.

of overall cardiac myocyte structure revealed architectural abnormalities with loss of structural integrity at the sarcomeric Z line, reduced sarcomere protein content, and disordered mitochondrial and sarcomeric organization (Figure 3B). Although mitochondria did seem altered, their morphology was preserved when compared with mitochondria of previously reported GSK-3 α homozygous knockout animals, which revealed severe mitochondrial swelling with disrupted cristae.^{17,18} Furthermore, mitochondria numbers were comparable between DKO and littermate controls.

Because of the prominent nuclear enlargement observed on H&E, we focused our electron microscopy analyses on the structural changes in the nucleus (Figure 3C and 3D). DKO hearts confirmed both nuclear enlargements as well as revealed alterations in nuclear composition and shape. The shape of the nuclear membrane was clearly different from control nuclei as evidenced by the extensive nuclear membrane invaginations. The nuclear membrane was thickened with increased space between membrane and cytoskeleton. Compositions of nuclei were variable and markedly different from control nuclei. DKO nuclei contained enlarged nucleoli and enhanced peripheral and central electron dense clumping indicative of

the extent of heterochromatin aggregation (Figure 3C and 3D). Taken together, these results demonstrate the severity of structural derangements in the DKO hearts as evidenced by cardiac myocytes with both gross and ultrastructural morphological abnormalities.

Microarray Analysis Reveals Alterations in Cell Cycle and Suggests G2/M Blockade in Cardiac Myocytes

To gain better insight into the processes that might contribute to the unexpected fatalities in DKO mice, a microarray analysis was performed at day 21 of the tam-timeline (Online Figure 1A–1C). Bioinformatics analyses revealed 419 coding transcripts that were upregulated and 204 transcripts that were downregulated by a greater than or lesser than 1.9-fold at a statistical significance of $P<0.05$ (Online Table 1A). Furthermore, we compared our microarray dataset with numerous other publically available datasets of DCM and other heart failure models.^{22,23} Indeed, along with the markers of heart failures, we uniquely observed an enrichment of differentially expressed genes related to cell cycle and checkpoint activation. Analysis of microarray data

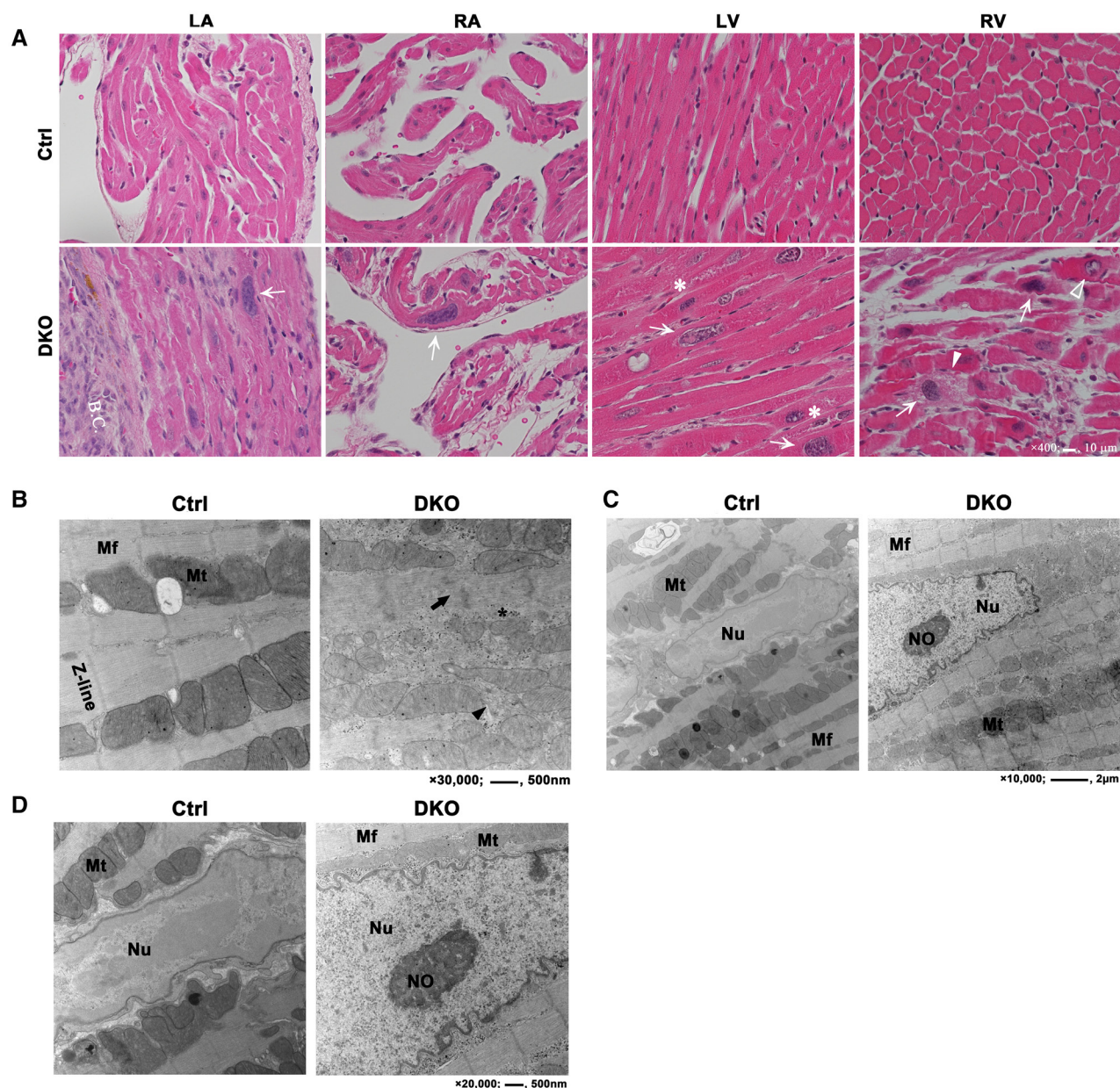


Figure 3. Double knockout (DKO) mice demonstrate ultrastructural defects and enlarged cardiac myocyte nuclei. **A**, Schematic representation of hematoxylin and eosin–stained heart sections from DKO versus control mice on day 35 of the tamoxifen (tam)–timeline showing enlarged nuclei in multiple chambers (arrows indicate enlarged cardiac myocyte nuclei, B.C indicates blood clot). **B**, Schematic representation of transmission electron micrographs of day 24 mouse hearts on tam–timeline demonstrate widened sarcomere Z line (arrow) and disrupted mitochondrial morphology (solid triangle). **C** and **D**, Schematic representation of transmission electron micrographs of day 24 mouse hearts on tam–timeline demonstrate enlarged nuclei (Nu) with nuclear aggregates (NO), abnormal sarcomeres (mf), and nuclear membrane invaginations.

through ingenuity pathway analysis indicated enrichment of differentially expressed genes related to fibrosis and cell cycle pathways (Online Table 1B). At 2 weeks of tamoxifen timeline, adult cardiac myocyte and fibroblast were isolated from DKO and littermate controls to delineate their specific role in observed phenotype. Western blot analysis reveals activation of cell cycle pathways and apoptosis specifically in cardiac myocytes only (Figure 4A–4E). Importantly, markers of cell cycle, apoptosis, and myofibroblast activation were comparable in isolated adult fibroblast from DKO and littermate controls (Figure 4A–4E). Taken together, at 2 weeks of tamoxifen timeline, fibrosis in DKO hearts and fibroblast

activation in an in vitro setting were comparable in DKO and controls. These data exclude fibroblast or fibrosis as the primary mechanism responsible for the observed phenotype. Hence, we focus on identifying the dysregulation of cell cycle pathway and associated mechanism. Specifically, assessment of cell cycle–related genes showed transcriptional changes in genes associated with G2/M (Table). Overall, these data revealed that adult cardiac myocyte *GSK3* deletion results in severe alterations in cell cycle control with subsequent activation of cell cycle checkpoints, thus providing insight into potential mechanisms by which this severe phenotype may be occurring.

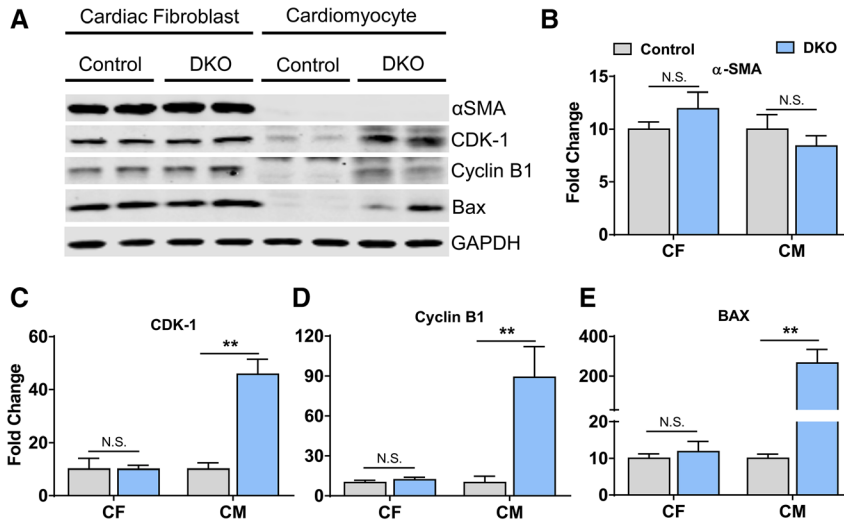


Figure 4. Double knockout (DKO) leads to activation of cell cycle and apoptosis in cardiac myocytes but does not in fibroblasts. **A**, At 2 weeks of tamoxifen timeline, cardiac myocytes and fibroblasts were isolated from DKO and littermate controls hearts and lysates were analyzed by Western blotting. **B**, Quantification of α -smooth muscle actin (α -SMA) shows unaltered myofibroblast activation. **C–E**, Western blot analysis reveals activation of cell cycle pathways and apoptosis specifically in cardiac myocytes only. ** $P < 0.005$. BAX indicates B-cell lymphoma 2-associated X protein; CDK-1, cyclin-dependent kinase 1; CF, cardiac fibroblast; CM, cardiac myocyte; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; and N.S., not significant.

GSK3 Deletion Induces Cell Cycle Re-Entry With Polyploidization and Multinucleation

The combined findings of nuclear abnormalities on histological analysis and cell cycle–related gene expression differences in the DKO prompted further investigation of the consequence of GSK-3 deletion on the cell cycle. Detailed histological analysis of cardiac myocyte nuclei in H&E-stained DKO heart sections revealed the severity of nuclear enlargement and alterations in nuclear shape (Figure 5A). Although both GSK-3 α and GSK-3 β cardiac myocyte–specific conditional knockouts heart displayed increased DNA synthesis on injury, this was not found in respective unstressed hearts compared with controls.^{7,14} In addition, such striking nuclear morphological abnormalities were not detected in isoform-specific conditional knockout hearts as well.

We performed bromodeoxyuridine-based assessment of DNA synthesis on DKO heart sections to provide insight on the observed nuclear abnormalities. Results showed increased DNA synthesis in the DKO cardiac myocytes compared with controls, indicating that loss of both GSK-3 isoforms stimulates S-phase entry at baseline (Figure 5B; Online Figure II). To assess the degree of DNA synthesis per nuclei, we utilized pericentriolar material 1 for flow cytometric analysis of nuclei extracted from DKO and control hearts. Pericentriolar material 1 is a centrosomal protein, which accumulates at the nuclear surface of mature cardiac myocytes and has frequently been utilized for quantification of DNA content.²⁴ In corroboration with reports in the literature, control cardiac myocyte nuclei were in a predominantly 2N state with a minimal number of nuclei with 4N content.^{25,26} However, DKO hearts revealed a reduction in nuclei with 2N and an increase in nuclei with $\geq 4N$ (Figure 5C). Although these results indicate an increase in overall cellular DNA content, they are not informative of the distribution of nuclear changes within the cardiac myocyte. To address this, we completed manual quantitation of the number of 4',6-diamidino-2-phenylindole* nuclei per isolated adult cardiac myocyte using confocal microscopy (Figure 5D). We found $\approx 50\%$ of the cardiac myocytes isolated from DKO hearts contained >2 nuclei with ≤ 8 nuclei in several cells (Figure 5E). Interestingly, the nuclei in the DKO did not

all seem to have completed karyokinesis, suggesting failed mitosis. These results demonstrate enhanced G1/S phase transition, with polyploidy, and multinucleation in DKO hearts.

DKO Cardiac Myocytes Show Mitotic Entry, DNA Damage, and Apoptotic Cell Death

With evidence of cell cycle progression with polyploidy, we were interested in characterizing the molecular mechanisms associated with these changes and characterizing later stages of the cell cycle. We completed Western blot analysis of representative markers of key modulators of the cell cycle. First we examined cyclin-dependent kinase 1 (CDK1)-CyclinB1, a critical regulatory complex for cellular commitment toward entering mitosis.²⁷ We find that CDK1 and its respective binding partner Cyclin B1 were significantly upregulated suggesting a cardiac myocyte mitotic entry (Figure 6A). Normally, adult cardiac myocytes do not express CDK1-Cyclin B1 and forced expression results in increased proliferative capacity.²⁸ However, at the end of G2 phase, cell division cycle 25 (CDC25) phosphatases activate Cyclin B1-CDK1 for nuclear entry. CDC25C phosphatase is a rate limiting inducer of mitosis and its loss results in mitotic blockade.²⁹ Because the DKO heart has a significant increase in Cyclin B1 accompanied with polyploidization (indicating inhibited mitosis), we assessed CDC25C activity. Western blot analysis revealed significant inactivation of CDC25C, and thus provides evidence for impaired mitotic progression in the DKO cardiac myocyte (Figure 6A and 6B).

The increase in CDC25C-pSer216 suggested increased DNA damage in the DKO cardiac myocytes. To examine this, we assessed phosphorylation of H2A histone family, member X (H2AX, γ H2A.X Ser139) that occurs on detection of DNA damage and is not normally found at baseline in hearts.^{30,31} Histological assessment of γ H2A.X in isolated adult cardiac myocytes from DKO mice demonstrated significant activation of DNA damage pathways (Figure 6C). In addition, Western blot analysis showed significant induction of activated checkpoint kinase 2 and γ H2A.X confirming double-stranded DNA damage (Figure 6D and 6E). We also assessed other DNA damage activated cell cycle inhibitors, p21 and p27^{kip1}.^{32,33} Western blot analysis showed

Table. Genes That Are Involved in Cell Cycle With Differential Expression in the Heart of DKO Versus Ctrl

Gene	Log (Ratio)	PValue	FDR	FC	GO Classification
Cks2	3.1766	0.0004	0.0197	9.0	Cell cycle, cell division
Ube2c	2.6760	2.87E-07	0.0014	6.4	Anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process, cell cycle
Knstrn	2.4585	1.22E-05	0.0044	5.5	Cell cycle, cell division
Cdkn3	2.4525	0.0004	0.0214	5.5	Cell cycle, cell cycle arrest
Esco2	2.3776	0.0005	0.0236	5.2	Cell cycle, chromosome segregation
Spc25	2.3373	1.27E-05	0.0044	5.1	Cell cycle, cell division
Ccnb1	2.3128	2.05E-05	0.0051	5.0	Cell cycle, cell division
Ckap2	2.2990	0.0001	0.0090	4.9	Apoptotic process, cell cycle
Aurka	2.2086	2.76E-05	0.0060	4.6	Anterior/posterior axis specification, cell cycle
Plk1	2.1487	4.09E-06	0.0028	4.4	Activation of mitotic anaphase-promoting complex activity, cell cycle
Cdk1	2.1427	1.90E-06	0.0024	4.4	Apoptotic process, cell aging
Anln	2.1270	0.0007	0.0277	4.4	Cell cycle, cell division
Cenpk	2.1182	0.0014	0.0388	4.3	Positive regulation of transcription from RNA polymerase II promoter
Fam83d	2.0174	1.31E-05	0.0044	4.0	Cell cycle, cell division
Cenpi	1.9967	0.0014	0.0380	4.0	Centromere complex assembly
Ccna2	1.9860	0.0003	0.0188	4.0	Cell cycle, cell division
Birc5	1.9349	1.98E-05	0.0051	3.8	Apoptotic process, cell cycle
Bub1	1.9272	0.0006	0.0248	3.8	Apoptotic process, cell cycle
Ccnb2	1.9143	1.10E-05	0.0043	3.8	Cell cycle, cell division
Oip5	1.9031	2.62E-06	0.0024	3.7	Cell cycle, cell division
Dlgap5	1.8876	0.0001	0.0112	3.7	Cell–cell signaling, cell cycle
Ndc80	1.8717	1.70E-05	0.0049	3.7	Attachment of spindle microtubules to kinetochore, cell cycle
Nuf2	1.8590	4.07E-05	0.0074	3.6	Attachment of spindle microtubules to kinetochore, cell cycle
Cenpn	1.8564	6.26E-06	0.0033	3.6	Centromere complex assembly, chromosome segregation
Spag5	1.8483	2.77E-05	0.0060	3.6	Cell cycle, cell division
Cdc25c	1.8178	0.0008	0.0289	3.5	Cell cycle, cell division
Mis18bp1	1.7971	0.0045	0.0685	3.5	Cell cycle, cell division
Kif11	1.7171	0.0003	0.0165	3.3	Cell cycle, cell division
Cdca3	1.6944	5.15E-06	0.0032	3.2	Cell cycle, cell division
Prc1	1.6129	0.0004	0.0209	3.1	Cell cycle, cell division
Racgap1	1.5941	2.20E-05	0.0052	3.0	Actomyosin contractile ring assembly, cell cycle
Cenpe	1.5798	0.0001	0.0091	3.0	Attachment of spindle microtubules to kinetochore, cell cycle
Cdc20	1.5736	2.08E-07	0.0014	3.0	Activation of anaphase-promoting complex activity, anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process
1500015010Rik	1.5627	0.0008	0.0292	3.0	Cellular senescence, cyclin catabolic process
Nusap1	1.5373	0.0009	0.0305	2.9	Cell cycle, cell division
Casc5	1.5355	0.0004	0.0219	2.9	Attachment of spindle microtubules to kinetochore, cell cycle
Mad2l1	1.5198	0.0004	0.0209	2.9	Cell cycle, cell division
Mki67	1.5100	0.0033	0.0587	2.8	Cell proliferation, meiotic nuclear division
H2afx	1.4946	0.0005	0.0233	2.8	Cell cycle, cellular response to DNA damage stimulus
4632434I1Rik	1.4844	0.0001	0.0115	2.8	Apoptotic process, cell cycle
Cenpp	1.4657	0.0003	0.0192	2.8	CENP-A containing nucleosome assembly at centromere

(Continued)

Table. Continued

Gene	Log (Ratio)	PValue	FDR	FC	GO Classification
Ncaph	1.4575	2.94E-06	0.0025	2.7	Cell cycle, cell division
Cdca8	1.4234	0.0003	0.0188	2.7	Cell cycle, cell division
2810417H13Rik	1.4151	0.0004	0.0213	2.7	Cellular response to DNA damage stimulus, centrosome organization
Ccne2	1.3900	0.0175	0.1432	2.6	Cell cycle, cell division
Ska3	1.3789	0.0080	0.0943	2.6	Cell cycle, cell division
Cenpw	1.3730	0.0003	0.0191	2.6	Cell cycle, cell division
Mlf1	-1.3126	0.0009	0.0302	2.5	Cell cycle, cell cycle arrest
Cep55	1.3094	0.0005	0.0233	2.5	Cell cycle, cell division
Mastl	1.2973	0.0017	0.0418	2.5	Cell cycle, cell division
Ube2t	1.2835	0.0019	0.0450	2.4	Cellular response to DNA damage stimulus, DNA repair
Bora	1.2738	0.0002	0.0154	2.4	Cell cycle, cell division
Aspm	1.2712	0.0001	0.0091	2.4	Brain development, cell cycle
Aurkb	1.2658	0.0012	0.0361	2.4	Cell cycle, cell division
Cenpm	1.2653	0.0003	0.0194	2.4	Biological process
Anapc1	1.2542	0.0237	0.1682	2.4	Cell cycle, cell division
Foxm1	1.2525	0.0000	0.0075	2.4	Cell cycle, cellular response to DNA damage stimulus
E2f1	1.2197	0.0031	0.0184	2.3	Anoikis, regulation of cell cycle
Gas2l3	1.2293	0.0003	0.0191	2.3	Actin cytoskeleton organization, cell cycle arrest
Ncapg	1.2231	0.0011	0.0339	2.3	Mitotic chromosome condensation
Zwilch	1.1995	0.0009	0.0313	2.3	Cell cycle, cell division
Bub1b	1.1990	0.0001	0.0111	2.3	Apoptotic process, cell cycle
Dsn1	1.1961	0.0017	0.0416	2.3	Cell cycle, cell division
Smc4	1.1627	0.0005	0.0235	2.2	Cell cycle, cell division
Mcm5	1.1357	0.0021	0.0476	2.2	Cell cycle, cell division
Dbf4	1.1328	0.0016	0.0400	2.2	Cell cycle, DNA replication
Ccne1	1.1311	0.0004	0.0203	2.2	Cell cycle, cell division
Clasp1	-1.1253	1.35E-05	0.0044	2.2	Cell cycle, cell division
Cenpa	1.0933	0.0013	0.0368	2.1	Establishment of mitotic spindle orientation, kinetochore assembly
Cdca5	1.0927	0.0002	0.0164	2.1	Cell cycle, cell division
Cep72	1.0822	0.0022	0.0478	2.1	γ -tubulin complex localization, spindle organization
Cdkn1a	1.0781	0.0073	0.0898	2.1	Cell cycle, cell cycle arrest
Fancd2	1.0642	0.0003	0.0180	2.1	Cell cycle, cellular response to DNA damage stimulus
Mcm7	1.0618	0.0005	0.0233	2.1	Cell cycle, cell proliferation
Smc2	1.0586	0.0038	0.0630	2.1	Cell cycle, cell division
Nek2	1.0441	1.75E-05	0.0049	2.1	Blastocyst development, cell cycle
Kif2c	1.0435	0.0012	0.0346	2.1	Cell cycle, cell division
Mcm6	1.0267	0.0009	0.0300	2.0	Cell cycle, DNA replication
Cdca2	0.9984	0.0004	0.0215	2.0	Cell cycle, cell division
E2f8	0.9885	0.0214	0.1595	2.0	Cell cycle, cell cycle comprising mitosis without cytokinesis
Ncapg2	0.9673	0.0029	0.0554	2.0	Cell cycle, cell division
Fam64a	0.9578	0.0015	0.0390	1.9	Cell cycle, cell division
Kif18b	0.9458	0.0015	0.0390	1.9	Cell cycle, cell division
Skil	0.9437	0.0003	0.0165	1.9	Blastocyst formation, cell cycle arrest

Ratio, mean from DKO mice to Ctrl ratio (n=4 biological duplicates). CENP-A indicates centromere protein A; DKO, double knockout; FC, fold change; FDR, false discovery ratio; and GO, gene ontology.

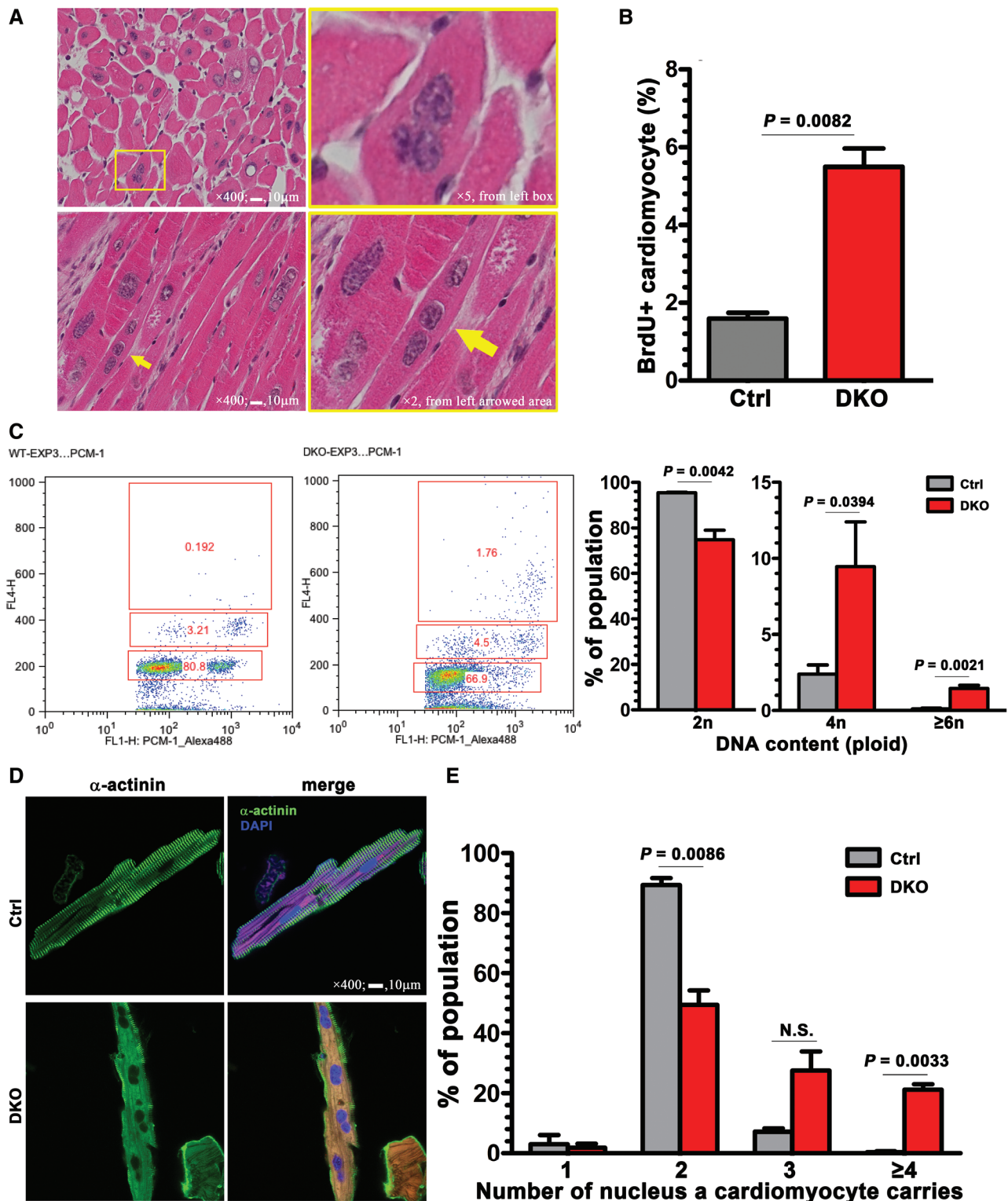


Figure 5. GSK3 deletion induces cell cycle re-entry with polyploidization and multinucleation. **A**, Schematic representation of hematoxylin and eosin images of double knockout (DKO) left ventricle from day 35 mice on the tamoxifen (tam)-timeline reveal the presence of multilobulated (box) and multinucleated cardiac myocytes (arrow). **B**, Quantitation of bromodeoxyuridine (BrdU)/ α -actinin⁺ cardiac myocytes from day 24 mice on the tam-timeline reveals statistically significant increases in DNA synthesis in DKO (n=3) cardiac myocytes compared with control (n=3). **C**, Flow cytometry analysis of pericentriolar material 1 (PCM-1)⁺ nuclei from isolated adult day 21 cardiac myocytes on the tam-timeline. Results indicate a statistically significant increase in nuclei with >4 nuclei in the DKO (n=3). **D**, Schematic representation of immunofluorescence images of adult day 21 cardiac myocytes mice on the tam-timeline stained with α -actinin and 4',6-diamidino-2-phenylindole (DAPI) revealed cardiac myocytes with ≤ 4 nuclei in karyokinesis in the DKO compared with control. **E**, Quantitation of number of DAPI⁺ nuclei in isolated adult cardiac myocytes from day 24 mice on the tam-timeline reveals a statistically significant reduction in binucleated cardiac myocytes and increase in cardiac myocytes with >4 nuclei in DKO (n=493 cells from 5 mice) versus controls (n=386 cells from 3 mice).

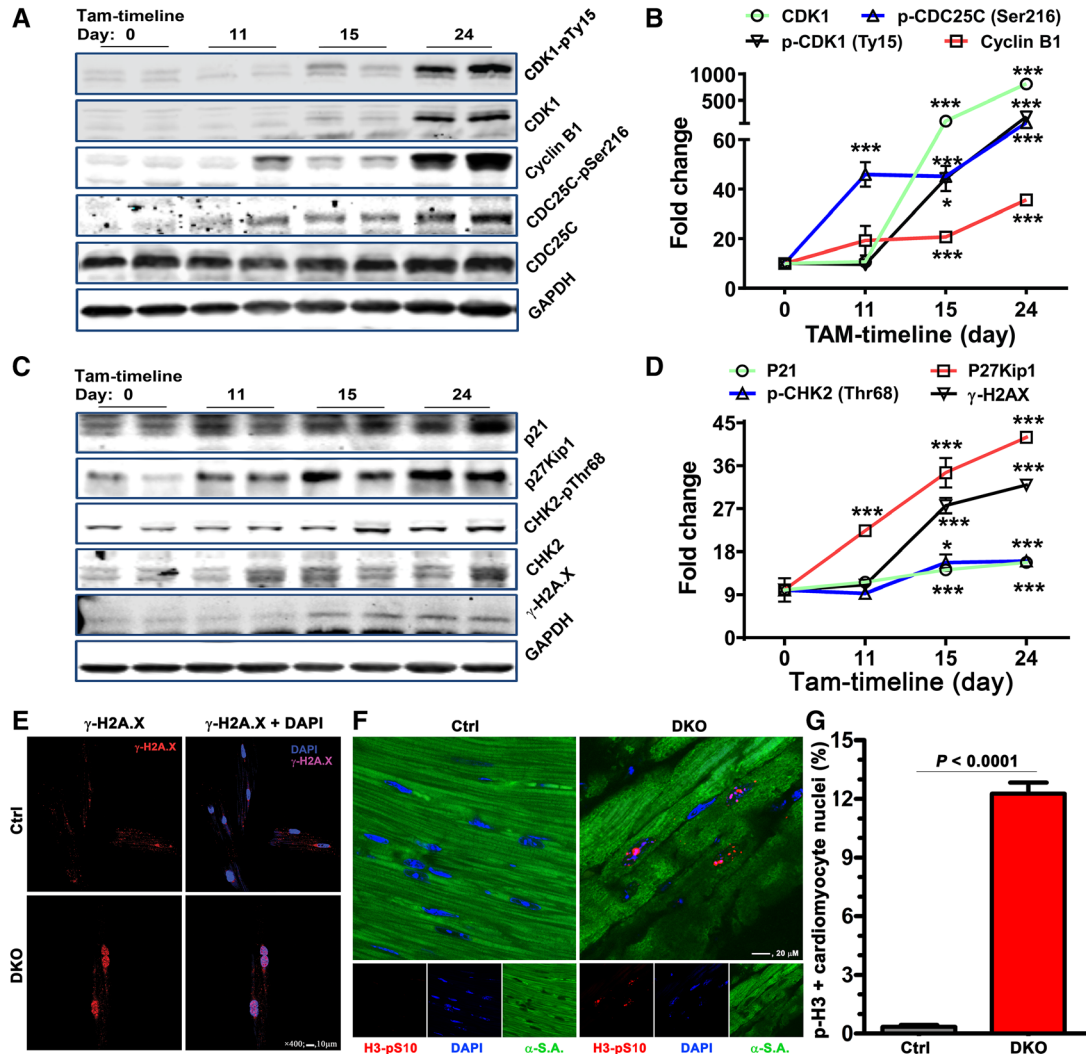


Figure 6. Double knockout (DKO) cardiac myocytes show mitotic entry and DNA damage. **A**, Schematic representation of Western blot analyses of various markers of the cell cycle. Results indicate increased protein expression of cyclin-dependent kinase 1 (CDK1), Cyclin B1, and CDC25C pSer-216 in DKO hearts compared with controls. **B**, Graph showing fold changes in CDK1, Cyclin B1, and CDC25C (pSer-216). **C**, Schematic representation of Western blots of DNA damage and cell cycle checkpoint markers γ -H2A histone family, member X (H2A.X), checkpoint kinase 2 (CHK2, pThr68), p21, and p27. **D**, Graph showing fold changes in γ -H2A.X and CHK2 (pThr68), p21 and p27 in the DKO hearts compared with controls. **E**, Schematic representation of immunofluorescence staining for γ -H2A.X in isolated adult cardiac myocytes from day 25 mice on the tamoxifen (tam)-timeline shows predominant detection of γ -H2A.X. **F**, Schematic representation of immunofluorescence staining for pH3-Ser10 in cardiac sections from day 21 mice on the tam-timeline shows predominant detection in α -actinin/pH3-Ser10 dual-positive nuclei in DKO cardiac myocytes. **G**, Quantitation of pH3-Ser10 (red) in α -actinin (green)/4',6-diamidino-2-phenylindole (DAPI) (blue) nuclei shows statistically significant increase in DKO cardiac myocytes ($n=3029$ nuclei from 3 mice) compared with controls ($n=3680$ nuclei from 3 mice). $^{*}P<0.05$; $^{***}P<0.005$. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.

a significant increase in p21 and p27^{Kip1} (Figure 6D and 6E). Together, these findings implicate an ongoing requirement for both GSK-3 α and GSK-3 β in the adult cardiac myocyte, which when perturbed results in DNA damage and cell cycle checkpoint activation.

As our results provide evidence for impaired mitotic entry, we measured various parameters associated with mitosis. Phosphorylation of histone H3-Ser10 is a common marker for assessing mitotic progression and is related to chromosome condensation.^{34,35} Histological assessment of DKO hearts showed increased number of pH3-Ser10-positive cardiac myocytes, indicating mitotic entry (Figure 6F and 6G). Quantification of cardiac myocytes dual-positive

for pH3-Ser10/ α -actinin revealed $\approx 12\%$ detection in DKO compared with negligible detection in controls (Figure 6G). Furthermore, microarray data provide evidence for upregulation of multiple markers of mitosis including: polo-like kinase 1 (Plk1), aurora B kinase (Aurkb), kinesin family member 4 (Kif4), anillin actin-binding protein (Anln), and centromere protein A (Cenpa; Online Table IA).^{36,37} These results demonstrate the presence of a large number of cardiac myocytes with condensed chromatin and are indicative of mitotic entry. However, the increased accumulation of pH3-Ser10⁺ cardiac myocytes in the presence of cell cycle checkpoint activation is highly suggestive of incomplete or delayed mitosis.

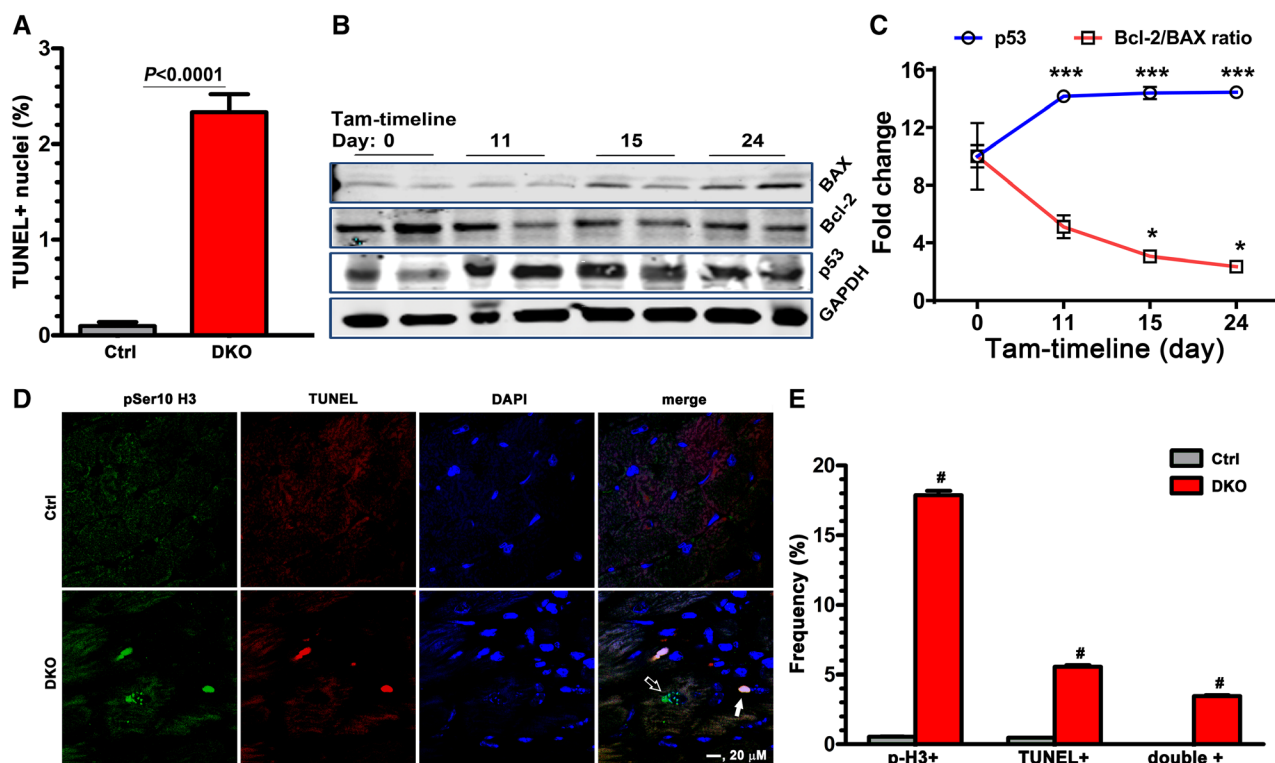


Figure 7. Mitotic entry of double knockout (DKO) cardiac myocytes leads to death by mitotic catastrophe. **A**, Quantitative terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) analysis on cardiac sections from day 35 mice on the tamoxifen (tam)-timeline shows a significant increase in TUNEL-positive cardiac myocytes in the DKO ($n=6513$ cells from 6 mice) compared with controls ($n=5211$ cells from 5 mice). **B**, Schematic representation of Western blots of apoptotic markers indicate a steady increase in p53 and an decrease in B-cell lymphoma 2 (BCL-2)/BCL-2-associated X protein (BAX) ratio after tam-administration in DKO hearts compared with controls ($n=4$). **C**, Graphical representation of the ratio of BCL-2/BAX in DKO hearts to control hearts during the tam-timeline. Results indicate a significant reduction in the antiapoptotic BCL-2 to proapoptotic BAX/protein ratio ($n=4$). $*P<0.05$; $***P<0.005$. **D**, Schematic representation of immunofluorescence staining for pH3-Ser10/TUNEL dual-positive nuclei from cardiac sections at day 21 mice on the tam-timeline shows predominant detection of dual-positive nuclei in DKO hearts. **E**, Quantitation of the distribution of pH3⁺/TUNEL⁺/4', 6-diamidino-2-phenylindole⁺ nuclei in the DKO versus control heart sections. Results indicate a significant proportion of triple⁺ nuclei in the DKO versus controls. $P<0.05$, χ^2 test. GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.

DKO Cardiac Myocytes Leads to Death by Mitotic Catastrophe

Because our findings provide evidence of cardiac myocyte mitosis in the DKO, we sought to identify the final fate of these cells. Although, as it was clear that the DKO hearts were in heart failure, we first characterized cardiac myocyte fate by assessing cellular death. Quantitative analysis of TUNEL-positive nuclei in cardiac myocytes revealed increased apoptotic cell death in the DKO hearts (Figure 7A). Western blot analysis of proapoptotic markers B-cell lymphoma 2 (BCL2)-associated X protein (BAX) and p53 indicated a significant induction of apoptotic cell death pathways (Figure 7B). The ratio of antiapoptotic BCL-2 to proapoptotic BAX member proteins is a major checkpoint in the common pathway of apoptotic cell death.^{38,39} Quantitation of the BCL-2/BAX ratio in the DKO indicates a reversal from high to low ratio, supporting the activation of an apoptotic cellular death (Figure 7C). Of more interest, we identified many cells that were positive for both pH3-Ser10 as well as TUNEL stain in DKO hearts versus none in the controls (Figure 7D and 7E). Quantification of histological findings shows that the majority of TUNEL⁺ cells are indeed also p-H3⁺. These findings provide strong evidence for the presence of apoptotic death in mitotically active cells. The combined morphological findings

and apoptotic cellular death in the presence of mitosis indicate the occurrence of mitotic catastrophe. Mitotic catastrophe is a mechanism for eliminating mitosis incompetent cells and occurs in the presence of inappropriate entrance into mitosis.^{40–42} A major classification of mitotic catastrophe indicates activation of cellular death in the presence of elevated cyclin B1.^{40,43,44} Our findings, which include striking nuclear aberrations on morphological assessment, elevated cyclin B1, and mitotic entry in the presence of cellular death, indicate the occurrence of mitotic catastrophe in cardiac myocytes.

Taken together, we conclude that GSK-3 in the adult cardiac myocyte is a critical suppressor of cell cycle induction and its loss leads to improper cell cycle re-entry, culminating in mitotic catastrophe. This associated loss of functional cardiac myocytes then results in impaired cardiac function with rapid onset of congestive heart failure and death in the DKO mice (Figure 8).

Discussion

In the DKO, we find that adult cardiac myocytes re-enter the cell cycle in the absence of cardiac stress. Although we previously demonstrated that cell cycle re-entry occurred in GSK-3 isoform-specific conditional knockout mice after cardiac injury, these mice were protected compared with

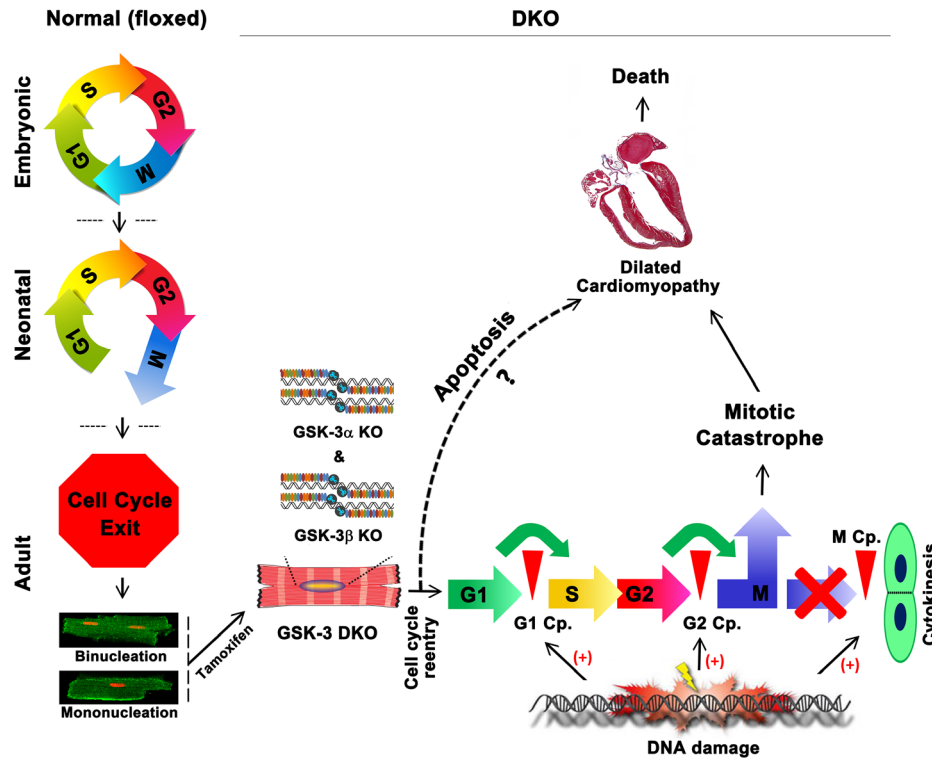


Figure 8. Schematic representation of the effects of glycogen synthase kinase 3 (GSK3) deletion on cardiac myocyte cell cycle.

During normal cardiac development cardiac myocytes retain the ability to proliferate. These cells are mononucleated during early development to birth but progress to a predominantly postmitotic, binucleated state by maturity. On deletion of GSK3, adult cardiac myocytes re-enter cell cycle. Cell cycle re-entry is opposed by cell cycle checkpoint activation and DNA damage. Although it is clear that double knockout (DKO) cardiac myocytes are able to bypass critical checkpoints to complete karyokinesis, these cells have impaired mitotic capacity and do not progress to cytokinesis. Instead, abnormal mitosis within these cells induces mitotic catastrophe resulting in a loss of functional cardiac myocytes. A possible alternative hypothesis showing loss of cardiac myocyte GSK-3 may lead to apoptotic cell death without cell cycle re-entry, which has been shown by dotted line. Mice develop dilated cardiomyopathy as a result of cardiac myocyte loss and ultimately succumb to death.

control littermates and exhibited a reduction in apoptosis.^{7,14} Strikingly, in the DKO hearts, the induction of cell cycle corresponded to a severe hypertrophic response with apoptotic cell death in the absence of injury. Cardiac myocytes were able to transit through G1/S and as well as partially through G2/M, however, did not complete cellular division because of mitotic catastrophe resulting in the ultimate development of fatal heart failure. This mode of cellular death has not yet been described in the setting of cardiomyopathy or connected to GSK-3 in the cardiac setting. The implications of these findings are broad including the potential consequence of chronic administration of GSK-3 inhibitors and our understanding of prolonged cardiac myocyte cell cycle reactivation in heart.

Currently, there is a major research focus on identifying pathways for activation of cardiac myocyte cell cycle re-entry. Many investigators have demonstrated successful cardiac myocyte cell cycle re-entry; however, the final fate of these cardiac myocytes is variable.^{45,46} Conditional adult cardiac myocyte-specific knockout mouse models have also successfully activated cardiac myocyte cell cycle re-entry, and some studies including ours have even demonstrated preserved cardiac function after injury.^{7,47–49} Although successful completion of adult cardiac myocyte cell cycle has been demonstrated,⁵⁰ the final outcome may include multinucleation and polyploidy.^{46,51,52} Furthermore, a majority of cardiac

myocytes in congestive heart failure and cardiac hypertrophy demonstrate polyploidy.^{53–56} The cell's ability to bypass mitotic completion and proceed through several rounds of DNA replication may be beneficial as this allows for increased transcription; however, the true relevance is unknown.⁵⁷ Thus far the number of molecules known to regulate cardiac myocyte polyploidy is limited and we demonstrate GSK-3 is a critical component of the cardiac myocyte polyploidization response. The mechanism by which GSK-3 loss results in polyploidy is unknown, but studies in pancreatic β cells have implicated p27 accumulation to be primarily responsible for polyploidy in that model.^{58,59} We speculate that the observed accumulation of p27 in the DKO cardiac myocytes may also play a role in polyploidization in the DKO cardiac myocyte. However, polyploidy in the DKO cardiac myocytes is accompanied by mitotic catastrophe, suggesting that the degree of cell cycle re-entry induced was detrimental and that therapeutic chronic activation of cardiac myocyte cell cycle could be maladaptive.

There is a limited body of literature that reports multinucleation and polyploidy similar to what is observed in the DKO in the absence of injury.^{46,47,60} Of most interest, none of these models report associated cellular death. Furthermore, cardiac-restricted Cyclin D1 overexpression induced G1/S transit, polyploidy, multinucleation, but demonstrated a down-regulation of mitotic markers including auroraB, pH3-Ser10,

Plk1, and mitotic arrest-deficient 2 (Mad2).⁴⁶ In contrast, DKO hearts showed increased auroraB, pH3-Ser10, and Plk1, thus indicating increased mitotic activity in the DKO. Cell death in the presence of mitotic entry is a defining characteristic indicative of mitotic catastrophe.

Mitotic catastrophe as a potential outcome for cardiac myocyte cell cycle re-entry is novel and not yet observed in the heart. A recent report linked GSK-3 β inhibition to mitotic catastrophe in cancer cells, further providing evidence for the occurrence of this phenomenon in pathology.⁶¹ This form of cellular death is defined to precede cellular death pathways and has been suggested to act as an onco-suppressive mechanism to suppress genomic instability.^{40,62} Mitotic catastrophe is a mechanism for eliminating mitosis incompetent cells and is triggered as a result of abnormal or inappropriate induction of mitosis. In stark contrast to activation of G2/M checkpoint induced cell death, mitotic catastrophe requires mitotic entrance. In the DKO, we provide sufficient evidence for activation of cell cycle checkpoints as well as the presence of mitotic entry in cardiac myocytes. Although, the morphological definition of mitotic catastrophe is incomplete, common findings have been suggested to be critical for assessing mitotic catastrophe.⁴² These include mitosis, polyploidy, and apoptotic cell death. Functionally, mitotic catastrophe has been described to include the activation of cell death machinery in the presence of elevated cyclin B1.⁴⁰ Our results provide strong evidence of cyclin B1 accumulation and induction of apoptotic cell death. Although the presence of mitotic catastrophe is indicated in the DKO, the reason for mitotic failure is not understood. It is currently thought that all types of mitotic catastrophe have a common underlying perturbation in the mitotic apparatus in the processes required for proper chromosome segregation.⁴⁰ Evidence supporting the role of GSK-3 in regulation of chromosome segregation is multifold in the literature.^{63–65} At the heart of chromosome, segregation is the spindle assembly checkpoint, which when prolonged is offered as a common mechanism by which cells trigger mitotic catastrophe.^{66,67} Microarray data from the DKO provides support of spindle assembly checkpoint dysregulation as indicated by significant upregulation of: Mad21l, budding uninhibited by benzimidazoles 1 (Bub1), Aurkb, Bub1b and Spc25. This is supportive of the notion that mitotic catastrophe in the DKO is associated with prolonged spindle assembly checkpoint activation. Overall, our data provide the first evidence for GSK-3-mediated regulation of cardiac myocyte polyploidy and mitotic catastrophe. Further studies are required to establish the mechanism by which GSK-3 deletion in the cardiac myocyte results in mitotic catastrophe.

Two previous reports with conditional deletion of both isoforms of GSK-3 demonstrate significant alterations in cell cycle but do not result in cellular death.^{68,69} In both studies, mice survived and there were no reported reductions in lifespan. Kim et al⁶⁸ investigated the consequences of combined GSK-3 α and GSK-3 β deletion in neural progenitor cells. These mice showed massive increases in neural progenitor cell proliferation without enhanced apoptosis when compared with controls. Jung et al⁶⁹ generated a conditional knockout mouse, in which GSK-3 α and GSK-3 β were deleted in

astrocytes. Astrocyte-specific GSK-3-DKO mice exhibited anxiety and altered social behavior with no effect on the lifespan. However, to date, only adult cardiac myocyte-specific deletion of both GSK-3 isoforms results in fatality, indicating that cardiac myocyte GSK-3 is essential for normal cardiac homeostasis and survival. The molecular mechanisms associated with the disparate fates for cells with cell cycle re-entry in these various GSK-3 knockout models are unknown and further studies are required to address this question.

The differences in tissue-specific consequences of GSK-3 deletion demonstrate the importance of GSK-3 in the cardiac myocyte as well as confer it as a preferential target during long-term GSK-3 inhibition. Indeed, optimism for developing GSK-3 inhibitors for clinical use remains high for treatment of a variety of severe pathological conditions, including progressive central nervous system disorders, cancer, metabolic disorders, and even ischemic cardiac injury.^{5,19–21} Current clinical trials are testing GSK-3 inhibitors for chronic treatment of patients with Alzheimer disease and supranuclear palsy.^{19,20} Although safety was achieved in 2 phase 1 clinical trials, our current data provide a cautionary note for the potential consequences of chronic pharmacological GSK-3 inhibition in the heart.

Herein, we have found that simultaneous deletion of both GSK-3 isoforms in cardiac myocytes results in increased cell cycle re-entry and progression but does not result in cardiac myocyte replication. The deletion of GSK-3 isoforms results in activation of cell cycle checkpoints with severe DNA damage and resultant mitotic catastrophe and cellular death. The subsequent excessive loss of cardiac myocytes impairs cardiac function and heart failure. In conclusion, loss of GSK-3 in the adult cardiac myocyte is incompatible with life because of cell cycle dysregulation that ultimately results in a severe fatal DCM.

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Novelty and Significance

What Is Known?

- Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed, serine/threonine kinase. In mammals, the GSK-3 family is encoded by 2 genes, GSK-3 α (*GSK3A*) and GSK-3 β (*GSK3B*).
- Isoform-specific deletion of either *GSK-3 α* or *GSK-3 β* specifically in cardiac myocytes leads to profound cardiac protection, post-myocardial infarction. The consequence of combined targeting of both GSK-3 isoforms in the hearts is unknown, and it is expected that targeting of both isoforms may provide synergistic cardiac protection.

What New Information Does This Article Contribute?

- GSK-3 is essential for cardiac myocyte cellular homeostasis and overall survival.
- GSK-3 in the adult cardiac myocyte is a critical suppressor of cell cycle induction as its loss leads to cell cycle re-entry, enhanced G1/S and S/G2 phase transition, activated cell cycle checkpoints, polyploidy and multinucleation.
- Aberrant cell cycle re-entry of GSK-3-deficient cardiac myocytes culminates to mitotic catastrophe, leading to severe fatal dilated cardiomyopathy.

Studies with isoform-specific mouse models have implicated the GSK-3 family as an attractive therapeutic target for management of cardiac diseases. However, the effect of complete loss of GSK-3 in cardiac myocytes is unknown. This is critical considering the fact that all GSK-3-targeted drugs, including drugs already in clinical trials target both GSK-3 isoforms, and none are isoform specific. To determine the effect of complete loss of GSK-3 α / β , we generated cardiac myocyte-specific knockout mice lacking both GSK-3 isoforms (double knockout). To our surprise, we found that cardiac myocyte-specific deletion of *GSK3* causes rapid heart failure and death. Double-knockout cardiac myocytes leads to abnormal cell cycle re-entry, increased DNA content, multinucleation leading to mitotic catastrophe. Mitotic catastrophe as a potential outcome for cardiac myocyte cell cycle re-entry is novel and previously unreported event in cardiac myocytes. In summary, GSK-3 is required for normal homeostasis of adult cardiac myocytes, and its deletion leads to cell cycle dysregulation resulting in a severe fatal dilated cardiomyopathy. These findings have important clinical implications and raise serious concerns over chronic administration of GSK-3 inhibitors.